Peripheral Nerve Demyelination and Neuropathic Pain

by

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DECLARATION

I hereby declare that the composition of this thesis and the work presented are entirely my own. Some of the studies presented have been published either as a research article or as a poster communication. Reprints and abstracts of all published work are included in the appendix.

Victoria C J Wallace BSc (Hons).

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ABSTRACT

Chronic neuropathic pain, characterised by allodynia (perception of innocuous stimuli as painful) and hyperalgesia (facilitated responses to painful stimuli), is poorly understood and is usually resistant to classical analgesics. Such abnormal pain phenomena can be associated with human demyelinating conditions such as Charcot-Marie-Tooth disease. Using mouse models of peripheral nerve demyelination, we have provided evidence for the consequent establishment of neuropathic pain and investigated possible underlying mechanisms.

The first model investigated was the *Prx*-null mouse. The murine periaxin gene (*Prx)* is expressed in Schwann cells and encodes L- and S-periaxin, two abundant PDZ-domain proteins thought to have a role in stabilisation of myelin in the peripheral nervous system (PNS). *Prx*-null mice show progressive demyelination in peripheral nerves and electrophysiological investigations indicate the presence of spontaneous action potential discharge; abnormal activity thought to be critical for the development of persistent pain states. Consistent with the time course of demyelination, *Prx*-null mice display an increased behavioural reflex sensitivity to cutaneous mechanical and noxious thermal stimulation.

To further investigate the link between demyelination of peripheral nerves and neuropathic pain, we have also characterised a novel model of focal peripheral nerve demyelinating neuropathy. Focal demyelination of the sciatic or saphenous nerve was induced with lysolecithin (lysophosphatidylcholine) and resulted in an increased behavioural reflex sensitivity to both thermal and mechanical tests, peaking at 9-14 days following treatment. Nerve morphology was investigated using light and electron microscopy, which revealed 30-40% demyelination of the treated nerve, (without lysolecithin-treated axon loss) coinciding with peak behavioural changes. Changes in the excitability of saphenous nerves were revealed, with spontaneous action potential discharge of 2-3 impulses per second present at peak behavioural change. No associated change in peripheral activation thresholds or conduction velocity was observed.

In both models, immunohistochemical investigations revealed no cell loss in the dorsal root ganglia (DRG) and no evidence for axonal damage. Similar methods revealed changes in the expression of neuropeptide Y, and the sodium channels $Na_v1.3$ and $Na_V1.8$ in DRG neurones. Such changes may account for increased nerve excitability and are known to occur in other models of nerve injury. However, these changes in the demyelinating models occur in a more restricted manner, specifically in the cells of formerly myelinated fibres. Intrathecal injections of the selective NMDA receptor antagonist, [R]-CPP, indicated that NMDA receptor-dependent changes are crucial for the development of a neuropathic pain–

like state following peripheral nerve demyelination. Intrathecal administration of pharmacological agents indicated a role for the transcription factor NFκB in the production of the behavioural reflex sensitivity of lysolecithin-treated mice, as well as identifying the endogenous cannabinoid system as an effective inhibitory regulator and potential analgesic target.

This study describes the first mouse models of peripheral nerve demyelination designed for the study of neuropathic pain and reveals phenotypic changes in DRG, which may contribute to the development of a neuropathic pain-like state. Therefore, these models may be useful for the evaluation of novel therapeutic targets for the treatment of demyelination-associated neuropathic pain.

ABBREVIATIONS

AC	Adenylate cyclase
AM 251	N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,3-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole proprionic acid
ANOVA	Analysis of Variance
ARCMT	Autosomal recessive CMT
BDNF	Brain derived neurotrophic factor
BFGF	Basic fibroblast growth factor
Ca^{2+}	Calcium
CAMIIK	Ca^{2+}/calmodulin-dependent protein kinase II
CAPE	Caffeic acid phenethyl ester
Caspr1	Contactin-associated protein
CCI	Chronic constriction injury
CGRP	Calcitonin gene related peptide
CMT1	Charcot-Marie-Tooth type I
CMT4	Charcot-Marie-Tooth type 4
CNS	Central nervous system
CVLM	Caudal ventrolateral medulla
Cx-32	Connexin-32
DAMGO	D-Ala_2, $MePhe_4$, Gly-olenkephalin
DDS	Dodecenyl Succinic Anhydride
DMP-30	2,4,6-Tri (dimethylaminomethyl) phenol
DOR1	Delta-opioid receptor
DRG	Dorsal root ganglia
DRP2	Dystroglycan-related protein 2
DS	Diagnostics Scotland
EM	Electron microscopy
FITC	fluorescein isothiocyanate
FRAP	Fluoride-resistant acid phosphatase
g	Grams
GABA	γ-aminobutyric acid
GAP-43	The growth associated protein -43
GBS	Guillain-Barré Syndrome

GDNF	Glial-derived-neurotrophic factor
HIV	Human immunodeficiency virus
HMSN	Hereditary motor and sensory neuropathy
HTM	High threshold mechanical
IASP	International Association for the Study of Pain
IB4	Isolectin B4
IL	Intraperiod Line
IL-1	Interleukin-1
K^+	Potassium ions
KOR1	Kappa-opioid receptor
LI	Lamina I
LII	Lamina II
LII_i	Lamina II inner zone
LII_o	Lamina II outer zone
LIII	Lamina III
LIV	Lamina IV
LV	Lamina V
LIF	Leukemia inhibitory factor
LLC	Large light cells
LM	Light microscopy
LTD	Long-term depression
LTP	Long-term potentiation
LV	Lamina V
m	Microtubules
M	Molar
MAG	Myelin-associated glycoprotein
MBP	Myelin basic protein
MDL	Major dense line
MGluRs	Metabotropic glutamate receptor
mls	Millilitres
MOR1	Mu-opioid receptor
MS	Multiple Sclerosis
Na^+	Sodium ions
Nd	Nodal region
NF	Neurofilaments

NFκB	Nuclear factor kappa B
NGF	Nerve growth factor
NK_1	Neurokinin 1 receptor
NMDA	N-methyl-D-aspartate
NOS	Nitric oxide synthase
NPY	Neuropeptide Y
NRM	Medullary nucleus Raphe magnus
Nrx-IV	Neurexin IV
NSAIDS	Non-steroidal anti-inflammatory drugs
°C	Degrees centigrade
P0	Protein zero
PACAP	Pituitary adenylate cyclase-activating polypeptide
PAG	Periaqueductal grey
PDGF-B	Platelet-derived growth factor B-chain
PKA	Protein kinase A
PKC	Protein kinase C
PMP22	Peripheral myelin protein-22 kda
PNS	Peripheral Nervous System
PRX	Human Periaxin gene
Prx	Murine Periaxin gene
PSDC	Postsynaptic dorsal column
PSNL	Partial sciatic nerve ligation injury
RA	Rapidly adapting
(R)-CPP	3-((R)-2-carboxypiperazine-4-yl)-propyl-1-phosphonic acid), (D-Ala_2, $MePhe_4$, Gly-ol)enkephalin
RF	Receptive Field
RVM	Rostral ventromedial medulla
SA	Slowly adapting
SC	Schwann cell
SCT	Spinocervical tract
SDC	Small dark cells
SEM	Standard error of the mean
SMT	Spinomesencephalic Tract
SNL	Spinal nerve ligation

SOM	Somatostatin
SP	Substance P
SSTR	SOM receptor
STT	Spinothalamic tract
TNF	Tumour necrosis factor
Tr	Trembler
TRITC	Tetramethyl rhodamine isothiocyanate
Tr-j	Trembler-j
TTX	Tetrodotoxin
µl	Microlitre
µm	Micrometer
VIP	Vasoactive intestinal polypeptide
VR	Vanilloid receptor
WIN 55,212-2	mesylate (R)-(+)-(2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo(1,2,3-de)-1,4-benzoxazin-6-yl)-1-naphthalenylmethanone

CHAPTER 1: 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... ".

(IASP Press 1994).

The sensation of pain is an extremely complex physiological phenomenon. It initially involves nociceptive processes, which lead to the transduction of information regarding potentially damaging stimuli from the point of stimulation to the brain. This information is then combined with cognitive, emotional and situational processing, producing the subjective individual experience of pain.
The capacity to experience pain serves a protective role to warn the animal or human of imminent or actual tissue damage and elicits co-ordinated reflex and behavioural responses to keep such damage to a minimum. Acute pain is therefore, a self-limiting normal sensation, which subsides upon cessation of the damaging stimulus.

Following tissue damage, excitability changes occur in the peripheral nervous system (PNS) and central nervous system (CNS) which establish a profound, but reversible pain hypersensitivity in the inflamed and surrounding tissue (Woolf and Mannion, 1999). This process assists wound repair as it promotes avoidance of the damaged area, and the pain experienced generally subsides once healing is complete.
However, persistent pain syndromes, in which the pain experienced outlasts the damaging stimulus and any associated healing processes, offer no such biological advantage. Such maladaptive, chronic pain can persist for weeks, months or even years, and typically results from damage to part of the nervous system resulting in hyperexcitability that produces spontaneous and hypersensitive pain syndromes. A major cause of chronic pain results from damage to a peripheral nerve. This is termed neuropathic pain, which is the main focus of this study. However, an understanding of the mechanisms of hyperexcitability requires first the consideration of the basic mechanisms of nociception leading to pain perception.

1.2 Introduction to Basic Mechanisms of Nociception

Somatosensory systems consist of highly specialised somatosensory nerve fibres, which provide information to the CNS regarding the external environment. Afferent axons run uninterrupted from their sensory endings in the peripheral target tissue to their central synaptic terminals in the CNS. The cell bodies lie in the dorsal root ganglia (DRG) near the spine or in the cranial nerve ganglia at the base of the skull.

Sensory impulse generation typically begins when a stimulus causes an increase in the permeability of the membrane of the sensory endings to various ions, particularly sodium ions (Na^+), depolarising the cell membrane. If the change in membrane depolarisation is sufficient, voltage-dependent ion channels open, resulting in the generation of an action potential and impulse propagation along the nerve to the CNS.

1.3 Peripheral Aspects of Nociception

Glabrous and hairy skin contain a complex array of sensory fibres, which are selectively sensitive to stimuli such as temperature and touch. Stimulation of the skin, evoking sensations such as vibration, stretch, touch, itch, pressure and pain, is detected by specialised receptors of primary afferent sensory axons, which transmit the information to the dorsal horn of the spinal cord in which afferents have highly structured termination patterns (Brown and Fuchs, 1975). There are three main types of cutaneous sensory afferent fibre, classified by their axonal diameter, conduction velocity and the specific modalities of sensory stimuli that evoke a response. They are broadly categorised as Aβ, Aδ and C-fibres. Aβ fibres are large diameter (>10μm), myelinated fibres and therefore have fast conduction velocities, in the range of 30-100m/s. Aδ-fibres are also myelinated, but have smaller axonal diameters (2-6μm) and therefore have a slower conduction velocity ranging from 12-30m/s. C-fibres are unmyelinated, small diameter (0.4-1.2μm) fibres with the slowest conduction velocities in the range of 0.5-2.0 m/s.

Mechanoreceptors and nociceptors relay information regarding mechanical stimuli (such as light touch, vibration and pressure) and painful stimuli (noxious pressure, pinch or low or high temperature), respectively (Lynn, 1994). They are associated with particular primary afferent axon types and have distinct activation thresholds, which must be achieved by a particular stimulus before activation occurs.

1.3.1 Non-nociceptive mechanoreceptors

Cutaneous mechanoreceptors, usually associated with myelinated primary afferent fibres, respond to a variety of innocuous tactile sensations. This diverse group of sensory receptors includes rapidly adapting ('RA') Meissner and Pacinian corpuscle receptors responding to light touch, pressure and vibration; slowly adapting ('SA') type I and type II mechanoreceptors associated with Merkel cells and Ruffini corpuscles respectively, which are involved in stretch perception and shape discrimination, (Willis and Coggeshall, 1991); and D, G and T-hair receptors which innervate hair follicles and respond to hair movement (Brown and Iggo, 1967;Lynn and Carpenter, 1982). Unmyelinated C-fibre mechanoreceptors have also been identified, which respond to gentle mechanical stimulation and cooling of the skin (Bessou et al., 1971).

1.3.2 Non-nociceptive thermoreceptors

Warm thermoreceptors are generally thought to be associated with unmyelinated axons (Iggo A, 1959) and respond only to slight warming of the skin in the non-noxious range (30-37°C). Non-nociceptive cold specific receptors respond to small variations in cold sensations and exist mainly associated with Aδ-fibres, although some may be associated with C-fibres (Iggo, 1959;Iggo, 1969).

1.3.3 Nociceptors

Nociceptors respond preferentially to stimuli that are potentially or actually injurious (Sherrington, 1906) (noxious) and are the least differentiated of the sensory receptors in the skin, where they terminate as free nerve endings. Nociceptors are functionally divided into two groups, comprising Aδ-fibre associated mechanical nociceptors or C-fibre associated polymodal nociceptors.

Aδ fibre nociceptors are primarily high threshold mechanoreceptors responding to high threshold mechanical (HTM) stimulation of the skin, such as pressure and pinch; however, they may also show weak responses to high intensity heat, cold (0 to -10°C) (Simone and Kajander, 1996;Simone and Kajander, 1997) and chemical stimuli. Their activation results in rapid, pricking or sharp sensations referred to as 'first pain', and perhaps aching.

C-polymodal nociceptors respond to multiple high threshold stimuli including heat (>45°C), high threshold mechanical and chemical stimulation (Bessou and Perl, 1969) and in some cases intense cold (Cervero and Iggo, 1980;Simone and Kajander, 1996;Willis and Coggeshall, 1991). Activity sufficient to activate C-fibre afferents results in a dull, diffuse or burning pain referred to as 'second pain'.

Other types of nociceptors have been identified including chemical nociceptors, which respond specifically to changes in extracellular pH and acidosis; and 'silent' nociceptors, which may be completely inactive until sensitised, for example, by the onset of inflammation (Besson and Chaouch, 1987;Bessou and Perl, 1969;Schmidt et al., 1995).

1.3.4 Sensory neurons: cell identification

The cell bodies of the sensory primary afferents lie in the DRG. They are characterised by their responsiveness to growth factors such as nerve growth factor (NGF) glial cell-derived-neurotrophic factor (GDNF) and by their expression of various substances, which can be used to identify and categorise them. For example, neuropeptides including calcitonin gene related peptide (CGRP) and substance P (SP) are normally expressed in a restricted pattern and will be dealt with in detail below (see section 1.7.4). Further markers expressed in a cell type restricted pattern include neurofilament (NF) protein, which can be composed of three subunits; the heavy chain, NFH (200 kDa), the medium chain NFM (155 kDa) and the light chain, NFL (68 kDa); nitric oxide synthase (NOS), the growth associated protein GAP-43, fluoride-resistant acid phosphatase (FRAP) as well as binding for the isolectin B_4 (IB_4) (Bergman et al., 1999; Da Silva et al., 1986; Petruska et al., 2000).

The primary sensory neurons of rats and mice have been classified into type 1/A, large light cells (LLC); type 2/B, small dark cells (SDC); and type 3/C cells on the basis of size and ultrastuctural and immunocytochemical characteristics. The LLC subpopulation is identified by immunostaining with the antibody to the phosphorylated form of NFH, RT97 (Kitao et al., 1996;Lawson et al., 1984;Perry et al., 1991) and accounts for approximately 35% of cells (Bergman et al., 1999). These cells do not express SP (Petruska et al., 2000) and are likely to be associated with Aβ-fibres. The SDC subpopulation can be defined by the binding of IB_4, a lectin from plant *Griffonia simplicifolia*, which has been found to recognise α-D-galactose carbohydrate residues on the surface of a subset of small DRG neurons accounting for approximately 50% of cells (Bergman et al., 1999). The neurons labelled by IB_4 binding in rat and mouse sensory ganglia are often regarded as non- NGF-dependent and non-peptidergic. However, a considerable number of IB_4-positive neurons in the DRG are also shown to be immunoreactive to SP and CGRP (Petruska et al., 2000), which are synthesized by NGF-dependent neurons and are likely to be associated with C-fibres. Furthermore, in situ hybridization histochemistry of DRG neurons demonstrated that 29% of NGF receptor expressing neurons and 66% of GDNF receptor expressing neurons were intensely labelled by IB_4 binding. This suggests IB_4 labelled neurons are peptidergic and growth factor dependent (Kashiba et al., 2001). The remaining subpopulation (approximately 15% of cells)

express RT97 and bind IB_4, do not express SP (Petruska et al., 2000) and are likely to be associated with Aδ-fibres. All three subpopulations, especially type 1, express CGRP many of which are also capable of synthesising galanin (Bergman et al., 1999). NOS is present in the RT97- subpopulations and frequently colocalize with CGRP (92%). GAP-43 is expressed in all three DRG subpopulations and colocalize with CGRP (88%), and/or NOS (22%) (Bergman et al., 1999). In contrast, FRAP appears to mark a subpopulation of DRG small cells which are non-peptidergic nociceptors demonstrated by studies of afferent terminal immunoreactivity (Da Silva et al., 1986).

1.4 Central Aspects of Nociception

The dorsal horn of the spinal cord, the first stage of processing of sensory information in the somatosensory system, plays an important role in transmitting information from nociceptive primary afferent neurons to the brain; however, our knowledge of its neuronal and synaptic organisation is still limited. Essentially, the dorsal horn consists of the central terminals of primary afferent fibres, intrinsic dorsal horn neurons and outputs and inputs to, and from, the rest of the CNS making it a complex, multisynaptic structure (Doubell and Mannion, 1999).

1.4.1 The dorsal horn of the spinal cord

The current view of the spinal cord is based on the work of Rexed (Rexed, 1952) who classified the 'butterfly' shape of the spinal cord grey matter, containing the neuronal cell bodies, into nine laminae that make up the dorsal and ventral horns of the spinal cord and a tenth lamina that surrounds the central canal. The central terminals of primary afferents in the dorsal horn occupy highly ordered spatial locations divided into five broadly parallel laminae in several mammalian species (Molander et al., 1984;Rexed, 1952) (see Figure 1a). Therefore only these five laminae will be considered for the purposes of the present investigation.

Dorsal horn organisation

Lamina I (LI), also known as the 'marginal zone' is the thin outermost layer of the dorsal horn and extends medially and laterally. It contains sparsely distributed small (5x5μm) and large cells (15x15μm), many of which are projection neurons and cells of origin of the spinothalamic tract (STT) (Giesler et al., 1976;Todd, 2002). LI cells send axons to various parts of the brain, including the caudal ventrolateral medulla (CVLM), parabrachial area, periaqueductal grey (PAG) and thalamus. A group of giant neurons named Waldeyer cells

can be identified in LI by the presence of high levels of the glycine receptor-associated protein gephyrin on their cell bodies and proximal dendrites (Puskar et al., 2001). Unlike many lamina I projection neurons which respond to excitatory input, Waldeyer cells either lack the neurokinin 1 (NK_1) receptor, or express it at a very low level and lie adjacent to γ-aminobutyric acid (GABA) containing terminals (Puskar et al., 2001), suggesting that they are influenced in an inhibitory manner. Lamina II (LII) also called the 'substantia gelatinosa', lies ventral to LI and consists of an outer zone (LII_o), which contains densely packed small cells (5x5μm) and a less compact inner zone (LII_i). LII contains mostly intrinsic interneurons with extensive local integration. Only a few (~1%) LII neurons project axons to the brainstem (Giesler et al., 1976;Willis et al., 1979), whilst some neurons project into LI. Together, LI and LII are known as the 'superficial dorsal horn'.

Nociceptive afferents, many of which contain SP, terminate mainly in laminae I and II of the spinal dorsal horn. Correspondingly, up to 80 % of lamina I projection neurons express the SP, receptor NK_1 (Todd, 2002).

Lamina III (LIII) lies ventral to LII and contains generally larger and less tightly packed cells. Primary afferents of large hair follicles, Pacinian corpuscles and RA mechanoreceptors terminate in LIII and cell dendrites project to LI and IV. LIII also contains cells of origin of the spinocervical tract (SCT) and the postsynaptic dorsal column (PSDC) tract (see Section 1.9) (Brown and Fyffe, 1981). Lamina IV (LIV) is a thicker layer, ventral to LIII and contains large, scattered cells (10x15μm) many of which project dendrites into LI –III and are therefore able to receive a direct primary afferent input from fibres that enter the superficial layers. A significant number of the larger LIV cells are also cells of origin of the SCT and the PSDC (Brown and Fyffe, 1981).

Within laminae III and IV, a population of large neurons express the NK_1 receptor, (Naim et al., 1997), project to the CVLM and parabrachial area (Todd, 2002) and may also target lamina I projection neurons (Sakamoto et al., 1999). These observations indicate that there are specific patterns of synaptic connectivity within the spinal dorsal horn.

Lamina V (LV) forms the neck of the dorsal horn and contains a heterogeneous neuropil including the largest cells of the dorsal horn laminae (15x20μm), many of which constitute the ascending projection to the thalamus as the STT (Giesler et al., 1976). Together, LIII, IV and the dorsal part of LV make up the 'nucleus proprius' (Brown and Fyffe, 1981). For schematic diagram of laminar organisation see Figure 1.1a.

1.4.2 Dorsal horn neurons

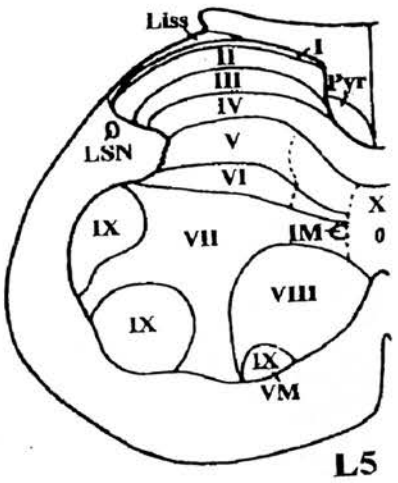
Nociceptive primary afferents terminate primarily in the superficial dorsal horn (LI and LII). C-fibres terminate predominantly in LII (Woolf and Doubell, 1994) while Aδ-afferents terminate in both the superficial laminae and send some projections deeper to lamina V (Jessell and Dodd, 1985). Aβ-fibres normally terminate only in the deeper laminae (III-IV) (Willis and Coggeshall, 1991) whilst many fine afferent fibres from muscle and viscera project directly in LV (Brown et al., 1981). The rostrocaudal and mediolateral location of the central terminals generate a somatotopic map of the body surface in the horizontal plane of the dorsal horn (Doubell and Mannion, 1999). For a schematic diagram of cutaneous afferent input into the dorsal horn see Figure 1.1b.

The neurons of the dorsal horn can therefore be categorised by their response characteristics to primary afferent fibre sensory input. They have been classified into three major groups; Class 1, non-nociceptive neurons, involved in innocuous information processing (Iggo and Ramsey, 1974); Class 2, multireceptive neurons, sensitive to cutaneous mechanoreceptive and nociceptive input; and Class 3, noci-specific neurons responding only to noxious stimulation of the skin (Cervero et al., 1976;Iggo and Ramsey, 1974). There is also a fourth class of dorsal horn neuron which are proprioceptive in nature, responding to joint movement and pressure on deep tissues and, as such, are beyond the scope of this work.

Dorsal horn neurons can also be broadly divided into three categories based on the destination of their axon projections. These are; projecting neurons, which relay their input to higher brain centres, local circuit neurons and local interneurons. The vast majority of dorsal horn neurons are interneurons and can be excitatory, containing glutamate as a neurotransmitter, thereby relaying excitatory afferent inputs to projection neurons, or inhibitory, containing the neurotransmitters GABA and/or glycine. Inhibitory interneurons synapse both presynaptically on primary afferents and postsynaptically on dorsal horn neurons so that the majority of primary afferent input into the dorsal horn occurs via polysynaptic pathways involving interneurons. For example, neurons in lamina III/IV receive numerous synapses from axons of local inhibitory interneurons which contain GABA and neuropeptide Y (NPY) (Todd, 2002) and some lamina I projection neurons receive a selective and dense input from GABAergic cells suggesting that these cells, which convey noxious information to the brain, are under powerful inhibitory control (Puskar et al., 2001). This leads to modulation of incoming somatosensory information before it is relayed to higher centres and is thought to inhibit much of the sensory input, implicating dorsal horn interneurons in several theories of pain and analgesia (Basbaum and Fields, 1984;Bennett, 1994;Jessell and Iversen, 1977;Melzack and Wall, 1965). Dorsal horn neurons also receive

inputs from descending neurons from the brain, which can exert further modulatory influences on spinal sensory processing. For example, descending serotonergic axons from the medullary raphe nuclei innervate LI and LIII/IV projection neurons (Todd, 2002). However, the present study has not addressed of the role of these descending inhibitory pathways and therefore, further description of such interactions will be kept to a minimum (see 1.6.3).

a)



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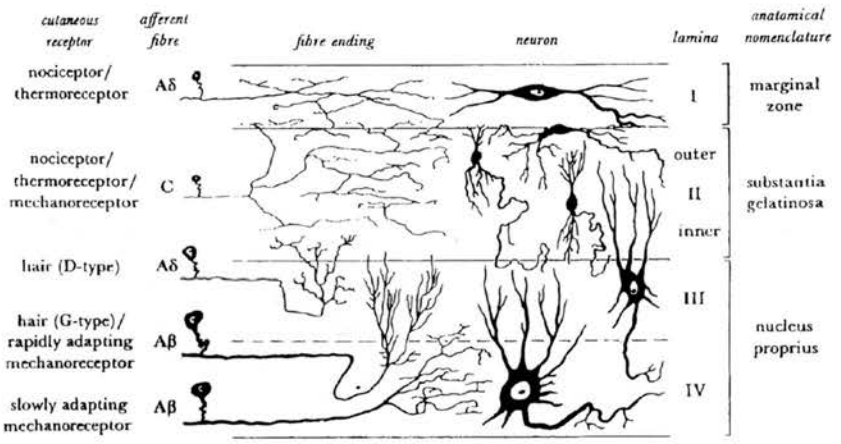


Figure 1. 1. Laminar organisation of the dorsal horn of the spinal cord and cutaneous afferent input to the spinal cord

a) Schematic representation of the laminar divisions of Rexed (Rexed, 1952) in the L5 segment of the rat spinal cord. (Molander et al., 1984) **Liss-** Lissauer's tract **IM-** intermedio-medial nucleus **Pyr-** pyramidal tract **VM-** ventro-medial nucleus **LSN-** lateral spinal nucleus **I-X-** Rexed's ten laminae. b) A hypothetical cross-section of the spinal dorsal horn illustrating the afferent fibres and neuronal endings in LI-IV. Rexed's (Rexed, 1952) laminar divisions are indicated on the right, with the afferent fibre types terminating in each region of the dorsal horn indicated on the left. Some neuron types are illustrated; a LI marginal cell, a LII limiting cell and two neurons of the nucleus proprius where dendrites of the LIII neuron illustrated projects into (Cervero and Iggo, 1980).

1.5 Sensory Projection Pathways

The projection cells of the spinal dorsal horn send their axons, via ascending tracts, to brainstem and midbrain regions, including thalamic nuclei and medullary structures. Reciprocally, these and other areas exert modulatory influences upon information processing in the spinal cord via descending control systems. Such inhibitory pathways contribute to endogenous analgesia systems, which will be briefly described below.

1.5.1 Ascending tracts

Spinothalamic Tract (STT)

STT cells of origin are located in LI, LII, LIV and LV from which information is carried to the contralateral side of dorsal horn and up the STT to the thalamic nuclei. In the rat, STT cells usually respond to noxious mechanical stimulation of the cutaneous receptive field, but they can also respond to innocuous mechanical stimulation and temperature (Giesler et al., 1976).

Spinomesencephalic Tract (SMT)

The SMT projects from cells located predominantly in LI, LV and LVI (Menetrey et al., 1982) to the mesencephalic tegmentum region including the PAG. A significant proportion of lamina I neurons contribute to the SMT many of which are likely to be nociceptive neurons (Menetrey et al., 1982).

Dorsal Column pathways

a) Postsynaptic Dorsal Column System (PSDC)

Axons of the PSDC originate in LIII/IV and project ipsilaterally through the dorsal funiculus to the nucleus gracilis and nucleus cuneatus of the medulla (Giesler et al., 1984). These cells are excited by innocuous hair movement, pressure, pinching and cooling as well as nociceptive stimuli (Angaut-Petit, 1975).

b) Spinocervical Tract (SCT):

SCT cells of origin are mostly located in the deeper laminae (LIV-LVII) and project ipsilaterally to the lateral funiculus (Cao et al., 1993). The SCT projects into the PSDC and horseradish peroxidase intracellular tracing has shown that a proportion of SCT cells also give rise to collaterals which project either to deeper regions in the grey matter, medially towards the central canal, across the mid-line in the ventral white commissure or in to the contralateral ascending ventral funiculus (Cao et al., 1993). The majority of SCT neurons

respond to tactile stimuli from hair follicles, however, many are multireceptive (Fleetwood-Walker et al., 1988) responding to innocuous stimuli as well as intense cutaneous pressure, pinch and thermal nociceptive stimulation (Cervero et al., 1979;Brown et al., 1983).

1.6 Endogenous Inhibitory Modulation

Endogenous systems involved in the modulation of pain were initially suggested by Melzack and Wall (Melzack and Wall, 1965) who proposed the 'Gate Control' theory of pain, in which spinal nociceptive transmission could be inhibited by non-nociceptive inputs. It is now clear that nociceptive processing is modulated not only via segmental control from inhibitory interneurons and activity in large diameter Aβ-fibres, but also by influences of descending pathways from supraspinal areas and endogenous analgesic compounds such as the cannabinoids. Together, these pathways influence activity in primary afferents, neurons of origin of ascending pathways and interneurons, providing a feedback system onto the dorsal horn.

1.6.1 Segmental control

Segmental control of spinal neurons to nociceptive stimulation is characteristically produced by cutaneous stimulation of large A-fibres, which can selectively inhibit C-fibre- and noxious stimulation-evoked excitation of dorsal horn neurons (Besson and Chaouch 1987;Woolf and Wall, 1982). Such inhibitory influences can be exerted on multireceptive and noci-specific dorsal horn neurons via the activation of spinal interneurons by primary afferent fibres, or indirectly by the activation of pathways descending from the brain involving the release of inhibitory transmitters such as the amino acids GABA and glycine and/or endogenous opioid peptides. In addition, monoamines such as serotonin and noradrenaline, are directly released by descending control systems to exert inhibitory effects (Besson and Chaouch, 1987).

GABA

GABA-like–immunoreactivity is shown by interneurons located predominately in LI-LIII (Barber et al., 1982;Todd and Sullivan, 1990). Ionophoretic application of GABA can inhibit dorsal horn neuron activity implicating GABA as a postsynaptic inhibitory neurotransmitter (Curtis et al., 1970;Zieglgansberger and Sutor, 1983).

Glycine

Glycine expression is found in LI-III of the spinal cord where it is often colocalised with GABA (Todd and Sullivan, 1990). Although thought of as an inhibitory neurotransmitter acting via the strychnine-sensitive glycine receptor (Budai et al., 1992), glycine can also exert an excitatory action via the *N*-methyl-D-aspartate (NMDA) receptor. However, ionophoretic application of glycine results in strong depression of dorsal horn neuron activity (Zieglgansberger and Sutor, 1983).

1.6.2 Endogenous opioids

Endogenous opioid peptides mimic narcotic analgesics in producing prolonged analgesic effects in animals. Opioid peptides such as enkephalin and dynorphin are present within synaptic terminals of the spinal dorsal horn (Cruz and Basbaum, 1985;Glazer and Basbaum, 1981;Willis and Coggeshall, 1991), such that they are highly associated with areas receiving small diameter afferent primary fibres (Cruz and Basbaum, 1985;Glazer and Basbaum, 1981), interneurons and in terminals synapsing with STT neurons, presumably providing the basis for an important inhibition of spinal nociceptive transmission (Ruda et al., 1984). The functional importance of this spinal modulatory system is well supported by the long-lasting analgesic effects seen in animals following the intrathecal administration of morphine (Yaksh and Reddy, 1981), which is further supported by studies in which ionophoretic application of opioids in the substantia gelatinosa produces strong inhibition of dorsal horn neuron responses to noxious stimulation (Fleetwood-Walker et al., 1988). Three members of the opioid receptor family were cloned in the 1990s, including the mouse delta-opioid receptor (DOR) (Evans et al., 1992;Kieffer et al., 1992), the mu-opioid receptor (MOR) (Chen et al., 1993;Thompson et al., 1993) and the kappa-opioid receptor (KOR) (Meng et al., 1993;Nishi et al., 1993). These receptors have been identified in the superficial dorsal horn of the spinal cord (Atweh and Kuhar, 1977) as well as on primary afferent terminals implying that their inhibitory action may occur via presynaptic sites or as an indirect action following activation of interneurons. All three receptors belong to the family of seven transmembrane G-protein coupled receptors, and share extensive structural homologies (Przewlocki and Przewlocka, 2001).

Various agonist and antagonist studies (Fleetwood-Walker et al., 1988;Gouarderes et al., 1996;Kalso et al., 1993;Sullivan et al., 1994) and more recently gene knockout models (for review see Kieffer and Gaveriaux-Ruff, 2002) have demonstrated a role for all three opioid receptors in the modulation of nociceptive processing in specific, yet distinct, areas of the spinal cord. Extracellular recordings made of the cutaneous sensory responses of SCT

neurons in the lumbar dorsal horn demonstrate that KOR agonists cause a selective reduction of the nociceptive responses of the neurons without effect on responses to innocuous stimuli (Fleetwood-Walker et al., 1988). When applied to the substantia gelatinosa, only MOR agonists showed a selective antinociceptive effect. Therefore, MORs and KORs can participate in the selective antinociceptive influence that opioids can exert over somatosensory information ascending to supraspinal levels, but at anatomically distinct sites.

The MOR 1 subunit is present on primary afferent axons and in a population of neurons in the superficial dorsal horn of the rat spinal cord, the majority of which are restricted to LII (Spike et al., 2002), where it is thought to play a role in opiate-induced analgesia via local inhibitory actions. 94% of MOR1 expressing cells do not express GABA or glycine, (Kemp et al., 1996) implying that most of the cells in the superficial dorsal horn which possess MOR1 are excitatory interneurons. Therefore, part of the action of MOR agonists, such as morphine, involves the inhibition of these excitatory interneurons interrupting the flow of nociceptive information through polysynaptic pathways in the spinal cord. Intrathecal administration of the morphine analogue DAMGO stimulates MOR internalization in LII neurons correlating precisely with the extent of analgesia produced (Trafton et al., 2000). However, it has been suggested that endogenous opioids do not initiate such MOR internalisation suggesting they may not act on interneurons but perhaps via presynaptic MORs or delta-opioid receptors (Trafton et al., 2000). However, this finding could be due to difficulties in detecting low levels of internalisation at such sites. Furthermore, noxious thermal stimulation of the hind paw induces c-Fos expression, indicative of activation, in approximately 15% of MOR-1 cells in the medial third of the ipsilateral dorsal horn at mid-lumbar level indicating postsynaptic activation of opioid responsive cells due to activation by nociceptive afferents (Spike et al., 2002). LII MOR expressing cells do not express NK_1 but receive many inputs from SP-containing afferents, the majority of which contain the mu-selective opioid peptide, endomorphin-2. Therefore, endomorphin-2 released from primary afferents is an endogenous ligand for pre- and postsynaptic MORs opposing the excitatory actions of co-localised SP, thereby serving as a major modulator of pain perception (Martin-Schild et al., 1998). This is supported by the fact that endomorphin-1 and -2 produce short acting, naloxone-sensitive antinociception in the tail flick test and inhibited the behaviour elicited by intrathecally injected substance P (Stone et al., 1997).

In general, the antinociceptive potency of opioids is greater against various noxious stimuli in animals with peripheral inflammation. Inflammation-induced enhancement of opioid antinociceptive potency is characteristic predominantly for MORs, since morphine elicits a greater increase in spinal potency of MORs than of DOR and KOR agonists (Hylden et al.,

1991). However, the reason for this is still relatively unknown as there are no apparent changes in receptor affinity or number (Cesselin et al., 1980;Stanfa and Dickenson, 1993).

1.6.3 Tonic descending modulation

Spinal nociceptive processing can also be modulated by descending pathways from supraspinal sites. Such systems can specifically modulate dorsal horn neuron responses to noxious input with little effect on innocuous input (Morton et al., 1983). Lesion studies have implicated the lateral reticular nuclei in the brainstem as sources of descending pathways (Foong and Duggan, 1986;Morton et al., 1983) and it is known that neurons in the medullary nucleus Raphe magnus (NRM) and adjacent structures of the rostral ventromedial medulla (RVM) are involved in descending control of nociceptive transmission. However, the exact origin of tonic descending inhibition is unclear. The NRM provides a major serotonergic input to the dorsal horn LI/II and V cells (Basbaum and Fields, 1978), electrical stimulation of which can produce analgesia to noxious stimuli (Guilbaud et al., 1977; Willis, 1977; Oliveras et al., 1974; Duggan and Griersmith, 1979). Electrical stimulation in the RVM produces a biphasic modulatory effect, showing facilitation at low intensities and inhibition at higher intensities (Calejesan et al., 2000). Fields and Heinricher (Fields and Heinricher, 1985) demonstrated two main classes of cell in the RVM namely; 'on-cells', which are constantly activated by noxious heat stimulation of almost anywhere on the body surface and 'off-cells', which are inhibited by the same stimulus (Hernandez and Vanegas, 2001).

Under mild noxious stimulation, RVM cells with inhibitory projections to the dorsal horn are not subject to strong GABAergic influence (Gilbert and Franklin, 2001) and are therefore capable of inhibiting spinal dorsal horn relay neurons. However, intense noxious peripheral stimulation may stimulate the release of GABA onto RVM cells, which in turn shuts off descending inhibitory fibres to allow transmission of nociceptor input through the dorsal horn (Gilbert and Franklin, 2001). The modulatory circuitry of the RVM may also be engaged physiologically in behaving animals via opioid-mediated activation of the RVM from higher brain centres such as the amygdala (McGaraughty and Heinricher, 2002), anterior cingulate cortex (Calejesan et al., 2000) synapsing via the periaqueductal gray (PAG). Such analgesia due to activation of 'off-cell' firing and depression of 'on-cells' has been demonstrated to involve the activation of both the DOR2 (Harasawa et al., 2000) and MORs (Wang and Wessendorf, 2002).

A similar division of cell type has been demonstrated in the parafascicular neurons (PF) of the thalamus (Zhang et al., 1997), which is modulated by ascending and descending influences of the locus coeruleus (LC) which provides noradrenergic input to the forebrain,

cerebellum, brainstem and spinal cord (Nygren, 1977). The LC exerts two different effects on nociceptive transmission via the PF; a predominantly inhibitory role on the nociceptive transmission at the spinal cord level by descending noradrenergic fibres, and a facilitatory role on the responsiveness of PF to noxious inputs by ascending fibres (Zhang et al., 1997). Therefore, descending modulation of nociceptive transmission in the spinal cord involves complex integration between various brain structures.

1.6.4 The endogenous cannabinoid system

An alternative spinal modulatory system that can exert selective antinociceptive effects is the endogenous cannabinoid system (Fox et al., 2001;Herzberg et al., 1997a;Richardson et al., 1998a). This is of particular interest to this study due to evidence that they may exert beneficial analgesic effects in chronic pain states including neuropathic pain and demyelinating diseases such as multiple sclerosis (Consroe et al., 1997;Robson, 2001).

Endocannabionoids

The endogenous cannabinoid receptor agonists, anandamide (arachidonylethanolamide) and 2-arachidonyl glycerol, were discovered in the 1990's. Both of these can act as neuromodulators or neurotransmitters, and are correspondingly synthesised by neurons and rapidly removed from the intercellular space following release (Di, V and Deutsch, 1998). Two types of cannabinoid receptor have been identified so far; CB_1 and CB_2 (Pertwee, 1997;Pertwee, 1998), both of which are coupled negatively to adenylate cyclase (AC) and positively to MAP kinase via $G_{i/o}$ proteins. These receptors have been shown to be present in the PNS and CNS, particularly in areas associated with nociceptive processing such as the superficial laminae of the spinal cord (Farquhar-Smith et al., 2000;Herkenham et al., 1991). CB_2 receptors are thought to be located mainly on peripheral immune cells whereas CB_1 receptors are found in particularly high concentrations within the CNS, on some peripheral neurons (Ahluwalia et al., 2000;Hohmann and Herkenham, 1999) and certain non-neuronal tissues. CB_1 receptors have also been reported to be located predominantly on spinal interneurons in the outer part of lamina II (Farquhar-Smith et al., 2000;Salio et al., 2002) and are also located on dorsal horn neurons of the spinal cord where they are likely to have an excitatory effect on neurotransmission due to closure of M-type potassium channels (Schweitzer, 2000). As well as this post-synaptic location, CB_1 receptors appear to be located both pre-synaptically on C-fibre and A-fibre primary afferent terminals where they probably modulate release of excitatory and inhibitory neurotransmitters. Autoradiographic studies of dorsal horn CB receptor binding in rats treated neonatally with capsaicin suggests

that only 16% of spinal CB receptors are located on C-fibre afferent endings (Hohmann and Herkenham, 1999) and in situ hybridisation studies have demonstrated the capability of predominantly medium and large sized neurons in the DRG to synthesise CB_1 receptors and insert them on primary afferent terminals (Hohmann and Herkenham, 1999).

A number of behavioural (Lichtman and Martin, 1997;Smith and Martin, 1992) and electrophysiological studies (Drew et al., 2000;Hohmann et al., 1998) have now demonstrated the ability of cannabinoids to inhibit acute nociception. It has been shown that under normal conditions, spinal CB receptors modulate C-fibre transmission and particularly facilitate post-discharge responses of dorsal horn neurons (Drew et al., 2000). Anandamide has been shown to inhibit capsaicin-evoked CGRP release into the dorsal horn of the spinal cord (Ramer et al., 1998) indicating a functional role for CB1 receptors located on C-fibre afferents presynaptically.

Understanding the role cannabinoids play in the modulation of nociceptive processing has been remarkably improved by the development of a variety of CB receptor agonists and antagonists. The mixed CB_1/CB_2 agonist, WIN55,212-2 has been shown to presynaptically inhibit GABAergic and glycinergic synaptic transmission in the superficial medullary DH (Jennings et al., 2001). Furthermore, putative selective CB_1 receptive antagonists such as SR141716A, at least partly block anti-nociceptive effects of spinally administered cannabinoid agonists (Drew et al., 2000) suggesting that they act predominantly via CB_1 receptors to modulate nociceptive processing.

Interactions of cannabinoids with other receptors

CB1 receptors are strongly colocalised with the vanilloid receptor, VR1, in sensory DRG (Ahluwalia et al., 2000). VR1's are pro-nociceptive receptors restricted to nociceptive afferents and mediate pain responses to noxious heat or changes in pH and are activated by the chilli pepper component, capsaicin. It is thought that the colocalisation of VR1 with CB1 may suggest VR1 is an endogenous target of anadamide (Di, V et al., 2000) and high concentrations of anadamide have been shown to excite central terminals of DRG afferent neurons via VR1 receptor activation (Tognetto et al., 2001). It has been suggested that such activation leads to desensitisation of the excitatory VR1 receptor producing antinociception (Iversen and Chapman, 2002). The relationship between the CB1 and VR1 receptors is an intriguing one, which is yet to be fully understood.

It is also possible that cannabinoids and opioids may interact in the control of nociception at the spinal level due to co-localization of CB1 and MORs in LII interneurons (Salio et al., 2001).

1.7 Neurotransmission from Primary Afferents

Stimulation of primary afferent fibres results in the release of a variety of neurotransmitters and neuropeptides from their nerve terminals in the spinal dorsal horn. These neurotransmitters and neuropeptides act upon dorsal horn neurons via their postsynaptic receptors. The major excitatory amino acid neurotransmitter from primary afferents is thought to be glutamate.

1.7.1 Glutamate

The excitatory amino acid, L-glutamate, appears to be the main neurotransmitter involved in primary afferent–mediated transmission. Electrical nerve stimulation causes glutamate release by terminals of both myelinated and unmyelinated axons (Davies et al., 1979;Duggan and Johnston, 1970) and correspondingly, activation of nociceptors causes glutamate release from nociceptive central terminals. Glutamate acts via a number of glutamate receptors which are divided into two main subtypes; ionotropic glutamate receptors, which are ligand-gated ion channels and metabotropic, G-protein linked glutamate receptor (mGluRs).

1.7.2 Ionotropic glutamate receptors

Based on their pharmacological response characteristics, the ionotropic receptors are subdivided into two main groups; N-methyl-D-aspartate (NMDA) receptors and non-NMDA receptors. The non-NMDA class of ligand-gated receptors are further subdivided into α-amino-3-hydroxy-5-methyl-4-isoxazole proprionic acid (AMPA) and kainate receptor subtypes. Glutamate produces a fast excitatory potential when acting at the AMPA receptor site and a long synaptic potential when acting at the NMDA receptor site.

High concentrations of AMPA receptor subunits have been demonstrated in neurons of the superficial laminae of the dorsal horn (Furuyama et al., 1993; Henley, Jenkins and Hunt, 1993; Tölle et al., 1993; Popratiloff, Weinberg and Rustioni, 1996) and NMDA receptors have been shown to be located throughout the brain and spinal cord particularly in the superficial dorsal horn (Greenamyre et al., 1985;Monaghan and Cotman, 1985). There is extensive evidence indicating a critical role of AMPA and NMDA receptors in spinal nociceptive processing, and in plastic changes in the spinal cord, which lead to persistent nociception (Coderre, 1993;Wilcox, 1991;Woolf and Thompson, 1991), such as in neuropathic pain. There is also compelling evidence to suggest that the NMDA receptor plays a crucial role in excitability changes such as long term potentiation (LTP)(Collingridge

et al., 1983;Morris et al., 1986) which is necessary for "spatial" learning and memory to occur in the hippocampus. Therefore, NMDA receptor dependent LTP is considered to be a possible parallel mechanism to that occurring in sensitisation of the spinal cord (Jia et al., 2001) and consequently the involvement of such mechanisms have been demonstrated in the generation of hyperalgesia (Dougherty et al., 1992;Zhou et al., 1996). Therefore, activation of the NMDA receptor is of particular relevance to this study and will be dealt with in more detail below (see 1.7.4).

1.7.3 Metabotropic glutamate receptors

The mGluRs are members of the super-family of seven transmembrane domain G protein-coupled receptors. Group I metabotropic receptors are concentrated in the superficial dorsal horn of the spinal cord (Berthele et al., 1999;Jia et al., 1999) and are of particular importance to nociception. This group includes mGluR1 and mGluR5, the activation of which leads to phosphoinositide hydrolysis, intracellular calcium (Ca^{2+}) mobilisation and possibly, to a lesser extent, increases in cyclic adenosine 3',5'-monophosphate (cAMP) accumulation (Abe et al., 1992;Aramori and Nakanishi, 1992), which all contribute to cell excitability.

Various roles for mGluRs in the induction of synaptic plasticity have been indicated. For example, activation of group I mGluRs in hippocampal slices of the CA1 region induces a form of long-term depression (LTD) of excitatory synaptic transmission which is thought to be via Ca^{2+} independent presynaptic mechanisms (Fitzjohn et al., 2001). There is also much evidence to suggest that group I mGluRs are involved in LTP at CA1 synapses within the hippocampus. Activation of class I-mGluRs can link them with intracellular kinases such as protein kinase C (PKC) and *src* which can phosphorylate and enhance the open probability of the NMDA receptor channel (Jia et al., 2001). This may lead to LTP involving the NMDAR, but not the AMPA receptor. However, evidence suggests that mGlu receptor-mediated potentiation of NMDA responses is not essential for the induction of LTP (Doherty et al., 2000) and that the role of mGluRs in the induction of LTP is fundamentally different from that of NMDA receptors (Bortolotto et al., 1994). NMDA receptors are ionotropic, and therefore, need to be activated each time a stimulus is delivered to initiate a linked molecular event involved in potentiation of the cells responsiveness. In contrast, mGluRs are coupled to G-proteins and therefore, a single activation can be sufficient activate a molecular switch negating the need for mGluR stimulation during the induction of LTP. This mGluR-activated switch is input-specific and can be turned off by a train of low-frequency stimulation (Bortolotto et al., 1994). Such a molecular switch has fundamental consequences for our understanding of synaptic plastic mechanisms such as those occurring in the spinal cord.

1.7.4 Neuropeptides

Various neuropeptides are synthesised in DRG neurons and are often co-released with glutamate from peripheral afferent terminals exerting a variety of synergistic effects on postsynaptic dorsal horn cells.

Substance P (SP)

SP is a member of the tachykinin family and is expressed by ~20% of DRG neurons which are mainly of small diameter (Hokfelt et al., 1975;Ju et al., 1987) When released, SP activates the NK_1 receptor located in the superficial dorsal horn of the spinal cord implicating it as a transmitter in primary sensory neurons.

In the rat, most SP-containing cells also contain CGRP and some also contain galanin (Battaglia and Rustioni, 1988;Ju et al., 1987). Functionally, SP and CGRP appear to facilitate transmission in the dorsal horn (Henry, 1978;Wiesenfeld-Hallin et al., 1991b) and it would appear that SP represents the main excitatory sensory peptide with a role in nociceptive transmission. Intrathecal injection of SP or an NK_1 receptor-selective agonist elicits biting and scratching behaviours in mice that may be indicative of pain sensation (Courteix et al., 1993;Hylden and Wilcox, 1981). However, NK_1 receptor antagonists are generally not effective at reducing dorsal horn neuron responses to brief noxious stimuli (Fleetwood-Walker et al., 1990;Yamamoto and Yaksh, 1991) and NK_1 receptor antagonists have not proved to be efficacious in clinical trials of chronic pain states (Hill, 2001) although this finding and the use of NK_1 receptor antagonists may be dependent upon the model of pain used.

Calcitonin gene-related peptide (CGRP)

CGRP is normally expressed by approximately 50% of DRG neurons with unmyelinated axons, as well as one-fifth of Aδ DRG neurons with myelinated axons (McCarthy and Lawson, 1990;Rosenfeld et al., 1983). Noxious thermal and mechanical stimulation of primary afferents causes CGRP release into LI, LII and LV of the spinal cord (Lee et al., 1985;Morton and Hutchison, 1989) where it plays an excitatory role. This is supported by reports that intrathecal application of CGRP decreases the nociceptive threshold for acute mechanical stimulation (Oku et al., 1987). Furthermore, CGRP plays a modulatory role in excitatory transmission to the spinal cord by inhibiting SP endopeptidase, potentiating the effect of co-released SP (Mao et al., 1992).

Vasoactive intestinal polypeptide (VIP)

Under normal conditions, VIP expression is seen only at very low levels in DRG neurons and in LI and LII neurons of the spinal dorsal horn (Gibson et al., 1981;Knyihar-Csillik et al., 1991). VIP activates the receptors $VPAC_1$, $VPAC_2$ and to a lesser extent PAC_1 (Hosoya et al., 1997;Ishihara et al., 1992). VIP generally appears to become more important following nerve injury and therefore will be discussed further below (see 1.12.1).

Pituitary adenylate cyclase-activating polypeptide (PACAP)

PACAP is a peptide similar in action to VIP as it is also normally present in low abundance in the DRG (Nahin et al., 1994;Zhang et al., 1995) and is a further agonist at $VPAC_1$, $VPAC_2$ and PAC_1, the latter of which displays a much higher affinity for PACAP as compared to VIP (Hashimoto et al., 1993;Shivers et al., 1991). Furthermore, PACAP is also thought to play a more significant role in somatosensory transmission following peripheral nerve injury, following which it is upregulated in the DRG cells (Noguchi et al., 1989;Zhang et al., 1996).

Galanin

Galanin is a peptide normally expressed at very low levels in sensory and sympathetic neurons and co-exists with several other neurotransmitters including CGRP and SP (Ju et al., 1987;Ma and Bisby, 1997). Galanin has widespread CNS distribution and can be found concentrated in LI-III of the dorsal horn, with moderate expression in IV-V and primary afferent terminals (Kar and Quirion, 1995). Galanin may act centrally as a modulator of excitatory peptides supported by evidence that intrathecal pre-administration of galanin antagonises the excitatory effects of SP and CGRP on the flexor withdrawal reflex (Wiesenfeld-Hallin et al., 1991b;Xu et al., 1990) and inhibits the analgesic effect of morphine on noxious thermal and mechanical stimuli (Wiesenfeld-Hallin et al., 1991a), whilst producing no effect alone on nociceptive inputs. As with VIP, the main actions of galanin in the PNS sensory transmission seem to occur following peripheral nerve injury and therefore will be discussed further below (see 1.12.2).

Neuropeptide Y (NPY)

NPY is not normally expressed by DRG neurons in normal animals (Lundberg et al., 1983). However, there is a high density of NPY-positive nerve fibres present in the superficial laminae of the dorsal horn arising from local cell populations or supraspinal sites (Wakisaka et al., 1991) where NPY may co-exist with galanin or GABA (Laing et al., 1994;Zhang et al., 1993). The functional role of NPY in the spinal cord is unclear, as at low doses intrathecal administration of NPY appears to be excitatory whereas at high doses, it produces inhibitory actions (Scherer et al., 1994). Again, the main actions of NPY of interest to this study occur following peripheral nerve injury and will therefore be dealt with further below.

Somatostatin (SOM)

SOM is a peptide expressed by small diameter primary afferent neurons which do not express SP (Hokfelt et al., 1976;Nagy and Hunt, 1982) and is thought to act, at least in part, by opposing the action of (SP) in neurogenic inflammation. In situ hybridisation has demonstrated SOM expression to be restricted to approximately 10% of lumbar DRG neurons of the rat all of which are intensely IB_4-labeled neurons and express the GNDF receptor (Kashiba et al., 2001). Application of GDNF to the rat dorsal horn of the spinal cord promotes activity-induced release of SOM from central terminals of sensory neurons (Lever and Malcangio, 2002) and GDNF induced inhibition of intrathecal SP-induced thermal hypersensitivity was reversed by the SOM antagonist (Lever and Malcangio, 2002). SOM expression in the spinal cord is restricted to LII (Hokfelt et al., 1976), however, its role in nociceptive processing is unclear with reports of both inhibitory and excitatory actions on dorsal horn neurons (Salt and Hill, 1983;Murase et al., 1982;Randic and Miletic, 1978). Intrathecal SOM application has been reported to increase the excitability of the spinal cord neurons following mechanical and thermal stimuli (Wiesenfeld-Hallin, 1985). In contrast, administration of somatostatin to the spinal cord or brain areas involved in nociception has been shown to result in analgesia and somatostatin sst2(a) receptors have been shown to be expressed by neurons closely associated with brain areas involved in analgesia and the modulation of nociception (Schindler et al., 1998). This inhibitory action of SOM especially in inflammatory pain, is supported by the fact that SOM receptor (SSTR) agonists produce analgesia following intrathecal or epidural administration in humans and that activation of peripheral SSTRs in the rat reduces both inflammatory pain and the activity of sensitized nociceptors (Carlton et al., 2001).

1.8 Chronic Pain

Chronic pain is currently a major health care problem throughout the world with reports of 14% of the UK population (Munglani et al., 1999) and 15-20% the USA population (Rudin, 2001) experiencing significant chronic pain. Common sources of chronic pain are cancer, inflammatory disorders, headache, low back pain, arthritic pain and neurogenic pain, which is defined as pain that arises from the nervous system (IASP press, 1994). Unfortunately, effective long-term therapies to treat chronic pain syndromes are limited in efficacy, making it a huge socio-economic problem.

1.9 Neuropathic Pain

Neuropathic pain is a common form of chronic pain resulting from a primary lesion or dysfunction in the nervous system (IASP Press 1994). Whether of central or peripheral origin, neuropathic pain is characterised by a neuronal hyperexcitability in damaged areas of the nervous system.

Peripheral neuropathic pain results from damage to the peripheral nerves, which may arise from polyneuropathy, entrapment neuropathy, or as a result of disease such as herpes zoster (Mohamed, Carr, 1994) or systemic illnesses such as diabetes, infection with human immunodeficiency virus (HIV) (Hewitt et al., 1997) or nutritional deficiencies. Although the causes of peripheral neuropathy are diverse, associated symptoms are consistent and include weakness, numbness, paraesthesias (abnormal sensations such as burning, tickling, pricking or tingling) and pain (Scadding, 1981). Such pain typically manifests as spontaneous, stimulus-independent pain and/or stimulus-evoked pain, which is typically characterised by the abnormal pain states of tactile allodynia; pain due to a stimulus which does not normally provoke pain (IASP Press 1994), and hyperalgesia; the increased response to a stimulus which is normally painful (IASP Press 1994) (Woolf et al., 1998).

1.10 Animal Models of Neuropathic Pain

Unravelling the mechanisms involved in neuropathic pain requires the use of laboratory animal models that replicate as far as possible, the different pathophysiological changes present in patients. For reasons of reproducibility and simplicity, most studies of neuropathic pain are based upon animal models of traumatic nerve injury, usually in the rat sciatic nerve (Boucher and McMahon, 2001). It is unlikely that any single animal model will include the full range of neuropathic pain mechanisms and therefore, several such models exist, employing a variety of nerve injury-inducing techniques. In the most frequently used models, a mixture of intact and injured fibres is created by loose ligation of either the whole sciatic nerve, known as a chronic constriction injury (CCI) (Bennett and Xie, 1988) or a tight ligation of a part of the sciatic nerve, known as partial sciatic nerve ligation injury (PSNL) (Seltzer et al., 1990). More recently a variant of partial denervation has been developed called the spared nerve injury model (Decosterd and Woolf, 2000), involving a lesion of two of the three terminal branches of the sciatic nerve (tibial and common peroneal nerves) leaving the remaining sural nerve intact. Therefore, in this model the co-mingling of distal intact axons with degenerating axons is restricted, permitting behavioural testing of the non-injured skin territories adjacent to the denervated areas. Probably the most commonly used model today involves the tight ligation of one or two spinal nerves just distal to the DRG, known as spinal nerve ligation (SNL) (Kim and Chung, 1992). This results in an all or none axotomy of DRG neurons within relevant ganglia. In all these animal models pronounced sensory changes are seen which are similar to those observed in many patients with neuropathic pain. Such changes are paralleled in the animal by behaviour indicative of such pain including guarding and avoidance of weight bearing on the appropriate paw, and escape behaviour to very light tactile stimuli, consistent with the development of allodynia. However, all of these models share the common feature of damage to, and degeneration of, some sensory fibres in a major somatosensory nerve.

1.11 Introduction to Mechanisms Underlying Neuropathic Pain

In peripheral neuropathic pain, damaged nerves exhibit abnormal spontaneous and/or increased evoked activity. Such hyperexcitability is thought to occur as a result of a series of molecular changes at the level of the peripheral nociceptor and in the DRG, which are thought to lead to similar molecular changes in the dorsal horn of the spinal cord. These changes include abnormal expression of Na^+ channel subtypes, changes in neuropeptide complement, increased activity in glutamate receptor sites, changes in GABA-ergic inhibition, and an alteration of calcium influx into cells. The next section will discuss some of these changes in more detail.

1.12 Changes in the Peripheral Nervous System

Sensory communication depends on sustained, yet controlled firing in afferents and subsequent pathways. Following injury to the nerve, primary afferent fibres may become sensitised to peripheral sensory stimulation, decreasing their threshold for activation and thereby producing an increased rate of firing to previously subthreshold stimuli. Perhaps more importantly for the generation of neuropathic pain is the generation of impulses at abnormal locations along the axon of sensory neurons (Devor and Seltzer, 1999). Such 'ectopic' activity can emerge due to various components of the injury, such as an area of demyelination (Baker and Bostock, 1992;Calvin et al., 1982;Rasminsky, 1978) or afferent sprouting and neuroma formation (Devor and Dubner, 1988). As a result, the damaged peripheral nerve acquires an ongoing, 'spontaneous' discharge. In addition to firing spontaneously, such ectopic pacemaker sites are often excited by mechanical forces applied to them during movement, resulting in spontaneous and movement-evoked pain (Devor and Seltzer, 1999). Spontaneous discharge can also occur in the DRG neurons, causing transmission not only to the central nervous system, but also antidromically towards the peripheral terminal, which can lead to further excitation of the peripheral axon (Kim et al., 1998;Xie et al., 1995).

1.12.1 Membrane remodelling

Repetitive firing capability depends on the distribution and gating properties of specific transmembrane ion channels (Devor and Seltzer, 1999). Nerve injury leads to the remodelling of the neuronal membrane including changes in expression and location of voltage-gated Na^+ and potassium ion (K^+) channels, which are responsible for afferent hyperexcitability.

Na^+ channels

High-density clusters of voltage-gated Na^+ channels are expressed at the nodes of Ranvier in myelinated fibres of the PNS where they are essential for saltatory propagation of action potentials. Following injury to the nerve, Na^+ channels are known to accumulate along the axon injury site (Devor and Seltzer, 1999), in aborted and regenerating sprouts and along regions of demyelination (Devor et al., 1989;Novakovic et al., 1998), rendering these sites as hyperexcitable (Matzner and Devor, 1992;Matzner and Devor, 1994;Omana-Zapata et al., 1997). Furthermore, expression of Na^+ channels in neurons of the DRG is highly dynamic, changing in various disease states, including some that are accompanied by pain (Waxman, 1999), making them important targets for the study of the molecular pathophysiology of pain.

In the normal state, DRG neurons express a complex repertoire of Na^+ channel transcripts (Waxman et al., 1999b), which are distinguished by their sensitivity to tetrodotoxin (TTX) (Black and Waxman, 1996). Some of these transcripts, such as TTX-resistant Na^+ channels SNS 1/Na_v1.8 (Akopian et al., 1996) and SNS 2/Na_v1.9 (Tate et al., 1998) are known to be specifically expressed by sensory neurons and are therefore implicated in pain states (Akopian et al., 1996;Novakovic et al., 1998). SNS 1/Na_v1.8, which is of particular interest to this study, has been shown to be expressed in approximately 50% of small diameter unmyelinated (Amaya et al., 2000) and in approximately 20% of medium to large diameter myelinated cells of the DRG, whereas SNS 2/Na_v1.9 is expressed only in neurons with unmyelinated axons (Amaya et al., 2000). Another Na^+ channel of interest to this study is the brain type III Na^+ channel/Na_v1.3, which is normally only found in DRG during development. However, following axotomy (Waxman et al., 1994b) and CCI (Dib-Hajj et al., 1999), changes are seen in the expression of such Na^+ channels resulting in changes in the properties of the Na^+ current. Specifically, the expression of type III/Na_v1.3 is upregulated in sensory neurons (Black et al., 1999) whereas, the two known TTX-resistant

channels in DRG neurons, SNS 1/Na_v1.8 and SNS 2/Na_v1.9 are downregulated (Decosterd et al., 2002;Waxman et al., 1999a).

The SNS class of Na^+ channel produce slowly inactivating currents (Akopian et al., 1996;Waxman et al., 2000a), therefore their decreased expression in DRG neurons may lead to a hyperpolarizing shift in resting potential, increasing the fraction of TTX-sensitive channels available for activation (Dib-Hajj et al., 1999;Waxman et al., 2000). Upregulation of the type III/Na_v1.3 Na^+ channel results in a switch in the properties of the TTX-sensitive currents in DRG neurons, with the emergence of a rapidly repriming current (Waxman et al., 1999c), which could sustain frequent ectopic discharges (Cummins and Waxman, 1997). Therefore, DRG neurons that express type III/Na_v 1.3 should be able to sustain higher firing frequencies, which may lead to hyperexcitability in the cell. The involvement of SNS 1/Na_v1.8 in the production of neuropathic pain is controversial. Partial knock down of the SNS 1/Na_v1.8 protein following spinal nerve injury via intrathecal administration of specific antisense oligodeoxynucleotides has been shown to reduce the slow-inactivating, TTX-resistant Na^+ current in DRG cells of animals and reverse neuropathic pain without effect on non-noxious sensation or response to acute pain (Lai et al., 2002). However, studies in SNS 1/Na_v1.8 knockout mice reveal that neuropathic pain behaviours are unchanged (Kerr et al., 2001), whereas earlier work suggests that the mutant demonstrates a pronounced analgesia to noxious mechanical stimulation with little deficit in thermoreception (Akopian et al., 1999). Studies have also argued against a role for the TTX-resistant Na^+ channels in the generation ectopic activity in injured primary sensory neurons (Dib-Hajj et al., 1999;Sleeper et al., 2000). Following partial nerve injuries, ectopic discharges are found in intact as well as injured neurons (Chan et al., 2001;Hanus et al., 1999;Liu et al., 2000a). However, it would appear that changes in TTX-resistant Na^+ channels are restricted to cells with injured afferents (Decosterd et al., 2002) making it unlikely that they contribute to ectopic activity recorded in uninjured afferents. However, activation of intracellular kinase pathways following inflammation lead to changes in the phosphorylation state of the SNS 1/Na_v1.8 channel which have been shown to alter the threshold and kinetic properties of the channel altering excitability contributing to sensitisation of the periphery (Gold et al., 1998;Khasar et al., 1999). This implies a mechanism by which the channel could lead to hyperexcitability following nerve injury in the neurons in which expression remains. Unfortunately, due to the lack of specific antagonists to the individual Na^+ channel subtypes (Wood and Baker, 2001), the significance of the role each plays has been hard to determine.

Ephaptic crosstalk

Cross-excitation, allowing excitatory interactions between afferents and among neurons can result in heightened activity following damage to the peripheral nerve. Normally, a single primary sensory neuron constitutes an independent signal conduction pathway. However, in the event of neuronal injury, an important form of cross-excitation in the PNS can occur, resulting in ephaptic (electrical) crosstalk between axons. Ephapsis can occur in neuromas, regenerating nerve and at patches of demyelination (Rasminsky, 1978) and is thought to result from the close apposition of adjacent axons in the absence of their normal insulation provided by Schwann cell myelination (Rasminsky, 1978). Electrically-coupled fibres are frequently of different types, thereby ephaptic crosstalk may cause normally nociceptive afferent neurons to be driven by electrical activity in low threshold afferents or even efferents (Devor and Seltzer, 1999) allowing innocuous stimuli or even movement to result in activity in the nociceptive pathway. This sort of cross excitation may account for changes in excitability of uninjured afferents that cannot be explained by ion channel changes alone.

As well as the changes in the peripheral nerve itself, a number of changes occur in the DRG and the spinal cord, which are thought to contribute to the initial development and then the maintenance of neuropathic pain (for reviews see Woolf, 1993;Woolf and Costigan, 1999).

1.12.2 Neuropeptide plasticity

Following traumatic injury to the peripheral nerve, a number of neurochemical and morphological changes may occur in peripheral nerve fibres, as well as centrally within the spinal cord (Dray et al., 1994;Hokfelt et al., 1994; Ma & Bisby, 1998). These are thought to contribute to altered sensory transmission, such as that associated with chronic pain states. A key event is the characteristic phenotypic changes that occur in many primary afferent neurons, reflected in the altered expression of neuropeptides in their cell bodies in the DRG (Hokfelt et al., 1994;Villar et al., 1991) and in their terminals and relevant receptors within the spinal dorsal horn (Dickinson and Fleetwood-Walker, 1999). For example, it is known that the expression of CGRP is downregulated following peripheral nerve injuries such as axotomy and CCI (Dumoulin et al., 1992;Noguchi et al., 1990) with a similar change observed in the expression of SP (Jessell et al., 1979;Nielsch et al., 1987), although there is evidence to suggest that SP may be upregulated in larger Aβ-fibre cell bodies (Malcangio et al., 2000), however the significance of such a change is controversial due to doubts regarding techniques identifying termination patterns of primary afferents in the dorsal horn following nerve injury (Bao et al., 2002) (see 1.13.3). In contrast, galanin is strongly upregulated

mainly in small and medium diameter neurons (Hokfelt et al., 1987;Villar et al., 1989), especially those that normally contain substance P and CGRP (Doughty et al., 1991;Kashiba et al., 1992), the majority of which are unmyelinated. Studies of galanin knockout mice have suggested that this upregulation may be important for the development of central sensitisation (Wynick et al., 2001) and intrathecal application of galanin has been shown to increase behavioural reflex sensitivity in naïve animals (Kerr et al., 2000). Similarly in large diameter primary sensory neurons, distinguished by the presence of the 200kDa subunit of neurofilament protein (NF-200) (Kashiba et al., 1994;Marchand et al., 1999), there is a marked increase in the expression of NPY after axotomy (Kashiba et al., 1994;Wakisaka et al., 1991), partial sciatic injury (Ma and Bisby, 1998b), CCI of the sciatic nerve (Munglani et al., 1995;Nahin et al., 1994) and following streptozocin-induced diabetic neuropathy (Rittenhouse et al., 1996). Levels of VIP and PACAP are also markedly increased predominantly in small to medium- sized neurons, especially those that normally contain SP and CGRP (Kashiba et al., 1992;Xu et al., 1990;Zhang et al., 1995) and it is thought that perhaps the upregulation of these latter peptides could reflect their capacity as mediators of sensory transmission in neuropathic pain states. Due to the timing of their changes in expression, it is thought that PACAP may be important in the regulation of the onset of the neuropathic pain state within the first 1-4 days, before pain behaviours have developed, whereas the more slowly rising levels of VIP, which peak at around 14 days following injury when pain behaviours have fully developed, may be more important in the maintenance of more prolonged pain states.

This phenotypic change may be primarily protective and restorative in the sense of attenuating the input and therefore the pro-nociceptive influence of SP and CGRP, in dorsal horn and by the trophic actions of VIP following injury (Hokfelt et al., 1994). However, VIP also causes enhanced transmission to the dorsal horn of the spinal cord (Wiesenfeld-Hallin et al., 1990) and is therefore likely to contribute to the induction of a sensitised state, which may underlie the development of chronic pain.

1.13 Changes in the Central Nervous System

1.13.1 Introduction to central sensitisation

Spontaneous discharge arising in some primary afferents following nerve injury provide constant input to the CNS, and thus may induce functional changes in the sensory processing mechanisms within the dorsal horn creating a phenomenon referred to as 'central sensitisation'. Central sensitisation can manifest in three ways: enlargement of the peripheral

receptive field of a neuron; increased response to a subthreshold input; and action potential discharge initiated by previously subthreshold inputs. These changes manifest as hypersensitivity to pain that spreads from the site of injury causing primary and secondary hyperalgesia, which can include tactile Aβ-fibre-mediated mechanical allodynia (Gracely et al., 1992;Woolf CJ, 1997).

When induced in response to tissue damage or peripheral sensitisation, central sensitisation can have a survival value as it helps to protect injured areas from further injury whilst healing proceeds. However, when occurring as a result of nerve injury, the pain endured persists even once healing processes are complete and therefore offers no such protective advantage. The persistence of neuropathic pain is thought to be due to structural reorganisation of the synaptic circuitry in the nervous system, which may include cell death (Coggeshall et al., 1997;Coggeshall et al., 2001), degeneration or atrophy of axon terminals (Castro-Lopes et al., 1990;Knyihar-Csillik and Rakic, 1987), appearance of new axon terminals (Okamoto et al., 2001) and structural modification of synapses (Doubell and Mannion, 1999). These structural changes within the dorsal horn may relate to a persistent change in sensory processing long after healing of the initial injury.

Underlying this functional plasticity are activity-dependent changes in synaptic efficacy within the spinal dorsal horn. In a variety of models of chronic pain, it is thought that mechanical allodynia and thermal hyperalgesia are characteristically underpinned by changes in NMDA receptor activation in the spinal dorsal horn, which, in a similar fashion to its involvement in LTP (Collingridge et al., 1983;Morris et al., 1986), is considered to be one of the key factors underlying central sensitisation (Chaplan et al., 1997;Dickenson and Sullivan, 1987;Woolf and Costigan, 1999).

1.13.2 NMDA-receptor dependent central sensitisation

There is extensive evidence indicating that ionotropic glutamate receptors such as the AMPA receptor (Nozaki-Taguchi and Yaksh, 2002) and especially the NMDA receptor, play a critical role in plastic changes underlying spinal nociceptive processing, which can lead to persistent nociception (Quartaroli et al., 2001;Wilcox, 1991;Woolf and Thompson, 1991) and much work has gone into developing NMDA receptor blocking drugs for the treatment of persistent pain states (Chizh et al., 2001;Parsons, 2001;Weinbroum and Ben Abraham, 2001).

At normal resting membrane potential, the NMDA receptor contributes relatively little to primary afferent-evoked synaptic currents in dorsal horn neurons due to voltage-dependent

block of the receptor ion channel by a magnesium ion. However, increased primary afferent input in response to injury or ectopic pacemaker activity, results in the increased release of glutamate and colocalised neuropeptides such as SP and neurokinin A. Via the activation of their various G-protein coupled metabotropic receptors, these neurotransmitters induce stimulation of intracellular PKC (Bleakman et al., 1992;Bond and Lodge, 1995) and potentially other kinases such as tyrosine kinases (Chen and Huang, 1992), which may mediate the phosphorylation of the NMDA receptor and facilitation of its activity. Such phosphorylation leads to changes in the receptor binding kinetics for the magnesium ion causing it to dissociate from the receptor at resting membrane potential. Consequently, pre-synaptically released glutamate activates the NMDA receptor, inducing an influx of calcium into the cell. Calcium entering the cell leads to many intracellular changes which may be amplified by depolarisation-induced voltage-sensitive calcium channel activation and calcium-induced release of calcium from intracellular stores. This increase in intracellular calcium causes the activation of kinases, such as PKC (Malmberg et al., 1997;Munro FE et al., 1994) and Ca^{2+}/calmodulin-dependent protein kinase II (CaMIIK) (Bortolotto and Collingridge, 1998;Silva et al., 1992), which have been shown to play significant roles in LTP and persistent pain states. Inputs from other receptors (and to a lesser extent Ca^{2+} elevation) may increase production of the intracellular second messenger; cyclic AMP (cAMP), a pathway capable of activating protein kinase A (PKA), which is also implicated in LTP and in spinal sensitisation (Cerne et al., 1992).

1.13.3 The importance of myelinated primary afferents in central sensitisation

The abnormal pain states of hyperalgesia and allodynia are thought to arise due to a combination of changes in different cell types; hyperalgesia is thought to arise due to abnormal processing of nociceptor input into the spinal cord, whereas, allodynia may be produced by the action of low threshold Aβ-afferents on an altered nervous system (Koltzenburg, 1998;Torebjork et al., 1992;Woolf, 1997) and/or by the reduction in the threshold of nociceptor terminals in the periphery.

In many circumstances, it is clear that abnormal activity in nociceptors, particularly C-fibre nociceptors, is capable of inducing central sensitisation (Coderre, 1993). However, evidence has begun to highlight the importance of activity in myelinated primary afferents in the induction of central changes underlying persistent pain states, which is of particular relevance to this study and has until recently been poorly explored in the context of neuropathic pain. For example, following L5 SNL, spontaneous activity arises almost exclusively in myelinated afferents for the time in which neuropathic pain develops and

becomes established (two weeks) (Bennett et al., 2000;Liu et al., 2000a), suggesting that ectopic activity in myelinated A-fibres may be sufficient to trigger tactile allodynia in some models of peripheral neuropathy (Liu et al., 2000a). This is further supported by evidence that infusion of local anaesthetic onto the L5 DRG after SNL blocks ectopic discharges and findings have indicated this reverses the hyperalgesia (Liu et al., 2000a), suggesting that activity in damaged Aβ fibres may maintain central sensitisation.

Activity in Aβ-fibres may also be capable of activating nociceptive primary afferents in areas of allodynia in a manner consistent with a pre-synaptic interaction (Garcia-Anoveros et al., 2001). Such dorsal root reflexes produced by a central pre-synaptic interaction of Aβ-fibres may conduct centrally, evoking allodynia, and peripherally, evoking neurogenic vasodilatation which may contribute to hyperalgesia as confirmed by studies of cutaneous blood flow following electrical stimulation of the sural nerve in a range that excites Aβ-fibres (Garcia-Nicas et al., 2001). Furthermore, input from non-noxious inputs via Aβ-fibres has been shown to enhance and extend the duration of previously developed secondary hyperalgesia via stimulation of undamaged areas of peripheral tissue (Kim et al., 2001b). This provides further support for the capability of large myelinated fibres to powerfully modulate central sensitisation in the dorsal horn.

Although in some clinical situations, Aβ-fibre mediated mechanical allodynia requires continual input to maintain central hyperexcitability, the involvement of proposed mechanisms such as phenotypic alterations and Aβ-fibre sprouting (see below) may result in Aβ-fibre mediated pain that is independent of a constant nociceptive drive (Woolf and Mannion, 1999).

Structural reorganisation: A-fibre sprouting and cell death

Central terminals of A-fibres occur in all laminae of the dorsal horn apart from lamina II of the superficial dorsal horn, which receives input exclusively from C-fibre nociceptors. However, following peripheral nerve injury, there may be induction of sprouting of Aβ-fibre central terminals into Lamina II (Lekan et al., 1996;Woolf et al., 1992). This may allow activity in innocuous mechanoreceptors to activate nociceptive second order neurons, which may lead to misinterpretation of the information by the nervous system. Therefore, A-fibre sprouting may be an anatomical substrate for mechanical allodynia (Woolf and Mannion, 1999). However, strong doubts have now emerged with regard to the degree of sprouting as assessed by the anterograde labelling technique using the cholera toxin B subunit (Tong et al., 1999) which has been used extensively in studies of A-fibre sprouting (Woolf et al., 1992). A recent study has demonstrated that that the cholera toxin B subunit also labels

unmyelinated, C-fibre afferents in LII and that there is little sprouting of Aβ-fibres into superficial laminae (Bao et al., 2002). Nonetheless, irrespective of the suitability of this method, it appears that Aβ-fibres gain the ability to excite monosynaptic neurons in superficial laminae after peripheral nerve damage (Kohama et al., 2000). Whole cell electrophysiological investigations have also demonstrated that following sciatic nerve transection, large myelinated A-afferent fibres establish synaptic contact with interneurons and transmit innocuous information to the substantia gelatinosa, representing a functional reorganization of the sensory circuitry which may constitute an underlying mechanism for associated sensory abnormalities (Okamoto et al., 2001).

Increased afferent drive may also lead to excitotoxicity due to excessive synaptic glutamate release, which is a feature of many neurological conditions in which neuronal death occurs. Primary afferent A-fibre activity, releasing glutamate into the dorsal horn, can induce neuronal cell death especially in areas of Aβ-fibre termination, the anatomical distribution of which depends on whether intact or injured fibres are activated (Coggeshall et al., 2001). Such stimulation-induced cell death may potentially contribute to the development of persistent pain. For example, it is known that peripheral nerve injury can lead to GABAergic cell loss, which coincides with the development of behavioural sensitivities (Ibuki et al., 1997). A role for such loss in enhanced sensitivity associated with neuropathic pain has been demonstrated with intrathecal injections of the glycine receptor antagonist, strychnine, and the GABA A receptor antagonist, bicuculline, both of which enhance thermal hyperalgesia following CCI (Yamamoto and Yaksh, 1993), reduce the cutaneous mechanical threshold, and increase the response to Aβ-inputs (Sivilotti and Woolf, 1994). This indicates that a decrease in the efficacy of spinal inhibitory circuits may contribute to the touch-evoked allodynia that occurs in pain hypersensitive states, where Aβ-inputs begin to produce pain.

Although sciatic nerve lesions do not actually result in detectable neuron loss in the dorsal horn, activation of Aβ-fibres in a previously sectioned sciatic nerve can cause substantial cell death not only in lamina III but also in laminae I and II, areas in which Aβ-fibre afferents do not normally terminate (Coggeshall et al., 2001). The expansion of the territory of Aβ-fibre afferent-evoked cell death is likely to reflect the sprouting of the fibres into these laminae after peripheral nerve injury.

Phenotypic changes

A-fibres may also contribute to central sensitisation following injury due to phenotypic changes including the upregulation of potential neuromodulators such as SP, brain-derived

neurotrophic factor (BDNF) and galanin. SP and CGRP are peptides normally expressed by nociceptor primary afferents and are strongly implicated in nociceptive transmission to the central nervous system. As mentioned above, following peripheral nerve injury the expression of these peptides in small afferents appears to be downregulated. However, following L5 SNL, large myelinated Aβ-fibres begin to express these neuropeptides (Miki et al., 1998) and stimulation of Aβ-fibres releases SP from primary afferent terminals (Malcangio et al., 2000). In inflammatory states, SP is thought to contribute to allodynic-like responses (Ma and Woolf, 1997), therefore this release may be involved in the generation of a central hyperexcitable state (Woolf and Mannion, 1999). Furthermore, following injury Aβ-fibres express BDNF (Michael et al., 1999;Zhou et al., 1999), which has also been shown to modulate dorsal horn excitability, playing a significant role in inflammation-induced hyperalgesia (Akopian et al., 1999;Mannion et al., 1999). Therefore, it is feasible that injury-induced phenotype changes in these fast conducting myelinated fibres render them capable of sensitising the CNS.

1.13.4 Spinal pathways in neuropathic pain

Although changes in local inhibitory spinal activity, such as the loss of spinal activity of GABA, have been shown to contribute to spinal nerve ligation-induced allodynia and hyperalgesia which is reversed by exogenous administration of GABA agonists (Malan et al., 2002), it is likely that tactile allodynia and thermal hyperalgesia involve separate spinal pathways. Complete and partial spinal cord lesions block allodynia, but not hyperalgesia, after SNL (Ossipov et al., 2000;Sun et al., 2001). Furthermore, lidocaine microinjected into dorsal column nuclei block only tactile allodynia (Sun et al., 2001). Therefore, tactile allodynia after peripheral nerve injury may be more dependent upon inputs to supraspinal sites whereas, thermal hyperalgesia is likely mediated predominantly through local spinal circuitry (Ossipov et al., 2000). This indicates that the interpretation that tactile allodynia following peripheral nerve injury is transmitted to the central nervous system by means of large diameter, myelinated fibres, which run on the ascending tracts further supporting the role for myelinated fibres in the transmission of neuropathic pain phenomena.

Abnormal activity in descending somatosensory pathways may also represent a mechanism of chronic pain (Kovelowski et al., 2000). For example loss of specific RVM cells, which project to spinal loci to inhibit or facilitate pain transmission, both prevents and reverses experimental neuropathic pain respectively (Porreca et al., 2001).

1.13.5 Opioids in neuropathic pain

A role for opioid peptides in the maintenance of persistent pain is supported by many clinical case reports which suggest that sustained opioid exposure can elicit unexpected, paradoxical pain and by the fact that subcutaneous delivery of morphine in the rat induces tactile allodynia and thermal hyperalgesia (i.e. 'opioid-induced pain') (Vanderah et al., 2001). Such opioid-induced pain may occur via interactions of descending pathways such as via tonic activation of bulbospinal facilitation from the RVM (Vanderah et al., 2001) or via local spinal opioid release. For example, abnormal, spontaneous afferent drive may also activate spinal dynorphin (Tang et al., 2000) which may play a role in the induction of abnormal pain states. Studies in prodynorphin knock-out mice suggest an early, dynorphin-independent phase of neuropathic pain followed by a later dynorphin-dependent stage (Wang et al., 2001). Correspondingly, antiserum to dynorphin given spinally blocks thermal hyperalgesia, after SNL, and also restores diminished morphine antinociception (Vanderah et al., 2000). This suggests that spinal dynorphin promotes abnormal pain and acts to reduce the antinociceptive efficacy of spinal opioids (i.e. tolerance). The data also identify a possible mechanism for previously unexplained clinical observations of the actions of administered

opioids and offer a novel approach for the development of strategies that could improve the long-term use of opioids for pain which have so far produced controversial results, especially in the treatment of neuropathic pain (Rowbotham, 2001).

1.14 Additional Factors that may Contribute to the Development of Neuropathic Pain

1.14.1 Neurotrophic factors: *friend or foe?*

Neurotrophic factors are molecules that promote survival, growth and maintenance of discrete populations of neurons. Some of these factors, such as the neurotrophins, are absolutely essential for normal neuronal development. The neurotrophin family includes NGF, BDNF, NT-3 and NT-4/5 which support the survival and growth of a distinct group of sensory neurons (Patel et al., 2000;Snider and Wright, 1996), determined by the specific expression of their relevant receptors, which include *trkA* (NGF), *trkB* (BDNF and NT4/5) and *trkC* (NT-3) and the p75 receptor, which is a low affinity receptor capable of binding all neuroptrophins. The neurotrophins also appear to regulate the development of normal functional properties of sensory neurons, such as their ability to respond to peripheral stimuli (Carroll et al., 1998).

A second family of neurotrophic factors important for survival of and normal function in sensory neurons, are the glial cell line-derived neurotrophic factor (GDNF) family of secreted proteins. These proteins are structurally related to GDNF and currently include GDNF, neurturin, artemin and persephin. They have particularly important effects on primary sensory neurons and all signal through *ret*, a transmembrane tyrosine kinase which forms complexes with one of four known binding proteins of the GDNF family receptor (GFR)α1-4 which confers specificity of action of the different GDNF family members.

Due to the expression of the relevant receptors, nociceptive afferents are likely to be sensitive to either NGF of GDNF, whilst large diameter mechanosensitive afferents are likely to be sensitive in particular to NT-3 and GDNF (Boucher and McMahon, 2001).

As discussed above, the nerve damage that precipitates neuropathic pain leads to many changes in sensory neurons, including alterations in putative neurotransmitters/modulators, receptors, ion channels, structural proteins and anatomical terminations. The relative contribution of each of these to neuropathic pain is currently unknown, however, neurotrophic factors have been shown to prevent several of these axotomy-induced changes (Bennett et al., 1998;Verge et al., 1995). Experimental evidence has implicated GDNF as playing a particularly important role as continuous intrathecal infusion with GDNF during partial sciatic nerve ligation or L5 SNL prevents the development of pain-associated

behaviours with no effect of NGF or NT-3 infusion (Bennett et al., 2000). Furthermore, following L5 SNL, spontaneous activity arising almost exclusively in myelinated afferents in the period of development and establishment of the neuropathic pain state (two weeks), is reduced following GDNF treatment (Bennett et al., 2000). In line with this, GDNF treatment reverses changes in Na^+ channel expression such that expression of type III/$Na_v1.3$ is completely suppressed paralleled by partial rescue of expression levels of SNS 1/$Na_v1.8$ and SNS 2/$Na_v1.9$ (Bennett et al., 2000). This suggests that perhaps a decrease in production of GDNF following nerve damage could contribute to the development of such hyperexcitability leading to central sensitisation and that increasing the local supply of GDNF may help to minimise the neuropathic pain.

However, the upregulation of various other neurotrophic factors following nerve injury may contribute to changes underlying increased excitability, enhancing the development of a neuropathic pain state. For example, synthesis of the leukemia inhibitory factor neurotrophic factor (LIF), is increased in lesioned peripheral nerves and has been shown to increase the expression of galanin in axotomized DRG neurons (Ozturk and Tonge, 2001). BDNF, which modulates dorsal horn excitability, playing a significant role in inflammation-induced hyperalgesia, has also been shown to increase in the uninjured L4 DRG neurons following SNL (Fukuoka et al., 2001) and in Aβ afferents following nerve injury (Michael et al., 1999) (Akopian et al., 1999;Mannion et al., 1999). NGF has also been shown to increase after SNL and evidence supports a role for both BDNF and NGF in the development of thermal hyperalgesia (Fukuoka et al., 2001). However, evidence also suggests that the reduction in levels of target-derived NGF in sympathetic and sensory neurons after axotomy is partly responsible for the subsequent changes in neuropeptide expression associated with nerve injury such as the decrease in SP and CGRP and upregulation of galanin and VIP (Shadiack et al., 2001). These apparently conflicting data suggest that a precise balance of neurotrophic factors is required for normal function in the nervous system. Therefore, dynamic alterations in the expression and content of BDNF, NGF and LIF in the injured and uninjured DRG neurons might be involved in the pathomechanisms of neuropathic pain.

The effects of growth factors in persistent pain states will be discussed further in relation to Schwann cell changes in Section C.

1.14.2 The sympathetic nervous system

There is little evidence for electrical communication between postganglionic sympathetic neurons and primary afferent neurons in the normal peripheral nervous system. However, in many cases of clinically severe and intractable neuropathic pain, the symptoms are

sympathetically maintained. Following nerve injury, such as in the CCI and SNL models of neuropathic pain, sympathetic axons sprout into the DRG from the injured nerve (Chung and Chung, 2001) with a time course that parallels the development of behavioural sensitivity changes (Ramer and Bisby, 1997). This may be an anatomical substrate for the sympathetic maintenance of pain, which may lead to sensitisation of neurons involved in chronic pain states (Janig, 1995;Ramer and Bisby, 1997). In support of this, guanethidine sympathectomy following CCI can reduce the heightened sensitivity, which develops in response to cold and heat (45°C). However, there is no modulation of any spontaneous activity or of increased sensitivity to mechanical stimulation (Attal et al., 1990;Perrot et al., 1993) implying that the sympathetic nervous system may only play a restricted role in the development and maintenance neuropathic pain. In recent years, there have been various mechanisms hypothesised to underlie the anatomical nature of nerve injury-induced sympathetic-sensory coupling. Several studies have been undertaken to determine what factor or factors are responsible for the sprouting, and for the formation of abnormal sympathetic terminal arborizations or 'baskets' around some DRG neurons (for review see) (Ramer et al., 1999). The details of such factors are beyond the scope of this study but include roles of NGF, BDNF (Deng et al., 2000;Zhang et al., 1998), and the cytokines; leukemia inhibitory factor (LIF) (Thompson and Majithia, 1998) and interleukin-6 (IL-6) (Ramer and Bisby, 1998), which may all be derived from the periphery as a result of Wallerian degeneration. A role of such factors and satellite cells (Shinder et al., 1999) within axotomized DRG, has also been suggested in which LIF or IL-6 may activate satellite cells in the DRG to bind neurotrophins to surface receptors for NGF which can then act as a sympathetic axon-guiding gradient by encouraging them to sprout into the DRG (Ramer et al., 1999).

1.14.3 A possible role of NFκB in neuropathic pain

Nuclear factor kappa B (NFκB) is a sequence-specific DNA binding protein/ transcription factor, which serves as a transducer between extracellular signals and gene expression. NFκB is known to exist in an inducible form in a wide range of eukaryotic cells, including neurons and glia throughout the PNS and CNS (Kaltschmidt et al., 1993) and is known to be a crucial signal for glial and neuronal cell function during inflammation injury and neural plasticity (see review) (O'Neill and Kaltschmidt, 1997). In unstimulated cells, NFκB exists in a latent form in the cell cytoplasm as a heterodimer most commonly composed of the subunits p50 and p65, which form a complex with an endogenous inhibitory protein of the IκB family. Extracellular signals such as cytokines and neurotrophins can activate NFκB via their receptors on the cell surface, phosphorylating IκB and releasing the bound NFκB,

thereby allowing translocation of the active form of NFκB to the nucleus. NFκB becomes able to specifically bind to consensus target sequences leading to the modulation of expression of the NFκB-regulated array of genes. Immunohistochemical studies have demonstrated the expression of NFκB in 30-45% of neurons of all sizes, particularly those of medium to large diameter, in L4 and L5 ganglia in the rat (Doyle and Hunt, 1997);(Ma and Bisby, 1998a), suggesting that NFκB is involved in intracellular signalling in sensory neurons under physiological conditions. Peripheral nerve injuries, especially those with the involvement of demyelination or Wallerian degeneration, can lead to production of inflammatory mediators by local Schwann cells and invading macrophages including cytokines, such as tumour necrosis factor (TNF) (Wagner and Myers, 1996), interleukin-1 (IL-1) (Lindholm et al., 1987) and neurotrophins such as NGF (Herzberg et al., 1997a). These factors have all have been shown to activate NFκB in various cell types in the PNS and CNS (Carter et al., 1996;Moynagh et al., 1994;Wood, 1995) and could therefore initiate the induction of multiple gene expression in DRG and spinal cord cells which may lead to events involved in the development of chronic pain. There are also various ways in which NFκB could be activated as a downstream event of cellular changes involved in neuropathic pain. A role for NMDA and non-NMDA ionotropic glutamate receptors has been demonstrated in the activation of NFκB in striatal and cerebellar neurons in the mammalian brain (Guerrini et al., 1997;Kaltschmidt et al., 1995;Nakai et al., 2000) and could therefore provide a further mechanism for the activation of NFκB in the spinal cord in which the NMDA receptor is known to play a crucial role in neuropathic pain, as discussed previously (see 1.13.2). Furthermore, it has been suggested that protein kinases such as PKA (Mosialos and Gilmore, 1993;Neumann et al., 1995) and PKCγ (Leitges et al., 2001;Shirakawa and Mizel, 1989) are capable of activating NFκB both of which have been implicated in persistent pain states (Cerne et al., 1993;Malmberg et al., 1997).

1.15 Clinical Treatments

The treatment of neuropathic pain conditions is currently extremely poor. The effectiveness of classical analgesics such as the opioids is controversial and have been reported to be poorly efficacious in many neuropathic circumstances (Bennett, 1994). However, opiods are currently used to treat many neuropathic conditions in the clinic and produce varying degrees of analgesia (for review see Rowbotham, 2001). However, they also produce a large number of detrimental side effects. A number of other recognised medications used in the management of chronic pain, such as the cyclo-oxygenase-targeting non-steroidal anti-

inflammatory drugs (NSAIDs) also have a relatively minor and highly variable effect on neuropathic pain.

The main problem experienced with current pharmacological agents used to combat neuropathic pain is associated side effects. For example, NMDA receptor antagonists, such as dextromethorphan or ketamine, initially appeared promising as therapeutic agents, however, toxicity, low safety margins and psychotropic side effects have limited the use of such drugs.

Anticonvulsant drugs such as carbamazepine and phenytoin have been investigated for the treatment of neuropathic pain due to the similarity of some aspects of the neuronal hyperexcitability with the electrical changes in certain forms of epilepsy (Backonja, 2000;Sindrup and Jensen, 2002). Studies have shown these agents can relieve painful diabetic neuropathy and paroxysmal attacks in trigeminal neuralgia (Charlton, 1993;McCleane, 1999;Zakrzewska., 1992). Furthermore, lamotrigine, a new anticonvulsant, probably acting by stabilising a slow inactivated conformation of a Na^+ channel and suppressing release of glutamate from presynaptic neurons (McNamara et al., 1996), is also effective in trigeminal neuralgia (Lunardi et al., 1997;Solaro et al., 2000) as well as painful peripheral neuropathy, and post-stoke pain (for review see Backonja, 2002). However, anticonvulsants have adverse effects including sedation and cerebellar symptoms, such as nystagmus, tremor and un-coordination and less commonly, haematological changes, and cardiac arrhythmias (Jensen, 2002).

Gabapentin, developed as a structural analogue for the GABA system has also been extensively studied for the treatment of pain conditions. The exact mechanism of gabapentin action is unknown as it has no actual effect on GABA receptors (Jensen et al., 2002;Lanneau et al., 2001) or any influence on GABA uptake. Several randomised pain studies in humans with postherpetic neuralgia (PHN) (Rice and Maton, 2001;Rowbotham et al., 1998) and painful diabetic neuropathy (Backonja, 2000;Morello et al., 1999) have indicated a potential for gabapentin in pain relief. Furthermore, a role for gabapentin in pain relief has been demonstrated when administered intrathecally in animal models of PHN (Takasaki et al., 2002) and following SNL (Cho et al., 2002). However, due to the difficulties associated with human studies (varied population size, patient age, and differential diagnoses) the results have been contradictory with some finding gabapentin to be no more useful than placebo (Gorson et al., 1999;Hemstreet and Lapointe, 2001). A recent study has suggested a facilitatory role for gabapentin in the efficacy of morphine in reducing signs of neuropathic pain following SNL (Matthews and Dickenson, 2002) suggesting that perhaps combined

regimes of drug therapy are likely to be a lot more efficacious. However, as with many other tried and tested drugs for pain relief, gabapentin does produce side effects such as dizziness, headache, peripheral oedema and sedation (Bosnjak et al., 2002;Rowbotham et al., 1998) and therefore, is still not the ideal drug therapy.

1.15.1 An endogenous analgesia target: the cannabinoid system

As mentioned previously, the endogenous cannabinoid system has received much attention of late due to its capacity to exert selective antinociceptive effects in both neuropathic and inflammatory hyperalgesia (Fox et al., 2001;Herzberg et al., 1997;Richardson et al., 1998b). Cannabinoids may exert beneficial analgesic effects in demyelinating diseases, such as multiple sclerosis (Consroe et al., 1997;Robson, 2001) and are currently under assessment as clinical therapeutic agents for this disorder (Robson, 2001). There is considerable evidence supporting a role for cannabinoids in the modulation of nociception and they have been shown to be effective in reversing hyperalgesia and touch evoked pain behaviour in animal models of inflammatory (Martin et al., 1999;Richardson et al., 1998a) and neuropathic pain (Bridges et al., 2001;Fox et al., 2001;Herzberg et al., 1997;Kim and Chung 1992). The majority of these effects appear to be mediated by the CB_1 cannabinoid receptors (Richardson et al., 1998c). However, a possible role of the CB_2 receptors as targets for the treatment of chronic pain conditions has recently been indicated (Malan, Jr. et al., 2001) and due to the peripheral location of these receptors, such treatment may avoid CNS complications associated with CB_1 receptor targeting.

The discovery of the involvement of the 'endogenous cannabinoid system' in relieving pain phenomena associated with chronic pain disorders, has led to the development of selective CB_1 and CB_2 receptor ligands, which may represent a valuable alternative strategy for the relief of chronic pain. However, the search for adequate analgesics to treat all types of neuropathic pain continues.

1.16. Peripheral Nerve Myelin

The myelin sheath is a multilamellar structure with a high lipid to protein ratio (80:20) which serves as an insulator of nerve fibres, reducing current flow across the internodal axonal membrane and permitting saltatory conduction of nerve impulses at the nodes (Scherer, 1999). Myelin is present in the CNS where it is produced by oligodendrocytes and in the PNS, where it is produced by Schwann cells. For the purpose of this study, only the latter will be considered.

1.16.1 Peripheral nerve myelination

The Schwann cell is the major glial cell of the vertebrate PNS, where it not only myelinates nerve fibres promoting rapid nerve impulse transmission but also has significant secondary roles providing trophic support for spinal motoneurons and DRG neurons (Riethmacher et al., 1997) and in promoting nerve regeneration in the PNS (Bunge, 1993;Zhang et al., 2002). During the development of the PNS, Schwann cell precursors arise from the neural crest, migrate and contact peripheral axons. These 'immature' Schwann cells invade and ensheath bundles of developing axons, at which stage they further differentiate into myelinating or non-myelinating Schwann cells (Webster, 1993). During this time, some Schwann cells establish a one-to-one association with an axon (promyelination stage), which is necessary for myelination to proceed. The thickness of the myelin sheath (Aguayo et al., 1977;Yin et al., 1998) appears to be directly related to the axon (Friede and Miyagishi, 1972) such that large diameter axons possess thicker myelin sheaths and thus a greater number of membrane turns of the sheath, implying that axons may regulate the action of Schwann cells and influence the process of myelination (Bunge et al., 1986).

To myelinate the axon, cytoplasm-filled processes extending from the myelinating Schwann cell align along and rotate around axons forming the internodal segments of the myelin sheath. These segments are divided by the nodes of Ranvier, which are relatively small (<1 μm in length), regularly spaced, unmyelinated regions of the axon (Morell et al., 1994). The myelin sheath can be divided into two domains; compact myelin, which forms the bulk of the myelin sheath and non-compact myelin (see Figure 2.1). Compact myelin is formed as the cytoplasm of the Schwann cell process is extruded, bringing adjacent cytoplasmic and extracellular membrane surfaces into apposition, resulting in the formation of a mature, compact myelin sheath. This sheath is largely composed of lipids, mainly cholesterol and sphingolipids (Arroyo and Scherer, 2000), but also contains a unique set of proteins including protein zero (P0), myelin basic protein (MBP) and peripheral myelin protein-22

kDa (PMP22) (Lemke, 1992;Lemke, 1993) which appear to be essential for normal myelination (see Figure 2.1). Non-compact myelin is found at the nodes of Ranvier and in Schmidt-Lanternman incisures, which form cytoplasmic channels through the myelin sheath (see Figure 2.2).

Adjacent to the node is the paranodal region at which the lateral edge of the compact myelin opens along the major dense line (MDL) -forming cytoplasm-filled lateral loops (paranodal loops), which spirals round the axon, forming the axo-glial junction (Ichimura and Ellisman, 1991). Here, structures known as transverse bands span the periaxonal space, apparently attaching the glial cell membrane to the axolemma allowing a close axo-glial association which probably impedes the passage of ions between the nodal region and periaxonal space facilitating saltatory conduction.

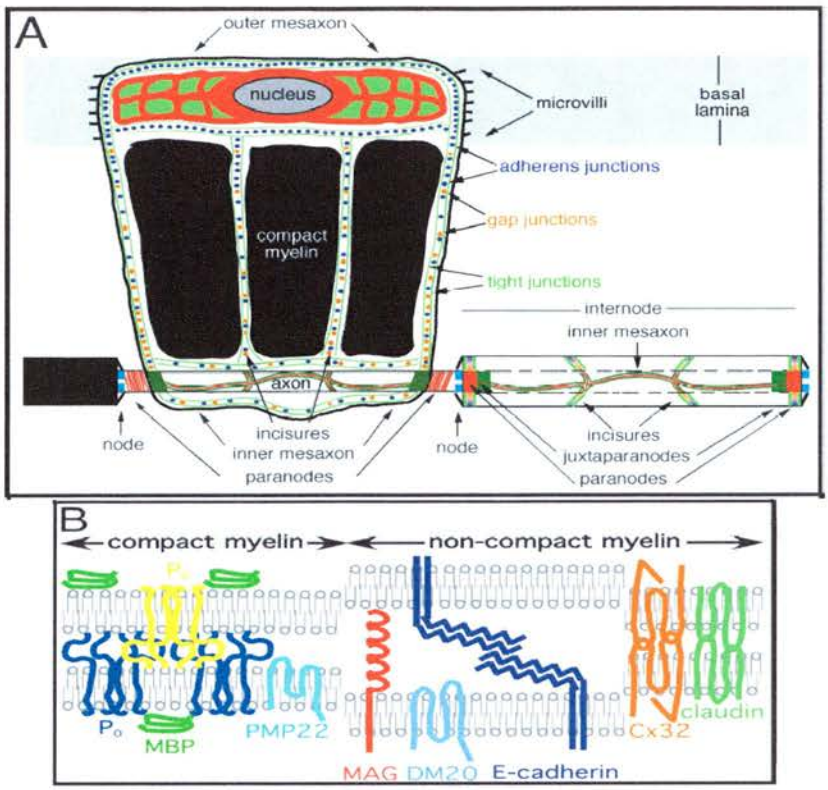


Figure 2.1. A) The organisation of the myelinated axon. An 'unrolled' Scwann cell, revealing the regions that form compact and non-compact myelin. The nodal, paranodal and juxtaparanodal regions are coloured red, green and blue respectively. B) **The proteins of compact and non-compact myelin.** (adapted from Arroyo and Scherer, 2002).

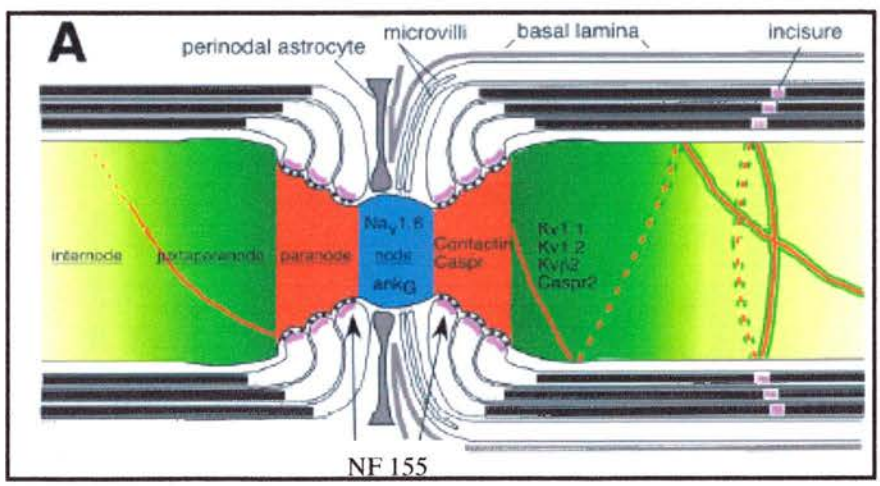


Figure 2.2. Schematic depiction of the node, paranode and juxtaparanode in the PNS myelin sheath. Na^+ channels are clustered in the nodal area, whereas K^+ channels accumulate at the juxtraparanodal region. This distribution allows saltatory conduction of action potentials to occur (Arroyo and Scherer, 2002).

1.17 The Role of Peripheral Myelination in Primary Afferent Function

1.17.1 Axon-glial iteractions

There are many ways in which the axon is thought to directly influence the process of myelination by interactions with the Schwann cell and myelin sheath. For example, Schwann cell proliferation and differentiation into ensheathing or myelinating type (Poduslo and Jang, 1984;Weinberg and Spencer, 1975), determination of myelin segment length, myelin thickness and basal lamina production (Bunge et al., 1986) are all important aspects of Schwann cell function known to be directly dependent upon axonal contact. Once completed, the maintenance of myelination depends on continued Schwann cell-axon interactions (Kamholz et al., 2000) as does the integrity, function and survival of the both myelinated and unmyelinated axons (Haney et al., 1999). It is this relationship, which is of particular interest to this study.

It is only in recent years that some of the molecules involved in the mediation of axonal-glial interactions have been identified. For example, myelin-associated glycoprotein (MAG), a member of the immunoglobulin gene super-family of cell adhesion molecules, and myelin galactolipids are both glial components implicated in mediating axo-glial interactions during the myelination process (Salzer et al., 1990), supported by various mutant mouse studies (Bosio et al., 1996;Coetzee et al., 1996;Dupree et al., 1998;Marcus et al., 2002). Various other molecules, such as ankyrin$_G$, neurofascin 155, Caspr1 and Caspr2, have recently been localised to the nodal and paranodal region, implicating them in axon-glial interactions especially in the regulation of ion channel localisation. The details of such proteins are beyond the scope of this study, however, a brief summary of their role in axo-glial interactions will be summarised below.

Voltage-gated sodium channel localisation

Myelinated nerves of the PNS express high-density clusters of voltage-gated Na^+ channels (1000's per μm^2) at the node, where their focal positioning allows for rapid, saltatory propagation of the action potential (Salzer, 1997). The nodal localisation of these channels is thought to be dependent on myelination (Dugandzija-Novakovic et al., 1995;Novakovic et al., 1996).

In the CNS and PNS, nodal microvilli contain molecules such as F-actin and cell adhesion molecules (CAMs), which may form complexes with axonal proteins to govern the clustering of voltage-gated Na^+ channels at the node. Ankyrin$_G$, located in the axolemma, is

known to interact with voltage-gated Na^+ channels, anchoring them to the nodal cytoskeleton (Davis et al., 1993; Kordeli et al., 1990). Also localised to the paranodal axolemma is the intrinsic membrane glycoprotein, contactin-associated protein (Caspr1), also known as paranodin (Dupree et al., 1999;Einheber et al., 1997;Peles et al., 1997). Caspr1 can bind to the actin cytoskeleton, indicating it may play a role in assembling signalling pathways within the axon (Arroyo and Scherer, 2000). In the Schwann cell paranodal glial loops, Neurofascin 155 interacts with contactin and Caspr1/paranodin to form septate-like junctions (Charles et al., 2002). As clusters of these proteins appear before $ankyrin_G$ and Na^+ channel clusters at the node in the development of the PNS (Lambert et al., 1997), it would seem likely that they may play a role in the mediation of their nodal localisation which is thought to be intricately related to the structure of the overlying myelin sheath (Arroyo and Scherer, 2000).

Voltage-gated potassium channel localisation

The Shaker-related potassium channels; $K_v1.1$, $K_v1.2$ and their associated β2 subunit, are concentrated in the region on the internodal side of the paranode; the juxtaparanode (Rasband et al., 1998;Vabnick and Shrager, 1998). These channels have been shown to contribute to rapid axonal membrane repolarisation during development (Vabnick et al., 1999) and are therefore thought to have an important physiological function in dampening the excitability of myelinated fibres, preventing repetitive firing due to re-excitation of myelinated fibres (Chiu et al., 1999;Zhou et al., 1998). The juxtaparanodal axonal membrane contains a second member of the Caspr family of proteins, Caspr2 with which $K_v1.1$, $K_v1.2$ are precisely colocalised (Menichella et al., 2001;Poliak et al., 1999). Therefore, it would appear that the Caspr family members differentiate myelinated axons distinct functional subdomains.

Therefore, an important symbiotic relationship exists between the myelin sheath and the axon, ensuring the restricted localisation of specific proteins that are essential for co-ordinated and controlled activity in the peripheral nerve axon and therefore the normal function of the peripheral nerves.

However, when this crucial relationship is disrupted (such as in peripheral nerve demyelinating disorders, which involve areas of damaged or dysfunctional myelin sheath, leading to demyelinated segments), abnormalities can develop which can lead to a dysfunctional neural function.

1.18. Peripheral Nerve Demyelination and Neuropathic Pain

Abnormal sensory phenomena including spontaneous pain, hyperalgesia and allodynia may be associated with human demyelinating neuropathies such as Multiple Sclerosis (MS), Charcot-Marie-Tooth types I and 4 (CMT1 & CMT4) and Guillain-Barré Syndrome (GBS) (see 1.22). MS is a chronic autoimmune demyelinating disease of the CNS (Pender, 2000), which involves central pain in approximately two-thirds of patients at some time during the course of their disease (Moulin, 1998;Nurmikko, 2000), the further discussion of which is beyond the scope of this study. CMT 1 is a slowly progressive, autosomal dominant, peripheral nerve demyelinating disorder characterised by diffuse muscle weakness and severe motor deficit as well as sensory deficits which, although usually less severe, may include pain (Carter et al., 1998). Similarly, GBS, often regarded as a predominantly motor neuropathy with fewer sensory features, can also have painful symptoms (Asbury, 1990). The next section will discuss possible mechanisms occurring following demyelination of the peripheral nerve that may lead to the development of neuropathic pain.

1.19 Proposed Mechanisms of Neuropathic Pain Following Demyelination

It is likely that pain and excessive sensitivity to touch in diseases characterised by segmental demyelination (particularly where there is no evidence for axonopathic changes) originate due to the presence of ectopic repetitive firing (Rasminsky, 1981;Tal and Eliav, 1996). Demyelinated segments can serve as foci for spontaneous multiple action potential generation (Baker and Bostock, 1992;Smith and McDonald, 1980) and the fact that there is good evidence that tactile allodynia is mediated by myelinated A-fibres (Bennett et al., 1998;Coggeshall et al., 1997) suggests that demyelination of A-fibres could lead directly to such abnormal phenomena. Nevertheless, the origin of tactile allodynia and neuropathic pain in human demyelinating disorders is still poorly understood (Carter et al., 1998;Rasminsky, 1981).

1.20 Changes to The Nerve Following Demyelination

1.20.1 Membrane remodelling

Demyelination of axons may initially result in partial or complete conduction block, decreasing or abolishing the capacity of the nerve to transmit action potentials to and from the CNS. This is thought to account for the negative sensory symptoms experienced, such as motor dysfunction and sensory loss (Rasminsky, 1981). However, secondary reorganisation of membrane electrical properties involving reorganisation of ion channels to allow action potential generation across the demyelinated region may occur to overcome this conduction block.

Voltage-gated Na^+ channels

Following peripheral nerve demyelination, Na^+ channel clusters lose their normal nodal localisation and appear throughout the internodal segments of demyelinated axons. It appears that that these channels are being newly recruited to these locations (England et al., 1990;Novakovic et al., 1996) and are not simply uncovered or relocated from the node (England et al., 1991). This would imply an increase in their synthesis within the cell body with subsequent transport down the axon to these new locations. Although this change in location is primarily a response of the nerve to recover conduction, areas of ectopic impulse generation are likely to form, leading to hyperexcitability in the demyelinated nerve.

Voltage-gated K^+ channels

Demyelination of the peripheral nerve also results in the delocalisation of K^+ channels to both nodal and paranodal regions (Rasband et al., 1998). Therefore, it is likely that their functional influence is considerably altered which may add to changes in excitability of the nerve.

1.21 Axon-Glial Interactions

1.21.1 Structural changes

The molecular abnormalities underlying heritable demyelinating neuropathies are generally intrinsic to proteins expressed by Schwann cells. However, most clinical manifestations are likely to result from changes in the properties of the axon (Griffin and Sheikh, 1999). Therefore, interactions between Schwann cells and axons appear to be crucial for the normal functioning of axons. A number of studies indicate that such Schwann cell-axonal interactions are disrupted in peripheral demyelinating neuropathies, such as CMT 1, causing significant changes to the nerve (Kamholz et al., 2000). Various studies utilising mouse models of PNS hypomyelination, have also demonstrated significant changes in axonal structure and function following disruption of the myelin sheath including decreased neurofilament and microtubule protein phosphorylation (de Waegh et al., 1992;Kirkpatrick and Brady, 1994) with resulting increased neurofilament density (de Waegh et al., 1992) and decreased axonal transport (de Waegh and Brady, 1990). These results indicate that the presence of a myelinated internode has important effects on the neurofilamentous cytoskeleton. Similar changes have been found in patients with CMT 1 (Watson et al., 1994) and grafting experiments show that this change is induced by abnormal Schwann cell contact (Aguayo et al., 1977). Furthermore, it is thought that the same principles of Schwann cell-axon contact may apply to the CNS (Brady et al., 1999), resulting in similar axonal changes in MS, which in later stages displays axonal interruption in areas of demyelination (Griffin and Sheikh, 1999). Unfortunately, as yet, the nature of the glial cell-derived signal influencing neurofilament phosphorylation, packing density, and axonal transport is not known.

Changes to the cytoskeleton of axons may have consequences significant for the development of neuropathic pain states. For example, targeting of axonal proteins such as voltage-gated ion channels (Brady, 1993;de Waegh et al., 1992) to their correct location may be dependent on an intact cytoskeleton. Furthermore, neurotrophic factors such as NGF often reach the afferent cell bodies in the DRG via retrograde axonal transport from peripheral target tissues which, when disrupted, as occurs following axonal damage, is thought to play a role in the changing expression of neuropeptides in the DRG such as VIP and NPY(McMahon and Priestley, 1995) (Verge et al., 1995). It seems plausible that changes in axonal transport due to demyelination may have similar effects.

1.21.2 Schwann cell-derived neurotrophic factors

Following injury to the peripheral nerve, Schwann cells often assist the axonal regeneration and restoration of function by secreting various factors, including neurotrophic factors (Ramer et al., 1999), in a manner often mimicking development. However, many of these factors may have additional negative functional consequences when upregulated and may contribute to mechanisms involved in the production of pain, such as influencing the phenotype of DRG neurons (Jongsma et al., 2001;McMahon and Priestley, 1995), sprouting of sympathetic afferents into the DRG (Ramer et al., 1999) and sprouting of regenerating afferent axons in to abnormal locations or neuromas (Kryger et al., 2001). Furthermore, any undamaged afferents are subsequently exposed to abnormal levels of neurotrophic factors, which may influence their function.

Neurotrophic factors produced by Schwann cells following injury include NGF (Taniuchi et al., 1988), GDNF (Bennett et al., 1999;Hoke et al., 2002), BDNF (Boyd and Gordon, 2002) and basic fibroblast growth factor (bFGF) (Grothe and Nikkhah, 2001).

GDNF is thought to play a protective role, thereby reducing symptoms of neuropathic pain (Bennett et al., 2000) and promoting axonal regeneration (Chen et al., 2001). Correspondingly, GDNF expression is rapidly upregulated in Schwann cells as early as 48 h after denervation of rat sciatic nerves (Hoke et al., 2002) however, levels decline a week after denervation, when pain behaviours are developing significantly. Therefore, a decrease or failure to maintain the upregulated expression of GDNF could contribute to the development of neuropathic pain.

Axotomy of sciatic nerve fibres in adult rats also induces expression of NGF receptors in the entire population of Schwann cells located distal to the injury (Taniuchi et al., 1988). The expression of Schwann cell NGF receptors is negatively regulated by axonal contact, being induced when axons degenerate and suppressed when axons regenerate. It is likely that NGF receptors bind NGF molecules upon the Schwann cell surface providing a substratum laden with trophic support and chemotactic guidance for regenerating neurons. However, altered input of NGF may cause phenotypic changes which may lead to induction of pain states (Jongsma et al., 2001;McMahon and Priestley, 1995) and has been associated with axon sprouting and neuroma formation (Kryger et al., 2001) (see 1.14.1).

In the DRG, molecules such as bFGF show a mainly neuronal expression, whereas following nerve injury, Schwann cells and invading macrophages have been shown to become the main cellular sources of bFGF with increasing levels of expression within a few hours of injury (Grothe et al., 2000;Scarlato et al., 2001). bFGF mediates rescuing effects on injured sensory neurons and supports neurite outgrowth of transected nerves (for review see)

(Grothe and Nikkhah, 2001) and therefore over-expression may lead to axonal sprouting, a mechanism implicated in abnormal pain phenomena (see 1.13.3). Additionally, BDNF, up-regulated in denervated Schwann cells following chronic axotomy, has been shown to inhibit axonal regeneration (Boyd and Gordon, 2002) and has previously been implicated in the induction of hyperalgesia (Miletic and Miletic, 2002;Ramer et al., 1999) therefore, increased release from Schwann cells may enhance such actions.

Together with changes in expression in neurons (see 1.14.1), neurotrophic factors produced by Schwann cells in response to nerve injury and associated demyelination seems likely to play a significant role in mechanisms leading to the development of persistent pain states.

1.22 Human Peripheral Demyelinating Disorders

1.22.1 Charcot-Marie-Tooth (CMT) disease

Charcot-Marie-Tooth (CMT) is one of the most common degenerative neurological disorders with a prevalence of 1 in 2500 (Skre, 1974). It is an hereditary motor and sensory neuropathy (HMSN) disease comprising a heterogeneous group of inherited peripheral neuropathies (Kamholz et al., 2000), the inheritance of which can be autosomal dominant, autosomal recessive (ARCMT) or X-linked. There are several different forms of HMSN, of which Type I (demyelinating form), Type II (neuronal form), Type IV (autosomal recessive demyelinating form) and the X-linked CMTX represent the CMT syndrome.

CMT Type 1

CMT 1, the most common form of CMT (Young and Suter, 2001) and the most common autosomal dominant neuropathy (Lynch and Chance, 1997), is associated with peripheral demyelination and is caused by mutations in one of several Schwann cell genes. CMT 1 is characterised by markedly reduced conduction velocities in peripheral sensory and motor nerves, segmental demyelination and Schwann cell hypertrophy with onion bulb formation in peripheral nerves (Calore et al., 1994).

There are at least three forms of CMT 1 based on their molecular genetics. The majority of CMT 1 patients, designated CMT 1A, have a duplication in the gene encoding the major PNS myelin proteins peripheral myelin protein 22 (PMP22) (Chance et al., 1993;Chance and Pleasure, 1993). CMT 1B is less common and is caused by mutations in the gene encoding the major myelin structural protein zero (P0) (Hayasaka et al., 1993;Kulkens et al., 1993). Several other gene mutations have also been associated with less common forms of CMT1 (Warner et al., 1998).

These mutations disrupt myelination and Schwann cell function in a number of ways producing a wide variety of clinical phenotypes. They may also produce secondary axonal damage, a major cause of weakness in CMT 1 (Kamholz et al., 2000).

Neuropathic pain is reportedly a significant problem for many CMT patients and impairment of nerve function in CMT1 is thought to be mediated mainly by changes in myelinated axons (Ericson and Borg, 1999).

CMTX

CMTX is associated with mutations in the gene for the gap junction protein, connexin-32, (Anzini et al., 1997) and is characterised by late onset peripheral demyelination.

CMT type 2

CMT 2, in which axonal neuropathy predominates, is often a less severe disease than CMT1 exhibiting axon loss and resultant Wallerian degeneration. Although motor and sensory action potential amplitudes are diminished, relatively normal conduction velocities remain (Carter et al., 1998), suggesting myelinated fibres are mildly affected and that nerve function impairment due to axonal damage to unmyelinated fibres dominates. Pain is not generally reported to be associated with CMT2 (Ericson and Borg, 1999), however, reports of incidence and severity differ and the fact that CMT1 and CMT2 are often clinically hard to distinguish makes the distinction between sensory problems associated with particular pathologies difficult to determine (Carter et al., 1998).

CMT type 4

CMT4, the autosomal recessive form of demyelinating CMT is also caused by mutations in various genes encoding myelin proteins, many of which are yet unidentified, but are known to include P0 and PMP-22. More recently and of particular interest to this study is the discovery of a mutation in the human Periaxin gene, *PRX*, underlying CMT in a large consanguineous Lebanese family (Guilbot et al., 2001). The *PRX* gene encodes L- and S-periaxin, which are proteins of myelinating Schwann cells and will be dealt with in more detail below (see 1.23.6). This mutation leads to an autosomal recessive demyelinating form of CMT, named CMT4F. Histopathological and immunohistochemical analysis of the sural nerve reveals supernumerary Schwann cells surrounding thinly myelinated or naked axons forming so-called 'onion bulb' structures and hypermyelinated regions of nerve. This morphology is very similar to the peripheral nerves in the mutant *Prx*-null mouse (Gillespie et al., 2000) which will be discussed further below (see 1.24). Of interest to this study is the

fact that patients with CMT4F display a variety of distal abnormalities including pain in both upper and lower extremities (Delague et al., 2000).

1.22.2 Guillain-Barré Syndrome (GBS)

GBS, or acute inflammatory polyneuropathy, is often regarded as being a predominantly motor neuropathy with few sensory features. However, pain is a common symptom (Asbury, 1990) with the incidence ranging from 54%-84%. Pathologically, there is inflammation of the peripheral nerve and spinal nerve root with lymphocytic and macrophage infiltration and demyelination (Moulin, 1998). Various types of pain are described in GBS patients, with dysaesthetic extremity pain, especially of the legs being common during the course of the illness and persisting indefinitely in 5-10% of patients (Moulin et al., 1997). It is thought that neuropathic pain of this type may be due to ectopic impulse formation at sites of demyelination, as well as axonal degeneration and regeneration along the peripheral nerve (Devor and Seltzer, 1999).

It has been generally argued that pain associated with demyelinating conditions results from axonal damage. The consequences of the lack of myelin alone have been largely ignored. However, demyelination is the common symptom of all these disorders, only some of which have been described above, and axonal pathology has not always been determined. Furthermore, animal models of neuropathic pain such as CCI, result in massive demyelination of the injured nerve, implying that this process may have an important role in the resulting chronic pain.

Therefore, it is of interest to investigate the mechanisms that underpin the production of neuropathic pain associated with demyelination of peripheral nerves. This may be achieved by utilising mouse models with mutations in various myelin genes, as well as experimental models of demyelination.

1.23 Mutant Mouse Models of Peripheral Demyelination

Several demyelinating neuropathies of genetic origin have now been modelled in mice by inactivating genes expressed in myelinating Schwann cells (Anzini et al., 1997;Suter and Snipes, 1995). Such animals are not only valuable for examining the biological function of the proteins encoded by these genes, but they also provide an opportunity to study the pathophysiology of the disease.

1.23.1 Protein zero (P0)

P0 is a myelin structural protein and the main adhesive molecule of peripheral myelin (Arroyo and Scherer, 2000), and is thought to be primarily concerned with myelin compaction (D'Urso et al., 1990;Giese et al., 1992). Mice deficient in P0 have changes in their peripheral myelin such as severe hypomyelination and decompaction of myelin followed by degeneration and onion bulb formation, which resemble some inherited human neuropathies, such as CMT 1B (Adlkofer et al., 1995;Giese et al., 1992).

1.23.2 Peripheral myelin protein 22 kDa (PMP22)

Axonal contact with a Schwann cell activates the insertion of PMP22 protein into the myelin membrane (Kamholz et al., 1999). Although less abundant than P0, its level of expression in compact myelin is critical (Murakami et al., 1996;Snipes and Suter, 1995). Due to the identification of the genetic mutations underlying the majority of CMT 1 cases, several transgenic mouse models have been based upon the alteration of expression of the *PMP22* gene. The mutant mice, *trembler (Tr)* and *trembler-j (Tr-j)*, are dominant natural mouse mutants with point mutations in one of the two *PMP22* genes, leading to classic signs of a demyelinating neuropathy (Adlkofer et al., 1995), including thinly myelinated axons, aberrant Schwann cell proliferation, onion bulb structure formation and abnormal Schwann cell-axon interactions (Robertson et al., 1997). Further transgenic mouse models with similar phenotypes have been created with an increased *PMP22* gene dosage, mimicking the intrachromosomal duplication containing the *PMP22* gene in human CMT 1A (Suter and Klaus-Armin, 1999).

1.23.5 Connexin 32

X-linked CMT disease is the second most common known cause of hereditary neuropathy (Scherer et al., 1998) and is caused by mutations in the gene for the gap junction protein connexin-32 (Cx-32). This protein is expressed in peripheral nerve and is present in

noncompact myelin in which it is likely to form channels. Cx-32 'knock-out' mice and transgenic mice expressing mutant Cx-32, develop a late onset demyelinating neuropathy similar to that observed in patients with CMTX (Anzini et al., 1997;Scherer et al., 1998), although specific pathology depends upon the nature of the mutation.

1.23.6 Periaxin

L-periaxin and the *Prx*-null mouse model of peripheral nerve demyelination have formed the basis for this study and therefore will be reviewed in more detail than the previously mentioned transgenic models.

L-Periaxin is a protein of myelinating Schwann cells thought to play a role in the later stages of myelination (Gillespie et al., 1994). The murine *Periaxin* (*Prx)* gene encodes two proteins with PDZ domains, L-periaxin (147 kDa) and S-periaxin (16 kDa) (Dytrych et al., 1998). L-periaxin is localised to the plasma membrane and S- periaxin to the cytoplasm. Both contain a single PDZ domain, typically present in proteins that interact with the cytoplasmic tail of plasma membrane proteins and with the cortical cytoskeleton suggesting that L-periaxin might be implicated in signal transduction pathways. During development and regeneration of the peripheral myelinated nerve, L-periaxin is predominantly localised to the adaxonal surface of Schwann cells, which apposes the axon. However, as the myelin sheath matures, L-periaxin becomes concentrated in the abaxonal plasma membrane, which apposes the basal lamina (Scherer et al., 1995). This translocation of L-periaxin to a predominantly abaxonal location is a feature of the maturation of the sheath and its expression coincides with the early events in the establishment of axo-glial contact (Dytrych et al., 1998;Scherer et al., 1995), suggesting that L-periaxin might participate in the recruitment of proteins to a cortical structure involved in transmembrane signalling (Dytrych et al., 1998;Scherer et al., 1995).

A role for L-periaxin in transmembrane signalling has been recently been supported by evidence for its interaction with dystrophin-related protein 2 (DRP2) which forms part of a dystroglycan-DRP2 complex at the surface of myelin-forming Schwann cells (Sherman et al., 2001). Such complexes in the PNS are believed to have structural and signalling functions (Bunge et al., 1986;Eldridge et al., 1989) and in the absence of L-periaxin, DRP2 is mislocalized and depleted, supporting a role for L-periaxin in associations with the cytoplasmic domains of plasma membrane proteins involved in adhesion. Once the mature myelin sheath is established, the amount of periaxin protein declines (Gillespie et al., 1994), suggesting that periaxin plays a role in ensheathment and spiralisation of Schwann cell myelin and is less involved in the maintenance of myelin in mature nerves.

1.24 The Periaxin-Deficient Mouse

A mutant mouse model has been characterised in which expression of the gene encoding periaxin has been prevented due to the deletion of exon 6 and part of exon 7 of the *Prx* gene (Gillespie et al., 2000). It has been demonstrated that the Schwann cells of mutant mice lacking the functional *Prx* gene initially ensheath and myelinate peripheral nerve axons in an apparently normal manner. However, this sheath later destabilises, resulting in the development of a severe demyelinating neuropathy (Gillespie et al., 2000). Animal models of CMTX are similar to the *Prx*-null mouse in that they myelinate normally and show a late onset pattern of demyelination with attempts to remyelinat. However, in contrast to the *Prx*-null mouse the CMTX mouse model displays mild deficits in nerve conduction and nerve fibres, such as in the saphenous nerve then show little evidence of demyelination (Anzini et al., 1997).

At 6 weeks of age, the sciatic nerves of *Prx*-null ($Prx^{-/-}$) mice are affected relatively mildly (Gillespie et al., 2000) compared to mice with mutations in the genes encoding P0 or PMP22, which display widespread derangements to the Schwann cell-axon unit from a much earlier age (Adlkofer et al., 1995;Giese et al., 1992). Furthermore, levels of major myelin proteins including MAG, P0 and MBP are normal in the $Prx^{-/-}$ sciatic nerve at 6 weeks however; there is evidence of focal thickenings (tomacula) and infoldings of internodal myelin (see Figure 1.3b). By 6 months of age, the sensory, motor and autonomic nerves of the $Prx^{-/-}$ show extensive segmental demyelination with profound disruption of axonal ensheathment and deranged Schmidt-Lanternman incisures. In contrast, mice heterozygous for the mutant allele of *P0* or *PMP22* show a propensity for sparing sensory versus motor nerves (Adlkofer et al., 1995). By 6-8 months, naked or thinly myelinated fibres are common in the $Prx^{-/-}$ sciatic nerve. These axons are often surrounded by redundant basal laminas and supernumerary Schwann cells, which form onion bulb structures, diagnostic of attempts to remyelinate fibres (see Figure 1.3a,b). There is little evidence for macrophage infiltrations indicating that this is not an inflammatory-mediated process. Importantly, there is no evidence for axonal degeneration or cell loss in the DRG of the *Prx*-null mouse at 6 weeks of age (Gillespie et al., 2000). As a result, this study has used the *Prx*-null mouse as a model in which to study neuropathic pain associated with peripheral demyelination.

1.25 The *Prx*-Null Mouse as a Model for Neuropathic Pain in Peripheral Demyelination

The homozygous *Prx*-null mouse is unique in the late onset and severity of the clinical phenotype, which shows a parallel with demyelinating peripheral neuropathies of adult onset (Dyck et al., 1993). It has been shown that the *Prx*-null mice have a marked reduction in their peripheral nerve conduction velocities (see Figure 1.4) (Gillespie et al., 2000) and electrophysiological investigations have identified the presence of spontaneous low frequency discharge (1-2Hz) in the saphenous nerve. Furthermore, the similarities of the human disease CMT4F, both clinically and histopathologically, to the phenotype of the *Prx*-null mouse are striking (Boerkoel et al., 2002;Gillespie et al., 2000;Guilbot et al., 2001), substantiating the utility of the *Prx*-null mouse as a model of CMT disease.

a) b)

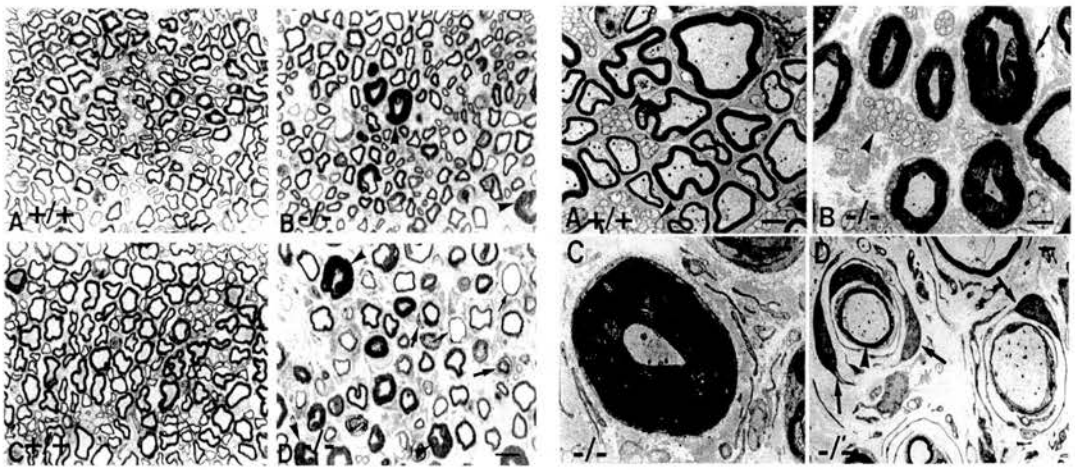


Figure 1.3 Light and electron microscopy of sciatic nerves from *Prx*-null mice. a). Resin sections (1μm) from sciatic nerves of normal (A and C) and mutant (B and D) mice at 6 weeks (A and B) and 6 months (C and D) of age. The null mutant shows features typical of a progressive demyelinating neuropathy with evidence of remyelination, as denoted by onion bulbs and thin myelin sheaths (thick and thin arrow, respectively). Disproportionately thick sheaths evident at 6 weeks of age are more numerous by 6 months of age (arrowhead) (adapted from (Gillespie et al., 2000). b) Electron microscopy of wild-type (A) and mutant saphenous nerves at 6 months (B and C) and at 8 months (D). C-fibre bundles appear normal (arrowhead), but myelinated fibres are grossly hypermyelinated (B and C), with evidence of myelin infolding at a tomaculum (arrow, D) (adapted from Gillespie et al., 2000).

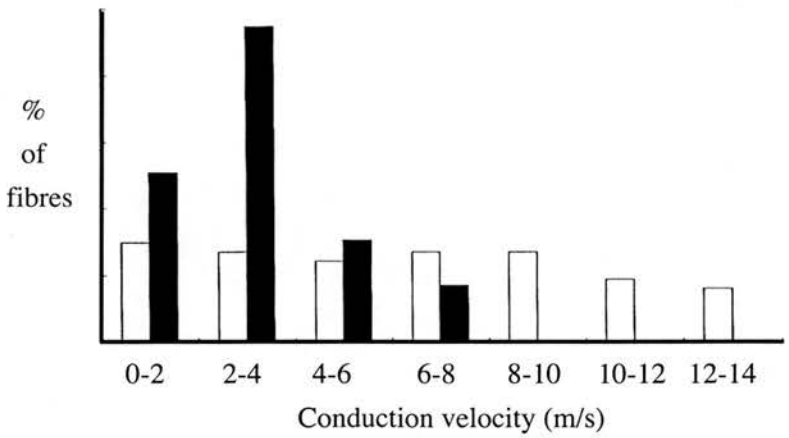


Figure 1.4. Conduction velocity profiles of saphenous nerves from *Prx*-null mice as compared to wild type littermates. The percentage of fibres was calculated displaying each conduction velocity for *Prx*-null Mice (■) as compared to wild type littermates (□) (adapted from Gillespie et al., 2000).

1.26 Focal Demyelination: Development of an Experimental Mouse Model

When using mutant mouse models, it is not possible to rule out indirect consequences of the altered genotype or related compensatory mechanisms on development. Furthermore, for purposes of this study, it was appropriate to refine the model of peripheral nerve demyelination to target individual sensory nerves, thereby eliminating any motor deficits and allowing specific investigation of sensory changes. To achieve this, we have developed a model of focal demyelination in the sciatic nerve and in the predominantly sensory, saphenous nerve using the non-toxic, myelinolytic agent, lysophosphatidyl choline (lysolecithin) (see Chapter 3), a group of monoacyl phosphoglycerides obtained by the hydrolysis of lecithin (van Deenen and De Haas, 1966).

The induction of demyelination in both peripheral and central nerves by micro-injection of lysolecithin into the perineurium of nerves is well-characterised in the literature (Hall and Gregson, 1971;Love et al., 1986). Lysolecithin has been used extensively in various animal models to produce demyelination in the sciatic nerve (Hall, 1973;Hall and Gregson, 1971), spinal cord (Hall, 1972), corpus callosum (Waxman et al., 1979) and nerve roots (Smith and Hall, 1980). The lysis of myelin is thought to be related to the detergent-like ability of lysolecithin to form complexes with lipids and proteolipids (Webster, 1961), rendering them soluble in an aqueous medium.

For any analysis of the mechanisms underlying neuropathic pain as a consequence of myelin loss it is of great importance to clarify whether or not there are any additional axonopathic changes in this model. The use of lysolecithin as the myelinolytic agent in this model appears to achieve this, since when injected via a glass micropipette of specific tip diameter (18-20 μm) in controlled amount (1-2 μl) and low concentration (1 %) into the sciatic nerves of adult mice, the action is rapid and restricted to the myelin sheath (Hall and Gregson, 1971). Reportedly, there was little damage to the Schwann cell cytoplasm or to the axon and the resulting demyelination is confined to the immediate vicinity of the site of injection and is of rapid onset (Love et al., 1986). Following topical application of lysolecithin to the peripheral nerve, the Schwann cells remain viable, and therefore proliferate and remyelinate the nerves (Gregson and Hall, 1973), and eventually the ensuing damage to the peripheral nerve is reversed and the nerve recovers.

1.27 AIMS

The aims of this study were to:

(1) utilise mouse models of peripheral nerve demyelination to investigate changes in sensory processing that may lead to abnormalities involved in the production of associated neuropathic pain phenomena. The models utilised were the *Prx*-null mouse and a model of focal peripheral nerve demyelination following topical application of lysolecithin. The latter model is a novel model of neuropathic pain.

(2) employ light and electron microscopy, as well as immunohistochemical approaches to characterise the morphology of the nerves and cell bodies in the DRG in these models.

(3) use well-characterised behavioural reflex testing techniques, to determine the presence of reflex behavioural changes characteristic of neuropathic pain in both models.

To examine functional changes in these models we have employed:

(4) peripheral nerve electrophysiological techniques to investigate activity changes in the saphenous nerve that may underlie the associated development of neuropathic pain.

(5) immunohistochemical techniques to investigate any changes in the expression of markers characteristic of other neuropathic pain models, such as the neuropeptides CGRP, Substance P, galanin, VIP and NPY and the sodium channels SNS 1/$Na_v1.8$ and brain type III/$Na_v1.3$ in the cell bodies of the affected peripheral nerves.

(6) intrathecal application of various pharmacological agents to examine the possible involvement of central changes in the behavioural reflex sensitivity displayed in both models. We investigated the effects of antagonists to the NMDA receptor and NFκB, and agonists and antagonists targeting the cannabinoid system.

CHAPTER 2. MATERIALS

2.1 Anaesthetics

- Halothane (Zeneca Ltd., Cheshire, UK)
- Sagatal (Rhone Merieux Ltd., Hertfordshire,UK)
- Urethane (Zeneca Ltd., Cheshire, UK),

2.2 Development of Animal Models

- 1ml syringe (Merck-BDH, UK)
- 25Gx1 needle (Terumo, Belgium)
- 4.0 suture thread (Ethicon Ltd., Edinburgh, UK)
- Glass capillaries (Clark Electromedical Ltd., Reading, UK)
- Lysolecithin (α-lysophosphatidylcholine; Sigma Chemical Co, Poole, UK)

2.3 Behavioural Reflex Testing

- Thermal stimulus (Hargreaves-Plantar apparatus, Ugo Basile, Italy) .
- Von Frey filaments (Stoelting Co., Wood Dale, Illinois).

2.4 Light and Electron Microscopy

- Araldite (Agar Scientific, Essex, UK)
- Dibutylpthalate (Agar Scientific, Essex, UK)
- Dodecenyl Succinic Anhydride (Agar Scientific, Essex, UK)
- Gluteraldehyde (Sigma Chemical Co, Poole, UK)
- Osmium tetroxide (Agar Scientific, Essex, UK)
- Paraformaldehyde (Sigma Chemical Co, Poole, UK)
- Propylene oxide (Agar Scientific, Essex, UK)
- Sodium cacodylate (Sigma Chemical Co, Poole, UK)
- Sodium cacodylate buffer (Agar Scientific, Essex, UK)
- Reichert OMU4 Ultracut microtome (Leica UK Ltd, Milton Keynes),
- Glass slides (Merck-BDH, UK)
- Philips BioTwain electron microscope (FEI UK Ltd, Cambridge,UK)
- 1mM calcium chloride solution (Sigma Chemical Co, Poole, UK).
- 2,4,6-Tri (dimethylaminomethyl) phenol (DMP-30) (Agar Scientific, Essex, UK)

2.5 G-ratio

- IP Lab Spectrum P programme (Scanalytics Corp, Fairfax,VA)

2.6 Electrophysiology

- AC coupled differential amplifier (3160, Digitimer Ltd, Welwyn Garden City, Herts UK)
- Claris Works 4.0 illustrator programme (Claris Corp, Smart Computing, Lincoln, NE).
- Digitimer (D100, Digitimer Ltd Welwyn Garden City, Herts UK)
- Dual beam oscilloscope (D13, Tektronix Inc., Beaverton, OR, USA),
- Ethyl chloride (Henry Stein, Glasgow, UK)
- Isolated stimulator (Mark IV, Lectromed Limited, St Quen, Jersey, Channel Islands)
- Powerlab/McLab Chart v3.6/s programme (AD Instruments Ltd, Oxford, UK)
- Spike processor (D.130, Digitimer, UK)

2.7 Immunohistochemistry

- Gelatine (Sigma Chemical Co, Poole, UK)
- Glass slides pre-coated with poly-L-lysine (Merck-BDH,UK)
- Heparin (Sigma Chemical Co, Poole, UK)
- Normal donkey serum (Diagnostics Scotland, Edinburgh, UK)
- Normal goat serum (Diagnostics Scotland, Edinburgh, UK)
- OCT mounting medium (Tissue Tek, UK)
- Paraformaldehyde (Sigma Chemical Co, Poole, UK)
- Sucrose (Sigma Chemical Co, Poole, UK)
- Triton X-100 (Sigma Chemical Co, Poole, UK)
- Vecta-Shield (Vector Laboratories, Burlingame, CA)

2.7.1 Antibodies

Primary

- Mouse monoclonal anti-neurofilament 200kDa (clone N52; Sigma, Chemical Co, Poole, UK)
- Mouse monoclonal anti-peripherin (Chemicon International Ltd, Harlow, UK)
- Rabbit polyclonal anti-ATF3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA)

- Rabbit polyclonal anti-brain type III sodium channel/$Na_v1.3$ (Alomone Labs, Jerusalem)
- Rabbit polyclonal anti-galanin (Advanced Chemtech Ltd, Cambridgeshire, UK)
- Rabbit polyclonal anti-NPY (Peninsula Laboratories Inc, Belmont, CA)
- Rabbit polyclonal anti-SNS/$Na_v1.8$ (supplied by S.Tate, GlaxoSmithKline, UK)
- Rabbit polyclonal anti-Substance P (Affiniti, Exeter, UK)
- Rabbit polyclonal anti-VIP (Incstar, Corp, Stillwater, MN,USA)
- Rabbit polyclonal anti-VIP (human, porcine,rat) (Peninsula Laboratories Inc, San Carlos, CA, USA)
- Sheep polyclonal anti-CGRP (Affiniti, Exeter, UK)

Secondary

- Donkey anti-sheep-FITC (Jackson ImmunoResearch Laboratories, Westgrove, PA)
- Goat anti-mouse-TRITC Southern Biotechnology Associates, Birmingham, Alabama)
- Goat anti-rabbit-FITC (Cappel, ICN Biomedical, CA, USA)

2.8 Intrathecal injections

- 25Gx1 needle (Terumo Belgium)
- Dimethylformamide (dmf) (Sigma Chemical Co, Poole, UK)
- Pontamine Sky Blue (Merck-BDH, UK)

2.8.1 Drugs used

- NMDA receptor antagonist: **(R)-CPP** (3-((R)-2-carboxypiperazine-4-yl)-propyl-1-phosphonic acid), (Tocris Cookson Ltd, Bristol, UK)
- mu opioid receptor agonist **DAMGO** (D-Ala_2, $MePhe_4$, Gly-olenkephalin) (Tocris)
- NFκB antagonists **Parthenolide** (Alexis, Exeter, UK); **CAPE** (Caffeic acid phenethyl ester) (Calbiochem, CN Bioscience, Nottingham,UK); **Sulphasalazine** (Sigma Chemical Co, Poole, UK)
- Mixed CB_1/CB_2 cannabinoid receptor agonist **WIN 55,212-2** (mesylate (R)-(+)-(2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo(1,2,3-de)-1,4-benzoxazin-6-yl)-1-naphthalenylmethanone), (Tocris Cookson Ltd, Bristol, UK)

- Selective CB_1 receptor antagonist **AM 251** (N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,3-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide) (Tocris Cookson Ltd, Bristol, UK)

2.9 Miscellaneous

- Freezing spray (Greenhill Chemical Products Ltd., Burton-on-Trent, UK)
- Glass coverslips (22 x 50mm) (Merck-BDH,UK)
- Isopentane (Merk Ltd., Leics, UK)
- Silica gel (Merck-BDH,UK)
- Sterile saline (Sigma Chemical Co, Poole, UK)

CHAPTER 3. MATERIALS AND METHODS

3. 1 Development of Animal Models

3.1.1 *Prx*-null mouse model

Background on creation of Prx -null mice (from (Gillespie et al., 2000)

To inactivate the mouse *Prx* gene, exon 6 and part of exon 7 were deleted by homologous recombination in embryonic stem cells (Gillespie et al., 2000). Cells of two clones were injected into C57/BL6 blastocysts and resulting heterozygous offspring were backcrossed to the parental C57/BL6 strain before inter-crossing at the F6 generation. Homozygous offspring were identified by Southern blotting, as previously described (Dytrych et al., 1998). RNA was extracted from 16 day old mutants (Cathala et al., 1983), resolved and probed with a cDNA fragment, of mouse periaxin cDNA which revealed that mutant mice lacked the 4.6 and 5.2 kb mRNAs found in the peripheral nerves of wild-type littermate. Protein levels were assessed in 3 month old *Prx*-null and wild-type littermate animals using isoform specific antibodies in Western blots as described previously (Dytrych et al., 1998), revealing that neither L- nor S- periaxin polypeptides were detectable in peripheral nerves (Gillespie et al., 2000).

For all experiments involving the *Prx*-null mouse, male mutant animals at either 6 weeks or 6 months of age were used as stated. All experiments were also carried out on age and sex matched wild-type littermates.

3.2.2 Lysolecithin-induced focal nerve demyelination

Male C57/BL6 mice over the age of 6 weeks were used for all lysolecithin-induced demyelination treatments and for all associated experiments. For the induction of demyelination of the peripheral nerve via the application of lysolecithin, animals were initially anaesthetised with 0.3 ml of 25% Sagatal (Rhone Merieux Ltd., Hertfordshire,UK) in sterile physiological saline (0.9%) and maintained on halothane (Zeneca Ltd., Cheshire, UK) and O_2, always ensuring that the pinch reflex of the hind limb was abolished. For all demyelination experiments, lysolecithin (Sigma Chemical Co, Poole, UK) was dissolved in sterile physiological saline (0.9%) to give a final concentration of 15 mg/ml.

Method 1: Microinjection into sciatic nerve

Glass capillaries (Clark Electromedical Ltd., Reading, UK) were stretched out on an electrode glass puller and subsequently knocked back on a curved glass rod under a light

microscope to give a final tip diameter of between 15 and 20 μm. Using a 1 ml syringe (Merck-BDH, UK), the glass pipette was filled with lysolecithin solution and attached to a positive displacement pipette via tight fitting rubber tubing. The sciatic nerve of the right hind leg (Figure 3.1) was dissected out at mid thigh level and the three branches; tibial, peroneal and sural, separated and laid over a stabilised pair of forceps held open at a separation of 5 mm. By clamping to a movable vice, the glass micropipette tip was injected just under the perineurium of the larger two branches (tibial and peroneal) of the sciatic nerve under a light dissection microscope. 1-2 μl of solution were then displaced from the glass pipette in 0.5 μl aliquots into the nerve over 10 minutes to prevent over spill onto surrounding tissue. The nerve was then rinsed with sterile saline and the overlying muscle and skin closed with 4.0 suture thread (Ethicon Ltd., Edinburgh, UK), marking the application site.

Method 2: Topical application to saphenous or sciatic nerve

a) Saphenous Nerve Demyelination

Whilst under anaesthesia, the saphenous branch of the femoral nerve was exposed at mid thigh level (Figure 3.2) and 5-10 μl of lysolecithin solution was applied topically via a 25Gx1 needle (Terumo, Belgium) over a length of 2-3 mm, ensuring that the nerve remained coated with the solution for 10 minutes. Excess lysolecithin solution was then washed off with sterile saline and the incision site closed with 4.0 suture thread, marking the application site.

b) Sciatic Nerve Demyelination

The sciatic nerve was dissected at mid thigh level and the two larger braches (tibial and peroneal) were carefully separated from the smaller sural branch which was subsequently excluded from the preparation. Lysolecithin was again applied topically to the nerve via a 25Gx1 needle over a length of 2-3 mm for 15 minutes; always ensuring the nerve remained moist. Excess lysolecithin solution was washed off and both overlying muscle and skin were then closed with 4.0 suture thread, marking the application site.

Sham control animals were prepared for both saphenous and sciatic nerve preparations by omitting lysolecithin from the solution so that the nerve was exposed as above and sterile saline applied topically for the relevant time.

Figure 3.1. Schematic diagram of anatomy of the medial aspect of the hind limb of the mouse with special reference to the saphenous nerve (adapted from (Popesko et al., 1990))

igure 3.2. Schematic diagram of anatomy of the lateral aspect of the hind limb of the mouse with special reference to the sciatic nerve (adapted from (Popesko et al., 1990))

3.2. Behavioural Reflex Testing

The threshold for hindpaw withdrawal in both animal models was measured in response to graded mechanical and noxious thermal stimulation in conscious animals. Behavioural reflex tests were carried out daily, over a period of 2 weeks, to determine stable thresholds for the *Prx*-null (n=8), wild-type littermates (n=8) and stock C57/BL6 mice designated for lysolecithin treatment of the saphenous (n=8) or sciatic (n=8) nerves.

3.2.1 Mechanical test

Mechanical thresholds were determined using von Frey filaments (Stoelting Co., Wood Dale, Illinois), which provide a calibrated indentation pressure against the hairless skin of the hindpaws. The threshold response was defined by the filament that caused foot withdrawal at least five times in every ten applications (Chaplan SR et al., 1997;Meyer, 1979).

3.2.2 Thermal test

The time for hindpaw withdrawal was measured in response to a quantified noxious thermal stimulus (Hargreaves-Plantar apparatus, Ugo Basile, Italy) of infra-red intensity setting 30 (>50°C) applied to the mid-plantar surface of each hind paw (Hargreaves et al., 1988). The withdrawal was characterised as a brief paw flick and the withdrawal latency was recorded in seconds.

For thresholds to both mechanical and thermal stimulation, measurements were repeated at least 6 times with a minimum time of 5 minutes between each test. From this the average threshold for each stimulus could be determined.

In sciatic or saphenous nerve lysolecithin-treated animals (n=16), behavioural reflex tests were carried out one day before and on the day of treatment to obtain baseline thresholds. Testing then re-commenced 5 days following surgery to allow for healing and was subsequently carried out every day to identify the development of any heightened reflex sensitivity in treated animals. Testing ceased once animals displayed recovery to normal baseline thresholds. This time course was also used to test sham control animals (n=4 for each nerve preparation). The threshold value at each time point tested was calculated as the mean ± SEM. Any statistically significant differences ($*P<0.05$) in paw withdrawal threshold from mechanical stimulation between a) *Prx*-null and wild-type littermate, b) paws contralateral and ipsilateral to lysolecithin treatment or c) paws contralateral and ipsilateral to sham treatment were determined by a Mann-Whitney Rank Sum test. Likewise any

statistically significant differences in withdrawal latency from thermal stimulation were determined by a Student's paired t-test.

3.3 Morphological Investigations

In order to test the integrity of lysolecithin-treated nerves, nerve morphology was examined in animals that previously had a) microinjection or b) topical application of lysolecithin to the sciatic nerve or c) topical application of lysolecithin to the saphenous nerve.

3.3.1 Processing and embedding

13 days post lysolecithin-treatment, the treated nerves were dissected from animals' euthanised by CO_2 inhalation. The lysolecithin-treated area, marked by a small suture during surgery, was selected and dissected with approximately 1 cm of normal nerve either side. The affected area could therefore be identified once processed and embedded. Control nerves were dissected at equivalent points.

Once dissected, the nerves were fixed for 4 hours in 2.5% glutaraldehyde solution containing 2.5% glutaraldehyde, 2% paraformaldehyde, 0.1 M sodium cacodylate buffer (pH 7.4) and 1mM calcium chloride solution. Solutions were all made up in ultra high purity water using reagents from Sigma-Aldrich (Poole, UK) unless otherwise indicated. Nerves were then washed twice in 0.1 M sodium cacodylate buffer for 20 minutes; osmicated in 1% osmium tetroxide for 1 hour; washed twice in sodium cacodylate buffer for 10 minutes and then dehydrated through increasing concentrations (10-100%) of alcohol in distilled water for 1 hour. Sections were then washed twice in 10 mls propylene oxide for 30 minutes followed by 10 mls 1:1 araldite mix (1:1 araldite and dodecenyl succinic anhydride (DDS)) with 10 mls propylene oxide for 1 hour and then overnight in araldite mix. Finally, the sections were washed twice in 55.2 mls araldite mix with 1.95 mls accelerator (dibutylphthalate & 2,4,6-tri (dimethylaminomethyl) phenol (DMP-30) for 2 hours in which they were then embedded and left to harden in a 50 °C oven for at least 48 hours. All materials used for processing and embedding were obtained from Agar Scientific (Essex, UK).

3.3.2 Light microscopy

For light microscopy, 1 μm resin sections of treated saphenous and sciatic nerve were cut from araldite embedded blocks on a Reichert OMU4 Ultracut microtome (Leica UK Ltd, Milton Keynes), heat dried to glass slides (BDH, Poole, UK) and subsequently stained with Toluidine Blue. From this, the level of myelination and hence the area of demyelination could be established by bright-field microscopy.

3.3.3 Electron microscopy

Ultrathin (80nm) sections were cut using the OMU4 microtome and stained with uranyl acetate and lead citrate to allow examination on a Philips BioTwain electron microscope (FEI UK Ltd, Cambridge, UK). Nerve counts were performed at the electron microscope level so that the amount of demyelination and integrity of large diameter, myelinated and small diameter, unmyelinated axons could be determined. C-fibres were identified as small diameter unmyelinated fibres, present in bundles of fibres surrounded by Schwann cell cytoplasm. From non-overlapping EM photographs of cross-sections of the entire nerve, the actual number of intact C-fibres was calculated. To minimise experimental bias and error, the images from all nerves (n=3 per group) were counted three times while blinded to the specific nerve from which each image originated. Once all counts were complete, the average number of fibres for each nerve was calculated. To count the number of myelinated A-fibres, axons with all levels of myelination including those that were completely demyelinated were considered. Therefore, A-fibres were identified as the larger diameter axons with myelin or smaller diameter unmyelinated fibres that were solitary. This allowed distinction of demyelinated fibres from unmyelinated fibres, which usually occur in bundles. The statistical significance (*$P<0.05$) of any difference between treated and naïve or sham nerves was determined using a Kruskal-Wallis One-Way Analysis of Variance on Ranks an all pairwise multiple comparisons procedure (Dunn's Method).

3.3.4 G-ratio

The G-ratio of an axon is a measure of myelin thickness calculated by dividing the axonal diameter by the overall diameter of the axon and myelin sheath. This gives an indication of the level of demyelination in axons that are not completely demyelinated by lysolecithin treatment. G-ratios of myelinated fibres were calculated from EM photographs of the whole cross sectional area of each nerve using the IP Lab Spectrum P programme (Scanalytics Corp, Fairfax,VA). This also calculates the number of myelinated axons in the sample. From this, the proportion of completely demyelinated axons per nerve was calculated by counting the number of myelinated fibres per photograph (area represented by each photograph = 292 μm^2). The statistical significance of any difference between treated and naïve or sham nerves (*$P<0.05$) was determined by Kruskal-Wallis One-Way Analysis of Variance on Ranks with an all pairwise multiple comparisons procedure (Dunn's Method).

3.4. Electrophysiology

To determine the effects of lysolecithin treatment on nerve fibre activity, electrophysiological recordings were carried out at different times following application of lysolecithin.

3.4.1 Preparation

Electrophysiological recording of peripheral nerve activity was performed on animals which had previously had focal demyelination induced in the saphenous nerve. Recording experiments were carried out on days 3- and 5-post surgery, times at which behavioural reflex sensitisation should not have developed and also on day's 11-,12- and 13-post surgery, corresponding to the peak time of development of behavioural reflex sensitisation, as tested on mice previously. Mice were assessed for the development of behavioural sensitisation to both thermal and mechanical tests, as described in section 3.2, and then anaesthetised with 5% urethane i.p. (1.25g/kg) and maintained at 36-37 °C with a radiant heat lamp. The saphenous nerve was exposed in the medial thigh and dissected from its associated vein and artery and cut centrally to the site of demyelination or at a corresponding level on the sham and naïve animals. After removal of the perineurium, further dissection under liquid paraffin enabled the identification of afferent preparations comprising a small number of units. The dissected afferents were placed over a bipolar recording electrode and the whole nerve preparation was submerged in liquid paraffin (Figure 3.3).

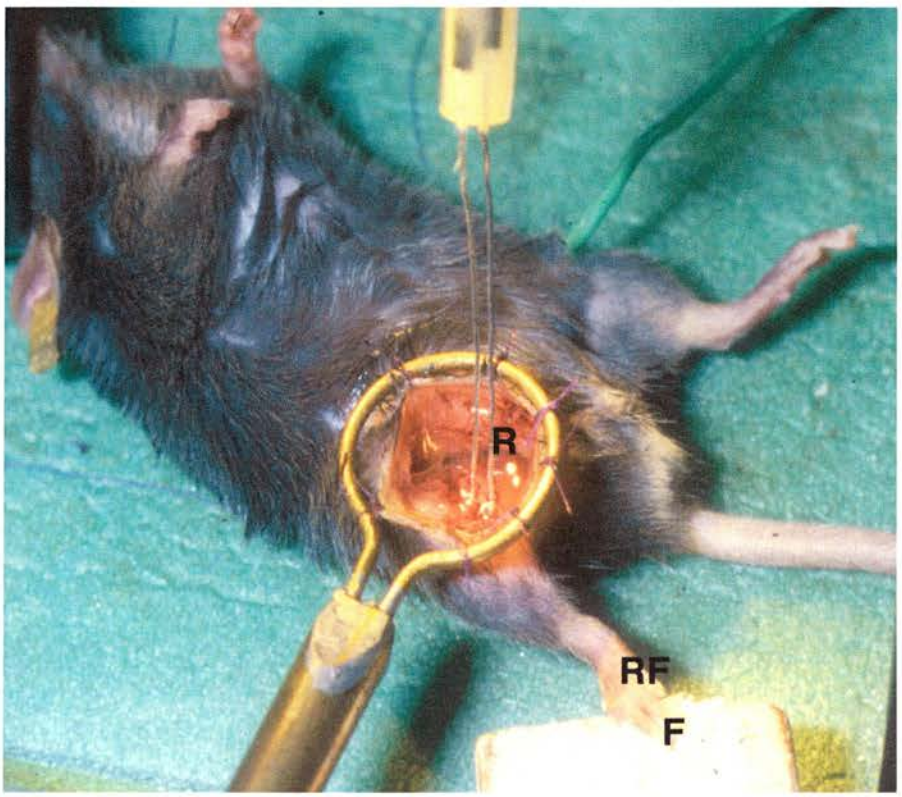


Figure 3.3. Nerve preparation for electrophysiological investigations of the saphenous nerve.

The saphenous nerve was dissected from its associated vein and artery and cut centrally to the site of demyelination or at a corresponding level on the sham and naïve animals. After removal of the perineurium, further dissection under liquid paraffin enabled the identification of afferent preparations comprising a small number of units. The dissected afferents were placed over a bipolar recording electrode (R) and the whole nerve preparation was submerged in liquid paraffin. The hind paw of the treated animal was secured to a footplate (F) using plaster of Paris coated gauze and the receptive field (RF) of the afferent preparation was identified by the induction of impulse activity via gentle brushing of the medial aspect of the hind paw.

3.4.2 Equipment

The electrophysiology apparatus was set up as represented in the box diagram (Figure 3.4). Afferent activity, detected by the bipolar recording electrodes, was amplified using an AC coupled differential amplifier (3160, Digitimer Ltd, Welwyn Garden City, Herts, UK) with a band width setting of 80 Hz to 2.5 kHz. Shapes and latencies of individual impulses were identified using the storage facility on the D13 oscilloscope (Tektronix Inc., Beaverton, OR, USA) and single frame or overlapping multiple frames converted to TTL (transistor-transistor logic) pulses using a spike processor (D.130, Digitimer, UK) which was connected to a) a second oscilloscope with audio display and b) the Powerlab/McLab Chart v3.6/s programme (AD Instruments Ltd, Oxford, UK). To stimulate the receptive field for thermal threshold recordings, a calibrated temperature source controlled the radiant heat source in a time dependent and temperature dependent manner. Conduction velocities were measured using electrical excitation of receptive fields with bipolar needle stimulating electrode. A Digitimer D100 (Digitimer Ltd) triggered an isolated stimulator (Mark IV, Lectromed Limited, St Quen, Jersey, Channel Islands) to deliver square-wave pulses (range 0.5 - 1.0 ms, 0.1 -100 volts) at levels selected to be just above threshold for few-unit preparations via the stimulating electrodes.

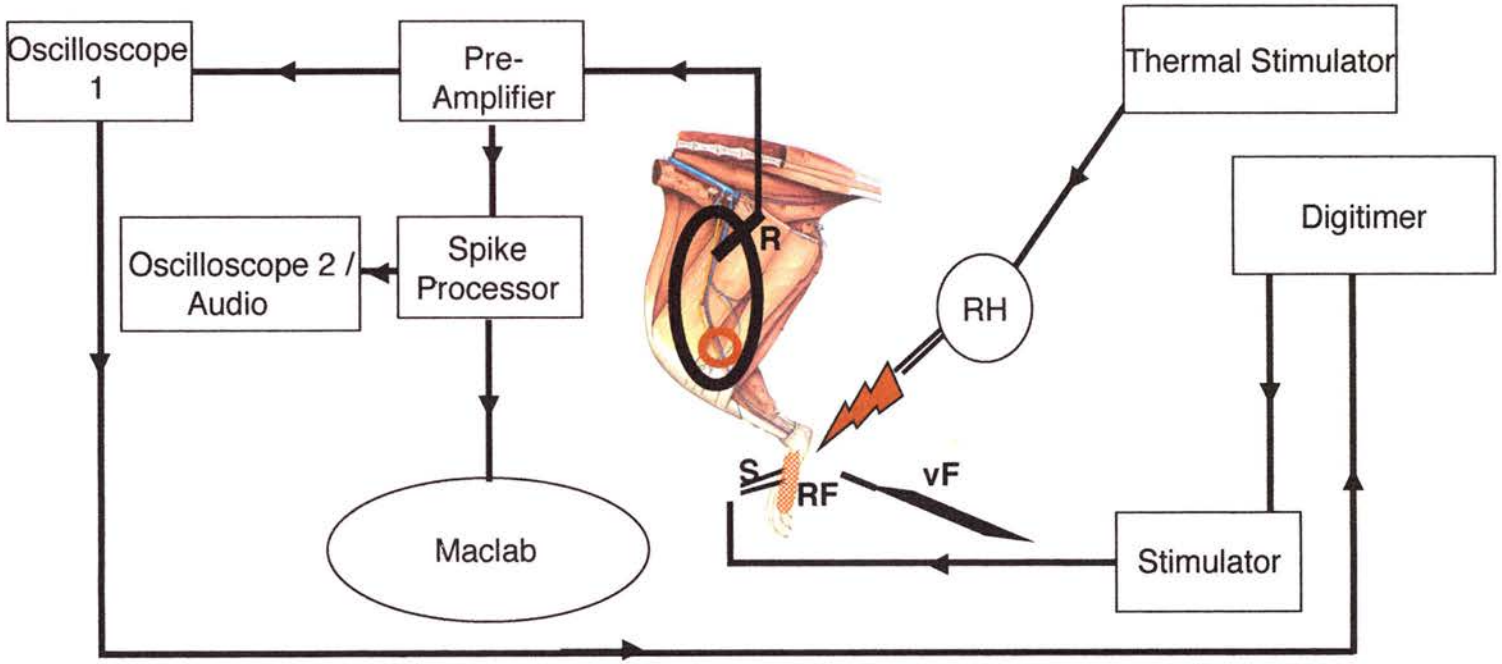


Figure 3.4 Schematic diagram of the electrophysiology equipment used to investigate properties of sapneous nerve folllowing lysolecithin-treatment. The Saphenous nerve was dissected (**0**) and central to the site of demyelination (o), afferent acitivity was measured via a bipolar recording electrode (R) and amplified by an AC coupled differential amplifier. Output from the amplifier was displayed on a dual beam oscilloscope (1), converted to TTL pulses using a spike processor which was connected to a) a second oscilloscope (2) with audio display and b) the Powerlab/McLab Chart v3.6/s programme (Maclab). To stimulate the nerve, for thermal threshold recordings, a calibrated temperature source controlled the radiant heat source (RH) onto the receptive field (RF) in a time dependant and temperature dependant manner. For mechanical threcholds, von Frey (vF) filaments were applied to the RF. Conduction velocities were measured using electrical excitation of receptive fields using bipolar needle stimulating electrodes, a Digitimer D100 triggered an isolated stimulator to deliver square-wave pulses (range 0.5 - 1.0 ms, 0.1 - 100 volts) at levels selected to be just above threshold for few-unit preparations via the stimulating electrode (S).

3.4.3 Characterisation of peripheral receptive fields

The hind paw of the treated animal was secured by the lateral two toes to a footplate using plaster of Paris coated gauze and the receptive field (RF) of the afferent preparation was identified by the induction of impulse activity via gentle brushing of the medial aspect of the hind paw. In some cases, a noxious pinch stimulus was also delivered to the identified RF using metal forceps to test the responsiveness of high threshold afferents. The mechano-sensitivity of sensory receptors with lower thresholds was measured using calibrated von Frey filaments and the thermal threshold with a calibrated radiant heat source (Sobair et al., 1997). Both mechanical and thermal response thresholds were defined in the central zone of the identified receptive field.

3.4.4 Conduction velocity

The conduction velocity of single identified afferent fibres was determined using bipolar electrodes and the peripheral stimulus technique (Iggo, 1958). Thereby, an electrical stimulus was delivered to the peripheral receptive field and any resulting action potential discharge was recorded from the nerve preparation (Figure 3.5). The distance between stimulating and recording electrodes, and the time taken for a spike to reach the recording electrode was measured by counting the time separation between the stimulating potential and peak action potential on the oscilloscope. The conduction velocity was calculated as distance/time. To obtain the full range of conductance velocities present, the procedure was repeated by moving the stimulating electrode to a variety of locations within the receptive field for each fibre. The percentage of axons in each conduction velocity range, from 0-3 m/s up to 18-21 m/s, was calculated and the statistical significance ($*P<0.05$) of any difference between each group determined by a Kruskal-Wallis One-Way Analysis of Variance on Ranks with all pairwise multiple comparisons procedure (Dunn's Method).

All recording traces of spike activity were obtained via a direct link of the oscilloscope to the Powerlab/McLab Chart v3.6/s programme (AD Instruments Ltd, Oxford, UK). This was subsequently analysed on the Claris Works 4.0 illustrator programme (Claris Corp, Smart Computing, Lincoln, NE).

Figure 3.5. Electrophysiology preparation for recording conduction velocities of afferent fibres in the saphenous nerve. The conduction velocity of single identified afferent fibres was determined using bipolar electrodes and the peripheral stimulus technique (Iggo, 1958). An electrical stimulus (S) is delivered to the peripheral receptive field (RF) and any resulting action potential discharge recorded from the nerve preparation (R).

3.5. Immunohistochemistry

3.5.1 Tissue preparation

For fixed-tissue immunohistochemistry, 6 week old *Prx*-null and wild-type littermate animals, and animals which had 13 days previously had lysolecithin treatment of the right sciatic nerve or saphenous nerve, were deeply anaesthetised with halothane and perfused through the heart with heparinised (Sigma) saline followed by 4% paraformaldehyde (Sigma) in 0.1M phosphate buffer (Sigma). For, *Prx*-null and wild-type littermate animals, all lumbar DRG were removed under a light microscope. For lysolecithin-treated animals, the L3, L4, L5 and L6 DRG, ipsilateral and contralateral to the lysolecithin-treated nerve or sham-operated nerve, were removed under a light microscope. All DRG were post-fixed with 4% paraformaldehyde for 2 hours, transferred through increasing concentrations of sucrose (Sigma) in 0.1M phosphate buffer for 1 hour and left overnight in 25% sucrose in 0.1M phosphate buffer. The DRG were then embedded in OCT mounting medium (Tissue Tek, UK), frozen over liquid nitrogen and stored at –70°C.

For non-perfused tissue immunohistochemistry, 6 week old *Prx*-null and wild-type littermate animals, and animals which had undergone lysolecithin treatment of the sciatic or saphenous nerve 13 days previously were euthanised by CO_2 inhalation. The same DRG were removed as in the fixed animals but were snap frozen over liquid nitrogen and embedded in OCT mounting medium. Cryostat sections of all DRG (10 μm) were thaw-mounted onto glass slides, which were pre-coated with poly-L-lysine (Merck-BDH).

For saphenous nerve-treated animals L3, L4 and L5 DRG were analysed, for sciatic-treated animals and for *Prx*-null and wild-type littermate animals, L4, L5 and L6 DRG were analysed.

3.5.2 Immunohistochemistry procedure

For localisation of the Na^+ channel, SNS 1/Na_v 1.8 or the peptides CGRP or NPY with the A-fibre cell body marker, neurofilament 200 kDa (NF-200) (Lawson and Waddell, 1991;Michael and Priestley, 1999) or the C-fibre cell body marker, peripherin (Goldstein et al., 1991), non-perfused DRG sections were immersion-fixed for 15 minutes with 4% paraformaldehyde in phosphate-buffered saline. Sections were pre-incubated in buffer (0.1M PBS, pH 7.4, containing 0.2% Triton X-100 (Sigma) and 2% gelatine (Sigma)) containing 10% normal goat serum (Diagnostics Scotland (DS), Edinburgh, UK) or 10% normal donkey serum (DS) (only for CGRP detection) for 1hr at room temperature and then incubated with primary antibodies diluted in buffer containing 4% goat or donkey serum overnight at 4°C.

Perfused tissue was used for detecting any expression of the brain type III Na^+ channel/$Na_v1.3$, the peptide galanin or the transcription factor ATF3 (Tsujino et al., 2000) with the A-fibre marker, neurofilament 200 kDa (NF-200}. The immunohistochemical procedure used was as for fresh tissue, omitting the immersion fixation step.

3.5.3 Antibodies

Antibodies were used at the following concentrations: rabbit polyclonal anti-SNS 1/$Na_v1.8$ (1:200; K107 supplied by S.Tate, GlaxoSmithKline, UK; the specificity of K107 having been tested by pre-incubating primary antibody with dilutions of the relevant antigenic peptide, (Amaya et al., 2000); rabbit polyclonal anti-brain type III sodium channel/$Na_v1.3$ (1:200, Alomone Labs, Jerusalem, (Black et al., 1999); sheep polyclonal anti-CGRP (1:1500; Affiniti, Exeter, UK, (Todd, 1997); rabbit polyclonal anti-substance P (1:1000 Affiniti) rabbit polyclonal anti-NPY (Peninsula Laboratories Inc, Belmont, CA 1:1000,(Polgar et al., 1999); rabbit polyclonal anti-galanin (1:2000; Advanced Chemtech Ltd, Cambridgeshire, UK); rabbit polyclonal anti-VIP (human, porcine,rat) (1:400; Peninsula Laboratories Inc, San Carlos, CA, USA); rabbit polyclonal anti-VIP (1:400, Incstar, Corp, Stillwater, MN,USA); rabbit polyclonal anti-ATF3 (1:400; Santa Cruz Biotechnology, Santa Cruz, CA); mouse monoclonal anti-neurofilament 200kDa (1:2000; clone N52; Sigma, (Bennett et al., 1998c); and mouse monoclonal anti-peripherin (1:1000; Chemicon International Ltd, Harlow, UK, (Amaya et al., 2000). Sections were then washed in buffer and incubated for 2 hours at room temperature with the appropriate secondary antibodies linked to either tetramethyl rhodamine isothiocyanate (TRITC) (goat anti-mouse-TRITC, 1:200; to detect all mouse primary antibodies; Southern Biotechnology Associates, Birmingham, Alabama) or fluorescein isothiocyanate (FITC) [goat anti-rabbit-FITC, 1:200; to detect all goat primary antibodies; (Cappel, ICN Biomedical, CA, USA) or donkey anti-sheep-FITC, 1:100; to detect anti-CGRP primary antibody; (Jackson ImmunoResearch Laboratories, Westgrove, PA)]. Three final washes in 0.1M PBS were conducted before cover-slipping with Vecta-Shield (Vector Laboratories, Burlingame, CA) for analysis. Control sections were processed as above omitting the primary antibodies. Observations were made and sections photographed on a Leitz microscope equipped for epifluorescence. Counts of profiles labelled for immunopositive cells were performed on 3-4 randomly selected 10 μm sections (separation of 100 μm) from each of four animals in each group and only neurons with clear nuclei were counted. Results were expressed either as a proportion of labelled profiles per section or the proportion per total number of single or double-labelled profiles from all 12 sections. The statistical significance of differences between groups was tested by Kruskal-

Wallis One-Way ANOVA on Ranks with all pairwise multiple comparison procedures (Dunn's Method).

3.6. Intrathecal administration of drugs

Animals which had previously had focal demyelination induced in the saphenous nerve via topical application of lysolecithin, were used for all intrathecal injection experiments. 6 week old *Prx*-null and wild-type littermate animals were also used for investigations using intrathecal injection of the NMDA receptor antagonist (R)-CPP. For all injections, baseline measurements for mechanical allodynia and thermal hyperalgesia were recorded over a period of up to 2 hours prior to injection. The mice were briefly anaesthetised with halothane and O_2 and injected intrathecally at the L4 level of the spinal cord using a 25Gx1 needle (Terumo) with the pharmacological agent of interest or the appropriate vehicle control. Injections were performed blinded to the pharmacological agent or vehicle used. Each drug was injected into at least 6 separate animals. A maximum of 4 animals were injected at one time and no individual animal was used on 2 consecutive days of injections.
To determine the effects of each drug on both mechanical allodynia and thermal hyperalgesia, behavioural reflex testing commenced 15 minutes following injection to allow recovery from anaesthesia and continued every 5 minutes thereafter until readings returned to baseline levels. For all drugs tested, the statistical significance ($p<0.05$) of any difference between ipsilateral and contralateral paw withdrawal thresholds following lysolecithin treatment or, between *Prx*-null and wild-type littermate paw withdrawal thresholds was tested by a Mann-Whitney Rank Sum test for mechanical stimulation or a paired Student's t-test for thermal stimulation. Any statistically significant difference ($p<0.05$) of the post-injection paw withdrawal from baseline paw withdrawal thresholds was determined by a One-Way Repeated Measures Analysis of Variance (ANOVA) with Dunnet's multiple comparisons versus control group post hoc analysis. Extensive control studies have shown that intrathecal injection of the vehicles, saline or 0.1-0.5% dimethylformamide (dmf) in saline have no effect on these behavioural reflex measures. Furthermore, dye injections using Pontamine Sky Blue (BDH) demonstrated that over various time periods up to 60 minutes after injection, there was no spread of dye to the PNS.

3.6.1 NMDA receptor antagonist

In lysolecithin-treated animals and *Prx*-null and wild-type littermate animals, the selective NMDA receptor antagonist (R)-CPP (3-((R)-2-carboxypiperazine-4-yl)-propyl-1-phosphonic acid), (Tocris Cookson Ltd, Bristol, UK) was injected at a concentration of 100 pmol in 10 µl saline.

3.6.2 mu-opioid receptor agonist

The selective mu-opioid receptor agonist, DAMGO (D-Ala_2, $MePhe_4$, Gly-olenkephalin) (Tocris) was injected at a concentration of 10 pmol in 10 µl saline

3.6.3 NFκB -pathway inhibitors

The actions of three structurally different NFκB pathway inhibitors were investigated;

a) parthenolide (Alexis, Exeter, UK) was injected at a concentration of 0.3 nmol in 10 µl saline with 0.3% dimethylformamide (dmf) (Sigma), b) caffeic acid phenethyl ester (CAPE) (Calbiochem, CN Bioscience, Nottingham,UK) was injected at a concentration of 0.3 nmol in 10 µl saline and c) sulphasalazine (Sigma) was injected at a concentration of 5nmol in 10 µl saline with 0.5% (dmf).

3.6.4 Cannabinoid receptor agonists

The mixed CB_1/CB_2 cannabinoid receptor agonist WIN 55,212-2 (mesylate (R)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone), (Tocris Cookson Ltd), was injected at a concentration of 60 pmol in 10 µl saline with 0.02% dmf. The highly selective CB_1 receptor antagonist AM 251 (N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,3-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide) (Tocris.), was injected at a concentration of 100 pmol in 10 µl saline with 0.3% dmf. Finally, a combination of WIN 55,212-2 plus AM 251 was injected at a concentration of 100 pmol in 10 µl saline with 0.3% dmf.

CHAPTER 4. CHANGES IN THE PERIPHERAL NERVOUS SYSTEM IN MODELS OF PERIPHERAL NERVE DEMYELINATION

4.1 Results

4.1.1. Behavioural Deficit

a) *Prx*-null mice

Investigations into the behavioural reflex sensitivities of 6 week old *Prx*-null mice, demonstrated that these mice have significantly lower thresholds for hindpaw withdrawal responses to mechanical (Fig 4.1a(i)) and noxious thermal (Fig 4.1 b(i)) stimuli, indicating the presence of heightened reflex sensitisation indicative of thermal hyperalgesia and mechanical allodynia, (Gillespie et al., 2000). Therefore, it was of interest to investigate if similar, or more pronounced, changes were apparent in 6 month old Prx-null mice by which age morphological derangement of myelinated nerves is more marked (Gillespie et al., 2000).

At 6 months of age, *Prx*-null mice display significantly lower thresholds when compared to the wild-type in reflex tests of cutaneous mechanical sensation using calibrated von Frey filaments (Fig 4.1a(ii)) and of thermal hyperalgesia using an infra-red beam applied to the mid plantar region of the hind paw (Fig 4.1 b(ii)) (Fig 4.1 c,d). Therefore, at 6 months the *Prx*-null mice display a heightened reflex sensitisation, similar to that displayed at 6 weeks of age, indicative of mechanical allodynia and thermal hyperalgesia.

b) Lysolecithin-induced demyelination

Mice which previously had topical application of lysolecithin to the saphenous or sciatic nerve displayed a markedly lowered ipsilateral threshold in reflex tests of cutaneous mechanical sensitisation using calibrated von Frey filaments (Fig 4.2a) and of thermal nociceptive sensitivity (Fig 4.2 c). The heightened reflex sensitivity to both stimuli was apparent from day 5 after lysolecithin treatment of saphenous or sciatic nerve but reached peak values from days 9 until day 15 post surgery. The threshold in both tests recovered to baseline levels by 23 days post treatment. Reflex responses from the contralateral hind paw of lysolecithin-treated mice showed no changes following treatment. Sham-operated mice showed no change from baseline reflex values following surgery (Fig 4.2b,d).

Figure 4.1. Development of mechanical allodynia and thermal hyperalgesia in 6 week and 6 month old *Prx*-null mice.

a) Minimum filament indentation pressure thresholds for paw withdrawal responses to mechanical stimulation with von Frey filaments were measured for (i) 6 week old (n=8) and (ii) 6 month old *Prx*-null mice (□) (n=8) and wild-type (WT) littermates (◆) (n=8). Baseline measurements were obtained at 2-day intervals. Statistical significance of differences between *Prx*-null and wild-type (WT) thresholds (*P<0.05) was determined by a Mann-Whitney Rank Sum test. Each value is the mean ± SEM.

b) The time taken for hindpaw withdrawal from a noxious thermal stimulus was measured for (iii) 6 week old (n=8) and (iv) 6 month (n=8) old *Prx*-null mice (□) and wild-type (WT) littermates (◆) (n=8). Baseline measurements were obtained at 2-day intervals. Statistical significance of differences between *Prx*-null and wild-type (WT) thresholds (*P<0.05) was determined by a paired Student's t-test. Each value is the mean ± SEM.

Figure 4.1.

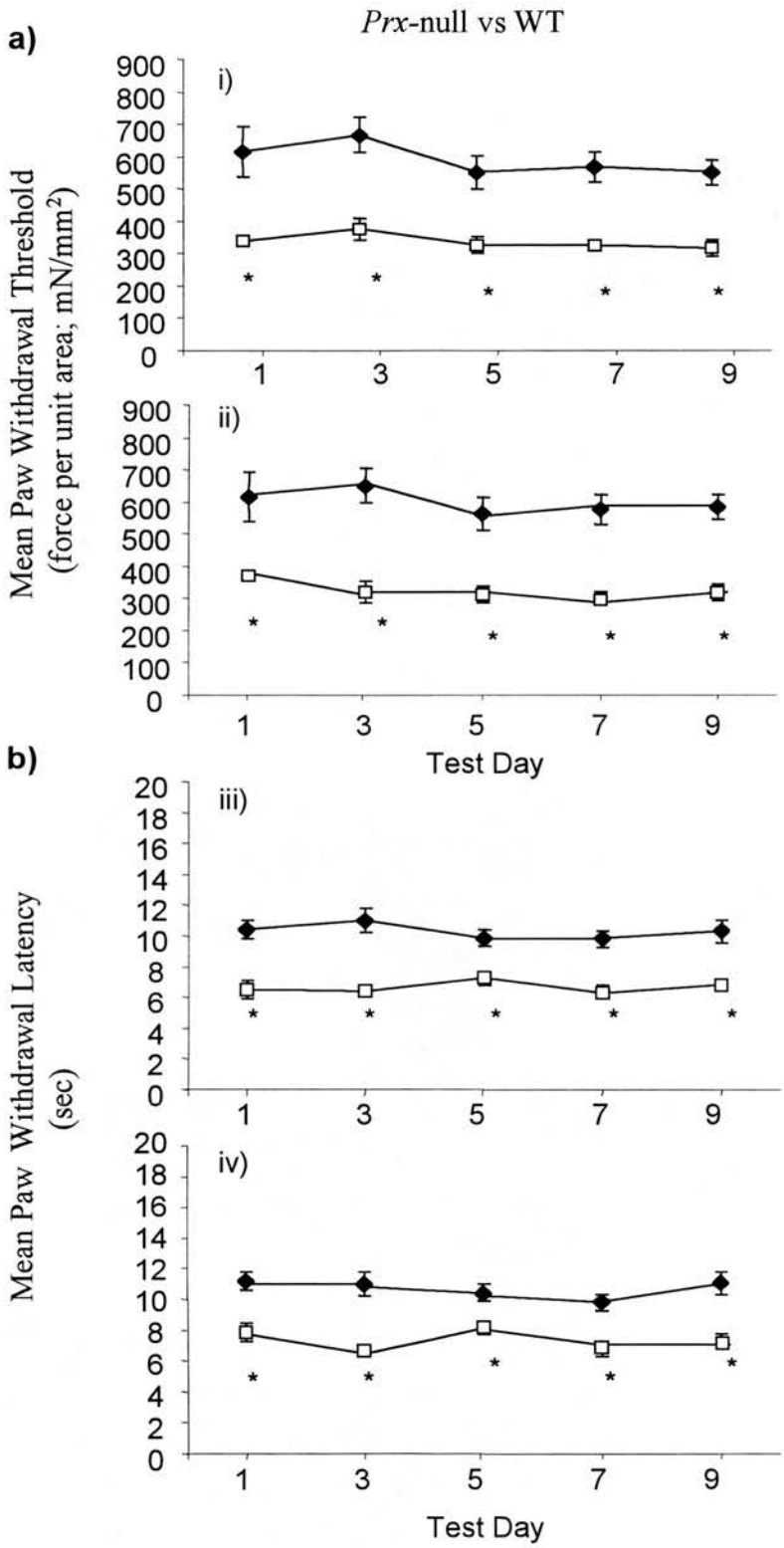

a)
Prx-null vs WT
Mean Paw Withdrawal Threshold (force per unit area; mN/mm²)
i)
ii)
b)
Mean Paw Withdrawal Latency (sec)
iii)
iv)
Test Day
Test Day

Figure 4.2. Development of mechanical allodynia and thermal hyperalgesia ipsilateral to lysolecithin treatment of the saphenous or sciatic nerve.

a) Minimum filament indentation pressure thresholds for repeat paw withdrawal responses to mechanical stimulation with von Frey filaments in conscious animals were measured for C57/BL6 mice following lysolecithin treatment (↑) to (i) one saphenous (n=6) or (ii) one sciatic nerve (n=6). Statistical significance of differences between ipsilateral (□) and contralateral (◆) paws (*P<0.05) was determined by a Mann-Whitney Rank Sum test. Each value is the mean ± SEM.

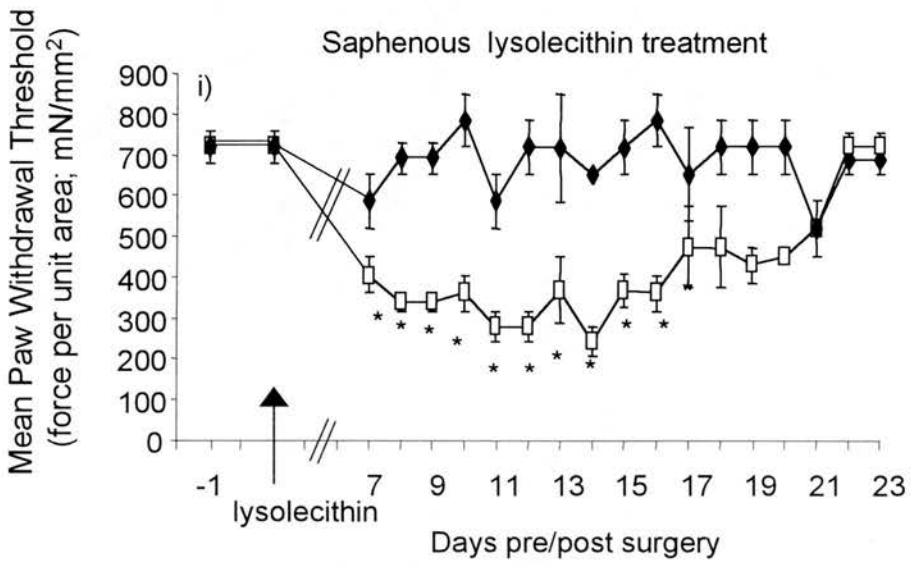
b) Minimum filament indentation pressure thresholds for repeat paw withdrawal responses to mechanical stimulation with von Frey filaments in conscious animals were measured for C57/BL6 mice following sham treatment (↑) to (i) one saphenous (n=4) or (ii) one sciatic nerve (n=4) commencing on post treatment day 5. Statistical significance of differences between ipsilateral (□) and contralateral (◆) paws (*P<0.05) was determined by a Mann-Whitney Rank Sum test. Each value is the mean ± SEM.

c) The time taken for hindpaw withdrawal from a noxious thermal stimulus was measured for C57/BL6 mice following lysolecithin treatment (↑) to (i) one saphenous (n=6) or (ii) one sciatic nerve (n=6). Statistical significance between ipsilateral (□) and contralateral (◆) paws (*P<0.05) was determined by a paired Student's t-test. Each value is the mean ± SEM.

d) The time taken for hindpaw withdrawal from a noxious thermal stimulus was measured for C57/BL6 mice following sham treatment (↑) to (i) one saphenous (n=4) or (ii) one sciatic nerve (n=4). Statistical significance between ipsilateral (□) and contralateral (◆) paws (*P<0.05) was determined by a paired Student's t-test. Each value is the mean ± SEM.

Figure 4.2.

a)


Saphenous lysolecithin treatment
i)
Mean Paw Withdrawal Threshold (force per unit area; mN/mm²)
900
800
700
600
500
400
300
200
100
0
-1
lysolecithin
7
9
11
13
15
17
19
21
23
Days pre/post surgery

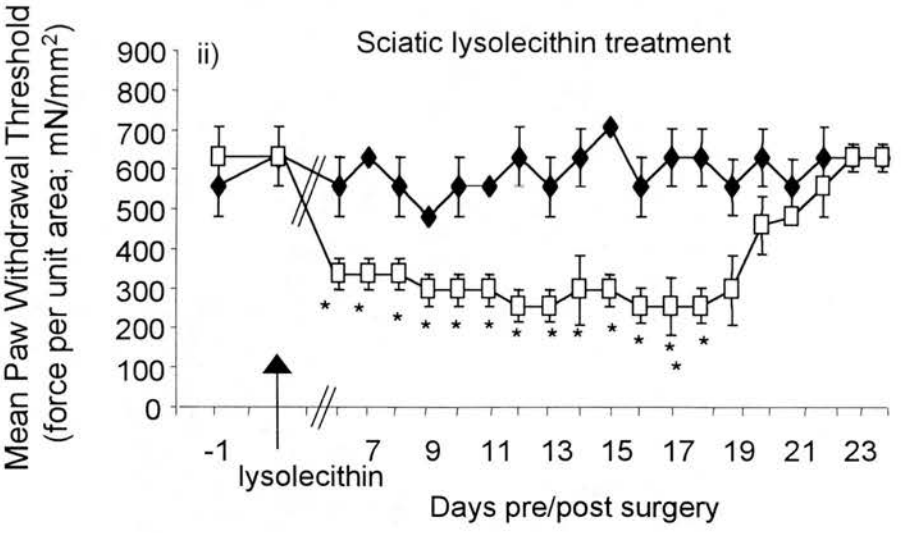

Sciatic lysolecithin treatment
ii)
Mean Paw Withdrawal Threshold (force per unit area; mN/mm²)
900
800
700
600
500
400
300
200
100
0
-1
lysolecithin
7
9
11
13
15
17
19
21
23
Days pre/post surgery

Figure 4.2. cont...

b)

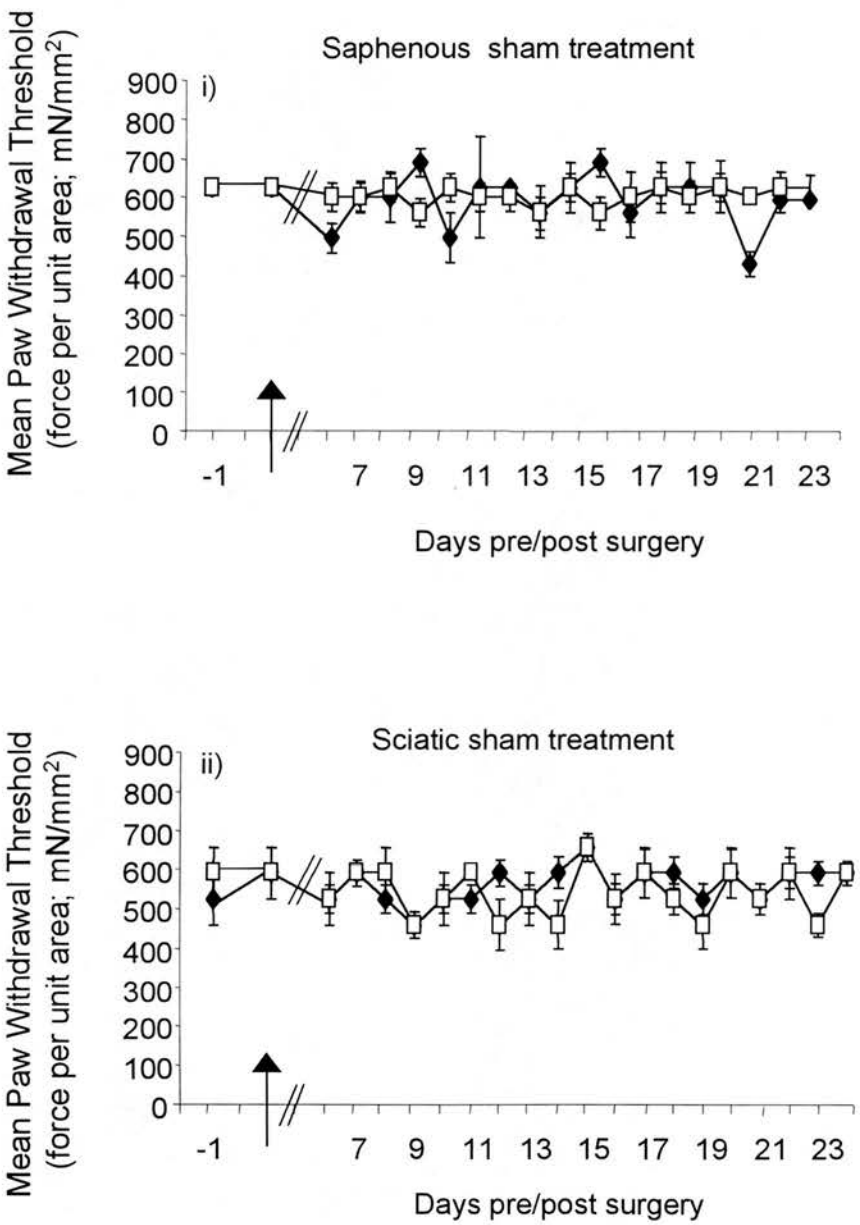
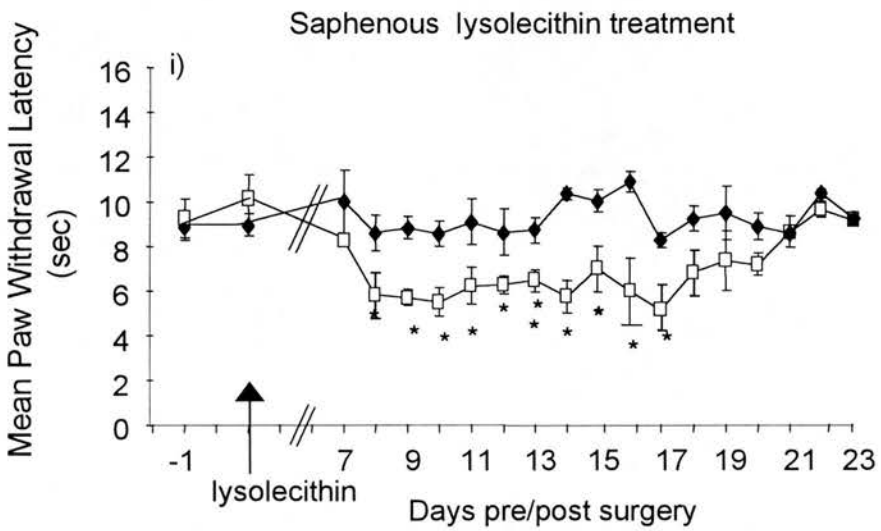


Figure 4.2. cont...

c)



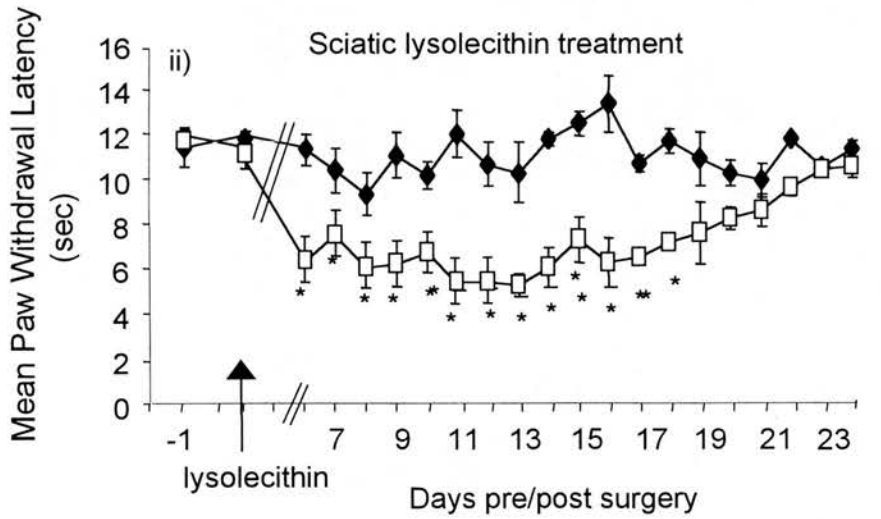
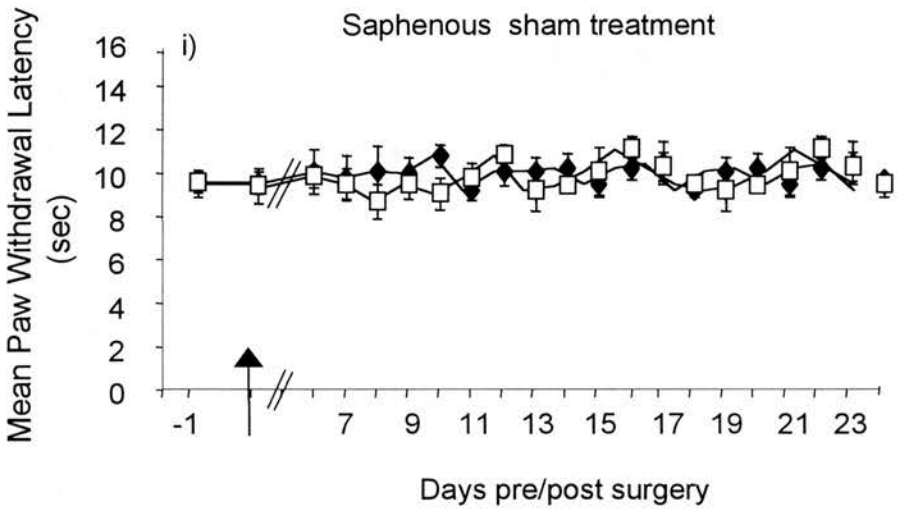
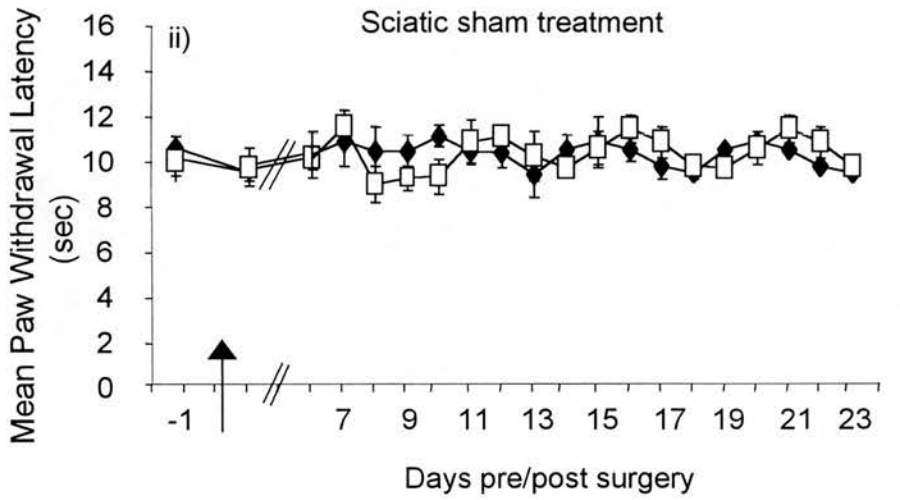


Figure 4.2. cont...

d)





4.1.2. Morphology of the lysolecithin-treated nerve

To determine the extent of demyelination as a result of lysolecithin treatment of the saphenous or sciatic nerve, we examined the treated region of the peripheral nerve (determined at time of embedding) of demyelinated, sham and naïve mice by light and electron microscopy at peak behavioural change (post-treatment day 11-13) and at behavioural recovery time (post-treatment day 23).

Method 1: Microinjection of lysolecithin

It was clear from LM and EM images of the sciatic nerve treated by microinjection of lysolecithin via a glass micropipette that structural damage of the axons had occurred. Therefore, we continued no further with our investigations of this model as it is of critical importance that there is no traumatic damage to axons in any model designed to assess demyelination-induced changes Therefore, from this point onwards, any reference to lysolecithin-treated nerves relates to those treated topically with lysolecithin (Method 2).

Method 2: Topical application of lysolecithin

a) Saphenous nerve: On days 11-13 post lysolecithin treatment, 40% of A-fibres in the saphenous nerves were completely demyelinated as compared to the contralateral nerve and many remaining myelinated fibres had myelin of reduced thickness. This was observed initially by light microscopy (Fig 4.3a(i,ii)) and then calculated from the number of myelinated fibres or formerly myelinated fibres present in each EM image (representing 292μm^2 of the nerve). Lysolecithin-treated saphenous nerves (n=3) contained 4.9 $\pm$ 0.9 myelinated axonal profiles per image (155 myelinated axonal profiles over 33 images analysed) and control nerves (n=3) contained 8.2 $\pm$ 1.7 myelinated axonal profiles per image (261 myelinated axonal profiles over 32 images analysed). Samples were taken from the entire nerve of 3 animals per group. The G-ratio, a relative measure of myelin thickness as a ratio of the axonal diameter to overall diameter (1:1 indicating no myelin sheath) was calculated for the remaining myelinated A-fibres following treatment with lysolecithin. The G-ratio significantly increased in the demyelinated nerve from 0.61 $\pm$ 0.01 in naïve nerves to 0.76 $\pm$ 0.01 in demyelinated nerves (Fig 4.2b) indicating a general decrease in myelin thickness of remaining myelinated fibres. There was no significant difference in the level of myelination in sham-operated animals or in recovery (day 23) animals from control naïve animals (Fig 4.3a,b) (*$P<0.05$) as determined by Kruskal-Wallis One-Way Analysis of Variance on Ranks with an all pairwise multiple comparisons procedure (Dunn's Method).

Figure 4.3. Effects of lysolecithin treatment on the myelination state of the saphenous nerve.

a) Resin sections (1μm) from the (i) ipsilateral and (ii) contralateral saphenous nerves of lysolecithin-treated animals on post-treatment day 13, (iii) ipsilateral saphenous nerve of lysolecithin-treated animals on post treatment day 23 (recovery), (iv) ipsilateral saphenous nerve of sham-treated animals post-treatment day 13 were analysed by light microscopy (n=3 in each case). Post lysolecithin treatment day 13 ipsilateral nerves display complete demyelination in about 40% of A-fibres with partial demyelination of many remaining myelinated fibres. Contralateral nerves from post lysolecithin treatment day 13, lysolecithin-treated nerves on post-treatment day 23 and nerves ipsilateral to sham treatment all appeared to be morphologically normal with consistent levels of myelination across the nerve. Scale bar: 10μm.

b) Quantification of the degree of myelination of A-fibres following lysolecithin treatment to the saphenous nerve was determined as a G-ratio from electron microscope images of (i) the contralateral and (ii) ipsilateral saphenous nerves of lysolecithin-treated animals on post-treatment day 13 and (iii) ipsilateral saphenous nerve of sham-treated animals post-treatment day 13 (n=3) in each case). Results are shown as the G-ratio of each fibre as a function of the axon diameter (i-iii) and the data are summarised as bar charts below (iv). The data show a general decrease in myelin thickness i.e. increased G-ratio following lysolecithin treatment, but not sham surgery. Statistical significance (*$p<0.05$) between conditions was determined by Kruskal-Wallis One Way Analysis of Variance on Ranks with an all pairwise multiple comparisons procedure (Dunn's Method).

Figure 4.3.

a)

Saphenous
lysolecithin treatment

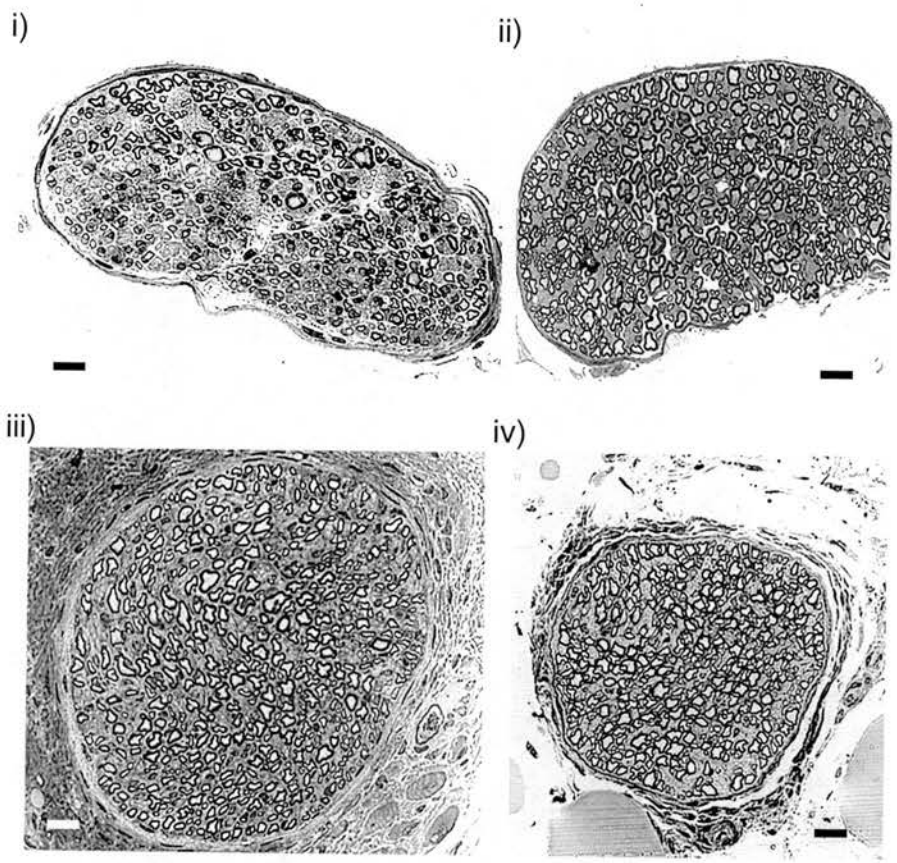
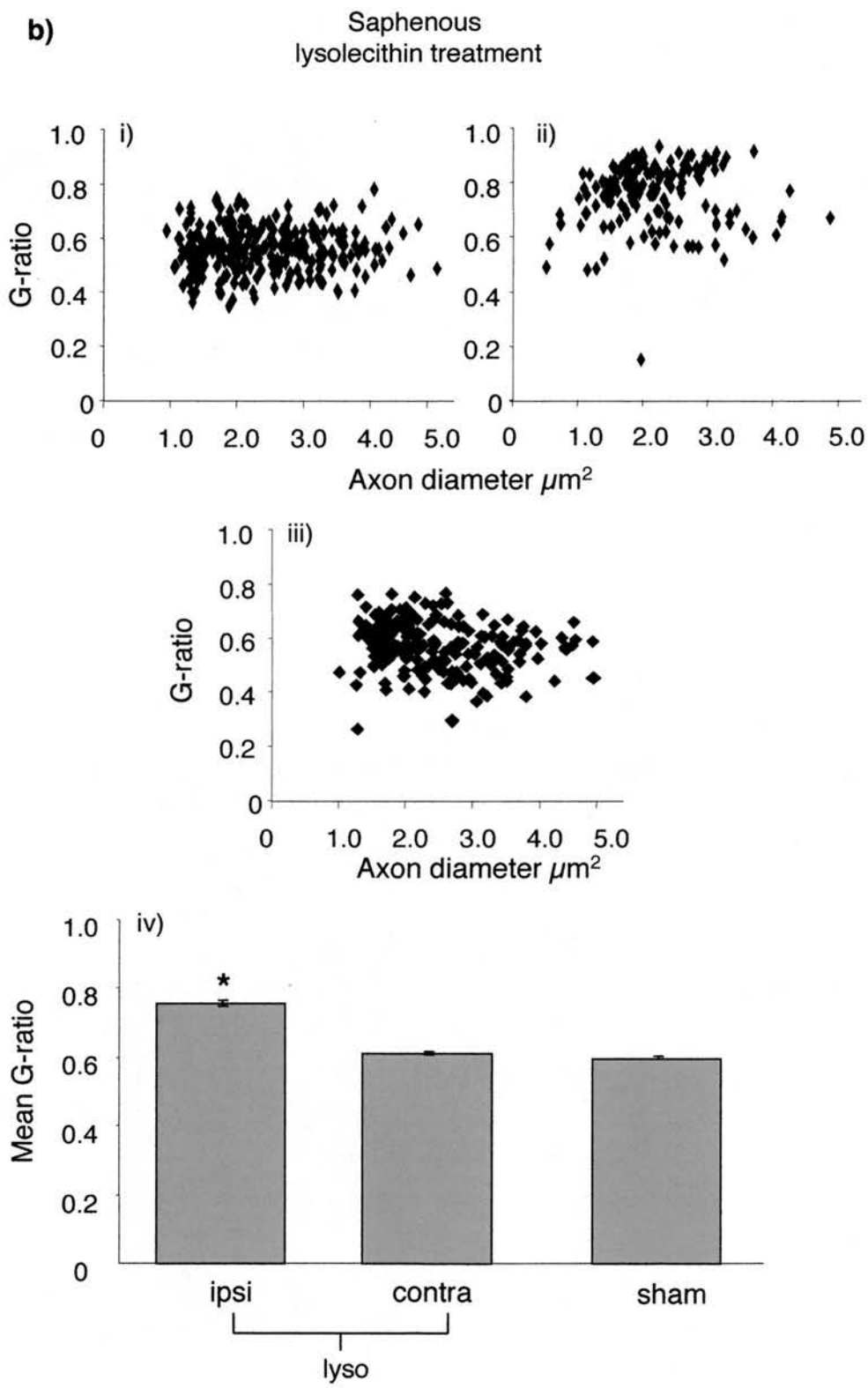


Figure 4.3. cont...

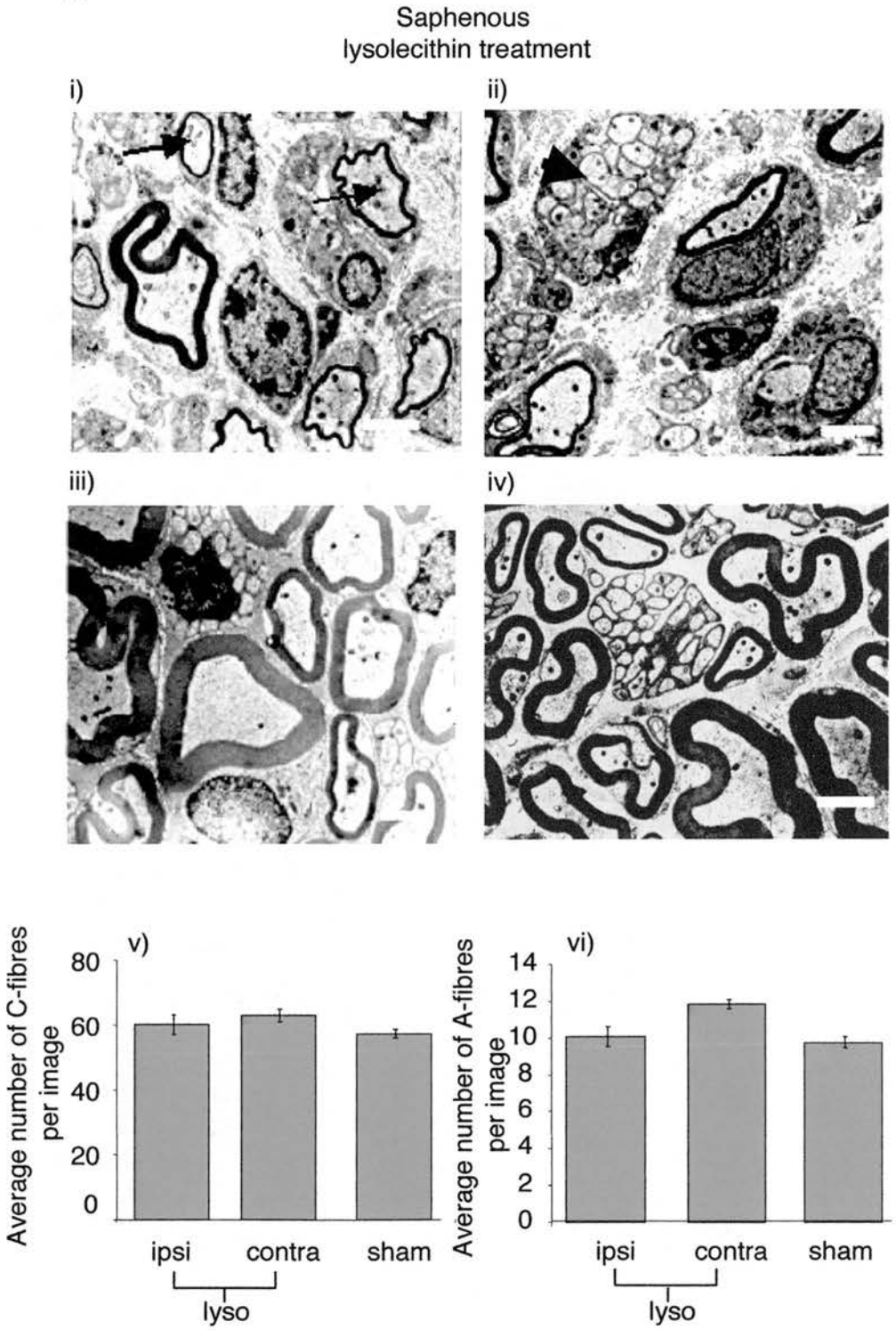


Electron microscopy (Figure 4.4) demonstrated that lysolecithin-treated saphenous nerves contained bundles of myelin debris and macrophages, which are involved in the process of myelin debris removal from the site and have been observed in other models of peripheral nerve demyelination (Carenini et al., 2001;Smith, 1999). However, unmyelinated C-fibre bundles appeared to be morphologically normal and fibre counts revealed that there was no statistically significant decrease in number of unmyelinated C-fibres (lysolecithin-treated nerve contained on average 60.1 ± 2.0 C-fibres per area sampled compared to 62.9 ± 1.9 in the control nerve and 57.3 ± 1.3 in sham control treated nerve) following treatment with lysolecithin. Similarly there was no detectable decrease in the number of larger A fibres that were either myelinated or demyelinated (lysolecithin-treated nerves contained on average 10.1 ± 0.5 A-fibres per area sampled compared to 11.8 ± 0.2 in the control nerve and 9.7 ± 0.3 in sham-treated nerves) (*$P<0.05$). Analyses were performed by Kruskal-Wallis One-Way Analysis of Variance on Ranks with an all pairwise multiple comparisons procedure (Dunn's Method) indicating that the treatment with lysolecithin does not affect the integrity of the axons (Figure 4.4).

Figure 4.4. Effects of lysolecithin treatment on afferent axons in the saphenous nerve.

Electron microscopy images of the contralateral and ipsilateral saphenous nerves of lysolecithin-treated animals on post-treatment day 13 and ipsilateral saphenous nerve of sham-treated animals post-treatment day 13 were compared (n=3 in each case). The images demonstrate typical sections from; (i & ii) the lysolecithin-treated nerve, which shows markedly reduced myelination of (i) A-fibres (arrow) while (ii) C-fibres (arrowhead) remain intact, and sections from (iii) contralateral, untreated nerve and (iv) ipsilateral sham treated nerve (x5000, Scale bar: 2 μm). Unmyelinated axons were identified as small diameter fibres devoid of any myelin sheath, which were present in bundles, surrounded by Schwann cell cytoplasm and were individually counted for all groups (v). A-fibres (with or without lysolecithin treatment) were identified as the larger diameter axons with myelin sheaths or smaller diameter thinly-myelinated fibres or occasionally those with no myelin which were solitary. All of these fibre types were individually counted (vi). Data for both fibre types are presented as average numbers of fibres per image (292 μm^2) ± SEM. A Kruskal-Wallis One Way ANOVA on Ranks showed no statistically significant differences between treatment groups.

Figure 4.4.


Saphenous
lysolecithin treatment
i)
ii)
iii)
iv)
v)
Average number of C-fibres per image
80
60
40
20
0
ipsi
contra
sham
lyso
vi)
Average number of A-fibres per image
14
12
10
8
6
4
2
0
ipsi
contra
sham
lyso

b) Sciatic nerve: From observations at the LM level of the sciatic nerve following topical application of lysolecithin, a similar degree of demyelination had occurred as in the saphenous nerve (Fig 4.5a). The G-ratio significantly increased in the demyelinated nerve from 0.56 ± 0.02 in naïve nerves to 0.81 ± 0.03 in demyelinated nerves (Fig 4.5b) indicating a general decrease in myelin thickness of remaining myelinated fibres. There was no significant difference in the level of myelination in sham operated animals from control naïve animals (Fig 4.5b) (*$P<0.05$) as determined by Kruskal-Wallis One-Way Analysis of Variance on Ranks with an all pairwise multiple comparisons procedure (Dunn's Method). At the EM level, as with the saphenous nerve, there was no indication of axonal damage to A- or C-fibres (Fig 4.6). As in the lysolecithin-treated saphenous nerve, electron microscopy demonstrated that lysolecithin-treated sciatic nerves contained bundles of myelin debris and macrophages, which are involved in the process of myelin debris removal from the site of demyelination. Unmyelinated C-fibre bundles appeared to be morphologically normal and fibre counts revealed that there was no statistically significant decrease in number of unmyelinated C-fibres (lysolecithin-treated nerve contained on average 45.1 ± 9.0 C-fibres per area sampled compared to 42.9 ± 8.9 in the control nerve and 39.3 ± 7.3 in sham control treated nerve) or larger A-fibres that were either myelinated or demyelinated (lysolecithin-treated nerves contained on average 6.8 ± 1.5 A-fibres per area sampled compared to 7.9 ± 2.2 in the control nerve and 6.7 ± 3.3 in sham-treated nerves) (*$P<0.05$) as determined by Kruskal-Wallis One-Way Analysis of Variance on Ranks indicating that the treatment with lysolecithin does not affect the integrity of the axons (Figure 4.6).

Figure 4.5. Effects of lysolecithin treatment on the myelination state of the sciatic nerve.

a) Resin sections (1 μm) from the (i) ipsilateral and (ii) contralateral sciatic nerves of lysolecithin-treated animals on post-treatment day 13 were analysed by light microscopy (n=3 in each case). Post lysolecithin treatment day 13 ipsilateral nerves display complete demyelination in about 40% of A-fibres with partial demyelination of many remaining myelinated fibres. Contralateral, nerves appear to be morphologically normal with consistent levels of myelination across the nerve. Scale bar: 10 μm.

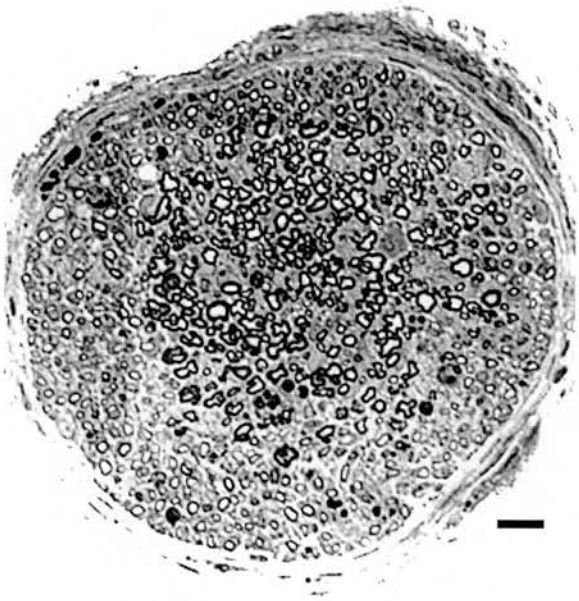
b) Quantification of the degree of myelination following lysolecithin treatment to the sciatic nerve was determined as a G-ratio from electron microscope images of (i) the contralateral and (ii) ipsilateral sciatic nerves of lysolecithin-treated animals on post-treatment day 13 and (iii) ipsilateral sciatic nerve of sham-treated animals post-treatment day 13 (n=3 in each case). Results are shown as the G-ratio of each fibre as a function of the axon diameter (i-iii) and the data are summarised as bar charts below (iv). The data show a general decrease in myelin thickness following lysolecithin treatment, but not sham surgery. Statistical significance ($*p<0.05$) between conditions was determined by Kruskal-Wallis One Way Analysis of Variance on Ranks with an all pairwise multiple comparisons procedure (Dunn's Method).

Figure 4.5.

a)

Sciatic

i)



ii)

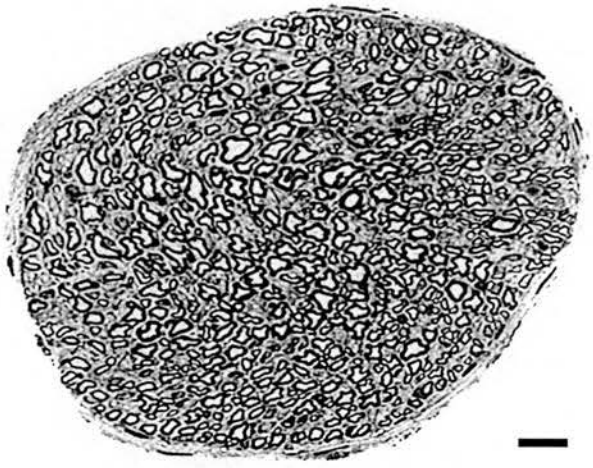


Figure 4.5. cont...

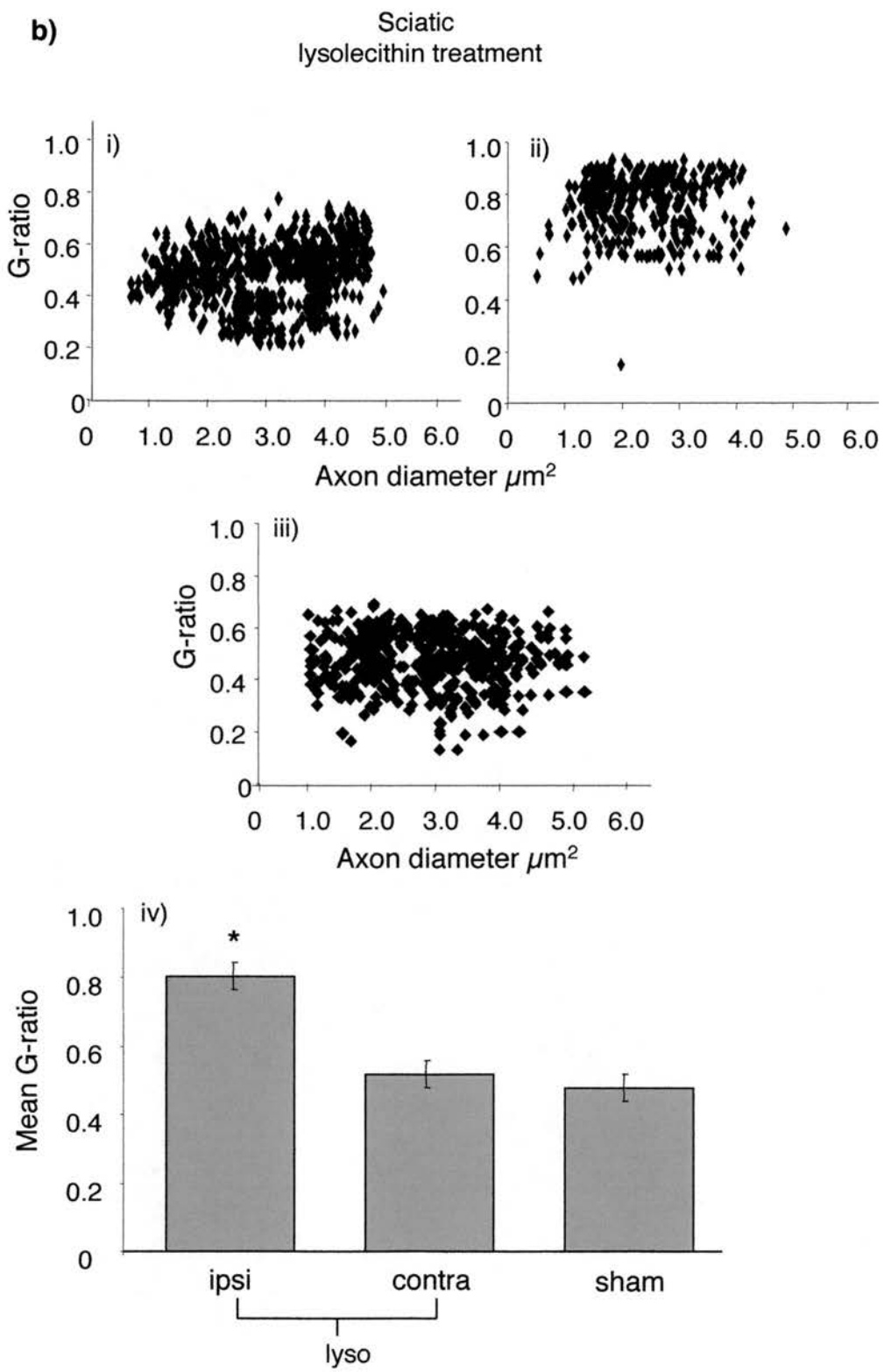
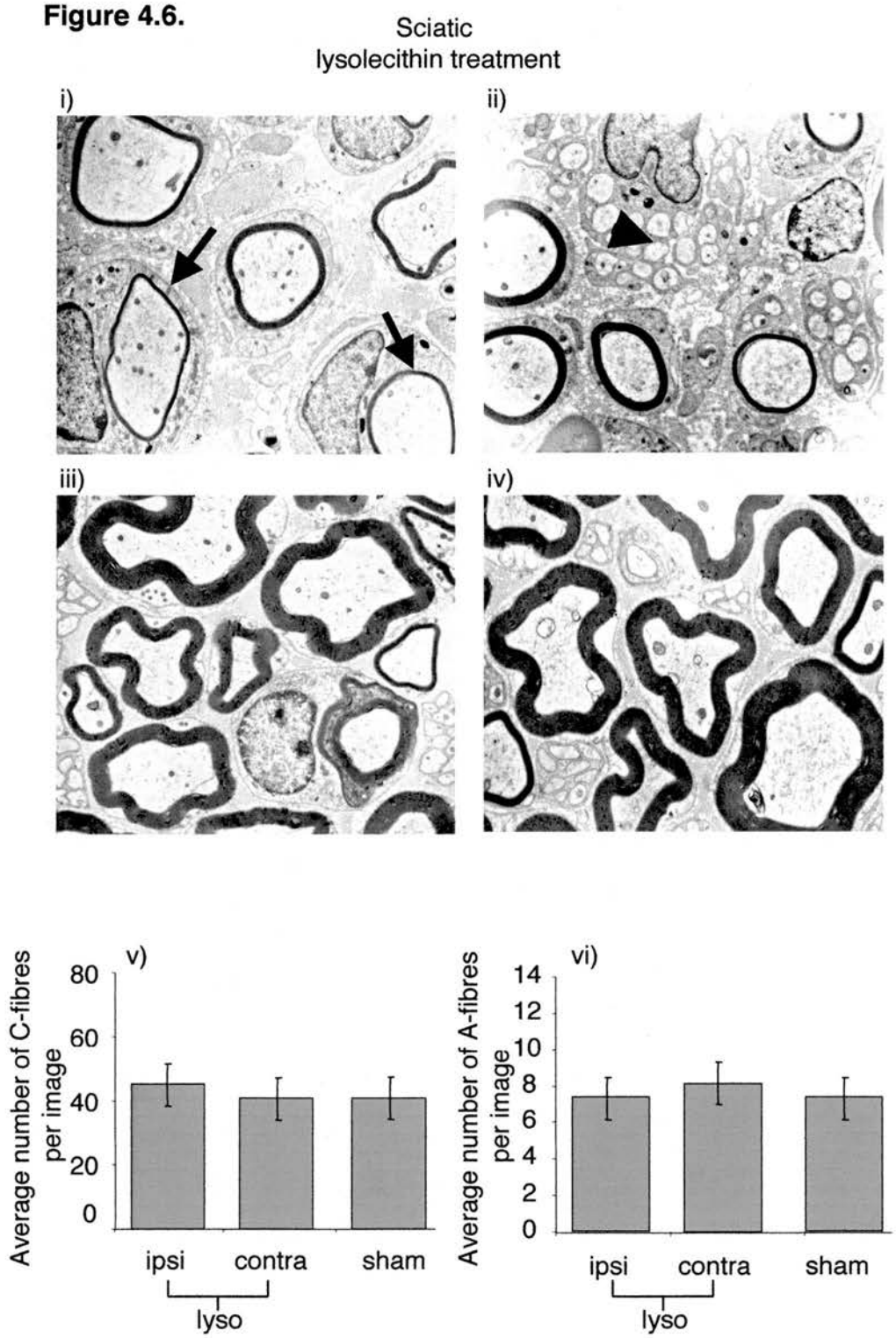


Figure 4.6. Effects of lysolecithin treatment on afferent axons in the sciatic nerve.

Electron microscopy images of the contralateral and ipsilateral sciatic nerves of lysolecithin-treated animals on post-treatment day 13 and ipsilateral sciatic nerve of sham-treated animals post-treatment day 13 were compared (n=3 in each case). The images demonstrate typical sections from; (i & ii) the lysolecithin-treated nerve, which shows markedly, reduced myelination of (i) A-fibres (arrow) while (ii) C-fibres (arrowhead) remain intact, and sections from (iii) contralateral, untreated nerve and (iv) ipsilateral sham treated nerve (x5000, Scale bar: 2 μm). Unmyelinated axons were identified as small diameter fibres devoid of any myelin sheath, which were present in bundles, surrounded by Schwann cell cytoplasm and were individually counted for all groups (v). A-fibres (with or without lysolecithin treatment) were identified as the larger diameter axons with myelin sheaths or smaller diameter thinly-myelinated fibres or occasionally those with no myelin which were solitary. All of these were individually counted (vi). Data for both fibre types are presented as average numbers of fibres per image (292 μm^2) ± SEM. A Kruskal-Wallis One Way ANOVA on Ranks showed no statistically significant differences between treatment groups.

Figure 4.6.


Sciatic
lysolecithin treatment
i)
ii)
iii)
iv)
v)
Average number of C-fibres per image
80
60
40
20
0
ipsi
contra
sham
lyso
vi)
Average number of A-fibres per image
14
12
10
8
6
4
2
0
ipsi
contra
sham
lyso

4.1.3. Electrophysiology of the lysolecithin-treated saphenous nerve.

a) 3 and 5 days following lysolecithin treatment, before development of behavioural change

At these time points, reflex testing showed no detectable change in either mechanically or thermally-evoked responses, indicating that a sensitised state had not yet developed. Correspondingly, there was no presence of any abnormal afferent fibre activity at either time point. A summary of results is displayed in Table 1.

b) Time of peak behavioural change following lysolecithin treatment.

At the time of peak behavioural change (11-13 days post treatment), the size of the peripheral receptive field and responsiveness to innocuous brush stimulation of the demyelinated saphenous nerve was normal as compared to naïve and wild-type animals. There was no significant difference between normal, sham and demyelinated nerves in the response thresholds to von Frey filament (threshold force of 8.41 mN/mm^2) (Fig 4.7a) or the response to thermal nociceptive stimulus which only produced a response to approximately 48-50°C (Fig 4.6b), indicating that any changes in peripheral transduction mechanisms are probably not responsible for the behavioural reflex sensitisation observed (Table 1). The focal nature of the demyelination and the integrity of the axons were reflected in a range of conduction velocities that were not discernibly different from those of sham and naïve nerves. The range included slow conduction velocities of less than $1ms^{-1}$ up to the fastest in the range of $20ms^{-1}$ and the number of units recorded for each conduction velocity did not differ significantly between the lysolecithin-treated, sham and naïve nerves (Fig 4.7c) ($*P<0.05$) as determined by Kruskal-Wallis One-Way Analysis of Variance on Ranks with all pairwise multiple comparisons procedure (Dunn's Method). A common feature of lysolecithin-demyelinated nerves over a range of 10-14 days post application was the presence of a spontaneous low frequency discharge (2-3 impulses/sec) (Fig 4.7d).

Table 1. Effects of topical lysolecithin treatment of the saphenous nerve on afferent fibre characteristics.

Electrophysiological recordings of peripheral nerve activity and cutaneous responsiveness were performed on animals 3, 5 (n=2 each) and 11, 12 & 13 days (n=6) following lysolecithin treatment to the saphenous nerve, 11-13 days after sham treatment (n=4) and on naïve animals (n=3). Any sensitisation of mechanical sensory receptors with high thresholds was measured using calibrated von Frey filaments and the presence of any sensitisations of thermally responsive receptors was measured with a calibrated radiant heat lamp. The conduction velocity range of afferent fibres was determined using the peripheral stimulus technique via bipolar electrodes. There was no difference in threshold for unit firing in response to mechanical or thermal stimulation or conduction velocity range between any of the groups. The lysolecithin-treated nerves, 11-13 days following treatment, displayed the presence of spontaneous afferent activity in the lysolecithin-treated nerves 11-13 days following treatment.

Table 1

	Receptive field	Spontaneous activity (imp/sec)	von Frey filament threshold (mN/mm^2)	Thermal threshold (^oC)	Conduction velocity range (ms^{-1})
Naïve (n=3)	normal	0	8.41-16.70	>48	1.4-20.0
Sham (day (11-13) (n=4)	normal	0	8.41-16.70	>48	0.9-18.2
Lysolecithin - treated (day 3-5) (n=4)	normal	0	8.41-16.70	>48	0.5-16.6
Lysolecithin - treated (day 11-13) (n= 6)	normal	2-3	8.41-16.70	>48	1.3-16.0

Figure 4.7. Effects of topical lysolecithin treatment of the saphenous nerve on afferent fibre characteristics.

Electrophysiological recording of peripheral nerve activity and function was performed on animals 3-5 days (n=2 each), 11- 13 days (n=6) following lysolecithin treatment to one saphenous nerve, 11-13 days after sham treatment (n=4) and on naïve animals (n=3). Examples of electrophysiological recordings from these nerves are displayed in each case for i) naïve, ii) sham, iii) 3-5 days post-lysolecithin, or iv) 11-13 days post-lysolecithin.

a) The mechanosensitivity of low threshold sensory receptors was tested using calibrated von Frey filaments ranging from 6.21 to 8.41 mN/mm^2. The traces shown are in response to threshold indentation pressure (8.41 mN/mm^2) in each test group. Application represented by arrow (↑).

b) The thermal sensitivity was measured with a calibrated radiant heat lamp. There was no difference in threshold for unit firing in response to thermal stimulation. Traces show the response of each nerve to a temperature range of maximum 50 °C over 10 seconds. There was no response to lower temperatures in any test group. The stimulus heat ramp is superimposed onto each trace recording to indicate the onset of threshold temperature (50°C) which is also indicated on the x-axis by an arrow (↑).

c) The lysolecithin-treated nerve of animals on days 11-13 post-treatment displayed a range of conduction velocities that were not discernibly different from those of naïve nerves (*$P<0.05$) as determined by Kruskal-Wallis One-Way Analysis of Variance on Ranks with all pairwise multiple comparisons procedure (Dunn's Method). The range included slow conduction velocities of less than $1ms^{-1}$ up to the fastest in the range of $20ms^{-1}$ and the number of units recorded for each conduction velocity did not differ significantly between the lysolecithin-treated (■), naïve (□) and sham (◧) nerves.

d) The lysolecithin-treated nerve of animals on days 11-13 post-treatment displayed spontaneous activity with a frequency of 2-3 impulses pre second, which was not observed in nerves of animals 3-5 days following lysolecithin treatment nor in the naïve nor sham-treated animals.

Figure 4.7.

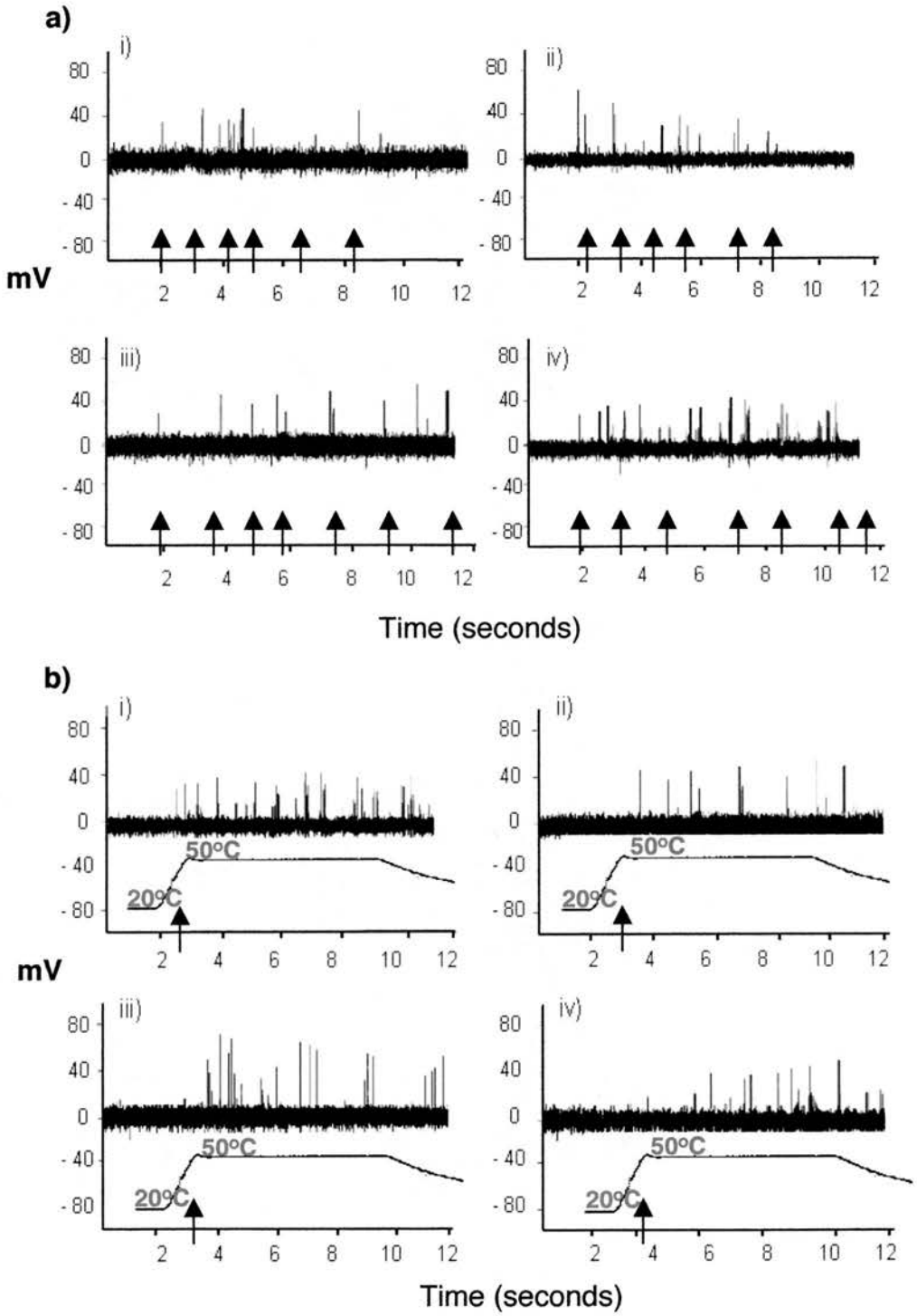
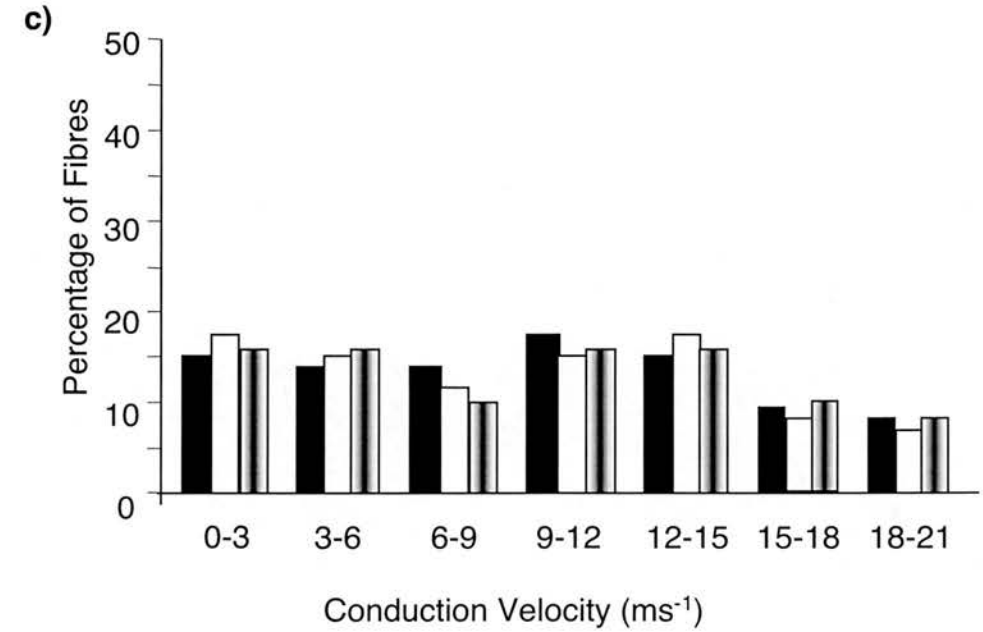
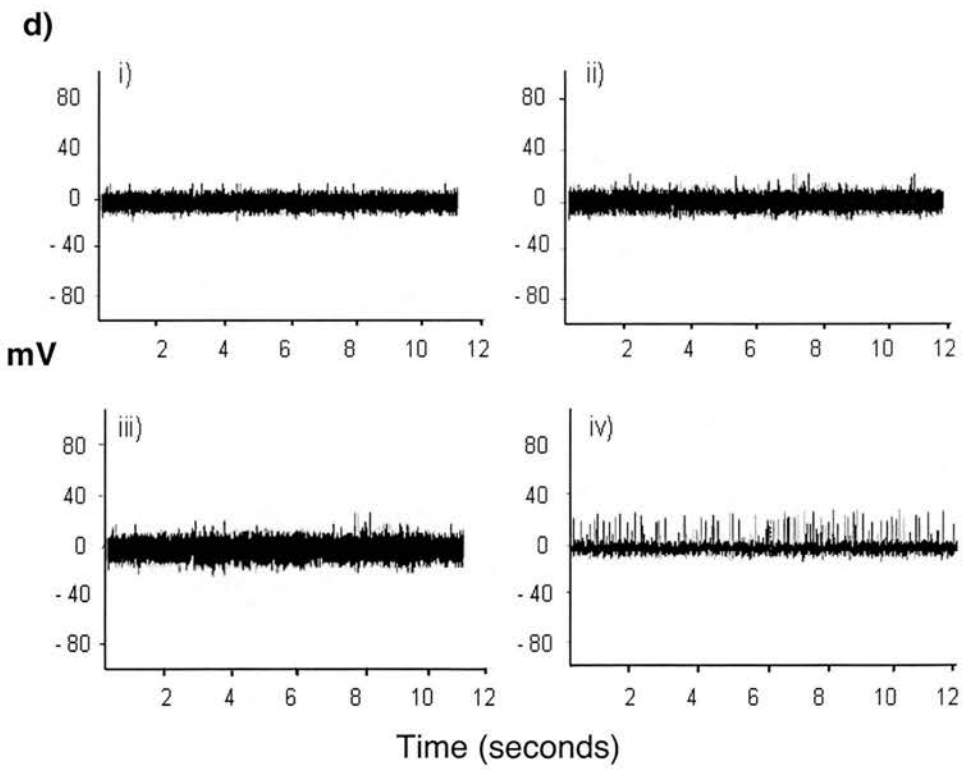

a)
i)
ii)
iii)
iv)
80
40
0
- 40
- 80
mV
2
4
6
8
10
12
Time (seconds)
b)
i)
ii)
iii)
iv)
50°C
20°C
mV
Time (seconds)

Figure 4.7. cont...





4.1.4. Immunohistochemical investigations into the phenotype of DRG cells of the *Prx*-null mouse and following lysolecithin treatment.

a) Expression of NF-200, peripherin and the indicator of axonal damage, ATF3.

In order to assess the integrity of the cell bodies of 6 week old *Prx*-null mice, 6 week old wild-type mice and of mice 13 days following lysolecithin treatment of the saphenous or sciatic nerve, we analysed the expression of NF-200, which labels the larger diameter, myelinated DRG cell population (Lawson and Waddell, 1991;Michael and Priestley, 1999) and peripherin, a type III intermediate filament, which is normally expressed selectively by unmyelinated sensory neurons (Amaya et al., 2000). No difference was observed in the average number of neurons per section immunoreactive for either peripherin or NF-200 in DRG ipsilateral to, versus DRG contralateral to lysolecithin treatment of the sciatic nerve (peripherin; ipsilateral, 129.7 ± 19.6, contralateral 119.7 ± 4.6); (NF-200; ipsilateral 78.5 ± 3.2, contralateral 77.3 ± 2.2) or of the saphenous nerve (peripherin; ipsilateral 120.4 ± 11.6, contralateral 124.7 ± 8.6); (NF-200; ipsilateral 67.1 ± 6.2, contralateral 74.3 ± 13.9) or sham treatment of the sciatic nerve (peripherin; ipsilateral 109.46 ± 9.6 contralateral; 103.9± 9.1) ; (NF-200; ipsilateral 68.1 ± 9.6;contralateral; 73.4 ± 2.7) (*$P<0.05$) as determined by Kruskal-Wallis One-Way Analysis of Variance on Ranks with all pairwise multiple comparisons procedure (Dunn's Method) (Fig 4.8a). Likewise, no difference was observed in the average number of DRG neurons immunoreactive for peripherin or NF-200 between *Prx*-null or wild-type mice (peripherin; *Prx*-null, 115.6 ± 14.4, wild-type 133.1 ± 9.8) (NF-200; *Prx*-null 89.8 ± 14.9, wild-type 104.4 ± 8.7) (*$P<0.05$) as determined by a Student's paired t-test (Figure 4.9a).

We also analysed the expression of the activating transcription factor 3 (ATF3); a member of the activating transcription factor/cAMP-responsive element binding protein (ATF/CREB) family (Hai et al., 1999) in the cell bodies of 6 week old *Prx*-null mice, 6 week old wild-type and mice 13 days following lysolecithin treatment or sham treatment of the sciatic nerve. ATF3 expression is induced in the DRG after peripheral nerve injury and can therefore be utilised as a marker identifying axotomised or injured neurons (Tsujino et al., 2000). As a positive control, we analysed the expression of ATF3 in the DRG of mice which were at the peak of neuropathic sensitisation following CCI to the sciatic nerve and found expression of ATF3 in 69 ± 16% of DRG neurons. However, there was no significant increase in the average proportion of ATF3-immunopositive cells in the DRG ipsilateral to lysolecithin treatment (7.3 ± 3.2%) as compared to the contralateral side (6.1 ± 1.4 %) or to sham treated

animals (ipsilateral 8.2 ± 3.4%;contralateral; 5.1 ± 3.2%) (*P<0.05) as determined by Kruskal-Wallis One-Way Analysis of Variance on Ranks with all pairwise multiple comparisons procedure (Dunn's Method) (Fig 4.8b) or in the DRG of *Prx*-null mice (8.2 ± 2.4%) as compared to wild-type mice (6.6 ± 1.7 %) (*P<0.05) as determined by a Student's paired t-test (Fig 4.9b). All control sections, which had the primary antibodies omitted, showed no immunofluorescence confirming the specificity of the secondary antibodies which were subsequently used for all immunohistochemistry. For a summary of results see Tables 2a and 2b.

Figure 4.8 Immunohistochemical assessment of NF-200 and peripherin in DRG cells 12 days post lysolecithin treatment of the saphenous or sciatic nerve, and the marker indicative of axonal damage, ATF3, in DRG cells 12 days post lysolecithin treatment of the sciatic nerve.

a) The expression of the neuronal structural markers peripherin (i,iii) and NF-200 (ii,iv) in ipsilateral and contralateral DRG sections from animals following lysolecithin treatment to the sciatic (i,ii) or saphenous (iii,iv) nerve on post treatment day 13 (n=4). Peripherin is expressed by small diameter unmyelinated cells and NF-200 by the medium to large diameter myelinated cells. As determined by a paired Student's t-test, there was no statistically significant difference in the number of cells expressing either marker following treatment with lysolecithin. Data are presented as the average number of immunopositive cells per DRG section ± SEM. Examples of cells immunopositive for peripherin are shown in images in Figures 4.10a and examples of cells immunopositive for NF-200 in Figures 4.8b, 4.9b and 4.10b-d.

b) The expression of the activating transcription factor 3 (ATF3) in ipsilateral and contralateral DRG sections from sciatic nerve lysolecithin-treated or sham treated animals on post treatment day 13 (n=4). There was no statistically significant increase in expression of ATF3 from that seen in control DRG following lysolecithin treatment or sham-treatment, although there was following CCI on day 12 corresponding to peak neuropathic change (Garry et al., 2001). The statistical significance (*$P<0.05$) as determined by Kruskal-Wallis One Way Analysis of Variance on Ranks with all pairwise multiple comparisons procedure (Dunn's Method). Images show ATF3-immunopositive cells labelled with FITC (green) and NF-200-immunopositive cells labelled with TRITC (red) in (i) ipsilateral DRG from CCI operated animals, (ii) ipsilateral and (iii) contralateral DRG from lysolecithin-treated animals. Scale bar: 20 μm.

Figure 4.8.

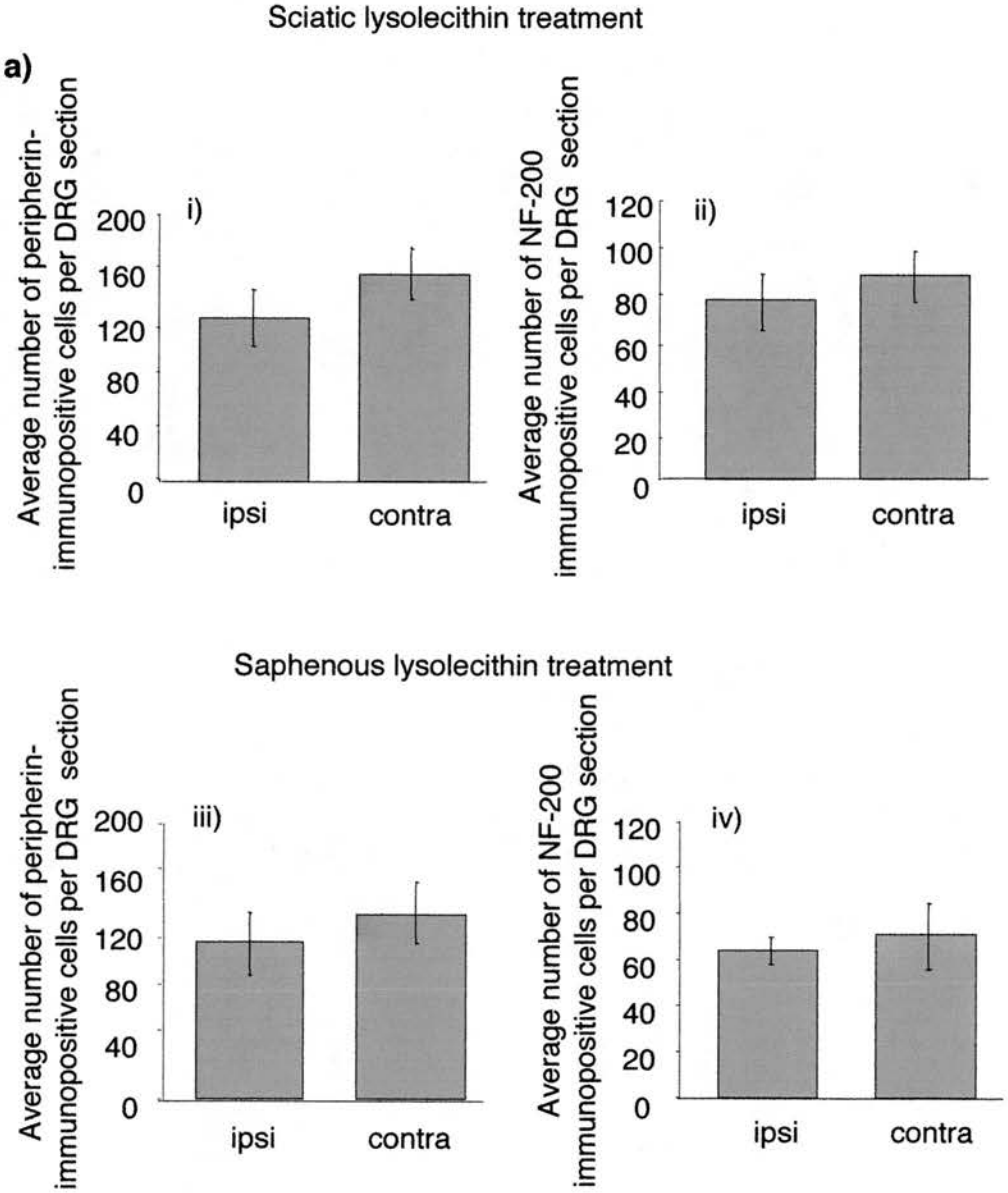

Sciatic lysolecithin treatment
a)
Average number of peripherin-immunopositive cells per DRG section
i)
200
160
120
80
40
0
ipsi
contra
Average number of NF-200 immunopositive cells per DRG section
ii)
120
100
80
60
40
20
0
ipsi
contra
Saphenous lysolecithin treatment
Average number of peripherin-immunopositive cells per DRG section
iii)
200
160
120
80
40
0
ipsi
contra
Average number of NF-200 immunopositive cells per DRG section
iv)
120
100
80
60
40
20
0
ipsi
contra

Figure 4.8 cont..

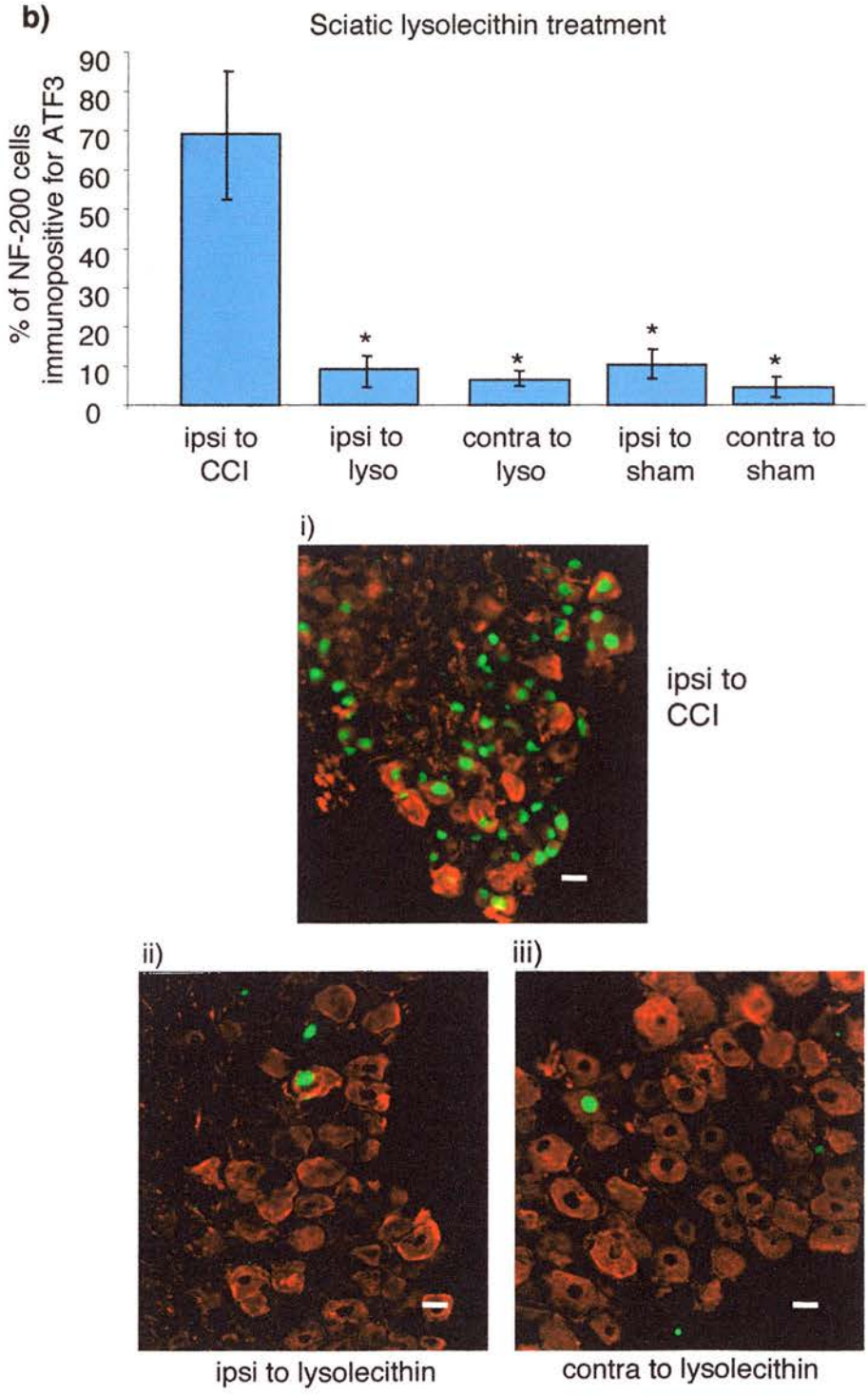


Figure 4.9. Immunohistochemical assessment of NF-200 and peripherin and the marker indicative of axonal damage, ATF3 in DRG cells of 6 week old *Prx*-null mice and wild-type littermates.

a) The expression of the neuronal structural markers peripherin (i) and NF-200 (ii) in DRG sections from 6 week old *Prx*-null mice (n=4) and wild-type (WT) littermates (n=4). Peripherin is expressed by small diameter unmyelinated cells and NF-200 by the medium to large diameter myelinated cells. As determined by a paired Student's t-test, there was no statistically significant difference in the number of cells expressing either marker following treatment with lysolecithin. Data are presented as the average number of immunopositive cells per DRG section ± SEM. Examples of cells immunopositive for peripherin are shown in images in Figure 4.11a and examples of cells immunopositive for NF-200 in Figures 4.11b,c and 4.12.

b) The expression of the activating transcription factor 3 (ATF3) in DRG sections from 6 week old *Prx*-null mice (n=4) and wild-type (WT) littermates (n=4). There was no significant increase in expression of ATF3 from that seen in control DRG following lysolecithin treatment or sham-treatment. The statistical significance of any difference was determined by a Kruskal-Wallis One Way ANOVA on Ranks with all pairwise multiple comparison procedures (Dunn's Method). Images show ATF3-immunopositive cells labelled with FITC (green) and NF-200-immunopositive cells labelled with TRITC (red) in DRG from (i) 6 week old *Prx*-null animals and (ii) wild-type (WT) littermates. Scale bar: 20 μm.

Figure 4.9.

a)

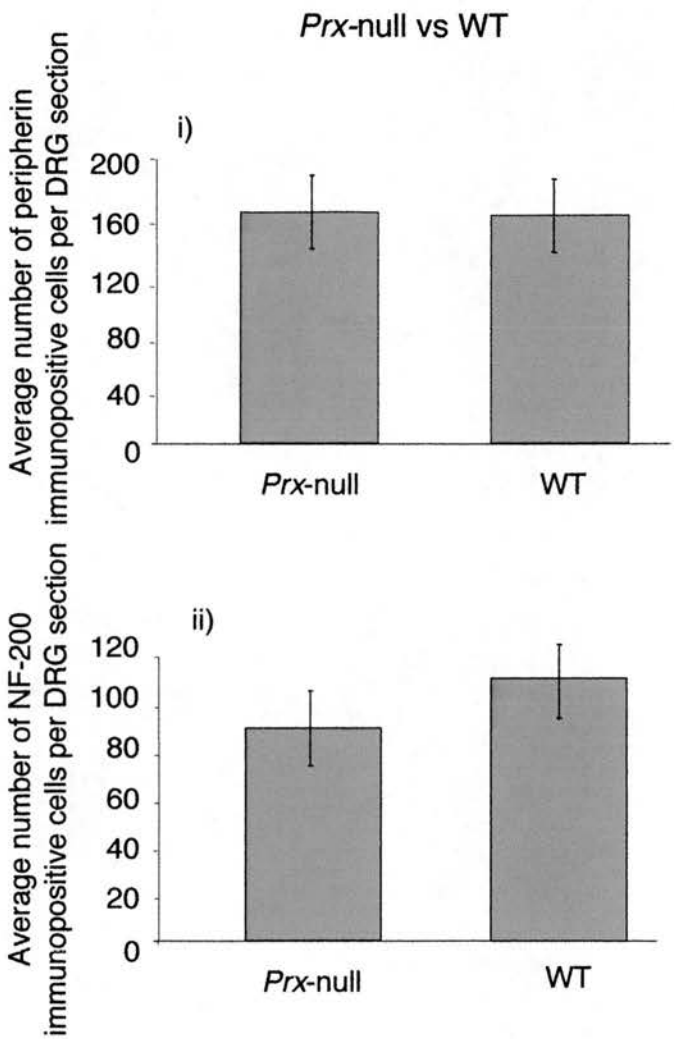
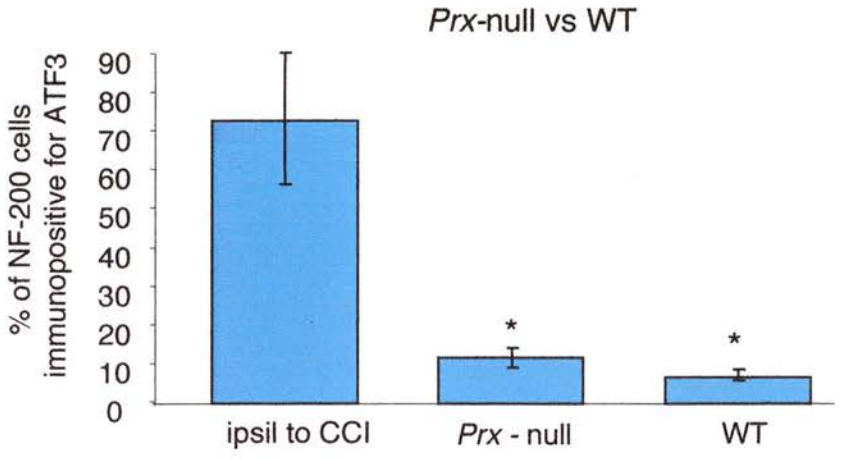

Prx-null vs WT
i)
Average number of peripherin immunopositive cells per DRG section
200
160
120
80
40
0
Prx-null
WT
ii)
Average number of NF-200 immunopositive cells per DRG section
120
100
80
60
40
20
0
Prx-null
WT

Figure 4.9. cont...

b)



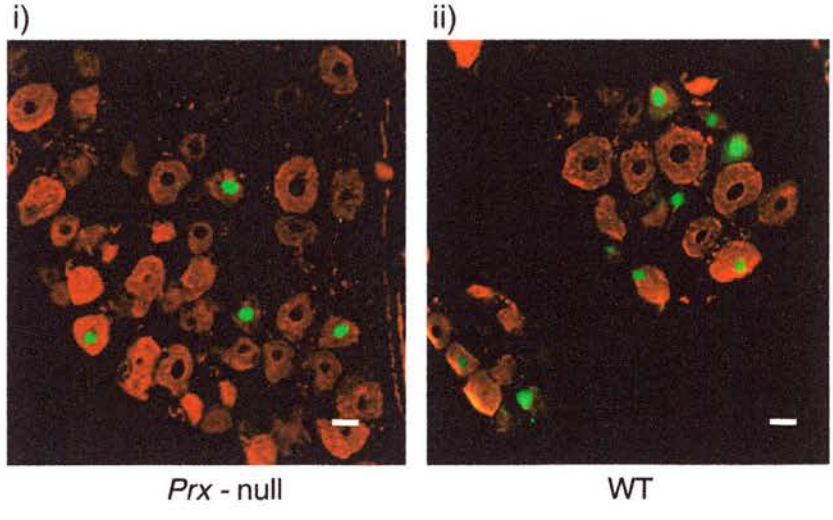


Table 2. Cell counts from immunohistochemical assessment of NF-200, peripherin and the indicator of axonal damage ATF3, in the DRG following lysolecithin treatment of the sciatic nerve and in the DRG of 6 week old *Prx*-null mice and wild-type littermates.

a) The expression of NF-200, peripherin and the indicator of axonal damage, activating transcription factor 3 (ATF3) in DRG cells ipsilateral and contralateral to either lysolecithin treatment of the sciatic nerve or saphenous nerve on post treatment day 13 (n=3 in each case). There was no difference in the number of cells expressing NF-200, peripherin or ATF3 following treatment with lysolecithin as compared to contralateral DRG as determined by a Kruskal-Wallis One-Way ANOVA on Ranks. Data are presented as the average number of immunopositive cells per DRG section ± SEM for NF-200 and peripherin or as average percentage of NF-200 immunopositive cells expressing ATF3 per DRG section ± SEM.

b) The expression of NF-200 and peripherin and the indicator of axonal damage activating transcription factor 3 (ATF3) in DRG cells from 6 week old *Prx*-null mice (n=4). There was no difference in the number of cells expressing NF-200, peripherin or ATF3 in cells from *Prx*-null mice as compared to wild-type (WT) littermates (n=4) as determined by Kruskal-Wallis One Way ANOVA on Ranks. Data are presented as the average number of immunopositive cells per DRG section ± SEM for NF-200 and peripherin or as average percentage of NF-200 immunopositive cells expressing ATF3 per DRG section ± SEM.

Table 2.

a)

Antigen	Average number of immunopositive cells per DRG section			
	Ipsilateral to lysolecithin	Contralateral to lysolecithin	Ipsilateral to sham surgery	Contralateral to sham surgery
NF-200	78.5 ±3.2	77.3 ±2.2	68.1 ± 9.6	73.4 ± 2.7
peripherin	129.7± 19.6	119.7± 4. 6	109.4± 9.6	103.9± 1.9
	Average number of immunopositive cells per DRG section as % of NF-200 immunopositve cells			
ATF3	7.3 ±3.2%	6.1 ± 1.4 %	8.2±3.4%	5.1 ±3.2%

b)

Antigen	Average number of immunopositive cells per DRG section	
	Prx -null	Wild Type
NF-200	89.8 ± 14.9	104.4 ± 8.7
peripherin	115.6±14.4	133.1 ± 9.8
	Average number of immunopositive cells per DRG section as % of NF-200 immunopositve cells	
ATF3	8.2 ± 2.4%	6.6 ±1.7 %

b) Expression of selected peptides in DRG; CGRP, galanin, VIP and NPY.

Following traumatic injury to the peripheral nerve, a number of neurochemical and morphological changes occur in the peripheral nerve fibres and centrally in the spinal cord (Dray et al., 1994;Hokfelt et al., 1994), which can contribute to altered sensory transmission such as that associated with chronic pain states. A key event is the characteristic phenotypic change that occurs in many primary afferent neurons, reflected in the altered expression of neuropeptides in their cell bodies in the DRG (Hokfelt et al., 1994;Villar et al., 1991). Following injury, there are known to be marked changes in the expression of several neuropeptides including CGRP, SP, galanin, VIP and neuropeptide Y (NPY). We therefore investigated whether some of these characteristic changes occur in our models of demyelination to compare the presence of any similarities to those occurring in other models of neuropathic pain in which axonal damage is a major factor.

CGRP is normally expressed by approximately 50% of unmyelinated DRG neurons as well as one-fifth of Aδ myelinated neurons (Hokfelt et al., 1994;McCarthy and Lawson, 1990;Rosenfeld et al., 1983). It is known that the expression of CGRP is downregulated following peripheral nerve injury such as axotomy and CCI (Dumoulin et al., 1992;Noguchi et al., 1990). However, there was no significant difference in the numbers of DRG cells expressing immunoreactive CGRP following lysolecithin treatment of one sciatic nerve (ipsilateral 145.9 ± 19.7, contralateral 154.5 ± 9.1) or of one saphenous nerve (ipsilateral 129.9 ± 8.7, contralateral 135.3 ± 7.4) (*$P<0.05$) as determined by a Student's paired t-test, suggesting that lysolecithin treatment caused no extensive damage to CGRP-expressing cells in the DRG (Fig 4.10a). Likewise, there was no significant difference in the numbers of DRG cells expressing immunoreactive CGRP in *Prx*-null mice as compared to wild-type mice (*Prx*-null 145.7 ± 27.0, wild-type 143.3 ± 20.1) (*$P<0.05$) as determined by a Student's paired t-test (Fig 4.11a). Furthermore, in line with previously reported patterns of CGRP expression in the DRG, we observed colocalisation of CGRP in approximately 50% of DRG-cells expressing peripherin as well as approximately 20% which were peripherin-negative in the DRG from lysolecithin-treated mice and *Prx*-null mice. As there was no change in expression of CGRP in either model when investigated immunohistochemically using peripherin as a co-stain, we did not repeat the immunostaining experiment with co-staining for NF-200. SP is normally expressed by ~20% of DRG neurons which are mainly of small diameter (Hokfelt et al., 1975;Ju G et al., 1987). Following peripheral nerve injury such as axotomy and CCI, the expression of SP is also dramatically downregulated (Jessell et al., 1979;Nielsch et al., 1987). However, there was no significant difference in the numbers of DRG cells expressing immunoreactive SP following lysolecithin treatment of one sciatic

nerve (ipsilateral 55.6 ± 4.2, contralateral 59.4 ± 5.4) or of one saphenous nerve (ipsilateral 61.1± 5.7, contralateral 57.2 ± 4.4) (*P<0.05) as determined by a Student's paired t-test (Fig 4.10a) suggesting that lysolecithin treatment also caused no extensive damage to SP-expressing cells in the DRG which correlates with the fact that most SP containing cells also contain CGRP. Likewise, there was no significant difference in the number of DRG cells expressing immunoreactive SP in *Prx*-null mice as compared to wild-type mice (*Prx*-null 49.7 ± 6.0, wild-type 52 ± 6.1) (*P<0.05) as determined by a Student's paired t-test (Fig 4.11b).

As we had demonstrated there to be no change in the number of NF-200 or peripherin-immunoreactive cell bodies in the DRG of either model of peripheral nerve demyelination, we decided to restrict further immunohistochemical investigations to the sciatic model of lysolecithin-induced demyelination and the *Prx*-null mouse (with appropriate controls). Furthermore, as there is no change in the expression of CGRP or SP following lysolecithin application to either the sciatic or saphenous nerve, it would appear that the effects of demyelination are similar regardless of the nerve affected by lysolecithin. We chose the sciatic model as opposed to the saphenous nerve model due to the sciatic nerve being a larger nerve with more afferent axons and therefore a larger proportion of cell bodies in the lumbar DRG as opposed to the saphenous nerve. Therefore, any effect of demyelination on the phenotype of associated cell bodies should be easier to detect in the model of sciatic nerve demyelination. Galanin is a peptide normally expressed at very low levels in sensory and sympathetic neurons. However, following peripheral nerve lesions, it is strongly upregulated mainly in small and medium sized neurons (Hokfelt et al., 1987;Villar et al., 1989) especially those that normally contain substance P and CGRP (Kashiba et al., 1992b), the majority of which are unmyelinated. However, we observed no significant immunoreactivity for galanin in DRG cells either ipsilateral or contralateral to lysolecithin-induced focal demyelination of one sciatic nerve or in the cells of the DRG of *Prx*-null mice. As a positive control to test the specificity of the antibody, we used DRG dissected from mice at peak neuropathic sensitisation following CCI treatment to the right sciatic nerve (Garry et al., 2001). These sections showed a significant proportion of mainly small diameter cells positive for galanin (ipsilateral 9.3 ± 2.4 cells per section; contralateral 0), verifying that the antibody could successfully detect the presence of galanin and its increased expression (Fig 4.10c) and that there is, in contrast, no significant upregulation following lysolecithin treatment or in *Prx*-null mice (no data presented due to lack of change).

VIP is normally expressed only at very low levels in DRG neurons and in neurons in LI and LII of the spinal dorsal horn (Gibson SJ et al., 1981;Rittenhouse et al., 1996). However, following nerve injury, levels of VIP are also markedly increased, predominantly in small to medium- sized afferent neurons, especially those that normally contain SP and CGRP (Kashiba et al., 1992;Xu XJ et al., 1990;Zhang et al., 1995). We attempted to investigate whether such changes were present in either model of demyelination. However, the immunostaining we achieved with different, recommended, VIP antibodies (see Chapter 3) was not of sufficient quality for the accurate analysis of data. Therefore, we have not included any results on VIP immunoreactivity in the DRG in either model of demyelination.

NPY is not normally expressed in DRG cells. However, the expression of NPY is strongly upregulated in DRG neurons after nerve injuries such as axotomy (Kashiba et al., 1994;Wakisaka et al., 1991) and CCI (Munglani et al., 1995;Nahin et al., 1994). This upregulation occurs mainly in large primary sensory neurons containing the 200kDa subunit of neurofilaments (Kashiba et al., 1994;Marchand et al., 1999). In line with this we observed significant NPY-immunoreactivity in 15.9 $\pm$ 2.2 % cells following demyelination of one sciatic nerve (185 NPY-positive cells from 1164 NF-200-positive cells counted over 26 sections (n=3)) as opposed to no detectable immunoreactivity in contralateral or sham nerves (Fig 4.10d) (*$P<0.05$) as determined by Kruskal-Wallis One-Way Analysis of Variance on Ranks with all pairwise multiple comparisons procedure (Dunn's Method). A similar, significant upregulation was observed in *Prx*-null mice, which demonstrated NPY-immunoreactivity in 13.6 $\pm$ 2.6 % of cells in the DRG (150 NPY-positive cells from 1104 NF-200-positive cells counted over 16 sections (n=3) 2 as opposed to no significant immunoreactivity in wild-type DRG cells (Fig 4.11c) (*$P<0.05$) as determined by a Student's paired t-test. All NPY-immunopositive cells were also immunoreactive for NF-200 indicating that they were cells with myelinated fibres and therefore, the immunostaining for NPY was not also repeated with co-staining for peripherin. For summary of results as compared to axotomy and CCI changes (described in Chapter 1) see Table 3.

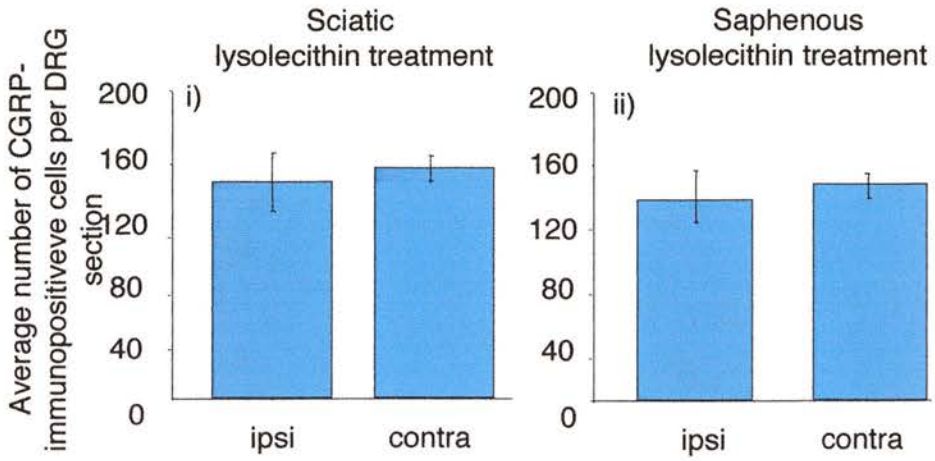
Figure 4.10. Immunohistochemical assessment of the neuropeptides CGRP and SP in DRG cells 13 days post lysolecithin treatment of the sciatic nerve.

a) The expression of the peptide CGRP in ipsilateral and contralateral DRG sections from animals following lysolecithin treatment to (i) the sciatic or (ii) the saphenous nerve on post treatment day 13 (n=4). The expression of CGRP is known to decrease following nerve injury in various models of neuropathic pain. There was no change in the number of cells expressing CGRP following treatment with lysolecithin. The statistical significance (*P<0.05) of any difference was determined by a paired Student's t-test. Data are presented as (i & ii) average number of CGRP-immunopositive cells per DRG section ± SEM and images show CGRP immunopositive cells labelled with FITC (green) and peripherin-immunopositive cells labelled with TRITC (red) (with colocalised immunoreactivity appearing yellow) in (iii) ipsilateral and (iv) contralateral DRG from sciatic lysolecithin-treated animals. Scale bar: 20 μm.

b) The expression of the peptide SP in ipsilateral and contralateral DRG sections from animals following lysolecithin treatment to (i) the sciatic or (ii) the saphenous nerve on post treatment day 13 (n=4). The expression of SP is known to decrease following nerve injury in various models of neuropathic pain. There was no change in the number of cells expressing SP following treatment with lysolecithin. The statistical significance (*P<0.05) of any difference was determined by a paired Student's t-test. Data are presented as (i & ii) average number of immunopositive cells per DRG section ± SEM and images show SP-immunopositive cells labelled with FITC (green) and NF-200 immunopositive cells labelled with TRITC (red) (with colocalised immunoreactivity appearing yellow) in (iii) ipsilateral and (iv) contralateral DRG from lysolecithin-treated animals. Scale bar: 20 μm.

Figure 4.10

a)


Sciatic
lysolecithin treatment
Saphenous
lysolecithin treatment
Average number of CGRP-immunopositiveve cells per DRG section
i)
ii)
200
160
120
80
40
0
ipsi
contra

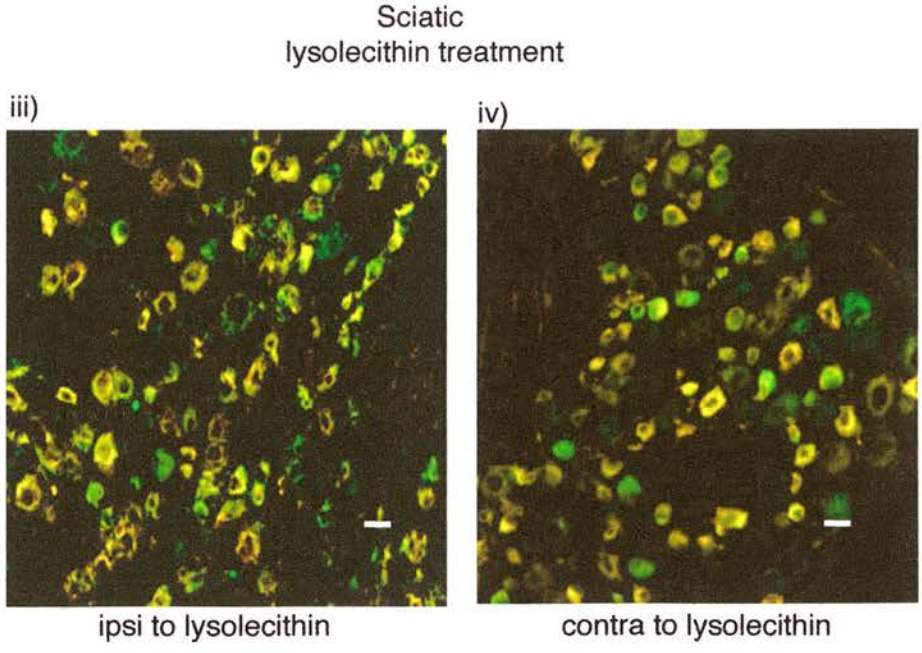
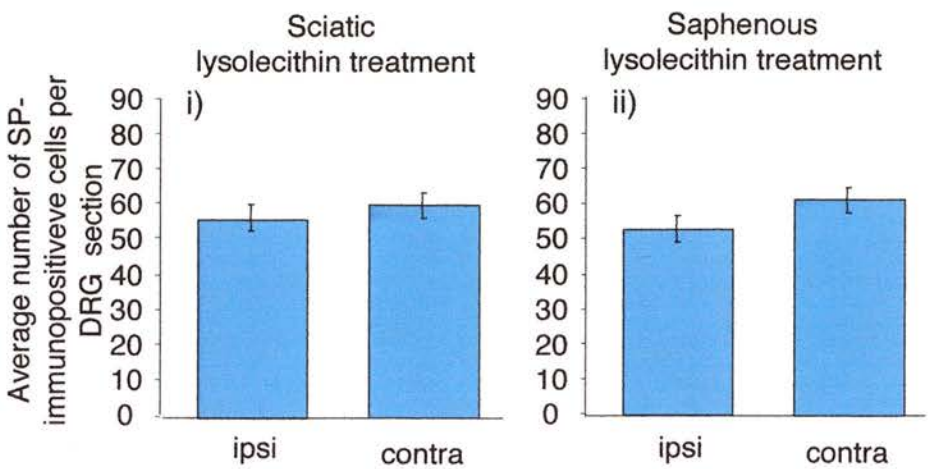

Sciatic
lysolecithin treatment
iii)
iv)
ipsi to lysolecithin
contra to lysolecithin

Figure 4.10 cont...

b)



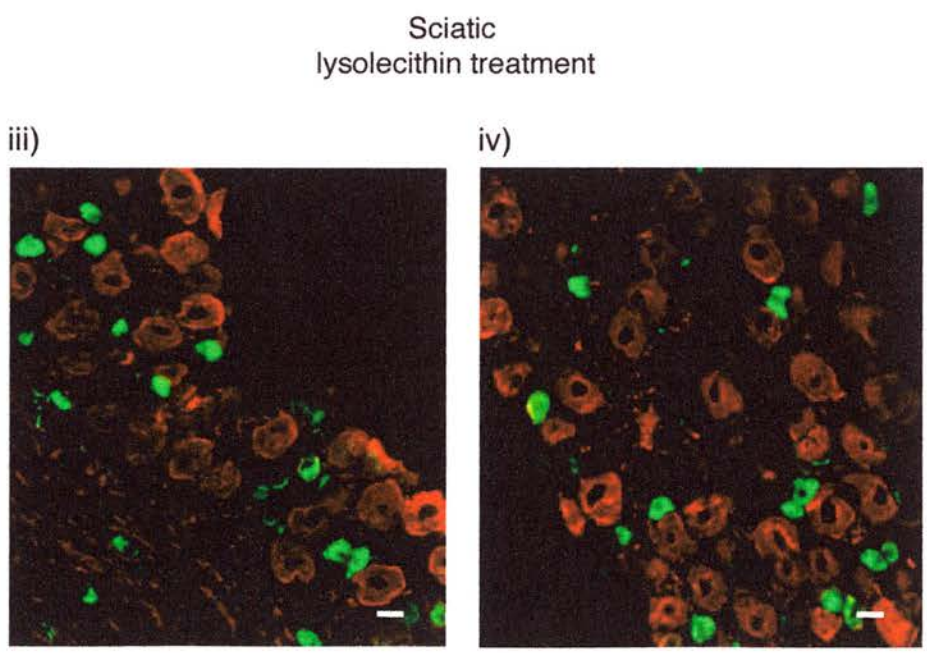


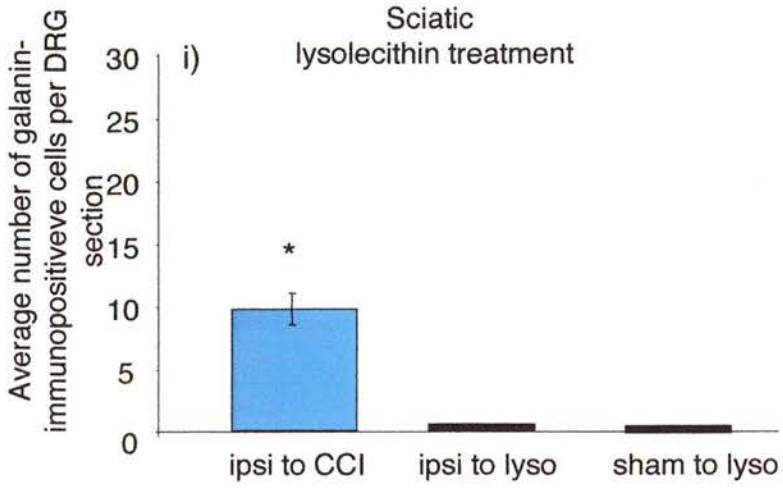
Figure 4.10 cont.... Immunohistochemical assessment of the neuropeptides galanin and NPY in DRG cells 12 days post lysolecithin treatment of the sciatic nerve.

c) The expression of the peptide galanin in DRG of animals at peak neuropathic sensitisation following lysolecithin treatment to the sciatic nerve on post-treatment day 13 (n=4) as compared to DRG from animals following CCI treatment to the right sciatic nerve on post treatment day 13. The immunopositive cells in the DRG from CCI-treated animals demonstrated the specificity of the antibody. No immunoreactivity was seen in the DRG of animals following lysolecithin treatment of the sciatic nerve (n=4). The statistical significance of any difference from CCI (*$P<0.05$) was determined by a paired Student's t-test. Data are presented as (i) average number of immunopositive cells per DRG section ± SEM and (ii) images shows galanin-immunopositive cells labelled with FITC (green) and NF-200 immunopositive cells labelled with TRITC (red) (with colocalised immunoreactivity appearing yellow) in the DRG of CCI-treated animals. Scale bar: 20 μm.

d) The expression of the peptide NPY in ipsilateral and contralateral DRG sections from animals following lysolecithin treatment to the sciatic nerve on post-treatment day 13 (n=4). Following lysolecithin treatment of the sciatic nerve we saw a significant number of NF-200 immunopositive cells expressing NPY (15.9 ± 2.2 %) (*$P<0.05$) as determined by Kruskal-Wallis One Way Analysis of Variance on Ranks with all pairwise multiple comparisons procedure (Dunn's Method). No NF-200 negative cells expressed NPY. There was no detectable expression of NPY in the DRG from naïve or sham treated animals. Data are presented as (i) average number of immunopositive cells per DRG section ± SEM and images show NPY-immunopositive cells labelled with FITC (green) and NF-200 immunopositive cells labelled with TRITC (red) (with colocalised immunoreactivity appearing yellow/green) in DRG (ii) ipsilateral to lysolecithin treatment as opposed to no positive cells in DRG (iii) contralateral to lysolecithin treatment. Scale bar: 20 μm.

Figure 4.10 cont...

c)



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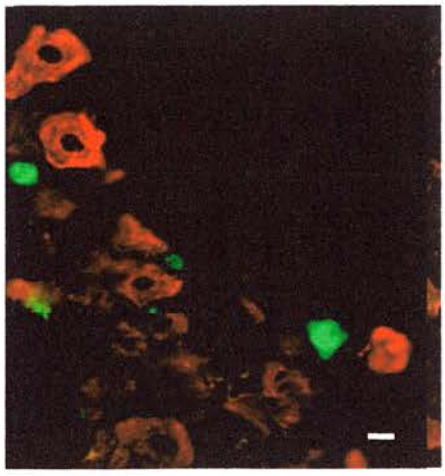
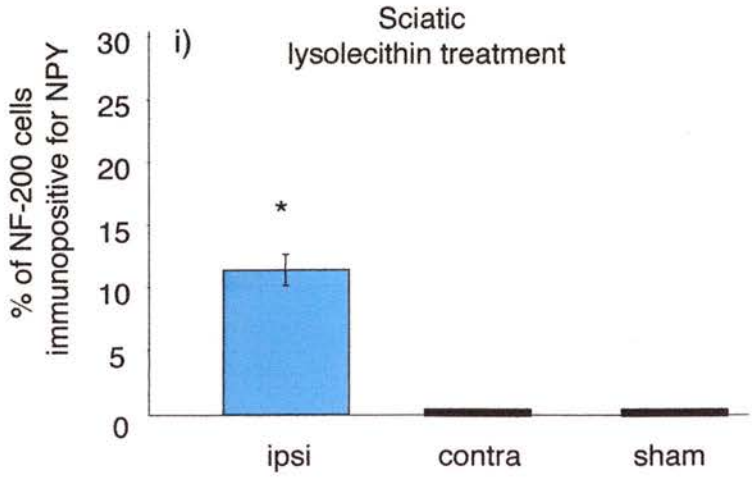


ipsi to CCI

Figure 4.10 cont...

d)



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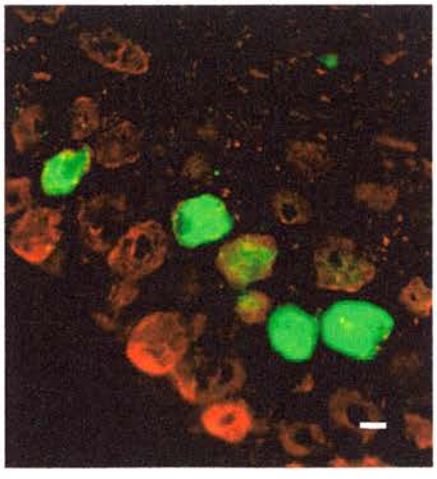
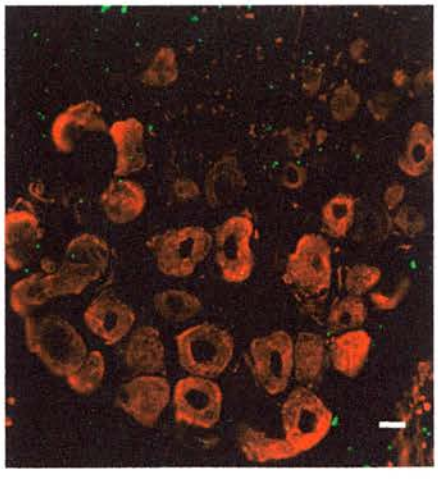


ipsi to lysolecithin

iii)



contra to lysolecithin

Figure 4.11. Immunohistochemical assessment of the neuropeptides CGRP, SP and NPY in DRG cells of 6 week old *Prx*-null mice and wild-type littermates.

a) The expression of the peptide CGRP in DRG sections from 6 week old *Prx*-null mice (n=4) and wild-type (WT) littermates (n=4).. There was no statistical difference in the number of cells expressing CGRP between *Prx*-null and wild-type DRG (*P<0.05) as determined by a paired Student's t-test. Data is presented as (i) average number of immunopositive cells per DRG section ± SEM and images show CGRP-immunopositive cells labelled with FITC (green) and peripherin-immunopositive cells labelled with TRITC (red) (with colocalised immunoreactivity appearing yellow) in DRG sections from (ii) 6 week old *Prx*-null mice and (iii) wild-type (WT) littermates. Scale bar: 20 μm

b) The expression of the peptide SP in DRG sections from 6 week old *Prx*-null mice (n=4) and wild-type (WT) littermates (n=4).. There was no change in the number of cells expressing SP between *Prx*-null and wild-type DRG (*P<0.05) as determined by a paired Student's t-test. Data are presented as (i) average number of immunopositive cells per DRG section ± SEM and images show SP-immunopositive cells labelled with FITC (green) and NF-200 immunopositive cells labelled with TRITC (red) (with colocalised immunoreactivity appearing yellow) in DRG sections from (ii) 6 week old *Prx*-null mice and (iii) wild-type (WT) littermates. Scale bar: 20 μm.

   The data regarding the expression of galanin is not shown as there were no immunopositive cells present in the DRG of *Prx*-null or wild-type (WT) mice. For counts and images of the positive control DRG from CCI-treated animals see Fig 4.8c.

c) The expression of the neuropeptide NPY in DRG sections from 6 week old *Prx*-null mice (n=4) and wild-type (WT) littermates (n=4). We saw a significant number of NF-200-immunopositive cells expressing NPY (13.6 ± 2.6 %) in the DRG of *Prx*-null mice (*P<0.05) as determined by a paired Student's t-test. No NF-200-negative cells expressed NPY. There was no expression of NPY in the DRG from wild-type (WT) animals. Images show NPY-immunopositive cells labelled with FITC (green) and NF-200 immunopositive cells labelled with TRITC (red) (with colocalisation appearing yellow) in DRG of (i) *Prx*-null mice as opposed to the lack of positive cells in DRG of (ii) wild-type (WT) mice. Scale bar: 20 μm.

Figure 4.11.

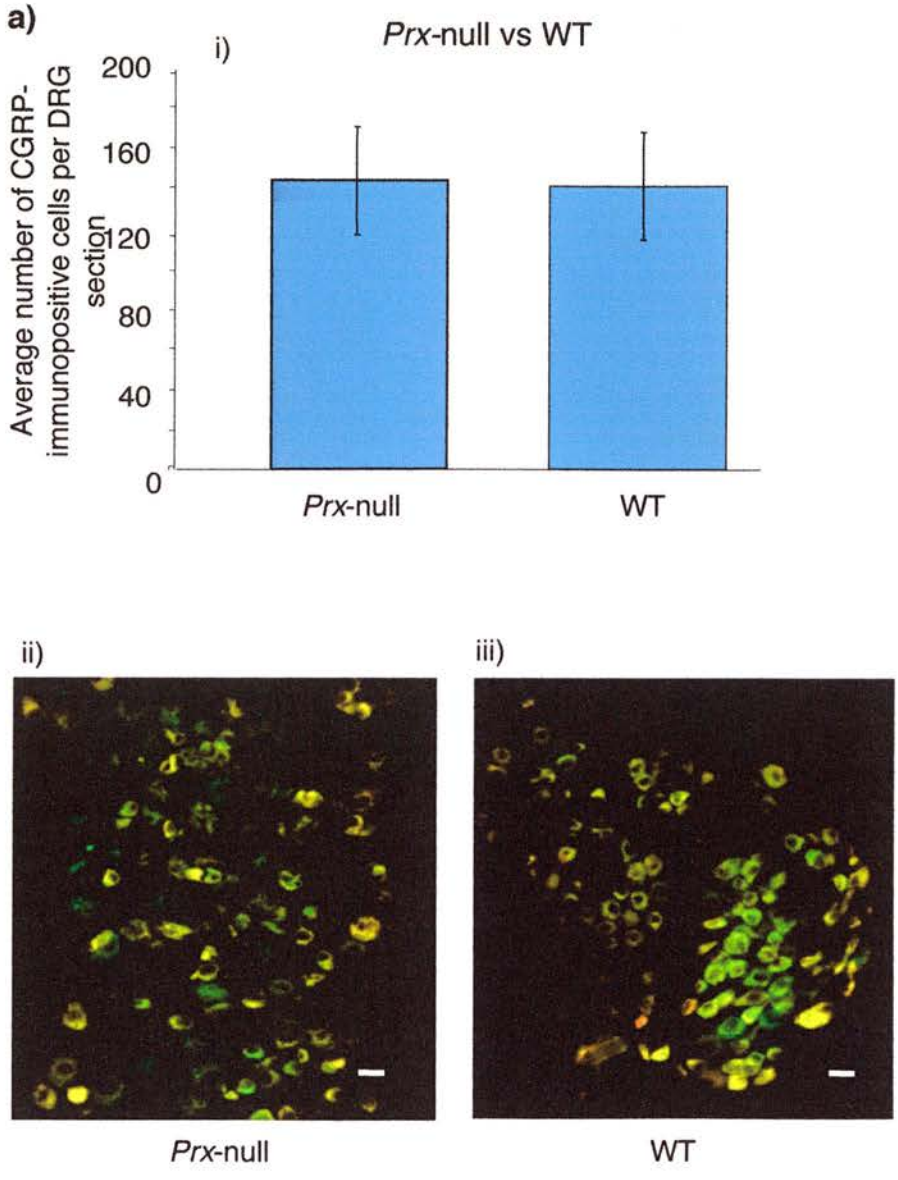
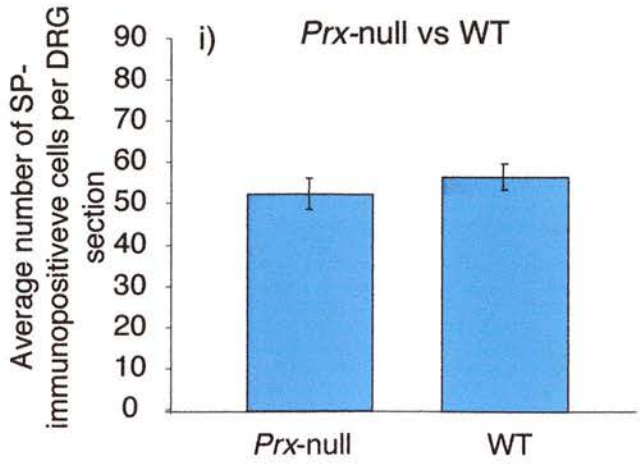

a)
i)
Prx-null vs WT
Average number of CGRP-immunopositive cells per DRG section
200
160
120
80
40
0
Prx-null
WT
ii)
iii)
Prx-null
WT

Figure 4.11 cont...

b)



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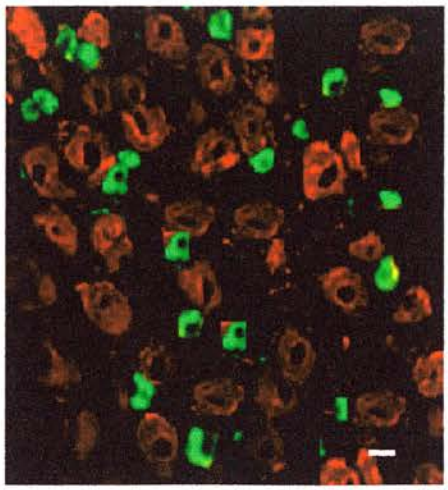
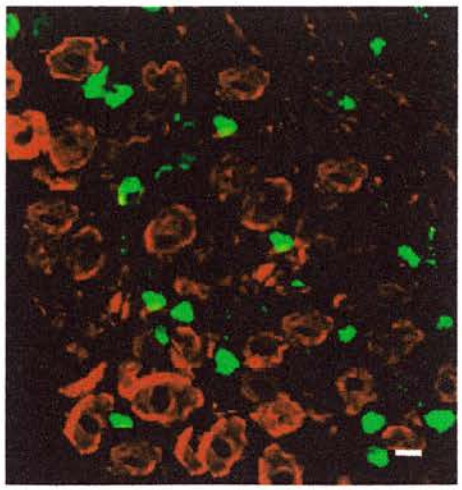


Prx-null

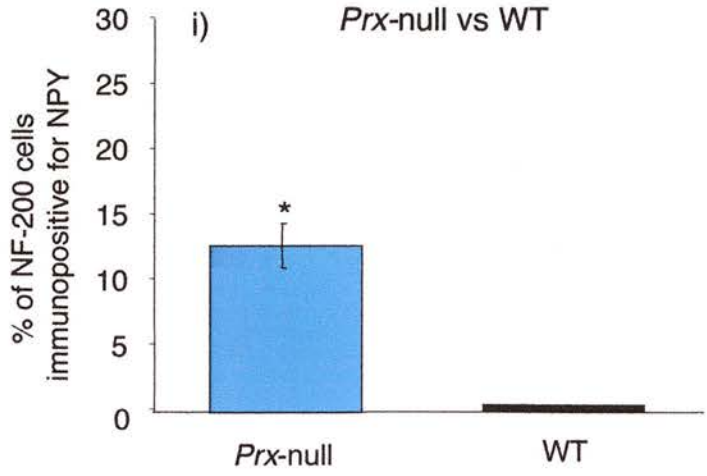
iii)

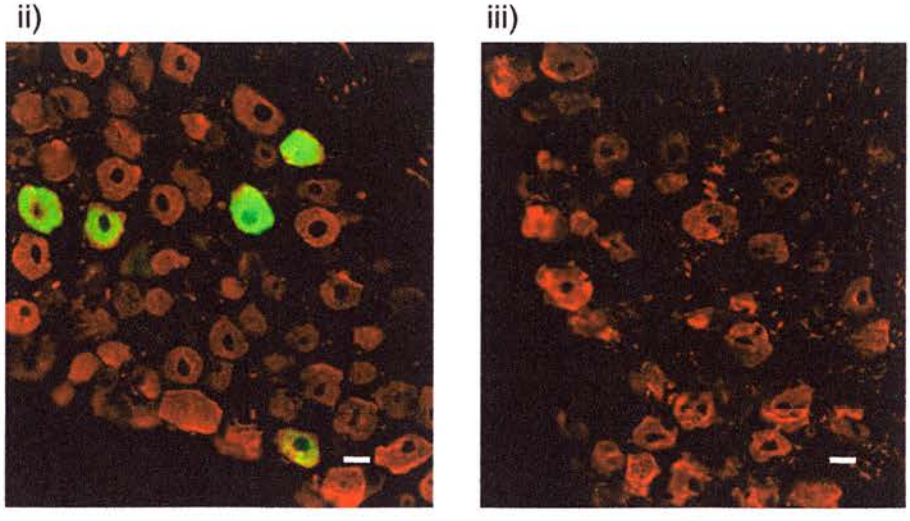


WT

Figure 4.11 cont...

c)





c) Expression of the Na^+ channels; SNS 1/ $Na_v1.8$ and brain type III/ $Na_v1.3$.

DRG neurons express a complex repertoire of sodium channel transcripts (Waxman et al., 1999b) distinguished by their sensitivity to tetrodotoxin (TTX) (Black and Waxman, 1996). The TTX-resistant sodium channel SNS 1/$Na_v1.8$ has been shown to be expressed normally in approximately 50% of cells with small diameter unmyelinated axons (Amaya et al., 2000) and in around 20% of medium to large diameter myelinated cells. Our results agree with this pattern of expression. In the contralateral DRG of lysolecithin-treated animals, we saw an average rate of SNS 1/$Na_v1.8$ expression in 1078 of 2130 (51%) peripherin-immunopositive cells sampled and 500 of 2086 (24%) NF-200 -immunopositive cells sampled. This is similar to levels of expression in the DRG of wild-type mice where we saw SNS 1/$Na_v1.8$ expression in 1090 of 2083 (57%) peripherin-immunopositive cells sampled and 389 of 1561 (25%) NF-200-immunopositive cells sampled. Likewise, in sham controls we saw SNS 1/$Na_v1.8$ expressed in 51% of peripherin-immunopositive cells ipsilaterally, 48% contralaterally and in 23% of NF-200-immunopositive cells ipsilaterally and 22 % contralaterally.

It has been shown previously that following axotomy and CCI, expression of SNS 1 is downregulated in neurons in the DRG (Dib-Hajj et al., 1999;Dray and Urban, 1996), a change that may contribute to the production of a neuropathic pain state. Therefore we have employed immunohistochemical techniques to detect the presence of any changes in expression of SNS 1/$Na_v1.8$ in the *Prx*-null mouse and following lysolecithin-induced demyelination of the sciatic nerve. In the L4, L5 and L6 DRG taken from animals 13 days post lysolecithin -treatment to the sciatic nerve, there was a 34 ± 6.3 % reduction of SNS 1/$Na_v1.8$ expression in the ipsilateral DRG (62.4 ± 3.4 SNS 1/$Na_v1.8$-immunopositive cells per section) versus the contralateral DRG (94.7 ± 4.7 SNS 1/$Na_v1.8$-immunopositive cells per section) and sham DRG (95.7 ± 6.0 SNS 1/$Na_v1.8$-immunopositive cells per section) (Fig 4.12a) as determined from sections co-stained with either peripherin or NF-200. Co-staining with these cell-type specific markers demonstrated that this reduction appeared to be restricted to NF-200-immunopositive cells, i.e. those presumed to have formerly myelinated axons (Fig 4.12a). Counts of SNS 1/$Na_v1.8$ immunostained sections costained for NF-200 revealed a significant reduction (by 38.4 ± 4.2 %) in the average number of SNS 1/$Na_v1.8$ -immunopositive cells per section which were immunoreactive for NF-200 in the DRG ipsilateral to the demyelinated sciatic nerve (15.7 ± 1.6 %), compared to the DRG contralateral to the demyelinated sciatic nerve (25.3 ± 2.6%) (Fig 4.12a), which accounted for all of the overall reduction in SNS 1/$Na_v1.8$ expression in the ipsilateral DRG. No change in the number of cells coexpressing SNS 1/$Na_v1.8$ and NF-200 was observed

between the ipsilateral DRG (22.5 ± 2.7%) or contralateral DRG (22.2 ± 2.9%) of sham control animals (Fig 4.12a). There was no decrease in the proportion of peripherin-immunoreactive cells expressing SNS 1/Na_v1.8 following lysolecithin treatment (ipsilateral 54.8 ± 3.7 %, contralateral 49.1 ± 6.3 %) (Fig 4.12a).

In the DRG from *Prx*-null mice there was a 42 ± 7.8 % reduction of SNS 1/Na_v1.8 expression (72.6 ± 7.8 SNS 1/Na_v1.8 -immunopositive cells per section) as compared to the wild-type mouse (124 ± 7.4 SNS 1/Na_v1.8 -immunopositive cells per section) (Fig 4.13a). Co-staining with cell-type specific markers also demonstrated that this reduction was restricted to NF-200-immunopositive cells thought to have formerly myelinated axons. As with the lysolecithin-treated animals, there was decrease in the proportion of peripherin-immunoreactive cells expressing SNS 1/Na_v1.8 (*Prx*-null mouse 55.8 ± 3.7 %, wild-type mouse 59.1 ± 6.3 %); and again the significant reduction by 48.4 ± 2.6 % in the average number of SNS 1/Na_v1.8-immunopositive cells per section that were immunoreactive for NF-200 in the DRG of *Prx*-null mice (13.3 ± 4.0 %) as compared to the DRG of wild-type mice (25.3 ± 2.7%) accounted for the overall reduction in SNS 1/Na_v1.8 expression (Fig 4.13a).

The brain type III sodium channel/Na_v1.3 is normally found in DRG only during development. However, its expression has been reported to increase in sensory neurons following axotomy (Black et al., 1999), CCI and spinal nerve ligation (Kim et al., 2001a). Therefore we have also employed immunohistochemical techniques to detect the presence of any changes in expression of the type III/ Na_v1.3 sodium channel following lysolecithin-induced demyelination of the sciatic nerve. There were, on average, 9.1 Na_v1.3-immunopositive cells out of 49.4 NF-200-immunoreactive cells per DRG section (18.5 ± 2.4%) ipsilaterally (Fig 4.12). Similarly, the cells in the DRG of *Prx*-null mice showed on average 9.6 Na_v1.3-immunoreactive cells out of 61.3 NF-200-immunoreactive cells per DRG section (15.6 ± 2.5%) (Fig 4.13). In both models, all brightly fluorescent sodium channel type III/Na_v1.3-immunoreactive cells were NF-200-positive (Fig 4.12b and 4.13b). In both cases, the upregulation of type III/ Na_v1.3 was significant compared to the lack of any obvious bright immunoreactivity above background levels observed in the DRG contralateral to lysolecithin treatment or in sham control DRG (*$P<0.05$) as determined by Kruskal-Wallis One-Way ANOVA on Ranks with all pairwise multiple comparisons procedure (Dunn's Method or in wild-type littermates of *Prx*-null mice (*$P<0.05$) as determined by a Student's paired t-test. For a summary of results as compared to CCI and axotomy changes (Hokfelt et al., 1994) see Table 3.

Figure 4.12. Immunohistochemical assessment of the sodium channels; SNS 1 / $Na_v1.8$ and brain type III/ $Na_v1.3$ in DRG cells 13 days post lysolecithin treatment of the sciatic nerve.

a) The expression of the sodium channel SNS 1/$Na_v1.8$ in ipsilateral and contralateral DRG sections from lysolecithin-treated animals on post-treatment day 13, and sham-treated animals post-treatment day 14 (n=3 in each case). Following lysolecithin treatment of the sciatic nerve we saw (i) a statistically significant decrease of 34 ± 6.3 % in the number of cells expressing SNS 1/$Na_v1.8$ in ipsilateral DRG compared to contralateral DRG, all of which were (ii) immunopositive for NF-200 and there was (iii) no decrease of SNS 1 expression in peripherin immunopositive cells. There was no significant decrease in expression following sham treatment. The statistical significance (*$p<0.05$) of any difference was determined by a Kruskal-Wallis One Way ANOVA on Ranks with an all pairwise multiple comparisons procedure (Dunn's Method). Data is presented as (i) average number of immunopositive cells per DRG section ± SEM and as (ii) % of NF200 cells immunpositive for SNS 1/$Na_v1.8$ or (iii) % of peripherin cells immunpositive for SNS 1. Images show SNS 1/$Na_v1.8$-immunopositive cells labelled with FITC (green) and NF-200 immunopositive cells labelled with TRITC (red) (with colocalised immunoreactivity appearing yellow) in (iv) ipsilateral and (v) contralateral DRG from lysolecithin-treated animals. Scale bar: 20 μm.

b) The expression of the brain type III sodium channel/ $Na_v1.3$ in ipsilateral and contralateral DRG sections from lysolecithin-treated animals on post treatment day 13 (n=4). We saw a statistically significant increase in expression of type III/$Na_v1.3$ expression ipsilateral to the lysolecithin treated nerve (*$p<0.05$) as determined by a paired Student's t-test. Data is presented as (i) % of NF200 cells immunpositive for type III/$Na_v1.3$ Images show type III/$Na_v1.3$-immunopositive cells labelled with FITC (green) and NF-200 immunopositive cells labelled with TRITC (red) (with colocalised immunoreactivity appearing yellow) in (ii) ipsilateral DRG from lysolecithin-treated animals as opposed to no type III/$Na_v1.3$-immunopositive cells in (iii) contralateral DRG from lysolecithin-treated animals. Scale bar: 20 μm.

Figure 4.12.

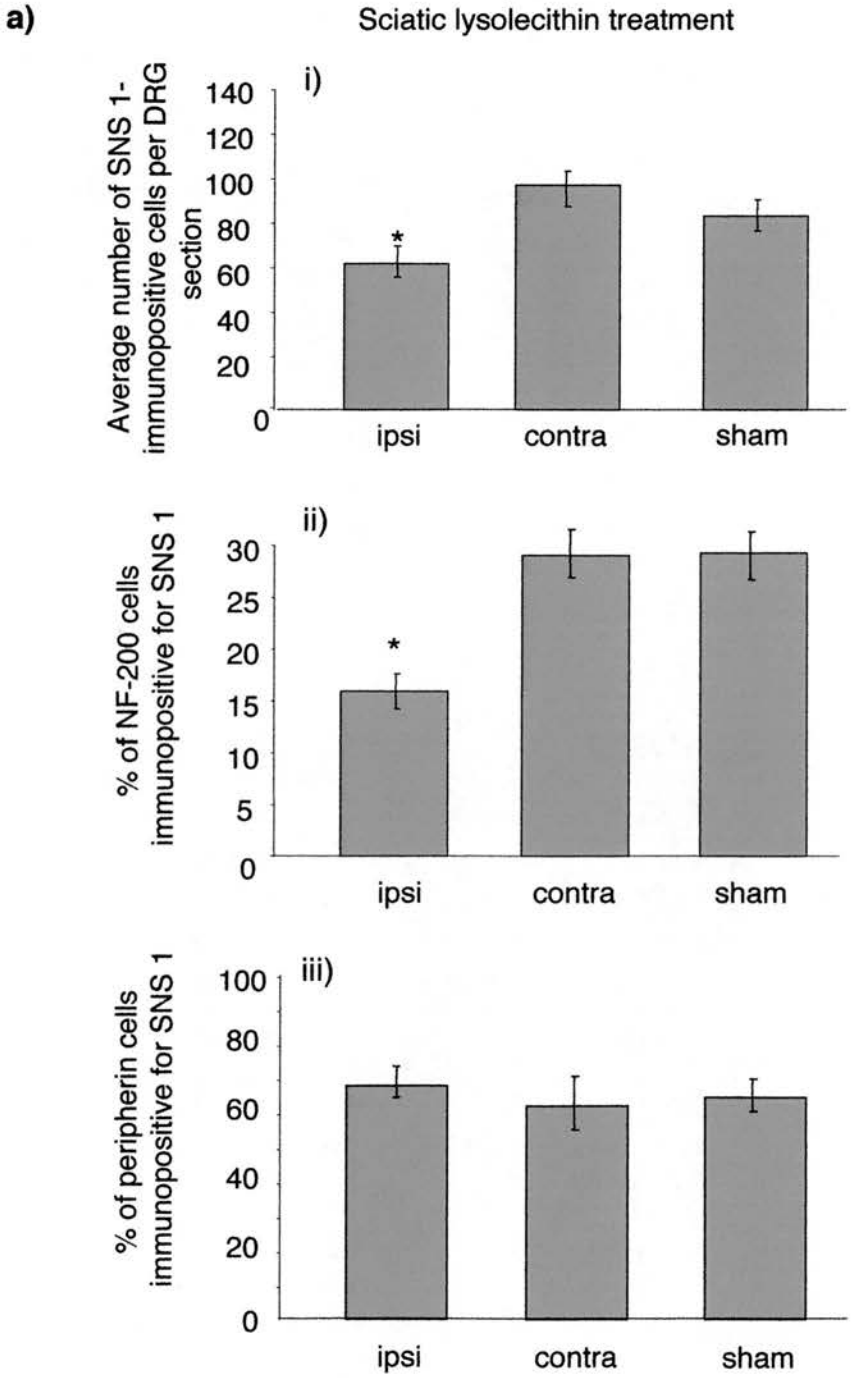
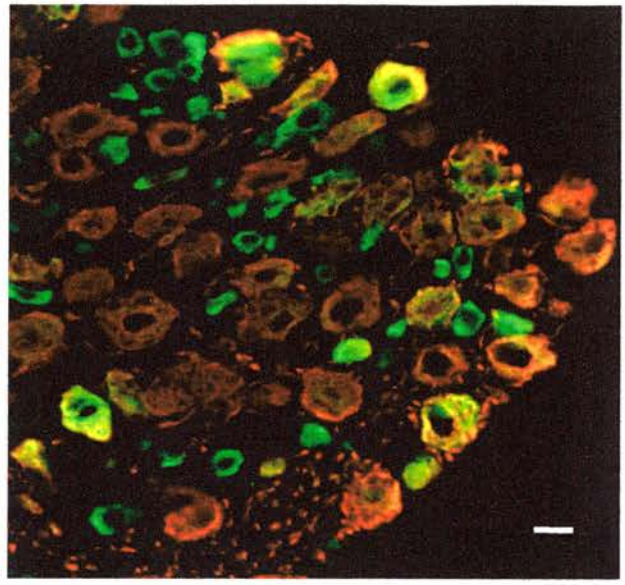

a)
Sciatic lysolecithin treatment
i)
Average number of SNS 1-immunopositive cells per DRG section
140
120
100
80
60
40
20
0
*
ipsi
contra
sham
ii)
% of NF-200 cells immunopositive for SNS 1
30
25
20
15
10
5
0
*
ipsi
contra
sham
iii)
% of peripherin cells immunopositive for SNS 1
100
80
60
40
20
0
ipsi
contra
sham

Figure 4.12. cont...

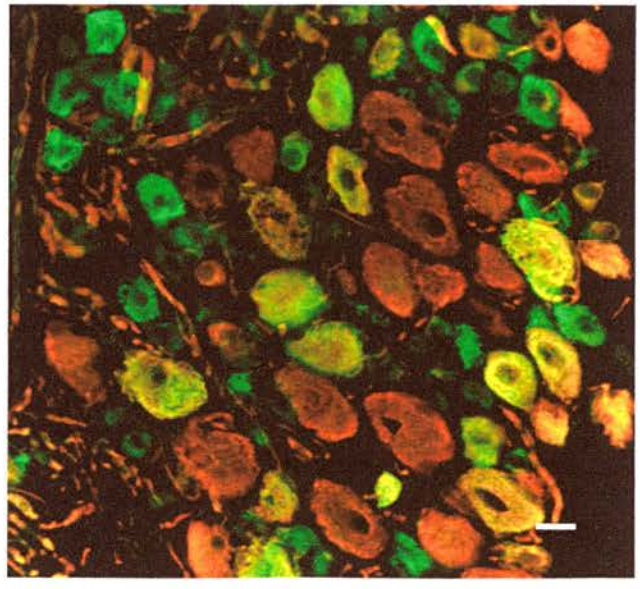
Sciatic lysolecithin treatment

iv)



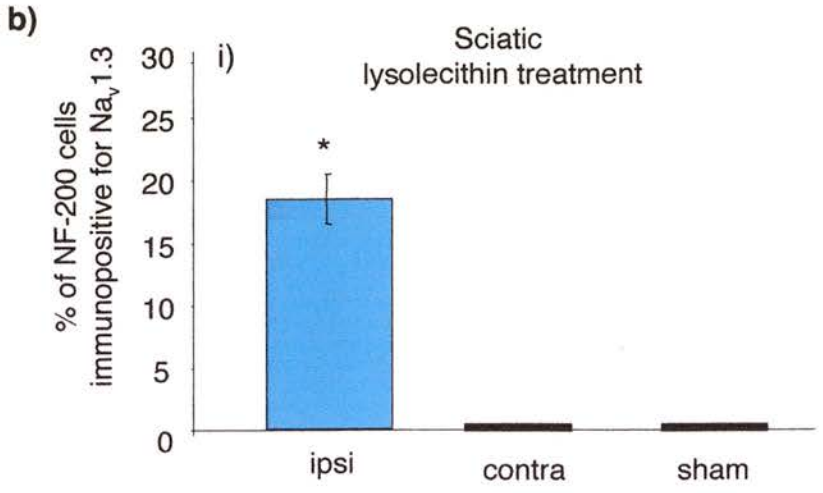
ipsi to lysolecithin

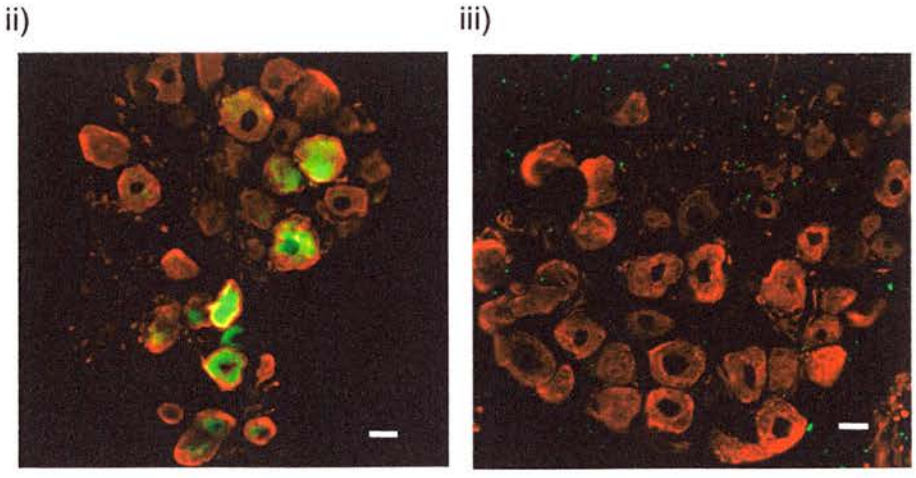
v)



contra to lysolecithin

Figure 4.12. cont...





ipsi to lysolecithin

contra to lysolecithin

Figure 4.13. Immunohistochemical assessment of the sodium channels; SNS 1/Na_v1.8 and brain type III/ Na_v1.3 in DRG cells of 6 week old *Prx*-null mice and wild-type littermates.

a) The expression of the sodium channel SNS 1/Na_v1.8 in DRG sections from 6 week old *Prx-null* mice (n=4) and wild-type (WT) littermates (n=4). (i) *Prx*-null mice showed a statistically significant decrease of 42% ± 7.8 in expression of SNS 1/Na_v1.8 as compared to wild-type (WT), all of which were (ii) immunopositive for NF-200 and there was (iii) no decrease of SNS 1/Na_v1.8 expression in peripherin immunopositive cells. The statistical significance (*$p<0.05$) of any difference was determined by a Kruskal-Wallis One Way ANOVA on Ranks with an all pairwise multiple comparison procedure (Dunn's Method). Data is presented as (i) average number of immunopositive cells per DRG section ± SEM and as (ii) % of NF-200 cells immunpositive for SNS 1/Na_v1.8 or (iii) % of peripherin cells immunpositive for SNS 1/Na_v1.8. Images show SNS 1/Na_v1.8 -immunopositive cells labelled with FITC (green) and NF-200 immunopositive cells labelled with TRITC (red) (with colocalised immunoreactivity appearing yellow) in DRG of (iv) *Prx*-null mice and (v) wild-type (WT) mice. Scale bar: 20 μm.

b) The expression of the brain type III sodium channel/ Na_v1.3 in DRG sections from 6 week old *Prx*-null mice (n=4) and wild-type (WT) littermates (n=4). We saw a statistically significant increase in expression of type III/ Na_v1.3 expression in DRG from 6 week old *Prx*-null mice (*$p<0.05$) as determined by a paired Student's t-test. Data is presented as (i) % of NF200 cells immunpositive for type III/ Na_v1.3. Images show type III/Na_v1.3-immunopositive cells labelled with FITC (green) and NF-200 immunopositive cells labelled with TRITC (red) (with colocalised immunoreactivity appearing yellow) in DRG of (ii) *Prx*-null mice as opposed to no detectable type III/ Na_v1.3 immunopositive cells in DRG from (iii) wild-type (WT) mice. Scale bar: 20 μm.

Figure 4.13.

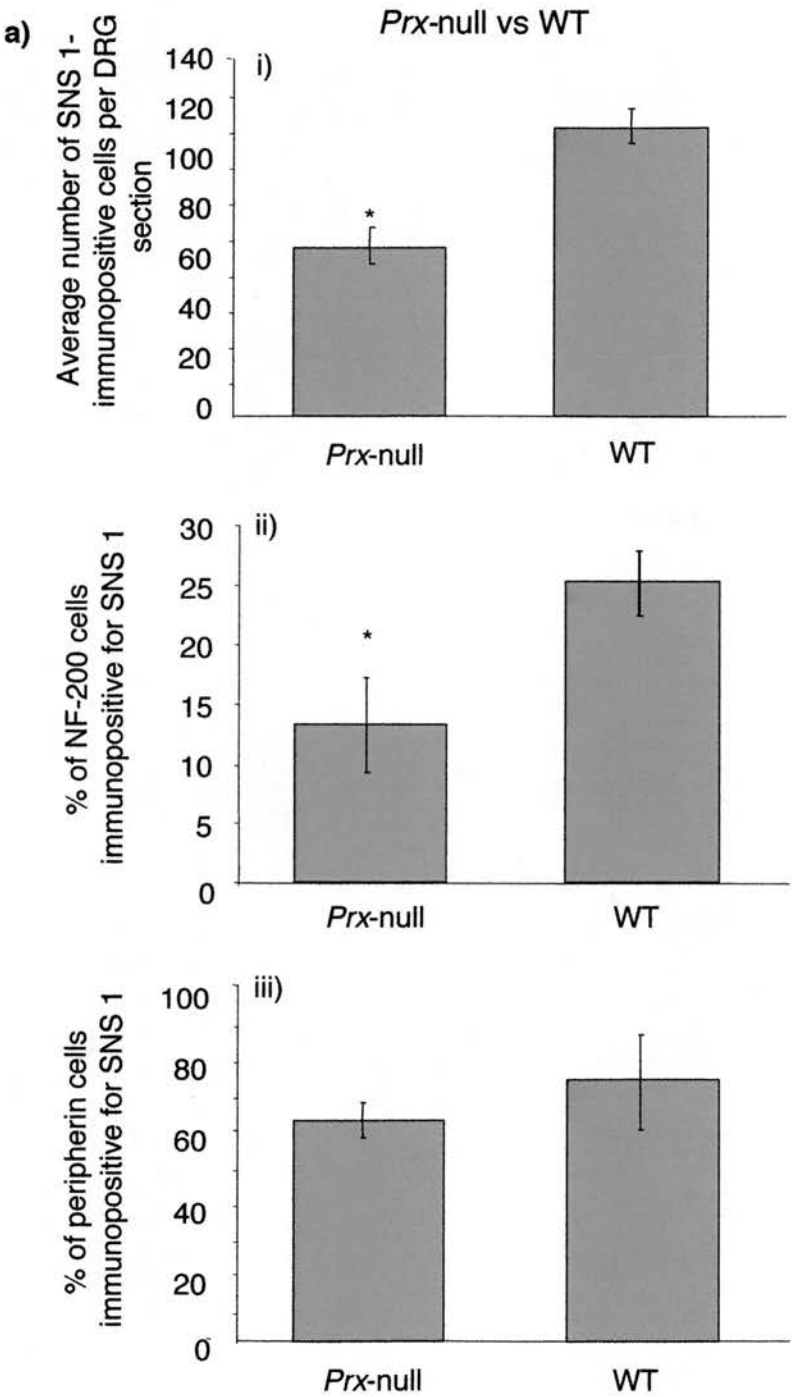
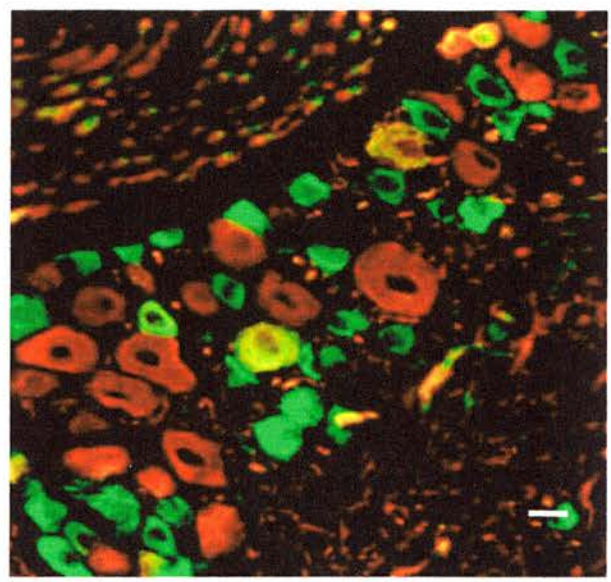

a)
Prx-null vs WT
i)
Average number of SNS 1-immunopositive cells per DRG section
140
120
100
80
60
40
20
0
*
Prx-null
WT
ii)
% of NF-200 cells immunopositive for SNS 1
30
25
20
15
10
5
0
*
Prx-null
WT
iii)
% of peripherin cells immunopositive for SNS 1
100
80
60
40
20
0
Prx-null
WT

Figure 4.13. cont...

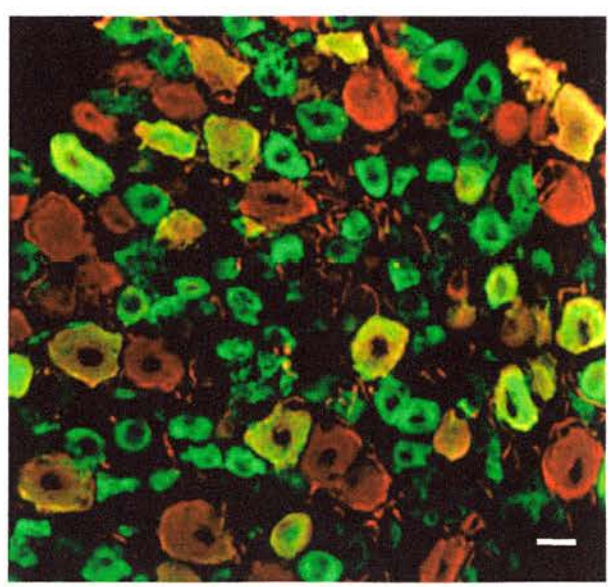
Prx-null vs WT

iv)



Prx-null

v)



WT

Figure 4.13. cont...

b)

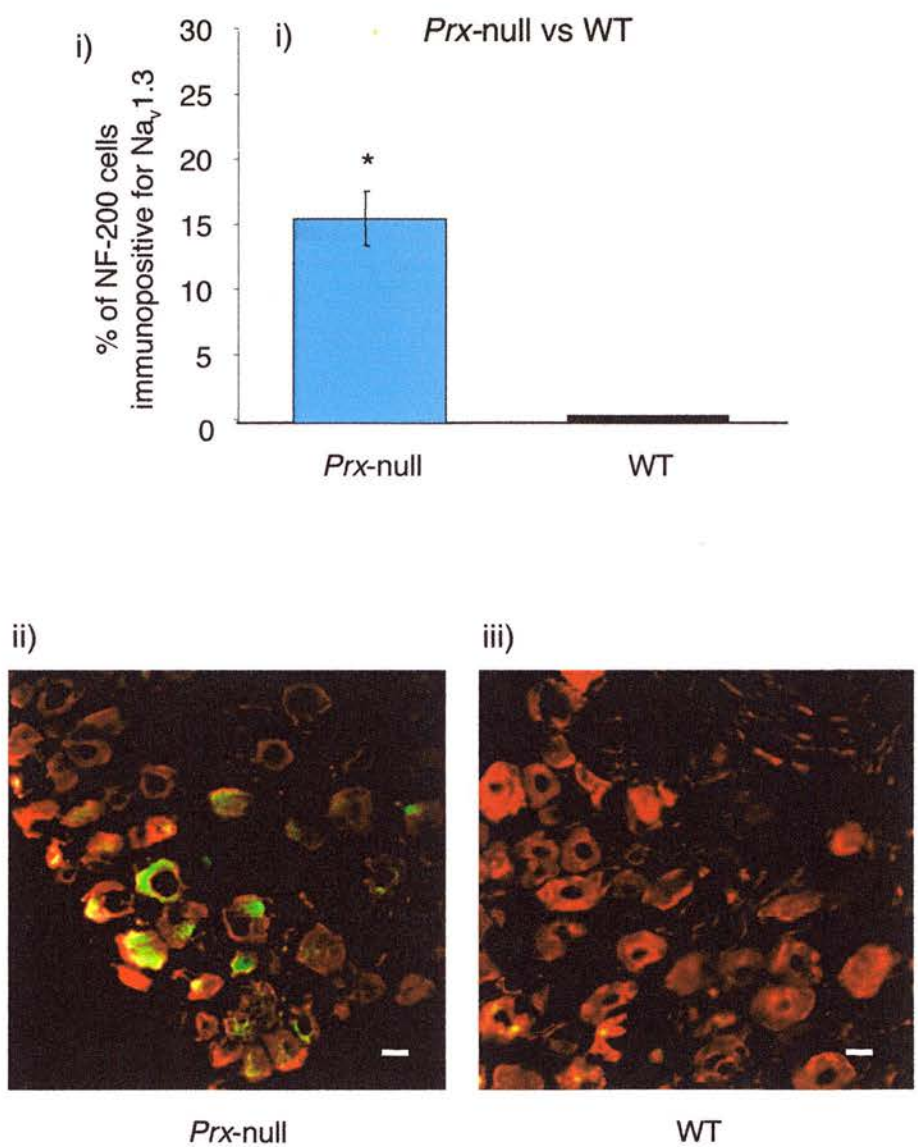


Table 3. Changes in expression of neuropeptides and the sodium channels in the DRG following lysolecithin treatment of the sciatic nerve and DRG of 6 week old *Prx*-null mice as compared to reported changes following axotomy and CCI.

The changes in expression of the peptides CGRP, SP, galanin and NPY and sodium channels SNS 1/ $Na_v1.8$ and brain type III/$Na_v1.3$ are compared for DRG sections ipsilateral to lysolecithin treatment on post-treatment day 13 (peak behavioural sensitisation) (n=4), DRG sections from 6 week old *Prx*-null mice and reported changes at times of peak behavioural sensitisation following nerve injury models axotomy and CCI. Downregulation of expression in small diameter cells of unmyelinated afferents is represented by (-u), in just medium to large diameter cells of myelinated afferents by (-m) and in both small and medium to large diameter cells by (--). Upregulation in just small diameter cells of unmyelinated afferents is represented by (+u), in just large diameter cells of myelinated afferents by (+m) and in both small and medium to large diameter cells by (++). No change in expression between treatment group and normal expression values is represented by 0.

Table 3.

Peptide/ Channel	Ipsilateral to axotomy (peak)	Ipsilateral to CCI (peak)	Ipsilateral to lysolecithin-treated sciatic nerve (peak)	*Prx*- null
CGRP	- -	- -	0	0
SP	- u +m	- u	0	0
Galanin	++	++	0	0
NPY	+m	+ m	+ m	+ m
SNS 1	- -	- -	- m	- m
Type III	+ +	+ +	+ m	+ m

4.2: Discussion

Correlates of neuropathic pain such as spontaneous pain, hyperalgesia and allodynia are often symptoms associated with human demyelinating neuropathies such as Multiple Sclerosis (MS), Charcot-Marie-Tooth type I disease (CMT) and Guillain-Barré Syndrome (GBS). Although sensory deficits are usually less severe than motor deficits, pain has been reported to be a problem in 70% of CMT sufferers (Carter et al., 1998) indicating it as a substantial clinical problem. Even though axonal pathology has not always been adequately investigated in demyelinating neuropathies, it is generally believed that pain associated with demyelinating conditions results from axonal damage. As a result, the consequences of the lack of myelin alone have, to date, been largely ignored. Furthermore, existing animal models of neuropathic pain, such as CCI, involve massive demyelination of the injured nerve, implying that this process could have an important role in the resulting chronic pain. Nevertheless, mechanisms underlying neuropathic pain which occur as a consequence of demyelination of the peripheral nerve are poorly understood (Rasminsky, 1981). Therefore, we have utilised a recently described model of peripheral nerve demyelination, the *Prx*-null mouse, and subsequently developed an animal model of focal peripheral nerve demyelination, using the myelinolytic agent lysolecithin, in order to study neuropathic pain following peripheral demyelination.

4.2.1. Altered afferent characteristics and behavioural reflex sensitivity in *Prx*-null mice.

The *Prx*-null mouse is unique in the late onset and the severity of its demyelinating phenotype, which shows parallels with demyelinating neuropathies of adult onset (Dyck et al., 1993). Furthermore, mutations in the human *PRX* gene have recently been linked to the condition, CMT4F, the clinical and histopathological features of which show striking similarities to the phenotype of the *Prx*-null mouse (Boerkoel et al., 2002;Gillespie et al., 2000;Guilbot et al., 2001). This evidence substantiates the *Prx*-null mouse as a model of CMT-like demyelinating disease (Guilbot et al., 2001) indicating it as a good model in which to study pain associated with demyelination. At 6 weeks of age, the function of the sciatic nerves of Prx-null mice are affected relatively mildly when compared to other mice in which myelin proteins have been altered (Gillespie et al., 2000). This is reflected in the lack of any overt motor deficit in these animals using the mechanical grip test (Meyer, 1979). However, afferent conduction velocities are reduced by approximately 50% indicative of demyelination of axons. By 6 months of age, extensive demyelination is observed in a

variety of myelinated nerves throughout the PNS of the mutant and conduction velocities in the sciatic and saphenous nerves of the mutant animal are reduced by 67% as compared to wild-type animals. Previous work, investigating the electrophysiological properties of the saphenous nerve in 6 week old *Prx*-null animals, revealed that although there is no evidence for changes in the stimulus-response relationship in C- or A- fibres to either cutaneous mechanical or thermal nociceptive stimulation, spontaneous discharge of 1-2 impulses per second is present in saphenous fibres across the conduction velocity range (Gillespie et al., 2000). This spontaneous activity is of interest to this study due to the fact that the generation of impulses at abnormal locations along the axon of sensory neurons (Devor and Seltzer, 1999) is thought to play an important role in the generation of neuropathic pain. Such 'ectopic' activity is known to emerge due to various components of the injury, which can include an area of demyelination (Baker and Bostock, 1992;Calvin et al., 1982;Rasminsky, 1978;Smith and McDonald, 1980), and is likely to be the origin of ectopic discharge in our models of peripheral nerve demyelination. Furthermore, although repetitive C-afferent axon stimulation is required to bring about a neuropathic pain state in many animal models of nerve injury (Coderre, 1993); (Amaya et al., 2000), it may also be maintained and promoted by Aβ-fibre input (Woolf and Costigan, 1999) and ectopic activity in A-fibres alone has been suggested to be sufficient to trigger tactile allodynia in some models of peripheral neuropathic pain (Liu et al., 2000; Wu et al., 2000). Therefore, this feature of the phenotype prompted us to investigate the behavioural reflex sensitivities of 6 week old and 6 month old *Prx*-null mice. We have demonstrated that at both ages, the Prx-null mice have significantly sensitised thresholds for hindpaw withdrawal responses to mechanical and noxious thermal stimuli, indicating the presence of behavioural correlates of abnormal thermal hyperalgesia and mechanical allodynia indicative of a neuropathic pain-like state.

4.2.2. Immunohistochemical investigation of evidence for DRG cell loss or axonal damage in the *Prx*-null mouse.

For any analysis of the mechanisms underlying neuropathic pain as a consequence of myelin loss it is of great importance that there are no axonopathic changes associated i.e. any axonal damage. Previous work characterising the phenotype of the 6 week old *Prx*-null mouse indicated no evidence for axonal degeneration or cell loss in the DRG (Gillespie et al., 2000). We confirmed this by investigating the presence of NF-200, which labels the larger diameter, myelinated DRG cell population (Lawson and Waddell, 1991;Michael and Priestley, 1999), and peripherin, a type III intermediate filament, which is normally expressed selectively by unmyelinated sensory neurons (Amaya et al., 2000). We demonstrated that there was no difference in the number of cells expressing either marker between the DRG of *Prx*-null and wild-type mice. Importantly there was also no evidence for significant expression of the indicator of axonal injury, ATF3 (Tsujino et al., 2000). Therefore we can find no evidence that this model of peripheral nerve demyelination shows any associated axonopathy or cell loss at 6 weeks of age.

4.2.3. Altered behavioural reflex sensitivity following lysolecithin-induced, focal demyelination of the peripheral nerve.

The emerging link between peripheral nerve demyelination and neuropathic pain in the *Prx*-null mouse prompted us to create a more refined model of peripheral nerve demyelination in which to address the study of neuropathic pain. Furthermore, we felt that it was important for such a study of neuropathic pain that firstly, the effects of demyelination in motor fibres were kept to a minimum; and secondly, that any whole body effects of inactivating a gene, in pre- and post-natal development, were eliminated. Therefore, we sought to refine a model of peripheral nerve demyelination to target individual sensory nerves with areas of focal demyelination. To achieve this we have developed a model of focal demyelination in the predominantly sensory, saphenous nerve, and in the sciatic nerve using the non-toxic, myelinolytic agent, lysophosphatidyl choline (lysolecithin) (Hall and Gregson, 1971). Following lysolecithin-treatment of either the saphenous or sciatic nerve, we have demonstrated the development of sensitised hindpaw withdrawal responses to mechanical and noxious thermal stimuli, indicative of the presence of thermal hyperalgesia and mechanical allodynia. This increased behavioural sensitivity peaked over days 9-14 following treatment and returned to baseline levels by 3 weeks. Therefore, we have demonstrated, for the first time, the development of pain-related behaviour following induction of focal demyelination using lysolecithin and of particular importance, following

changes in the sensory saphenous nerve, a nerve which is rarely manipulated in models of neuropathic pain.

4.2.4. Morphology of the demyelinated nerve.

For any analysis of the mechanisms underlying neuropathic pain as a consequence of myelin loss it is again of great importance that there are no additional axonopathic changes in this model. We chose lysolecithin as our myelinolytic agent as, based on previous reports on the use of lysolecithin to induce demyelination of the sciatic nerves of adult mice, its action is rapid and restricted to the myelin sheath (Hall and Gregson, 1971) with resulting demyelination remaining confined to the site of application. Importantly, there is little damage to the Schwann cell cytoplasm or to the axon (Love et al., 1986). Nevertheless, as this is the first time that lysolecithin application to the peripheral nerve has been used to study demyelination of sensory nerves associated with neuropathic pain, we have conducted thorough morphological investigations to ensure that i) significant demyelination occurs following lysolecithin-treatment and ii) that there is no evidence that axon damage has occurred. Based on previously reported methods of delivery to the peripheral nerve, we initially developed two methods of lysolecithin application; sub-perineurial injection and topical application. However, when injected into the nerve via a glass micropipette, it was clear from EM images that mechanical damage of the nerves had occurred. This is in line with a report of small diameter fibre damage following injection of lysolecithin into the nerve (Mitchell and Caren, 1982) and we therefore could continue no further with our investigations using this method. We sought to avoid this problem by employing a topical application technique. In our model, such controlled topical application of lysolecithin to the saphenous or sciatic nerve produces a focal region of essentially complete demyelination of approximately 40% of the previously myelinated axons (plus partial losses in others) at times corresponding to peak behavioural reflex sensitisation. This demyelination occurred without any detectable axon damage or loss as assessed by light and electron microscopy of afferents. Therefore, this suggests we have developed a novel model of a neuropathic pain state associated with peripheral nerve demyelination that does not seem to be accompanied by axonal loss.

4.2.5. Immunohistochemical investigation of evidence for DRG cell loss or axonal damage in the lysolecithin-treated saphenous/sciatic nerves.

Corresponding to LM and EM investigations of topical lysolecithin application to either the sciatic or saphenous nerve, immunohistochemical investigations demonstrated no loss of neuronal somata in the DRG as identified by the medium to large diameter cell marker, NF-200, or the small diameter cell marker, peripherin. Importantly there was also no evidence for significant expression of the indicator of axonal injury, ATF3 (Tsujino et al., 2000). Therefore, as in the 6 week old *Prx*-null mouse, the lysolecithin-induced model of peripheral nerve demyelination appears to be unaccompanied by axonopathy or cell loss and demonstrates that we have developed a novel experimental model of a neuropathic pain state associated with peripheral nerve demyelination.

4.2.6. Electrophysiological characteristics of the lysolecithin-demyelinated saphenous nerve.

Demyelination of peripheral nerve axons initially results in conduction block rendering the nerve incapable of action potential transmission to and from the CNS, which presumably accounts for symptoms experienced, such as motor dysfunction and sensory loss (Rasminsky, 1981). However, reorganisation of membrane electrical properties, involving redistribution of ion channels allows action potential conduction through the demyelinated segment. However, such reorganisation may lead to hyperexcitability, reflected in spontaneous action potential discharge, mechanosensitivity, and sustained after-discharge (Baker and Bostock, 1992), which may lead to the development of persistent pain. Therefore, we have investigated the electrophysiological characteristic of the lysolecithin-demyelinated saphenous nerve at times corresponding to peak behavioural change.

Based on evidence suggesting that peripheral sensitisation can be a significant contributor to neuropathic pain behaviour in a variety of models of central (spinal) sensitisation (Koltzenburg, 1998;Woolf and Costigan, 1999), we initially investigated the presence of any changes in peripheral transduction thresholds following lysolecithin treatment of the saphenous nerve. We could find no evidence for significant changes in the threshold for action potential generation in either A or C fibres to either cutaneous mechanical or thermal nociceptive stimulation.

We were therefore interested to investigate what changes, if any, in afferent fibre properties could be contributing to the behavioural reflex sensitisation observed in animals following lysolecithin treatment. As mentioned previously, ectopic repetitive firing has been proposed

to be the origin of abnormal sensation in diseases characterised by segmental demyelination, particularly where there is no evidence for axonopathic changes (Rasminsky, 1981;Tal and Eliav, 1996) which is the situation represented by our model of lysolecithin-induced focal demyelination. Demyelinated segments can serve as foci for spontaneous action potential generation (Baker and Bostock, 1992;Calvin et al., 1982;Smith and McDonald, 1980) and there is good evidence that mechanical hypersensitivity observed in tactile allodynia is mediated by myelinated A-fibres (Koltzenburg, 1998;Woolf, 1997), a fact that may be of great importance in the development of neuropathic pain following demyelination. Furthermore, it has been suggested that spontaneous activity in myelinated afferents is sufficient to induce neuropathic pain behaviours in animal models of nerve injury (Liu et al, 2000; Wu et al., 2000). Therefore, it is of much interest that we observed the presence of spontaneous low frequency discharge (2-3 impulses/sec) in the primary afferents of the focally demyelinated nerve, only at times which corresponded with peak behavioural reflex sensitivity. This is similar to the spontaneous activity shown to be a feature of the *Prx*-null mouse. Although we have not dissected out which afferents are firing spontaneously, due to the fact that there appears to be no damage to the unmyelinated afferents, it is likely that such activity may be arising from formerly myelinated afferents in both models. We cannot, however, rule out the possibility of ephaptic transmission of spontaneous activity to unmyelinated afferents at sites of demyelination (Rasminsky, 1978). Ephaptic crosstalk may cause normally nociceptive afferent neurons to be driven by electrical activity in low threshold afferents (Devor and Seltzer, 1999) such as the demyelinated A-fibre afferents in our preparation, which may, in turn, further sensitise the spinal dorsal horn neurons (see Chapter 6). It would be of interest to follow this discovery with further investigations into electrophysiological changes resulting from demyelination of the peripheral nerve. Firstly, it is important to reveal the specific fibre type in which this spontaneous activity is occurring. Also, as the preparation used for these investigations involved decentralisation of the peripheral nerve to eliminate any activity occurring in any efferent axons or in axons of the sympathetic nervous system, the electrophysiological consequences of demyelination of the peripheral nerve proximal to the point of decentralisation, were also eliminated. For example, any changes in characteristics of DRG neurons could be of significance as they have been shown to fire spontaneously in other models of neuropathic pain (Abdulla and Smith, 2001;Amir et al., 2002;Liu et al., 2000c). Also recording from dorsal roots may give a more accurate indication of the magnitude of any hyperexcitablity arising for the peripheral nerve. It is likely that such investigations would reveal activity of a greater magnitude than has been demonstrated here, which could likely play a major role in enhanced transmission

to the dorsal horn of the spinal cord, inducing cellular changes thought to bee associated with the development of central sensitisation (see Chapter 6).

Nevertheless, we have demonstrated the presence of abnormal afferent electrical properties following lysolecithin-induced demyelination, which may be of a frequency great enough to be a likely contributor to the development of a neuropathic pain state (see Chapter 6).

4.2.7. Phenotypic Changes in the DRG

a) Neuropeptides

Following peripheral nerve lesions, prominent changes are seen in the expression of peptides in primary sensory neurons and in the spinal cord. The changes observed are thought to represent primarily adaptive, and restorative responses of the nervous system to limit damage to the organism and to promote survival and recovery of the neuron. However, secondary actions of upregulated peptides may cause enhanced transmission to the dorsal horn of the spinal cord contributing to the induction of a sensitised state, which may underlie the development of the chronic pain (Hokfelt et al., 1994). We therefore decided to investigate whether some of these characteristic changes occur in our models of demyelination in order to assess whether the pain behaviours displayed are associated with changes similar to those seen in other models of neuropathic pain. The question of whether any changes are restricted to specific cell types is also of interest, since our model appears to affect the morphology of only the larger myelinated fibres.

CGRP and SP

We initially investigated the expression of CGRP, which is normally expressed by approximately 50% of unmyelinated DRG neurons as well as a proportion of medium diameter myelinated neurons (Gibson et al., 1984;Hokfelt et al., 1994;Rosenfeld et al., 1983). Following traumatic peripheral nerve injury, the expression of CGRP is dramatically downregulated (Dumoulin et al., 1992;Noguchi et al., 1990). However, we could find no evidence of a decrease in CGRP expression in the DRG cells of the *Prx*-null mouse at 6 weeks when compared to the DRG of wild-type littermates, or in DRG ipsilateral to demyelination of one saphenous or one sciatic nerve, when compared to the DRG contralaterally or of sham controls. This prompted us to also investigate the expression of SP in both models, the expression of which is also dramatically downregulated following axotomy or CCI (Jessell et al., 1979;Nielsch et al., 1987). We demonstrated no changes in SP expression levels in either model. The lack of a change in the expression of either peptide may be a reflection of the lack of damage to the small unmyelinated cells or the

axons of the larger A-class neurons. It has been suggested that the decrease in CGRP expression is due to interrupted retrograde transport of NGF from peripheral target tissue (Shadiack et al., 2001). Therefore, the fact that we see no decrease in CGRP expression suggests that such retrograde transport may not be affected by the demyelination, implying that the axons are still functionally intact (see section 5.2.3).

Due to there being no change in the number of cell bodies in the DRG of either model of peripheral nerve demyelination, we decided to restrict further immunohistochemical investigations to the model of lysolecithin-induced demyelination of the sciatic nerve, and to the *Prx*-null mouse. As mentioned in Chapter 3, we chose the sciatic model as opposed to the saphenous nerve model because it is a larger nerve and therefore has a larger proportion of cell bodies in the lumbar DRG as opposed to saphenous nerve. We have demonstrated that there is no damage to A- or C-fibres following lysolecithin application to either nerve as well as no indication of cell loss in the DRG or change in the expression of CGRP or SP suggesting that lysolecithin results in a comparable condition following application to either nerve. Therefore, any effect of demyelination on the phenotype of associated cell bodies should be comparable but easier to detect in the model of sciatic nerve demyelination.

Galanin

We investigated the presence of any changes in the expression of galanin following demyelination of the sciatic nerve as it is known to increase in cells of the DRG following axotomy and CCI (Hokfelt et al., 1987;Villar et al., 1989). We could detect no increased expression in cells of DRG from mice following lysolecithin treatment or from the *Prx*-null mouse at 6 weeks of age. This again suggests that the axons in the preparation are supplying sufficient levels of NGF from the periphery which is also thought to influence the expression of galanin (Shadiack et al., 2001).

The alterations of CGRP, SP and galanin in various models of neuropathic pain have been proposed to be involved in the development of neuropathic pain. However, we saw no change in these peptides in our models of peripheral nerve demyelination, which have associated behaviours indicative of neuropathic pain. This suggests that although these neuropeptides can play a functional role in enhancing the production of a sensitised state (Wynick et al., 2001), changes in their expression are perhaps not critical for the production of neuropathic pain. Therefore, their role may be of less significance than previously thought, which may help to explain why therapeutics targeting such peptides have yet to be

proved efficacious (Bennett et al., 2000). Furthermore, it is of importance that changes characteristic of nerve injuries that usually occur in the small diameter cells of unmyelinated axons, are not observed in either of our models. This further substantiates the evidence that in our models of neuropathic pain there is a lack of involvement of the unmyelinated fibres that are thought to be critical for the production of chronic pain in many other models and the fact that changes in myelinated fibres alone may be sufficient for the development of a neuropathic pain state

NPY

It has been demonstrated that following axotomy (Kashiba et al., 1994;Noguchi et al., 1993;Wakisaka et al., 1991), partial sciatic injury (Ma and Bisby, 1998), CCI of the sciatic nerve (Munglani et al., 1995;Nahin et al., 1994) and following streptozotocin-induced diabetic neuropathy (Rittenhouse et al., 1996), the expression of NPY increases in large diameter primary sensory neurons, distinguished by the presence of the 200kDa subunit of neurofilament protein (Kashiba et al., 1994;Marchand et al., 1999). In line with such studies, we observed an upregulation of the expression of NPY in large NF-200-positive cells in the *Prx*-null mouse and ipsilateral to the focal demyelination of one sciatic nerve.

It is of particular importance that this is a change also restricted to NF-200-positive cells on all models of neuropathic pain as this suggests that via demyelination, we have induced comparable changes to such models in myelinated neurons, whilst eliminating the changes associated with unmyelinated neurons. The increase in expression of NPY following nerve transection varies from 16% (Shi et al., 2001) to 30% (Kashiba et al., 1994) in the literature. Therefore, the increase in NPY expression we observe (10% in *Prx*-null DRG; 16% in DRG ipsilateral to lysolecithin) is lower than in most models of sciatic nerve transection which may correspond to the lack of axon damage involved in both our models of demyelination compared to more severe models of neuropathic pain.

Upregulation of NPY may represent an adaptive response by primary sensory neurons to traumatic injury, regardless of the initiating event. This implies that in our model of demyelination, NPY has been upregulated, in the cells of demyelinated axons, as an adaptive response of the nervous system to damage of the myelin sheath. How this upregulation is specifically controlled is unknown. The upregulation of NPY may have additional consequences upon primary afferent actions upon cells in the dorsal horn supported by evidence for the centrifugal transport of NPY in primary afferents to the spinal cord (Zhang et al., 1993;Wakisaka et al., 1991). NPY acts via two metabotropic receptors; Y_1 and Y_2. The actions of NPY via the Y_1 receptor are usually excitatory via its actions upon voltage-

gated ion channels and the release on intracellular calcium via activation of IP_3. In contrast the actions of NPY via the Y_2 receptor are inhibitory due to the inhibition of calcium entry into the cell (Bleakman et al., 1991;Ewald et al., 1988). A role for NPY in pain perception is suggested by the fact that following nerve injury, NPY upregulation follows the time-course of injury-induced Aβ-fibre firing (Frisen et al., 1992), thought to be related to the appearance of allodynia (Woolf and Doubell, 1994). Furthermore, levels of NPY in the spinal cord, in axons of myelinated sensory input, relates to the degree of hyperalgesia displayed by the animal (Munglani et al., 1995). This would suggest that a similar role of NPY might be occurring in our models of peripheral nerve demyelination, in which myelinated fibre changes are produced, and implies a role for NPY in the production of some of the behavioural reflex sensitisation we observe. Furthermore, the fact that in all nerve injury models NPY expression is associated with cells of myelinated fibres provides a logical explanation for why it is the only peptide in which we observe an expression change.

Intrathecal application of NPY suggests that Y_1 receptors are located on dorsal horn neurons (Duggan et al., 1991). Therefore, an increase in NPY release from primary afferents fibres may lead to an increase in intracellular calcium in these cells, a change crucial for the production of central sensitisation (see Chapter 6). However, the overall role of NPY in nociception is controversial. Levels of NPY have been demonstrated to increase in animals without any development of behavioural sensitisation (Benoliel et al., 2001). Furthermore, mice lacking the Y_1 receptor develop hyperalgesia and mechanical sensitivity (Naveilhan et al., 2001) suggesting a role for the receptors in the production of analgesia and it has been suggested that NPY plays an antinociceptive role via its actions upon Y_2 receptors peripherally (Hokfelt et al., 1997). Nevertheless, theory would imply that the actions of NPY on spinal Y_1 receptors might play a role in sensitisation of dorsal horn cells indicating a possible role for its upregulation in the behavioural sensitisation displayed by our animal models of peripheral nerve demyelination.

A further mechanism by which peripheral NPY release may play a role in the maintenance of neuropathic pain, is via it interactions with the sympathetic nervous system, with a possible role in potentiating algesic effects of released noradrenaline (Edvinsson et al., 1984).

b) Na^+ channel expression

In models of neuropathic pain such as following axotomy (Waxman et al., 1994b) and CCI (Dib-Hajj et al., 1999), changes are seen in the expression of several Na^+ channel genes leading to changes in the properties of the Na^+ current in sensory neurons. These changes include the down regulation of the tetrodotoxin-resistant channel SNS 1/ $Na_v1.8$ (Akopian et al., 1996;Dib-Hajj et al., 1996;Novakovic et al., 1998;Sangameswaran et al., 1996;Waxman et al., 1999b), paralleled by the upregulation in expression of the (brain) type III Na^+ channel/ $Na_v1.3$ (Waxman et al., 1994;Waxman, 1999). We have demonstrated that in line with other models of neuropathic pain, there is a selective decrease in the expression of SNS 1/ $Na_v1.8$ channels in the DRG of the *Prx*-null mouse (48% decrease) and following lysolecithin-induced, focal demyelination of the sciatic nerve (34% decrease). However, in contrast to the axotomy and CCI models, following demyelination this decrease in SNS 1/ $Na_v1.8$ expression appears to be restricted to NF-200-positive cells, all of which have myelinated or now demyelinated axons. This further supports the role of changes in A-fibres in the production of neuropathic pain and suggests that mechanisms underlying sensitisation and pain associated with demyelination of the peripheral nerve may differ in some respects from other models of neuropathic pain. The results of the type III Na^+ channel/ $Na_v1.3$ localisation experiments suggest that there is an upregulation of the type III Na^+ channel in around 16% and 18% of the NF-200-immunoreactive neuron population of the DRG of the *Prx*-null mouse and the demyelinated sciatic nerve respectively. The population of cells upregulating the type III Na^+ channel/ Na_v 1.3 following nerve injury in previous reports appears to vary with the model used. Following sciatic nerve transection, type III Na^+ channel/ Na_v 1.3 immunoreactivity was observed in most small diameter neurons (Black et al., 1999). In contrast to this, following spinal nerve ligation, type III/ Na_v 1.3 immunoreactivity was observed in 15-21% of medium to large DRG neurons (Kim et al., 2001a). These studies used the same antibody as we have employed here. We are aware that the specificity of this antibody has been questioned previously, and agree that background fluorescence is observed when using this antibody. Nevertheless, there was a clear increase in immunofluorescence in specific cells when compared to others from the same DRG and to those in control sections, which was of a sufficient magnitude to indicate a clear difference of type III/ Na_v 1.3 expression in the cells. It is of interest that the changes we observe in our model of peripheral nerve demyelination are restricted to myelinated cells with a similar proportion as that observed in the SNL study (Kim et al., 2001a). This further supports the importance of A-fibres in the development of neuropathic pain and that changes in their phenotype alone may be as sufficient for the induction of changes underlying persistent pain

as changes occurring in both myelinated and unmyelinated fibres in other models of neuropathic pain.

The changes that we observed in the expression of the SNS 1/ $Na_v1.8$ and type III/ Na_v 1.3 Na^+ channels may be of functional importance, and due to their differing channel current properties, are likely to be involved in the production of spontaneous action potential discharge displayed in the demyelinated nerves of both models. The SNS class of Na^+ channels produce slowly inactivating current (Akopian et al., 1996;Sangameswaran et al., 1996;Waxman et al., 2000). Therefore their decreased expression in DRG neurons may lead to a hyperpolarizing shift in resting potential increasing the fraction of TTX-sensitive channels available for activation (Dib-Hajj et al., 1999;Waxman et al., 2000). Upregulation of the type III/ Na_v 1.3 channel results in a switch in the properties of the TTX-sensitive currents in DRG neurons, with the emergence of a rapidly repriming current (Waxman et al., 1999c) Therefore, DRG neurons that express it should be able to sustain higher firing frequencies which may lead to hyperexcitability in the cell. This hyperexcitability may be sufficient to generate sustained ectopic discharge necessary to maintain central sensitisation (Porreca et al., 1999). A further possible functional correlate of the changing expression of sodium channels in the DRG neurons, is the relocation of SNS 1/ $Na_v1.8$ channels to the demyelination site along with the *de novo* expression and distribution of type III/ Na_v 1.3 channels. It is known that following demyelination associated with nerve injury, Na^+ channels accumulate along patches of demyelination (Devor et al., 1989;Novakovic et al., 1998). Immunohistochemical studies have demonstrated the accumulation of abnormal aggregations of Na^+ channels at the distal tips of injured axons (Devor et al., 1989; England et al., 1994;Renganathan et al., 2000) including type III/ Na_v 1.3 (Black et al., 1999) and SNS 1/ Na_v 1.8 (Novakovic et al., 1998). Therefore, it is possible that the loss of SNS 1/ Na_v 1.8 immunoreactivity in L4-L6 DRG may represent subsequent redistribution and accumulation of channel protein in nerve proximal to injury (Novakovic et al., 1998c) or in this case, demyelination. Changes in the distribution and type of channel expressed in the axon are generally considered to lead to changes in distinct kinetic properties that could contribute to hyperexcitability and ectopic pacemaker activity (Cummins and Waxman, 1997;Devor et al., 1994;Matzner and Devor, 1992;Matzner and Devor, 1994;Omana-Zapata et al., 1997;Waxman, 1999) which again may be necessary to evoke and/or maintain central sensitisation. Evidence that Na^+ channel-blocking agents can be effective analgesics not only in experimental neuropathic pain conditions but also in chronic neuropathic pain in humans (Devor M et al., 1992;Chabal et al., 1992;Omana-Zapata et al., 1997;Rizzo, 1997), suggests that changes in expression of specific Na^+ channel subunits in our model of focal peripheral

nerve demyelination may indicate useful therapeutic targets for the treatment of pain associated with demyelination. Furthermore, although currently available Na^+ channel-blocking agents appear to lack the specificity required to target neuropathic pain effectively without the complications of side effects, work is in progress to create more specific Na^+ channel-blocking agents. Therefore, it may be of interested to follow this study with investigations to detect the localisation of the SNS 1/ $Na_v1.8$ and type III/ Na_v 1.3 Na^+ channels in the demyelinated peripheral nerves of both our models followed by investigating the effects of applying Na^+ channel-blocking agents to the demyelinated nerve on both the electrophysiological properties of the demyelinated nerve, as well as upon the behavioural reflex sensitisation displayed. These investigations may give a clearer insight into the level of the involvement of Na^+ channels in our model of neuropathic pain.

4.3 Summary

The investigations into the changes occurring in the PNS following demyelination of the peripheral nerve, have indicated that changes previously shown to be of importance in the production of neuropathic pain following nerve injury, such as axotomy and CCI, are also likely to play a role in our models of peripheral nerve demyelination, however, they are not entirely comparable. Of interest, we could only detect the presence of phenotypic changes in the cells of myelinated fibres. Moreover, changes known to occur in unmyelinated fibres following nerve injury models, such as axotomy and CCI, were not present in the DRG cells from either of our models of peripheral nerve demyelination. These results suggest that demyelination of the peripheral nerve has induced no changes in unmyelinated axons or their cell bodies. This highlights the important functional role that A-fibres are capable of playing in the induction of neuropathic pain and that conversely, C-fibre involvement, although associated with previous models of neuropathic pain, may not be necessary for its emergence. Of interest, we observed a change in the expression of Na^+ channel subtype in the cells of myelinated axons, a change that may have an important functional consequence in the development of neuropathic pain.

These investigations have also indicated that there are no obvious differences between the effect of genetic demyelination, as created in the *Prx*-null mouse model, and focally induced demyelination as in the lysolecithin model. This not only aids to assure that deleting the *Prx* gene has little indirect consequence on developmental factors relevant to this study, but strengthens the evidence that peripheral nerve demyelination, be it focal or widespread, can have consistent consequences on the nervous system and its function and likely plays an important role in all models of neuropathic pain.

Importantly, in both models, the demyelinated nerve displayed the presence of ectopic, spontaneous activity, a mechanism thought to be crucial for the induction of changes in the spinal cord that underlie persistent pain states. Therefore, the focus of this study moved centrally, to the spinal cord, to investigate the involvement of various specific changes that may indicate potential therapeutic targets.

CHAPTER 5. CHANGES IN THE CENTRAL NERVOUS SYSTEM IN MODELS OF PERIPHERAL NERVE DEMYELINATION:

5.1 Results

5.1.1. Intrathecal administration of drugs

Various drugs selective for receptors or signal transduction pathways were intrathecally administered to *Prx*-null mice or to mice displaying peak behavioural reflex changes following lysolecithin treatment to the saphenous nerve. The saphenous nerve preparation was used due to the focus of this study being upon the sensory aspects of demyelination and therefore our studies have aimed to concentrate on the effects following demyelination of the sensory saphenous nerve where possible.

a) The role of the NMDA receptor in the behavioural sensitisation displayed by *Prx*-null mice or following lysolecithin treatment.

A key change in neuropathic pain states involves sensitisation of cells in the dorsal horn in the spinal cord, which is thought to involve activation of the NMDA receptor. A role for spinal NMDA receptors in the mechanical allodynia displayed by 6 week old *Prx*-null animals and in the ipsilateral hindpaw in animals with focal demyelination of one saphenous nerve was demonstrated by the reversal of the behavioural sensitisation to von Frey filaments following spinal administration of the highly selective NMDA receptor antagonist (R)-CPP (Lehmann et al., 1987) (Figs 5.1a and 5.2a). Similarly, the reduction in thermal nociceptive response latency displayed by both groups was reversed by (R)-CPP without effect on wild-type littermates or in naïve animals (or in the case of lysolecithin-treated animals without effect on the contralateral side) (Fig 5.1a and 5.2a). With the exception of the first 20 minutes following injection on thermal withdrawal responses in the *Prx*-null mouse, equivalent injections of the saline vehicle had no significant effect on reflex responses in the *Prx*-null mouse or wild-type littermates or, ipsilateral and contralateral to lysolecithin treatment (Fig 5.1b and 5.2b). This apparent effect of saline on the thermal withdrawal latency in the *Prx*-null mouse does not correlate with the time course of the effect of (R)-CPP and is therefore likely to be a behavioural anomaly. The statistical significance of differences from pre-injection baseline values following all injections was determined by a One-Way Repeated Measures ANOVA) with Dunnet's multiple comparisons versus control group post-hoc analysis (*$P<0.05$).

Figure 5.1. Effects of intrathecal administration of the NMDA receptor antagonist (R)-CPP on the sensitised behavioural reflex responses to von Frey filaments and to radiant heat in *Prx*-null mice.

Paw withdrawal thresholds, in response to cutaneous mechanical stimulation with von Frey filaments and cutaneous noxious thermal stimulation, were measured in 6 week old *Prx*-null and wild-type (WT) littermate animals.

For *Prx*-null investigations, *Prx*-null (□) and WT (◆)values are displayed before and after intrathecal administration of each pharmacological agent. All paw withdrawal thresholds are displayed in mN/mm^2 for mechanical stimulation or mean paw withdrawal latency (seconds) for thermal stimulation. Each value is the mean ± SEM and any statistically significant (*$P<0.05$) difference at each time point post injection from pre-injection baseline values was determined by a One-Way Repeated Measures Analysis of Variance (ANOVA) with Dunnet's multiple comparisons versus control group post-hoc analysis.

a) The NMDA receptor antagonist R-CPP (100 pmol in 10 µl) reversed the behavioural reflex sensitisation to mechanical and noxious thermal stimulation observed in 6 week old *Prx*-null animals (n=8) for up to 55-60 minutes after intrathecal application.
b) Saline resulted in no significant change in the behavioural reflex sensitisation to mechanical and noxious thermal stimulation in *Prx*-null animals (n=4).

Figure 5.1.

a)

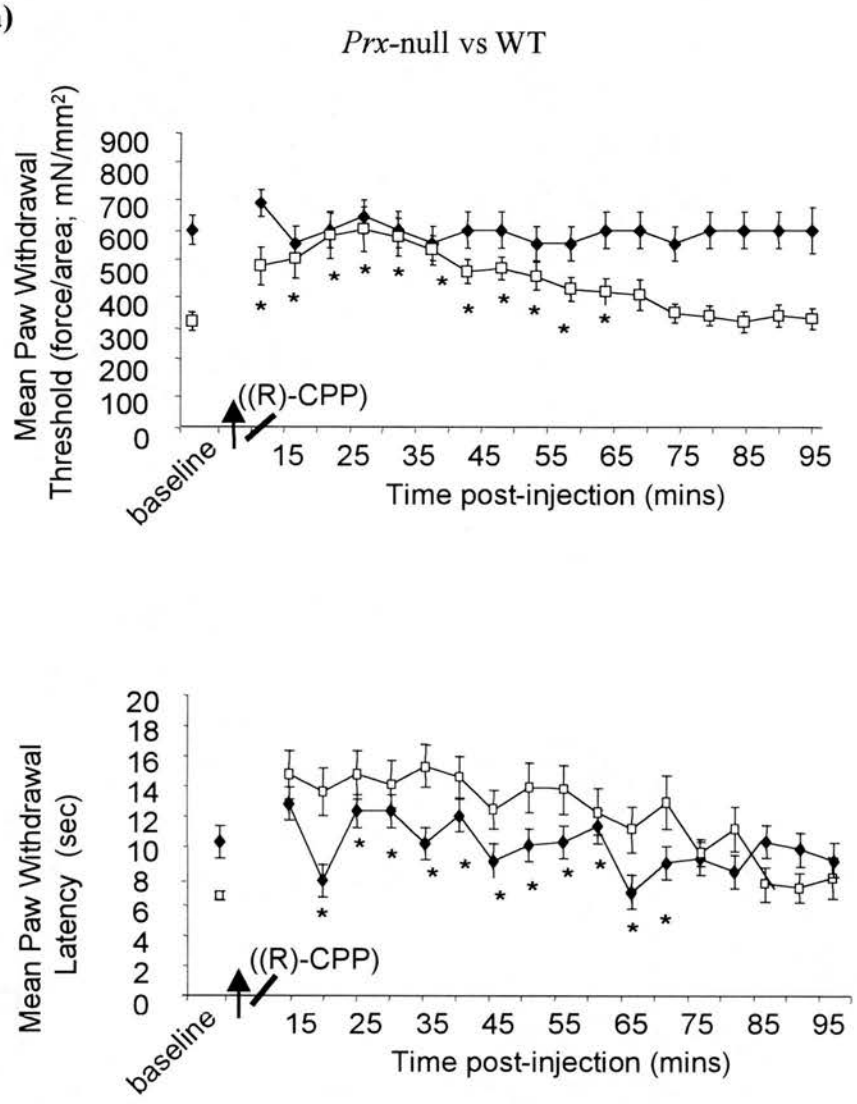

Prx-null vs WT
Mean Paw Withdrawal Threshold (force/area; mN/mm²)
900
800
700
600
500
400
300
200
100
0
((R)-CPP)
baseline
15 25 35 45 55 65 75 85 95
Time post-injection (mins)
Mean Paw Withdrawal Latency (sec)
20
18
16
14
12
10
8
6
4
2
0
((R)-CPP)
baseline
15 25 35 45 55 65 75 85 95
Time post-injection (mins)

Figure 5.1 cont...

b)

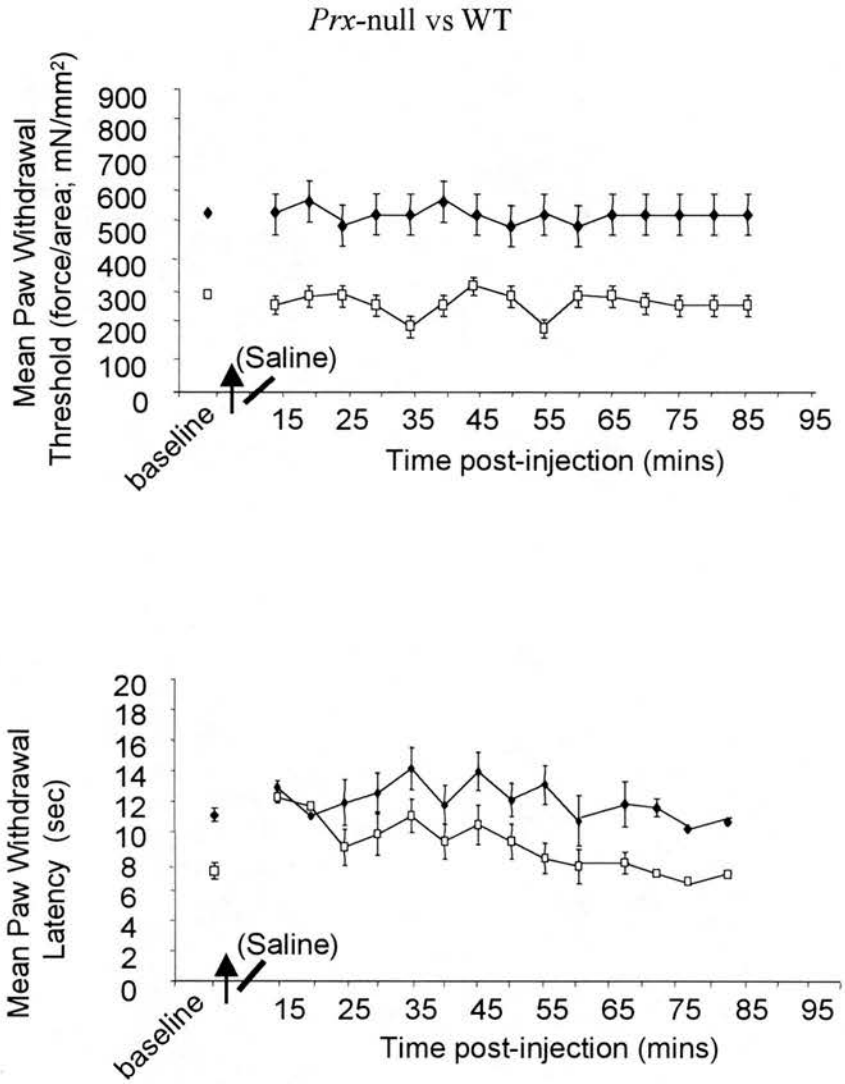


Figure 5.2. Effects of intrathecal administration of the NMDA receptor antagonist (R)-CPP on the sensitised behavioural reflex responses to von Frey filaments and to radiant heat in animals following lysolecithin treatment.

Paw withdrawal thresholds, in response to cutaneous mechanical stimulation with von Frey filaments and cutaneous noxious thermal stimulation, were measured in animals at their peak behavioural reflex sensitivity following topical application of lysolecithin to one saphenous nerve. Ipsilateral (□) and contralateral (◆) values are displayed. All paw withdrawal thresholds are displayed in mN/mm^2 for mechanical stimulation or mean paw withdrawal latency (secs) for thermal stimulation. Each value is the mean ± SEM and any statistically significant ($*P<0.05$) difference at each time point post-injection from pre-injection baseline values was determined by a One-Way Repeated Measures Analysis of Variance (ANOVA) with Dunnet's multiple comparisons versus control group post-hoc analysis.

a) The NMDA receptor antagonist R-CPP (100 pmol in 10 μl) reversed the behavioural reflex sensitisation to mechanical and noxious thermal stimulation observed in lysolecithin-treated animals (n=8) for up to 60-70 minutes after intrathecal application.
b) Saline resulted in no change the behavioural reflex sensitisation to mechanical and noxious thermal stimulation in lysolecithin-treated animals (n=4).

Figure 5.2.

a)

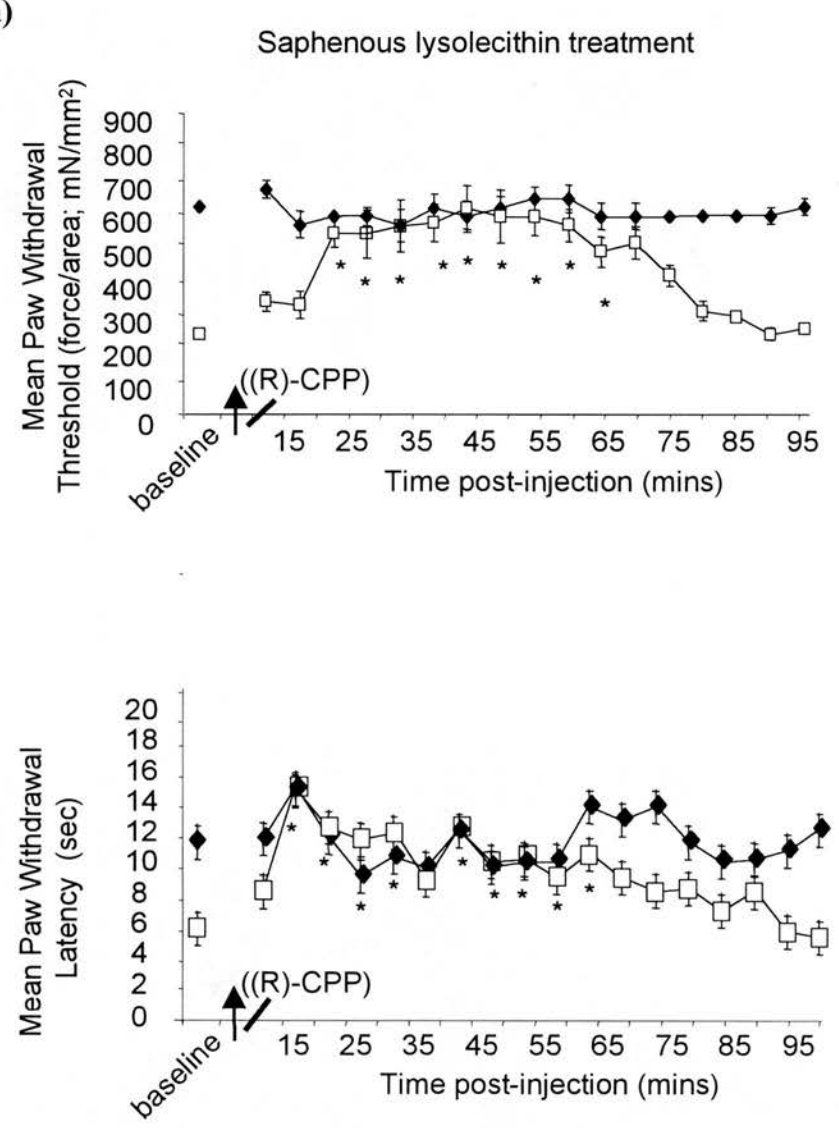
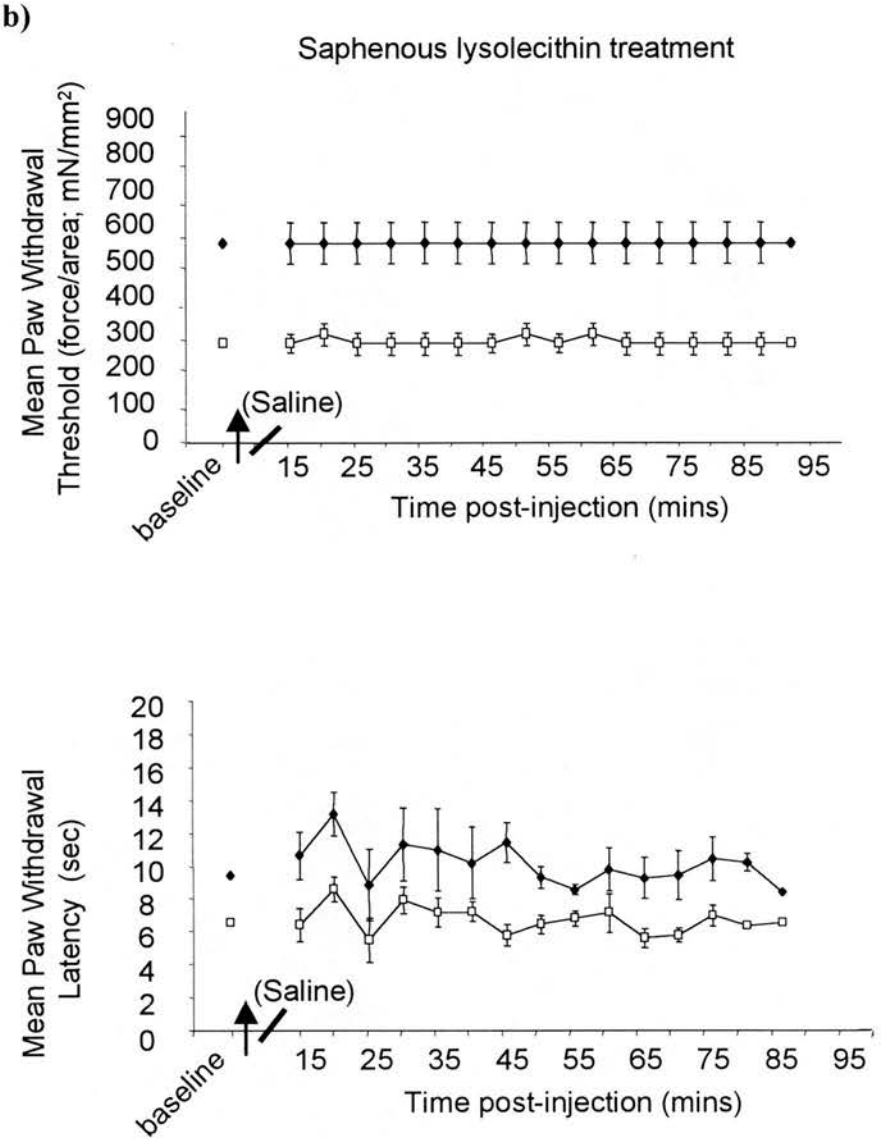

Saphenous lysolecithin treatment
Mean Paw Withdrawal Threshold (force/area; mN/mm²)
900
800
700
600
500
400
300
200
100
0
((R)-CPP)
baseline
15 25 35 45 55 65 75 85 95
Time post-injection (mins)
Mean Paw Withdrawal Latency (sec)
20
18
16
14
12
10
8
6
4
2
0
((R)-CPP)
baseline
15 25 35 45 55 65 75 85 95
Time post-injection (mins)

Figure 5.2 cont...

b)



b) The effect of the morphine analogue DAMGO on lysolecithin-induced central sensitisation

Since animal models of neuropathic pain states often show a relative insensitivity to spinal opioid analgesia compared to acute or peripheral inflammatory pain states (Arner and Meyerson, 1988;Fleetwood-Walker SM et al., 1988;Yaksh and Harty, 1988), we assessed the effects of the potent and selective μ-opioid receptor agonist DAMGO in the lysolecithin model. At the highest dose of intrathecal DAMGO that could be administered before overt psychomotor side effects were seen (10pmol in 10 μl), there was no specific reversal of the mechanical allodynia or thermal hyperalgesia seen ipsilateral to lysolecithin treatment (Fig 5.1c). Significance (*$P<0.05$) from pre-injection baseline values following all injections was determined by a One-Way Repeated Measures Analysis of Variance (ANOVA) with Dunnet's multiple comparisons versus control group post-hoc analysis. Further investigations may be necessary to explore the possible effects of a full dose-curve of DAMGO on the behavioural reflex sensitisation displayed by animals following lysolecithin treatment of the saphenous nerve. Nevertheless, these investigations suggest that focal demyelination with lysolecithin is a model of neuropathy without involvement of any significant μ-opioid-sensitive component of the chronic pain such as would be expected to be observed in inflammatory pain states.

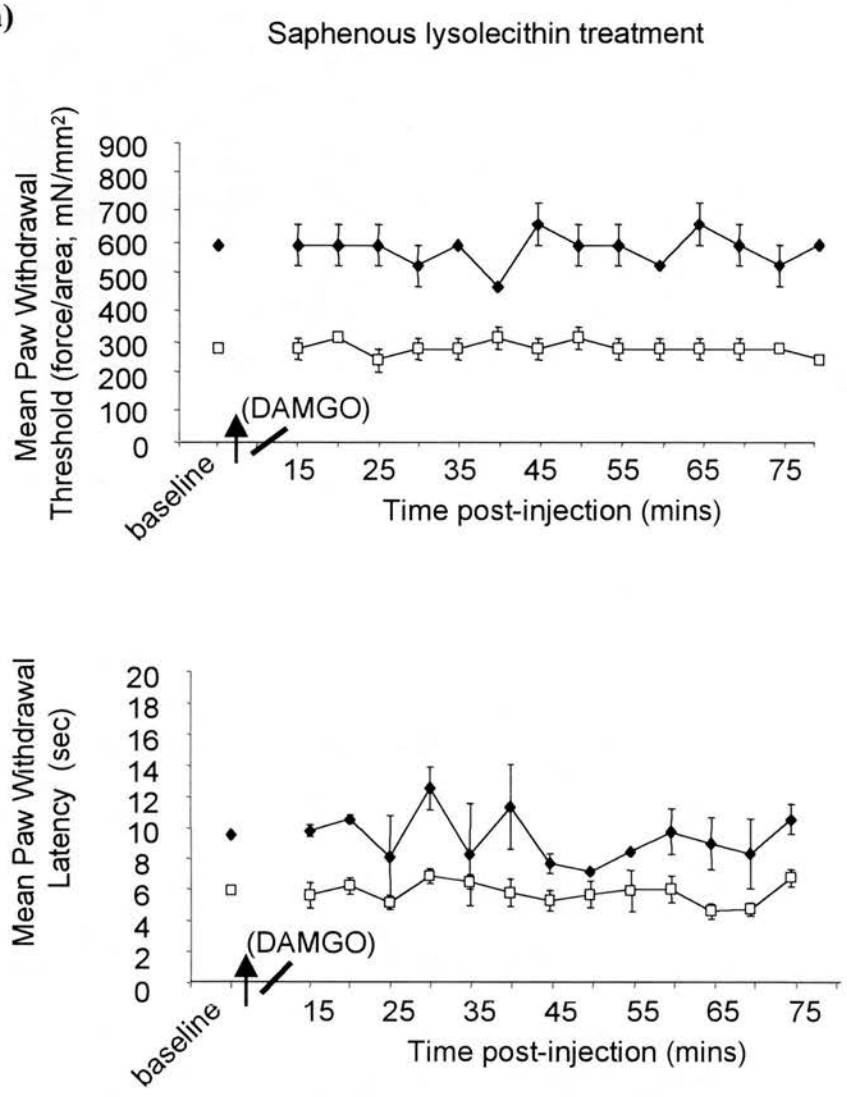
Figure 5.3. Effects of intrathecal administration of the selective mu-opioid receptor agonist, DAMGO on the sensitised behavioural reflex responses to von Frey filaments and to radiant heat in animals following lysolecithin treatment.

Paw withdrawal thresholds, in response to cutaneous mechanical stimulation with von Frey filaments and cutaneous noxious thermal stimulation, were measured in animals at their peak behavioural reflex sensitisation following topical application of lysolecithin to one saphenous nerve. Ipsilateral (□) and contralateral (◆) values are displayed. All paw withdrawal thresholds are displayed in mN/mm^2 for mechanical stimulation or mean paw withdrawal latency (secs) for thermal stimulation. Each value is the mean ± SEM and any statistically significant (*$P<0.05$) difference of each time point post injection from pre-injection baseline values was determined by a One-Way Repeated Measures Analysis of Variance (ANOVA) with Dunnet's multiple comparisons versus control group post-hoc analysis. For saline control see Fig 5.1b.

a) The selective mu-opioid receptor agonist, DAMGO (10 pmol in 10 µl) resulted in no change the behavioural reflex sensitisation to mechanical and noxious thermal stimulation observed in lysolecithin-treated animals (n=3).

Figure 5.3.

a)


Saphenous lysolecithin treatment
Mean Paw Withdrawal Threshold (force/area; mN/mm²)
900
800
700
600
500
400
300
200
100
0
(DAMGO)
baseline
15
25
35
45
55
65
75
Time post-injection (mins)
Mean Paw Withdrawal Latency (sec)
20
18
16
14
12
10
8
6
4
2
0
(DAMGO)
baseline
15
25
35
45
55
65
75
Time post-injection (mins)

c) A potential role for $NF_{\kappa}B$ in lysolecithin-induced central sensitisation

NFκB is known to be a crucial signal for glial and neuronal cell function during inflammation injury and neural plasticity (see review) (O'Neill and Kaltschmidt, 1997). It is likely that NFκB is involved in intracellular signalling in sensory neurons under physiological conditions and furthermore, factors such as TNF, IL-1 and NGF which may be produced by Schwann cells and macrophages following partial nerve injury, can cause NFκB activation in the PNS and CNS (Carter et al., 1996;Moynagh et al., 1994;Wood, 1995) (Laughlin et al., 2000) which may lead to events involved in the development of chronic pain (Ma and Bisby, 1998a). Furthermore, it has been suggested that changes which may occur in dorsal horn cells following peripheral nerve injury including the activation of NMDA and non-NMDA ionotropic glutamate receptors (Guerrini et al., 1997;Kaltschmidt et al., 1995;Nakai et al., 2000) and intracellular activation of protein kinases such as PKA (Mosialos and Gilmore, 1993;Neumann et al., 1995) and PKCγ (Leitges et al., 2001) may be capable of activating NFκB. Therefore, it was of interest to investigate whether the activation of NFκB in the spinal cord is associated with the behavioural reflex changes displayed in our model of lysolecithin-induced peripheral nerve demyelination. Individual spinal administration of 3 separate and distinctly acting antagonists of NFκB activation; parthenolide, sulfasalazine and CAPE have indicated a significant role for NFκB in the sensitised pain induced by our animal model of peripheral nerve demyelination. All three antagonists caused a complete reversal of mechanical allodynia and thermal hyperalgesia (Fig 5.1d-f). This effect persisted for at least 55 minutes post injection of each drug returning to baseline levels after approximately 70 minutes without any effect on contralateral paw withdrawal threshold. Vehicle control injections showed no effect on the withdrawal thresholds to either mechanical or thermal stimuli. Statistical significance of differences ($*P<0.05$) from pre-injection baseline values following all injections was determined by a One-Way Repeated Measures Analysis of Variance (ANOVA) with Dunnet's multiple comparisons versus control group post-hoc analysis.

It must be considered that all of these drugs are unspecific in their actions upon NFκB and in theory may be acting via different mechanisms to affect the behavoural reflex sensitisation displayed in our model of lysolecithin-induced peripheral nerve demyelination. However, the fact that all three act commonly to block the activation of NFκB suggests that this is the key pathway by which they are acting to reverse the behavioural reflex sensitisation as is observed.

Figure 5.4. Effects of intrathecal administration of the selective NFκB activation antagonists parthenolide, CAPE and sulphasalazine on the sensitised behavioural reflex responses to von Frey filaments and to radiant heat in animals following lysolecithin treatment.

Paw withdrawal thresholds, in response to cutaneous mechanical stimulation with von Frey filaments and cutaneous noxious thermal stimulation, were measured in animals at their peak behavioural reflex sensitisation following topical application of lysolecithin to one saphenous nerve. Ipsilateral (□) and contralateral (◆) values are displayed. All paw withdrawal thresholds are displayed in mN/mm^2 for mechanical stimulation or mean paw withdrawal latency (secs) for thermal stimulation. Each value is the mean ± SEM and any statistically significant (*$P<0.05$) difference of each time point post injection from pre-injection baseline values was determined by a One-Way Repeated Measures Analysis of Variance (ANOVA) with Dunnet's multiple comparisons versus control group post-hoc analysis.

a) The NFκB antagonist parthenolide (0.3 nmol in 10μl) reversed the behavioural reflex sensitisation to mechanical and noxious thermal stimulation observed in lysolecithin-treated animals (n=8) for up to 65-70 minutes after intrathecal application. The vehicle saline resulted in no change the behavioural reflex sensitisation to mechanical and noxious thermal stimulation when injected alone (see Fig 5.1b).
b) The NFκB antagonist CAPE (0.3 nmol in 10μl) reversed the behavioural reflex sensitisation to mechanical and noxious thermal stimulation observed in lysolecithin-treated animals (n=8) for up to 55-60 minutes after intrathecal application. The saline vehicle resulted in no change in the behavioural reflex sensitisation to mechanical and noxious thermal stimulation when injected alone (see Fig 5.1b).
a) The NFκB antagonist sulphasalazine (5 nmol in 10μl) reversed the behavioural reflex sensitisation to mechanical and noxious thermal stimulation observed in lysolecithin-treated animals (n=8) for up to 55 minutes after intrathecal application.
b) The vehicle (2% dmf in saline) resulted in no change in the behavioural reflex sensitisation to mechanical and noxious thermal stimulation in lysolecithin-treated animals (n=4).

Figure 5.4.

a)

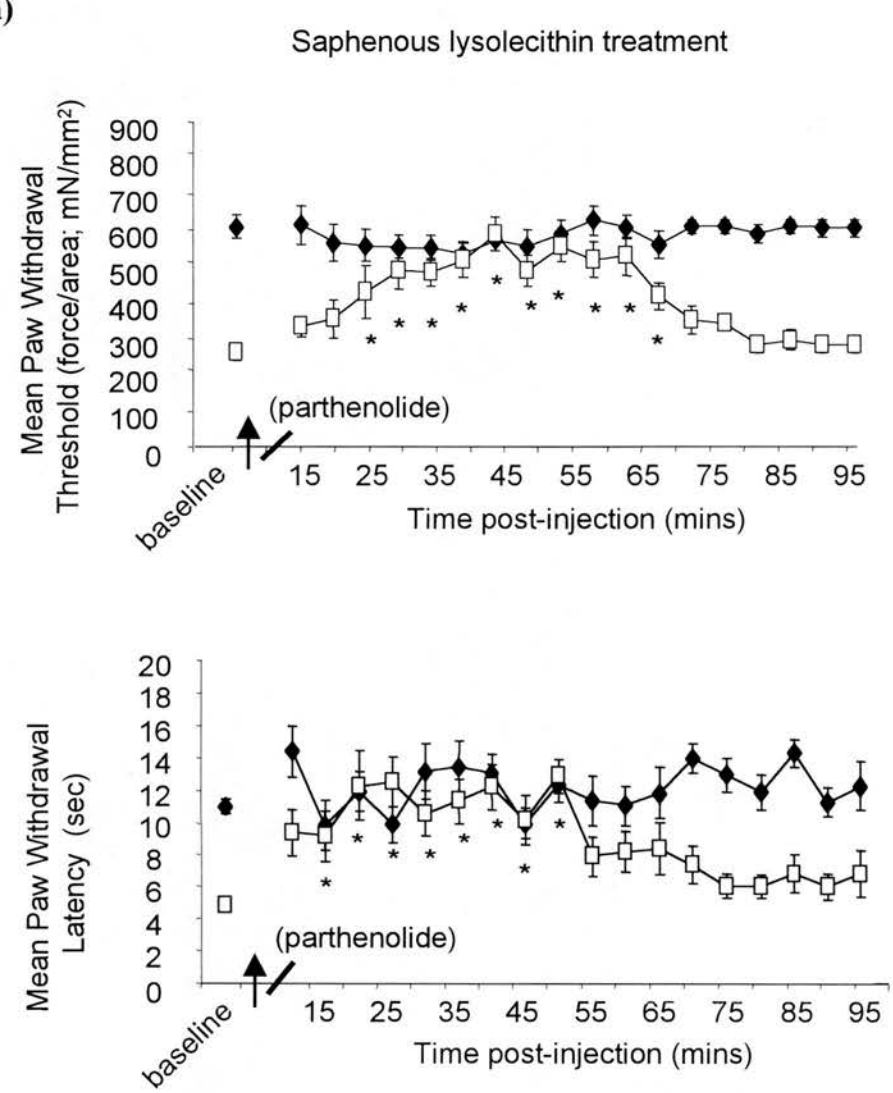

Saphenous lysolecithin treatment
Mean Paw Withdrawal Threshold (force/area; mN/mm²)
900
800
700
600
500
400
300
200
100
0
(parthenolide)
baseline
15 25 35 45 55 65 75 85 95
Time post-injection (mins)
Mean Paw Withdrawal Latency (sec)
20
18
16
14
12
10
8
6
4
2
0
(parthenolide)
baseline
15 25 35 45 55 65 75 85 95
Time post-injection (mins)

Figure 5.4. cont...

b)

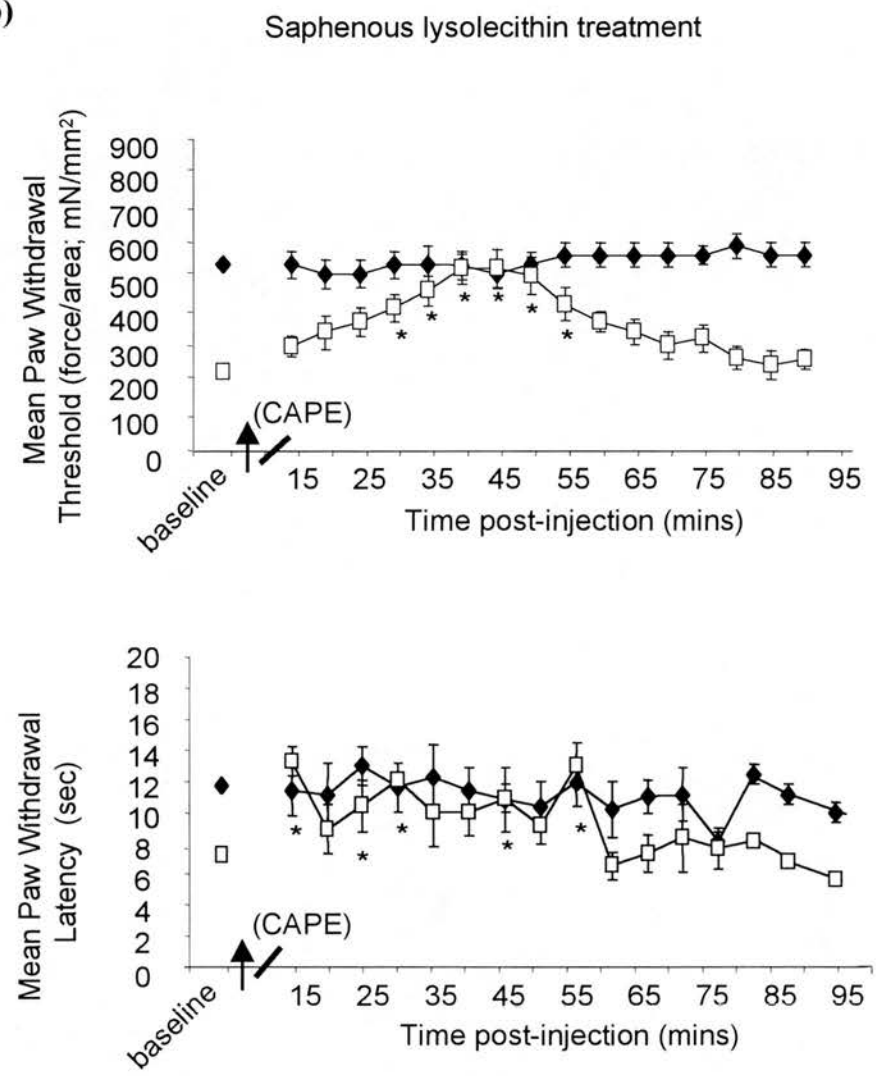


Figure 5.4. cont...

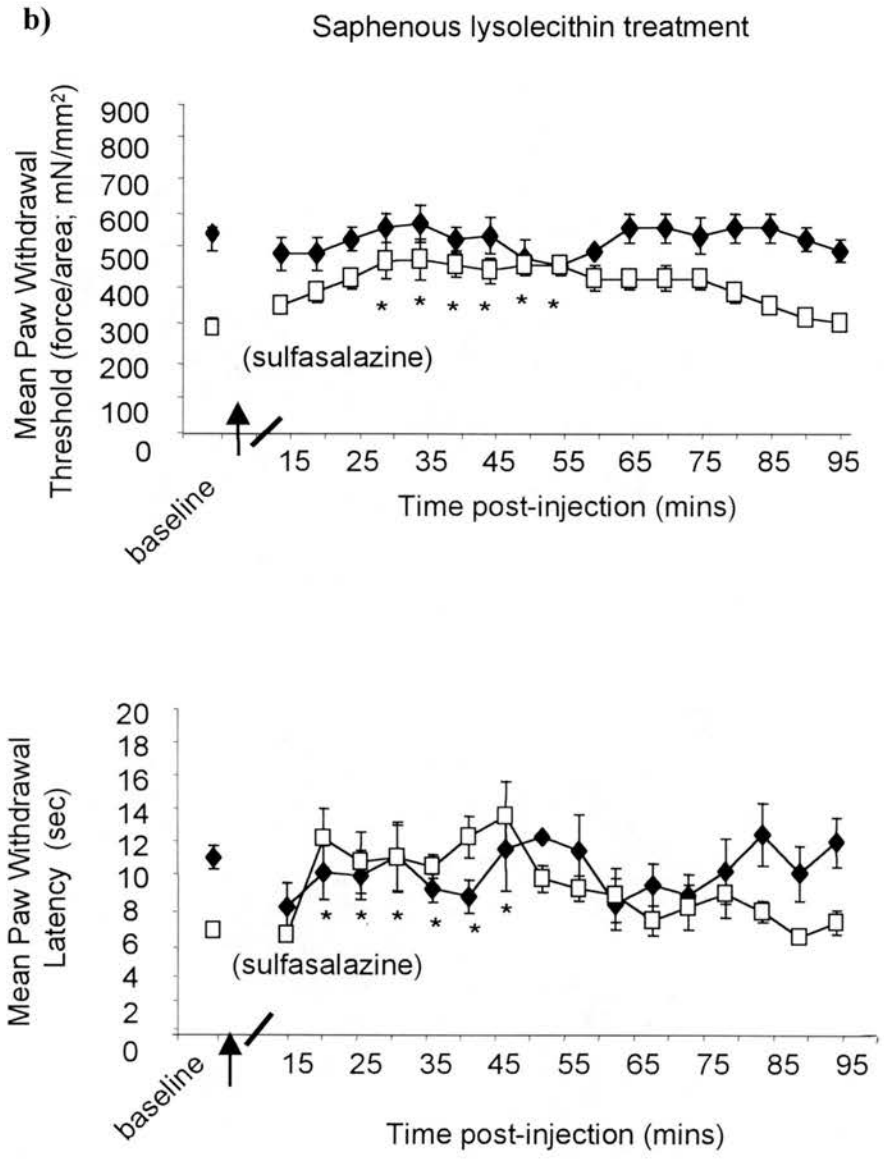
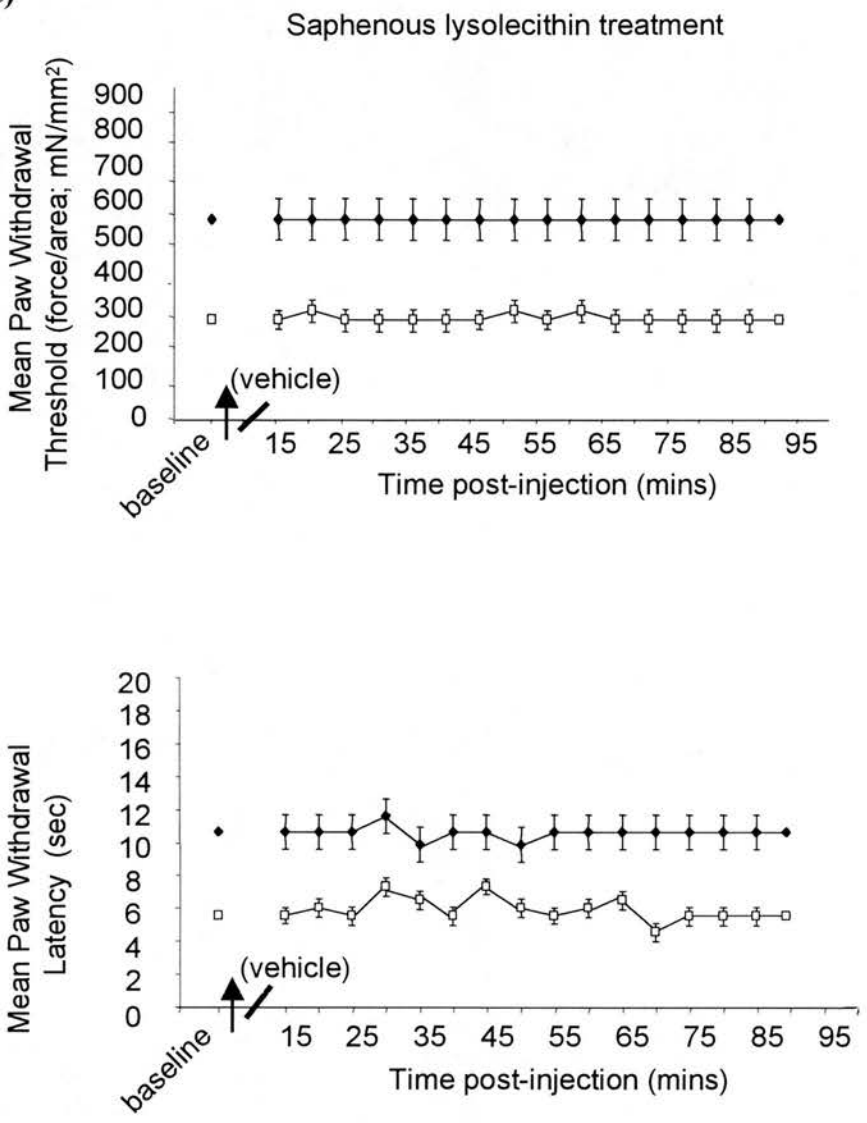


Figure 5.4. cont...

c)



d) Effects of the endogenous cannabinoid system on lysolecithin-induced central sensitisation.

An alternative spinal modulatory system that can exert selective antinociceptive effects in both neuropathic and inflammatory hyperalgesia is the endogenous cannabinoid system (Fox et al., 2001;Herzberg et al., 1997;Richardson et al., 1998). This is of particular interest because of evidence that cannabinoids may exert beneficial analgesic effects in demyelinating diseases such as multiple sclerosis (Consroe et al., 1997;Robson, 2001) and are under assessment as clinical therapeutic agents for this disorder (Robson, 2001). We were able to show a clear modulatory role for the cannabinoid system in the sensitised pain behaviour induced in our animal model of peripheral nerve demyelination. Spinal administration of the cannabinoid receptor agonist WIN 55,212-2 attenuated the reduction in thermal reflex latencies and mechanical nociceptive thresholds that were displayed ipsilateral to lysolecithin (Fig 5.1h). This attenuation appeared to be more marked on thermal than on mechanical sensitised responses, but in both cases changes in responses were seen only on the sensitised side, ipsilateral to lysolecithin. A role for the CB_1 receptor in particular was demonstrated by spinal administration of the selective CB_1 receptor antagonist AM 251, which completely reversed the effects of WIN 55,212-2 on mechanical sensitisation and partially reversed its effects on thermal sensitisation (Fig 5.1i). However, when administered alone, AM 251 had no significant effect, indicating that endogenous spinal CB_1 receptors had not become tonically activated as a result of the lysolecithin treatment (Fig 5.1j).

Figure 5.5. Effects of intrathecal administration of targets of the endogenous cannabinoid system; WIN 55,212-2 and AM 251 on the sensitised behavioural reflex responses to von Frey filaments and to radiant heat in animals following lysolecithin treatment.

Paw withdrawal threshold, in response to cutaneous mechanical stimulation with von Frey filaments and cutaneous noxious thermal stimulation, were measured in animals at their peak behavioural reflex sensitisation following topical application of lysolecithin to one saphenous nerve. Ipsilateral (□) and contralateral (◆) values are displayed. All paw withdrawal thresholds are displayed in mN/mm^2 for mechanical stimulation or mean paw withdrawal latency (secs) for thermal stimulation. Each value is the mean ± SEM and any statistically significant (*$P<0.05$) difference of each time point post injection from pre-injection baseline values was determined by a One-Way Repeated Measures Analysis of Variance (ANOVA) with Dunnet's multiple comparisons versus control group post-hoc analysis.

a) The mixed CB_1/CB_2 cannabinoid receptor agonist WIN 55,212-2 (60 pmol in 10 μl) partially reversed the behavioural reflex sensitisation to mechanical and fully reversed the behavioural reflex sensitisation to noxious thermal stimulation in lysolecithin-treated animals (n=7) for up to 60 minutes after intrathecal application. The vehicle (0.5% dmf in saline) had no effect on behavioural reflex sensitisation to either mechanical or thermal stimulation (see Fig 5.4d)
b) The CB_1 receptor antagonist AM 251 (100 pmol in 10 μl) resulted in no change of the behavioural reflex sensitisation to mechanical and noxious thermal stimulation in lysolecithin-treated animals (n=7). The vehicle (0.5% dmf in saline) had no effect on behavioural reflex sensitisation to either mechanical or thermal stimulation (see Fig 5.4d)
c) When injected in combination, WIN 55,212-2 and AM 251 produced no significant effect on the behavioural reflex sensitisation to noxious thermal stimulation in lysolecithin-treated animals (n=8) suggesting that the effect of WIN 55,212-2 was prevented by AM 251 acting at the CB_1 receptor. The vehicle (0.5% dmf in saline) had no effect on behavioural reflex sensitisation to either mechanical or thermal stimulation (see Fig 5.4d).

Figure 5.5.

a)

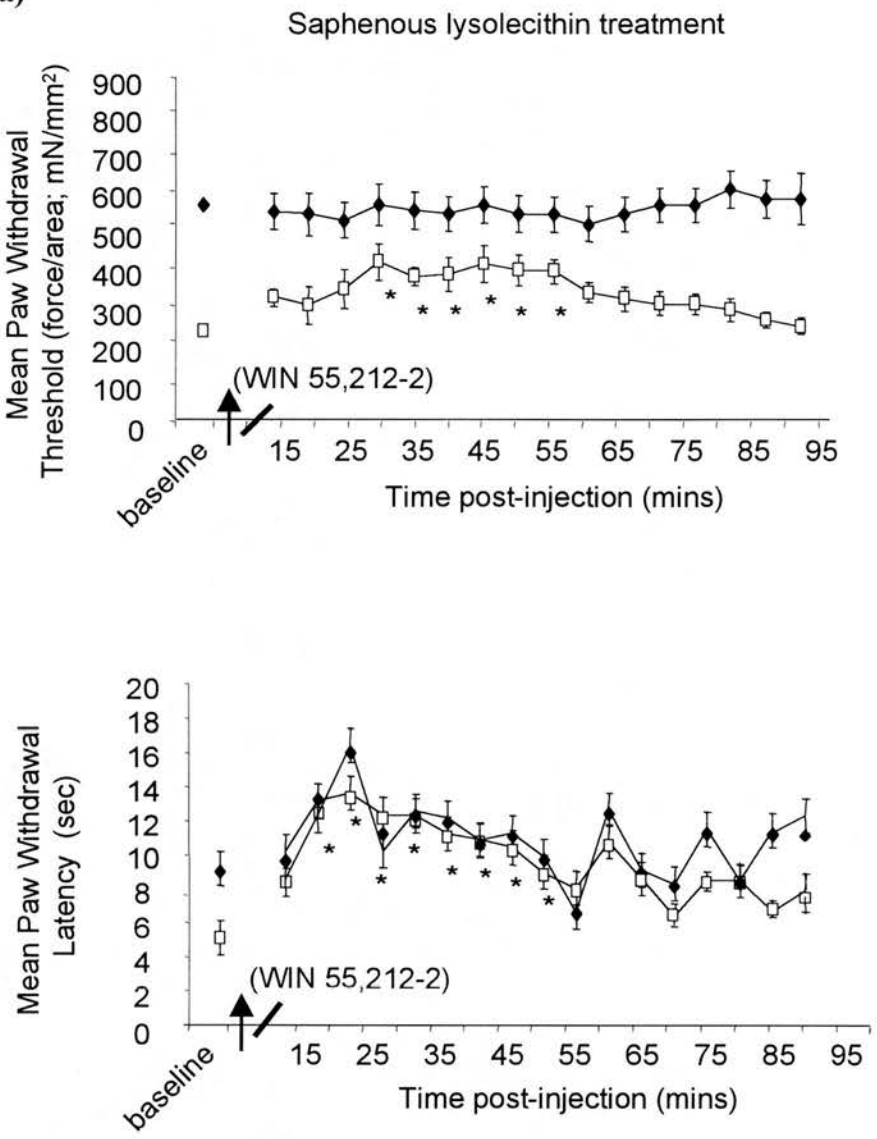

Saphenous lysolecithin treatment
Mean Paw Withdrawal Threshold (force/area; mN/mm²)
900
800
700
600
500
400
300
200
100
0
(WIN 55,212-2)
baseline
15 25 35 45 55 65 75 85 95
Time post-injection (mins)
Mean Paw Withdrawal Latency (sec)
20
18
16
14
12
10
8
6
4
2
0
(WIN 55,212-2)
baseline
15 25 35 45 55 65 75 85 95
Time post-injection (mins)

Figure 5.5. cont...

b)

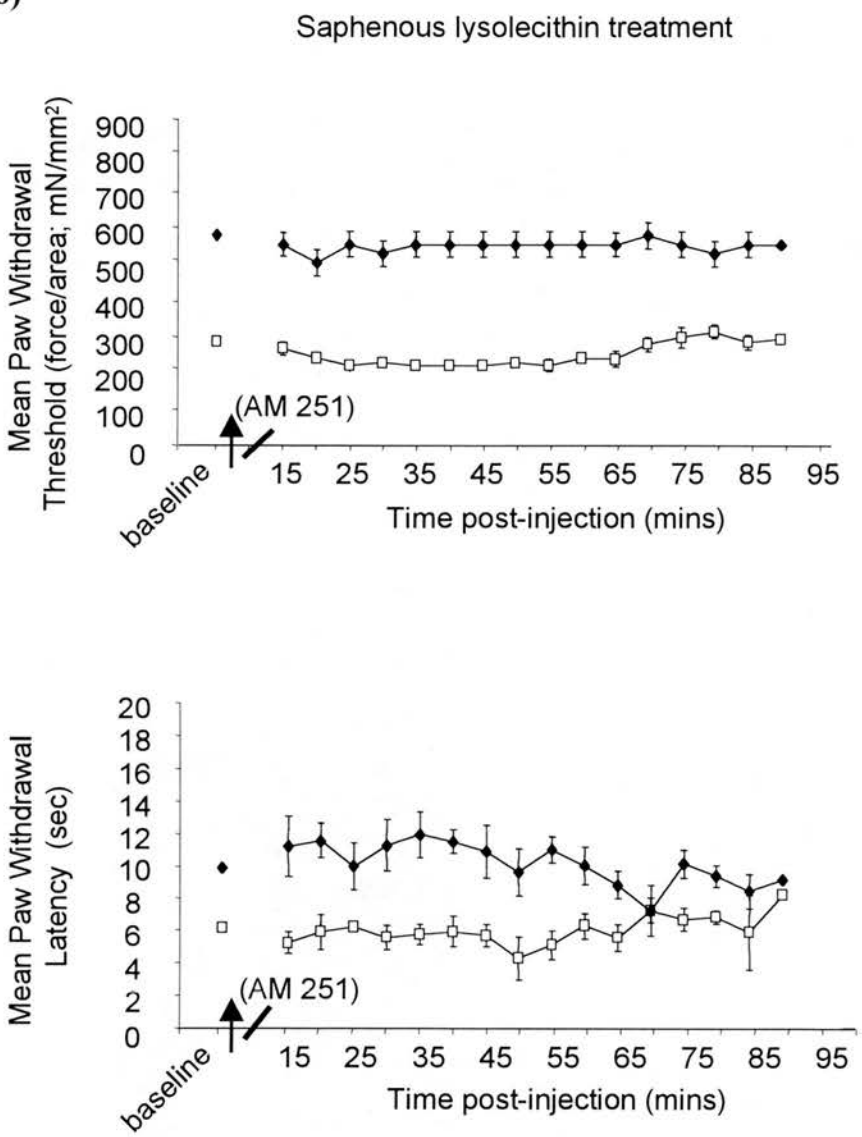
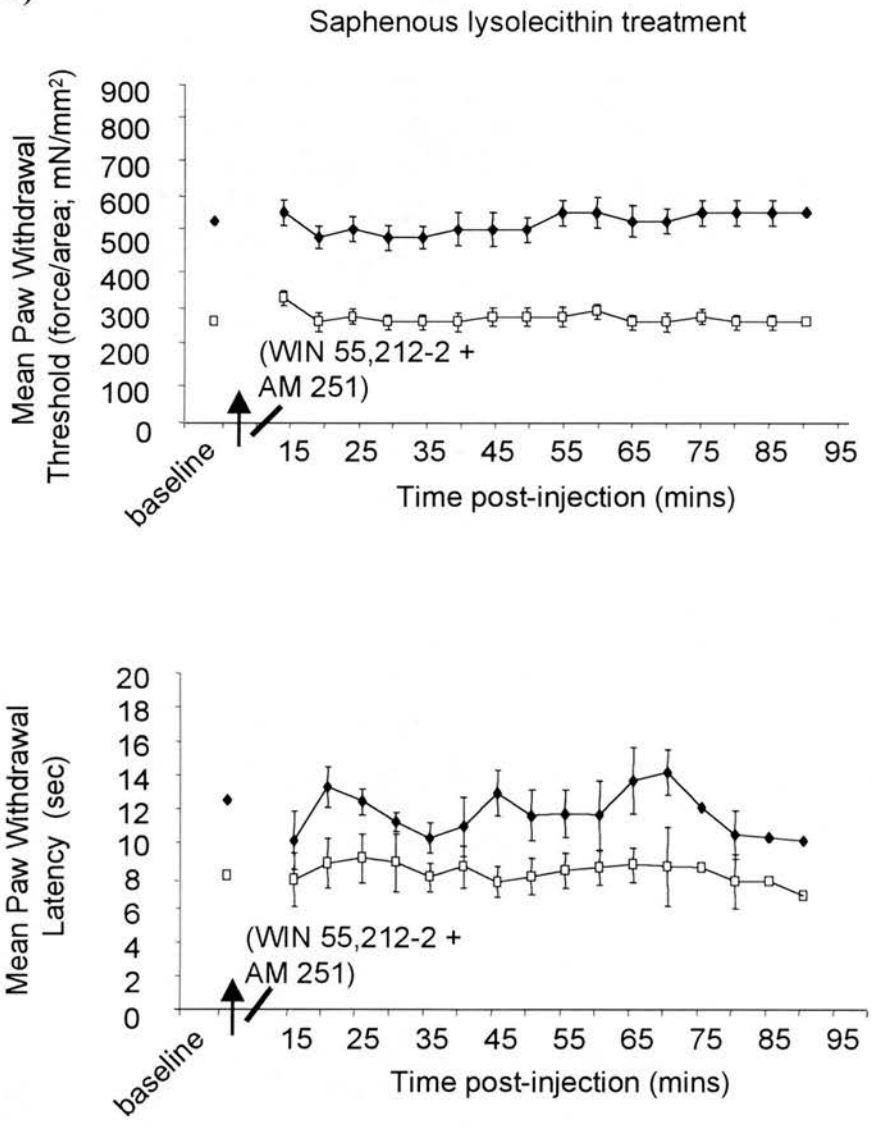


Figure 5.5. cont...

b)



5.2: Discussion

5.2.1. NMDA receptor-dependent central sensitisation

Afferent hyperexcitability and spontaneous action potential generation, as displayed by the saphenous nerves of *Prx*-null mice and normal saphenous nerves treated with lysolecithin, provides a basis for ongoing input to the CNS. Such input may induce functional changes in the sensory processing mechanisms within the dorsal horn creating a phenomenon referred to as 'central sensitisation'. These changes manifest behaviourally as hypersensitivity to pain that spreads from the site of injury causing primary and secondary hyperalgesia, which can include tactile Aβ-fibre-mediated mechanical allodynia (Gracely et al., 1992;Woolf, 1997); behavioural phenomena that we have shown to be part of the phenotype of both models of peripheral nerve demyelination. In a variety of models of chronic pain, it is thought that mechanical allodynia and thermal hyperalgesia, similar to that seen in our models of peripheral nerve demyelination, are underpinned by changes in NMDA receptor activation in the spinal dorsal horn. This is considered to be one of the key factors underlying central sensitisation (Bennett, 1994;Chaplan et al., 1997;Dickenson and Sullivan, 1987;Mao et al., 1993;Woolf and Costigan, 1999) (see Chapter 6).

Using the intrathecal application of pharmacological agents, we have demonstrated that NMDA receptor-dependent events at a central site, in the spinal cord, were essential for the phenomena of mechanical allodynia and thermal hyperalgesia, both of which become manifest in *Prx*-null mice and in mice following lysolecithin treatment of the saphenous or sciatic nerve. This indicates that central changes including NMDA-receptor-dependent central sensitisation are involved in the production of the mechanical allodynia and thermal hyperalgesia observed. This implies that as in other models of neuropathic pain, changes are occurring in the spinal cord, which are comparable to those occurring in hippocampal LTP (Sandkuhler, 2000). Therefore, by taking example of the extensive work being carried out in to the mechanisms of LTP in the hippocampus, we can formulate many hypotheses of the pathways involved in the induction of central sensitisation (see Chapter 6).

5.2.2 Lack of analgesic effects of mu–opioid receptors in demyelination-induced pain models

Neuropathic pain models display a relative insensitivity to spinally administered opioid analgesics (Arner and Meyerson, 1988;Lee et al., 1995;Yaksh and Harty, 1988) which are much more effective for the treatment of inflammation associated pain conditions (Lombard and Besson, 1989). The fact that intrathecal administration of the mu –opioid receptor agonist DAMGO failed to affect the behavioural reflex sensitisation displayed by lysolecithin treated mice corresponds with other models of neuropathic pain and suggests that our model does not involve a significant inflammatory component, verifying its use as a model of neuropathic pain. However, a more thorough study using various doses of the spinal mu-opioid receptor antagonist would be appropriate to investigate any possible role that spinal mu-opioid receptors may play in inducing analgesia in both of our models of peripheral nerve demyelination. This may highlight a potential clinical use for opiods in the treatment of pain associated with demyelinating conditions in humans.

5.2.3. A possible role of NFkB in demyelination induced neuropathic pain

The transcription factor, NFκB, is known to be a crucial signal for glial and neuronal cell function during injury and neural plasticity (O'Neill and Kaltschmidt, 1997). Inducible NFκB is expressed in 30-45% of neurons of all sizes, but particularly by medium to large neurons, in L4 and L5 ganglia in the rat (Doyle and Hunt, 1997;Ma and Bisby, 1998a), suggesting that it may be involved in intracellular signalling in sensory neurons under physiological conditions. After nerve injury, NFκB (p65/p50 heterodimer) expression reportedly decreases acutely in both large and small cells (Doyle and Hunt, 1997). However, the proportion of activated NFκB (i.e. that released from IκB and therefore capable of translocation to the nucleus where it can regulate gene transcription) has been reported to increase within ipsilateral DRG neurons, particularly in medium diameter cells (Ma and Bisby, 1998a), which presumably have myelinated axons. This increase occurs 2 weeks after nerve injury (Ma and Bisby, 1998a) when behavioural signs of neuropathic pain have become established implying a role for NFκB in neuropathic pain; an area in which NFκB involvement has been little investigated, with much focus remaining upon its role in modulation of inflammatory processes. The activation of NFκB, leading to the induction of upregulation of, or the new expression of, multiple genes, can occur as a consequence of various events, which are associated with nerve injury. Of particular relevance to this study is the evidence for the activation of NFκB following NMDA and non-NMDA ionotropic glutamate receptor activation (Guerrini et al., 1997;Kaltschmidt et al., 1995;Nakai et al.,

2000). We have demonstrated a role for spinal NMDA receptors in the behavioural reflex sensitisation displayed by both models of demyelination. Therefore, their activation could subsequently lead to the activation of NFκB in the dorsal horn neurons of the spinal cord and the subsequent induction of multiple gene expression, which may lead to events involved in the development of chronic pain (see Chapter 6). It has also been demonstrated that NFκB may be stimulated via the activation of metabotropic, G-protein coupled receptors (Cowen et al., 1997;Kravchenko et al., 1995). Many receptor subtypes of this family, including metabotropic glutamate receptors and neuropeptide receptors, such as NK_1, are involved in transmission of nociceptive information from the PNS to the spinal cord and are thought to play an important role in the induction of persistent pain states. Therefore, it is plausible that such receptors could provide a further substrate for the activation of NFκB following peripheral nerve injury. Furthermore, it has been suggested the protein kinases such as PKA (Mosialos and Gilmore, 1993;Neumann et al., 1995) and PKCγ (Leitges et al., 2001) are capable of activating NFκB. Both kinases have been implicated in persistent pain states (Cerne et al., 1993;Malmberg et al., 1997;Sluka and Willis, 1997) and may be stimulated in dorsal horn cells following nerve injury via second messenger pathways of metabotropic receptors (Bleakman et al., 1992;Bond and Lodge, 1995;Bliss and Collingridge, 1993). Therefore, evidence would suggest a strong possibility for the activation of NFκB in response to changes in the activity of receptors and their intracellular pathways following nerve injury. An extensively researched area of NFκB activation involves factors such as TNF, IL-1 and NGF which are capable of activating NFκB in cells of the PNS and CNS (Carter et al., 1996;Moynagh et al., 1993;Moynagh et al., 1994;Wood, 1995). Following partial nerve injury, TNF, IL-1 and NGF are produced in increased quantities by Schwann cells. It is plausible that such factors produced by Schwann cells may not only act upon cells of the DRG to activate NFκB, but may also be capable of acting upon cells in the dorsal horn via their secretion into the spinal cord at central termination sites. It seems likely that a similar upregulation may occur following demyelination, in which Schwann cells are activated to remyelinate the axon. In line with these proposed mechanisms of NFκB activation, and the evidence for NFκB activation in previous models of nerve injury, we have demonstrated a role for spinal NFκB in the behavioural reflex sensitisation, which develops following lysolecithin treatment to the saphenous nerve, as indicated by effects of three structurally distinct NFκB pathway inhibitors; parthenolide, CAPE, and sulphasalazine. It must be kept in mind that these inhibitors are unspecific in their actions upon NFκB in that they can also act as anti-inflammatory agents due to their affects on other mechanisms (Song

et al., 2002; Uchi et al., 2002). For example, sulphasalazine is also used to treat inflammatory conditions due to its NSAID-like properties (O'Dell et al., 2002) and CAPE has been shown to directly inhibit catalytic activity of inducible nitric oxide synthase (Song et al., 2002) as well as its expression via its actions on NFκB. Such actions could be involved in the reversal of the behavoural reflex sensitisation displayed in our model of lysolecithin-induced peripheral nerve demyelination, However, the fact that all three drugs act commonly to block the activation of NFκB suggests that this is the key pathway by which they are acting to reverse the behavioural reflex sensitisation as is observed.

Following this discovery, it would be of interest to localise the specific cells in which NFκB is activated and whether or not any activation is restricted to cells of damaged afferents. It may also be of interest to investigate any activation of NFκB in DRG cells to i) see if this correlates with previous work, which is so far limited, and ii) if any activation occurring is restricted to cells of myelinated afferents. Furthermore, it would be of interest to investigate which genes NFκB may be regulating and how their expression might relate to the development and/or maintenance of neuropathic pain associated with nerve injury and demyelination. Such dissection of specific gene regulation could potentially point the way to new therapeutic targets for the treatment of neuropathic pain.

Due to the implications of various roles of growth factors not only in the activation of NFκB but the production of pain in general, this may be a good point at which to suggest further investigations into changes in, and the possible role of, growth factors in our models of peripheral nerve demyelination. It would be of interest to measure levels of expression of growth factors such as TNF, NGF and GDNF and their receptors, as well as inflammatory mediators such as IL-6, in Schwann cells, DRG cells and locally in the dorsal horn of the spinal cord. These investigations may provide insight in to which growth factors, if any, are changing and at which location they might be acting. Using blocking agents for their receptors would give an idea of the importance of their role in the development of behavioural reflex sensitisation in our models of peripheral nerve demyelination and thereby may indicate possible targets for therapeutic intervention.

The measurement of NGF and GDNF expression would be of particular interest in light of other work investigating their effects on neuropathic pain and related mechanistic changes. NGF expression levels in our models would be of interest as the results from the work investigating neuropeptide expression in the DRG would suggest that the demyelination does not result in changes in peptides, such as CGRP and SP down-regulation, thought to result from NGF deprivation. However, NGF deprivation is also thought to play a role in the modulation of expression of Na^+ channels, resulting in the decreased expression of SNS 1/

$Na_v1.8$ and the corresponding upregulation of type III/ $Na_v1.3$ (Black et al., 1997;Dib-Hajj et al., 1998;Okuse et al., 1997), changes which we have demonstrated to be a feature of our models of demyelination. Therefore, our results of phenotypic changes in the DRG following peripheral nerve demyelination may present a conflict in theory with regard to the role of NGF in neuropathic pain. GDNF may also play a role in the regulation of sodium channel expression (Fjell et al., 1999) (Bennett et al., 2000) and evidence indicates that GDNF treatment may reverse changes in Na^+ channel expression whilst preventing the development of pain-associated behaviours in various animal models of neuropathic pain (Bennett et al., 2000). Of particular relevance to this study is evidence indicating a potential role for the use of GDNF treatment in reducing spontaneous activity arising almost exclusively in myelinated afferents in the period of development and establishment of the neuropathic pain state following SNL (Bennett et al., 2000). This suggests that perhaps GDNF might be of use to target the spontaneous activity arising in the fibres of the demyelinated nerves in our models of peripheral nerve demyelination. Therefore, investigations into the levels of GDNF in our models of neuropathic pain may indicate whether or not any changes in its expression levels have occurred as a result of demyelination and therefore, whether increasing the local supply of GDNF may help to minimise the neuropathic pain.

5.2.4. Cannabinoids

Reports of the therapeutic use of cannabis (Δ^9-tetrahydrocannabinol) date back as far as 5000 years ago when reports from China recommended its use for malaria, constipation, rheumatic pains and childbirth (Mechoulam, 1986). It was not until the 19^{th} century that cannabis became a mainstream medicine in Britain following observations of its use as an analgesic as well as an anticonvulsant, anti-spasmodic and anti-emetic drug in India (Robson, 2001). It became a popular drug and was, (and according to anecdotal reports still is), used to treat pain, muscle cramps and spasms, associated with conditions such as MS (Consroe et al., 1997). There are also anecdotal reports of benefits in bone and joint pain, cancer pain and labour pain (Grinspoon and Bakalar, 1993). Cannabis was available over the counter in pharmacies until 1928 when it was outlawed and by 1971, the prescription of cannabis was prohibited. However, the therapeutic effects of cannabis are still acknowledged and its considerable potential in neuropathic pain treatment recognised (Joy et al., 1999) which has led to much research to develop cannabinol analogues to combat conditions such as chronic pain.

The discovery of the 'endogenous cannabinoid system' has led to the development of selective CB_1 and CB_2 receptor ligands, which may represent a valuable alternative strategy for the relief of chronic pain. There is considerable evidence supporting a role for cannabinoids in the modulation of nociception and many reports have shown them to be effective in reversing hyperalgesia in models of chronic inflammatory pain (Martin et al., 1999;Richardson et al., 1998c) and neuropathic pain (Bridges et al., 2001;Fox et al., 2001;Herzberg et al., 1997a;Kelly and Chapman, 2001). The majority of these effects appear to be mediated by CB_1 cannabinoid receptors (Richardson et al., 1998c) which are located at both peripheral and central sites.

Strong evidence is emerging to link the efficacy of cannabinoids in the treatment of neuropathic pain and, of particular relevance to this study, their actions upon myelinated A-fibres. For example, evidence suggests that in the PNS cannabinoid receptors are synthesised predominantly by medium and large sized DRG cell bodies i.e. those with myelinated axons (Hohmann and Herkenham, 1999). This suggests that aberrant activity in A-fibres which underlies allodynia (Gold, 2000) may be a possible target for modulation by cannabinoids. However, of particular interest to this study are reports indicating that spinal CB_1 receptors may be predominantly located on spinal interneurons which suggests that cannabinoids may also play a crucial role in regulating dorsal horn activity. This suggests that at a central level, cannabinoids can be involved in the modulation of incoming afferent activity arising from various fibre types. A recent study has suggested that the potent cannabinoid agonist, HU210, has selective effects on the responses of dorsal horn neurons in nerve-injured rats (Chapman, 2001; Kelly and Chapman, 2001). This study claims that following SNL, the normal inhibitory effects of the agonist on C-fibre mediated responses of spinal neurons were lost, yet inhibitory influences upon Aδ-fibre mediated responses remained. It is suggested that under normal conditions, spinal cannabinoid receptors predominantly regulate the activity of C-fibre input to the spinal cord modulating the transmission of nociceptive input. However, the distinction between C- and Aδ-fibre afferent inputs to the spinal cord cells is perhaps harder to define than is implied due to the overlap in conduction velocity range of the two fibre groups. However, these claims do suggest that changes occur in the efficacy of the cannabinoid system in the SNL model of neuropathic pain. Such a hypothesis may be reflected in the results of behavioural studies in various models of neuropathic pain (Bridges et al., 2001;Fox et al., 2001;Herzberg et al., 1997a) in which C-fibre activity is likely to be elevated and cannabinoid agonists show a marked reduction of mechanical allodynia with more limited effect on thermal hyperalgesia. However, in a situation in which there is no increase in the activity of C-fibre input, and where any constant afferent drive is

of low frequency, as may be the case in our models of demyelination, cannabinoids may be more effective in reducing all pain-related behaviours.

In line with this hypothesis, we found that intrathecal administration of the CB receptor agonist, WIN 55,212-2, attenuated the behaviour indicative of mechanical allodynia and thermal hyperalgesia seen in mice with lysolecithin treated saphenous nerves. The effects of WIN 55,212-2 were largely reversed by the selective CB_1 receptor antagonist AM 251, although, in agreement with similar investigations in other models of neuropathic pain, AM 251 alone did not heighten the allodynia or hyperalgesia (Kemp et al. 1996) . In contrast to previous studies of cannabinoids in models of neuropathic pain, we observed a substantial attenuation of thermal hyperalgesia, which was apparently greater than the effect upon mechanical allodynia. This suggests that because C-fibre primary afferents are unaffected in our model of demyelination-associated neuropathic pain, the central antinociceptive effect of cannabinoids is maintained, unlike the situation following nerve injury involving damage to axons. As a result, sensitisation of dorsal horn neurons to incoming C-fibre activity would still be suppressed via activation of the CB_1 receptor at these sites. This implies that in situations in which central sensitisation is induced by activity in A-fibres alone, the sensitisation of the CNS to incoming noxious thermal stimulation (reflected by behavioural correlates of thermal hyperalgesia) may also be effectively targeted by cannabinoids.

It may be of interest to further investigate the expression of CB_1 receptors in the DRG cells and spinal cord, in our models of afferent demyelination-induced pain to see whether they differ from neuropathic pain models that involve axonal damage. While contrasting to a degree with other nerve injury models, our observation of a reversal of both mechanical and thermal sensitivities has important implications for the treatment of conditions which arise due to peripheral nerve demyelination.

The fact that AM 251 alone did not produce any changes in behavioural reflex thresholds suggests that the endogenous cannabinoid system does not appear to be tonically activated in response to demyelination-induced neuropathic changes. However, our results suggest that it could be effectively targeted as a possible therapeutic intervention site in demyelinating disorders.

The use of cannabinoid analogues and CB receptor agonists may therefore prove very useful in the treatment of pain associated with peripheral nerve demyelination.

5.3 Summary

The investigations into changes in the spinal cord following peripheral nerve demyelination have indicated the presence of a number of key changes. This work could not only lead to the development of possible therapeutic targets but could aid the direction of further studies of central changes which may play important roles in the production of neuropathic pain.

CHAPTER 6. SUMMARY DISCUSSION AND CONCLUSIONS

Neuropathic pain is a condition often associated with peripheral demyelinating neuropathies such as Charcot-Marie-Tooth disease, types I and IV (CMT1 & CMT4). However, mechanisms that may link neuropathic pain to peripheral nerve demyelination are poorly understood (Rasminsky, 1981). To address this problem, we have investigated two models of peripheral nerve demyelination. The first model is the previously characterised *Prx*-null mouse, in which the peripheral nerve myelin protein, L-periaxin, is disrupted creating a late onset peripheral demyelinating neuropathy. The second model is a novel animal model of neuropathy that we have developed by inducing focal demyelination of the peripheral nerve, using the myelinolytic agent lysolecithin.

We have demonstrated that both animal models display behaviour indicative of the development of a neuropathic pain state and that neither have any evidence for axonal damage or cell loss in the DRG. Therefore, both can be considered as new models in which to study pain associated with demyelination alone. In both models there is no evidence for changes in peripheral stimulus transduction thresholds and it would appear that the behavioural reflex sensitivity displayed is due to central changes involving the NMDA receptor, known to be crucial for development of the central sensitisation, which forms the basis for persistent pain.

Electrophysiological investigations have revealed the presence of spontaneous activity in the peripheral nerve of both models that is likely to be capable of inducing of central changes involved in the development and maintenance of a centrally sensitised state. We have demonstrated changes in the PNS including in the expression of sodium channel subtypes that might potentially account for such hyperexcitability. Importantly, we have demonstrated that these changes differ from those associated with other models of neuropathic pain in which axons of all types are damaged pointing towards an important role for myelinated fibres in the production of neuropathic pain.

Central sensitisation of dorsal horn cells is thought to be analogous in many ways to mechanisms of LTP in the hippocampus, which exists in homosynaptic and heterosynaptic forms. Both forms of LTP are likely to occur at synapses in the spinal cord. It has been demonstrated that conditioning stimulation of afferents at intensities sufficient to activate Aδ-fibres and at frequencies ranging from 2-100 Hz induces LTP at synapses of the active afferents in the spinal cord; an example of homosynaptic LTP (Randic et al., 1993).

However, prolonged burst-like stimulation of the sciatic nerve at Aδ-fibre strength has been shown to induce LTP at synapses of C-fibres (Liu and Sandkuhler, 1998), suggesting that heterosynaptic LTP can also be evoked in the superficial spinal dorsal horn. This mechanism could potentially be involved in our models of demyelination-induced pain, in which although spontaneous activity appears to arise from, demyelinated A-fibres, the animals behaviourally demonstrate signs indicative of increased sensitivity to C-fibre mediated thermal stimuli suggestive of sensitisation/LTP at C-fibre synapses.

A further hypothesis that could help to account for the induction of C-fibre related hypersensitivity in our models, in which there is no indication of C-fibre damage, is the fact that potentiation of excitatory synaptic transmission may occur between A-fibres and spinal interneurons, which may in turn presynaptically regulate central C-fibre terminals. Therefore, the activation of interneurons by A-fibres could indirectly activate C-fibre receptors causing sufficient depolarisation of C-fibre terminals to release neurotransmitters into the spinal dorsal horn (Sandkuhler, 2000).

The spontaneous action potential discharge we have observed is of a relatively low frequency compared to frequencies used to induce LTP in the hippocampus, which require s a brief high frequency input (Woolf, 1996). However, it has been shown that low frequency trains of stimuli are sufficient to induce excitability changes such as those used in the experimental induction of windup (Mendell & Wall, 1965; Mendell, 1966), which although using C-fibre stimulation intensities, occurs maximally following stimulation at 1-2Hz (Schouenborg, 1984). Potentiation of synapses, as induced in windup, manifests only during a train of repetitive inputs due to the summation of synaptic potentials (Thompson, et al. 1993) . Therefore, repetitive afferent drive of low frequency (as is a feature of the demyelinated nerves in our models) may be sufficient for inducing persistent changes in dorsal horn cell sensitivity due to a similar summation of synaptic potentials which could provide a basis for the development of central sensitisation.

For central sensitisation to occur, key changes are necessary in dorsal horn neurons, which sensitise them to primary afferent input. Such changes can include increased activity in intracellular second messenger cascades via the activation of cell surface G protein-coupled, metabotropic receptors such as mGluRs and NK_1, which can in turn result in an increase in the intracellular calcium concentration ($[Ca^{2+}]_i$) the activation of intracellular kinases such as CaMKII, PKC (and perhaps even PKA) and transcription factors. Together such changes can lead to an increased response of the cell to normal primary afferent input forming the basis for central sensitisation (Woolf, 1996). For example, a large enough increase in ($[Ca^{2+}]_i$) will produce sufficient depolarisation of the cell to remove the Mg^{2+} block of the NMDA

receptor ion channel (Woolf, 1996). This allows NMDA receptor activation by incoming glutamate, resulting in the influx of Na^+ and Ca^{2+} into the cell, which will cause further depolarisation of the cell and remove the Mg^{2+} block from additional NMDA receptors. Voltage-dependent Ca^{2+} channels and activated protein kinases can further potentiate the activation of the NMDA channel. Therefore, relatively small changes in afferent input may be sufficient to trigger the induction of changes in the NMDA receptor on dorsal horn neurons, an event that can ultimately trigger a cascade of events resulting in the induction of hypersensitivity of the whole system. The spontaneous action potential generation, which is a feature of the demyelinated nerves in our models of neuropathic pain, is therefore likely to cause the release of afferent glutamate into the dorsal horn and initiate many of these changes. Even when such activity is restricted to A-fibres, ephaptic mechanisms may trigger C-fibre release of glutamate and colocalised neuropeptides such as SP, which we have shown to be expressed in the DRG of the demyelinated nerve. We have demonstrated that the activation of the NMDA receptor is involved in the sensitised behaviours displayed in our animal models of neuropathic pain suggesting that events such as those described above are induced in response to changes in the demyelinated nerve.

LTP-like changes occurring in dorsal horn neurons as a result of increased primary afferent input may subsequently lead to delayed changes in gene expression which may contribute to late phases of hyperalgesia (Neumann et al., 1996;Dubner and Ruda, 1992;Woolf and Costigan, 1999), the process of which involves activation of transcription factors such as NFκB. This perhaps explains why we see a reversal of signs of behavioural reflex sensitisation upon antagonism of NFκB at up to 2 weeks following induction of demyelination. Furthermore, the possibility that activation of NFκB is occurring as a consequence of activation of receptors such as the NMDA receptor (Guerrini et al., 1997;Kaltschmidt et al., 1995;Nakai et al., 2000), would provide an explanation for the fact that spinal antagonism of either mediators reverses signs of behavioural sensitisation displayed in our model of neuropathic pain. Clearly other transcription factors will also be involved in the altered pattern of gene expression following demyelination.

Mechanisms of central sensitisation such as these may account for the relatively delayed onset of behavioural sensitisation. Brief but strong excitation of C-fibres, such as occurs in severe axotomising nerve injuries, rapidly induces hyperalgesia. However, less severe injury, as is the case in our models of peripheral nerve demyelination, is likely to result in a slower induction of increased afferent input resulting in a slow summation of synaptic potentials, building up intracellular changes over a prolonged period of time. This would result in a delayed onset of central sensitisation indicated by the later onset of behavioural

correlates of neuropathic pain. However, the increased input to the spinal cord triggers a chain of events maintaining the central sensitisation for at least a number of weeks, as demonstrated in the lysolecithin model of peripheral nerve demyelination, or if the injury is intractable, as in the case of the *Prx*-null mouse, for an extensive period of time.

Once such changes in gene transcription have begun, it may become less likely that pharmacological targeting of changes in the periphery will combat the central sensitisation underlying the persistent pain. Furthermore, due to a lack of specificity, currently available Na^+ channel blocking agents have yet to be proven as satisfactory in eliminating established neuropathic pain. This suggests that the search for adequate therapies needs to focus upon central targets. Our studies in the CNS have pointed to two potential targets for therapeutic intervention in demyelination-induced pain. Firstly, by blocking its activation, we indicated that the transcription factor, NFκB, is involved in the production of the behavioural reflex sensitisation at times corresponding to peak behavioural changes following lysolecithin treatment of the peripheral nerve. Therefore, additional investigations into the cell population in which it is activated, and subsequently which genes it is regulating, may give an indication of possible targets for therapeutic intervention. Secondly, we have demonstrated that agonists of the endogenous cannabinoid system are effective in reversing the neuropathic pain behaviour displayed following the induction of focal demyelination. The endogenous cannabinoid system appears to be a very promising target for the development of pharmaceutical agents to treat chronic pain conditions and our investigations suggest that this may indeed be a very effective target in combating pain induced following peripheral nerve demyelination.

In conclusion, we have developed two models in which neuropathic pain results from either general or focal peripheral nerve demyelination, apparently without the involvement of axonal damage. The lysolecithin model is a novel model of neuropathic pain and is unique in the production of pain-related behaviour from a focal region of demyelination in the purely sensory saphenous nerve. Possible mechanisms underlying the production of pain in this model are similar to other models of neuropathic pain but are not identical. This model should therefore prove valuable not only for the study of pain associated with demyelination but also for the ongoing search for therapies to combat the distressing clinical problem of neuropathic pain.

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APPENDIX: Publications arising from research.

Gillespie,C.S., Sherman,D.L., Fleetwood-Walker,S.M., Cottrell,D.F., Tait,S., Garry,E.M., Wallace,V.C., Ure,J., Griffiths,I.R., Smith,A., and Brophy,P.J. (2000). Peripheral demyelination and neuropathic pain behavior in periaxin-deficient mice. *Neuron* **26,** 523-531.

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Wallace,V.C., Garry,E.M., Sherman,D.L., Cottrell,D.F., Brophy,P.J., Fleetwood-Walker,S.M. (2001). Ouch!!-Touched a raw nerve? Submitted for Society for Neuroscience Annual Meeting Press Book as a lay summary.

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Peripheral Demyelination and Neuropathic Pain Behavior in Periaxin-Deficient Mice

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Summary

The *Prx* gene in Schwann cells encodes L- and S-periaxin, two abundant PDZ domain proteins thought to have a role in the stabilization of myelin in the peripheral nervous system (PNS). Mice lacking a functional *Prx* gene assemble compact PNS myelin. However, the sheath is unstable, leading to demyelination and reflex behaviors that are associated with the painful conditions caused by peripheral nerve damage. Older $Prx^{-/-}$ animals display extensive peripheral demyelination and a severe clinical phenotype with mechanical allodynia and thermal hyperalgesia, which can be reversed by intrathecal administration of a selective NMDA receptor antagonist. We conclude that the periaxins play an essential role in stabilizing the Schwann cell–axon unit and that the periaxin-deficient mouse will be an important model for studying neuropathic pain in late onset demyelinating disease.

Introduction

The Schwann cell is the major glial cell of the vertebrate peripheral nervous system (PNS), where its prime function is to myelinate nerve fibers and to promote rapid nerve impulse transmission. Schwann cells have a significant secondary role in providing trophic support for spinal motoneurons and dorsal root ganglion neurons (Riethmacher et al., 1997) and in promoting nerve regeneration in the PNS (Bunge, 1993). Hence, these versatile cells play a vital role not only in the normal development of the PNS but also in the process of repair.

Extracellular signals, including those from the axon, are believed to be responsible for the induction of the genes required for myelination in the Schwann cell, and they are also thought to determine the thickness of the myelin sheath (Aguayo et al., 1977; Lemke and Chao, 1988; Yin et al., 1998). As in other cell types, the downstream pathways that transduce these signals are likely integrated by the cytoskeleton (Tapon and Hall, 1997) since it has an essential role in mediating both the changes in cell shape and the regulation of gene expression required for axonal ensheathment (Fernandez-Valle et al., 1997). Hence, it was of interest that biochemical analysis of the cytoskeleton-associated proteins of myelinating Schwann cells revealed periaxin as a protein with a possible role in the later stages of myelination (Gillespie et al., 1994). As myelin sheaths mature, periaxin becomes concentrated in the abaxonal plasma membrane, and this shift in localization suggested that it might participate in the recruitment of proteins to a cortical structure involved in transmembrane signaling (Scherer et al., 1995).

Consistent with a role in cortical signaling, the murine *Prx* gene encodes two proteins with PDZ domains, L-periaxin and S-periaxin, of 147 and 16 kDa, respectively (Dytrych et al., 1998). PDZ motifs are protein interaction modules of ~90 amino acids named after the three proteins in which they were first identified, namely postsynaptic density protein PSD-95, *Drosophila discs large* (*dlg*) tumor suppressor, and the tight junction-associated protein ZO-1 (Kornau et al., 1995). The PDZ motif is present in a variety of proteins believed to have an organizing function at sites of cell–cell contact, where they are implicated in the assembly of macromolecular signaling complexes (Kornau et al., 1997; Tsunoda et al., 1997). Such proteins can multimerize homotypically and heterotypically (Hsueh et al., 1997; Srivastava et al., 1998). They may also interact with the cytoplasmic tail of transmembrane proteins, such as glutamate receptors (Dong et al., 1997; Sheng and Wyszynski, 1997; Srivastava et al., 1998).

The evidence so far suggests that the periaxins participate in the later stages of axonal ensheathment, possibly in the stabilization of the myelin sheath (Scherer et al., 1995). If this is correct, inactivation of the *Prx* gene should destabilize the relationship of the axon with its ensheathing Schwann cell. We have generated mice that lack a functional *Prx* gene, and we demonstrate that their Schwann cells ensheath and myelinate peripheral nerve axons in an apparently normal manner but that this sheath destabilizes, and the mice develop a severe demyelinating neuropathy. These data suggest that the periaxins play an essential role in the establishment of a stable Schwann cell–axon unit in the myelinated fibers of the vertebrate PNS.

In addition to reducing the rates of nerve impulse transmission, segmental demyelination in human disease can be associated with tactile allodynia, the perception of normally innocuous stimuli, such as touching or brushing, as painful, and hyperalgesia, a heightened response to painful stimuli; however, the mechanisms of neuropathic pain in demyelinating disease are poorly understood (Rasminsky, 1981). Importantly, we show that in addition to a marked reduction in their peripheral nerve conduction velocities, periaxin null mice display

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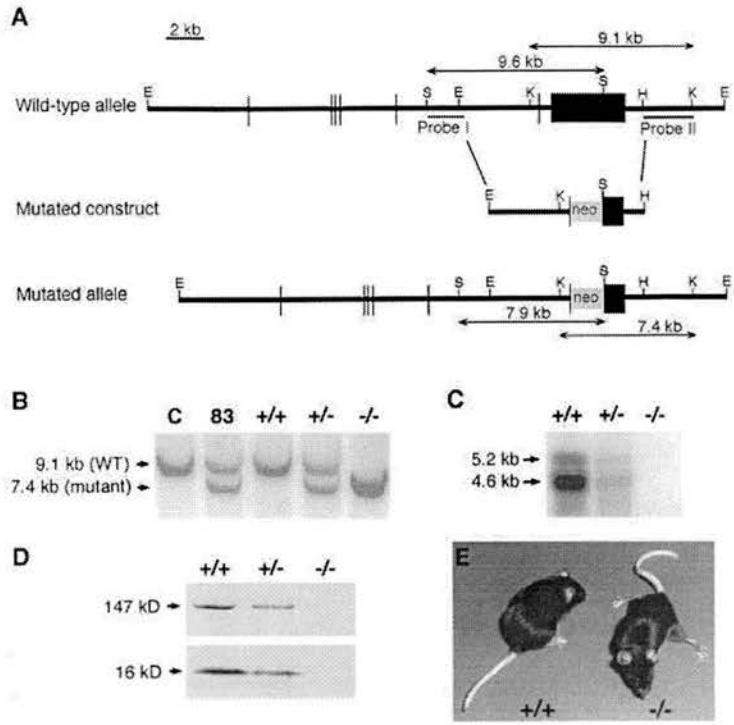

Figure 1. Targeted Disruption of the *Prx* Gene

(A) Structure of wild-type and targeted *Prx* loci.

(Top) A partial restriction endonuclease map showing the position of SacI (S) and KpnI (K) sites in the murine *Prx* gene. A β-actin promoter–*neo* gene cassette was inserted between the SmaI site in exon 6 and the SacI site in exon 7. Digestion of DNA with SacI identified the targeted allele by the presence of a 7.9 kb fragment instead of a 9.6 kb when probed with external probe I, and by a 7.4 kb instead of 9.1 kb when DNA was digested with KpnI and probed with external probe II.

(B) DNA from a control ES clone (C), from a homologous recombinant ES clone (83), and from tail clips of 3-week-old littermates obtained by heterozygous intercrosses was digested with KpnI and probed with external probe II.

(C) Northern blot analysis of RNA (3 μg) from sciatic nerves of 16-day-old $Prx^{+/+}$, $Prx^{+/-}$, and $Prx^{-/-}$ mice demonstrates the absence of the *Prx* wild-type mRNAs in mice homozygous for the mutant allele.

(D) Western blot of sciatic nerve homogenate protein (10 μg) from 1-month-old mice demonstrates the absence of both L- and S-periaxin (147 and 16 kDa, respectively) from homozygous mutants.

(E) $Prx^{-/-}$ mice at 8 months are unable to support themselves on their hindlegs.

reflex behaviors associated with allodynia and hyperalgesia. We suggest that these animals will prove to be valuable in identifying the pathophysiological basis of neuropathic pain.

Results

Generation and Clinical Phenotype of *Prx* Null Mice

To inactivate the *Prx* gene, we deleted exon 6 and part of exon 7, corresponding to 611–3440 bp of the mouse cDNA, by homologous recombination in embryonic stem (ES) cells (Figure 1A); 2 of 130 lines, clones 57 and 83, were heterozygous for insertion at the *Prx* locus, and clone 83 was selected for further study (Figure 1B). Homozygous offspring lacked the 4.6 and 5.2 kb mRNAs found in the peripheral nerves of $Prx^{+/+}$ mice (Figure 1C), and neither L- nor S-periaxin polypeptides were detectable in the peripheral nerves of mutant mice (Figure 1D). The genotypes of the progeny of mice heterozygous for the mutant allele were as expected from Mendelian laws, which indicated that embryonic development was not compromised by inactivating the *Prx* gene. Mice homozygous for the mutant allele appeared grossly normal for up to 6 weeks, with the exception that when lifted by the tail, they all displayed a distinctive clasping of the hindlimbs from about 4 to 6 weeks of age, and some animals displayed a slight tremor. By 6–9 months of age, there was a pronounced unsteadiness in the mutants' gait, and they had great difficulty supporting themselves on their hindlimbs, which were often splayed (Figure 1E). Older animals (>6–9 months) lost weight rapidly, owing to an inability to feed, and their breathing was labored. As soon as the animals were in obvious distress, they were euthanized.

Structure of the Schwann Cell–Axon Unit

We next examined the peripheral nerves of periaxin-deficient mice by light microscopy to determine if the myelin sheath was affected. At 6 weeks of age, the sciatic nerves of periaxin null mice contained focal thickenings (tomacula) and infoldings of internodal myelin (Figure 2B). Nevertheless, Western blotting showed that the levels of the major myelin proteins, myelin-associated glycoprotein, P0, and myelin basic protein, were normal in the sciatic nerves of mutant animals, which confirmed that at this age there had not been extensive demyelination (data not shown). A variety of spinal, cranial, and autonomic nerves also displayed limited abnormalities at this age. However, by 6 months, sensory, motor, and autonomic (vagus) nerves were extensively demyelinated, and most internodes in the sciatic nerves of periaxin null mice contained focal thickenings or infoldings of the sheath (Figure 2D). Profound disruption of axonal ensheathment and segmental demyelination was apparent in teased sciatic nerve fibers at this age (Figures 2E and 2F), and their Schmidt-Lanterman incisures, which are normally visible as cytoplasm-filled structures along the length of the internodes, were deranged (Figures 2G and 2H).

Electron microscopy demonstrated that saphenous nerves, which are predominantly sensory, were extensively hypermyelinated but that unmyelinated C fiber bundles appeared to be morphologically normal, which is consistent with the absence of *Prx* gene expression in

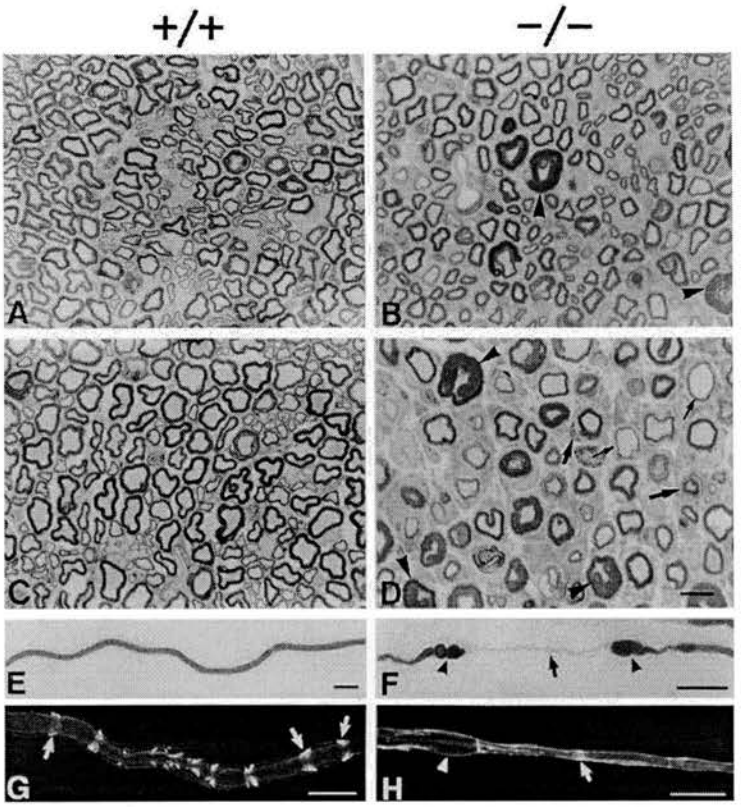

Figure 2. Light Microscopy of Sciatic Nerves from *Prx* Null Mice

Resin sections (1 μm) from the sciatic nerves of normal (A and C) and mutant (B and D) mice at 6 weeks (A and B) and at 6 months (C and D) were compared. The null mutant (B and D) showed features typical of a progressive demyelinating neuropathy with evidence of remyelination, as denoted by onion bulbs and thin myelin sheaths (thick and thin arrows, respectively). Disproportionately thick sheaths evident at 6 weeks are much more numerous at 6 months (arrowhead). A low-power view of wild-type (E) and mutant (F) teased sciatic nerve fibers from 8-month-old $Prx^{-/-}$ mice shows focal thickenings of the myelin sheath (arrowheads) flanking a demyelinated segment. A higher-power view of nerves stained with TRITC-phalloidin (G and H) reveals the loss of Schmidt-Lanterman incisures in the sheath (arrows); a tomaculum is visible in outline (arrowhead). Scale bar, 10 μm (A–D); 50 μm (E and F); 25 μm (G and H).

non-myelin-forming Schwann cells (Scherer et al., 1995) (Figures 3A–3C). Hypermyelination resulted in sheath infolding (Figure 3B) and axonal compression (Figure 3C). By 8 months, naked or thinly myelinated axons were common in sciatic nerve fibers, often surrounded by redundant basal laminae and supernumerary Schwann cells, which formed onion bulb structures, diagnostic of attempts to remyelinate demyelinated fibers (Figures 3D). There was little evidence for macrophage infiltration, indicating that demyelination was not the result of a macrophage-mediated inflammatory process. The damage seemed to be confined to the myelin sheath in that there was no evidence of axonal degeneration, and there was no difference in the number of dorsal root ganglion neurons (L5) between wild-type controls (8655 ± 127, n = 3) and periaxin-deficient mice (8668 ± 619, n = 3). Furthermore, neuronal cell bodies in spinal gray matter and spinal ganglia showed no evidence of degenerative changes (data not shown). Mice heterozygous for the null allele ($Prx^{+/-}$) showed occasional focal thickenings of the myelin sheaths, a feature that did not increase with age, at least up to 10 months, and no evidence of demyelination was noted.

The ability of periaxin-deficient mice to remyelinate was determined after crush lesion. Electron microscopy of sciatic nerves distal to the lesion site 1 month post crush revealed that 4-month-old $Prx^{-/-}$ mice differed markedly from their wild-type littermates, in spite of their being able to assemble apparently normal compact myelin in the early developing PNS. In comparison with the myelinated reinnervated axons of control nerves, remyelination was retarded in the periaxin null mice, with many incompletely ensheathed axons and abnormally thin sheaths (Figures 3E and 3F).

Peripheral Nerve Electrophysiology in $Prx^{-/-}$ Mice

At 6–8 months, the extensive pathology noted in peripheral nerves was reflected in reduced conduction velocities in nerves from control and $Prx^{-/-}$ mice. Both the peak compound action potential (CAP) and the electromyogram from single identified skeletomotoneurons of the sciatic nerve were reduced by ~60% (Table 1). The values derived from both techniques were similar, suggesting that sensory and motor branches were equally affected in this mixed nerve, a conclusion that was supported by morphological examination of the ventral and dorsal spinal roots. The spike shape of the CAP in mutants was distorted, which may reflect a degree of ephaptic cross-activation caused by extensive demyelination (Smith and McDonald, 1980) (data not shown).

It was of particular interest to analyze sensory nerves since they have been reported to be spared in animals deficient in other PNS myelin proteins (Adlkofer et al., 1995; Martini et et al., 1995; Anzini et al., 1997). The conduction velocities of hair follicle mechanoreceptor afferents in the saphenous nerve were tested by focal displacement of single identified hairs with von Frey filaments. Disruption of the myelinated fibers of these afferents in 6-week-old mutants was reflected in a significant reduction in their mean conduction velocities from 7.6 ± 3.3 (5 animals, 52 mechanoreceptors) to 3.9 ± 1.6 ms^{-1} (6 animals, 42 mechanoreceptors) ($p < 0.0001$ by

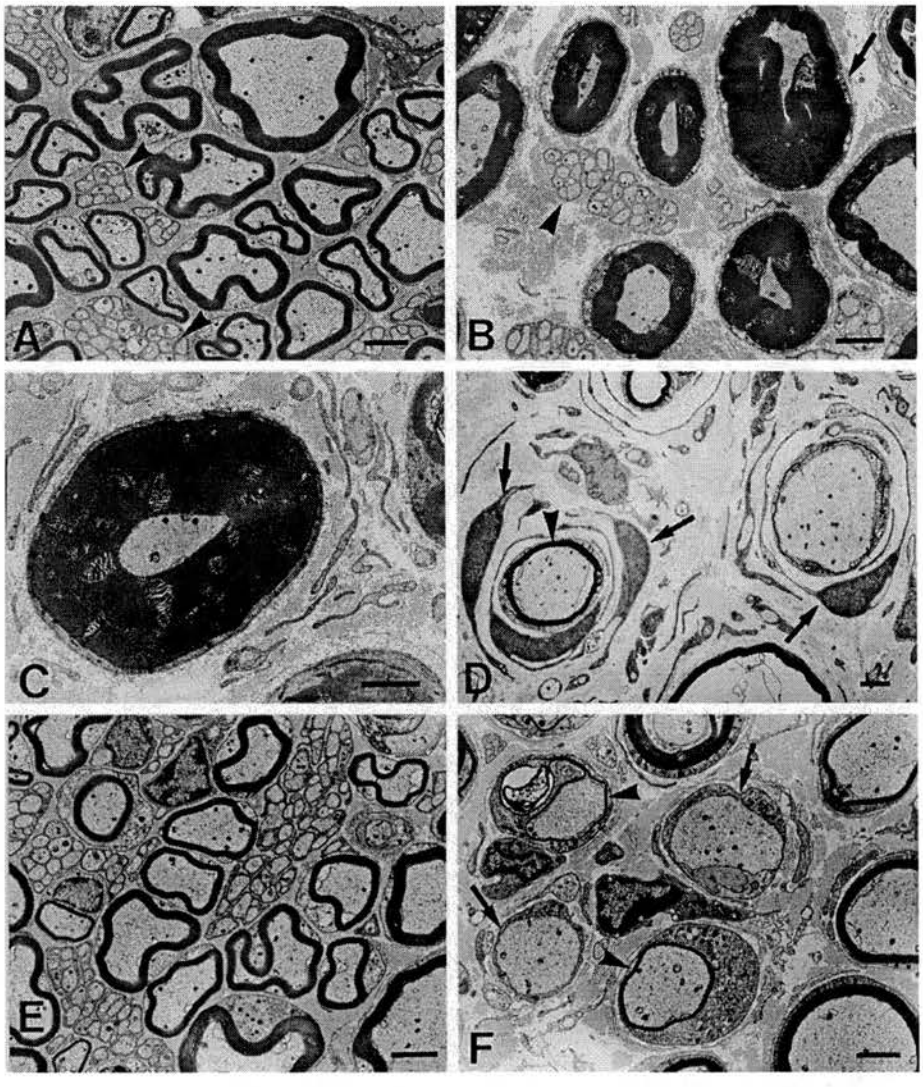

Figure 3. Structure of Schwann Cell–Axon Units in Nerves from *Prx* Null Mice

Electron microscopy of wild-type (A) and mutant (B and C) saphenous nerves at 6 months. Unmyelinated C fiber bundles appear normal (arrowhead), but myelinated fibers are grossly hypermyelinated (B and C), with evidence of myelin infolding at a tomaculum (arrow, [B]) and axonal compression (C). By 8 months, the sciatic nerves of mutant animals (D) display the onion bulb pathology of supernumerary Schwann cells (arrows) characteristic of a demyelinating neuropathy with attempts at remyelination that results in disproportionately thin myelin sheaths (arrowhead). In 4-month-old wild-type animals, robust reinnervation and remyelination were observed in sciatic nerves distal to the crush site 1 month post crush (E). In contrast, ensheathment of mutant nerves (F) was highly retarded (arrows), with numerous abnormally thin sheaths (arrowheads). Scale bars, 2 μm.

Student's t test). Only 8% of recorded afferents in mutant animals had conduction velocities >6 ms^{-1}, and none were above 8 ms^{-1}, in marked contrast to wild-type animals (Figure 4). The vast majority of these were in the A fiber conduction velocity range, with a conduction velocity above 2 ms^{-1}, indicating that they were myelinated (Koltzenburg et al., 1997); only a minor population, in each case, displayed conduction velocities characteristic of unmyelinated fibers (Figure 4). Similar results were obtained at 6 months, but the reduction in conduction velocities (67%) was more severe (data not shown).

The unmyelinated fibers were also specifically investigated in 6-week-old animals, and there was no significant difference in the mean conduction velocity in mutants compared with normal littermates ($Prx^{+/+}$, 1.46 ± 0.10 ms^{-1}, n = 11 afferent units; $Prx^{-/-}$, 1.17 ± 0.13 ms^{-1}, n = 15 afferent units; three mice in each case and $p > 0.05$ by Student's t test); nor was there a significant difference between normal and mutant mice in the von Frey filament response thresholds of the A fibers or the thermal nociceptive response thresholds of the C fiber population (Table 2).

A common feature of the saphenous nerves of both mutant animals and their wild-type littermates at 6 weeks was the presence of a spontaneous low-frequency discharge (1–2 Hz) in fibers throughout the

Table 1. Nerve Conduction Velocities in Sciatic Nerves at 8 Months of Age

	Peak CAP	EMG
Wild-type	25.0 ± 0.7	27.5 ± 1.6
Mutant	10.9 ± 2.0	8.6 ± 1.0
	$p < 0.005$	$p < 0.0001$

Compound action potential (CAP) and electromyogram (EMG) values (ms^{-1}) were measured after electrical excitation of the peripheral nerve and are mean ± SEM for four animals in each group. Statistical significance was determined by Student's t test.

range of conduction velocities. However, more mutants (100%, n = 14) displayed this activity in comparison to their wild-type counterparts (28%, n = 15). This spontaneous activity occurred across the conduction velocity range.

Peripheral Nerve Conduction and Behavioral Deficit

The spontaneous low-frequency discharge observed in saphenous fibers prompted us to analyze the response of the mice in well-characterized models of nociceptive reflex behavior since it has been suggested that ectopic activity might contribute to pain following nerve injury (Rasminsky, 1981; Tal and Eliav, 1996). At 6 weeks of age, mutant mice displayed a markedly lower threshold in a reflex test of cutaneous mechanical sensation using calibrated von Frey hairs applied to the saphenous territory of the hindpaw (Table 3). This was in spite of the fact that we had already shown that the mean threshold response of hindpaw mechanoreceptors to natural mechanical stimuli, measured electrophysiologically in the saphenous nerve at 6 weeks, was unchanged (Table 2).

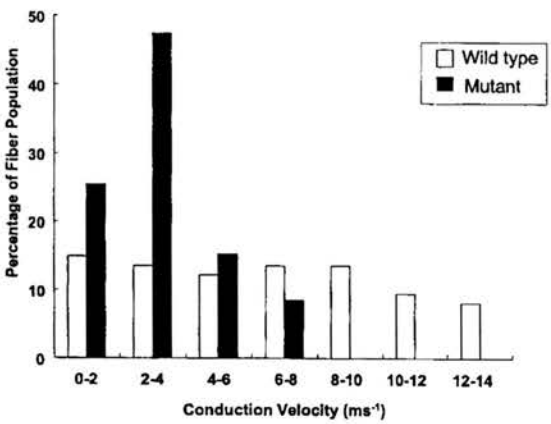

Figure 4. Frequency Range of Conduction Velocities in Saphenous Nerve Fibers

Nerve conduction velocities were measured from single afferent fibers in the saphenous nerves of 6-week-old mice. All units with conduction velocities above 2 ms^{-1} were tested and found to be mechanosensitive, and demonstrated a majority of low-threshold, rapidly adapting units, with very few slowly adapting units. The locations of peripheral receptive fields for natural mechanostimulation and electrical excitation were identical. Receptive fields were focal, confined to between one and two hairs, and afferent units were not excited when stimulating electrodes were slightly displaced from the natural receptive field.

Table 2. Threshold of the Response of Teased Saphenous Nerves to Mechanical and Thermal Stimulation with von Frey Filaments or Noxious Radiant Heat at 6 Weeks of Age

	Threshold of Activation	
	Mechanical ($mNmm^{-2}$)	Noxious Thermal (°C)
Wild-type	23.8 ± 6.6	48.2 ± 0.7
Mutant	23.4 ± 4.2	48.0 ± 0.3

Fibers tested by mechanical stimulation had conduction velocities above 2 ms^{-1} (n = 5 in both groups); those tested by noxious thermal stimulation had conduction velocities below 2 ms^{-1} (n = 3 in both groups). Each value is the mean ± SEM, and there was no statistically significant difference by Mann-Whitney Rank Sum test.

To determine if this diminished response threshold was specific to receptors with myelinated afferents, we also measured the response threshold in a reflex test of thermal nociceptive sensitivity for which the relevant afferents are largely unmyelinated C fibers (Hargreaves et al., 1988; Ahlgren et al., 1997; Koltzenburg et al., 1997). *Prx* null mice had significantly lower response thresholds, which indicated the presence of thermal hyperalgesia, as well as mechanical allodynia (Table 3). As pointed out earlier, there was no evidence for sensory neuron loss that might have led to altered reflex behavior.

In contrast to the lowered threshold in the nociceptive reflex tests, mutant mice at 6 weeks displayed responses in the normal range in a mechanical grip test (Meyer et al., 1979), which indicates a lack of any overt motor deficit at that age (data not shown).

The mechanical response thresholds (Figure 5) and nociceptive response latencies (Table 4) were also significantly reduced in 6-month-old mutants when morphological derangement of myelinated nerves was more pronounced. In these animals, a role for spinal NMDA receptors in the mechanical allodynia shown by *Prx* null mice was demonstrated by reversal of this behavioral sensitization following spinal administration of the highly selective NMDA receptor antagonist 3-([R] 2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid ([R]-CPP) (Lehmann et al., 1987) (Figure 5). Similarly, a reduction in thermal nociceptive response latency was reversed in $Prx^{-/-}$ mice without affecting the responses of normal littermates (Table 4).

Table 3. Hindpaw Withdrawal Response to Mechanical and Noxious Thermal Stimulation at 6 Weeks of Age

	Mechanical Withdrawal Threshold ($mNmm^{-2}$)	Withdrawal Latency from Noxious Heat (s)
Wild-type	460 ± 40	11.5 ± 2.2
Mutant	274 ± 23*	6.1 ± 1.1**

Minimum filament indentation pressure thresholds for repeat paw withdrawal responses to mechanical stimulation with von Frey filaments in conscious animals were measured for four animals in each group. Statistical significance (*$P < 0.05$) was determined by Mann-Whitney Rank Sum test. The time taken for hindpaw withdrawal from an infrared beam was measured for six normal littermates and seven mutant animals. Statistical significance (**$P < 0.05$) was determined by Student's t test. Each value is the mean ± SEM.

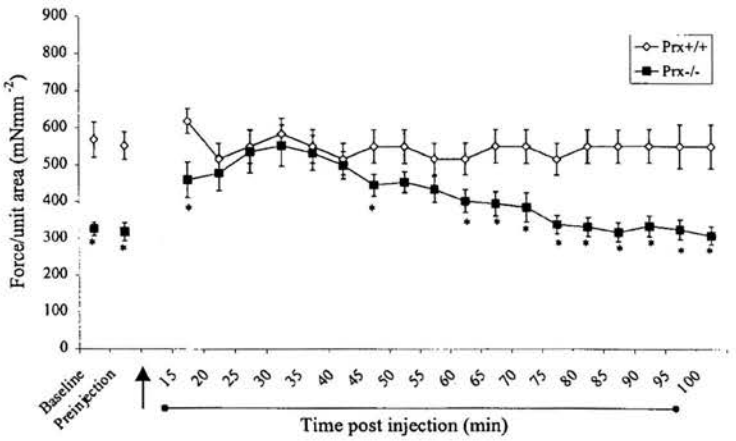

Figure 5. The Effect of Intrathecal Injection of (R)-CPP on Mechanosensitive Paw Withdrawal Threshold

The paw withdrawal threshold was measured in response to cutaneous mechanical stimulation with von Frey filaments in $Prx^{+/+}$ (n = 3) and $Prx^{-/-}$ (n = 8) mice after intrathecal injection of (R)-CCP (↑, 100 pmol in 10 μl). Statistical significance of differences from wild-type animals (*$p \leq 0.05$) was determined by Mann-Whitney Rank Sum test, and each value is the mean ± SEM. Intrathecal injection of the saline vehicle had no significant effect in either $Prx^{+/+}$ or $Prx^{-/-}$ (n = 5 for both).

Discussion

Several demyelinating neuropathies of genetic origin have now been modeled in mice by inactivating genes expressed in myelinating Schwann cells (Suter and Snipes, 1995; Martini, 1997). Such animals are not only valuable for examining the biological function of the proteins encoded by these genes but they also provide an opportunity to study the pathophysiology of disease. However, the homozygous periaxin null mouse is unique in the late onset and the severity of the clinical phenotype, which shows parallels with demyelinating peripheral neuropathies of adult onset (Dyck et al., 1993). Furthermore, sensory deficits have not been a significant feature of mouse models of peripheral demyelinating neuropathy thus far even though they are of relevance to human disease.

Prx Is Required for Stable Myelination in the PNS

This work suggests that the periaxin PDZ domain proteins have an essential role in myelin-forming Schwann cells once the sheath has been assembled around the axon. In *Prx* null mice, the earliest evidence for morphological disruption of the Schwann cell–axon unit is the focal thickening of the sheath. The ensuing overproduction of myelin is followed by demyelination and remyelination. The mechanism by which the thickness of the myelin sheath is signaled is not known, but it is likely that it is regulated by the axon (Lemke and Chao, 1988). It may be significant that the *Prx* gene itself is regulated by axonal contact (Scherer et al., 1995).

Many of the other well-characterized proteins expressed by differentiating, myelin-forming Schwann cells are incorporated as structural components of compact myelin. In contrast, L-periaxin becomes concentrated in the abaxonal plasma membrane of the Schwann cell, which suggested that it may recruit proteins to a cortical structure involved in transmembrane signaling (Gillespie et al., 1994; Scherer et al., 1995; Dytrych et al., 1998). The periaxins may associate with the cytoplasmic domains of plasma membrane proteins involved in adhesion since their expression coincides with the early events in the establishment of axo–glial contact and since the translocation of L-periaxin to a predominantly abaxonal location is a feature of the maturation of the sheath (Gillespie et al., 1994; Scherer et al., 1995; Dytrych et al., 1998).

Structure and Electrophysiology of the PNS in Mutant Animals

At 6 weeks of age, the sciatic nerves of periaxin-deficient mice are affected relatively mildly compared with mice lacking the myelin proteins P0 and PMP22, which display widespread derangements to the Schwann cell–axon unit from a much earlier age (Giese et al., 1992; Adlkofer et al., 1995). Mice lacking P0 also display clasping of their hindlimbs, but, at 4–6 weeks, they are much more clinically affected than are *Prx* mutants (Giese et al., 1992). This foot clasp response is also observed in mutant mice with neurological disease of purely CNS origin (Chiesa et al., 1998).

Similar to periaxin null mice, animal models of Charcot-Marie-Tooth disease Type X (CMTX) deficient in the gap junction protein connexin 32 myelinate normally and show a late onset pattern of demyelination with attempts

Table 4. Hindpaw Withdrawal Response to Noxious Thermal Stimulation following Intrathecal Administration of the NMDA Receptor Antagonist (R)-CPP in 6-Month-Old Mice

		Treatment		
	Preinjection	Vehicle	(R)-CCP	Recovery
Wild-type	9.1 ± 0.7 (5)	11.9 ± 1.6 (6)	10.0 ± 1.3 (5)	9.0 ± 1.9 (5)
Mutant	6.6 ± 0.3* (8)	9.1 ± 1.4* (6)	12.9 ± 1.3 (8)	6.7 ± 0.8* (8)

The effect of intrathecal injection of (R)-CCP (100 pmol in 10 μl) was measured as the mean threshold (latency to paw withdrawal) ± SEM of values determined between 20 and 50 min following injection and, at recovery, between 100 and 110 min following injection. Statistical significance from wild-type values with the same treatment (*$p \leq 0.05$) was determined by Student's t test. Number of animals is in brackets.

to remyelinate (Anzini et al., 1997). However, these animals display very mild deficits in nerve conduction; furthermore, the saphenous nerve, which is predominantly sensory, shows little evidence of demyelination in CMTX-deficient mice (Anzini et al., 1997). Mice heterozygous for the mutant alleles of P0 and PMP22 also show a propensity for sparing sensory versus motor nerves (Adlkofer et al., 1995; Martini et al., 1995). There was no evidence for such sparing in *Prx* mutants since the profiles of ventral and dorsal roots in cross section were similar. The reason for the differential susceptibility of sensory versus motor nerves in other demyelinating conditions remains unclear.

When deprived of their intimate association with axons during Wallerian degeneration, myelin-forming Schwann cells revert to a premyelinating phenotype (Bunge, 1993; Mirsky and Jessen, 1996). This transition is accompanied by the removal of myelin debris, the downregulation of genes that encode myelin proteins, and the initiation of Schwann cell proliferation (Poduslo and Windebank, 1985; Lemke and Chao, 1988; Scherer and Salzer, 1996). Reinnervation is normally accompanied by remyelination. However, the process of repair appears to be compromised in the sciatic nerves of periaxin null mice. Although the reason for this impairment is not clear, it is of relevance to the cumulative residual deficits caused by repeated mechanical compression of peripheral nerves in such diseases as hereditary neuropathy with liability to pressure palsy (Lynch and Chance, 1997).

By 6 months of age, conduction velocities in the sciatic and saphenous nerves of mutant animals were reduced to 44% and 33% of the respective normal values. This was entirely consistent with the extensive demyelination observed in a variety of myelinated nerves throughout the PNS. The frequent incidence of demyelinated axons engulfed by supernumerary Schwann cells was strikingly similar to the onion bulb pathology characteristically found in human CMT disease Type IA (CMTIA) (Dyck et al., 1993).

Demyelination, Allodynia, and Hyperalgesia

Damage to sensory nerves is linked to pain and excessive sensitivity to touch in human peripheral demyelinating disease (Devor, 1989; Pentland and Donald, 1994), and there is good evidence that the mechanical hypersensitivity observed in tactile allodynia is mediated by myelinated A fibers (Woolf, 1997; Koltzenburg, 1998). In a variety of models of central (spinal) sensitization, there is evidence that peripheral sensitization may be a significant contributor to neuropathic pain behavior (Koltzenburg et al., 1994; Woolf and Costigan, 1999). Nevertheless, we could find no evidence for significant changes in the stimulus–response relationship in either A or C fibers in $Prx^{-/-}$ mice with either cutaneous mechanical or thermal nociceptive stimulation in periaxin null animals; nevertheless, the spinal cord reflex responsiveness to this traffic may be affected by sensitization, especially at central sites. A contributor to central sensitization may be the presence of spontaneous activity in saphenous afferents. While repetitive C-afferent stimulation is normally required to bring about this state, in conditions of peripheral inflammation, it may be maintained or promoted by Aβ input (Woolf and Costigan, 1999).

Furthermore, our evidence that NMDA receptor–dependent events at a central site were essential for the phenomena of mechanical allodynia and thermal hyperalgesia behavior to become manifest in $Prx^{-/-}$ mice indicates that some form of central change, probably sensitization, is a feature of the phenotype. NMDA receptor–dependent central sensitization characteristically underpins the mechanical allodynia and thermal hyperalgesia seen in a variety of other models of chronic pain states (Mao, 1993; Bennett, 1994; Dickenson and Sullivan, 1987; Chaplan et al., 1997; Woolf and Costigan, 1999).

Ectopic repetitive firing has been proposed to be the origin of abnormal sensation in diseases characterized by segmental demyelination, particularly when there is no evidence for axonopathic changes (Rasminsky, 1981; Tal and Eliav, 1996). Demyelinated segments can certainly serve as foci for spontaneous multiple spikes or for those evoked by mechanical stimuli (Smith and McDonald, 1980; Calvin et al., 1982; Baker and Bostock, 1992). Nevertheless, the origin of tactile allodynia and neuropathic pain in human demyelinating disorders is still poorly understood (Ropper and Shahani, 1984; Carter et al., 1998). The periaxin-deficient mouse is a promising new model in which to address this problem.

Experimental Procedures

Generation and Characterization of $Prx^{-/-}$ Mice

The genomic locus was targeted by homologous recombination using a cassette comprising the human β-actin promoter (Frederickson et al., 1989) and the neomycin resistance (*neo*) gene flanked by 4.3 kb and 2.5 kb of strain 129 DNA. The cassette was inserted between the SmaI site in exon 6 and the SacI site in exon 7 and subcloned into the plasmid pGEM11Zf. The construct was excised from the plasmid by digestion with NotI and SalI and electroporated into the ES cell line CGR8, maintained in the absence of a feeder layer (Mountford et al., 1994). After selection in G418, the homologous recombinants were identified by Southern blotting using probes external to the construct. The targeted alleles were identified by the presence of a 7.9 kb fragment instead of a 9.6 kb when DNA was digested with SacI and probed with external probe I, and by a 7.4 kb fragment instead of 9.1 kb when DNA was digested with KpnI and probed with external probe II (Figure 1A). Two clones, 57 and 83, were injected into C57BL/6 blastocysts, and their progeny were typed by Southern blotting of tail DNA. Heterozygous offspring were backcrossed to the parental C57BL/6 strain before intercrossing at the F6 generations. Control animals were age- and generation-matched $Prx^{+/+}$ mice or $Prx^{+/+}$ littermates. Mice homozygous for the mutant allele were identified by Southern blotting as described previously (Dytrych et al., 1998).

RNA and Protein Analysis

RNA was extracted from the sciatic nerves of 16-day-old mice (Cathala et al., 1983), and 3 μg was resolved and probed with a cDNA fragment comprising bases 1132–1549 of mouse periaxin cDNA as described previously (Dytrych et al., 1998). L- and S-periaxin proteins in sciatic nerves from 3-month-old animals were detected with isoform-specific antibodies in Western blots as described previously (Dytrych et al., 1998).

Microscopy and Cell Counts

Mice were perfused intravascularly with 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.3). Nerves were removed, fixed for 2 hr in the same fixative, postfixed in OsO_4, and embedded in araldite. Ultrathin sections were stained with uranyl acetate and lead citrate and examined on a Phillips BioTwin electron microscope. The following nerves were sampled: sciatic, tibial, lumbosacral and lumbar plexi, cervical and lumbar

nerve roots, saphenous, vagus, and trigeminal. Blocks of cervical and lumbar spinal cord were also examined. The sciatic nerves of anesthetized mice were crushed as previously described (Scherer et al., 1995). For light microscopy, 1 μm resin sections were stained with toluidine blue. For teased fiber preparation, sciatic nerves either were postfixed with 2% OsO_4 in 0.1 M sodium cacodylate buffer for 2 hr, dehydrated, infiltrated in Spurr medium, teased in Spurr embedding medium, and examined by bright-field microscopy or were teased in 0.1% Triton X-100 in phosphate-buffered saline, stained with TRITC-phalloidin (Sigma) (0.1 mg/ml) for 1 hr, and examined with a Leica TCS4D confocal microscope to visualize microfilaments. For cell counts on dorsal root ganglia, mice were perfused with neutral buffered formalin, and ganglia were embedded in paraffin after further immersion fixation in formalin overnight. Sections (8 μm) were stained with fast cresyl violet, and neurons with nucleoli in clear nuclei were counted in every fifth section.

Electrophysiology

Mice were anesthetized with 25% urethane i.p. and maintained at 36°C–37°C with a radiant heat lamp. The saphenous femoral nerve was exposed in the medial thigh and dissected from its associated vein and artery. Further dissection under liquid paraffin enabled the identification of afferent preparations comprising a small number of units. For fibers with conduction velocities above 2 ms^{-1}, the mechanosensitivity of individual sensory receptors with high sensitivity was measured in hairy and hairless skin using calibrated von Frey hairs. The mechanical threshold was defined as the pressure required to cause units to fire 50% of the time by direct application of von Frey filaments to identified receptive fields. The conduction velocity of single identified afferent fibers was determined using bipolar electrodes and the peripheral stimulus technique (Iggo, 1958). The cutaneous responsiveness of fibers with conduction velocities below 2 ms^{-1} was tested following isolation of few-unit preparations, and a focal heat stimulator was set to deliver a preset focal radiant heat stimulus to the receptive field at an intensity determined by a thermocouple for a preset period of 10–15 s (Sobair et al., 1997). Interstimulus intervals >10 min were allowed between applications to mitigate receptor sensitization. The CAP was measured over a length of sciatic nerve. The femoral length of the sciatic nerve was exposed in an isolated solder ring pool that was flooded with liquid paraffin. Bipolar recording electrodes were positioned on the sciatic nerve at the level of the femoral head, and bipolar stimulating electrodes were placed on the sciatic nerve in the pool, near the knee. A second pair of stimulating electrodes was positioned over the skin covering the tibial nerve at the ankle. The peak of the fastest conducting wave of the CAP was measured, providing a mean value for the Aα fibers. The electromyogram caused by activation of a single skeletomotoneuron was measured in the interosseus muscles with a concentric needle electrode using just-threshold levels of excitation at two locations in the sciatic nerve (Sharma and Thomas, 1974).

Behavioral Tests and Drug Treatment

The threshold for hindpaw withdrawal in response to graded mechanical stimulation was measured in conscious animals at 6 weeks of age with von Frey filaments, which provide a calibrated indentation pressure against the hairless skin of the hindpaws. The threshold response in this test was defined as the filament that causes foot withdrawal five times in every ten applications (Meyer et al., 1979; Chaplan et al., 1994). The time for hindpaw withdrawal in response to a quantified noxious thermal stimulus provided by an infrared beam to the hairless surface of the paws was measured (Hargreaves et al., 1988). Before determining the effect of an NMDA receptor antagonist, mice were tested daily over a period of 2 weeks prior to injection. A minimum of five measurements were made per animal. Mice were briefly anesthetized with halothane and oxygen after determining baseline measurements for mechanical allodynia and thermal hyperalgesia and were injected intrathecally at the L4 level of the spinal cord with 100 pmol of (R)-CPP in saline (10 μl). Testing commenced after a 15 min period of recovery from anesthesia. Mice were then tested every 5 min.

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Abstract #1:poster presentation

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PERIPHERAL DEMYELINATION AND NEUROPATHIC PAIN

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Chronic neuropathic pain is poorly understood and is usually untreatable by classical analgesics. Neuropathic pain is characterised by allodynia (perception of innocuous stimuli as painful) and hyperalgesia (facilitated response to painful stimuli). Pain is a symptom often associated with human demyelinating conditions such as Charcot-Marie-Tooth disease or multiple sclerosis. Using a novel mouse model, we have recently provided evidence for the establishment of neuropathic pain as a result of peripheral demyelination.

The *Prx* gene in Schwann cells encodes L- and S-periaxin, two abundant PDZ-domain proteins thought to have a role in stabilisation of myelin in the peripheral nervous system (PNS). *Prx-null* mice show progressive demyelination in peripheral nerves and display an increased behavioural reflex sensitivity to cutaneous mechanical and noxious thermal stimulation. This sensitisation can be reversed by intrathecal injection of the NMDA receptor antagonist, [R]-CPP, indicating that key changes occur within the spinal cord.

In peripheral nerves morphological studies revealed compromised myelination and electrophysiological investigations indicate the presence of spontaneous action potential discharge; abnormal activity which may be a consequence of demyelination.

This investigation provides the first direct evidence linking the presence of peripheral demyelination to the production of neuropathic pain and present experiments are seeking to elucidate the mechanisms by which this may occur.

Experiments complied with the Animals (Scientific Procedures) Act 1986

Work supported by the Wellcome Trust

Abstract #2:poster presentation

International Association for the Study of Pain; 10th World Congress on Pain, San Diego, CA, USA. August 2002

A NOVEL MODEL OF FOCAL PERIPHERAL DEMYELINATION AND NEUROPATHIC PAIN

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Focal demyelination of the sciatic or saphenous nerve was induced with lysolecithin (lysophosphatidylcholine). The behavioural reflex sensitivity of treated mice was assessed using a thermal stimulus and von Frey filaments. Nerve morphology was investigated using light and electron microscopy and peripheral nerve recordings were made to assess changes in excitability. Intrathecal injections of the selective NMDA receptor antagonist, [R]-CPP, were carried out to explore the role of NMDA receptor-dependent changes in the spinal cord in the behavioural reflex sensitivity.

Results

Lysolecithin treatment resulted in 30-40% demyelination of the treated nerve without axon loss. This was associated with an increased behavioural reflex sensitivity, in both thermal and mechanical tests, which was reversed by the NMDA receptor antagonist, [R]-CPP. At peak behavioural change, spontaneous action potential discharge of 2-3 impulses per second was present.

Conclusions

This study describes the first mouse model of peripheral nerve focal demyelination designed for the study of neuropathic pain. Changes in the peripheral nerve result in altered nerve excitability, which may lead to the changes observed in sensory processing in the spinal cord. This model may be useful for the evaluation of novel therapeutic targets for the treatment of demyelination associated pain.

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