Neuro-glia and the delayed onset of pain-like hypersensitivity following infant nerve injury

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Declaration

The work in this thesis was conducted in the Department of Neuroscience, Physiology and Pharmacology at University College London.

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

Rebecca McKelvey

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Abstract

Peripheral nerve injuries in adults can trigger neuropathic pain which coincides with alterations in dorsal horn neuronal activity and glial cells residing in the dorsal horn, and infiltrating T-lymphocytes. These cells synthesise and release pro-inflammatory mediators that can directly and indirectly sensitize dorsal horn neurones and contribute to neuropathic pain. Neuropathic pain is rare in infants and studies presented in this thesis show that a spared nerve injury (SNI) at postnatal day (P) 10 does not result in pain-like behaviour but triggers an anti-inflammatory immune response in the dorsal horn, not observed in nerve injured adults. However, if infant SNI treated animals are intrathecally administered with the pro-inflammatory mediator tumor necrosis factor (TNF) or lipopolysaccharide-activated microglia they develop pain-like behaviour while blockade of anti-inflammatory activity after infant SNI 'unmasks' neuropathic pain-like behaviour. Thus, nerve injury induced pain-like hypersensitivity in infants is actively suppressed by dominant anti-inflammatory neuro-immune activity. The anti-inflammatory response can also be evoked by direct C-fibre nerve stimulation in the infant, but not in adult rodents.

However, mechanical hypersensitivity does eventually develop following early life nerve injury in the rat at adolescence (Vega-Avelaira et al., 2012). Longitudinal studies presented in this thesis indicate that hypersensitivity emerges in response to not only mechanical stimulation but also following innocuous and noxious cold stimulation of the hind paw, and contralateral weight bearing. The emergence of behavioural hypersensitivity at adolescence coincides with an increase in spontaneous and evoked- activity of wide dynamic dorsal horn neurons, that is absent in sham controls. In addition, the immune response in the dorsal horn switches from an anti-inflammatory response to pro-inflammatory, characterised by an increase in the expression of TNF and Brain-derived neurotrophic factor. This explains why neuropathic pain is rare in infants, but complex regional pain syndromes can emerge, for no observable reason at adolescence.

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Abbreviations

ACC	anterior cingulate cortex
APC	antigen presenting cells
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ATP	adenosine triphosphate
BDNF	Brain-derived neurotrophic factor
BBB	blood-brain barrier
BSA	bovine serum albumin
BSpCB	blood-spinal cord barrier
CCI	chronic constriction injury
CCL2	chemokine (C-C motif) ligand 2
CD	cluster of differentiation
CGRP	calcitonin gene related peptide
CNS	central nervous system
CRPS	complex regional pain syndromes
CVLM	caudal ventrolateral medulla
DAMP	damage associated molecular patterns
DAPI	4'6- diamidino-2-phenylindole
DRG	dorsal root ganglia
EMG	electromyography
EPSC	excitatory postsynaptic currents
ERK1	extracellular signal-regulated kinase 1
FITC	fluorescein isothiocyanate
FJC	fluoro-Jade C
GABA	gamma-aminobutyric acid
GAP-43	growth associated protein 43
GAPDH	glyeraldehyde 3-phosphate dehydrogenase
GFAP	glial fibrillary acidic protein
GLT-1	glutamate transporter
IB4	isolectin B4
IBA-1	marker ionized calcium-binding adapter molecule 1
IC	insular cortex
ICAM-1	intercellular adhesion molecule 1
IFN-γ	interferon gamma
IFNR	interferon receptor
iNOS	inducible nitric oxide synthase
JNK	c-Jun N-terminal kinases
KCC2	potassium chloride co-transporter
Lpb	lateral parabrachial
LPS	lipopolysaccharide
LTP	long term potentiation
MAPK	mitogen activated kinase

MHC	major histocompatibility complex
NKCC1	sodium potassium ion exporter 1 channel
NMDA	N-methyl-D-aspartate
NMDAR	N-methyl-D-aspartate receptor receptors
MMP-9	matrix metallopeptidase 9
mRNA	messenger RNA
Na+	sodium ion
NeuN	neuronal marker
NF-κβ	nuclear factor kappa β
NGF	nerve growth factor
NGS	normal goat serum
NKCC1	Na^+K^+ exporter 1 channel
NO	nitric oxide
NST	nucleus of the solitary tract (NTS)
P2X4R	P2X4 receptors
PAG	periaqueductal grey
PAMPS	pathogen associated molecular patterns
PB	Phosphate buffer
PBS	phosphate buffer saline
PGE2	prostaglandin E2
PKC-γ	protein kinase C gamma
PNS	peripheral nervous system
PSL	partial sciatic ligation
qPCR	real time quantitative polymerase chain reaction
RNA	ribonucleic acid
RVM	rostro-ventral medulla
sIPSC	spontaneous inhibitory postsynaptic currents
SNI	spared nerve injury
SNL	spinal nerve ligation
SOCS3	suppressor of cytokine signalling 3
SS	somatosensory
STAT	signal Transducer and Activator of Transcription
TGF-β	transforming growth factor beta
TLR	toll-like receptors
TNF	tumour necrosis factor
Trk	tyrosine kinase receptor
TRP	transient receptor potential
WDR	wide dynamic range

Chapter One

General Introduction

1.1 Introduction

The perception of pain is essential for survival and to avoid harm. It is defined by the International Association for the Study of Pain (IASP) as ‘an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage’. This suggests that pain is an experience with both sensory and affective components to the physiological sensation of noxious, or potentially noxious stimuli. The term nociception is defined by IASP as ‘the neural process of encoding noxious stimuli’. Therefore nociceptive pain occurs in the presence of an acute noxious stimulus and serves a protective role preventing against injury by generating both a reflex withdrawal from the stimulus and an unpleasant sensation resulting in avoidance of the noxious stimuli, and is typically described in animal studies.

In mammals the nociceptive system enables noxious or high threshold stimuli to activate high threshold primary sensory neurons, termed nociceptors (Fig 1.1). Nociceptors transmit information about the noxious stimulus from the periphery to the dorsal horn spinal cord, the first point of integration in the central nervous system (CNS). Within the dorsal horn sensory information can be modulated by excitatory and inhibitory interneurons and descending pathways from the brain before it is transmitted onto higher brain centres where pain is perceived. Therefore, the modulation of sensory input in the dorsal horn spinal cord plays a key role in pain perception. Importantly nociceptive pain is typically short in duration and stops when noxious stimulus is removed such as upon tissue repair (Basbaum et al., 2009; Woolf and Ma, 2007).

The nociceptive system is also highly plastic and when noxious input is repeated or is particularly intense the nociceptive system can become sensitized so that the threshold for its activation is reduced and responses are amplified leading to behavioural hypersensitivity (Ji et al., 2003; Woolf and Salter, 2000; Woolf and Walters, 1991). In normal nociceptive processing these processes return to normal once the tissue has healed which is the case of inflammatory pain caused by inflammation associated with peripheral tissue

injury, which is not permanent and is the expression of 'use-dependent' synaptic plasticity triggered in the CNS by nociceptive input (Latremoliere and Woolf, 2009).

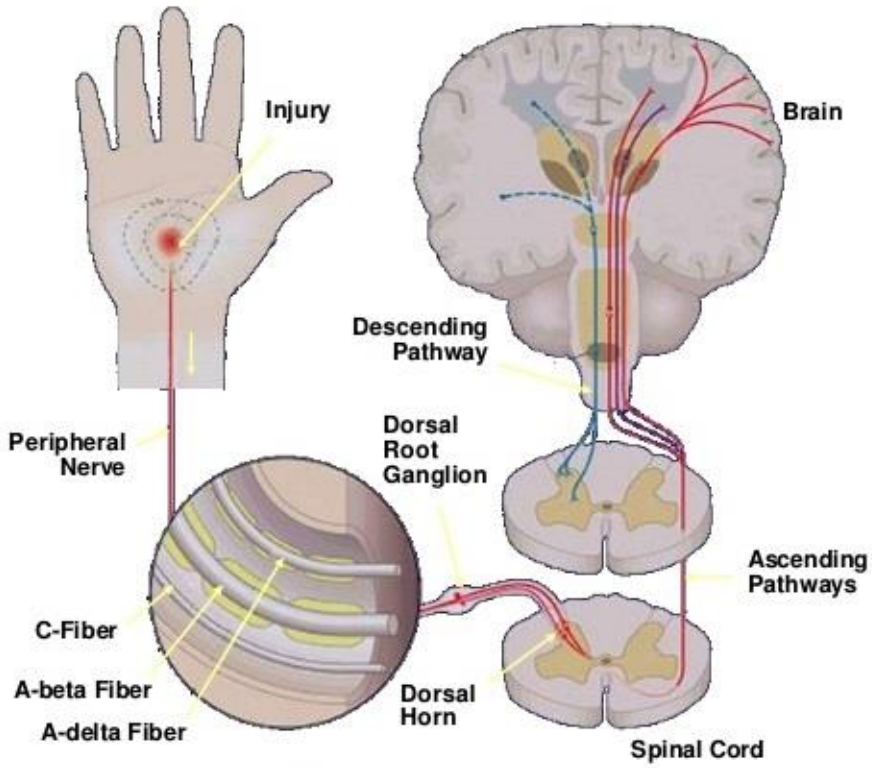


Fig 1.1 The nociceptive system

Nociceptors have specialised transduction molecules that generate inward currents in the nociceptor's peripheral terminal in response to a noxious stimulus. If the current exceeds the threshold value an action potential is generated enabling the transmission of information from the periphery, centrally into the dorsal horn spinal cord. Sensory information maybe modulated in the dorsal horn by interneurons or by descending pathways before being relayed to higher centres of the brain. Adapted with permission from WebMD Scientific American® Medicine. 1/1/2015

Sensitization also underlies pathological pain states which can persist, even once the injury has healed, and therefore fails to serve a protective or useful biological role. Although inflammatory pain can develop into pathological pain, one key example is neuropathic pain which is pain caused by direct lesion of the nervous system such as following traumatic injury or surgical intervention (Clifford J Woolf and Mannion, 1999). Therefore, a painful insult leads to inflammation and an acute phase of 'nociceptive pain' followed by a recovery period, healing, and the return of function. In nerve injured patients' pain can persist resulting in a state of chronic neuropathic pain characterised by

debilitating symptoms that can severely affect a patient's quality of life (Woolf and Mannion, 1999).

Neuropathic symptoms include both positive symptoms (hypersensitivity) which are often combined with hyposensitivity due to denervation. Patients may experience spontaneous pain (pain in the absence of noxious stimuli), allodynia (the reduction in the threshold required to elicit pain so that innocuous stimuli can produce pain) and hyperalgesia (enhanced amplitude and duration in response to noxious stimuli) (Baron et al., 2010; Scholz and Woolf, 2002; Von Hehn et al., 2012).

Several animal models that mimic peripheral nerve injury have been developed to elucidate mechanisms underlying neuropathic pain (Bennett and Xie, 1988; Decosterd and Woolf, 2000; Ho Kim and Mo Chung, 1992; Seltzer et al., 1990). Much of the research suggests that the development and maintenance of chronic pain is an expression of neural plasticity in both the peripheral nervous system (PNS) i.e. peripheral sensitization (hyperexcitability and sensitization of primary sensory afferents) and central nervous system (CNS) i.e. central sensitization (enhancement of excitatory synaptic transmission in the dorsal horn spinal cord) (Basbaum et al., 2009; Costigan et al., 2009; Gold and Gebhart, 2010; Ji et al., 2003; Latremoliere and Woolf., 2009; Von Hehn et al., 2012; Woolf and Salter., 2000; Porreca et al., 2002; Ruscheweyh et al., 2011). Most current pain management strategies that focus on dampening peripheral and central abnormal neuronal activity produce undesirable side-effects and lack efficacy (Baron et al., 2010; Varrassi et al., 2010; Kingery., 1997; Nikolajsen et al., 1997).

The last decade has brought compelling evidence that neuropathic pain is not confined to just neuronal alterations but involves the activation of immune and immune like glial cells. For example, in the dorsal horn spinal cord, resident glial cells including microglia and astrocytes transform into a 'pain related enhanced state' and T-cell lymphocytes infiltrate into the dorsal horn. These cells release inflammatory cytokines, chemokines and growth factors that alter neuronal activity and contribute to neuropathic pain (Austin et al., 2012; Clark and Malcangio, 2012; McMahon and Malcangio, 2009; Taves et al., 2013; Tsuda et al., 2013; Xu et al., 2006).

In the infant, both animals and humans display clear acute pain responses to noxious stimuli and also develop pain hypersensitivity upon inflammation of the skin, joints and viscera (Andrews and Fitzgerald, 1994; Barr, 1998; Cornelissen et al., 2013; Fitzgerald, 1991; Fitzgerald et al., 1989; Hedo et al., 1999; Lidow et al., 2001; Marsh et al., 1999; Ruda et al., 2000; Slater et al., 2007; Thompson et al., 1995; Walker et al., 2003). However, infant's exhibit lower thresholds and reflexes to noxious stimulation are un-coordinated and involve whole body movements (Andrews et al., 2002; Jennings and Fitzgerald, 1998) and studies into the postnatal development of the nociceptive pathway suggest that there are profound alterations in the nociceptive system in the first few weeks of life that underlie these baseline thresholds (Andrews et al., 2002; Andrews and Fitzgerald., 1999; Fitzgerald and Swett., 1983; Beggs et al., 2002).

Despite infants displaying robust nociceptive responses neuropathic pain is rare in infants before the age of 5-6 years of age (Anand and Birch, 2002; Howard et al., 2014; Marsh et al., 1999; Sethna et al., 2007; Walco et al., 2010; Walker et al., 2005) and this has been confirmed in nerve injury infant rat and mice models (Costigan et al., 2009; Howard et al., 2005; Moss et al., 2007; Vega-Avelaira et al., 2009). So the question remains as to why there is specifically no neuropathic pain immediately following peripheral nerve injury in infants.

Recent longitudinal studies in rats have revealed a previously unsuspected aspect of infant neuropathic pain, that animals that undergo nerve injury in early life do eventually develop pain hypersensitivity, but only when they reach adolescence (Vega-Avelaira et al., 2012). This suggests that early life nerve injury can affect pain processing later in life. Studies aimed to identify the mechanism underlying these age dependent differences indicate that the inflammatory immune response, which is robust in the adult dorsal horn spinal cord following a nerve injury, maybe absent in the dorsal horn spinal cord of infants (Barr and Hunter., 2014; Costigan et al., 2009; Moss et al., 2007; Vega-Avelaira et al., 2007).

To date both the neuronal and inflammatory profile in the dorsal horn both in the early period after infant nerve injury (in the absence of pain like-behaviour)

and in the later period following infant nerve injury (at the onset of pain-like behaviour), remain to be elucidated and is the subject of this thesis. This introduction gives a brief background to pain including the sensory circuitry required to transmit and process nociceptive inputs from the periphery to the central nervous system and its postnatal development. As this thesis is concerned with the processing of neuropathic pain in the infant nervous system, the mechanisms underlying neuropathic pain and the evidence for long-term changes in pain processing following early life injury is also examined.

1.2 The nociceptive system

Research into elucidating key cells and molecules that underlie normal acute pain is important to understanding the mechanisms underlying pathological pain hypersensitivity. Therefore this section will outline the processing of acute pain, with a particular focus on signalling in the dorsal horn.

1.2.1 Nociceptors

Nociceptors are a group of sensory fibres that are distinguished by their relatively high thresholds, being activated by noxious tissue threatening stimuli (Campbell et al., 1988; Sherrington, 1906) and convey information regarding the intensity, duration and location of the stimulus (Handwerker et al., 1987; Peschanski et al., 1981).

These afferents have cell bodies located either in dorsal root ganglia (DRG) or the trigeminal ganglia and transduce information via specialised transducer receptor/ion channel complexes from free endings located in peripheral skin and subcutaneous tissue to the dorsal horn spinal cord (Lawson and Waddell, 1991; Mesulam and Brushart, 1979).

Sensory neurons are known as primary afferent fibres and are functionally, anatomically and neurochemically diverse and can be classified according to their conduction velocity, axon diameter and myelination status as well as responses to different stimuli (Hjerling-Leffler et al., 2007; Lawson, 2002) and can be classified into three different subtypes (Aβ, Aδ and C-fibres) accordingly (Boyd and Kalu, 1979; Hunt and Rossi, 1985).

1.2.3 A-fibres

Myelinated Aδ- and Aβ-fibres were first identified in cats as a group of sensory neurons responding to noxious mechanical stimulation (Burgess et al., 1968; Burgess and Perl, 1967), although the ability of Aβ-fibres to conduct noxious information is frequently overlooked (Djouhri and Lawson, 2004). A-fibres conduct more rapidly than C-fibres and mediate acute well localized 'first' or fast pain while the slower C-fibres are responsible for the dull poorly localized 'second' or slow pain following noxious stimulation (Handwerker et al., 1987; Julius and Basbaum, 2001; Woolf and Ma, 2007), although a smaller proportion of nociceptors are A-fibres.

Aδ-fibres are small and thinly myelinated fibres (2-5μm) and conduct information about noxious inputs such as pin prick and non-noxious input from hair follicles at 12-30ms^{-1} (Djouhri and Lawson, 2004; Lawson and Waddell, 1991; Menétrey et al., 1977).

Aβ-fibres are larger than Aδ-fibres (5-14μm) and carry information about light touch, brush, vibration and other innocuous stimuli conducting at approximately 14-30ms-1 (Djouhri and Lawson, 2004; Harper and Lawson, 1985).

Aδ-fibres can be subdivided into two subtypes I and II. Type I Aδ-fibres are high threshold rapidly conducting mechanoreceptors that are also responsive to high intensity heat (>53^{O}C), cold and chemical stimuli (Handwerker and Kobal, 1993; Simone and Kajander, 1997; Treede et al., 1995). If the heat stimulus is maintained, these afferents will also respond at lower temperatures (Treede et al., 1995). These fibres sensitize following injury, and underlie pinprick pain (Magerl et al., 2001). Type II have lower heat thresholds and are slower conducting (Lawson et al., 1997; Lawson, 2002; Treede et al., 1998, 1995).

1.2.4 C-fibres

C-fibres are thin and unmyelinated fibres (0.4-1.2μm), with a conduction velocity of around 2m/s^{-1} (Woolf and Fitzgerald, 1983) and are sub-classified based on their receptive properties. The majority of C-fibres are polymodal, responding to a variety of noxious stimuli including mechanical, thermal and

chemical stimuli but can also be mechano-insensitive and play a crucial role in mediating itch and pain (Bessou and Perl, 1969; Djouhri and Lawson, 2004; Han et al., 2013; Perl, 2007; Schmelz et al., 1997; Torebjörk, 1974; Torebjörk and Hallin, 1974). A small subset of C-fibres respond to cooling and another to low threshold stimulation (brush and light touch) and play a critical role in mechanical hypersensitivity caused by injury (Olausson et al., 2007; Seal et al., 2009).

C-fibres can also be classified neurochemically (Snider and McMahon, 1998) into a subpopulation that are peptidergic and release substance P and calcitonin gene related peptide (Hunt and Rossi, 1985; Nagy and Hunt, 1982) and express the neurotrophic tyrosine kinase receptor type 1 (TrkA) the receptor for nerve growth factor, and a subpopulation that are termed non-peptidergic (Kaplan et al., 1991; Snider and McMahon, 1998). A large percentage of non-peptidergic fibres bind isolectin B4 (IB4) and project to distinct laminae in the dorsal horn spinal cord in comparison to peptidergic neurons (see below) (Plenderleith and Snow., 1993; Dong et al., 2001).

Nociceptive afferents, in addition to the aforementioned neurotransmitters also synthesize and release glutamate (a major nociceptor neurotransmitter), , adenosine triphosphate (ATP), nitric oxide (NO) and others, at their terminals in the dorsal horn and are thus also involved in central transmission and modulation of nociceptive information (Millan, 1999).

1.2.5 Tansduction and transmission

Nociceptors express an array of transducer receptor/ion channel complexes including ligand-gated ion channels and G protein coupled receptors on the surface of the peripheral terminal which when activated generate depolarizing currents and, if large enough, the initiation of action potentials that enables the encoding of high intensity noxious stimuli to the dorsal horn spinal cord. This distinguishes nociceptors from sensory neurons which transmit innocuous information and express low threshold transducers.

Nociceptor transduction molecules include the transient receptor potential (TRP) family of cation channels (Ramsey et al., 2006). For example TRP vanilloid 1 (V1) is the molecular target of capsaicin but also responds to heat

and protons (Caterina et al., 1999, 1997). TRPV1 is activated by heat and TRPV3 and TRPV4 are activated by chemical, osmotic and heat stimuli and have a threshold of 32-39^{0}C and 27-34^{0}C respectively and participate in the perception of warmth (Güler et al., 2002; Lee and Caterina, 2005).

Cold sensation in the skin is mediated by the expression of ion channel TRP melastatin 8 (M8) which is activated by cooling the skin to below 30^{0}C and also responds to other cooling chemicals (Dhaka et al., 2007; McKemy et al., 2002; Peier et al., 2002). Acid sensing ion channels (ASICs) are activated by protons which are increased during tissue inflammation and therefore contribute to inflammatory pain (Deval et al., 2010). The molecular mechanisms that underlie mechanosensory transduction has been difficult to elucidate (Gillespie and Walker, 2001). Although ASICs were candidate transducer molecules, this was disproven (Drew et al., 2004). However, a recent study using a conopeptide analogue termed noxious mechanosensation blocker-1 (NMB-1) lead to elucidation for a role of Epac1, a cyclic AMP sensor that potentiates mechanotransducer Piezo2 and contributes to mechanical allodynia (Eijkelkamp et al., 2013).

The generation of action potentials in nociceptors requires the activation of voltage gated ion channels including sodium channels (VGSC) and potassium channels. At the central terminal voltage gated calcium channels enable the release of neurotransmitters into the dorsal horn spinal cord and thus convey nociceptor signals to postsynaptic dorsal horn neurons. As VGSC contribute to generator potentials in the periphery and action potentials in the axon and release of central neurotransmitters, they are a prime target for investigation and therapeutic approaches for pain including Nav1.7 and 1.8 (Akopian et al., 1999; Benarroch, 2007; Benn et al., 2001; Dib-Hajj et al., 2008; Estacion et al., 2008; Minett et al., 2012; Nassar et al., 2004; Raouf et al., 2010; Wood et al., 2004).

1.2.6 Projection of primary afferents into the dorsal horn spinal cord

Primary afferents project to the dorsal horn through dorsal roots and synapse onto dorsal horn spinal cord neurons, which is the first site of integration of sensory information in the central nervous system. During acute pain, the

release of glutamate into central terminals in the dorsal horn from nociceptors primarily activates the ligand gated channels of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate subtypes of ionotropic glutamate receptors that generate excitatory postsynaptic currents (EPSCs). Summation of EPCSs on the postsynaptic neuron can result in action potential firing and transmission to higher order neurons.

The dorsal horn is organised into anatomically and electrophysiologically distinct laminae (Basbaum and Jessell, 2000). Information arriving at the dorsal horn maybe processed by both excitatory and inhibitory interneurons, and may also be modulated from descending control from the brain stem (Sivilotti and Woolf, 1994). The majority of these neurons within the spinal dorsal horn correspond to propriospinal and local circuit interneurons, but approximately 10% have projecting axons that terminate in the brainstem and thalamus (Todd, 2010).

The grey matter is divided into ten laminae (Molander et al., 1989, 1984; Rexed, 1954, 1952) which can be subdivided into superficial laminae I and II, and deep laminae III-X and together is known as the dorsal horn (Fig 1.2). Primary afferents project into the dorsal horn in a highly organised and ordered somatotopic fashion based on both the sensory modality they transmit and body region that they innervate (Molander and Grant, 1985).

C-fibres terminate predominantly in lamina II outer (IIo). Peptidergic fibres, (expressing CGRP and innervating deeper parts of the skin in addition to joints and viscera) project to both lamina I and II while non-peptidergic fibres (expressing IB4 and innervate the epidermis) project centrally to lamina II (Lawson et al., 1997; Todd, 2010, 2002). Aδ nociceptors project to lamina I, some to IIo and branch to V and X. Aδ hair follicle afferents terminate between lamina II and III. Aβ tactile and hair afferents innervate lamina III-IV and some to lamina V-IV (Light and Perl, 1979) although one study identified Aβ terminating within laminae I-V (Woodbury et al., 2008).

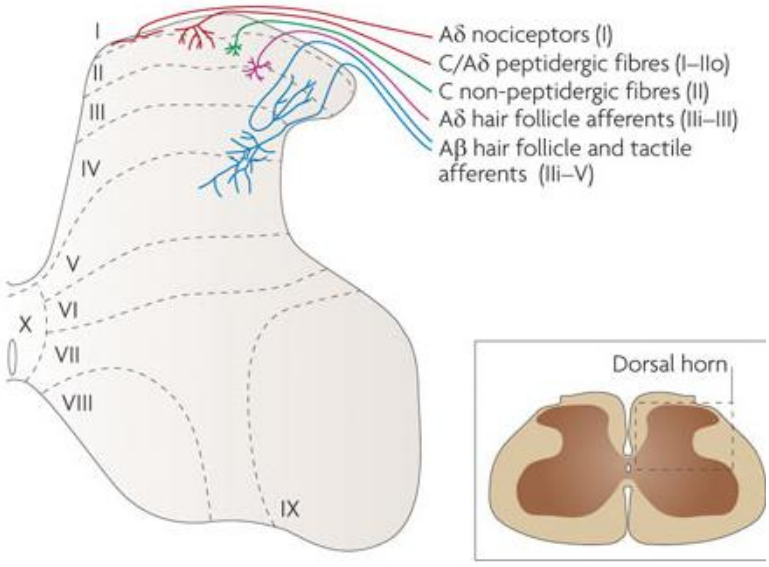


Fig 1.2 Primary afferent innervation of the dorsal horn spinal cord.

Primary afferents terminate in a highly ordered manner. Aβ tactile and hair afferents arborize in lamina III-V and some to lamina II inner. Aδ nociceptors terminate mainly in lamina I although some branch to lamina V and lamina X. Aδ hair follicle afferents arborize between lamina II and III. C-fibre peptidergic afferents arborize in LI and LII outer and most non-peptidergic C-fibres arborize centrally in LII. Adapted by permission from Macmillan Publishers Ltd: Nature reviews neuroscience (Todd., 2010), copyright (2010)

The ventral horn contains motor neurons that are responsible for the initiation and coordination of movement including withdrawal reflexes associated with noxious stimulation.

1.2.7 Laminae organisation of the dorsal horn spinal cord

The somatotopic distribution of primary afferents into the dorsal horn enables areas on the skin to be represented via their central termination patterns in the spinal cord (Swett and Woolf, 1985). A study by Molander and Grant, 1986 utilised horseradish peroxidase tracing in the hind limb nerves of rats. They found that although the central projections of primary afferents overlap, they are well delineated in three dimensional areas in a symmetrical longitudinal pattern in the dorsal horn. Further studies showed that these nerve terminals are organised in a medio-lateral direction with nerve terminals from afferents innervating hind paw skin territories terminating more laterally then those innervating the plantar hind paw, which terminate in the medial dorsal horn and those that innervate the lateral hind paw terminating caudally to those

innervating the medial hind paw (Molander and Grant, 1986; Woolf and Fitzgerald, 1986). As a result, dorsal receptive fields (an area of the body that when stimulated, evokes action potentials in a given neuron) are organised somatotopically (Jr and Coggeshall, 2004).

In naïve animals, stimulation of the skin outside the receptive fields (or 'low probability firing fringe') can influence the interneurons within the dorsal horn (via polysynaptic inputs) that may be either excitatory or inhibitory but are not sufficient to evoke an action potentials (Brown and Fyffe, 1981; Woolf and Fitzgerald, 1986; Woolf and King, 1990). Following injury, such as with the chemical irritant mustard oil, the surrounding area receptive fields expand so that the low probability firing fringes induces action potentials (Cook et al., 1987; Devor et al., 1979; Devor and Wall, 1981; Suzuki et al., 2000; Wilson and Kitchener, 1996; Woolf and King, 1990). This demonstrates that the receptive field size, while intimately determined by the primary afferent input, is normally under inhibitory control and can expand rapidly.

1.2.8 Dorsal horn neurons

Dorsal horn neurons are responsible for encoding information from primary afferents and passing information to reflex circuits or supraspinal sites. Although the modality to which a dorsal horn neurone will respond is determined to some extent by the peripheral input to that cell (for example a large number of neurons in lamina I respond to noxious input) electrophysiological studies in the dorsal horn spinal cord have enabled identification and classification of dorsal horn neurons based on their responses to mechanical peripheral stimulation. The major types have been described (Menétrey et al., 1977).

1. Low threshold neurons, responding solely to innocuous brush, touch and pressure. These are mostly located in deeper laminae II, III and a few in lamina I (Millan, 1999).
2. Wide dynamic range (WDR) neurons respond to both innocuous and noxious stimuli and respond to input from all primary afferents and are therefore activated in response to a range of modalities including mechanical, heat and chemical stimuli (Coghill et al., 1993; Handwerker

et al., 1975; Maixner et al., 1986; Menétrey et al., 1977). WDR neurons are the most prevalent cell type, predominantly found in deep laminae IV to VI but are also encountered in laminae I and II (Coghill et al., 1993). WDR neurons located in deeper laminae are implicated in C-fibre sensitization and amplification and therefore pain. Other properties of WDR neurons include their ability to fire action potentials in a graded fashion and, unlike nociceptive specific neurons (see below) maintain high rates of impulse discharge throughout repetitive nociceptive stimulation and are also sufficient to encode intensity in addition to spatial and qualitative aspects of pain (Coghill et al., 1993; Maixner et al., 1986). WDR also exhibit wind-up (Cata et al., 2006; Nackley et al., 2004).

3. Nociceptive specific neurons respond to noxious stimulation only. They are predominantly located in laminae I and IIo but are also located in laminae V and VI. The ability of these neurons to encode stimulus intensity is limited (Coghill et al., 1993).
4. Neurons that are able to respond to joint movement or pressure.

The majority of the neurons within the dorsal horn are locally projecting interneurons which pay a key role in modulating the output in response to primary afferent input.

Interneurons can be subdivided into excitatory and inhibitory types depending on the expression of neurotransmitters with glutamate being the main excitatory transmitter. Gamma-aminobutyric acid (GABA) and glycine are the main inhibitory transmitters that activate ligand gated ion channels $GABA_A$ receptor and glycine receptors respectively. Although both neurotransmitters are released by descending inhibitory pathways from the brainstem, they are released predominantly by local interneurons (Kato et al., 2006; Millan, 1999).

The second major type of dorsal horn neuron are the projection neurons that relay information to supraspinal sites, where pain and other somatic sensations are perceived. Dorsal horn projection neurons are located largely in lamina I but also in lamina III-V and receive monosynaptic inputs directly from primary afferents, or polysynaptic inputs from interneurons (Coggeshall

and Carlton, 1997; Dubner and Bennett, 1983; Jankowska and Lindström, 1972; Spike et al., 2003).

1.2.9 Ascending pathways

In general, projection neurons in lamina I receive monosynaptic input from Aδ and C-fibres and polysynaptic information from Aβ fibres via excitatory interneurons from deeper laminae (Coggeshall and Carlton, 1997; Dubner and Bennett, 1983, 1983; Jankowska and Lindström, 1972; Todd et al., 1994). Projection neurons are the origin of multiple of ascending pathways including the spinothalamic, spinoreticular, spinoparabrachial and spinomesencephalic tracts which terminate in a variety of forebrain structures.

Retrograde and anterograde tracing have shown that lamina I projection neurons have two main supraspinal targets. These are that 1) the lateral parabrachial area (LPb) that projects to areas of the brain responsible for the affective components of the pain experience including the amygdala and hypothalamus (Gauriau and Bernard, 2002) and 2) the lateral thalamus that then projects to the insular (IC) and somatosensory (SS) cortices involved in the sensory-discriminative aspects of pain (Gauriau and Bernard, 2004).

However, lamina I projection neurons also project to the caudal ventrolateral medulla (CVLM), the nucleus of the solitary tract (NTS) and the periaqueductal grey matter (PAG) and nuclei in the thalamus (Bourgeais et al., 2003; Todd, 2010).

Lamina I projection neurons respond to noxious stimuli, and a few to innocuous cooling and have dendrites that generally remain within the lamina (Bester et al., 2000; Han et al., 1998; Willis et al., 1974; Zhang et al., 2005). The projections from deeper laminae (although fewer in number) terminate in the medial thalamus and projects to other areas including the anterior cingulate cortex (ACC) that is implicated in the attentional aspect of pain and the reticular nuclei that may be involved in the motor responses (Gauriau and Bernard, 2002). Human imaging experiments indicate that the most common area activated during noxious stimulation are the primary and secondary somatosensory, insular, anterior cingulate and prefrontal cortices and thalamus (Apkarian et al., 2005). However, there is no one brain area activated

solely by noxious stimulation as the areas aforementioned may also be activated during non-noxious stimulation (Iannetti and Mouraux, 2010; Tracey and Mantyh, 2007).

1.2.10 Descending pathways

Sensory information arriving in the dorsal horn from primary afferents can also be modulated by descending control in the dorsal horn spinal cord, an example of top-down modulation by the brain. Although not the subject of this thesis, it is important to understand how these systems are organised and impact on dorsal horn processing.

The PAG and rostro-ventral medulla (RVM) are important structures that mediate descending modulation by exerting both inhibitory and facilitatory effects on spinal excitability and pain sensitivity in the adult (Eippert et al., 2009; Tracey and Mantyh, 2007). The PAG receives input from dorsal horn projection neurons as well as from the limbic forebrain, amygdala and the diencehphalon (Fields et al., 1991). Stimulation of the PAG can reduce dorsal horn responses, inhibit reflex responses to noxious stimuli and produce analgesia, all of which can be eliminated by spinal cord transection (Basbaum et al., 1977; Fields et al., 1977; Mayer et al., 1971). This descending inhibitory system is activated in situations of stress and fear when noxious input does not produce pain (Terman et al., 1984).

Although the PAG exerts inhibitory effects on nociception via a descending pathway to the spinal cord, it is mostly relayed via the RVM, the main output nucleus to the dorsal horn (Ossipov et al., 2010; Pomeroy and Behbehani, 1979). Unlike the PAG, the RVM exerts inhibitory and excitatory effects on nociception in the dorsal horn (Fields and Heinricher, 1985). There are three cell types in the RVM identified by firing in response to noxious stimulation preceding the tail-flick reflex i) cells that fire immediately before the tail flick test are termed ON cells, ii) cells that decrease in firing immediately before the tail-flick are termed OFF cells and iii) those that remain unaltered are termed neutral cells (Fields et al., 1991; Fields and Heinricher, 1985). These studies suggest that the population of ON cells are associated with facilitating the reflex responses while the OFF cells do the converse. Furthermore, low

intensity stimulation of the RVM facilitates spinal nociception and high intensity stimulation can have inhibitory effects (Hathway et al., 2009; Urban and Gebhart, 1999; Zhuo and Gebhart, 1997, 1992). Fields and colleagues identified that both ON and OFF cells project to laminae I, II and V of the spinal cord where nociceptive primary afferent fibres terminate (Fields et al., 1995). The role of neutral cells in the nociceptive reflex is unclear. The cell types cannot be separated anatomically within the RVM and all contain 5-hydroxytryptamine (5-HT) (Bardin, 2011; Marinelli et al., 2002) or GABA (as well as glycine) (Hossaini et al., 2012; Kalyuzhny and Wessendorf, 1998) and are not always functionally distinct.

1.3 Sensitization in pain state

When noxious stimulation is repeated, or in the presence of tissue injury, the nociceptive system can become sensitized so that the threshold for its activation is reduced and responses are amplified and behavioural hypersensitivity exhibited. This can occur in both the peripheral and central nervous system (Ji et al., 2003; Woolf and Salter, 2000; Woolf and Walters, 1991).

1.3.1 Peripheral sensitization

Peripheral sensitization typically develops as a consequence of tissue damage that causes a local inflammatory response associated with changes in the environment of the nerve fibre following tissue injury and inflammation. Such insults can induce the release of molecules such as neurotransmitters, cytokines, chemokines, growth factors, ion channel activators termed the ‘inflammatory soup’ (Basbaum et al., 2009; Chiu et al., 2012; Milligan et al., 2001; Woolf and Ma, 2007). These molecules activate intracellular signalling pathways in the nociceptor that can lead to phosphorylation of receptors and ion channels resulting in changes in threshold and kinetics that alter the sensitivity and excitability of the nociceptor. The nociceptor may now be sensitive to normally non-noxious stimuli as well as exhibiting enhanced responses to noxious stimuli.

One example is nerve growth factor (NGF) that is released upon tissue injury and is a key component of the 'inflammatory soup' (Woolf et al., 1994). NGF acts via its receptor TrkA on peptidergic C nociceptors (Snider and McMahon, 1998) and produces heat hypersensitivity. NGF-TrkA activates downstream signalling pathways via Src kinase that directly phosphorylates and potentiates TRPV1, a transducer of heat, on peripheral nociceptor terminals (Caterina et al., 1999). Activation of TRPV1 leads to depolarisation and a rapid change in heat sensitivity (Zhang et al., 2005). In addition retrograde transport of NGF to the nucleus of nociceptors promotes an increase in proteins including substance P, TRPV1, and Nav1.8 (Ji et al., 2002). These changes lead to enhanced excitability of the nociceptor and amplifies the inflammatory response.

ATP and bradykinin can also bind to their receptors on primary afferents and modulate TRPV1 via intracellular signalling pathways while other inflammatory agents function by directly binding to TRPV1 to modulate the channel and include extracellular protons and lipids. These interactions result in a decrease in the channel's thermal activation threshold and increase in the magnitude of the response providing a direct mechanism by which inflammatory meditators induce allodynia and hyperalgesia respectively (Basbaum et al., 2009).

Immune cells that are recruited by tissue injury release inflammatory mediates that can act directly on primary afferents to cause peripheral sensitization and subsequent release of neuropeptides that further activate immune cells, inducing a positive feedback loop that drives inflammation (Chiu et al., 2012). Two of the most common cytokines released by immune cells (such as macrophage and neutrophils) are tumour necrosis factor (TNF) and interleukin (IL)-1 beta (-1β) (Binshtok et al., 2008; Zhang et al., 2011a) which are sensed directly by nociceptors via receptors and lead to increases in membrane excitability by intracellular signalling pathways. For example, intraplantar administration of TNF induces thermal sensitivity that can be reversed by anti-NGF administration (Woolf et al., 1997). In addition, exposure of TNF and IL-1β to voltage gated sodium channels 1.7-1.8 generate and propagation action potentials and sensitize these channels (Linley et al., 2010).

Peripheral sensitization can therefore present as a reduction in threshold and an amplification in the responsiveness of primary afferents leading to pain in response to normally innocuous stimuli and enhanced pain responses to noxious stimulation respectively at the site of inflammation (primary hyperalgesia) (Bessou and Perl, 1969; Gold and Gebhart, 2010) and represents an important mechanism underlying nociception during acute tissue injury and inflammation.

However, peripheral sensitization cannot explain a number of aspects of clinical pain hypersensitivity including the spread of sensitivity outside the areas of tissue injury (secondary hypersensitivity), why repeated stimuli at a constant intensity leads to an increase in pain and why pain may outlast a peripheral stimulus (Pfau et al., 2011; Seal et al., 2009; Woolf and Salter, 2000). These aspects of pain hypersensitivity are instead explained by central sensitization.

1.3.2 Central sensitization

Central sensitization is a form of long-lasting activity dependent synaptic plasticity in the dorsal horn that is initiated by nociceptive fibres and leads to pre and post synaptic changes resulting in an increase in post synaptic membrane excitability and the facilitation of nociceptive processing (Woolf, 1983a). This can be driven by a nociceptive conditioning input such as sustained activation of C-fibres by heat, electrical or chemical activation that leads to homo- and hetero-synaptic potentiation. In this way a normally subthreshold input starts to activate dorsal horn neurons due to a spread of change in synaptic strength from activated to neighbouring non-activated synapses. This can alter the receptive field spatial, temporal and threshold properties (Ji and Suter, 2007a; Latremoliere and Woolf, 2009; Woolf and King, 1990) generating long lasting responses to inputs from low threshold primary afferents and from topographically different locations (Cook et al., 1987; Woolf, 1983a; Woolf and King, 1990; Woolf and Thompson, 1991; Woolf and Wall, 1986). Specifically, heterosynaptic potentiation may explain how a nociceptive high threshold input enables input from low threshold sensory fibres activated by light touch to activate normally high threshold nociceptive

neurons, thus reducing the pain threshold as a consequence of an increase in synaptic efficacy and increase in the excitability of dorsal horn neurons.

Multiple mechanisms have been implicated in central sensitisation including an alteration in glutamatergic/ N-methyl-D-aspartate receptor (NMDAR) mediated neurotransmission, disinhibition and glial-neuronal interactions (Basbaum et al., 2009).

1.3.3 Glutamatergic/NMDAR mediated neurotransmission

During nociceptive pain glutamate will bind to AMPA and kainate receptors while the NMDAR remains silent, blocked by a magnesium ion in its receptor pore. However, upon injury sustained release of glutamate and neuropeptides substance P and calcitonin gene related peptide (CGRP) leads to sufficient membrane depolarisation to force the magnesium ion to leave the pore enabling glutamate to bind to the receptor and the subsequent generation of an inward calcium current (Mayer et al., 1984).

NMDA is essential for central sensitization and administration of NMDAR antagonist MK-801 attenuates behavioural hypersensitivity following cutaneous application of mustard oil, sural nerve stimulation and nerve injury -induced behavioural hypersensitivity (Woolf and Thompson, 1991).

Postsynaptic cytosolic calcium activates intracellular signalling pathways and secondary messenger systems leading to the phosphorylation and alteration in NMDA and AMPA receptors conductance's and membrane trafficking. This results in an increase in the density of ion channels in the postsynaptic neuron and enhanced excitatory transmission in the dorsal horn and thus a functional change that manifests as central sensitization (Carvalho et al., 2000; Lau and Zukin, 2007).

Calcium is multifunctional, inducing the formation of NO and prostaglandin E2 (PGE2) that can increase dorsal horn excitability (Samad et al., 2001; J. Wu et al., 2001) along with activating signalling pathways that leads to downstream transcriptional changes contributing to increases in AMPA and NMDA currents and reductions in potassium currents (Hu et al., 2003; Ji et al., 2009, 1999).

1.3.4 Disinhibition

A reduction in the synthesis, release or activity of inhibitory transmitters leads to a state of disinhibition. Inhibitory interneurons in the dorsal horn are essential for modulating information as observed by spinal administration of GABA or Glycine receptor antagonists that produce behavioural hypersensitivity (Malan Jr et al., 2002; Sivilotti and Woolf, 1994). Evidence suggests that disinhibition decreases tonic inhibition to enhance depolarisation and excitation of dorsal horn projection neurons leading to enhanced neuronal output in response to noxious and innocuous stimulation (Keller et al., 2007; Torsney and MacDermott, 2006). For example, mice lacking protein kinase C gamma (PKCγ) interneurons, located in lamina II fail to develop nerve injury induced pain-like behaviour (Malmberg et al., 1997).

In naïve mice, blockade of glycinergic inhibition caused innocuous brushing of the hind paw to activate PKCγ interneurons indicating that disinhibition leads to hypersensitivity. Non-neuronal cells located in the CNS can also lead to disinhibition (see below) acting through a variety of mechanisms and contribute to the plasticity observed in pathological pain.

1.3.5 Glial-neuronal interactions

Microglia and astrocytes are both non-neuronal cells that reside in the CNS and have been implicated in altering the excitability of spinal cord circuits. The diversity of receptors on both microglia and astrocytes enable them to sense and react to their surroundings via activation of intracellular pathways, such as the mitogen activated kinase (MAPK) family, that leads to the release of mediators that modulate synaptic activity. The MAPK family includes 3 major members; extracellular signal-regulated kinase 1 and 2 (ERK1/2), p38 and c-Jun N-terminal kinase (JNK) and play a key role in intracellular signalling in glia (but also neurons) that lead to the synthesis of inflammatory mediators that modulate both excitatory and inhibitory synaptic transmission (Fig 1.3) and are required for the genesis of persistent pain (Gao et al., 2010a; Ji et al., 2009, 1999; Zhuang et al., 2005).

1.3.5.1 Microglia

Microglia are the resident immune cells of the CNS, constituting as much as 10-15% of cells in adulthood and are heterogenous with respect to both morphological and functional properties (McMahon and Malcangio, 2009; Olah et al., 2011). Microglia are derived from a restricted subpopulation of yolk sac erythromyeloid progenitors that express the transcription factors SPI1/Pu.1+ and Irf8+ (Alliot et al., 1999; Kierdorf et al., 2013).

Microglia interact with synapses and have been implicated in circuitry formation as well as phagocytosis of newborn neurons, synaptic terminals and also play a role in shaping neuronal circuitry in the early postnatal stages (Paolicelli et al., 2011; Schafer et al., 2012; Stephan et al., 2012; Tremblay et al., 2010). The surveillance role of microglia relies on several pattern recognition receptors expressed by microglia that also recognize pathogen associated molecular patterns (PAMPs) and damage associated molecular patterns (DAMPs) including soluble and insoluble factors released by damaged cells enabling them to sense neurotransmitters, neuropeptides and neuromodulators and thus neuronal activity.

Following an insult, microglia transition into a 'pain related enhanced state' characterised by alterations in morphology, expression of cell surface receptors and secretion of mediators including cytokines, chemokines, ATP, glutamate and NO that are all capable of enhancing excitatory transmission via pre-, post- and extrasynaptic mechanisms (Ji et al., 2013) promoting ongoing inflammation and central sensitization (Beggs and Salter, 2010; Chessell et al., 2005; Clark et al., 2007; Suter et al., 2007; Taves et al., 2013; Tsuda et al., 2009; Ulmann et al., 2008; Zhang et al., 2007). This has been examined most extensively in lamina II dorsal horn neurons where incubation of TNF, IL-1β and chemokine (C-C motif) ligand 2 (CCL2) in spinal cord slices rapidly increases spontaneous EPSC (sEPSC), as does exposure of cultured dorsal horn neurons with interferon gamma (IFN-γ) indicating a role in presynaptic modulation (Kawasaki et al., 2008; Vikman et al., 2005, 2003; Zhang et al., 2010).

These mediators also act to increase EPSC amplitude caused by enhanced signalling of AMPA glutamate receptors in post-synaptic sites (Ji et al., 2013).

Extrasynaptic TNF, IL-1β and CCL2 act to induce central sensitization by enhancing NMDAR currents in LII neurons (Gao et al., 2009; Kawasaki et al., 2008). Although the contribution of microglia is mostly demonstrated in neuropathic pain, a role has been implicated in other pain states (Cho et al., 2012; Clark et al., 2012; Raghavendra et al., 2004).

1.3.5.2 Astrocytes

Astrocytes are derived from the neuroectoderm and form physically coupled networks that are closely associated with neuronal synapses where they contribute to synaptic plasticity by altering the excitability of dorsal horn circuits, as described in both inflammatory and neuropathic pain (Gao et al., 2009; Gao and Ji, 2010). Astrocytes can facilitate intercellular transmission of calcium signalling and exchange of cytosolic contents. It has also been shown that increases in astrocytic calcium can modulate neural networks by uptake of potassium, thus determining the resting membrane potential of neurons and neuronal activity (Wang et al., 2012).

Stimulation of astrocytes in pain states induces JNK signalling pathways that lead to the production of mediators including CCL2 and IL-1β that modulates synaptic transmission and contributes to central sensation (Gao et al., 2009). Astrocytes contain the glutamate transporter (GLT-1) that buffers the glutamate released into synapses to prevent excessive activation of postsynaptic glutamate receptors. The release of glutamate at nerve terminals activates metabotropic glutamate receptors on astrocytes, leading to the increase of intracellular calcium and release of glutamate, D-serine and ATP that can increase neuronal sensitivity (Hamilton and Attwell, 2010). D-serine acts on synaptic NMDA receptors while glutamate binds to extrasynaptic NMDA receptors. If the glutamate transporter GLT-1 is down regulated, such as after nerve injury (Sung et al., 2003) this leads to an increase in glutamate and increase in dorsal horn excitability that contributes to persistent pain (Nie and Weng., 2009; Ren., 2010).

In astrocytes TNF induces phosphorylation of intracellular JNK and activation of nuclear factor kappa β (NF-κβ) leading to the release of CCL2 that binds to

its receptor on neurons and interacts positively with neuronal NMDA and AMPA receptors (Gao et al., 2010a; Gao and Ji, 2010; Wang et al., 2011).

1.3.5.3 Modulation of inhibitory synaptic transmission.

Central sensitization can also be the result of a decrease in inhibitory control (disinhibition) (Baba et al., 2003; Coull et al., 2005; Moore et al., 2002; Zeilhofer, 2008). Glial mediators including BDNF, cytokines, chemokines and PGE2 can all modulate inhibitory synaptic transmission. For example IL-1β and IL-6 act pre-synaptically to inhibit the frequency of spontaneous inhibitory postsynaptic currents (sIPSCs) in lamina II neurons with IL-1β also reducing the amplitude of sIPSCs.

One of the most established mechanisms by which microglia increase spinal excitability is via the release of BDNF. Under normal conditions, intracellular concentrations of chlorine ion (Cl^-)are maintained by the effect of the Cl^- co-transporter potassium ion (K^+)Cl^- exporter 2 channel (KCC2) and sodium ion (Na^+)K^+ exporter 1 channel (NKCC1) that together maintain a Cl^- concentration gradient so that opening of Cl^- channels causes Cl^- entry into a neuron and hyperpolarisation. BDNF can reduce the KCC2 expression so that activation of $GABA_A$ receptors by GABA results in diminished or reduced Cl^- entry and disinhibition. TNF can also suppress the activity of Glutamate decarboxylase (GAD) 67 positive neurons in spinal cord slices and CCL2 and IFN-γ inhibits GABA-induced responses in dorsal horn neurons (Gosselin et al., 2005; Zhang et al., 2010). Anti-inflammatory cytokines such as IL-10 and IL-4 can also regulate synaptic activity via suppression of pro-inflammatory cytokine production (Ji et al., 2013).

1.4 Neuropathic pain

Neuropathic pain results from pain caused by lesion or disease of the nervous system and affects up to 8% of the European population characterised by stimulus-independent pain (such as shooting, burning, lancinating or dysesthesias) and stimulus-evoked pain (such as mechanical, thermal or chemical hyperalgesia) which can occur alongside depression, mood and

sleep changes (Bourquin et al., 2006; Torrance et al., 2006). Neuropathic pain is often chronic, causing substantial disability and a loss of quality of life and is among the most challenging pain disorders to treat and is therefore associated as having a high economic impact on society (Attal et al., 2006; Bouhassira et al., 2008; Dworkin et al., 2003; Finnerup et al., 2005; Hunt and Koltzenburg, 2005; Koltzenburg, 1998; Toth et al., 2009). Estimates of the life-time prevalence of neuropathic pain are as high as 10%. There are many different classification schemes for peripheral neuropathic pains. Firstly, clinicians will classify painful neuropathies as asymmetrical (affecting individual nerves) and symmetrical types (affecting many nerves simultaneously for example as in a glove distribution). Patients are then sub-grouped into causes.

A third group with complex regional pain syndrome type I is also included in 'neuropathic pain' which is a heterogenous disease that presents without demonstrable nerve lesion (Hunt and Koltzenburg, 2005).This classification presents difficulties and no 'framework' for the clinical management of the pain (Woolf and Mannion, 1999) because within each group the pain experienced by patients maybe heterogeneous and thus this scheme does not identify the mechanism underlying the generation of pain.

It has been suggested that grouping patients based on their sensory symptoms, as opposed to aetiology would be more effective (Baron, 2006; Koltzenburg, 1998) and enable the identification of distinct sensory symptoms that can be related to peripheral or central mechanisms and may lead to better targeted therapeutics (von Hehn et al., 2012). The advantage of this approach is it will lead to a therapeutic focus on targeting the maladaptive plasticity (von Hehn et al., 2012). For example, a patient experiencing pin prick hypersensitivity in an area outside the receptive field of injury to the skin or mechanical allodynia suggests central sensitization as an underlying mechanisms. Pharmaceutical targets such as post-synaptic NMDA receptors maybe targeted such as ketamine or by mimicking and enhancing inhibition via the administration of tricyclic antidepressants and opioids. However, the use of these therapeutics are limited due to the side effects of these neuronal targets agents.

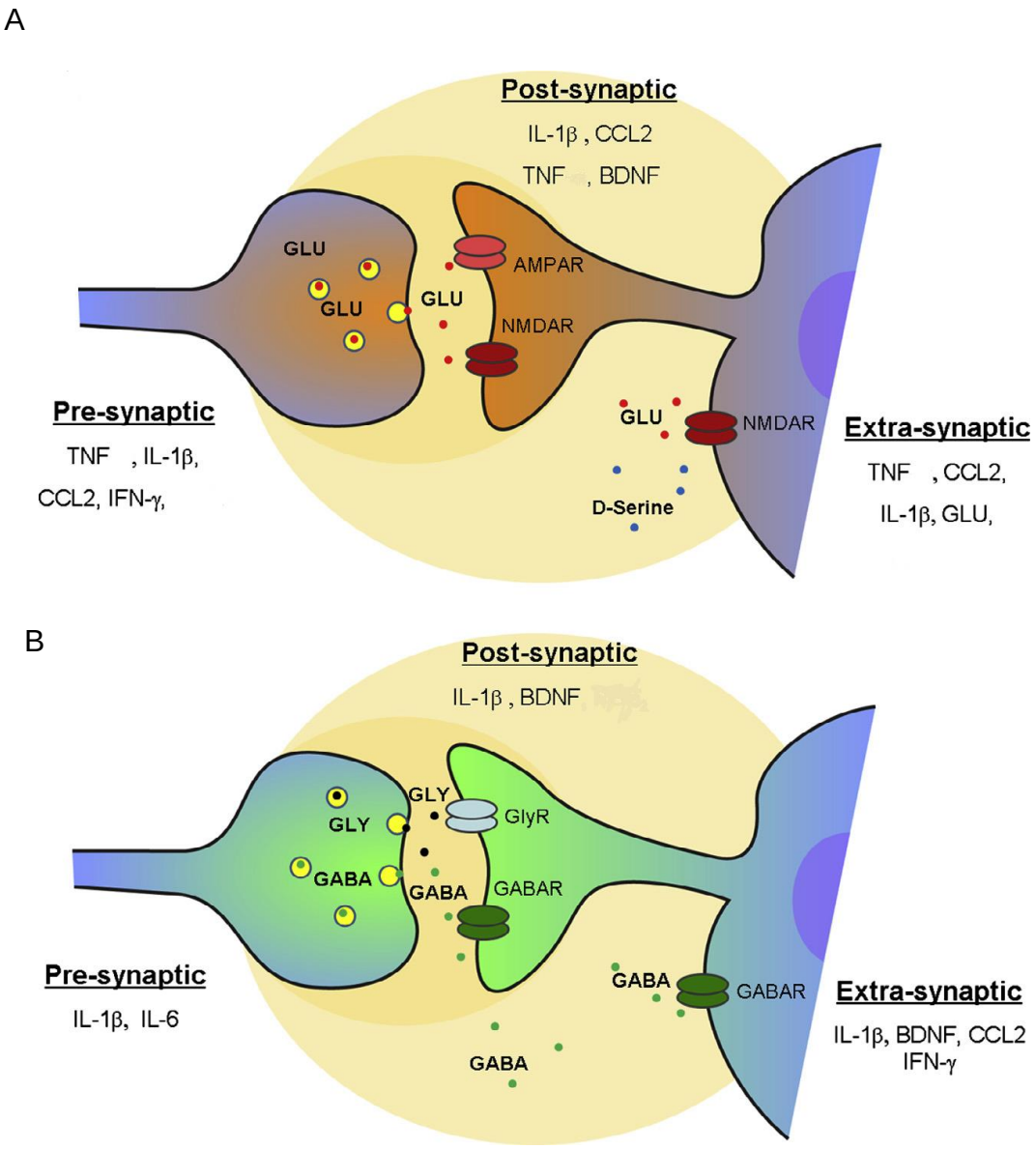


Fig 1.3 Glial mediated modulation of excitatory and inhibitory synaptic transmission in the dorsal horn spinal cord.

(A) **Modulation of excitatory synaptic transmission**. Presynaptically TNF, IL-1β, CCL2 and IFN-γ increase glutamate release and enhance EPSC frequency. Postsynaptically acting mediators increase AMPAR activity and extrasynaptic acting mediators increase NMDAR activity and NMDA-induced currents while glutamate released from astrocytes can induce NR2B inward currents in neurons (B) **Modulation of inhibitory synaptic transmission**. Presynaptically IL-1β and IL-6 decrease GABA and glycine release and IPSC frequency. Postsynaptically acting IL-1β and BDNF decrease GABA/GLYR activity and IPSC amplitude. Extrasynaptic acting IL-1β, CCL2 and IFN-γ suppress GABA and/or glycine-induced currents. TNF inhibits action potentials in inhibitory neurons. In lamina I neurons BDNF causes disinhibition. Reprinted from Pain, Glia and pain Is chronic pain a gliopathy, 154, Ji, Berta and Nedergaard, S10-S28, (2013).

1.4.1 Animal models of pain

The development of animal models has been an invaluable tool with which to investigate key mechanisms underlying different pain conditions and aid drug discovery. However, as non-human animals are unable to self-report, instead hypersensitivity to a noxious stimulus is characterised by a reflex withdrawal as a surrogate measure (Mogil et al., 2006).

Administration of thermal, cold, or mechanical stimuli applied to an inflamed or injured region (usually the paw) and the time taken for hind-limb withdrawal or the amplitude of the reflex can then be measured and compared to controls (either the contralateral uninjured side or a sham control animal). However, one criticism of animal models is that ongoing pain is not easily assessed and this is universally exhibited in chronic pain patients (Backonja and Stacey, 2004). A number of attempts to measure spontaneous pain include monitoring innate behaviour including asymmetrical directed behaviour such as shaking, guarding and flinching but are difficult to measure (Mogil, 2009). As a result hypersensitivity measured by a reflex withdrawal from noxious stimuli is the most common measure in pain research.

1.4.2 Animal models of neuropathic pain

Although there is an abundance of neuropathic pain models (including central pain, drug induced neuropathy and disease induced neuropathy models) to investigate the pathophysiology of traumatic nerve injury and the ontogeny of neuropathic pain (Sorkin and Yaksh, 2009), the peripheral nerve injury model is most frequently used (Pradhan et al., 2010; Jaggi et al., 2011). In rodents peripheral nerve injury normally involves injuring the sciatic nerve, or branches or central nerve spinal nerves allowing the evaluation of changes in the hind paw reflex response compared with baseline and the contralateral paw (Fig 1.4).

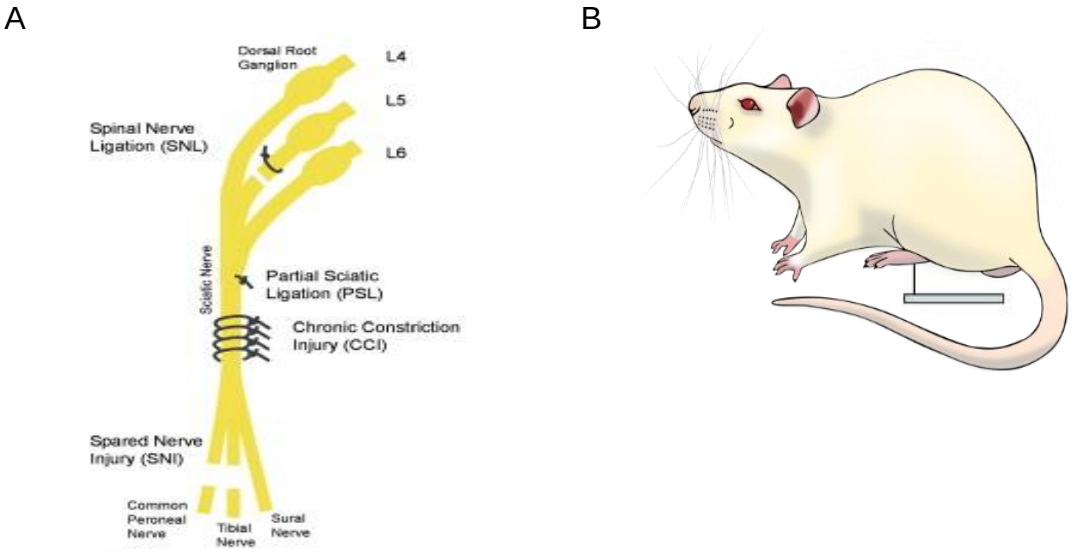


Fig 1.4 Animal models of peripheral nerve injury

(A) Four different nerve injury models are shown including the spared nerve injury (SNI), chronic constriction injury (CCI), partial sciatic ligation (PSL) and the spared nerve ligation (SNL) model. (B) Nerve Injury models leads to hypersensitivity such as an enhanced response to mechanical stimulation with Von Frey hair monofilaments (Vfh). Vfh's are applied to the plantar surface of the hind paw. The threshold force for a paw withdrawal decreases following nerve injury (Kim and Chung., 1992; Seltzer et al., 1991; Decosterd and Woolf., 2000). Reprinted from Cell, 52, Campbell and Meyer, Mechanisms of neuropathic pain, 77-92 (2006) with permission from Elsevier.

The first neuropathic pain models involved complete transection of the sciatic nerve (Wall et al., 1979) and resulted in a neuroma and behavioural autotomy. However, the major limitation is that complete amputation is usually only observed as phantom limb pain whereas clinically neuropathy involves partial lesions to peripheral nerves.

Subsequent models share the commonality of degeneration of some, but not all sensory fibres so that a peripheral target is partially de-innervated and innervated. Bennett and Xie developed the chronic constriction injury (CCI) involving application of 3-4 loose ligatures of chronic gut (or silk) tied around the sciatic nerve till a brief twitch is observed. Animal behavioural signs of pain-like behaviour include autotomy, guarding, excessive licking, limping, contralateral weight bearing (De Vry et al., 2004). The onset of mechanical, thermal and cold hypersensitivity develop within one week and persist for seven weeks post-surgery. However, the CCI model has a greater inflammatory component than other nerve injury models and due to the subjectivity of the tightness of the constriction there is considerable variation in the aforementioned behavioural outcomes (Pradhan et al., 2010).

The partial sciatic nerve ligation (PSL) model or Seltzer model (Seltzer et al., 1990) involves the tight ligation of one-third of the sciatic nerve distal to the nerve branches. Signs of spontaneous pain have been reported and cold, and mechanical hypersensitivity develop one week after surgery, persisting for six weeks. As in the CCI model there is variation in the duration and magnitude of responses depending on the material used. The spinal nerve ligation model (SNL) involves ligation of the L5 and L6 nerves (Kim and Chung, 1992) and results in the development of spontaneous pain like behaviour and mechanical, cold and thermal hypersensitivity develops 24-48 hours and persists for 10-16 weeks. Although the ligation is more easily reproducible compared to the above methods, the close proximately to the L4 nerve means that inflammatory process may play a key role in this neuropathic animal model.

The spared nerve injury model, developed by Decosterd and Woolf., 2000 involves the ligation and subsequent axotomy of the tibial and common peroneal nerves while keeping the sural and saphenous nerve intact. Therefore the damaged sensory fibres innervate a more restricted area and testing is performed in the innervated skin region adjacent to the denervated area of the limb. In both rats and mice, mechanical hypersensitivity develops 4 days after surgery and persist for up to 6 months (Bourquin et al., 2006; Decosterd and Woolf, 2000; Shields et al., 2003).

Responsiveness to noxious and innocuous mechanical and cold stimuli is increased in the sural and, to a minor extent the saphenous region (Decosterd and Woolf, 2000). However, thermal thresholds remain unaltered although responses to noxious thermal stimulation is exaggerated. These changes are robust, substantial and prolonged and closely mimic many features of clinical neuropathic pain (Shields et al., 2003). In addition, this model enables investigation of responses to both non-injured skin territories that adjoin de-innervated areas enabling simultaneous investigations in both injured and non-injured primary afferents that both make a contribution to neuropathic pain. Importantly the surgical procedure for creating this model is simple in comparison to the SNL, PNL and CCI and thus, there is less variability in the degree of damage (Decosterd and Woolf, 2000; Lindia et al., 2005).

Together nerve injury animal models have been key to the elucidation of the neuronal and non-neuronal mechanisms underlying neuropathic pain-like behaviour in the PNS and CNS.

Following peripheral nerve injury primary afferents exhibit ectopic activity and extensive transcriptional changes in both the injured and non-injured sensory neurons that lead to alterations in membrane properties and transmitter function. These alter the presynaptic release of new transmitters and neuromodulators into the dorsal horn that induce activity dependent plastic changes in the CNS involving neuronal and non-neuronal mechanisms (Bester et al., 2000; Costigan et al., 2002; Fukuoka et al., 2001; Kohno et al., 2003; Latremoliere and Woolf, 2009; Noguchi et al., 1995). There are several reviews outlining nerve injury induced peripheral changes that contribute to neuropathic pain but in this thesis I focus upon the changes that take place in the CNS with a focus on the dorsal horn spinal cord (Campbell and Meyer, 2006; Devor, 1991; Sommer and Kress, 2004; Woolf and Mannion, 1999).

1.4.3 Changes in the CNS underlying neuropathic pain

Following peripheral nerve injury abnormal activity from primary afferents can induce activity dependent plastic changes in the CNS. For example, in naïve conditions noxious stimulation is required to activate extracellular signal-regulated kinases (ERK) in superficial dorsal horn neurons but following peripheral nerve injury low threshold stimulation acquires this capacity (Ji et al., 1999; Matsumoto et al., 2008) that mimics the allodynia observed in patients with peripheral neuropathic pain.

Central sensitisation therefore provides a mechanism for how i) hypersensitivity occurs following nerve injury in the absence of peripheral sensitization ii) how low threshold primary afferent input can produce pain (allodynia) and iii) why there is a spread of sensitivity beyond areas outside the damaged nerve territory (secondary hyperalgesia) (Campbell and Meyer, 2006; Perl, 2007; Seal et al., 2009; Woolf, 2011; Woolf and Salter, 2000).

Mechanisms underlying these sensations include alterations in synaptic circuitry, disinhibition and alterations in brainstem regulation. Numerous studies also indicate that the enhanced output of dorsal horn neurons is not

solely dependent upon neuron to neuron transmission but glia to neurons interactions also play a key role in initiation and maintenance of pain hypersensitivity following nerve injury coining the idea that neuropathic pain is a neuro-immune disorder (Austin and Moalem-Taylor, 2010)

1.4.4 Central neuronal mechanisms of neuropathic pain

Peripheral nerve injury induces structural, physiological and expression changes in the dorsal horn spinal cord. Following nerve injury low threshold Aβ and C fibre mechanoreceptors which usually convey information about pleasant touch and project onto protein kinase C-γ (PKC-γ) positive interneurons start to convey pain (Perl, 2007; Seal et al., 2009). In the dorsal horn PKCγ, which is restricted to ventral lamina II and III in the dorsal horn is up-regulated following nerve injury and although mice lacking PKCγ show normal acute pain responses they do not develop hypersensitivity following nerve injury (Malmberg et al., 1997; Mao et al., 1995; Polgár et al., 1999).

Dynorphin may also play a role in the maintenance of neuropathic pain. Dynorphin is up-regulated in the spinal cord several days after the onset of nerve injury-induced pain-like behaviour and intrathecal administration of dynorphin causes mechanical hypersensitivity, while antibodies blocking dynorphin are able to reverse hypersensitivity (Kajander et al., 1990). Furthermore, pain-like behaviour is initiated but not maintained in mice lacking dynorphin (Wang et al., 2001).

Following a nerve injury postsynaptic changes in the dorsal horn occur which include phosphorylation of NMDA subunits and increased AMPA receptor density as a result of an increase in trafficking and synthesis of ion channels and scaffold proteins that are important in synaptic plasticity and indicative of central sensitization (Iwata et al., 2007; Latremoliere and Woolf, 2009; Miyabe et al., 2006; Takasusuki et al., 2007; Tao et al., 2003).

In vivo recordings in nerve injured rodents indicate alterations in the activity of dorsal horn neurons to a range of stimuli. However, the response of dorsal horn neurons to heat stimulation remain unaltered, despite the development of thermal hypersensitivity in these animals (Laird and Bennett, 1993; Palecek et al., 1992). These studies show no changes in the mechanical threshold or

responses to wind up in nerve injured animals which are comparable to sham controls (Chapman et al., 1998a), more neurons respond to innocuous mechanical stimulation and there is an enlargement of receptive fields compared to sham controls (Laird and Bennett, 1993; Palecek et al., 1992; Suzuki et al., 2000).

Patch clamp recordings in spinal cord slices indicate a facilitation of NMDA receptor currents in the SNL animal model while following CCI and nerve transection the threshold for eliciting EPSCs is reduced and there is an increase in mono-and poly-synaptic Aβ-fibre evoked EPSCs (Kohama et al., 2000; Okamoto et al., 2001).

A loss of inhibition (or disinhibition) also contributes to the increase in excitatory synaptic strength and neuronal excitability in the dorsal horn. Following nerve injury a reduction in IPSC in lamina II neurons occurs as a consequence of reduced presynaptic GABA release in the superficial dorsal horn (Moore et al., 2002). Inhibiting GABA and glycine release increases A-fibre mediated excitatory transmission in the superficial dorsal horn and induces mechanical hypersensitivity (Baba et al., 2003; Sivilotti and Woolf, 1994).

Behavioural investigations also suggest that descending pathways, involving the descending facilitatory projections from the PAG and RVM are importance for the maintenance, but not induction of neuropathic pain behaviour and injection of lidocaine into these brain areas can attenuate mechanical hypersensitivity in nerve injured animals (Burgess et al., 2002; Pertovaara et al., 1996). Inhibitory neurotransmission (norepinephrine, 5-hydroxytryptamine and endogenous opioids) following nerve injury can also alter so that tonic noradrenergic inhibition is suspended and the descending serotonergic input changes from inhibition to facilitation (Bee and Dickenson, 2008; Rahman et al., 2008; Vera-Portocarrero et al., 2006).

Histological studies suggest that ipsilateral dorsal horn neurons undergo degeneration following CCI as observed with TUNEL staining, and are possible GABAergic neurons (Azkue et al., 1998). However, it is possible that

these cells maybe non-neuronal and there is some controversy about this (Polgar et al., 2003).

A key clinical problem in neuropathic pain patients is mechanical allodynia and a number of investigations into the mechanisms underlying this symptom have been undertaken. Spinal lamina I output neurons are not organised to receive direct input from low threshold afferent (their dendrites are restricted to lamina I) and low threshold afferents terminate in deeper laminae (Bester et al., 2000; Keller et al., 2007; Miraucourt et al., 2007). However, there are excitatory interneurons that are presynaptic to lamina I projection neurons that have dendrites ventrally and receive input from deeper layers (Cordero-Erausquin et al., 2009).

Polysynaptic pathways have also been identified that are normally repressed by inhibition (via feed-forward glycinergic interneurons) that represses the relay of information via an excitatory interneuron that expresses PKC-γ from low threshold mechanosensitive afferents to lamina I projection neurons (Baba et al., 2003; Keller et al., 2007; Lu et al., 2013; Miraucourt et al., 2007; Torsney and MacDermott, 2006). However, following peripheral nerve injury the nociceptive neurons that respond to innocuous touch can be replicated via antagonising glycine and/or $GABA_A$ mediated currents pathways suggesting dis-inhibition as a key mechanism underlying mechanical allodynia. Disinhibition also provides a mechanism by which glial activation can regulate pain processing via altering Cl^- mediated inhibition (Ferrini and De Koninck, 2013).

1.4.5 Central non-neuronal mechanisms of neuropathic pain

Following nerve injury neurotransmitters and other mediators are released in to the dorsal horn spinal cord such as glutamate, fractalkine, ATP, misfolded proteins, complement components and nuclear factors that stimulate microglia, the recruitment of T-lymphocytes and at later time points, astrocytes. These non-neuronal cells alter the expression of cell surface receptors, signalling pathways that ultimately leads to the release of pro- and anti-inflammatory mediators, the balance of which determines if neuropathic pain

is initiated and maintained (McMahon and Malcangio, 2009; Scholz and Woolf, 2007; Tanga et al., 2004).

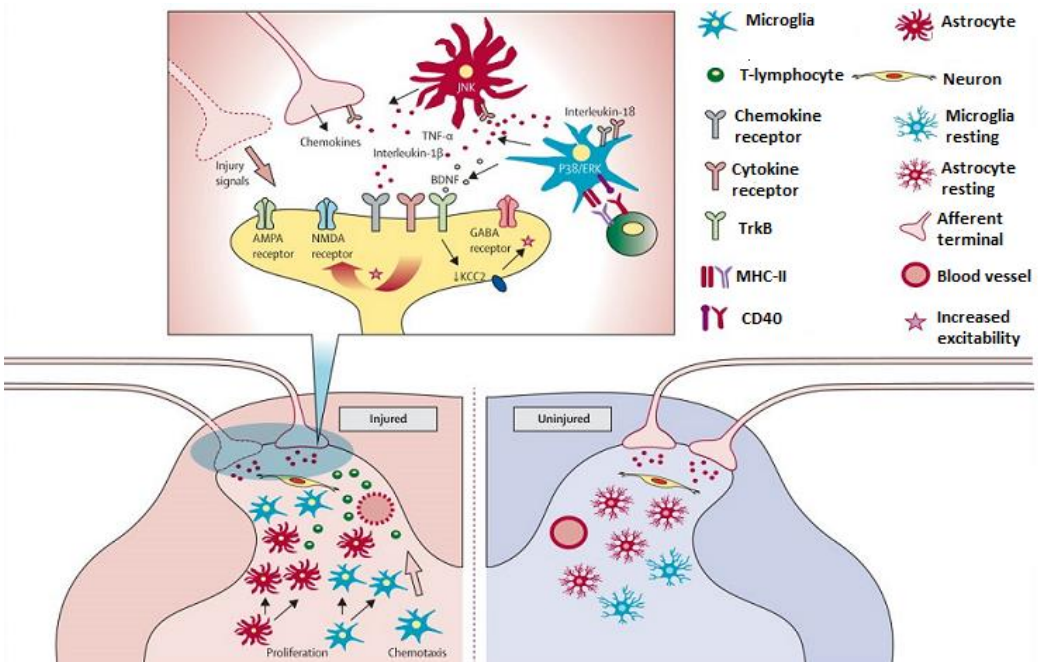


Fig 1.5 Microglia, astrocyte and T-cells transition to a pain-related enhanced state in the dorsal horn following peripheral nerve injury.

Signals conveying a nerve injury arrive via primary afferents in the dorsal horn and stimulate the release of chemokines, proteases, growth factors, neurotransmitters and cathepsin-S that induces the proliferation and movement of microglia to the site in the dorsal horn where injured primary afferents arborize. Microglia express MHC-II and, together with co-stimulatory molecule CD40 present antigens to T-lymphocytes. Astrocytes also proliferate. The induction of intracellular signalling of MAPKs in astrocytes and microglia, induce the release of pro-inflammatory mediators such as TNF, BDNF and IL-1β which act pre-synaptically to enhance neurotransmitter release from primary afferents and post-synatpcially on dorsal horn neurons which together increase the activity of excitatory neurons and reduce the activity of inhibitory neurons. Reprinted from The Lancet, 11, Calvo, Daves and Bennett, The role of the immune system in the generation of neuropathic pain, page 629-642., Copyright (2012), with permission from Elsevier.

1.4.5.1 Microglia

In the adult, the most highly regulated genes following nerve injury are expressed in spinal microglia (Griffin et al., 2007) which are the first spinal cord glia cell type to be stimulated, within 4 hours of peripheral nerve injury (Tanga et al., 2004). Initially microglia undergo rapid proliferation and migrate to the site where injured terminals terminate as well as in the ventral horn around motor neuron cell bodies of injured motor neurons (Beggs and Salter, 2007; Suter et al., 2007).

Three main signalling pathways mediate the recruitment of resident microglia to the dorsal horn and including the chemokine fractalkine acting on the CXCR1 receptors, CCL2 on CCR2 and Toll-like receptors (TLRs) (Kim et al., 2007; Tanga et al., 2005; Verge et al., 2004; White et al., 2005) although a range of mediators both released by primary afferents and the products of tissue injury including ATP, misfolded proteins, and nuclear factors are sensed by microglial cell surface receptors that also initiate the transition into an enhanced pain response state (Liu et al., 2000; Ma et al., 2003; Wu et al., 2001).

In addition to nerve injury, dorsal horn sensitization can also be evoked by intense C-fibre stimulation. A study by Hathway et al showed that C-fibre stimulation of the sciatic nerve in adult rats induced hypersensitivity and increases in microglia marker expression in the dorsal horn that could be prevented by administration of minocycline (Hathway et al., 2009). If the nerve is completely blocked, mechanical hypersensitivity and microglial activation is stopped, although C-fibre inhibition alone does not prevents SNI nerve injury or microglia activation (Suter et al., 2009; Wen et al., 2007; Xie et al., 2009).

The transition of microglia into a ‘pain related enhanced state’ is characterised by alterations in cell morphology, gene expression, the expression of surface proteins, regulation of receptors and channels and activation of intracellular singling cascades (including phosphorylation MAPK such as p38 and ERK and the Src-family kinases Src, Lck and Lyn) and the subsequent release of inflammatory mediators.

Although an over simplification, microglia polarisation can be categorised into classical M1 and alternative M2 activation states. Lipopolysaccharide (LPS) is known as a representative M1 polarisation inducer that leads to increases in pro-inflammatory molecules including TNF, IL-1β, and IFN-γ and NO. In contrast IL-4 induces M2 polarisation leading to the expression IL-4, cluster of differentiation (CD)206 and IL-10 and neuro-protective effects (David and Kroner, 2011; Kigerl et al., 2009; Kobayashi et al., 2013; Liao et al., 2012; Ponomarev et al., 2007). Following nerve injury in adult rodents, microglia exhibit a predominantly pro-inflammatory M1 response characterised by the release of TNF, IFN-γ, and growth factors such as BDNF that alter excitatory

synaptic transmission and reduces inhibitory transmission and thus induce central sensitisation via distinct mechanisms (Coull et al., 2005; Ledeboer et al., 2005; Svensson et al., 2005; Tanga et al., 2004). While neurotransmitters such as glutamate and GABA produce synaptic alterations at μM concentrations, cytokines, chemokines and growth factors alter synaptic activity at nM concentrations (Coull et al., 2005; Gao et al., 2009; Kawasaki et al., 2008).

The actions of these mediators are characterised by increased excitatory synaptic transmission and decreased inhibitory synaptic transmission leading to central sensitization of dorsal horn neurons and the development of persistent pain. To counterbalance this effect M2 polarised anti-inflammatory cytokines including IL-4, IL-10, IL-13 and Transforming growth factor beta (TGF-β) act on homeostasis restoration and promote repair mechanisms. However, following adult nerve injury in the dorsal horn the balance is tipped towards a predominant pro-inflammatory response and pain-like behaviour. Multiple studies have examined the role of cytokines and chemokines on neuronal activity. For example, Gruber-Schoffnegger and colleagues showed that activation of spinal glial were essential for the induction of long term potentiation (LTP) induction at C-fibre synapses in LI neurons and the cytokines TNF and IL-1β are individually sufficient and necessary for LTP induction, amplifying both AMPA and NMDA mediated currents acting indirectly via IL-1 receptors and TNF receptors expressed on glial cells in the dorsal horn that lead to the subsequent release of inflammatory mediators (Gruber-Schoffnegger et al., 2013; Jin et al., 2003; Katsura et al., 2006; Suter et al., 2007; Tsuda et al., 2004; Zhuang et al., 2005). These inflammatory mediators act in a positive feedback loop to further recruit microglia, stimulate astrocytes, and promote the sensitization of the CNS nociceptive circuits and neuropathic pain (Jin et al., 2003; Katsura et al., 2006; Suter et al., 2007; Tsuda et al., 2004; Zhuang et al., 2005).

Microglia also play a key role in T lymphocyte recruitment as part of the adaptive immune system that have a role in the maintenance of hypersensitivity following nerve injury (Cao and DeLeo, 2008; S. M. Sweitzer et al., 2002). For example, nerve injured stimulated microglia act as local

antigen presenting cells (APCs) and direct and modify T-cells by expressing major histocompatibility complex (MHC) class II and co-stimulatory molecule CD40 that regulate leukocyte infiltration (Grace et al., 2011; Kazansky, 2008). Finally, blocking microglial responses or promotion of anti-inflammatory responses in animal models of neuropathic pain prevents and in some cases reverse hypersensitivity (Coull et al., 2005; Ledeboer et al., 2005; Raghavendra et al., 2003; Tsuda et al., 2003)

1.4.5.2 T-cells

T-cell lymphocytes (T-cells) are the major cellular components of the adaptive immune response and can be divided into helper T (Th) cells, cytotoxic (Tc) cells and regulatory T cells (Tregs). Th cells can be sub-divided into type 1 (Th1) and Th2 that broadly have different patterns of cytokine production, originally described in a CD4+ T-cell and are classified on their cytokine production and associated effector function (Mosmann and Coffman, 1989; Mosmann and Sad, 1996). Th1 cells produce mostly IFN-γ, an inflammatory cytokine important in the response to microbial infections. Th2 cells secrete IL-4 participating in immunity against parasites and encourage antibody production as well as IL-10. The cytokine productions of Th1 and Th2 cells are mutually inhibitory for both the differentiation and effector functions of the reciprocal phenotype. For example, IFN-γ inhibits the proliferation of Th2 cells and IL-10 inhibits the synthesis by Th1 cells (Fiorentino et al., 1989; Mosmann et al., 1990; Mosmann and Coffman, 1989). T-cells can infiltrate healthy and diseased CNS across the choroid plexus, blood-brain barrier (BBB) and blood-spinal cord barrier (BSpCB) to identify antigens (Engelhardt, 2006; Engelhardt and Ransohoff, 2005; Hickey, 2001; Ransohoff et al., 2003).

Accumulating evidence suggests that seven days after peripheral nerve injury CD4+ T-cells infiltrate the spinal cord, due to an increase in blood-CNS barrier permeability, where they interact with activated glia and exhibit a Th1 response which contributes to pro-inflammatory mediators that sensitise neurons (Beggs et al., 2010; Cao and DeLeo, 2008; Costigan et al., 2009; Hu et al., 2007; Sweitzer et al., 2002). In addition to nerve injury, permeability of the BSpCB can be increased 24 hours after electrical stimulation of the sciatic nerve or

capsaicin application which can be reduced by lidocaine prior to surgery (Beggs et al., 2010).

The use of knock out rodents has provided important evidence for the involvement of lymphocytes in the maintenance of neuropathic pain behaviour. For example, hypersensitivity in nerve injured mice can be attenuated in nerve injured MHC class II and microglia CD40 null mice (Cao et al., 2009; Sweitzer et al., 2002). Athymic rats lacking T-cells and T-cell deficient nude mice exhibit reduced hypersensitivity following peripheral nerve injury (Cao and DeLeo, 2008; Moalem et al., 2004) and mice lacking functional T-cells (deficient in the recombinant activating gene-1) exhibit attenuated thermal and mechanical hypersensitivity following nerve injury (Costigan et al., 2009; Kleinschnitz et al., 2006).

In adult neuropathic rodents, IFN-γ is a prominent pro-inflammatory mediator up-regulated in the dorsal horn spinal cord that alters neuronal excitability (see below). As a result of the antagonist function of a Th1 and Th2 response, there is a strong bias towards either a Th1 or Th2 response. As CD4 null mice still exhibit increases in the expression of microglia markers, but not astrocyte markers, suggests that the interaction of T-cells and microglia may facilitate the activation of astrocytes (Raghavendra et al., 2003).

1.4.5.3 Astrocytes

Nerve injury stimulates astrocytes at a later time point than microglia, around 4 days post injury (Colburn et al., 1999; Tanga et al., 2004) and these play a key role in the maintenance of neuropathic pain. Glial fibrillary acidic protein (GFAP) antisense treatment in neuropathic rats reverses established pain (Kim et al., 2009). Similarly to microglia, astrocytes express a variety of receptors and following nerve injury, leads to the activation of intracellular signalling pathways of the JNK member of the MAPK family that is persistently activated in spinal cord astrocytes following nerve injury (Ma and Quirion, 2002; Zhuang et al., 2006). Astrocytes also release a plethora of mediators many of which overlap with microglia and T-cells including NO, excitatory amino acids, ATP and pro-inflammatory cytokines including IFN-γ and CCL2 (Gao et al., 2009; Gosselin et al., 2005; Liu et al., 2000; Milligan et al., 2001;

Queiroz et al., 1997; Racz et al., 2008). Although fewer studies have investigated the role of astrocytes in nerve injury induced pain, astroglial toxins and astrocyte proliferation inhibitors can attenuate neuropathic pain like behaviour in animal models (Milligan et al., 2003).

Together this data suggests that nerve injury induces the release of microglial-derived mediators that contribute to a pro-inflammatory environment in the first synapse in the spinal cord that persists beyond the original nerve injury and spreads to remote sites and hence, the development of pain hypersensitivity and long term maintenance of pain.

1.5 Postnatal development of pain pathways

Even before birth, the infant nervous system responds to noxious stimulation; chemical, heat or mechanical cutaneous stimuli can evoke activity in dorsal horn spinal cord neurons and reflexes in the foetus (Fitzgerald, 1987c; González and Angulo, 1932). However, the neonatal nervous system undergoes profound postnatal developmental alterations reflected in both the lower reflex thresholds, larger reflex receptive fields and exaggerated and un-coordinated responses following noxious stimulation in the neonate. This continues until approximately postnatal day 10 (P10) in the rat and gradually refines over the first few weeks of postnatal development (Andrews et al., 2002; Andrews and Fitzgerald, 1999, 1994; Ekholm, 1967; Fitzgerald et al., 1988; Waldenström et al., 2003, 2003).

These alterations in behaviours arise from fine tuning of excitatory and inhibitory synaptic connections and neuronal circuitry in the dorsal horn over the postnatal period so that the processing of noxious inputs differ in infant and adult animals (Fitzgerald, 2005a; Fitzgerald and Walker, 2009). For example, the properties and receptive fields of dorsal horn neurons reflect the same properties of large receptive fields, low thresholds and prolonged action potential after-discharge in the early postnatal period (Fitzgerald, 1988, 1985a; Torsney and Fitzgerald, 2002; Ririe et al., 2008). Furthermore sensitization of the spinal cord to repeated C-fibre input does not occur in infant animals (Hathway et al., 2009) but electrical stimulation of cutaneous A-fibres sensitizes dorsal horn neurons in P6 rats (Jennings and Fitzgerald, 1998).

These functional properties in the newborn rodent are also observed in the kitten and in human neonates (Andrews et al., 2002; Andrews and Fitzgerald, 1994; Ekholm, 1967).

Compared to humans, the rat pup is born at an immature stage and the first postnatal week corresponds to human preterm development from 24 weeks to full term 40 post conception weeks (Fitzgerald, 1991). By P7 a rat pup can be considered an infant and by P21, when the rat is weaned, they are considered a young adolescent (McCutcheon and Marinelli, 2009; McGrath et al., 2013) and are considered an adult by the age of 8 weeks.

This section describes how sensory systems develop over the postnatal period with particular attention to dorsal horn spinal cord processing. Within the dorsal horn postnatal developmental changes in the balance of inhibitory and excitatory signalling influence acute responses to sensory input and also underlies longer term alterations in sensory processing following tissue injury in early life.

1.5.1 Primary afferent connections to the dorsal horn

In the rat, by birth much of the nociceptive circuitry is in place. For example, sensory neurons grow out from the dorsal root ganglia towards the periphery, reaching the epidermis and dorsal horn and the lumbar spinal cord by E20 (Fitzgerald, 1987a; Reynolds et al., 1991). *In vivo* dorsal horn recordings in the rat foetus at E20 show that activity can be evoked in the dorsal horn spinal cord in response to noxious cutaneous chemical, heat and mechanical stimulation (Fitzgerald, 1987b). However, during development A-and C-fibres in the immature dorsal horn undergo reorganisation in the postnatal period (Benn et al., 2001; Fitzgerald et al., 1991; Fitzgerald, 1987a; Fitzgerald and Swett, 1983; Jackman and Fitzgerald, 2000; Mirnics and Koerher, 1995; Woodbury and Koerber, 2003) with maturation occurring through the first 2-3 postnatal weeks in the rodent, a period that extends throughout infancy and early childhood in humans (Beggs et al., 2002; Cornelissen et al., 2013, 2013; Jennings and Fitzgerald, 1998).

By E15 A-fibres penetrate the dorsal horn in a somatotopic appropriate manner and have made synaptic contacts (Coggeshall et al., 1996; Fitzgerald et al.,

1994). Initially however, A-fibre terminals spread widely into the superficial dorsal laminae extending into LI and LII which in the adult is occupied mostly by C-fibres. These terminals form functional connections with immature superficial dorsal horn neurons which has a significant influence on the physiological responses of dorsal horn neurons. Stimulation at Aβ-fibre strength stimulation can evoke c-Fos expression (a marker of neuronal activation) and prolonged activity in LII which is absent by P21 (Andrews and Fitzgerald, 1994; Jennings and Fitzgerald, 1998; Nakatsuka et al., 2000; Park et al., 1999) and repetitive Aβ fibre stimulation before P21 sensitizes dorsal horn neurons (Jennings and Fitzgerald, 1998). As a result dorsal horn cell cutaneous mechanical thresholds are lower and a larger proportion of neurons respond to low threshold stimulation (Fitzgerald, 1987b; Fitzgerald and Jennings, 1999; Torsney and Fitzgerald, 2002).

Over the first three postnatal weeks A-fibres withdraw to deeper laminae in an activity dependent manner and exhibit a mature pattern by P21(Beggs et al., 2002; Fitzgerald et al., 1994; Fitzgerald and Jennings, 1999; Granmo et al., 2008; Jennings and Fitzgerald, 1998, 1996).

In contrast to A-fibres, C-fibres are the last to enter the dorsal horn grey matter directly into LI-LII at E19 although the full complement of C fibres (including the IB4+ subset) are detected as late as P5 (Fitzgerald et al., 1987a; Fitzgerald and Swett., 1983). During the early postnatal period A and C-fibres overlap which partly explains the sensitivity of infant animals to low-threshold input. Although C-fibres form functional synapses at birth, in the first postnatal week, they are unable to evoke spike activity in dorsal horn neurons until P9 (Baccei et al., 2003; Fitzgerald, 1988; Jennings and Fitzgerald, 1998). For example, C-fibre irritant mustard oil induces only a weak flexion reflex and c-Fos expression in dorsal horn neurons (Fitzgerald and Gibson, 1984; Williams et al., 1990) and although application of capsaicin to spinal cord slices increases glutamate release in the dorsal horn by P0, these is a significant increase between P5 and P10 showing that C-fibre synaptic input matures steadily from birth (Baccei et al., 2003). These experiments show that although nociceptive responses are present at birth, neurotransmitter release is asynchronous and thus does not readily induce spike activity *in vivo* (Fitzgerald et al., 2005).

Therefore, in the early postnatal period, nociceptive responses are largely mediated through Aδ-fibres which may assume a different role in the transmission of information to the spinal cord to accommodate for the immaturity of C-fibre connections.

C-fibre input in the early postnatal period is important for the development of nociceptive processing and innocuous input processing and the withdrawal of A-fibre terminals from the superficial laminae is halted in animals without C-fibre terminals (Beggs et al., 2002; Torsney et al., 2000).

1.5.2 Excitatory neurotransmission in the immature dorsal horn

Most of the excitatory transmission in the dorsal horn is mediated by glutamate acting on postsynaptic ionotropic receptors including NMDA, Kainate and AMPA receptors. These receptors show considerable postnatal regulation exhibiting differences in expression and subunit stoichiometry with age. Initially these receptors are highly expressed and gradually become restricted to the superficial dorsal horn (Jakowec et al., 1995; Pattinson and Fitzgerald, 2004).

In the first postnatal weeks NMDA receptors (NMDAR) have a higher affinity for NMDA and are more abundant at birth declining in the first two postnatal weeks (Gonzalez et al., 1993; Green and Gibb, 2001). Receptors also exhibit a more calcium permeable stoichiometry in the neonate and neonatal rat LI-II neurons have increased AMPAR-dependent calcium influx due to a reduced expression of subunit GluR2 compared to mature rats which have a reduced calcium permeability (Hartmann et al., 2004). Activation of calcium dependent intracellular cascades alters synaptic strength and is also important in neuron growth (Gu and Spitzer, 1995; Li et al., 2013; Li and Baccei, 2011). The restriction of expression of glutamate receptors and changing of subunit composition coincides with a dampening of general spinal cord excitability.

1.5.3 Inhibitory neurotransmission in the immature dorsal horn

Inhibitory circuits and brainstem descending controls play an equally essential role in modulating nociceptive signals and also show considerable postnatal developmental changes. GABA and glycine are the major inhibitory neurotransmitters in the dorsal horn that act via activation of ligand gated

chlorine channels ($GABA_A$ and glycine receptor). In the neonatal dorsal horn GABAergic signalling dominates in lamina II neurons for the first two postnatal weeks while glycinergic miniature IPSC are absent, although their receptors are present (Baccei and Fitzgerald, 2004; Dougherty et al., 2009; Ma et al., 1992; Schaffner et al., 1993). In contrast glycinergic inhibition develops later in the second postnatal week coinciding with an alteration in glycinergic receptor expression to a mature α1/β glycine receptor composition (Baccei and Fitzgerald, 2004; Koch et al., 2012).

In the immature dorsal horn tactile responses are facilitated rather than inhibited by glycinergic activity although this period ceases in the second postnatal week upon the development of glycinergic inhibition coinciding with the maturation of C-fibre spinal input (Koch and Fitzgerald, 2014). The combination of greater excitatory neurotransmission and a lack of glycinergic inhibition plays a key role in the excitability of the dorsal horn, a trend that favours synaptic strengthening and is important for the maturation of the pain pathway.

1.5.4 Descending neurotransmission in the immature dorsal horn

Experiments in adult mammals indicate that descending control exerts inhibitory effects on dorsal horn spinal cord processing, as blocking descending control results in a fall in mechanical reflex thresholds and exaggerated responses to noxious stimulation (Sherrington, 1910).

Stimulation of the dorsolateral funiculus that contains descending fibres from the brainstem, produces analgesia in adults by inhibiting responses and c-Fos activation of dorsal horn neurons in response to hind paw stimulation but not in animals younger than P9 (Boucher et al., 1998; Fitzgerald and Koltzenburg, 1986). From P12 stimulation inhibits around 50% of cells and by P18 causes widespread inhibition in the dorsal horn (Fitzgerald and Koltzenburg, 1986) and stimulation of the PAG does not inhibit dorsal horn cells until P21 (van Praag and Frenk, 1991).

The ablation of the RVM in animals up to the age of P21 results in an increase in mechanical thresholds whereas the same treatment in adults results in a fall in mechanical thresholds (Hathway et al., 2009). A study by Hathway et al.,

demonstrated that this top-down control on dorsal horn neurons changes markedly in the first few postnatal weeks from a unimodal facilitatory control in neonatal rats (before P21) to inhibitory at older ages (Hathway et al., 2009, 2012). In addition, in contrast to adults, brainstem descending inhibition of C fibre inputs is absent at P21 while the facilitation of A-fibre input in early life is likely to enhance non-noxious inputs to the dorsal horn in the early postnatal weeks, promoting activity-dependent development of sensory networks (Koch and Fitzgerald, 2014).

The mechanism underlying the switch is likely to be associated with the maturation of RVM and PAG circuitry. Interestingly, the postnatal development of GABAergic neurons in the rat PAG correlates with the development of descending control with an increase from P5 to P10, maturation at P14 and established after P20 (Barbaresi, 2010).

1.6 The development of persistent and chronic pain

While mammalian nociceptive pathways are functional at birth and robust behavioural and physiological responses to noxious stimuli can be measured, the response of the nervous system to more prolonged injury is less well understood (Fitzgerald, 2005a, 1991; Slater et al., 2007).

Both neurobiological and behavioural studies in animal models show that during critical periods in the postnatal period a ‘normal’ sensory experience (i.e. non-noxious stimulation) is essential for the postnatal development of the nociceptive circuitry indicating activity depending shaping of neuronal circuits (Beggs et al., 2002; Fitzgerald, 2005a; Koch et al., 2012).

The use of different animal models to mimic injuries such as inflammation, surgical injury and visceral insults has shown that when noxious stimulation is repeated or persistent the effects may outlast the period of stimulation. This may have long-lasting alterations on pain processing later in life which is critically dependent on the time at which the injury occurs and the type, severity and duration of injury (Fitzgerald., 2005).

1.6.1 Long-term effects of early life injury

Insults such as acute carrageenan inflammation or surgical incision in the hind paw during the first postnatal week, a critical period for many insults, are associated with a general hyposensitivity (i.e. decreased sensitivity) that emerges 4-5 weeks later. The area around the injury maintains an enhanced sensitivity so that a new injury (such as a repeat of CFA, capsaicin, formalin but not nerve injury) to the same region causes an enhanced hyperalgesia that is greater in amplitude and duration compared to controls (Beggs et al., 2012; Chu et al., 2007; LaPrairie and Murphy, 2009; Ren et al., 2004; Sternberg et al., 2005; Walker et al., 2009). The hypersensitivity following a second insult is apparent shortly after the initial injury but also in adult hood (even in 125 day old rats), long after the initial injury is resolved (Ren et al., 2004). Importantly, the effects of these relatively mild insults are constrained to the early neonatal period, the critical period being in the first postnatal week and disappearing if the initial injury is completed after P9.

If the injury is more extensive, such as following neonatal visceral injury e.g. exposing the bladder or bowel to a chemical irritant, animals display long-lasting hypersensitivity and secondary hyperalgesia while re-injury in adulthood results in enhanced hyperalgesia (Christianson et al., 2010; DeBerry et al., 2010; Randich et al., 2006; Wang et al., 2008).

The two different changes in pain sensitivity i) a global reduction in baseline sensitivity (hyposensitivity) or ii) increased pain and hyperalgesia in the region of neonatal injury must be due to two different mechanisms. The hyposensitivity following carrageenan is generalized to all hind paws and must involve pathways beyond the dorsal horn. Due to the maturational switch of the RVM from a facilitatory before P21 to a dominating inhibition, brain stem-mediated alterations in descending modulation maybe a key mechanism. In support of this evidence in animal models shows that following neonatal hind paws carrageenan insult, adults exhibit enhanced inhibition from the RVM in response to noxious inputs and alterations in the PAG including opioid-mediated responses and increased expression of 5HT receptors (Anseloni et al., 2005; Wang et al., 2004; Zhang et al., 2010). Therefore a permanent

change in RVM circuitry or signalling as a result of early injury may alter descending control and subsequently nociceptive dorsal horn networks.

Mechanisms underlying the increased hyperalgesia are segmentally restricted to the previously injured paw and thus represent different mechanisms to those underlying hyposensitivity. During these insults afferent input following neonatal injury is likely to be intense as *in vivo* electrophysiological recordings of dorsal horn neurons during skin incision show that the initial afferent evoked activity is greater in infants compared to adults (Ririe et al., 2008) and early life injury causes changes in dorsal horn nociceptive circuitry, similar to central sensitisation (Al-Chaer and Traub, 2002; Beggs et al., 2012; Wang et al., 2008).

Neonatal skin wounds cause increases in spinal flexion reflex electromyographic excitability, enlarged dorsal horn receptive fields 6 weeks later that are NMDAR dependent (Beggs et al., 2012; Chu et al., 2007; Torsney and Fitzgerald, 2003) and patch clamping studies in dorsal horn slices of adults following infant tissue injury show a decrease in inhibitory signalling in lamina II neurons (Li et al., 2013). As the maturation of dorsal horn glycinergic circuits are dependent on C-fibre activity in the postnatal period, insults during this period may alter glycinergic neurotransmission (Koch et al., 2012; Koch and Fitzgerald, 2014).

Long-term enhancement of pain activity might also be maintained by the neuro-immune system. Of particular interest is the incision model, where incision activates microglia in the dorsal horn in the adult, but prior neonatal skin incision greatly enhances hypersensitivity that is mirrored by alterations in time course and degree of microglial expression in the dorsal horn compared to controls. This 'primed' state can be reversed by intrathecal administration of minocycline at the same time as adult incision (Beggs et al., 2012).

As microglia in adults are known to release pro-inflammatory mediators this 'priming' may increase the release after re-injury, thus enhancing spinal dorsal horn excitability and behavioural hypersensitivity. Furthermore, microglia are long lived cells they are well suited to a role in persistent alterations. Evidence for this was shown by direct C-fibre stimulation, not injury, in the neonate, that

also ‘primed’ microglia showing that alterations are maintained in the dorsal horn circuitry. However, novel neuro-immune interactions in the neonate have been implicated in long term effects of neonatal nerve injury which differs substantially from neonatal inflammation and tissue injury with respect to both the critical period and behavioural responses.

1.6.2 Nerve injury at infancy

Neuropathic pain is rare in infants and only very few reports exist before 5-6 years of age in humans (Anand and Birch, 2002; Howard et al., 2014; Sethna et al., 2007; Walco et al., 2010). For example, thirteen years is the median age of onset for paediatric neuropathic pain syndromes such as phantom pain, complex regional pain syndrome and peripheral neuropathy pain (Walco et al., 2010). The reasons for this are unknown, although it is apparent that pain is more likely to be observed in late childhood and adolescence than at younger ages (Anand and Birch, 2002; Atherton et al., 2008).

Traumatic severe traction nerve injury at birth can cause brachial plexus palsy and would be expected to result in pain but studies show a full recovery of sensory function with little evidence for neuropathic pain (Anand and Birch, 2002). Similarly traumatic brachial plexus injury pain occurs in older children, but not infants (Dumontier and Gilbert, 1990; El-Gammal et al., 2003) and distal nerve injury leads to neuropathic pain in children older than 5 years, with an increase in severity in teenagers (Atherton et al., 2008; Hwang et al., 2008). In addition, clinical investigation into adolescents that had phantom limb pain show that children with the earliest amputations had a delayed onset of phantom pain, emerging after a mean of 7 years (Melzack et al., 1997) suggesting long term alterations in pain processing occurs as a result of trauma sustained at infancy.

1.6.3 Animal model of infant nerve injury

Very early studies into the effect of infant nerve injury used neonatal peripheral nerve section (usually of the sciatic nerve) to show that axotomy in the early neonatal period (P0) caused growth retardation of the rat dorsal horn, substantial DRG cell death and sprouting of adjacent nerve terminals which began to occupy inappropriate somatotopic termination patters (Aldskogius

and Risling, 1981; Cheema et al., 1984; Fitzgerald and Shortland, 1988; Himes and Tessler, 1989; Yip et al., 1984). However, further studies show that these effects were absent if sectioning took place at P10 or later (Beggs., 2000; Coggeshall et al., 1997; Fitzgerald, 1985b; Himes and Tessler, 1989; Li et al., 1994). More recent studies using peripheral nerve injury models (as opposed to axotomy) show that the absence of neuropathic pain in early life is confirmed in rodent nerve injury models. SNI, CCI, PNL and in the first 2-3 weeks of postnatal life do not produce the same degree of acute mechanical hypersensitivity as observed in adults (Howard et al., 2005; Lee and Chung, 1996; Ririe and Eisenach, 2006). This is very different to other insults where the 'critical period' in rodents is observed only if the insult occurs in the first postnatal week of life. Furthermore, infant rats and humans clearly respond to acute and chronic pain behaviour from an early neonatal age suggesting that the mechanisms underlying neuropathic pain behaviour in infants must be differentially regulated.

Interestingly the genes most differentially regulated in the dorsal horn spinal cord following infant and adult nerve injury are immune related (Costigan et al., 2009). Immunohistochemical analysis indicates that although there is a significant increase in microglial markers in the infant dorsal horn spinal cord following nerve injury, this was substantially less than in adults (Moss et al., 2007; Vega-Avelaira et al., 2012). Whereas nerve injury in adults is associated with an infiltration of T-cells associated with the Th1 pro-inflammatory response in the dorsal horn spinal cord this occurs to a substantially lesser extent, if at all, in infants following nerve injury (Costigan et al., 2009). Finally, a key characteristic of adult neuropathic pain is an induction of a pro-inflammatory response and the release of pro-inflammatory immune mediators in the dorsal horn, critical for sensitization and pain-like hypersensitivity (Taves et al., 2013; Tsuda et al., 2013) that is absent when the same surgery is performed before P21 at least (Costigan et al., 2009; Moss et al., 2007; Vega-Avelaira et al., 2007).

These studies have ascribed the absence of neuropathic pain behaviour following nerve injury in infant rodents to immature neuroimmune pathways, rather than a failure in pain circuitry which are summarised in Fig 1.6A

(Costigan et al., 2009; Moss et al., 2007; Vega-Avelaira et al., 2012). Recent longitudinal studies in rats have revealed a previously unsuspected aspect of neuropathic pain, that animals that undergo nerve injury in early life do eventually develop pain hypersensitivity but only when they reach adolescence, age P31 (Vega-Avelaira et al., 2012). A similar delayed onset was observed in neuropathic autonomy in infant animals (Wall et al., 1979).

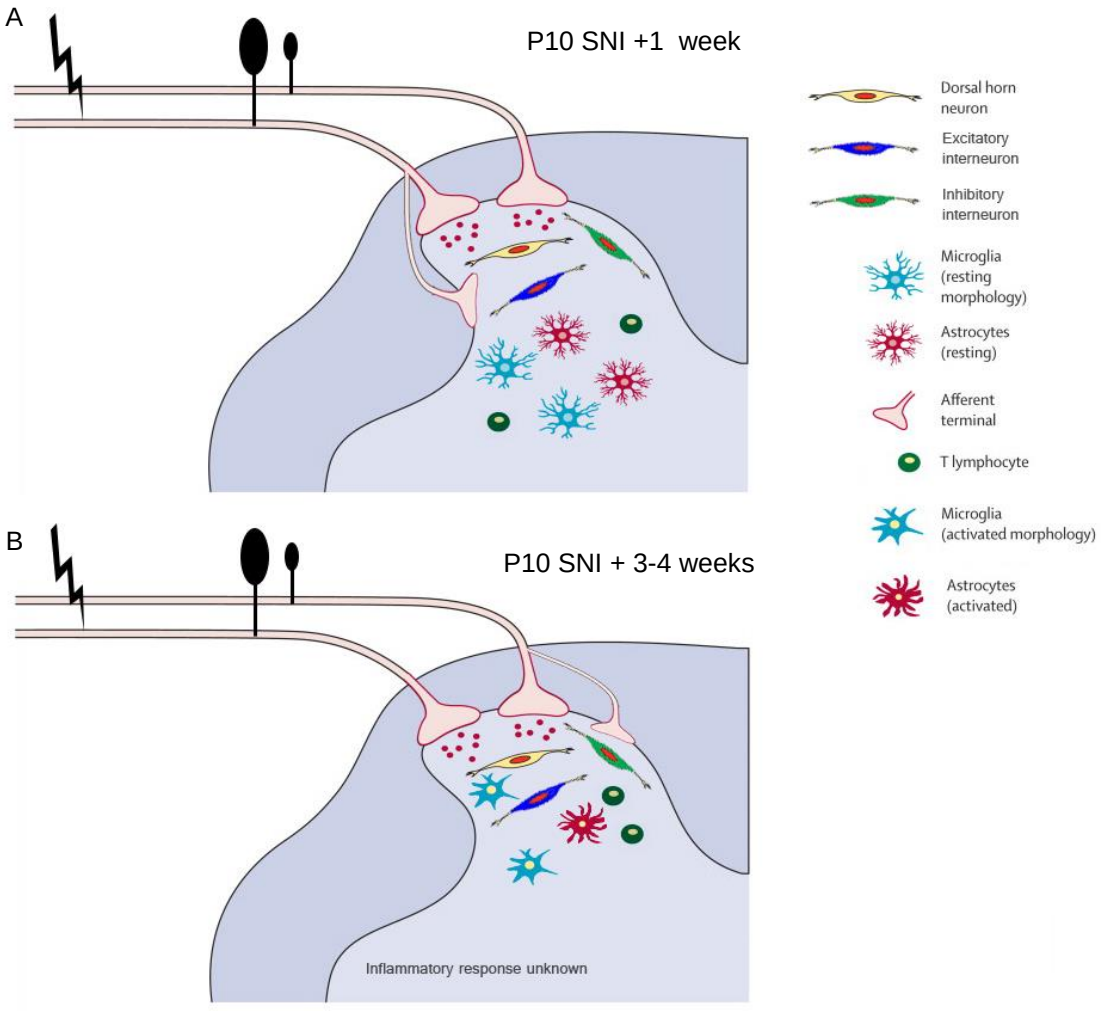


Fig 1.6 Cellular activity in the dorsal horn in the early and late phase after infant SNI.

A) 1 week after P10 SNI (when the animal is P17) A-fibres are withdrawing from the superficial dorsal horn. Pro-inflammatory mediators are absent. Some astrocyte and microglial makers are up-regulated, others remain the same compared to controls. B) 3-4 weeks after P10 SNI (when the animal is P31-P38) A-fibres have withdrawn from the superficial dorsal horn. Astrocyte (GFAP) and microglial (IBA-1) immunohistochemistry markers appear up-regulated. Reprinted from The Lancet, 11, Calvo, Daves and Bennett, The role of the immune system in the generation of neuropathic pain, page 629-642., Copyright (2012), with permission from Elsevier.

Together these data raises the intriguing possibility that neuropathic pain is not so much absent in the infant but suppressed until the animal reaches adolescence. At this time there is an increase in immune-reactivity of IBA-1 and GFAP (microglial and astrocytes respectively). However the presence and inflammatory mediators at this age has not yet been investigated (see Fig 1.6b)

The understanding of postnatal maturation of T-cells may provide some underlying clues as although initially it was thought that neonatal T-cells were unresponsive in the neonatal period, leading to the susceptibility of new-borns to microbial infection (Gammon et al., 1986; Lawn et al., 2005) this interpretation did not explain the vulnerability of infants to immune mediated allergic reactions. In the 1980s it was recognised that T-cells could be classified into Th1 or Th2 and it was only then that it was recognised that infants are capable of mounting an immune response (Ridge et al., 1996; Sarzotti et al., 1996) but has a strong bias towards a Th2 function (Adkins et al., 2000; Forsthuber et al., 1996; Min et al., 2000; Powell and Streilein, 1990; Singh et al., 1996). Furthermore, under certain conditions neonates may also mount a potent Th1 response such as following some infections (Forsthuber et al., 1996; Jupelli et al., 2010; Sarzotti et al., 1996). This raises the following questions; is the immune response immature and unable to respond to insult? Or are immune processes under postnatal developmental regulation?

Although little work has been undertaken to identify whether microglia alter their phenotype and functions over postnatal development recent studies show that microglia play varied and key physiological roles in the postnatal development of the nervous system including remodelling and refining the developing nervous system by removing excess axonal projections and promoting neurogenesis (Baskar Jesudasan et al., 2014; Lai et al., 2013; Schafer et al., 2012; Scheffel et al., 2012; Tremblay et al., 2010). These studies highlight that the phenotype of immune mediators alter depending on the age of the animal and region in the CNS.

1.7 Aims of the thesis

The aims of the experiments presented in this thesis were as follows;

1. To examine the early and delayed behavioural consequences of a peripheral nerve injury sustained at infancy to a range of stimuli.
2. To characterise the properties of sensory dorsal horn cells *in vivo* during the early and delayed period following early life peripheral nerve injury and to identify if changes in dorsal horn neuronal activity parallel changes in behaviour.
3. To characterise the immune profile in the dorsal horn in the early and late period following infant peripheral nerve injury and test if the absence of pain-like behaviour is caused by underlying immune alterations.

Chapter Two

The delayed onset of pain-like behaviour following infant nerve injury

2.1 Introduction

Animal models of nerve injury have been an essential tool, enabling the elucidation of some of the key mechanisms underlying a variety of neuropathic pain symptoms. More recently, these have also been replicated in infant rats to illustrate that if a nerve injury is performed before P33, pain-like behaviour is absent, which is in stark contrast to the robust hypersensitivity modelled in adult rodents following the same nerve injury(Howard et al., 2005, Decosterd and Woolf., 2000). A study by Vega-Avelaira et al., showed that following infant nerve injury (SNI), mechanical hypersensitivity does eventually emerge when the animal reaches adolescence (Vega-Avelaira et al., 2012). Before the mechanisms underlying this phenomena were investigated in subsequent chapters of this thesis, this chapter further tests the delayed onset of pain-like behaviour model and identifies if hypersensitivity develops later to other sensory modalities, in addition to mechanical stimulation. The delayed onset of pain-like hypersensitivity model following infant nerve injury (SNI) is also tested in mice. As nerve transection in the early neonatal period in rodents can result in extensive neuronal loss of DRG and interneurons, the degree of neuronal loss following infant SNI is also investigated seven days after surgery (Cheema et al., 1984; Oliveira, A et al., 1997; Yip et al., 1984).

2.1.1 The SNI model of neuropathic pain

The SNI model of neuropathic pain was first described by Decosterd and Woolf and mimics partial denervation, a common cause of neuropathic pain in patients (Decosterd and Woolf., 2000; Koltzenburg, 1998). The surgery comprises of exposing the sciatic nerve, ligation and lesion of two of the three terminal distal branches of the sciatic nerve (the tibial and common peroneal nerves) while leaving the sural nerve intact (Fig 2.1A). This permits behavioural testing of the non-injured skin territories in the area innervated by the sural nerve which is located on the lateral plantar (Fig 2.1B) and dorsal paw surfaces and is adjacent to the denervated areas.

The SNI model has a number of advantages over earlier nerve injury animal models such as the Seltzer partial sciatic nerve ligation (PSL) model and the Bennett chronic constriction injury (CCI) model (Bennett and Xie, 1988; Seltzer et al., 1990). In these models ligation/chronic constriction of the sciatic nerve induces swelling and strangulation which leads to the development of thermal and mechanical hypersensitivity in the ipsilateral hind paw.

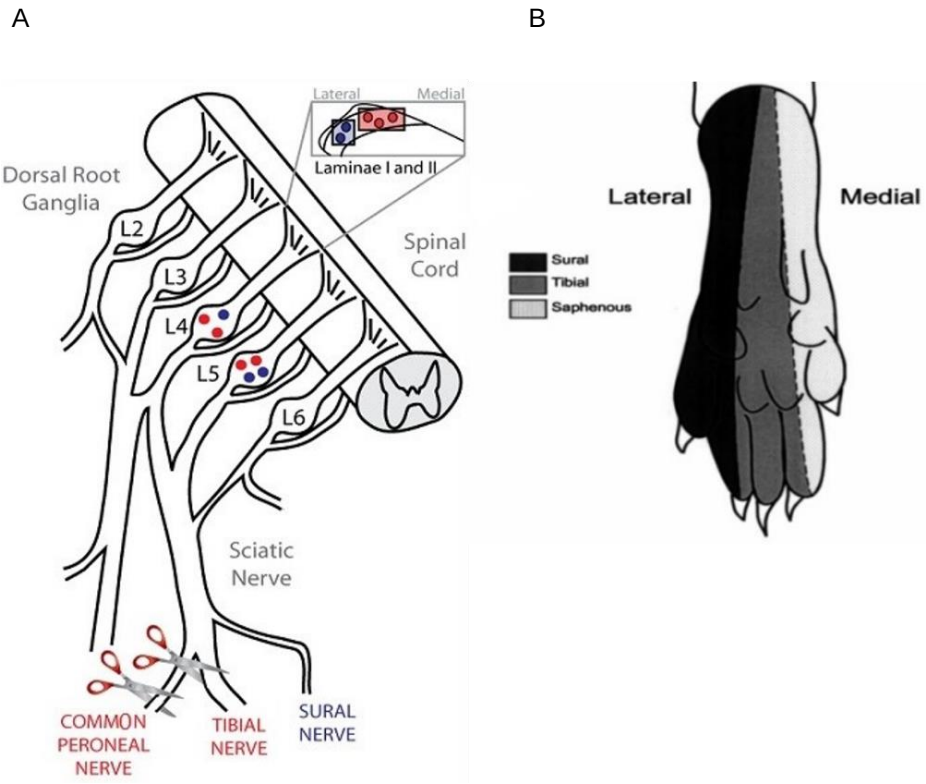


Fig 2.1 Spared nerve injury model of neuropathic pain.

(A) Diagram of the sciatic nerve, terminal branches, dorsal root origins (L4-L6 of the dorsal root ganglion) and dorsal horn innervation pattern. (B) Different areas on the plantar surface of the paw innervated by the sciatic nerve. Reprinted from Pain, Spared nerve injury: an animal model of persistent peripheral neuropathic pain, 87, Decosterd and Woolf, 149-158, (2000).

However, inevitably the extent of damage is difficult to reproduce and leads to variability in behavioural outcomes (Jaggi et al., 2011). Another widely used model, the Chung spinal nerve ligation (SNL), which involves putting a tight ligature of two spinal segmental nerves (L5 and L6) close to the intact DRG and L4 spinal nerve (Ho Kim and Mo Chung, 1992). Although this model enables behavioural assessments of the intact dermatomes of the paw, there is a risk of damage and/or exposure of the intact L4 spinal nerve to inflammation. In contrast to the aforementioned peripheral nerve injury animal

models, the SNI model is advantageous as the surgery is simple, requiring the transection of two sciatic nerve branches, which is less invasive and does not give rise to excessive inflammation. Importantly this surgery ensures a long lasting injury and has a 100% success rate (Decosterd and Woolf, 2000; Pertin et al., 2012). The consistency of this model is particularly helpful when comparing behavioural changes in animals of different ages and sizes as was completed in this chapter. Adult mice also develop hypersensitivity following SNI surgery while younger animals do not (Bourquin et al., 2006; Pertin et al., 2012; Shields et al., 2003).

It is widely believed that gender differences in pain exist and that this is mediated by gonadal steroid hormones (Craft et al., 2004; Greenspan et al., 2007) with female human and non-human animals exhibiting lower pain thresholds and more hypersensitivity than males (Aloisi et al., 1999; Wiesenfeld-Hallin, 2005). However, both male and female rats and mice develop robust neuropathic pain-like behaviour following nerve injury (Bourquin et al., 2006; Casals-Díaz et al., 2009). However, as this study involves the longitudinal assessment of behaviour from infancy to adolescence and the effects of sex remain relatively untested in this nerve injury model, the decision was made to carry out all experiments in male rats and mice only.

2.1.2 Behavioural responses in the adult rodent following SNI

Symptoms reported by patients suffering nerve injury include spontaneous pain, tactile and cold hypersensitivity and pinprick hypersensitivity, all of these are replicated in animals that undergo SNI (Decosterd and Woolf., 2000; Pertin et al., 2012). These hypersensitivities in the rodent have a rapid onset (<24h post-surgery) and prolonged duration (at least 6 months) (Decosterd and Woolf, 2000; Erichsen and Blackburn-Munro, 2002; Leith et al., 2010; Woolf and Mannion, 1999). In SNI treated rodents sensitivity to basal heat remains unchanged which is in contrast to other nerve injury models (such as CCI and SNL) which give rise to thermal hypersensitivity and is a reflection of a reduction in the activation threshold of nociceptor peripheral terminals (i.e. peripheral sensitization) (Baron, 2006; Koltzenburg, 1998; LaMotte et al., 1982; Woolf and Mannion, 1999). However, a number of studies suggest that

thermal hyperalgesia is not a typical feature of clinical neuropathic pain (Baron, 2006; Koltzenburg, 1998; LaMotte et al., 1982; Woolf and Mannion, 1999).

2.1.3 Behavioural responses in the infant rodent following SNI

Compared to adult models of nerve injury, comparatively little is known about the behavioural consequences and the time course following infant nerve injury. A relatively recent study has shown that nerve injury in infant rat pups fails to induce hypersensitivity if performed in rat pups before 4 weeks of age (Howard et al., 2005; Ririe et al., 2006; Vega-Avelaira et al., 2007). Only when nerve injury is performed at postnatal day 33 does mechanical hypersensitivity develop in the affected limb 7 days after surgery, as in adult rodents. A recent longitudinal study which examined the long term consequences of early-life nerve injury upon mechanical hypersensitivity showed that SNI at postnatal day 10 (P10) did not induce mechanical hypersensitivity for the first three weeks after surgery (Vega-Avelaira et al., 2012). However, mechanical hypersensitivity did develop on the ipsilateral hind paw when the animal reached P38 (Vega-Avelaira et al., 2012) and was maintained for as long as testing continued (44 days). As in adults, SNI in infant rats did not change thermal behavioural thresholds at any time after surgery (Decostered and Woolf, 2000). This suggests that the delayed development of mechanical hypersensitivity was not due to a non-specific motor deficit but was a sensory response to early life peripheral nerve injury.

These data led to the hypothesis that nerve injury in early life can affect pain processing later in life. The work in this thesis aims to decipher the possible mechanisms that underlie these age dependent changes in pain perception following nerve injury. However, before these mechanisms can be investigated a more thorough analysis of behavioural modifications and their time course was required, testing responses to a range of sensory modalities. This would enable the paradigm to be applied to further investigate the mechanisms underlying age dependent differences in pain-like behaviour following nerve injury. In extension to this work it is currently unknown if mice also display a delayed onset of mechanical hypersensitivity following infant nerve injury. Therefore this paradigm was also applied to mice to further validate the model and test the hypothesis that mice would display a delayed onset of mechanical

hypersensitivity following infant nerve injury. This novel application to mice is favourable so that future experiments can take advantage of genetically altered mice.

2.1.4 Neuronal degeneration of interneurons in adults following nerve injury

In adults there is some evidence that proposes that adult peripheral nerve injury causes neuronal degeneration in the DRG and dorsal horn spinal cord (Moore et al., 2002; Polgár et al., 2005; Scholz et al., 2005; Tandrup et al., 2000; Whiteside and Munglani, 2001). These studies have used a combination of complementary methods to study neuronal degeneration and include labelling of dorsal horn sections with Terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL), that detects DNA fragmentation from apoptotic signalling cascades and cells that have suffered severe DNA damage (Gavrieli et al., 1992) as well as labelling of Caspase-3, a protease that mediates apoptosis and precedes DNA damage (Degterev et al., 2003; Polgár et al., 2005).

In addition to labelling, stereological analysis of the numbers of neurons in a specified volume of the dorsal horn (packing density) has also been utilised (Coggeshall, 1992). However these studies draw different and conflicting conclusions. While all studies report no significant increases in TUNEL or Caspase-3 positive cells on the contralateral side to nerve injury, a number report significant increases in cell death 7 days after SNI surgery (Moore et al., 2002; Scholz et al., 2005; Whiteside and Munglani, 2001). These significant increases described equates to an increase of 1 and a quarter TUNEL or Caspase-3 positive cells per section. Scholz et al (2005) reported that the cell death observed in the superficial dorsal horn (lamina I-III) occurred specifically in GABAergic inhibitory interneurons together with a marked decrease in inhibitory postsynaptic currents, 4 weeks after SNI (Scholz et al., 2005).This paper suggests that 7 days after peripheral nerve injury the greatest degree of cell death is observed in interneurons, with no cell death occurring after 21 days. Transplantation of GABAergic neuronal precursors from the mouse medial ganglionic eminence into the spinal cord of mice 7 days after SNI

reduced SNI-induced mechanical hypersensitivity within two weeks of injury (Bráz et al., 2012).

However, an extensive study by Polgar et al., (2005) refutes this, reporting no detectable loss of neurons from laminae I-III in the ipsilateral dorsal horn 1 or 4 weeks after SNI in any of the three methods used (Polgár et al., 2005). This group also showed that following adult nerve injury, both Caspase-3 and TUNEL staining did not co-label with NeuN, but with the astrocyte marker glial fibrillary acidic protein (GFAP) and a microglia marker ionized calcium-binding adapter molecule 1 (IBA-1) (Gehrmann et al., 1995; Polgár et al., 2005) which would suggests a significant loss of neurons from the dorsal horn is not necessary for the development of pain-like behaviour in the SNI model.

2.1.5 Naturally occurring neuronal degeneration in infants

The extent of neuronal degeneration of spinal interneurons, occurring naturally over postnatal development, or following nerve injury, is not as well described as that of more clearly defined and quantifiable neuronal groups, such as the dorsal root ganglion (DRG) or spinal motor neurons. These systems have relatively few synaptic connections and projections, making them simple to manipulate and investigate (Harris and McCaig, 1984; Himes and Tessler, 1989; Kashihara et al., 1987; Tandrup et al., 2000). In contrast, cell death of spinal interneurons, a heterogeneous group in terms of number, connections, and spatial relationship, have been relatively neglected (Lowrie and Lawson, 2000).

During development, it is now well established that both DRG neurons and motor neurons undergo a period of naturally occurring neuronal degeneration which is regulated by signals from their inputs and synaptic targets. Evidence for this largely comes from the fact that the occurrence of neuronal degeneration is at the time when contact is being made with the main synaptic targets to enable size matching of synaptically linked populations (Coggeshall et al., 1994; Lowrie and Lawson, 2000). Naturally occurring cell death in DRG cells occurs between E 17-19, with some continuing till P5. In motor neurons, degeneration occurs exclusively prenatally during a critical period that peaks at E16, coinciding with the formation of neuromuscular connections

(Coggeshall et al., 1994; Hamburger and Oppenheim, 1990; Harris and McCaig, 1984). Interestingly, if a target organ (limb bud) is removed or added before this critical period, natural motor neuron cell death is increased or decreased respectively (Hamburger, 1958; Oppenheim, 1991). In contrast, studies into naturally occurring degeneration of interneurons are conflicting. A number of early studies in the spinal cord and brain nuclei of the chick found no evidence for interneuron cell death during development (McKay and Oppenheim, 1991). However, one study in the rat pup by Lawson et al., observed neuronal degeneration in the dorsal horn spinal cord on and just after birth that fell sharply until P10, where it was negligible (Lawson et al., 1997). As these cells were widely distributed throughout the grey matter of the dorsal horn, it suggests that the low level of degeneration is not restricted to a subset of interneurons.

2.1.6 Neuronal degeneration in infants following nerve injury

It is widely established that disruption of synaptic connections during an early postnatal age, a critical period of plasticity, can have extensive consequences. For example, similarly to the developmental studies, the effect of nerve injury on neuronal degeneration in neonates is most commonly described following P0 peripheral axotomy in the DRG, (as opposed to interneurons) which induces extensive (between 50-75%) degeneration of axotomised DRG cells, occurring rapidly (in a 24 hour period) (Aldskogius and Risling, 1981; Himes and Tessler, 1989; Yip et al., 1984). Following this, lesioned cells withdraw from the central terminals while the undamaged adjacent collaterals sprout into the denervated region (Bondok and Sansone, 1984) and form synaptic connections in inappropriate laminae in the dorsal horn spinal cord (Fitzgerald et al., 1990; Fitzgerald, 1985; Shortland and Fitzgerald, 1994).

However, the extent of neuronal degeneration following peripheral injury is age dependent with early neonatal animals exhibiting more extensive neuronal degeneration than older animals. For example, axotomy in P10, P21 or adult animals does not cause any DRG cell loss 7 days after injury (Beggs., 2000; Coggeshall et al., 1997; Himes and Tessler, 1989; Li et al., 1994). This difference may be due to the requirement of peripheral sensory neurons for neurotropic support in the early postnatal period (Himes and Tessler, 1989;

Lewis et al., 1998; Schmalbruch, 1984; Tandrup et al., 2000; Whiteside et al., 1998).

Even less is known about the effects of early life nerve injury on interneurons. One study indicated that nerve crush at P2 did cause a low but significant increase in interneuron degeneration 4 days after injury compared to un-operated animals, but not compared to the contralateral dorsal horn (Lawson and Lowrie, 1998). Importantly, different nerve injuries are also likely to cause a variation in the extent of neuronal degeneration of these interneurons. For example, in adults, sciatic nerve transection, nerve crush, SNI and CCI induced interneuron degeneration to a significant but varying extent, with SNI inducing the least and SNL the greatest interneuron cell loss in the L5 spinal segment (Li et al., 1995; Parrilla-Reverter et al., 2009; Scholz et al., 2005).

Together these studies indicate that the extent of neuronal degeneration is dependent on the type of nerve injury (axotomy, SNI, CCI), the age that the injury occurred (P0, P10, adult) and the group of cells involved (DRGs, motor neurons, interneurons). As studies in the adult suggest that interneuron degeneration following peripheral nerve injury is observed greatest at 7 days after SNI and that a nerve injury at P0 causes extensive neuronal cell death, it is important to first establish if extensive neuronal degeneration of interneurons occurs following SNI at P10.

2.2 Aims of the chapter

In adult rats, the behavioural modifications following SNI is well described (Bourquin et al., 2006; Decosterd and Woolf, 2000), and interneuron degeneration may occur 7 days following nerve injury (Polgár et al., 2005; Scholz et al., 2005). However, in the infant behavioural modifications and their time course following the same injury is relatively unexamined and it is unknown if nerve injury induces extensive interneuron death in the dorsal horn spinal cord. The behavioural modifications in infant mice following peripheral nerve injury also remains to be elucidated. The key objectives of this chapter are to:

1. Complete the longitudinal behavioural profile in infant (P10) rats following SNI to a range of sensory stimuli including mechanical, innocuous cold, noxious cold, thermal stimulation and weight bearing.
2. Establish if the same pattern of delayed SNI-evoked mechanical hypersensitivity that occurs in infant rats is also observed in infant mice.
3. To examine the extent of neuronal degeneration following infant SNI in rats to include dorsal horn interneurons.

The immunostaining experiments in this chapter were completed with the assistance of BSc student Liam O'Leary

2.3 Materials and Methods

2.3.1 Animals

Surgery was performed on male Sprague–Dawley rats, obtained from UCL Biological Services or male CD1 mice, from Charles River. Surgery was performed either on young adult rodents (rats and mice) at postnatal day 33 (P33), which were housed in littermates of five or in infant rodents at P10, that were housed with their mother and littermates (Moss et al., 2007; Vega-Avelaira et al., 2012, 2007). Weaning of both rats and mice were completed at P21. All animal procedures were licensed by the UK Home Office (London, United Kingdom) and experiments were performed in accordance with the UK Animal (Scientific Procedures) Act 1986.

2.3.2 Animal Surgery

Spared nerve injury (SNI) and sham surgery were performed on P10 and P33 animals under general anaesthesia with 2-3% isoflurane in oxygen (Abbott Animal Health, Queensborough, UK) with antiseptic conditions (Chaplan et al., 1994). SNI surgery was performed by moving the biceps femoris muscle to expose the sciatic nerve and its three terminal branches in the upper lateral thigh. The common peroneal and tibial branches were tightly ligated with a 5.0 silk suture and transected distally while the sural nerve was left intact. In sham animals the sciatic nerve was exposed but not ligated. In all cases care was taken not to touch, stretch or damage intact nerves. Muscle and skin were then closed in two layers. After surgery, animals were returned to their cages and litters and maintained on a 12 hour light/dark cycle at constant ambient temperature in the Biological Services Unit. Animals had free access to food and water until the next procedure.

2.3.3 Animal behaviour

Both rats and mice were habituated to the testing environment for four days prior to sensory testing. Behavioural acclimation was allowed until cage exploration and major grooming activities ceased. In rats, sensory reflex withdrawal thresholds of the hind paw to mechanical, thermal radiant heat stimulation, acetone and ethyl chloride stimulation were measured in the

ipsilateral and contralateral paw, together with weight bearing 1 day prior to surgery (baseline) and every 7 days after surgery, not extending beyond 35 days post-surgery (Fig. 2.2). In mice only mechanical thresholds were tested until there was a drop in the mechanical threshold of the ipsilateral paw. For all tests stimuli were applied to the lateral plantar surface of the hind paw in the sural nerve innervated region. Each sensory test was followed by at least a 10 minute interval and all tests were performed during the day portion of the circadian cycle only (06:00-18:00 h).

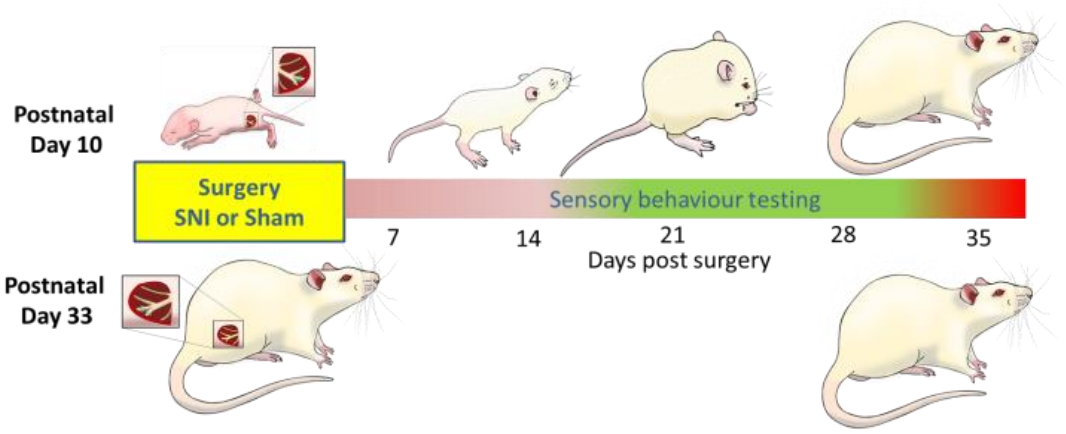


Fig 2.2 schematic diagram showing the design of the longitudinal behavioural study in rats.

Behavioural testing was carried out every 7 days following P10 or P33 SNI or sham surgery.

2.3.4 Mechanical sensory testing

Animals were placed into Perspex boxes on an elevated metal mesh floor until they had acclimated to the environment. Hind-limb flexion withdrawal reflex thresholds in response to mechanical stimulation of the hind-paw were determined using von Frey hairs (vFh) (Stoelting, Woodvale, Il). VFhs are calibrated nylon monofilaments that exert a reproducible stimuli measured in grams that increase logarithmically in stiffness (0.02–2.56 g, Stoelting). A vFh was presented perpendicular to the dorsolateral plantar surface of the ipsilateral or contralateral hind paw in the sural nerve territories and held for 2 seconds with enough force to bend the filament (Decostered and Woolf, 2000). The 50% paw withdrawal threshold was determined using the up-down method (Chaplan et al., 1994). The starting vFh used for mice was vFh #5 and for rats,

vFh #9. Based on the first response (negative or positive) the next filament was then presented which was either a higher or lower filament respectively (up and down). For example, if the animal did not respond to filament #5 the next filament presented was #6. If the animal did respond to #6 by a withdrawal of the hind paw, the next filament presented would be #5 again. Following the first change in direction (up to down or down to up) filaments were applied 4 more times, regardless of whether the animal responded or not. Using this pattern of positive responses (represented by an X) and negative responses (represented by a 0) the result was tabulated and the 50% response threshold was interpolated using the formula as outlined by Chaplan et al, 1994:

$$50\% \text{ g threshold} = (10^{(Xf+k\delta)}) / 10{,}000$$

Where Xf = value (in log units) of the final vFh used; kappa = tabular value for the pattern of positive/negative responses; and δ= mean difference (in log units) between stimuli which is 0.224.

2.3.5 Heat sensory testing

To test heat withdrawal latency, rats were placed in the same Perspex cages used for mechanical sensory testing. The lateral plantar surface of the hind paw was exposed to a beam of radiant heat (Hargreaves et al., 1988) and the withdrawal latency (seconds) was recorded. This was repeated twice at an interval of 5 minutes on each paw and the mean of each paw calculated. A cut off latency of 20 seconds was set to avoid tissue damage (Kiguchi et al., 2010).

2.3.6 Cold sensory testing

Rats were kept in the same Perspex boxes as above and two different cooling stimuli were applied to the lateral paw, acetone (Fisher Scientific) and ethyl chloride (Acorus Therapeutics LTD) to examine cold responses. Acetone was applied to the hind paw by using a syringe with tubing attached to create an acetone bubble which was placed on the lateral plantar surface for one second (Flatters and Bennett, 2004). Ethyl chloride was applied as a spray to the lateral planter surface of the hind paw for one second. Both coolants spread over the whole hind paw and the rat's response was measured on a four point scale as described by Flatters and Bennett, (2004). Over the first 20 seconds

if, no response was seen the trial was recorded as 0. If the animal did respond, behaviour was assessed for another 20 seconds (to a total of 40 seconds). This length of time was used as the behaviour evoked by acetone can be in the form of an episodic sequence and to ensure that only cooling pain behaviour was measured, as opposed to the startle response. Responses were graded on a four point scale; 0, no response; 1, a quick withdrawal, flick or stamp of the paw; 2, prolonged withdrawal or repeated flicking (>2) of the paw; 3, repeated flicking of the paw with licking directed at the ventral side of the paw. The test was repeated 5 minutes later and the mean response of each paw was calculated.

2.3.7 Weight bearing evaluation

Hind limb weight bearing was measured using an incapacitance tester (Churchill Electronic Services Ltd). The rats were placed in a Plexiglas box chamber designed so that each hind paw rested on a separate transducer pad with front paws resting on a slope. For rat pups the forelimbs were supported by a gloved finger in the chamber. The load on each transducer was recorded over a 5 second period. For each rat three readings from each paw were taken and then averaged. The results are presented as the percentage of ipsilateral weight of the total weight bearing on the ipsilateral and contralateral paws.

2.3.8 Spinal cord preparation and sectioning

Spinal cords were harvested from rats 7 days after P10 SNI or sham surgery for immunohistochemistry analysis or FJC staining (a total of 18 rats). Rats were terminally anaesthetised with an intraperitoneal overdose of pentobarbital (Euthatal, 100 mg/kg, Merial Animal Health Ltd, UK) and transcardially perfused with heparinised saline (0.5% heparin; Monoparin, CP Pharmaceuticals, UK) in 0.9% NaCl (Baxter, Belgium) followed by 4% paraformaldehyde in 0.1M phosphate buffered saline (PBS). A laminectomy was completed and the spinal cords were carefully dissected and removed with the dura intact to minimize any physical trauma to the cord. The fourth and fifth lumbar segments (L4 and L5) of the spinal cord were then cut and a slice was made into the contralateral ventral surface of the cords using a razor. This enabled the easy identification of the ipsilateral and contralateral spinal

cord of sham and SNI tissue during the analysis stage. Spinal cords were then post fixed for 6 hours in 4% paraformaldehyde and transferred to 20% sucrose in 0.1 M PBS for 18–36 h. Finally, spinal cords were stored at 4°C in 30% sucrose in 0.1 M PBS until staining procedures were carried out.

To section, spinal cords were frozen rostral cord up onto a microtome (Leitz Wezlar, Germany) using 30% sucrose and dry ice. Serial transverse sections of spinal cord were then cut (14µm for Fluoro-Jade C staining and 40µm sections for immunohistochemical analysis) and stored in a 0.1 M phosphate buffer (PB) solution containing 5% sucrose and 0.02% azide.

2.3.9 NeuN and IB4 immunohistochemical staining

To identify if there was a change in the overall density of neurons in the spinal cord 7 days following P10 SNI, an antibody against neuronal nucleic marker (NeuN) was used that binds to a neuron specific nuclear protein and is expressed from early in development (Sarnat et al., 1998). An antibody against isolectin B4 (IB4) was also used to label the non-peptidergic subpopulation of C-fibres that project principally to the interior of lamina II of the dorsal horn (Wang et al., 1994), from the early postnatal period (Jennings and Fitzgerald, 1998).

First, 40µm free floating sections were blocked in 0.1 MPB solution containing 10% normal goat serum (NGS, Vector) and 0.3% Triton-X (BDH) for 1 hour. Sections were then incubated in primary antibody mouse polyclonal antibody anti-NeuN (1:5000, Chemicon, USA) and anti-IB4 (Griffonia simplicifolia) labelled with fluorescein isothiocyanate (FITC, 1:1000; Sigma-Aldrich Inc.) for 24 hours at room temperature. Sections were then washed three times in 0.1M PB for 10 minutes and then incubated with the secondary goat anti-mouse polyclonal antibody Alexa-593 (1:500, Invitrogen, Eugene, Oregon, USA). After two washes in 0.1 M PB, sections were mounted onto gelatinized slides and left to air dry for 50 minutes. Finally, slides were cover slipped in fluoromount (Sigma-Aldrich Inc). Antibody specificity was ensured by excluding the primary antibody to test for antibody specificity and by excluding the secondary antibody to identify any primary antibody fluorescence. Both controls were negative in accordance with published data (Cavallaro et al.,

2008; Fang et al., 2006). Following staining, slides were kept in the dark for 20 hours to normalize the background and then imaged using on a microscope at 10X magnification with OpenLab® software. Slides were coded and examined under the appropriate wavelength fluorescent microscopy and the images were visualised using image J 1.36 (NHS) software.

2.3.10 Fluoro-Jade C staining

Fluoro-Jade C (FJC) is a sensitive and high resolution marker for degenerating neurons (Bian et al., 2007; Schmued et al., 2005; Schmued and Hopkins, 2000) which has been used to identify neurodegeneration in a range of tissues, including the infant rat spinal cord (Walker et al., 2010; Westin et al., 2010). As FJC only stains neurons, it does not generate false positives, unlike more general stains of dying cells including caspase-3 and TUNEL (Polgár et al., 2005).

First, 14 µm L4-L5 spinal cord sections were placed into nets and rinsed in distilled water and then immersed in 1% sodium hydroxide in 80% ethanol for 5 minutes. This was followed by immersion in 70% ethanol for 2 minutes and 2, 2 minute rinses. Sections were placed in 0.06% potassium permanganate solution for 10 minutes followed by 10 minutes in 0.0002% solution of FJC (Chemicon, Temecula, CA) and 0.01% of 4'6- diamidino-2-phenylindole (DAPI; Molecular Probes, Eugene, OR) dissolved in 0.1% acetic acid vehicle. Sections were rinsed in distilled water and sections were mounted onto gelatinized slides and left to air dry for 50 minutes before cover slipping with DPX non-fluorescent mounting media. Staining specificity was confirmed by the absence of staining in controls where FJC had been excluded from the protocol. Following staining, slides were kept in the dark for 20 hours to normalize. Microscope images were acquired at a 40X magnification with OpenLab® software. Slides were coded and examined under the appropriate wavelength fluorescent microscopy. The images were visualised using image J 1.36 (NHS) software and analysis was performed under blind conditions. For each section, the number of FJC cells that co-labelled with DAPI were counted under blind conditions. DAPI was used to identify the location of sections under the microscope and ensure the FJC stain was only apparent in the soma of cells. For each animal, a positive co-localised cell was drawn as a dot on to a

spinal cord diagram to enable comparisons between ipsilateral, contralateral sides and dorsal versus the ventral horn. Finally, the counts from 10 sections containing the highest number of FJC counts were averaged for each animals and used for statistical analysis.

2.3.11 Data analysis

Data was analysed and graphs plotted using GraphPad Prism software (version 6.00, GraphPad Software, San Diego, CA, USA, www.graphpad.com). All behavioural data was first tested for normality and homogeneity of variance using Shapiro-Wilk and Levene tests respectively. If data was not normally distributed, logarithmic transformation (log 2) was carried out and all paw withdrawal thresholds (g) before statistical analysis as previously described (Geranton et al, 2009 Baumgartner et al, 2002; Sens et al, 2012). Behavioural data was then analysed by a repeated measure two-way analysis of variance (ANOVA) (time and surgery), adjusted for multiple comparisons using the Bonferroni correction analysis where appropriate. A two tailed student's *t-test* was used to compare FJC counts between SNI and sham animals. The criterion for statistical significance was $p < 0.05$. All data are presented as mean±standard error of the mean unless otherwise stated.

2.4 Results

The effects of infant (P10) and adult (P33) SNI on the responses of the hind paw to mechanical, innocuous cold (acetone), noxious cold (ethyl chloride), thermal cutaneous stimulation and contralateral weight bearing were investigated in a longitudinal behavioural study.

2.4.1 Longitudinal study of mechanical paw withdrawal thresholds following SNI in infant and adult rats.

SNI in adult rats causes a well-established, significant and persistent mechanical hypersensitivity on the ipsilateral hind paw 7 days post-surgery compared to controls (SNI contralateral paw and the sham ipsilateral paw) (Fig 2.3A) when the mechanical threshold of the ipsilateral SNI paw drops to 3.73g±0.72 compared to the SNI contralateral paw (14.00g±2.8, $P<0.0001$, Bonferroni post-test) and the sham treated ipsilateral paw (11.75g±2.43, $P=0.0007$, Bonferroni post-test) which was maintained for at least 3 weeks post-surgery (Decosterd and Woolf, 2000).

Similarly SNI in adult mice causes a significant mechanical hypersensitivity on the ipsilateral hind paw post-surgery compared to controls (Fig 2.3C). Four days post SNI, the mechanical thresholds on the ipsilateral paw were significantly lower (0.44g±0.14) compared to the SNI contralateral paw (1.74g±0.27, $P<0.0001$, Bonferroni post-test) and the sham treated ipsilateral paw (1.89g±0.38, $P<0.0001$, Bonferroni post-test). Time was only significantly different in the SNI ipsilateral paw from 0 to 3 days post SNI surgery ($P<0.01$, Bonferroni post-test).

The same SNI surgery in infant rats had no effect on the ipsilateral hind paw mechanical threshold for the first three weeks after infant surgery (Howard et al, 2005) (Fig 2.3B). Only at 28 days after SNI, when infant rats reached P38 was there a significant difference. At this time point the mechanical threshold of the SNI ipsilateral hind paw fell (5.47g±1.2) significantly below the SNI contralateral hind paw mechanical threshold (15.65g±2.8, $P<0.001$ Bonferroni post-test) and sham ipsilateral hind paw mechanical threshold (12.89±3.75 $P<0.01$; Bonferroni post-test) (Vega-Avelaira et al., 2012). The mechanical hypersensitivity on the ipsilateral paw was maintained until the end of the

experiment (35 days post-surgery). As previously described, mechanical thresholds increase normally with postnatal age from P10, P17, P24, P31 to be significantly greater at P38 in both the SNI contralateral and sham ipsilateral paws ($P < 0.05$, Bonferroni post-test). There was no effect of time on the SNI ipsilateral paw (Howard et al., 2005; Vega-Avelaira et al., 2007).

Infant mice also showed a delay in the development of mechanical hypersensitivity following infant SNI (Fig 2.3D) remaining similar to controls for the first three weeks after surgery. Only at 21 days post-surgery, when infant mice reached P31 did mechanical thresholds in the SNI treated ipsilateral paw fall 5 fold below controls. In infant mice there is a significant increase in the mechanical thresholds in control groups from P10, P17, P24 to P31 and not the SNI ipsilateral paw ($P<0.001$, Bonferroni post-test).

2.4.1.1 Longitudinal study of weight bearing, cool, cold and thermal hypersensitivity following SNI in infant and adult rats.

The delayed-onset of infant hypersensitivity post-nerve injury was not restricted to mechanical tests. Fig 2.4 B, D and F show that other tests of hind paw sensitivity including weight bearing and cold sensitivity (measured by application of acetone and ethyl chloride) displayed a similar pattern of delayed adolescent onset hypersensitivity. Only hind paw heat sensitivity was unaffected in both infant and adult groups compared to controls (Fig 2.4H).

Mechanical (vFh)

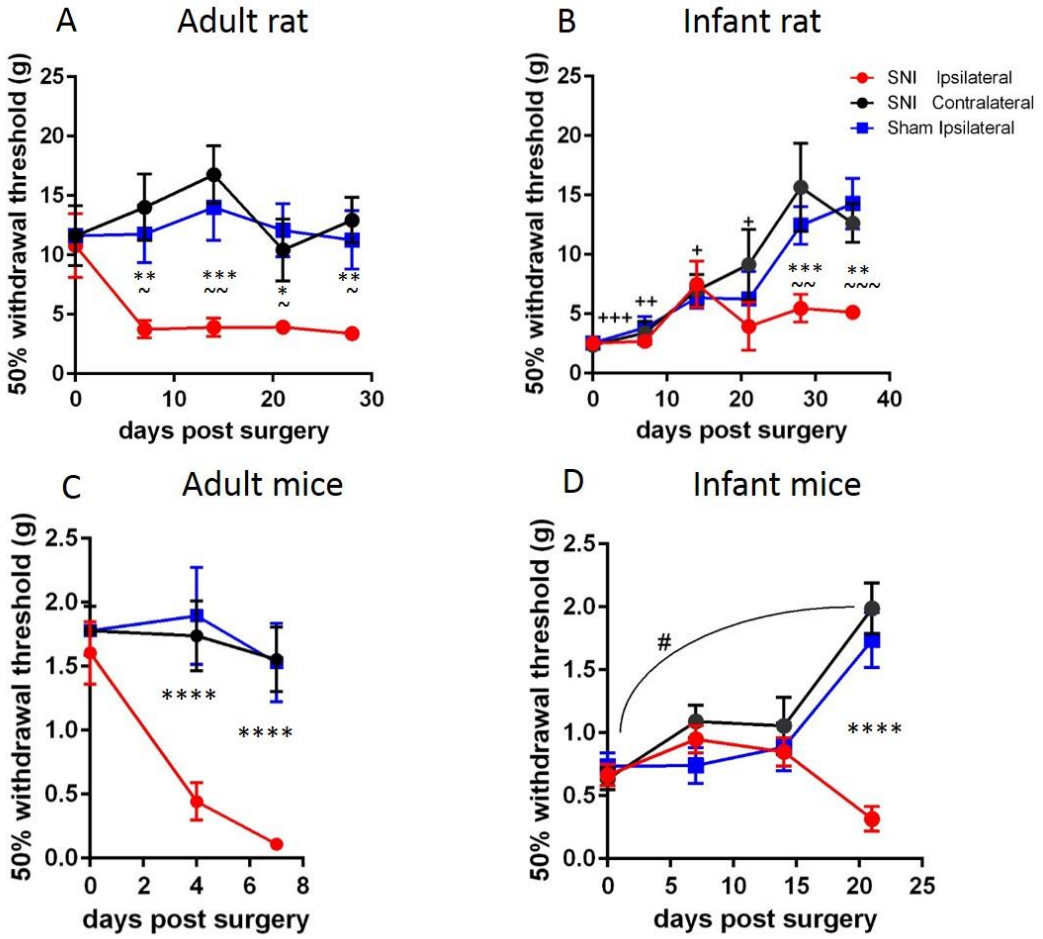


Fig 2.3 Mechanical withdrawal thresholds in rats and mice following infant and adult SNI or sham surgery

(A) In adult rats the mechanical withdrawal threshold of the ipsilateral paw is significantly reduced below that of controls (SNI contralateral paw and the Sham ipsilateral paw) 7 days after SNI surgery and is maintained (2-way ANOVA, $F(2,15)=9.616$, $P=0.002$, SNI surgery: n=6, sham surgery: n=6, i.e. n=6 per group) (B) Mechanical withdrawal thresholds in control infant rat paws increase with age from day 0, 7, 14, 21 days post-surgery today 28 post-surgery (P38) (2-way ANOVA, $F(5,90)=13.75$, $P<0.0001$). 28 days post infant SNI is there a significant decrease in the mechanical threshold of the ipsilateral hind paw compared to controls (2-way ANOVA, $F(2, 19)=8.487$, $P=0.0023$, n=7 per group) and interaction between time and surgery ($F(10, 90)=2.361$, $P=0.0158$) (c) Adult mice display a significant reduction in mechanical withdrawal thresholds on the ipsilateral paw 4 days post SNI surgery compared to controls (2-way ANOVA, $F(2,12)=23.28$, $P<0.0001$, time $F(2,24)=12.83$, interaction $F(4,24)=2.224$ n=5 per group). (D) Mechanical withdrawal thresholds in infant control mice increase with age (2-way ANOVA, surgery $F(3, 95)=10.68$, $P<0.0001$,). 21 days post infant SNI there is a significant decrease in the mechanical thresholds in the ipsilateral hind paw compared to controls (2-way ANOVA, $F(2,16)=8.872$, $P<0.0026$, n=6 per group). There was also an interaction of time (2-way ANOVA, $F(6, 95)=8.020$, $P<0.0001$. Bonferroni post-test). Rats: SNI ipsilateral vs SNI contralateral (and sham ipsilateral if only * is shown) *P<0.05; **P<0.01, ***P<0.001, ****P<0.0001 and SNI ipsilateral vs sham ipsilateral paw ~P<0.05 ~~P<0.01 ~~~P<0.001. Mice SNI ipsilateral vs SNI contralateral and sham ipsilateral ****p<0.0001. Bonferroni post-test between days post-surgery comparisons group +++ P<0.001, ++ P<0.01, +P<0.05

2.4.2 Infants

Twenty one days post infant nerve injury there is a significant reduction in ipsilateral weight bearing (17.1±2.1%) compared to that of sham controls (44.7±0.88%, P<0.001, Sidak post-test, Fig 2.4B) that was maintained until the end of testing. There was a significant effect of time on weight distribution weight bearing only in the SNI ipsilateral paw between 0-14 days post-surgery to 21 days post-surgery (P<0.0001, Sidak post-test).

Application of acetone (Fig 2.4D) and ethyl chloride (Fig 2.4F), did not alter cold scores following infant surgery for the first 3 weeks post-surgery. Only 28 days after infant surgery did cold scores significantly differ when the ipsilateral paw showed significantly greater cold scores (1.20±0.38 cold score) compared to the SNI contralateral hind paw (0.38±0.18 cold score, P<0.05, Bonferroni post-test) and the sham ipsilateral hind paw (0.25±0.16 cold score, P<0.05, Bonferroni post-test) following acetone application. Similar increases in cold scores upon ethyl chloride application were observed on the nerve injured ipsilateral paw 28 days after SNI surgery (SNI-ipsilateral 2.0±0.93 cold score) compared to controls (SNI-contralateral 0.75±0.16 cold score, sham-ipsilateral 0.63±0.18 cold score, P<0.001, Bonferroni post-test) which was maintained for at least 35 days post-surgery.

SNI at infancy did not alter thermal thresholds compared to controls (Fig 2.4H). However, as previously reported, there was a significant fall in thermal thresholds with postnatal age between P10 and P17 to P24 on all paws (2-way ANOVA showed a main effect of time, P=0.0001; P<0.05, Bonferroni post-test, n=4/group) (Falcon et al., 1996; Huang et al., 2010; Vega-Avelaira et al., 2012), confirming the absence of any generalised loss of spinal cord function in these animals.

2.4.3 Adults

In contrast SNI in adult rats develop reduced ipsilateral weight bearing and hypersensitivity in response to acetone and ethyl chloride stimulation (cool and cold hypersensitivity) within 7 days of surgery (Fig 2.4A, C, E) but not thermal hypersensitivity (Fig 2.4G). 7 days after SNI surgery the percentage of ipsilateral paw load drops (17.12%±2.29) compared to the ipsilateral paw of

sham controls (48.87%±7.17, P<0.0001, Sidak post-test) which is maintained for at least 28 days. Time was significantly different in the SNI ipsilateral paw only, between P0 and 7 days post SNI surgery (P<0.001, Sidak post-test).

As previously published, adult rats develop cold hypersensitivity 7 days after SNI surgery in response to innocuous cold (acetone) (Decosterd and Woolf, 2000; Leith et al., 2010). At day 7 the cold score on the SNI ipsilateral paw was significantly higher (Fig 2.4C, 1.42±0.30 cold score) than that of the SNI contralateral and sham ipsilateral paw (both which had a 0.0±0.0 cold scores P<0.0001, Bonferroni post-test) and persisted until the end of testing. Responses of SNI in adults to hind paw noxious cold stimulation (ethyl chloride) showed similar results (Fig 2.3C).

Seven days post SNI surgery the cold score on the ipsilateral hind paw was significantly higher (1.86±0.5 cold score) compared to the SNI treated contralateral paw (0.57±0.39 cold score, P<0.05; Bonferroni post-test) and the sham ipsilateral paw (0.43±0.30 cold score, P<0.01; Bonferroni post-test) and maintained for at least 28 days post SNI (Decosterd and Woolf, 2000).

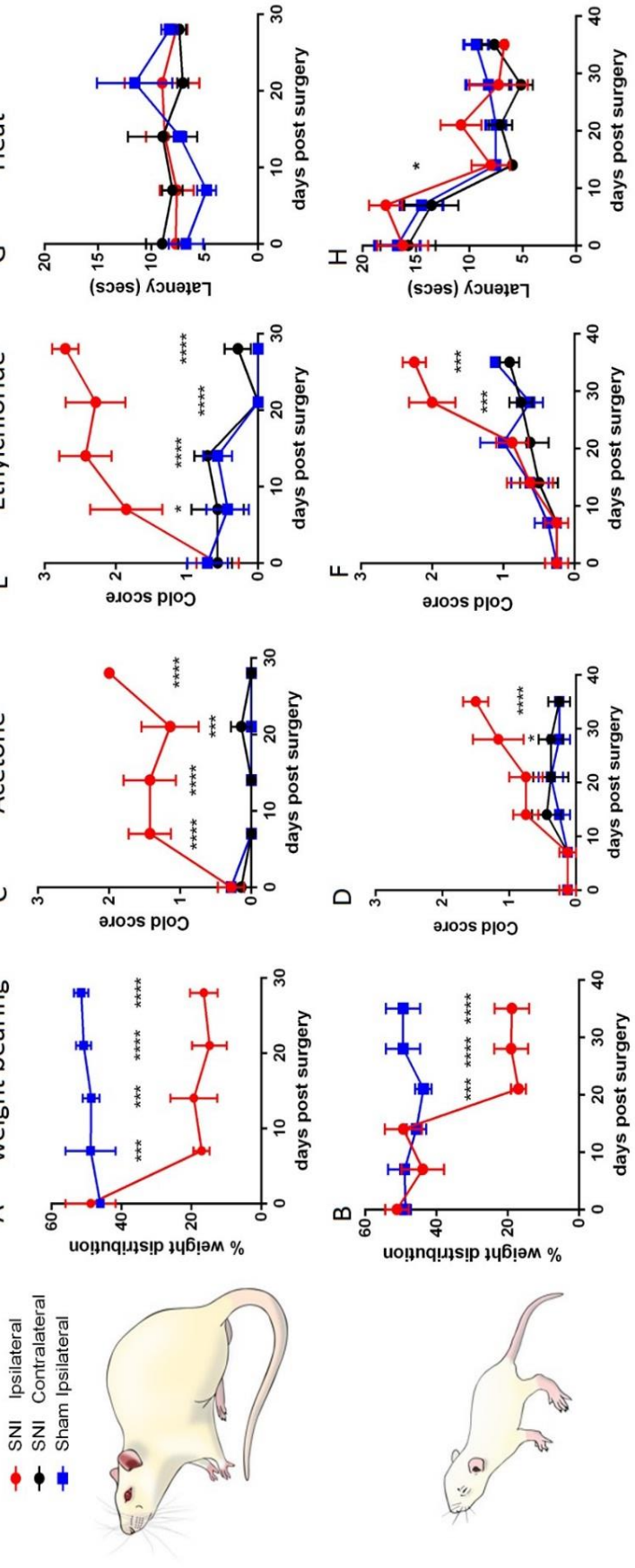


Fig 2.4 Behavioural testing in rats following P10 and P33 SNI or sham surgery

(A) In adults contralateral weight bearing developed 7 days following SNI but not sham surgery (2-way ANOVA, F (1, 6) =80.37, P=0.0001, n=4 per group). (B) Infant rats maintain equal weight bearing on both ipsilateral and contralateral hind paws until 21 days post-surgery when contralateral weight bearing develops and is maintained till 35 days post-surgery (2-way ANOVA, surgery F(1,10)=28.13, P=0.0003, time F(1,30)=35.91, interaction F(5,30)=7.831, n=6 per group) (C) 7 days after SNI, adult rats develop a significant increase in cold scores on the ipsilateral paw compared controls following both acetone (2-way ANOVA, F(2,18)=46.83, P<0.0001, n=7 per group) and (E) ethyl chloride (F(2,18)=75.29, P<0.0001, n=7 per group) stimulation. Infant rats show no significant differences in cold scores following acetone (D) or ethyl chloride (F) stimulation for 3 weeks following SNI and sham surgery. Cold hypersensitivity eventually develops on the ipsilateral paw 28 days after infant SNI surgery in response to both acetone (surgery, F(2,21)=7.65, P=0.0033; time, F(5,105)=5.145; interaction, F(10,105)=2.558, P=0.0083, n=8 per group,) and ethyl chloride (2-way ANOVA, F(2,21) = 8.719, P=0.0018, n=8 per group) (G) There are no significant differences in heat thresholds in adult rats following SNI and sham surgery (n=3/group) or (H) in infant rats. However, there is a significant reduction in thermal latency over time between P10 and P24 (2-way ANOVA, F (5, 54) =17.75, P<0.0001, n=4 per group). Bonferroni post-test; *P<0.05, ***P< 0.001, ****P<0.0001.

2.4.4 The effect of SNI upon spinal neuronal degeneration in the infant rats.

A preliminary test into the effect of SNI surgery at infancy (P10) on the neuronal composition of the dorsal horn, 7 days after infant SNI or sham surgery is shown in Fig 2.5. NeuN (red) immuno-staining on L4/L5 dorsal horn sections reveals no obvious or gross difference in the NeuN neuronal staining pattern or density in the dorsal horn of sham and SNI treated animals. IB4 immune-staining (in green) shows that non-peptidergic C-fibre terminals disappear in the SNI derived section and shows the location of the lesioned afferents (Beggs and Salter, 2007; Molander et al., 1996; Molliver et al., 1997; Plenderleith et al., 1992).

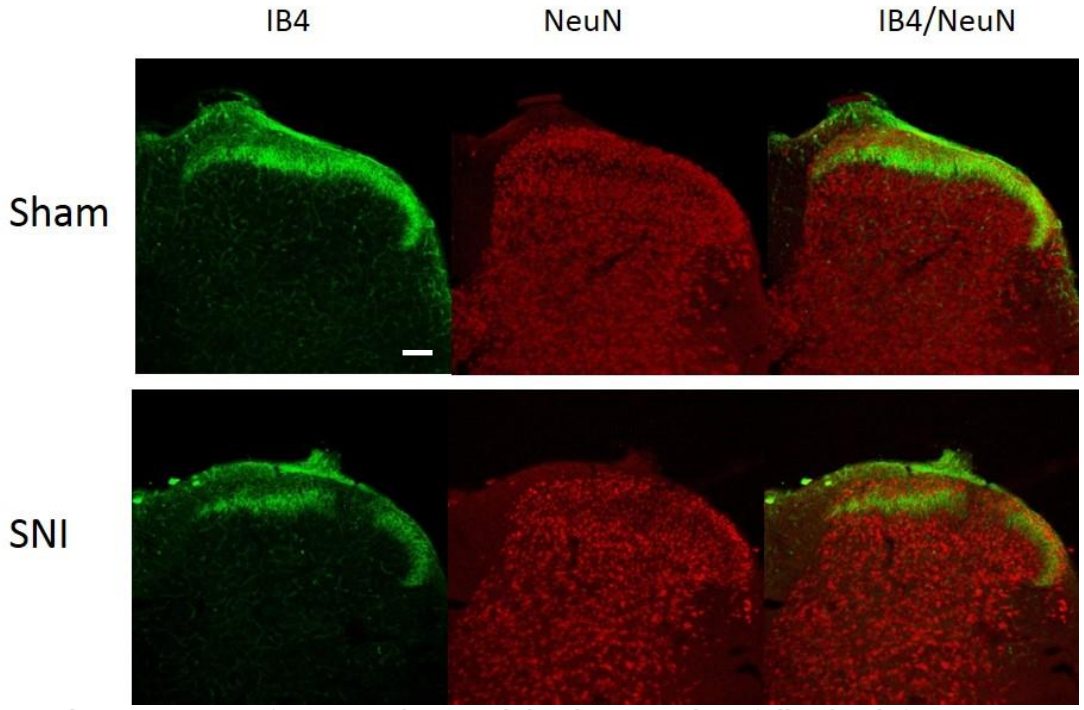


Fig 2.5 Images of NeuN and IB4 staining in rats 7 days following infant SNI or sham surgery.

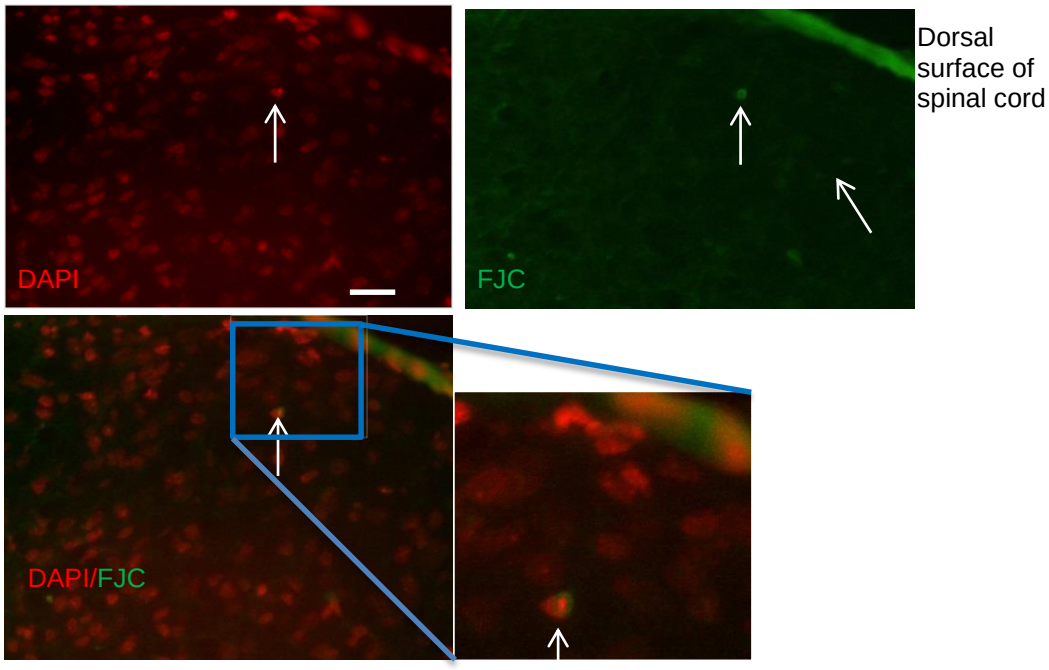
Patterning of neuronal staining (NeuN in red) shows no obvious difference in SNI and Sham groups. IB4 staining (green) was used to identify the termination area of damaged non-peptidergic C-fibre terminals. n=3/treatment group. Images were taken at 10 x magnification. Scale bar, 100µm.

2.4.5 Fluoro-Jade C staining

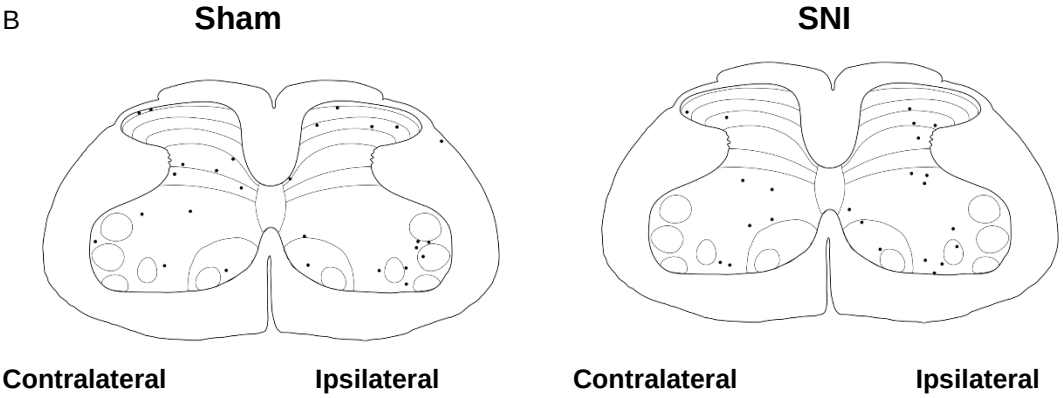
Fluoro-Jade C (FJC) stains degenerating neurons and has successfully been used to identify neuronal degeneration in the spinal cord tissue of infant rats (Walker et al., 2010; Westin et al., 2010). To identify if infant SNI induced neuronal degeneration, FJC positive cells in the L4/L5 lumbar region of the

spinal cord collected from rats 7 days after infant SNI or sham surgery were counted in a blind manner. FJC staining was observed in a few DAPI labelled cells with a distinctive disrupted soma (Fig 2.6A). FJC/DAPI positive cells from 10 sections which contained the highest FJC counts per animal were mapped onto a spinal cord diagram (Fig 2.6B) and the average number of FJC/DAPI co-labelled cells in 10 sections/animal were counted in SNI and sham treated groups (n=6 animals/treatment group). Fig 2.6C and D shows there is no significant differences in the mean number of degenerating neurons 7 days post infant SNI and sham surgery in either the dorsal horn or the ventral horn (SNI ipsilateral-59±5.2 compared to sham treated control 45±4.2, unpaired T-test). Furthermore, FJC positive neurons were distributed throughout the ipsilateral and contralateral dorsal and ventral horn (Fig 2.6B) suggesting no obvious pattern in distribution.

A



B



C D

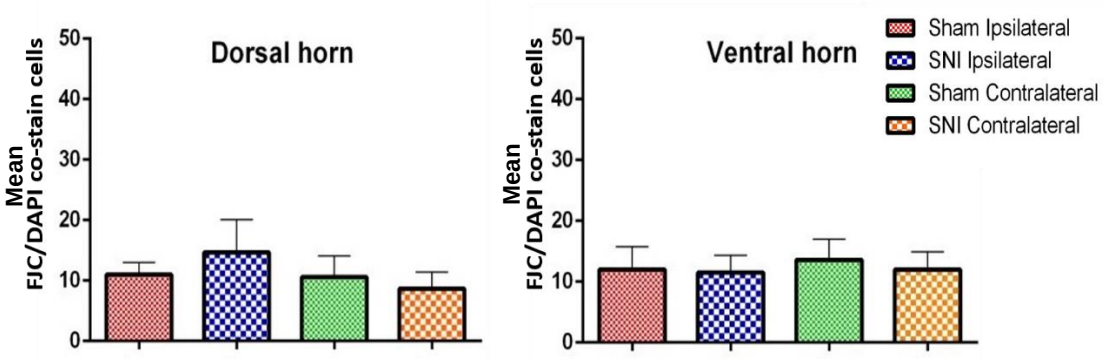


Fig. 2.6 FJC staining in the L4/L5 lumbar spinal cord of rats 7 days following SNI or sham surgery.

(A) Fluoro Jade C (green) co-stains with DAPI (red) in the L3/L4 spinal cord as illustrated with arrows. The co- stain image is magnified to show clear co-labelling. (B) Mapping of FJC positive cells in each animal revealed no patterns in distribution in sham or SNI treated animals (diagrams are representative images from 1 animal). (C-D) The number of FJC positive cells were counted from 10 sections from rats 7 days after infant SNI or sham surgery. No significant differences were observed in FJC/DAP counts taken from the ipsilateral and contralateral dorsal (C) and ventral horn (D). (10 sections from each animal; n=6 animals/treatment group; data was normally distributed, unpaired Student's t-test was not significant). Images are taken at 40x total magnification, Line with error bars represent mean±s.e.m. Scale bar=40µm.

2.5 Discussion

Data presented in this chapter confirms previous studies to show that infant rodents do not develop mechanical hypersensitivity in the early period following a peripheral nerve injury, emerging 3-4 weeks after surgery, when the rat reaches P38 (Howard et al., 2005; Ririe and Eisenach, 2006; Vega-Avelaira et al., 2007,2012). In extension to the published data, these studies extend beyond mechanical hypersensitivity to cool and cold hypersensitivity and weight bearing which develop within a few days following adult SNI but are absent when SNI is performed at P10. Clinical studies also suggest that neuropathic pain symptoms in infants are rare before the ages of 5 and 6 but increase in incidence with age (Sethna et al., 2007; Walco et al., 2010) and also that early-life nerve injury may have long term consequences upon pain processing later in life. One study by Melzack et al, (2007) investigating phantom limb pain in adolescents documented that children with the earliest loss of limbs have a delay in the onset of phantoms, which emerge after a mean of 7 years (Melzack et al., 1997).

2.5.1 Delayed onset of hypersensitivity following infant SNI to mechanical, cool, cold but not thermal stimulation

Mechanical thresholds were tested using vFh, which deliver a punctate mechanical stimulus, detected by mechanonociceptors that are densely innervated in the extremities of glabrous skin (e.g. hind paws) to elicit pin-prick pain (Boada et al., 2010). Experiments involving selective nerve fibre block indicate that sensitivity to punctate mechanical stimuli is mediated primarily by thermal sensitive, capsaicin insensitive A-fibre nociceptors (Aδ fibres) (Magerl et al., 2001; Ziegler et al., 1999). The use of ketamine, an NMDAR antagonist, in adult rats following nerve injury attenuates mechanical hypersensitivity (Qian et al., 1996; Suzuki et al., 2001). An earlier study completed in the Fitzgerald laboratory indicated that administration of ketamine also reverses mechanical hypersensitivity that arises at adolescence following infant nerve injury (Vega-Avelaira et al., 2012). This finding suggests that the delayed onset of mechanical hypersensitivity is mediated by NMDA facilitated central sensitization. Central sensitization leads to enhanced neuronal activity

occurring as a result of opening of NMDA channels that allow calcium entry into the cell (Latremoliere and Woolf, 2009; Woolf, 1983), vital for both the initiation and maintenance of activity dependent central sensitization (Ma and Woolf, 1995).

Studies in the adult rodent show that PKC-γ positive neurons play a key role in the development of mechanical hypersensitivity (Lu et al., 2013; Malmberg et al., 1997; Seal et al., 2009) although the expression of PKC-γ is present in the dorsal horn spinal cord by P10 (Malmberg et al., 1997). Pro-inflammatory mediators, such as TNF, are also known to interact positively with NMDA subunits in the dorsal horn spinal cord of nerve injured rats, promoting neuronal activity and pain-like behaviour (Daulhac et al., 2006; Zhang et al., 2011a) and initial studies suggest that infants may not mount a robust inflammatory response as adults following nerve injury (Costigan et al., 2009; Moss et al., 2007; Vega-Avelaira et al., 2007). However, the inflammatory profile in the dorsal horn spinal cord at the onset of pain behaviour following infant nerve injury remains to be elucidated.

For the first time data presented here indicates that infant mice also display the same delay in mechanical hypersensitivity, emerging 21 days after SNI, which until now has only been examined in the rat. Interestingly the onset of mechanical hypersensitivity in mice was quicker after surgery than in rats. This is consistent with a study by Wall and colleagues which also described this time course difference between rats and mice following sciatic nerve transection where mice displayed an earlier onset on the 7^{th} and 14^{th} day after nerve transection while rats displayed autotomy 7 days later (Wall et al., 1979).

Hind paw behavioural testing also shows that following infant SNI, rats display a delayed onset of hypersensitivity to both innocuous (acetone) and noxious (ethyl chloride) cold and contralateral weight bearing. Both coolants generate rapid changes in both the surface and subsurface skin temperatures. Acetone lowers the surface temperature of the skin by 7.7 ^{O}C, while ethyl chloride causes a decrease by 25.4^{O}C (Leith et al., 2010). These temperatures activate different receptors and primary afferent subsets. For example innocuous cold substances activate the TRP melastatin 8 (TRPM8), while noxious cold (<15^{o}C) activates the TRP ankyrin 1(TRPA1) channel (Caterina et al., 1997;

Story et al., 2003) although there is disagreement as to whether native or recombinant TRPA1 is intrinsically cold sensitive (Bautista et al., 2006; Karashima et al., 2009). While innocuous cooling with acetone activates subsets of A δ and C-fibres low-threshold afferents, more intense cold stimuli with ethyl chloride additionally activate populations of nociceptive afferents including C and A δ fibres (Cain et al., 2001; Campero et al., 1996).

These afferents project to different laminae in the dorsal horn with C-fibres mainly located in lamina I and II, A δ to laminae I, III and V (Lamotte, 1977; Light and Perl, 1979). These differences are also reflected in the differential behavioural response to these coolant, as stimulation of the rat hind paw with ethyl chloride, but not acetone, evokes robust withdrawal reflexes in lightly anesthetized animals (measured as hind limb EMG activity) (Leith et al., 2010). Another difference between these coolants is that spinal responses of ethyl chloride and acetone are also differentially modulated by descending control systems originating in the PAG, that selectively modulates the transmission of noxious (evoked by ethyl chloride) but not innocuous (evoked by acetone) information (Leith et al., 2010). As the delayed cold hypersensitivity emerges following both acetone and ethyl chloride in the same pattern the differences in the processing of these two coolants do not underlie the onset of cold pain hypersensitivity.

Contralateral weight bearing is not a stimulus-evoked reflex response, but an example of ‘guarding’ behaviour. This type of behaviour is highly appropriate in preventing potentially injurious mechanical stimuli to the actual site of injury and avoids interference with the progress of wound healing. Therefore, a delay in onset of contralateral weight bearing also suggests an underlying centrally mediated mechanism associated with an increase in the activity of the somatosensory system and an associated central adjustment which is displayed here as contralateral weight bearing (Mogil et al., 2010; Mogil and Crager, 2004).

In contrast to mechanical, non-noxious and noxious cold stimulation and contralateral weight bearing, the withdrawal latency to heat stimuli following infant SNI developed normally, similarly to sham controls for all time points tested and is consistent with a previous study. The latencies of withdrawal from

a heat stimulus are longer in infants and shorten during postnatal development (Vega-Avelaira et al., 2012). A δ (type II) and C-fibres are both necessary for the perception of thermal pain near the pain thresholds (Torebjörk and Hallin, 1973). One ion channel involved in the transduction of heat (52^{o}C) is the neuronal TRP vanilloid receptor 1, (TRPV1), as illustrated by noxious heat responses being inhibited by vanilloid receptor antagonists (Kirschstein et al., 1999; Nagy and Rang, 1999). Clinical studies have shown that changes in temperature thresholds (measured here as withdrawal latency) is not a usual symptom displayed by neuropathic pain patients (Decosterd and Woolf, 2000; Jørum et al., 2003; LaMotte et al., 1982; Magerl et al., 2001) and when it does present, is usually an indicator of peripheral sensitization (Hunt and Koltzenburg, 2005).

Interestingly, *in vivo* electrophysiological studies in adult rodents indicate that nerve injury does not induce an increase in the activity of dorsal horn neurons in response to heat stimuli, despite the presence of heat hypersensitivity in these animals (Laird and Bennett, 1993; Palecek et al., 1992) which is in contrast to alterations in response to other sensory modalities such as following mechanical stimulation (Palecek et al., 1992; Suzuki et al., 2000). To measure changes in central processing, the reflex duration from a heat source (the time the animal lifts its paw following thermal stimulation) can be measured and an exaggerated reflex would reflect an increase in hypersensitivity of neurons in the CNS (Decosterd and Woolf, 2000; Treede et al., 1992). In this study the reflex response duration to heat stimuli was not tested as, due to the longitudinal nature of the study, constant testing to suprathreshold heat stimuli in itself may lead to damage and/or sensitization. However, as adult SNI rats do display a longer reflex response compared to sham controls, it is expected that P10 rats would also display an increase in the reflex response duration to heat stimulation 3-4 weeks after SNI, although this still needs to be confirmed.

The behavioural data from this study suggests a number of interesting points. Firstly, the early absence of pain-like behaviour following infant SNI is unique to nerve injury as P0-P10 rodents demonstrate robust hypersensitivity to peripheral inflammatory insults including formalin, carrageenan and full

thickness skin wounds (Alvares et al., 2000; Barr et al., 2003; Marsh et al., 1999; Ren et al., 2004; Reynolds and Fitzgerald., 1995; Torsney and Fitzgerald, 2002; Walker et al., 2003). Secondly, the data suggests that the delayed hypersensitivity is not due to a general sensory or motor deficits. Having extended behavioural sensory testing in rats and consolidated the delay in mechanical hypersensitivity following infant nerve injury in mice, the next aim was to identify the mechanisms that may underlie the delay in pain-like behaviour following infant nerve injury.

2.5.2 Neuronal degeneration following P10 SNI

Although axotomy of the sciatic nerve in the immediate postnatal period (P0) causes extensive loss (75%) of DRG cells, occurring within 24 hours of injury (Aldskogius and Risling, 1981; Cheema et al., 1984; Himes and Tessler, 1989; Yip et al., 1984) at older ages axotomy of the sciatic nerve including P10, P21 and adult animals does not cause any DRG cell loss 7 days after injury (Beggs., 2000; Lewis et al., 1999; Li et al., 1994). The data described in this chapter indicates that P10 SNI does not induce neuronal degeneration in the spinal cord of rats compared to sham controls, at the 7 day time point following P10 SNI. The absence of neuronal degeneration at this age maybe due to the fact that interneurons have abundant connections from different sources and may undergo adjustment rather than degeneration. Furthermore, with age these neurons change in the requirement of peripheral sensory neurons for neurotrophic support in the immediate postnatal period (Bennett et al., 1996, 1988; Himes and Tessler, 1989; Lewis et al., 1998; Schmalbruch, 1984; Oliveira et al., 1997: Tandrup et al., 2000; Whiteside et al., 1998). In addition, although axotomy of the sciatic nerve results in central sprouting, this is not observed in animals beyond P10 (Fitzgerald, 1985b; Fitzgerald and Shortland, 1988; Shortland and Fitzgerald, 1994) and together with the data presented in this thesis suggests that nerve injury in the infant is not associated with extensive neuronal cell loss in the spinal cord.

As the neonatal nervous system undergoes considerable postnatal changes (as outlined in Chapter One), alterations occurring, particularly at the time of pain-like behaviour onset, may provide a clue to the underlying mechanism.

2.5.3 Postnatal development of the peripheral nervous system.

By birth both A and C sensory fibres are distributed to all body regions and, although fibres initially penetrate the epidermal surface, they retract and become organised in late embryonic stages (Fitzgerald, 2005; Jackman and Fitzgerald, 2000). Age related changes in receptor function, distribution and firing frequency can alter sensitivity to different stimuli. Nociceptors are responsive to stimuli and respond to tissue damage by birth including in response to intense mechanical, thermal and chemical skin stimulation and the expression of TRPV1, TRPA1 and TRPM8 mRNA is present in the DRG by birth (Fitzgerald, 1987a, b; Hjerling-Leffler et al., 2007) as are voltage gated sodium channels Nav1.8 and 1.9, which regulate neuronal hyper-excitability, and by P7 they are expressed at adult levels (Benn et al., 2001). In addition, the maturation of primary afferent input in to the dorsal horn occurs at an earlier time point than the onset of pain like behaviour (Beggs et al., 2002; Coggeshall et al., 1996; Fitzgerald et al., 1994; Jennings and Fitzgerald, 1998, 1996). For example, small diameter afferents, which are the last afferent type to enter the dorsal horn, grow into the rat dorsal horn by P5 and A fibres withdraw to mature locations in the first 3 weeks (Baccei et al., 2003; Beggs et al., 2002; Benn et al., 2001; Fitzgerald et al., 1994; Fitzgerald, 1987a; Fitzgerald and Shortland, 1988; Fitzgerald and Swett, 1983; Nakatsuka et al., 2000; Park et al., 1999; Woodbury and Koerber, 2003). This suggests that the postnatal maturation of sensory transduction and transmission mechanisms and central innervation are unlikely to underlie the delayed onset of pain behaviour following infant SNI.

2.5.4 Postnatal development of central processing

The functional properties of the dorsal horn network are also highly age dependent and undergoes considerable postnatal development (See Chapter One). This includes the fine-tuning of both excitatory and inhibitory synaptic connections and neuronal circuitry which in the naïve animal coincides with the behavioural changes observed in the first postnatal weeks. For example, in neonates the concentrations of NMDARs are high and widely distributed in the dorsal horn but by P21 these are restricted to laminae I and II (Watanabe et al., 1994) and both receptor affinity for NMDA and NMDA evoked calcium

influx decline to adult levels by P15 (Hori and Kanda, 1994). A similar trend is observed with AMPAR, and metabotropic glutamate receptor subtypes (mGluR) 3 and 5 that are also expressed at a high level in the neonatal dorsal horn and decrease to adult levels in the first 3 weeks of postnatal life (Berthele et al., 1999; Jakowec et al., 1995).

GABAergic control is present from birth, although glycinergic inhibition emerges only in the second postnatal week, contributing to the refinement and dampening of responses to touch at this time (Baccei and Fitzgerald, 2004; Koch and Fitzgerald, 2014). Furthermore, both GABA and glycinergic inhibition exhibit mature properties and spinal cord distribution by P23 (Keller et al., 2001; Schaffner et al., 1993).

Descending activity from the brainstem also contributes to the excitation and inhibition in spinal nociceptive circuits which is immature at birth and the rostroventral medulla (RVM), exclusively facilitates spinal pain transmission and only later does the influence of the RVM exhibits biphasic facilitation and inhibition (Hathway et al., 2009). As the role of descending control in the neonate is one of excitation, it is unlikely that the developmental change in descending control underlies the delayed onset of pain hypersensitivity.

Overall, the neonate is more excitable than the adult and underlies the differences observed in the behaviour of the naïve animal (Andrews and Fitzgerald, 1999, 1994; Falcon et al., 1996; Fitzgerald, 1985; Fitzgerald and Shortland, 1988; Jennings and Fitzgerald, 1996; Pattinson and Fitzgerald, 2004; Teng and Abbott, 1998; Torsney and Fitzgerald, 2002). The normal development of thermal thresholds suggest that the delay is not due to a general sensory or motor deficit at this age and it is also unlikely that the delay is due to changes occurring during postnatal development, as these occur at an earlier time point than to be relevant to the delayed emergence of pain-like behaviour although a centrally mediated mechanism is possible.

A previous study by Vega-Avelaira et al., shows that the delayed onset of mechanical hypersensitivity following infant nerve injury can be reversed by administration of an NMDAR antagonist, which further suggest pain-like behaviour may be centrally mediated (Vega-Avelaira et al., 2012). In adults,

electrophysiological recordings from nerve injured animals show that dorsal horn neurons develop profound changes including a significant increase in activity of wide dynamic range neurons with a larger percentages of neurons responding to innocuous mechanical stimuli (Palecek et al., 1992) which also have larger receptive field sizes (Suzuki et al., 2000). To date *in vivo* electrophysiological studies in infant animals following nerve injury are yet to be completed but may elucidate if the absence of hypersensitivity following infant nerve injury and its subsequent onset at a later time point reflect alterations in the activity of dorsal horn spinal cord neurons. This is investigated in the next chapter.

2.6 Conclusion

The data presented in this Chapter confirms and extends published work to indicate that P10 rats do not display hypersensitivity to mechanical, innocuous and noxious cold stimulation or contralateral weight bearing for the first two to three weeks following infant SNI surgery. However, pain-like behaviour does eventually emerge but later when the rat reaches adolescence.

Chapter Three

Alterations in dorsal horn neuronal activity following infant nerve injury

3.1 Introduction

Chapter Two described the longitudinal behavioural alterations that occur following infant (P10) peripheral nerve injury (SNI) to show that nerve injury at infancy does not cause hypersensitivity to mechanical, innocuous and noxious cold stimuli, in the early period following surgery, but does eventually develop along with contralateral weight bearing 3-4 weeks later. This is in contrast to adult nerve injury models where neuropathic pain-like behaviour develops within 7 days of nerve injury (Decosterd and Woolf, 2000). This Chapter aims to investigate if these behavioural alterations following infant SNI are accompanied by changes occurring centrally, in the dorsal horn spinal cord. Experiments presented in this Chapter use *in vivo* electrophysiology to examine dorsal horn neuronal activity in the early period after P10 SNI (when pain-like behaviour is absent) and 28 days later (when pain-like behaviour emerges) (Vega-Avelaira et al., 2012).

Peripheral nerve injury in the adult rodent leads to changes in both the peripheral and central nervous system which contributes to the initiation and maintenance of neuropathic pain behaviour. In the periphery, primary afferent fibres become more excitable upon nerve injury and electrophysiological studies have shown that ectopic action potential discharge is generated from within the neuroma a the sits of injury or dorsal root ganglia (Amir et al., 2005; Kajander et al., 1992; Kajander and Bennett, 1992; Petersen et al., 1996; Study and Kral, 1996; Xie and Xiao, 1990). The irregular firing of sensory neurons is the result of multiple molecular changes in the neurons, including altered expression of sodium and potassium channels (Kim et al., 2008; Rose et al., 2011; Roza et al., 2003), the growth associated protein (GAP)-43 and neurotransmitters such as calcitonin gene-related peptide (CGRP) and substance P (Bennett et al., 1989; Cameron et al., 1991). Together these peripheral changes result in abnormal input to the dorsal horn spinal cord, which in turn contribute to postsynaptic changes in the dorsal horn that drive central sensitization.

As the dorsal horn serves as a vital first point in sensory integration for incoming peripheral afferent signals and transmission of sensory information, abnormal signals arriving from the periphery following nerve injury alter dorsal horn neuronal excitability that have consequences on the spinal processing of cutaneous inputs, and subsequently the perception of sensory information. A number of studies have investigated nerve injury induced changes in spinal somatosensory processing in the spinal cord dorsal horn of the rat and monkey (Chapman et al., 1998a; Laird and Bennett, 1993; Leem et al., 1995; Palecek et al., 1992; Suzuki et al., 2000; Takaishi et al., 1996). However, the effect of nerve injuries on spinal processing in neonatal animals are not currently known.

3.1.1 Peripheral nerve injury in adults results in alterations in dorsal horn neuronal activity.

In vivo electrophysiological studies in adult rodents following peripheral nerve injury of the sciatic nerve indicate two consistent changes of dorsal horn neurons and include i) an absence of detectable peripheral receptive fields due to partial deafferentation (Laird and Bennett, 1993; Takaishi et al., 1996) and ii) abnormally high levels of spontaneous activity (Chapman et al., 1998b; Laird and Bennett, 1993; Palecek et al., 1992; Suzuki and Dickenson, 2006; Takaishi et al., 1996; Walczak et al., 2006). Interestingly, the majority of studies using partial nerve injury models have failed to demonstrate altered dorsal horn responses to heat stimuli, despite the presence of heat hypersensitivity in these animals (Palecek et al., 1992; Laird and Bennett, 1993). While the characteristics described above are prominent features in all experimental neuropathic pain animal models, other alterations in dorsal horn responses to different stimuli vary depending on the nerve injury model used and time point analysed. Changes in the mechanical sensitivity to dorsal horn neurons are subtle. For example, Laird and Bennett (1993) reported that 9-11 days post chronic constriction injury (CCI) there were no differences in the size of cutaneous receptive fields, thermal or mechanical thresholds, or the magnitude of responses to acetone (cold) compared to sham controls. However, other studies, following spared nerve ligation (SNL) in the adult rat, have shown that a larger percentage of wide dynamic range (WDR) neurons

respond to innocuous mechanical stimuli, and these neurons also have enlarged receptive fields (Palecek et al., 1992; Suzuki et at., 2000). A larger percentage of neurons exhibited high spontaneous firing rates following SNL (Chapman et al., 1998; Takaishi et al., 1996: Suzuki and Dickenson, 2006; Palecek et al., 1992) although thermal and mechanical thresholds remained comparable 7-14 days post-surgery and wind-up responses are also comparable to controls (Chapman et al., 1998b).

In vitro recordings from slice preparations in the substantia gelantinosa indicate that N-methyl-D-aspartate receptor (NMDAR) currents are facilitated in the SNL model (Isaev et al., 2000). In addition, following both sciatic nerve transection and CCI, there is a decrease in the threshold for eliciting excitatory post synaptic currents (EPSCs) and increased prevalence of mono and poly synaptic Aβ fibre evoked EPSCs (Kohama et al., 2000; Kohno et al., 2003; Okamoto et al., 2001). Other studies have shown that primary afferent evoked inhibitory postsynaptic currents (IPSCs) are reduced in duration and magnitude after partial nerve injury and it has been suggested that this is due to a reduction in the release of GABA acting on pre-synaptic $GABA_A$ receptors (Moore et al., 2002). These findings further indicate that neurons within the dorsal horn are hyperexcitable following nerve injuries.

To date studies into the responsiveness of spinal neurons following SNI in the adult remain unexplored. This is surprising as studies into nerve injury associated spinal cord glial cell activation and dorsal horn cell death predominantly utilize the SNI model (Basbaum et al., 1991; Bennett and Xie, 1988; Carlton et al., 1991; Ho Kim and Mo Chung, 1992; Munger et al., 1992; Seltzer et al., 1990). As illustrated in Fig3.1, an advantage of the SNI model for *in vivo* electrophysiological recordings is that the branches of the sciatic nerve innervate the dorsal horn spinal cord in a specific somatotopic map, enabling the sampling of dorsal horn neurons located in the spared sural nerve territory while the area innervated by sectioned branches can be excluded (Molander and Grant, 1986; Woolf and Fitzgerald, 1986). This is in contrast to the CCI models where the experimenter would be unaware if they are sampling dorsal horn neurons with an unclassified input, i.e. damaged or spared by constriction.

Reconstruction of superficial dorsal horn illustrating areas innervated by nerves from the hind paw.

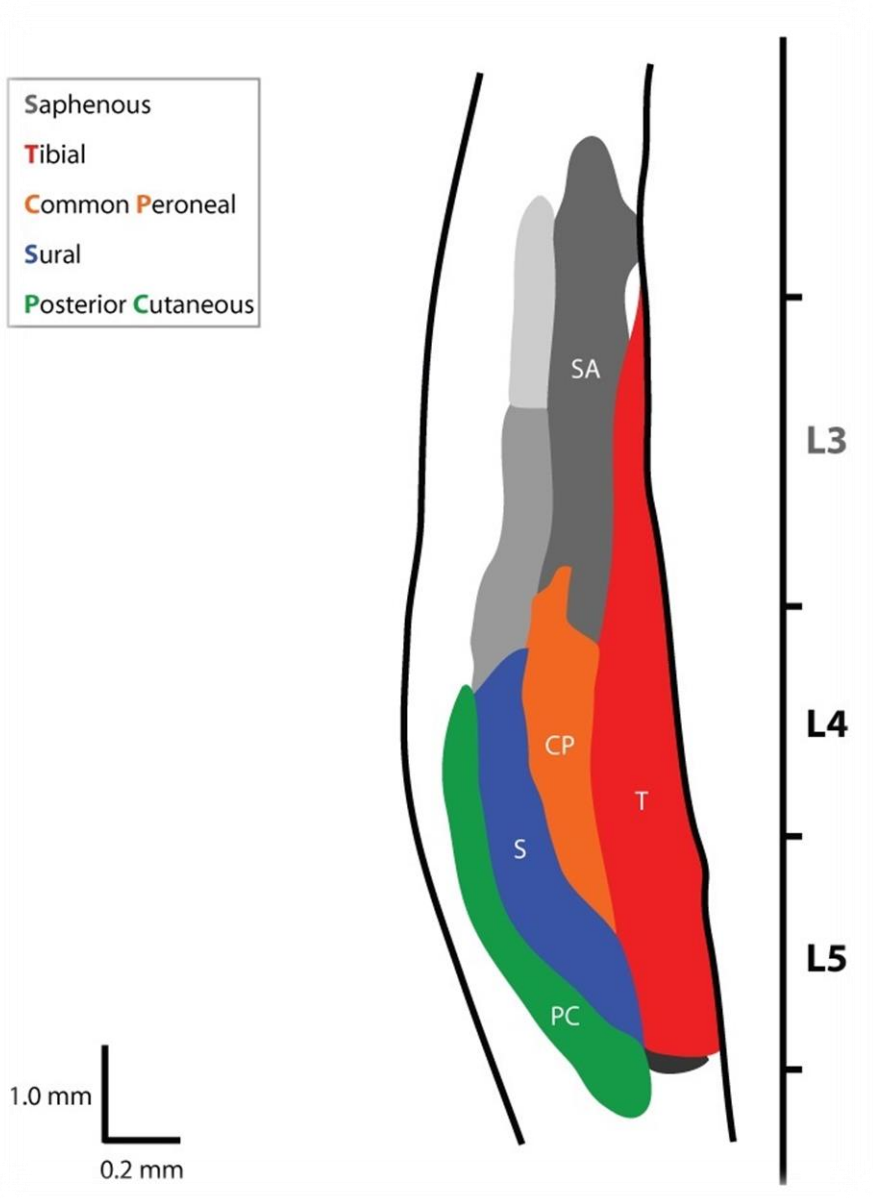


Fig 3.1 Schematic dorsal view map of lamina II of the dorsal horn spinal cord.

Diagram illustrates the density centres of projections from different nerves that innervate the hind paw T, tibial, CP, common peroneal, S, sural, PC posterior cutaneous. Reprinted from Neuroscience, 19, Molander and Grant, Laminar distribution and somatotopic organization of primary afferent fibres from hind limb nerves in the dorsal horn. A study by transganglionic transport of horseradish peroxidase in the rat, 297-312 (1986) with permission from Elsevier.

3.1.2 Alterations in dorsal horn neurons following infant nerve injury

If neonatal nerve injury is performed soon after birth (P0-5) it can permanently alter central spinal cord connections of afferents (Fitzgerald et al., 1990; Shortland and Fitzgerald, 1991). For example, sciatic nerve section at birth (P0) induces the sprouting of the saphenous nerve terminal field caudally from mid L4 to L4-L5 while the axotomised and invading A-fibres sprout dorsally into the deafferented substantia gelatinosa (Fitzgerald et al., 1990; Fitzgerald and Shortland, 1988). These changes are triggered by primary sensory neuron cell death in the DRG and subsequent deafferentation of the dorsal horn. However by P10 the sprouting observed following sciatic nerve transection is absent (Fitzgerald, 1985b).

To date there has been only one study investigating alterations in dorsal horn activity following partial denervation peripheral nerve injury at infancy (Li et al., 2009). In this study patch clamp recordings demonstrated that SNI at P6 failed to elicit spontaneous excitatory neurotransmission or neuronal excitability 3-5 days after surgery which was in contrast to adults (Li et al., 2009). The effect of infant nerve injury on dorsal horn neuronal activity *in vivo* in the early period in the absence of pain-like behaviour and later at the onset of pain like behaviour have not been investigated.

3.2 Aims of the Chapter

While it is documented that peripheral nerve injury in adult rodents leads to alterations in dorsal horn responses and central processing, this has not been characterised in the SNI neuropathic model in adult or infants. To test whether the delayed onset of behavioural hypersensitivity following infant SNI coincides with changes in the dorsal horn sensory circuits, responses of WDR neurons will be recorded in infant rats following peripheral nerve injury, in the early period where pain-like behaviour is absent, and in the later period when pain behaviour emerges. The key objectives of the experiments described in this chapter are to:

1. Characterise spontaneous and cutaneous evoked responses of dorsal horn neurons in adult rats, 7 days after SNI and sham surgery, when neuropathic pain-like behaviour is established.
2. Characterise spontaneous and cutaneous evoked responses of dorsal horn neurons in young rats, 7 days after P10 SNI and sham surgery, when there is no neuropathic pain-like behaviour.
3. Characterise spontaneous and cutaneous evoked responses of dorsal horn neurons in rats, 28 days after P10 SNI and sham surgery, when neuropathic pain-like behaviour has emerged.

3.3 Methods

3.3.1 Animals and surgery

Male Sprague-Dawley rats were obtained from UCL Biological services unit. Rat pups were housed with mother and their littermates while animals over P21 were caged in littermates of 5. All animals had free access to water and were housed in 12 hour light/dark cycles. SNI or sham surgery was completed on P10 or P33 rats and mechanical sensory testing was completed as outlined in Chapter Two. All experiments were conducted in accordance with the United Kingdom animals Scientific Procedures Act of 1986.

3.3.2 *In vivo* electrophysiology

All electrophysiology experiments were conducted on rats at the following time points:

1) 7 days post infant (P10) SNI or sham surgery (when rats reach P17)
2) 28 days post infant (P10) SNI or sham surgery (when rats reach P38)
3) 7 days post adult (P33) SNI or sham surgery (when rats reach P40)

P17 and P40 naïve rats were also used as non-injured controls.

3.3.2.1 Animal preparation

The animals were anaesthetised under isoflurane-anaesthesia (5% in medical oxygen, Univentor unit 400, Royem Scientific, UK) via a nose cone to achieve areflexia and placed on their back on a heating blanket. A cannula was inserted into the trachea and sutured to ensure stability. The animals were artificially ventilated by connecting the tracheal cannula via a Y connector to a ventilator pump (small animal ventilator, model 687, Harvard Apparatus Inc) at 80 breaths per minute. The air flow was adjusted according to the rat's size and heart rate (350-400 beats per minute) and monitored using an electrocardiogram. The animal was then fixed onto a stereotaxic frame (Kopf Instruments, CA) using ear and hip bars. The body temperature was maintained using a feedback electric blanket with a rectal probe maintained at (37^{o}C) and for smaller rats a heating lamp was also used. A laminectomy was

performed to expose the lumbar enlargement taking care not to touch the spinal cord.

3.3.2.2 Laminectomy

The skin on the back of the animal was cleaned, and hair flattened with alcohol. An incision to the skin was made using a scalpel along the vertebrae two centimetres rostral and caudal to the base of the rib cage to allow access to the lumbar segment (L) 4-L5 of the spinal cord. Fat and connective tissue was then removed to enable visualisation of the vertebrae. Rat tooth forceps were used to lift the column and vertebrae were removed using rongeurs. Any bleeding was immediately stopped by applying pressure to the bone. The dura was then lifted up with fine forceps and cut using iris scissors. Once the surgery was completed, the vertebral column was secured using a pair of rat tooth forceps clamped perpendicularly to the stereotaxic frame. Throughout the experiments warm mineral oil was used to cover the exposed spinal cord and prevent excessive heat and fluid loss, subcutaneous injections of saline were given to each animal to maintain hydration.

3.3.2.3 Single unit extracellular recordings

Isoflurane-anaesthesia was reduced to 1.8% for extracellular recordings that were made using a 10µm tipped glass-coated tungsten microelectrode (Ainsworks, Welford, Northants) which was lowered onto the dorsal horn. A reference electrode was also inserted into the muscle close to the recording area for differential recordings. The recording system was grounded through the stereotaxic frame and animal.

Neuronal activity was passed through a x1 head stage amplifier and amplified by 5000 via a x5k differential amplifier (NeuroLog, Digitmer, UK). This signal was passed through low and high pass filters, set at 1 kHz and 10 kHz respectively and onto a spike trigger and set manually to produce output pulses for spikes above a particular voltage. The spike trigger unit generated all-or-nothing impulses from the raw data signal with the threshold for the generation of spikes set on the front panel of the unit during each experiment. The signal was fed into an audio amplifier (TDS 2012 digital storage oscilloscope,

Tektronix) and speaker so that a click could be heard each time an impulse was produced. Filtered signal was then fed into a PowerLab system which converted the signal from analogue to digital (4SP, AD Instruments, UK) and recorded onto a computer. Signals of raw data were displayed on to an oscilloscope to allow constant viewing of the data signal. Spike discrimination and analysis was carried out using Chart 7 software (ADI instruments, Oxfordshire, UK)

Recordings were made from dorsal horn neurons with an input from the 'spared' sural nerve, identified as having a receptive field in the sural nerve territory. To identify these cells, the electrode was moved rostrocaudally along the cord, adjacent to the central vein. Once in the correct area, the electrode was manually lowered through the cord in vertical tracks in 10µm steps. To isolate individual dorsal horn cells the electrode was manually lowered onto the dorsal surface of the spinal cord and stroking of the ipsilateral (to the surgery) plantar skin of the hind paw (in the sural nerve territory) was used as the search stimulus. The threshold of the spike trigger unit was continuously set above background activity so that clicks were formed by the action potentials of cells and facilitated their isolation. Cells in the deep dorsal horn were selected once a stable action potential spike amplitude (usually 50µV) and shape could be distinguished from background noise levels (usually 15µV) (Torsney and Fitzgerald, 2002). These parameters were monitored throughout to ensure the same cell was always being recorded.

3.3.2.4 Cell Characterisation

Only cells with receptive fields within the sural nerve territory on the plantar region of the hind paw were used for recordings. Wide dynamic range (WDR) neurons were selected by ensuring each neurons responded to both cutaneous touch and pinch of the receptive field (Fig 3.2).

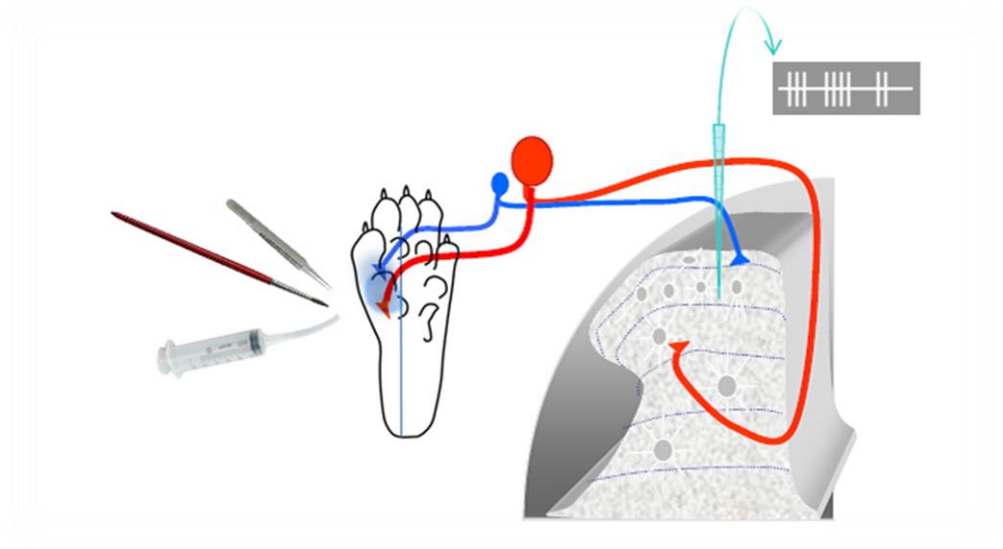


Fig 3.2 Diagram showing *in vivo* electrophysiological recordings in the dorsal horn spinal cord

An electrode in laminae III-IV of the dorsal horn was used to record background activity of cells in the sural nerve territory (denoted by the blue line on the paw). Receptive fields of isolated cells were mapped in response to pinch and brush followed by recording of a range of stimulus-evoked neuronal activity in response to stimulation of the receptive field.

First, background activity was recorded over a 3 minute period. If the recorded spike was not stable over this recording period, the cell was rejected. The plantar paw receptive field of a cell was then characterised by mapping the cells response to pinch (using forceps) and dynamic brush (using a paint brush) of the skin. This was drawn out onto a representative image of the plantar paw and digitally scanned. Receptive fields were analysed using ImageJ software and presented as a percentage of total paw area (Ririe et al., 2008).The properties of each cell was then characterized by applying stimuli to the centre of the cutaneous receptive field for three seconds and the number of spikes in 3 seconds were measured unless stated (Fig 3.2).

Recordings during the stimulation of the receptive field included:

1. Dynamic brush (no.3 paintbrush)
2. Noxious pinch with calibrated forceps
3. Acetone drops
4. Ethyl chloride spray
5. 33g and 66g calibrated pressure forceps:
6. Calibrated von Frey hairs (Threshold that elicited firing)
7. Application of threshold von Frey hair
8. Application of suprathreshold, 6.3g von Frey hair

Each stimuli was applied 3 times with at least 5 minute intervals between each stimulus (Chapman et al., 1998b). The mean number of spikes evoked per second to each stimulus was then calculated and used for analysis. All animals were euthanized with an overdose of sodium pentobarbitone (intraperitoneal injection) at the end of each experiment.

3.3.3 Data Analysis

Background spikes were counted in a fixed window duration of 3 minutes. Stimulus evoked spikes were counted with a fixed window duration of 3 seconds (so as to exclude any discharge). All data are presented as mean. The number of spikes was analysed and graphed using GraphPad Prism software (San Diego, CA, USA). Comparisons of proportions of cells exhibiting specific firing characteristics were made using the Chi-Square (X^2) test. The non-parametric Mann–Whitney U test was used to test for significance between treatments at a given age unless stated. For all data a significance-value of less than 0.05% was deemed statistically significant.

3.4 Results

3.4.1 Electrophysiological properties of dorsal horn neurons in infants and adults following the spared nerve injury.

In this study a total of 114 single unit extracellular recordings were made from dorsal horn neurons from 50 rats. A summary table for the number of cells recorded in each age and treatment groups is provided in Table 3.1. A pilot study was also undertaken using naïve P17 and P40 rats which were used to establish any differences in the recordings between sham animals and age matched naïve controls.

	Number of cells (number of animals)		
	SNI	Sham	Naïve
Infant surgery +7 days	10 (5)	17 (5)	11 (4)
Infant surgery +28 days	10 (5)	15 (5)	
Adult surgery +7 days	17 (6)	20 (11)	14 (9)

Table 3.1 Number of cells recorded in each experimental group

Table showing the number of cells, and in brackets the number of animals used, recorded in each experimental group at each age.

All cells used in the study had cutaneous mechanoreceptive fields located on the plantar surface of the hind paw in the sural nerve territory. Only WDR neurons were selected for recording while neurons with only low or high threshold input were rejected (low threshold cells did not respond to pinch, high threshold cells did not respond to brush). Multiple cells were recorded in each animal but not all stimuli were tested in each cell.

There was no significant difference in the depth of recordings between SNI and sham treated animals within a postnatal age (Fig 3.3). The average depth of all cells recorded from the spinal cord surface from P17 rats was 477±11.54µm and from P38-P40 rats 654.9±21.40µm which are classified as cells from deep III, IV and V laminae (Torsney and Fitzgerald, 2002; Urch and Dickenson, 2003).

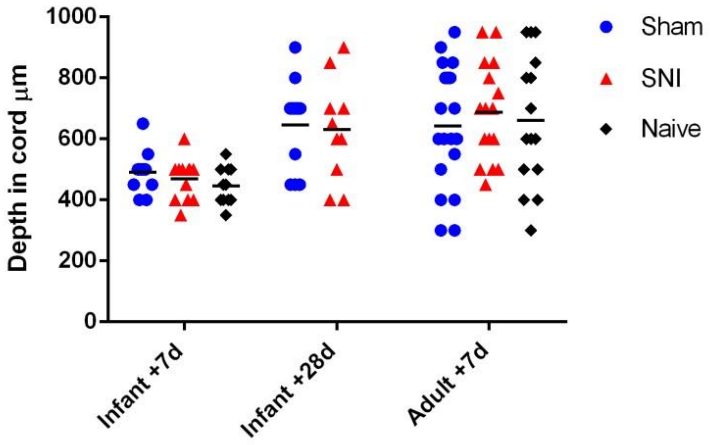


Fig 3.3 Depth of recorded dorsal horn cells at different ages

Scatter plot shows the depth of recorded cells from the surface of the spinal cord. There is no significant difference in the depth of recorded cells between different treatment groups (SNI or sham) within a postnatal age.

3.4.2 Dorsal horn activity in infant and adult rats following sham surgery is comparable to age matched naïve controls.

To test for any effects of sham surgery on dorsal horn activity a pilot study was initially conducted to compare the spontaneous and evoked-activity (to innocuous dynamic brush and noxious pinch) of dorsal horn neurons in sham animals and age matched naïve controls. Infant sham surgery did not significantly alter the mean spontaneous or evoked activity of dorsal horn neurons compared to naïve rats at either 7 days (Fig 3.4A) or 28 days (Fig 3.4B) after P10 surgery. As the activity of cells from sham and naïve animals are not significantly different within a postnatal age group, any significant differences observed between SNI and sham surgery treatment groups are due to peripheral nerve injury.

A B

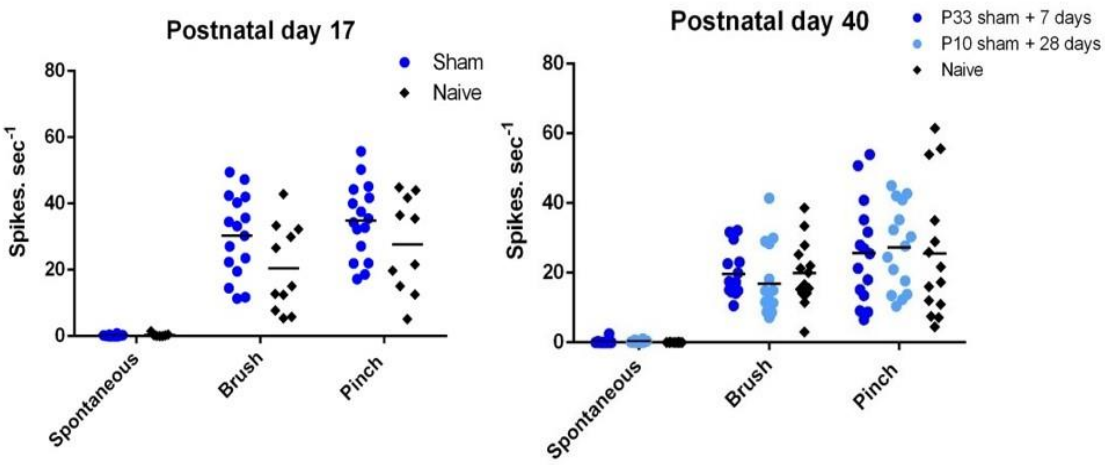


Fig 3.4 Activity of dorsal horn neurons in infant and adult sham treated rats is comparable to naïve age matched controls.

(A) 7 days after infant (P10) sham surgery rats display comparable activity to naive age matched controls to spontaneous activity infant sham: n=16 cells, infant naïve: n=7cells; brush (infant sham n=16 cells; infant naïve n=11 cells) and pinch-evoked activity (infant sham: n=17 cells; infant naïve: n=10 cells) (B) 7 days after adult (P40) sham surgery and 28 days after infant sham surgery (P38) rats display comparable spontaneous activity (infant sham +28 days n=10 cells; adult sham n=14 cells; adult naïve n=11 cells) and brush (infant sham +28 days n=15 cells; adult sham n=15 cells; adult naïve n=14 cells) and pinch-evoked activity (infant sham +28 days n=15 cells; adult+7 days sham n=15 cells, to age matched (P40) naïve controls (n=14). P10+7d sham n=5 animals, P17 naive n=4 animals, P40 naive rats n=9 animals, P33sham+7d n=11 animals, P10sham+28d n=5 animals.

3.4.3 Dorsal horn activity following infant and adult SNI and sham surgery

3.4.3.1 Spontaneous activity

Fig 3.5 shows that in infants spontaneous activity of WDR neurons was not significantly different between SNI and sham treated rats 7 days after surgery. However, 28 days after infant SNI, spontaneous activity had increased significantly compared to sham-treated rats (mean firing rate, sham = 0.3±0.11 spikes. sec^{-1}; SNI = 15.3±8.5 spikes. sec^{-1}; Mann Whitney test $P<0.0001$). An increase in spontaneous activity was also observed in adults 7 days post SNI (3.5±1.03 spikes. sec^{-1}) compared to sham controls (0.2±0.17 spikes. sec^{-1}) (Mann Whitney test $P<0.01$) consistent to previous reports (Chapman et al., 1998b; Laird and Bennett, 1993). A higher proportion of neurons exhibited spontaneous activity (over 0.1 spikes per second) in adults following SNI (sham 42%; SNI 69%, although not significant) and in rats 28 days after P10 SNI (sham 50%; SNI 100%; $\chi 2$ analysis, $P<0.05$). In infants 7 days after SNI

there was no significant difference in the percentage of neurons exhibiting spontaneous activity compared to sham controls (sham 56%; SNI 43%).

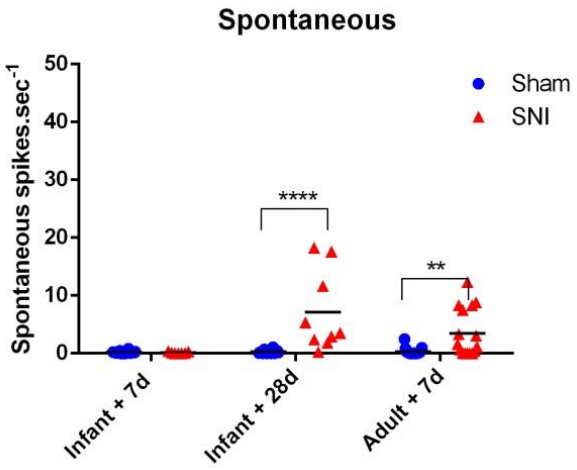


Fig 3.5 Spontaneous dorsal horn activity in infant and adult rats following SNI or sham surgery

(A) Infants did not show any significant differences in spontaneous neuronal activity 7 days after SNI surgery compared to sham controls (sham n=16; SNI n=7 cells). A significant increase in activity did occur in infants 28 days after SNI (sham=10; SNI=9) and in adult SNI treated rats 7 days after SNI surgery compared to sham controls (sham=19; SNI=16;). Mann Whitney test** P<0.01, **** P <0.0001. Infant sham +7d n=5 animals per group, Infant SNI +7d n=5 animals per group, Infant sham +28d, n=5 animals per group, Infant SNI+28d, n=5 per group, Adult sham +7d, n=11 animals per group, Adult SNI +7d, n=6 animals per group.

3.4.4 Cutaneous receptive field sizes.

Fig 3.6A and B show the effect of SNI and sham surgery on the receptive fields of brush and pinch respectively. The cutaneous receptive field size was measured as a percentage of the total foot area in order to correct for large differences in plantar foot area in the different postnatal ages tested (Koch et al., 2012; Torsney and Fitzgerald, 2003). Although not statistically compared here receptive fields appeared larger in infant compared to older sham treated adults which is consistently reported in other studies (Andrews and Fitzgerald, 1994; Beggs et al., 1992; Fitzgerald, 1985a). SNI at infancy significantly reduces the average brush and pinch receptive field compared to sham controls 7 days after surgery (Brush: sham 12.7±1.37%, SNI 7.6±0.71%, Mann Whitney test P<0.01; Pinch: sham 16.0±1.86 %, SNI 7.4±1.12% Mann Whitney test, P<0.001). However, by 28 days after infant SNI, brush and pinch - receptive fields significantly increase compared to sham surgery (Brush: sham 5.6±1.86%, SNI 11.4±1.05%, Mann Whitney test, P<0.05; Pinch: sham 8.5±1.10 %, SNI14.2±1.57%, Mann Whitney test, P<0.001). In adults both

brush and pinch receptive fields in SNI and sham treated rats are not significantly different as previously reported in peripheral nerve injured adult rodents (Laird and Bennett, 1993).

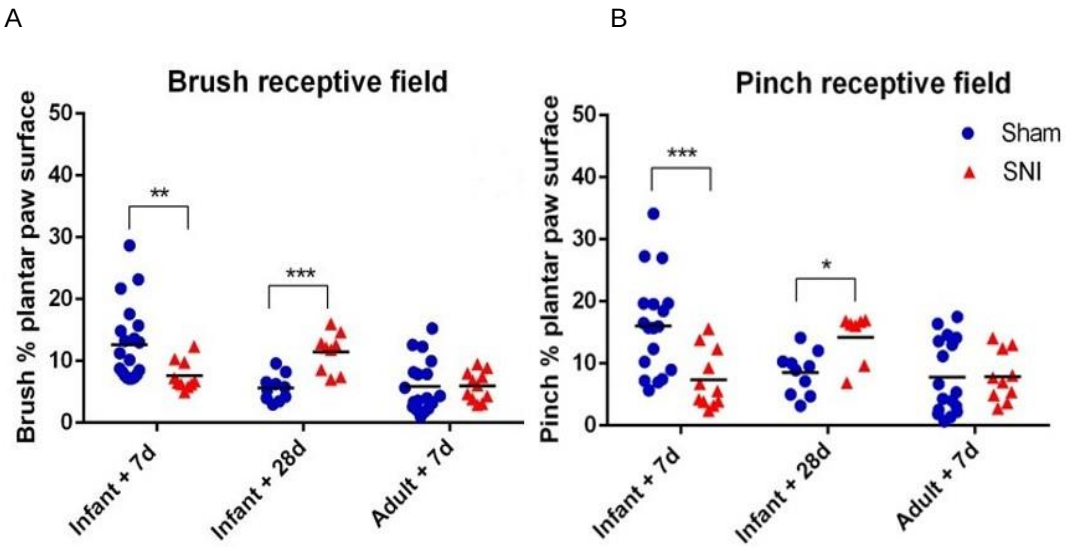


Fig 3.6 Receptive fields of brush and pinch in infant and adult rats following SNI or sham surgery.

(A) Brush receptive fields in infant animals 7 days after SNI (n=10 cells) decreased significantly compared to sham controls (n=20) but 28 days after infant SNI (n=9) increased significantly compared to sham control (n=10). The receptive field of brush in adult SNI (n=11) and sham treated animals (n=19) were similar. (B) Pinch receptive fields in infant animals 7 days after SNI (n=10) also decreased compared to sham controls (n=18) while 28 days after infant SNI pinch (n=7) receptive fields increased compared to sham controls (n=10). Pinch receptive fields in adult SNI (n=11) and sham treated animals (n=18) were similar. Mann Whitney test *P<0.05, ** P<0.01, *** P<0.001. Infant sham +7d, n=5 animals per group; Infant SNI+7d, n=5 animals per group, Infants sham +28d, n=4 animals per group, Infants SNI +28d, n=4 animals per group and Adult sham +7d, n=11 animals per group, Adult SNI +7d, n=6 animals per group.

3.4.5 Pinch and brush-evoked activity

Fig 3.7A shows that the activity of neurons evoked by pinching the receptive field did not significantly alter in SNI treated rats compared sham controls within any age group. In addition, brush evoked activity was not significantly different in infant and adult SNI from sham treated rats 7 days after surgery (Fig 3.7B). However, brush evoked activity of dorsal horn neurons was significantly increased in rats 28 days after infant SNI with a mean firing rate of 34.7±9.56 spikes. sec^{-1} compared to 16.9±2.69 spikes. sec^{-1} following sham surgery (Mann Whitney test P=0.0009).

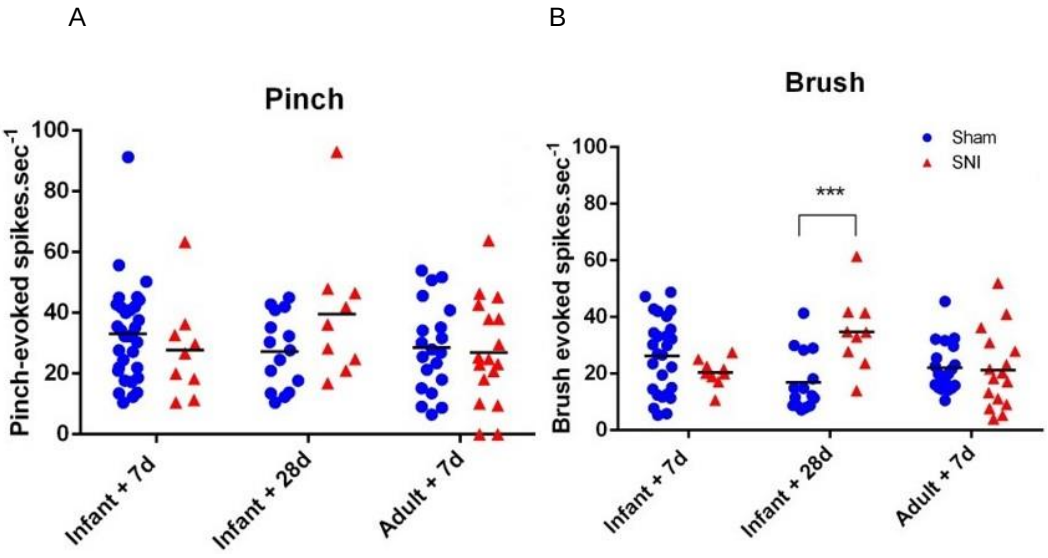


Fig 3.7 Pinch and brush evoked dorsal horn activity in infant and adult rats following SNI or sham surgery.

(A) Infant and adult SNI and sham treated rats exhibited comparable pinch evoked-activity in each age group (Infant+7days, sham n=17, SNI n=9 cells; Infant+28days, sham n=15, SNI n=9; Adult+7days, sham=15, SNI n=17). (B) Infant and adult SNI and sham treated rats exhibited comparable brush evoked activity 7 days after surgery (Infant+7days, sham n=29, SNI n=9; Adult+7days, sham=15 SNI n=16). Brush evoked activity significantly increase 28 days after SNI (sham n=15, SNI n=9; Mann Whitney test Pt<0.001). Infant SNI+7d, n=5 animals per group, Infants sham +28d, n=5 animals per group, Infants SNI +28d, n=5 animals per group and Adult sham +7d, n=11 animals per group, Adult SNI +7d, n=6 animals per group.

3.4.6 Acetone and ethyl chloride evoked activity

Although cellular responses to cold stimulation in adult rodents have been characterised in naïve and SNI treated animals, this is the first study to date that has investigated responses in infants. Fig 3.8A shows that in infants 7 days after SNI or sham surgery, there was no significant difference between the percentage of cells that responded to acetone (SNI 80%, Sham 88%). Although not statistically tested it also appears that infant rats maybe naturally more responsive to acetone than mature rats. 28 days after infant SNI surgery a significantly larger proportion of cells responded to acetone in SNI treated animals (90% of cells) compared to sham (36% of cells) surgery ($\chi 2$ analysis, $P < 0.01$). In addition, SNI treated animals exhibited a significantly higher activity (10.52±3.05.03 spikes. sec^{-1}) compared to sham controls (0.8±0.34 spikes. sec^{-1}, Mann Whitney test, $P<0.0001$). Similarly, in adults, a larger proportion of cells responded to acetone 7 days after SNI (70%) compared to sham controls (22%) ($\chi 2$ analysis, $P<0.05$) and rats also displayed significantly higher mean acetone evoked activity following SNI (6.3±1.80 spikes. sec^{-1})

compared to sham animals (0.08±0.05 spikes. sec^{-1}; Mann Whitney test, P<0.05).

The activity of WDR neurons in response to ethyl chloride applied to the receptive field was also measured. In infants 7 days after surgery, all cells in both sham and SNI treated animals responded to ethyl chloride (sham; n= 16 cells; SNI n=3 cells). However as the n of cells was low, the magnitude of the responses was not graphed or significance calculated. In animals 28 days after infant SNI or sham surgery a similar proportion of cells responded to ethyl chloride (SNI 100%, Sham 83%) but the mean neuronal activity of SNI treated animals was significantly higher (40.9.1±10.90 spikes. sec^{-1}) than sham controls (6.6±1.95 spikes.sec^{-1}, Mann Whitney test, P<0.001). In adults both the proportion of cells and neuronal activity of cells were not significantly different 7 days after SNI and sham surgery (SNI 100%, Sham 70%).

A B

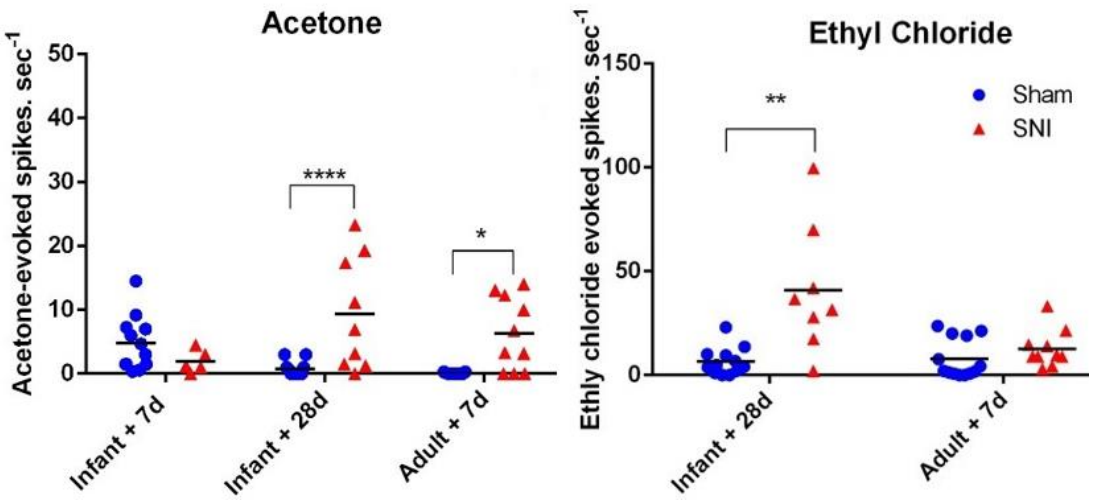


Fig 3.8 Acetone and ethyl chloride evoked dorsal horn activity in infant and adult rats following SNI or sham surgery.

(A)Infant rats exhibited comparable neuronal activity in response to acetone application 7 days post sham (n=12) and SNI (n=5) surgery. 28 days after infant SNI (n=9) acetone evoked activity significantly increased compared to sham (n=11) controls and in adults 7 days after SNI (n=10) compared to sham surgery (n=9). (B) Similarly 28 days after infant SNI (n=9) ethyl chloride evoked responses significantly increased compared to sham (n=13) controls. Mann Whitney test *P<0.05, ** P<0.01, **** P<0.0001. Infant sham+7d, n=4 animals per group, SNI+7d, n=3 animals per group, Infants sham +28d, n=4 animals per group, Infants SNI +28d, n=3 animals per group and Adult sham +7d, n=9 animals per group, Adult SNI +7d, n=3 animals per group.

3.4.7 Pressure evoked activity

Dorsal horn activity to 33g and 66g pressure stimulus applied with forceps to the centre of the receptive field was also tested. Every cell tested responded to both 33g and 66g pressure application. Fig 3.9A and 3.9B shows that 28 days after infant SNI dorsal horn neuronal activity is increased in response to 33g and 66g pressure compared to sham controls (Mann Whitney test, P<0.05, P<0.01). Responses of adult dorsal horn neurons to 33g and 66g pressure in rats 7 days following SNI was not significantly different from sham controls. Responses to pressure were not measured in P17 rats due to their comparatively small paws which made application of the pressure device difficult and readings varied widely with replication.

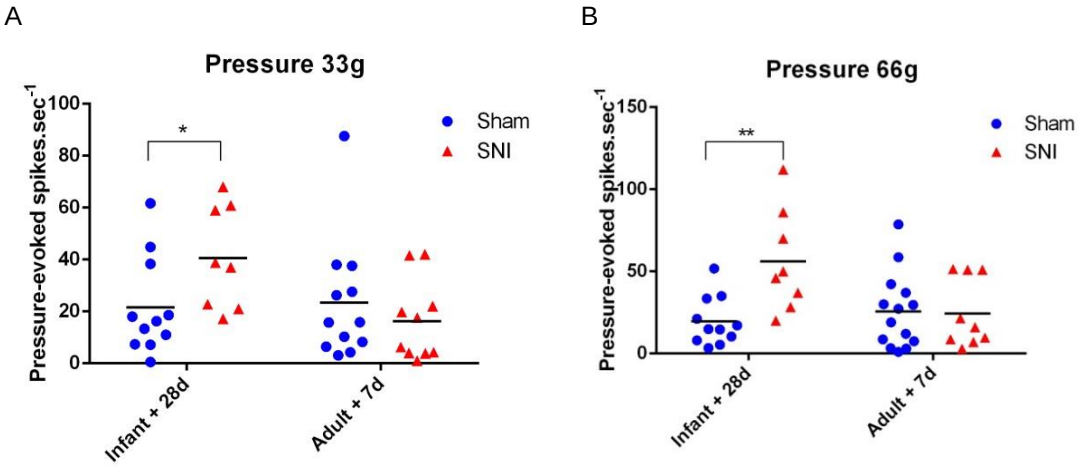


Fig 3.9 33g and 66g pressure evoked dorsal horn activity in infant and adult rats following SNI or sham surgery.

(A) 33g pressure induces an increase in dorsal horn activity in rats 28 days following SNI (n=8) relative to sham controls (n=11) but the activity of dorsal horn neurons in adults 7 days after SNI (n=10) remain comparable to sham controls (n=12). (B) 66g pressure evoked activity in rats 28 days after SNI (n=8) increased relative to sham treated rats (n=11) but remained comparable in SNI (n=9) and sham treated rats (=14) 7 days after adult surgery. Mann Whitney test *P<0.05, **P<0.01. Infants sham +28d, n=4 animals per group, Infants SNI +28d, n=3 animals per group and Adult sham +7d, n=9 animals per group, Adult SNI +7d, n=5 animals per group.

3.4.8 Von Frey hair thresholds and evoked activity

Mechanical thresholds were determined by applying vFh's to the cutaneous receptive field in ascending order, where the lowest force required to induce action potential firing was the 'threshold' hair and at this point recordings were made. Fig 3.10A shows that following SNI, the threshold vFh at each age group is not significantly different than the vFh threshold of sham treated rats.

However, the mean neuronal activity evoked by the application of the threshold vFh in infant rats 7 days after infant SNI (Fig 3.10B) was significantly reduced compared to age matched sham controls (Sham 22.7±2.04 spikes. sec^{-1}, SNI 10.5±1.90 spikes. sec^{-1} , Mann Whitney test, P<0.001). Fig 3.10C shows that 7 days after infant SNI significantly lower activity is evoked in WDR neurons following stimulation with a 6.3g vFh (11.6±2.35 spikes. sec^{-1}) compared to sham controls (31.8±5.44 spikes. sec^{-1},Mann Whitney test, P<0.01). Differences in mean activity of dorsal horn neurons were not observed to either a threshold or 6.3g vFh application at any other age.

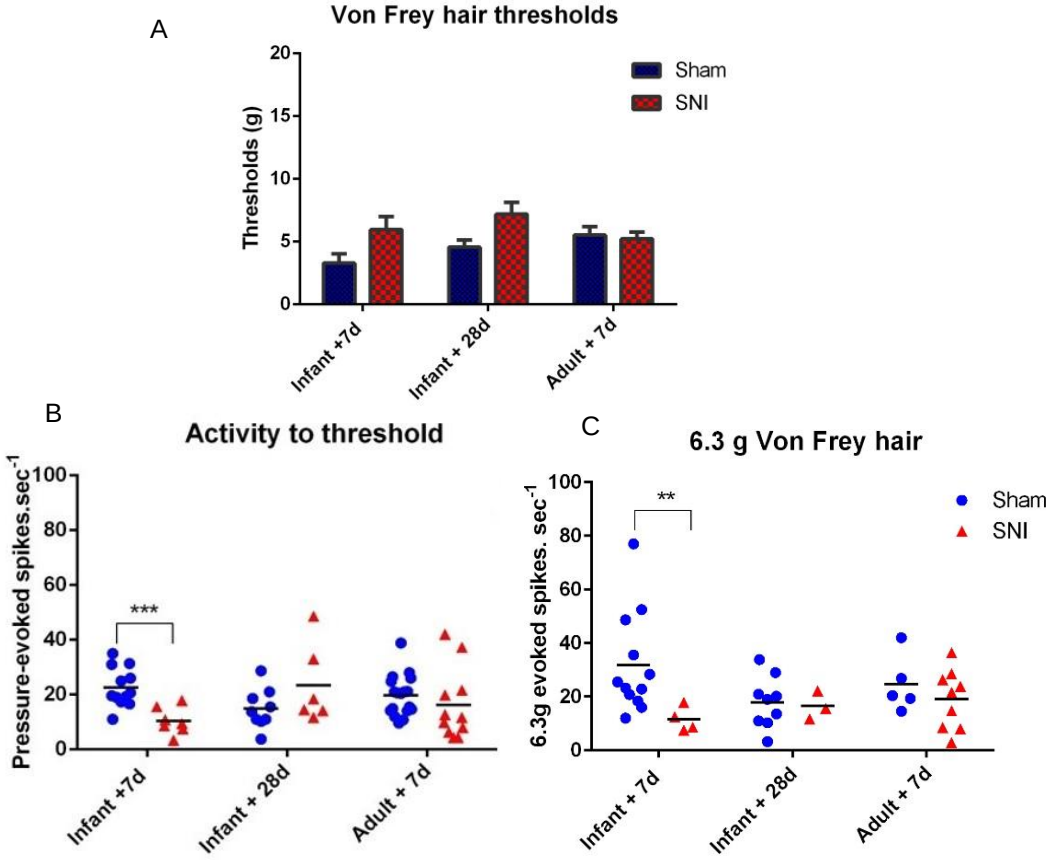


Fig. 3.10 Von Frey hair threshold and evoked activity in infant and adult rats following SNI and sham surgery.

(A) In all age groups VFh thresholds were similar in SNI and sham treated animals (Infant +7days, SNI n=7 and sham n=13 cells; Infant +28 days, SNI n=6 and sham n=18; Adults +7days, SNI n=8 and sham n=19). (B) Activity of dorsal horn neurons was significantly lower in infant rats 7 days after infant SNI (n=7) compared to sham controls (n=12) but comparable in infants 28 days after SNI (n=6) and sham surgery (n=9) and in adults 7 days after SNI (n=11) and sham surgery (n=18). (C) Activity of dorsal horn neurons to a 6.3g VFh was reduced in infant rats 7 days after SNI (n=4) compared to sham controls (n=12) but comparable in infants 28 days after SNI (n=3) and sham(n=9) surgery and adults 7 days after SNI (n=9) and sham (n=5) surgery. Mann Whitney test *P<0.05,** P<0.01.Anaimals/group: Infant sham+7d, n=4, SNI+7d, n=3, Infant sham +28d, n=4, Infant SNI +28d, n=3 and Adult sham +7d, n=10, Adult SNI +7d, n=4.

3.5 Discussion

Chapter 2 demonstrated that in contrast to adults, SNI at infancy (P10) does not induce pain-like behavioural alterations 7 days post-surgery but emerges 28 days later. This is the first study to examine the physiological properties of dorsal horn neurons at these early and late stages of post infant nerve injury and demonstrates that peripheral nerve injury at infancy induces long lasting physiological changes in dorsal horn processing of sensory information.

In adult rodents the use of NMDA antagonists in peripheral nerve injury animal models, can significantly reduce hypersensitivity (Carlton and Hargett, 1995; Christensen et al., 1999; Latremoliere and Woolf, 2009; Mao et al., 1993) suggesting that nerve injury induces central sensitisation and thus alterations in the spinal processing of sensory processing which underlie the pain-like behaviour (Chaplan et al., 1997; Shields et al., 2003). This has been confirmed in a number of peripheral nerve injury models including CCI and SNL, where alterations in spontaneous and evoked responses of dorsal horn neurons were observed alongside typical neuropathic pain-like behaviour (Chapman et al., 1998b; Laird and Bennett, 1993; Suzuki and Dickenson, 2006; Takaishi et al., 1996; Walczak et al., 2006). However, different animal models of peripheral nerve injury display similar, but not identical behavioural changes (Pradhan et al., 2010).

3.5.1 Spontaneous activity in adults and infants following SNI.

Although behavioural responses to evoked stimuli can be readily measured in animal models of nerve injury, ongoing activity is more difficult to quantify and only a few studies characterize ongoing pain (Tappe-Theodor and Kuner, 2014) despite a large proportion of neuropathic pain patients reporting this as a symptom (Rasmussen et al., 2004). As a result measuring spontaneous activity using *in vivo* electrophysiological recordings is a useful tool to reveal physiological changes underlying spontaneous pain, which is difficult to identify using behavioural measures (Suzuki and Dickenson, 2006).

In infants, no difference in either the mean spontaneous activity or proportion of cells exhibiting spontaneous activity was observed in rats 7 days after SNI

surgery compared to sham controls. However, 28 days later a significantly greater proportion of cells in SNI treated rats exhibited spontaneous activity of greater magnitude (more than 17 fold) compared to age matched sham controls. Similarly in adults SNI induced a mean increase (by more than 14 fold) in spontaneous activity to 3.5 spikes.sec^{1} compared to sham controls, paralleling the time course of the evoked behavioural hypersensitivity which is well characterised in other studies of nerve injury. Dorsal horn spontaneous activity is well documented in the literature as being a dominant characteristic in animal nerve injury models which also exhibit spontaneous firing patterns with an average frequency of 3.5 spikes.sec^{-1} (Chapman et al., 1998a, 1998b; Laird and Bennett, 1993; Walczak et al., 2006). Although not significant, there was also trend for a higher proportion of neurons in the adult SNI group to exhibit spontaneous activity compared to sham controls at this age. This is similar to previous studies (Chapman et al., 1998a, 1998b; Palecek et al., 1992) which found that 50% of neurons in SNL treated rats and monkeys exhibited spontaneous activity, significantly higher than sham controls.

Although the origin of spontaneous activity of spinal neurons in the dorsal horn is unknown, it maybe secondary to the generation of ectopic activity from the site of injury and from the dorsal root ganglion, occurring 12 hours post-surgery and persisting for 53 days (Chapman et al., 1998b; Sun et al., 2005; Tal and Eliav, 1996; Wall and Devor, 1983; Wu et al., 2001). Supporting this, previous studies suggest that ectopic discharge shows a good correlation with neuropathic pain behaviour in rodents in the early stage after nerve injury (Chul Han et al., 2000; Sun et al., 2005) and prevention of sensory neuronal ectopic activity (by blocking sodium channels or using local anaesthetic) can attenuate behavioural signs of allodynia (Boucher et al., 2000; Sukhotinsky et al., 2004). Therefore ectopic activity in afferents could promote the hyperexcitability in spinal neurons which in turn contribute to the development and maintenance of neuropathic pain behaviour (Sheen and Chung, 1993; Tal and Eliav, 1996; Wall and Devor, 1983; Yoon et al., 1996) although this is debated as neuropathic pain-like behaviour in adult rats following SNI is not prevented by a peripheral nerve block (Suter et al., 2003). Furthermore, if the periphery was the origin of spontaneous activity observed in dorsal horn neurons in rats 28

days after P10 SNI, the initiation of ectopic activity of primary afferents would be at least 10 days after surgery, which is a substantially longer time frame than observed in adults, which is initiated 12 hours post injury if not sooner (Sun et al., 2005).

Substantial evidence suggests that glial cells may also alter central neurotransmission following nerve injury via the release of glial and neuronal signalling molecules such as cytokines, chemokines and growth factors that exert pathological effects including neuronal hyperexcitability and spontaneous activity. For example incubation of spinal cord slices with TNF, IFN-γ, IL-1β and CCL2 increase spontaneous EPSC frequency (Gao et al., 2009; Kawasaki et al., 2008; Vikman et al., 2003; H. Zhang et al., 2010) and blocking pro-inflammatory responses in adults following nerve injury can alleviate pain behaviour (Ledeboer et al., 2005; Svensson et al., 2005; Xu et al., 2010) However, the presence of pro-inflammatory cytokines in the dorsal horn 28 days after infant SNI remains to be explored.

Regardless of the mechanism, this marked contrast in the occurrence of spontaneous activity 28 days after P10 SNI, but not 7 days may underlie behavioural hypersensitivity as proposed in adults (Chapman et al., 1998b; Suzuki and Dickenson, 2006).

3.5.2 Alterations in evoked-neuronal activity in adults following SNI.

Only receptive fields in the ‘spared’ sural nerve territory were mapped in both sham and SNI treated animals and there was no evidence of discontinuous or oddly shaped receptive fields at any age. Similarly to previous findings in other rodent nerve injury models, SNI in adults did not induce alterations in receptive fields (Laird and Bennett, 2011; Palecek et al., 1992). In addition, mechanical thresholds and evoked activity to brush, pinch, pressure, ethyl chloride, threshold and 6.3g von Frey hair application were not altered by SNI which is consistent to previous studies (Chapman et al., 1998b; Laird and Bennett, 1993; Palecek et al., 1992; Takaishi et al., 1996). Following SNI acetone evoked activity in a higher proportion of cells and to a higher frequency compared to sham surgery. This result is important clinically, as heightened cold sensitivity is frequently reported by patients with neuropathic pain (Jørum

et al., 2003; Leith et al., 2010; Ochoa and Yarnitsky, 1994). Interestingly there was no change in either the frequency of responses or proportion of cells in SNI treated animals using ethyl chloride. Electrophysiological studies have demonstrated that WDR and nociceptive specific dorsal horn neurons are excited by cold stimuli and encode intensity to noxious cold temperatures (Brignell et al., 2008) with subsets of spinal dorsal horn neurons responding to innocuous (e.g. acetone) and or noxious cold (e.g. ethyl chloride).

A recent study has shown that complement factors and their receptors, including complement-5 (c5), are some of the most differentially regulated genes in the dorsal horn in a number of adult nerve injury models and are only expressed by microglia (Griffin et al., 2007). Furthermore, intrathecal injection of C5 synthetic peptide induces cold hypersensitivity (enhancing responses to acetone) while blockade of C5 receptor reduces cold evoked pain behaviour in rats following SNI.

3.5.3 Alterations in evoked-neuronal activity in infants 7 days following SNI.

In contrast to adults, infant SNI induced a significant reduction in both brush and pinch receptive fields 7 days after surgery compared to sham controls. It has been well documented that cutaneous receptive fields are larger relative to the size of the paw in P3 rats than in P21 animals (Fitzgerald, 1985a; Fitzgerald and Jennings, 1999; Torsney and Fitzgerald, 2002) due to immature glycinergic inhibition of cutaneous evoked activity, that undergo activity dependent maturation in the second postnatal week (Koch et al., 2012). A potential mechanism underlying the decrease in receptive field size could be injury evoked-acceleration of the maturation of glycinergic neurotransmission in the cord, and warrants further investigation. While brush and pinch evoked activity and von Frey hair thresholds in infants were similar between treatment groups, the number of spikes evoked by von Frey hairs was reduced in the infants 7 days after SNI. An explanation for the decrease in firing observed could be due to a decrease in receptive field size. This would cause a reduction in the number of dorsal horn neurons activated by a given stimulus leading to an increase spatial discrimination while decreasing responses to vFh stimulation. Since mechanical thresholds were unchanged, it is unlikely that

changes in mechanical transduction processes underlie this decrease in activity. Furthermore, behaviourally P10 SNI exhibited similar responses to sham controls and there were no signs of hyposensitivity (Howard et al., 2005; Vega-Avelaira et al., 2012). The reduction in activity of WDR neurons following SNI may seem surprising, but is consistent to the responses observed in adult models of nerve injury (Chapman et al., 1998b; Palecek et al., 1992). One confounding factor maybe a reduction in peripheral drive into the dorsal horn spinal cord resulted from a complete injury. However, as the model used in this study was the SNI, all WDR neurons recorded had receptive fields in the sural nerve area which was spared. In support of this Li et al., used patch clamp slice recording of dorsal horn spinal cord neurons taken from rats 3-6 days after P6 SNI to show that infant nerve injury does not significantly modulate synaptic transmission or neuronal excitability in the dorsal horn (Li et al., 2009). The data in this thesis also suggests that nerve injury in infants does not increase dorsal horn neuronal activity.

As previously mentioned neuronal glia cell interactions may also influence the overall activity of the dorsal horn neuronal activity and the failure to evoke mechanical hypersensitivity in infant rats may reflect a weaker glial response in the infant spinal cord completed to adults (Moss et al., 2007; Vega-Avelaira et al., 2007, 2012). In addition, as glia can also be beneficial and release anti-inflammatory factors which act to restore normal pain signalling and protect against neurotoxicity, it is possible that a different immune response may occur between infants and adults which in the infant is protective.

3.5.4 Alterations in evoked-neuronal activity in animals 28 days following infant SNI

28 days after infant SNI, at the onset of pain-like behaviour WDR neuronal activity is profoundly altered and significantly greater compared to sham controls to range of stimuli including brush, acetone, ethyl chloride, threshold von Frey hair, 33g and 66g pressure. There is also an increase in both brush and pinch receptive fields in SNI treated animals. Importantly, these data suggest that the onset of pain-like behaviour is maintained by alterations occurring centrally. This supports a previous study showing that the pain-like

behaviour following infant SNI can be reversed by the administration of an NMDA receptor antagonist (Vega-Avelaira et al., 2012).

There are a number of possible mechanisms that may underlie the observed increase in receptive field and stimulus-induced increases in dorsal horn activity. One possibility is that nerve injury at infancy induces collateral sprouting of cutaneous sensory terminals into adjacent inappropriate regions of the dorsal horn outside their usual terminal areas, as in neonates following sciatic nerve section. However sprouting is not observed if transection is performed at P10 and is unlikely it would occur following partial peripheral denervation (Fitzgerald 1985b, 1990; Shortland and Fitzgerald, 1991, 1994). It is also possible that the balance of inhibitory and excitatory activity in the spinal cord are altered. For example, following nerve injury in the adult dorsal horn, glutamate uptake activity is reduced and a loss of GABAergic transmission may occur acting to enhance neuronal transmission supporting the induction and maintenance of neuropathic pain (Moore et al., 2002; Sung et al., 2003).

Peripheral nerve injury can also cause the activation of silent synapses (Devor and Wall, 1981; Hylden et al., 1989) between primary afferents and dorsal horn neurons and induce changes in the physiological characteristics of these cells. For example, in nerve injured adults, heterosynaptic facilitation can occur and input from primary afferent Aβ fibres transmitting non-noxious stimuli such as light touch can engage in pain transmission circuits resulting in pain in response to innocuous stimuli. In support of this, a recent study identified a feed forward glycinergic interneurons that in naïve animals represses the relay of innocuous input to lamina I by a lamina IIi excitatory interneurons that expresses PKC-γ (Lu et al., 2013). Although in naive animals LI neurons do not receive input directly from non-nociceptive primary afferents, after nerve injury the majority becomes responsive to innocuous touch (Keller et al., 2007).

This can also be replicated by inhibiting either glycine or GABA inhibition showing that central disinhibition can unmask interconnections between separate sensory path ways (Keller et al., 2007; Miraucourt et al., 2007). However, GABAergic inhibition is present early in postnatal development and glycinergic inhibition matures by the second postnatal week and in this context it would be more likely for increases in activity to be observed 7 days after

infant SNI (Koch and Fitzgerald, 2013). Microglia activation following nerve injury can lead to hypersensitivity also through the modulation of chlorine mediated inhibition (Ferrini and De Koninck, 2013).

As the genes most differentially regulated in infants and adults following nerve injury are immune related, with infants failing to mount a pro-inflammatory response in the dorsal horn 7 days after nerve injury (Costigan et al., 2009; Vega-Avelaira et al., 2009, 2007). One hypothesis is that a switch in the profile of inflammatory mediators in the dorsal horn may underlie the onset of pain behaviour 28 days after P10 SNI. A recent study suggests that at this time point, glial cell markers are increased in the dorsal horn compared to sham controls (Vega-Avelaira et al., 2009) although the presence of mediators that modulate dorsal horn neuronal activity remains to be elucidated.

3.6 Conclusion

While behaviour alone may reflect changes in the peripheral or motor system, the results presented in this chapter indicate that nerve injury induces alterations in the processing of sensory information by directly effecting dorsal horn neuronal sensitivity.

The profound alterations of neuronal activity coinciding with the onset of behavioural hypersensitivity suggests that the mechanism for the delayed onset of hypersensitivity lies within the dorsal horn. This data also confirms that early life nerve injury can cause prolonged changes in the central processing of sensory information in the dorsal horn that persist into adulthood.

Chapter Four

Inflammatory mediators in the dorsal horn following infant nerve injury

4.1 Introduction

Despite infant rat pups not displaying pain-like behaviour in response to neuropathy in the first few weeks of life, behavioural hypersensitivity does eventually emerge at adolescence (Vega-Avelaira et al., 2012). Chapter 3 indicates that the onset of pain-like behaviour following infant nerve injury coincides with an increase in both spontaneous and evoked dorsal horn neuronal activity suggesting that central changes in sensory processing in the dorsal horn spinal cord may underlie behavioural hypersensitivity. This corroborates a previous study showing that the late onset of mechanical hypersensitivity following infant nerve injury can be reversed by administration of an NMDA antagonist (Vega-Avelaira et al., 2012). As behavioural hypersensitivity coincides with an increase in microglia (IBA-1) and astrocyte markers (GFAP) in the dorsal horn spinal cord (Vega-Avelaira et al., 2012) this Chapter aims to elucidate the immune profile in the dorsal horn spinal cord in the early and late period following infant nerve injury in the absence and onset of pain-like behaviour respectively.

4.1.1 Adults exhibit a pro-inflammatory response in the dorsal horn spinal cord following peripheral nerve injury.

The dorsal horn spinal cord is the primary relay site for the processing of somatosensory information and central sensitization following nerve injury, contributing to the onset of neuropathic pain (See Chapter One). Following peripheral nerve injury in the adult infiltrating immune cells (e.g. macrophages and T-lymphocytes), glial cells in the CNS (e.g. microglia and astrocytes) and neurons release predominantly pro-inflammatory mediators (including cytokines, chemokines and growth factors) into the dorsal horn. Together these cells form an integrated network that modulates dorsal horn sensory neuronal excitability leading central sensitization and pain behaviour and has led to the notion that neuropathic pain is a neuro-immune disorder (Austin and Moalem-Taylor, 2010; Beggs and Salter, 2007; Clark et al., 2007; Costigan et al., 2009; Tsuda et al., 2003; Zhuang et al., 2005). Electrical stimulation of the sciatic nerve and dorsal root, in the absence of nerve injury, can also stimulate

the release of fractalkine, induce the transition of microglia into a pain-related enhanced state, and the subsequent release of pro-inflammatory mediators leading to behavioural hypersensitivity that mimics neuropathic allodynia (Clark et al., 2009; Hathway et al., 2009). Thus, the activity of the primary afferents that synapse in the dorsal horn is a pivotal component in the release of pro-inflammatory mediators that lead to dorsal horn neuron excitability and pain behaviour via a number of mechanisms (Wen et al., 2007; Xie et al., 2009).

4.1.2 Microglia

Microglia are regarded as macrophages equivilants in the central nervous system and act as the first and main form of immune response. Increasing evidence suggests spinal microglia are prominent players in the genesis of persistent pain, by releasing the inflammatory mediators (Taves et al., 2013). Nerve trauma causes microglia to display characteristic signs of reactivity within 4 hours of nerve injury in the area where these nerves terminate (Beggs and Salter, 2007; Hathway et al., 2009; Ji et al., 2013; Suter et al., 2009; Tanga et al., 2004).

A number of signalling molecules have been identified that enable direct communication between injured primary afferents and microglia including fractalkine, chemokine ligand 2 (CCL2), neuregulin-1 and matrix metallopeptidase 9 (MMP-9) (Calvo et al., 2010; Clark et al., 2010, 2009, 2007). In addition, products of tissue injury including adenosine triphosphate (ATP) which act through purinergic receptors, proteins that act through TLRs, complement components and reactive oxygen species can all induce a microglia response (Abbadie et al., 2003; Tanga et al., 2005; Tsuda et al., 2004, 2003).

Reactive microglia exhibit increased phosphorylation MAPK p38 and ERK1/2 that induce proliferation and secretion of inflammatory mediators (cytokines, chemokines and growth factors). Like T-cells, microglia can be functionally polarised under different conditions (Durafourt et al., 2012; Kobayashi et al., 2013; Taves et al., 2013). The M1 phenotype (classical/pro-inflammatory) occurs in response to pro-inflammatory cytokines (IFN-γ and TNF) as well as

pathogen-associated molecular patterns (PAMPS) such as LPS, while interleukin IL-10, IL-4 or IL-13 differentiate microglia towards an M2 (alternative/anti-inflammatory phenotype) (Fairweather and Cihakova., 2009; Gadani et al., 2012; Michelucci et al., 2009; Ponomarev et al., 2007).

Nerve injury in adults, stimulates the transition of microglia into an M1 phenotype and 'pain-related enhanced response state' and release of pro-inflammatory mediators including TNF, IL-1β and BDNF from the primary afferent fibre terminals (McMahon and Malcangio, 2009). These mediators can depolarise post synaptic neurons, thus contributing to central sensitization and pain-like behaviour (Coull et al., 2005; Ji and Suter, 2007b; Kawasaki et al., 2008; Ledeboer et al., 2005; Milligan et al., 2003; Tsuda et al., 2004; Zhuang et al., 2005).

TNF is synthesized and released by a multitude of cell types and is a prototypic pro-inflammatory cytokine due to its primary role in initiating the activation of other cytokines and growth factors including IL-1β, IL-6 and IL-8 (Hide et al., 2000; Wagner and Myers, 1996). In the dorsal horn TNF enhances the amplitude of glutamate-induced excitatory currents by increasing the frequency of spontaneous EPSC and the amplitude of AMPA and NMDA-induced currents (Kawasaki et al., 2008; Zhang et al., 2011). This dual role in increasing neuronal excitability and promoting ongoing inflammation make TNF a central mediator of neuropathic pain behaviour. This is confirmed by interference of TNF signalling through neutralising anti-bodies or receptor antagonists that reverses pain hypersensitivity in models of peripheral nerve injury, even if the treatment is delivered after the pain is established (Marchand et al., 2009; Svensson et al., 2005; Sweitzer et al., 2001).

BDNF, released from microglia following ATP activation via P2X4 receptors (P2X4R) acts via its tyrosine protein kinase B (trkB) receptor on Lamina I dorsal horn neurons to reverse the polarity of currents activated by GABA, which switch from hyperpolarizing or inhibitory to depolarizing or excitatory. This leads to the disinhibition of lamina I neurons which are a major group of nociceptive output neurons in the dorsal horn and increase pain-like behaviours in animal models of neuropathic pain (Coull et al., 2005; Kawasaki et al., 2008; Trang et al., 2009).

4.1.3 Astrocytes

Astrocytes are another subtype of glial cell in the CNS which envelope synapses and have many important physiological properties, many of which relate to the maintenance of homeostasis (Dong and Benveniste, 2001) but are also immunocompetent cells and are activated following peripheral nerve injury in adults. Activation markers including JNK and ERK, that are up-regulated in the dorsal horn 1 week after peripheral nerve injury and may persist for 3 months post injury (Colburn et al., 1999; Coyle, 1998; Ji et al., 2013; Ma and Quirion, 2002; Mika et al., 2009; Zhuang et al., 2006, 2005). Following nerve injury astrocytes also release a plethora of mediators such as nitric oxide, excitatory amino acids, IL-1β and TNF that mediate hypersensitivity and are associated with the maintenance phase of neuropathic pain (Duan et al., 2003; Kawasaki et al., 2008; Liu et al., 2000; Malcangio et al., 1996; Queiroz et al., 1997).

4.1.4 T-cells

Following nerve injury T lymphocytes (but not B or NK cells) infiltrate the dorsal horn spinal cord within 7-10 days by transendothelial migration (Cao and DeLeo, 2008; Costigan et al., 2009; Engelhardt, 2006; Grace et al., 2011; Hu et al., 2007; Zhang et al., 2009). Depending on the environmental context, naïve T cells proliferate and differentiate into T helper (Th) subsets Th1, Th2 and Th17 that are classified by their distinctive cytokine profiles and effector function (Mosmann and Coffman, 1989; Mosmann and Sad, 1996; Palmer and Weaver, 2010).

Th1 cells produce mediators mostly associated with pro-inflammation such as IFN-γ which is important in responses against microbial infection, while Th2 cells secrete anti-inflammatory mediators including IL-4 and IL-10. Two transcription factors T-bet and GATA-3 are required for the transcription of Th1 and Th2 cytokine genes respectively. When cluster of differentiation 4 (CD4) T-cells are activated under Th1 skewing conditions, GATA3 is down regulated while in Th2 conditions (such as in the presence of IL-4) GATA3 is up regulated. The opposite is true for T-bet (Zheng and Flavell, 1997). Therefore, in addition to promoting Th2 cell differentiation GATA3 also inhibits Th1 cell

differentiation and IFN-γ production (Ouyang et al., 1998; Zheng and Flavell, 1997). Evidence also indicates that Th2 responses also suppress macrophage and microglia M1 phenotypes and expression of pro-inflammatory mediators (Aloisi et al., 1999; Chao et al., 1993; Durafourt et al., 2012; Kopf et al., 1993; Kuhn et al., 1991; Lord and Lamb, 1996; Milligan and Watkins, 2009; Ponomarev et al., 2005; Üçeyler et al., 2009).

In adults, following nerve injury a Th1 response dominates in the dorsal horn and interferon gamma (IFN-γ) is significantly up regulated (Costigan et al., 2009). IFN-γ is released from infiltrating T-cells (as well as astrocytes and damaged neurons) and intrathecal administration of IFN-γ in adult rats induces pain-like behaviour, together with spontaneous firing, increased wind up and a reduction in inhibitory tone of dorsal horn neurons, thus promoting central sensitization and neuropathic pain like behaviour in rodents (Tsuda et al., 2009). IFN-γ signalling is also critical in transforming resting microglia via its IFN receptor (IFNR) into an activated morphology, increasing the expression of key markers and release of pro-inflammatory mediators associated with an enhanced pain-related response state (Costigan et al., 2009; Tsuda et al., 2009). In adult rats disruption of this signalling pathway can reverse pain-like behaviour and IFN-γR null mutant mice also exhibit reduced neuropathic pain like behaviour (Costigan et al., 2009; Racz et al., 2008; Vikman et al., 2005). In addition, adoptive transfer of Th1 cells into *nude* rats following nerve injury increases pain like hypersensitivity while adoptive transfer of CD4+ (cluster of differentiation 4) T-cells into CD4-/- mice induces mechanical hypersensitivity to wild type levels (Cao and DeLeo, 2008; Moalem et al., 2004).

4.1.5 Inducing an anti-inflammatory response in adults after nerve injury

Anti-inflammatory mediators can regulate and limit potentially damaging effects of excessive inflammatory reactions by down-regulating the synthesis of pro-inflammatory cytokines (such as TNF, IL-1β and IL-6) and recruitment of anti-inflammatory immune cells in the spinal cord (Hu et al., 1999; Ledeboer et al., 2005; Poole et al., 1995).

Many studies have shown that by inducing a response in favour of an anti-inflammatory pathway following peripheral nerve injury can disrupt the

development of chronic neuropathic pain. For example, IL-4 (released from T-cells in the dorsal horn) and IL-10 (released from both T-cells and microglia in the dorsal horn) are hallmark potent anti-inflammatory cytokines that are anti-nociceptive in models of chronic pain and act to suppress pro-inflammatory mediators including IL-1β, IL-6, nitric oxide (NO) and TNF and microglia/macrophage responses (Poole et al., 1995). IL-10 cytokine acts via the induction of suppressor of cytokine signalling 3 (SOCS3) to inhibit genes associated with the janus kinase (JAK) and signal transducer and activator of transcription (STAT) 3 pathway that inhibit the transcription factor nuclear factor kappa-B (NF-Kb) important for the expression of pro-inflammatory mediators including IL-1β and TNF (Driessler et al., 2004; Inagaki-Ohara et al., 2003; Mosser and Zhang, 2008; Ogawa et al., 2008). Intrathecal administration of IL-10 protein or adenovirus vectors (that produce IL-10) can prevent and reduce pre-established pain related behaviours following peripheral nerve injury and is correlated with a decrease in pro-inflammatory cytokine expression in the dorsal horn spinal cord (Milligan et al., 2005a,b). Treatment with glatiramer acetate, which enhances the expression levels of IL-10 and IL-4, can reverse hypersensitivity following nerve injury (Leger et al., 2011). Plasmid DNA encoding IL-10 encapsulated into micro particles on a synthetic polymer permits high but slow release of IL-10 to effectively reduce mechanical hypersensitivity following CCI for 70 days (Soderquist et al., 2010).

Together these studies indicate that, in adults, the predominant release of pro-inflammatory mediators in the dorsal horn spinal cord act as potent neuromodulators to induce dorsal horn sensitization and neuropathic pain. Clearly an important factor in the development and duration of pain following nerve injury is the balance between pro and anti-inflammatory mechanisms, and altering the balance towards an anti-inflammatory response maybe a therapeutic strategy for neuropathic pain (Costigan et al., 2009; Kawasaki et al., 2008; Ledeboer et al., 2005; Milligan et al., 2003).

4.1.6 Infants do not exhibit a pro-inflammatory response in the dorsal horn spinal cord following nerve injury.

As described in Chapter Two, peripheral nerve injury in infant rat pups is not associated with the development of mechanical hypersensitivity 7 days after

surgery, and the up-regulation of glia cell markers in the dorsal horn spinal cord is significantly less than observed in adults (Costigan et al., 2009; Moss et al., 2007; Vega-Avelaira et al., 2007). Microarray comparison of adult and infant L4/L5 DRG and lumbar dorsal horn spinal cord tissue 7 days after infant nerve injury indicate that the genes most differentially regulated are those associated with the peripheral and central immune system (Costigan et al., 2009; Vega-Avelaira et al., 2009). This led to the concept that the absence of pain like behaviour in infants following peripheral nerve injury maybe due to an endogenous 'immature' and 'unresponsive' immune response (Costigan et al., 2009; Moss et al., 2007; Vega-Avelaira et al., 2007).

However, further studies in the infant show that responses are more complex. Intrathecal administration of exogenous ATP-stimulated microglia and direct C-fibre stimulation does not result in reduced behavioural mechanical thresholds or robust expression of microglia markers or T-cell markers, in contrast to adults (Hathway et al., 2009; Moss et al., 2007; Vega-Avelaira et al., 2007). Indeed, studies suggest that infants can mount an immune response but this is dependent on the type of the injury (Forsthuber et al., 1996; Moss et al., 2007). Furthermore, in response to insults that induce a pro-inflammatory response in adults, infants can mount anti-inflammatory responses in the periphery (Adkins et al., 2000, 2000; Min et al., 2000; Powell and Streilein, 1990).

These studies suggest that an infant may mount a dominant anti-inflammatory dorsal horn response in the early phase following nerve injury. As the animal reaches adolescence this response may subside or even switch to a pro-inflammatory response, unmasking pain behaviour. Currently little is known about the expression and balance of inflammatory mediators in the infant dorsal horn in the early and late periods after nerve injury and warrants further investigation.

4.2 Aims of the chapter

In adults peripheral nerve injury leads to an immune response characterised by the release of pro-inflammatory mediators that underlie neuropathic pain sensitization. However, comparatively little is known about the immune response in the dorsal horn spinal cord of infants after peripheral nerve injury. This is important because the onset of pain behaviour is delayed in infants and only emerges when the animals reach adolescence. The key objectives of this chapter are to:

1. Identify the immune profile in the dorsal horn of infants 7 days after P10 SNI, in the absence of pain behaviour.
2. Determine if a similar inflammatory response is induced in the infant following C-fibre stimulation.
3. Establish if the immune profile in the dorsal horn of infants changes 21 days after P10 SNI, at the onset of pain behaviour.
4. To test whether manipulation of the immune response in the early post nerve injury period in infant mice can ‘unmask’ pain-like behaviour.

4.3 Materials and Methods

4.3.1 Animals, surgery and behavioural testing

Male CD1 mice were obtained from Charles River. Mice pups were housed with mother and their littermates while animals over P21 were caged in littermates of 5 and allowed free access to water and housed in 12 hour light/dark cycles. SNI or sham surgery was completed on P10 and P33 CD-1 mice and mechanical sensory testing was completed as outlined in chapter 2. All experiments were conducted in accordance with the United Kingdom animals Scientific Procedures Act of 1986.

4.3.2 Tissue extraction of dorsal horn spinal cord tissue for use in cytokine array and real time qPCR

Mice were transcardially perfused with PBS. The spinal cord was exposed by making an incision to the skin using a scalpel along the vertebrae and cutting through the vertebrae vertically, four centimetres rostral to the base of the ribcage, and three centimetres caudal to the base of the rib cage to ensure the lumbar segment (L) 4-L5 of the spinal cord remained intact. Fat and connective tissue was then removed via scalpel to enable visualisation of the vertebrae. Toothed forceps were used to lift the column and the vertebrae was removed using rongeurs. The rostral spinal cord was lifted out using small toothed forceps. The ipsilateral and contralateral L4/L5 lumbar dorsal horn quadrants were separated using a scalpel and placed immediately into labelled Eppendorf tubes and stored at -80°C until required.

4.3.3 Cytokine array

4.3.3.1 Protein extraction from spinal cord

Ipsilateral and contralateral lumbar dorsal horn quadrants of mice 21 days after P10 SNI (n=5) were defrosted on ice and homogenized separately using a pestle tissue grinder (Corning life science, #7724T-1) in 20µl lysis buffer (containing 1/10 RIPA; Millipore, #20-188; 1/100 of protease and phosphatase inhibitors; Sigma., # P5726, P0044, P8340, P7626, adjusted to 1ml with double distilled water and kept at 4^0C). Homogenised tissue was made up to a volume of 100-200µl with lysis buffer, vortexed (10 seconds) and kept on ice for 30

minutes (Zhuang et al., 2005). Samples were centrifuged at 8,000 rpm for 20 minutes at 4^0C to remove any remaining insoluble material, and the supernatant was transferred in new tubes and subsequently stored at -80^0C.

4.3.3.2 Total protein quantification

Protein quantity per sample was determined using a bicinchoninic acid (BCA) assay (Pierce # PI23227). Six bovine serum albumin (BSA) standards were prepared by using a 50:50 dilution curve beginning at 1mg/ml (Table 4.1). In a 96 well plate 10µl of each BSA standard or 2µl of protein sample with 8µl of water was added to each well. 200µl of BCA working reagent was then added to each well, covered and placed on a shake plate for 10 seconds and kept in an incubator set at 37^0C for 30 minutes. The plate was re-shaken for 10 seconds, uncovered and read at 562nm absorbance. The colour emitted by the known samples enabled a standard curve to be generated and the unknown protein concentrations to be calculated.

Number	Concentration (mg/ml)	Standard protein (µl)	Water (µl)
1	0.00	0	400
2	0.25	50	350
3	0.50	100	300
4	1.00	200	200
5	1.50	300	100
6	2.00	400	0

Table 4.1 Table showing volumes of solutions used to make six BSA standards.

Standard protein was added with water volumes to form concentrations of solution and used to form a standard curve.

4.3.3.3 Incubation of samples with cytokine blots

The mouse cytokine array kit was purchased from R & D Systems (Mouse cytokine array panel A; # ARY006) and performed according to the protocol of the manufacturer as previously described (Gao et al, 2009). The protein concentration from ipsilateral or contralateral tissue obtained from 5 animals (21 days after P10 SNI) were added together to produce one 400µg protein sample. Each sample was then incubated with a separate blot (array) pre-coated with 40 cytokine/chemokine duplicate antibodies for 24 h at 4°C on a

rocker. Blots were washed in buffers provided and incubated for 30 minutes using Streptavidin-horse radish peroxidase (HRP)-conjugated secondary antibody, and developed using enhanced chemiluminescence (ECL) solution (Thermo scientific, #34076). Both blots were then exposed onto Hyperfilm at the same time (GE Healthcare) for 1–10 minutes. The intensity of the duplicate bands were analysed using image J 1.36 (NHS) software. Duplicates were averaged and the background subtracted to calculate the mean pixel density for each protein.

4.3.4 Quantitative real-time RT-PCR.

Dorsal horn spinal cord tissue for real time quantitative polymerase chain reaction (qPCR) analysis was defrosted on ice. Total ribonucleic acid (RNA) was extracted using RNeasy Plus Mini kit (Qiagen) according to the manufacturer's protocol as follows. Tissue was placed in 300µl buffer RLT containing β-mercaptoethanol (10µl/ml) and was disrupted and homogenized using a pestle tissue grinder and vortexed for 10 seconds. The lysate was added into a Qiashredder (Qiagen) column and collection tube and centrifuged for 3 minutes at maximum speed (13,000 rpm). Tissue lysate was then centrifuged at full speed for 3 minutes, removed and transferred via pipette into an Eppendorf tube. Samples were processed by further centrifugation steps as described in the manufacturer's protocol. To ensure complete removal of ethanol from RNA extract following the final centrifugation, columns were placed on a heat block for 1 minute at 55^0C and RNAse free water was eluted in the step. The quantity of RNA obtained was measured using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific).

4.3.4.1 Reverse transcription and q-PCR

Total RNAs (0.5-1 µg) were reverse-transcribed using the SuperScript III reverse transcriptase (Invitrogen) or QuantiTect RT kit (Qiagen) according to the protocol of the manufacturers. Specific primers including glyeraldehyde 3-phosphate dehydrogenase (GAPDH) control were designed using IDT SciTools Real-Time qPCR software (Integrated DNA Technologies). Firstly RNA was added to 2µl of gDNA wipe-out buffer and made up to 12µl with RNase-free water and set into a heat block at 37^0C. The cDNA concentration

of each sample was then measured using a Nanodrop and stored at -20ºC until required. Specific primers were designed using IDT SciTools Real-Time PCR software (Integrated DNA technologies) and purchased from Sigma® and diluted to a concentration of 100μM using RNAfree water. Forward and reverse primer sequences are described in Table 4.2. Gene-specific mRNA analysis was performed using a MiniOpticon Real-Time or CFX96 Real-time systems (Bio-Rad). Quantitative PCR amplification reactions contained the same concentration of reverse transcription product in a final volume of 15 μl (7.5μl of 2x Kapa SYBR Master Mix (Kapa Biosystems), 1.5μl of forward and 1.5μl of reverse primer and 4.5μl of cDNA dilution). Samples were added into wells and centrifuged for 5 minutes at 5,000g. The thermal cycling conditions comprised of 3 minutes of polymerase activation at 95°C, 45 cycles of 10 second denaturation at 95°C, and 30 second annealing and extension at 60°C. A DNA melting curve was included to test the amplicon specificity. GAPDH was used as an internal control.

	Forward	Reverse	Gene bank no
GAPDH	TTGATGGCAACAATCTCCAC	CGTCCCGTAGACAAAATGGT	NM_001289726
TNF	CAAGGGACAAGGCTGCCCCG	GCAGGGGCTCTTGACGGCAG	NM_013693
BDNF	GCCCTTCATGCAACCGAAGTA	TGAGTCTCCAGGACAGCAAA	NM_001048139
IBA-1	GGACAGACTGCCAGCCTAAG	GACGGCAGATCCTCATCATT	NM_019467
GFAP	GAATCGCTGGAGGAGGAGAT	GCCACTGCCTCGTATTGAGT	NM 010277
CD2	CACAGGTCAGGGTTGTGTTG	AATGGGATGACTAGGCTGGA	NM_013486
IL-10	TGTCAAATTCATTCATGGCCT	ATCGATTTCTCCCCTGTGAA	NM_010548
IL-4	GGTCTCAACCCCCAGCTAGT	GCCGATGATCTCTCTCAAGTGAT	NM_021283
TGF-β	TGGAGCAACATGTGGAACTC	CAGCAGCCGGTTACCAAG	NM 01557
T-BET	AACCGCTTATATGTCCACCCA	CTTGTTGTTGGTGAGCTTTAGC	NM_011486
GATA3	AGGATGTCCCTGCTCTCCTT	GCCTGCGGACTCTACCATAA	NM_008091
IL-13	CACACTCCATACCATGCTGC	TGTGTCTCTCCCTCTGACCC	NM_008355

Table 4.2 Sequences of the primers used for quantitative real-time RT-PCR.

4.3.5 C-fibre stimulation

C-fibre stimulation of mice was performed as previously described in rats (Hathway et al., 2009). P10 or P33 mice were anesthetized with isoflurane (5%

induction) and the left sciatic nerve was exposed via an incision through the thigh, the overlying muscle was moved aside using blunt dissection and the nerve dissected free of perineural membranes. Isoflurane was reduced to 3% and exposed nerve was electrically isolated from the surrounding muscle and other tissues by placing a small piece of plastic under the nerve. Two silver wire electrodes were placed under the exposed sciatic nerve. Care was taken to ensure that the electrodes were only in contact with the nerve and that the sciatic nerve was never stretched. Isoflurane was reduced to 2.3% and trains of electrical stimuli were applied for 5 min at 10 Hz (500 μs, 6 mA) to recruit C-fibre afferents as described elsewhere (Kerr et al., 2001). Sham controls underwent surgery and electrode placement but not electrical stimulation. Stimuli were generated using a Neurolog (Digitimer, Welwyn Garden City, UK) NL300 pulse generator, an NL510 pulse buffer and an NL800 stimulus isolator. Following stimulation, electrodes were removed and the muscle and skin sutured using 5.0 Mersilk (Ethicon, Edinburgh, UK). Animals were then returned to their home cage to recover with free access to food and water. Identical procedures were followed in both P10 and P33 mice. Mechanical sensitivity was tested 3 and 24 hours post C-fibre stimulation and tissue collection taken at the 24 hour time point for q-PCR analysis. Q-PCR for this experiment was performed by Dr Berta.

4.3.6 Microglial culture and activation

Microglia cultures were prepared from cerebral cortices of 2-day-old postnatal mice (n=10). Tissues were then minced into approximately 1 mm pieces, added to 2mls of Hank's balanced salt solution (HBSS, Invitrogen, #141175-095) and centrifuged at 300g for 5 minutes. Supernatant was discarded and replaced in 3ms of Dulbecco's modified eagle medium (DMEM, Invitrogen, # 11995-065). The cell pellets were dissociated with a pipette and poured through a cell strainer and centrifuged at 500g for 5 minutes. Supernatant was discarded and the pellet was re-suspended in 3mls of DMEM. Cell suspension was then filtered through a 100μm and 10 μm filter (BD Bioscience, USA) and volume adjusted to 25mls of DMEM and mixed. 5ml of DMEM were added to flasks to cover the bottom and 5ml of re-suspended cells were added to each flask and incubated at 37^0C at 5% CO_2 level for 2 days before adding 5mls of

fresh DMEM. Media was maintained for 3 weeks with culture media (containing 10% foetal bovine serum, heat inactivated; invitrogen #10010-023, Fungizone; Invitrogen #15295-018 in high-glucose DMEM) which was then changed with DMEM every 3 days. The mixed glia were shaken at 37^0C for 4 hours (200 rpm), and the floating cells were collected and centrifuged at 300g for 15 minutes and plated at a density of $5x10^5$ cells per well on 13mm diameter uncoated glass coverslips. After 48 hours the plated cell medium was exchanged with HEPES buffer (10mM pH 7.4 containing: NaCl, 150 mM; KCl, 5 mM; $MgCl_2$, 1 mM; CaCl,1mM and D(+) glucose, 5.55 mM). The cell density of microglia was measured using a cell counter and the volume of PBS adjusted to give a final density of 1000cells/10µl. This method resulted in greater than 95% purity of microglia (Nakajima et al., 1992). The cells were subsequently incubated with Lipopolysaccharide (LPS, 1mg/mL; E.coli; Sigma, UK) or an equivalent volume of PBS for three hours prior to intrathecal injection. LPS-activated or PBS-non-activated microglia were kept at 37^0C and vortexed prior to each injection. Microglia culture production and stimulation was performed by Dr Elisabeth Old.

4.3.7 Drug and microglia administration and post behaviour testing

Anti-IL-10, TNF and LPS-activated microglia preparations or controls were administered via intrathecal (i.t) injection to male mice 7 days after P10 SNI or sham surgery (Fig 4.1). Mechanical thresholds were tested before and at various time points following i.t injection (Table 4.3).

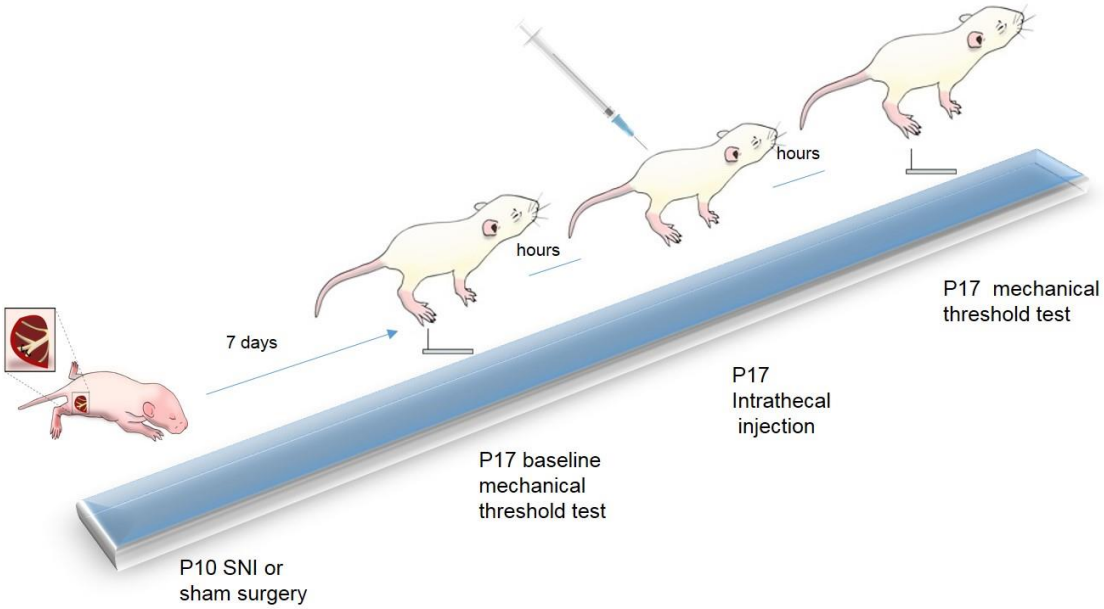


Fig 4.1 Diagram showing the time course for the intrathecal delivery of drugs and microglia cells.

Mice were given SNI or sham surgery at P10. 7 days later (when the mice reached P17) intrathecal injections were administered and mechanical thresholds were then tested at various times following injections.

Rat IL-10 monoclonal antibody (R&D systems, Mineapolis, MN# JES052A5) or isotype control antibody (R&D systems, Mineapolis, MN# MAB005) were reconstituted (0.5mg/ml) in sterile phosphate buffer saline (PBS) and stored at -20^{0}C until use. Prior to injection, solution was kept out of the fridge to reach room temperature and then intrathecally administered (10µg). This dose was based on a previous study (Lin et al, 2010). Recombinant rat TNF (R&D systems, Mineapolis, MN #510-RT) or vehicle control were reconstituted (10µg/ml, pH7) in sterile PBS containing 0.1% bovine serum albumin and intrathecally administered (20ng).

Reagent	Concentration and volume	Number of injections	Mechanical threshold testing
Anti-IL-10 or Isotype control	10ug, 3.5 µl	1 per day for 3 days (ie days 7,8,9 post surgery)	1 hour following each injection and one day after injection 3
TNF or Isotype control	20ng, 3.5µl	1	30mins, 3 hours, 24 hours later
LPS activated or PBS non- activated microglia	350cells, 3.5µl	1	1 hour for 6 hours following injection

Table 4.3 Table showing the doses of anti-IL10, TNF and microglial preparations administered and time course of mechanical threshold testing

Intrathecal injections containing anti-IL-10, TNF, LPS-activated microglia or controls were administered at 7 days post P10 SNI or sham surgery. IL-10 was administered for 3 consecutive days while one i.t injection of TNF and microglia were administered. Mechanical thresholds were tested at different time points following i.t injections for each reagent.

This dose was based on previous study (Gao et al., 2009; Zhang et al., 2011). LPS-activated microglia or control (PBS non-activated microglia) were kept in a water bath maintained at 37^0C, and vortexed before being intrathecally injected. To ensure accurate volumes of reagents were injected, injectable solutions were measured with a pipette and put onto parafilm for easy transfer to 1ml insulin syringes. All injections were performed under brief isoflurane anaesthesia (2.7%) by spinal cord puncture with a 1 ml insulin syringe between the L5 and L6 level to deliver reagents (3.5µl) to the cerebral spinal fluid (Beringue, 2013; Westin et al, 2010; Hylden and Wilcox, 1980; Zhang et al., 2011). Drugs and microglial preparations were administered for different numbers of days and mechanical thresholds were tested at different time points following administration (see table 4.3).

4.3.8 Statistical analyses.

Data was analysed and graphs plotted using GraphPad Prism software (version 6.00, GraphPad Software, San Diego, CA, USA, www.graphpad.com). All behavioural data was checked for skewness and kurtosis and the Shapiro-Wilk normality test completed before statistical

testing. A normal distribution of 50% mechanical threshold (g) was achieved by logarithmic transformation (log2) of data before statistical analysis as previously described (Baumgärtner et al., 2002; Géranton et al., 2009; Sens et al., 2012). Behavioural data was then analysed by a repeated measure two-way analysis of variance (ANOVA) and adjusted for multiple comparisons using the Bonferroni correction analysis where appropriate for within age group comparisons (Sens et al, 2012). Behavioural data is presented as mean±standard error of the means unless otherwise stated. QPCR data was analysed using a 2-tailed student's t test and expressed as fold change compared to sham control. The criterion for statistical significance was $p < 0.05$.

4.4 Results

4.4.1 Three weeks after infant SNI, an increase in inflammatory mediators occurs in the ipsilateral dorsal horn.

To determine if the delayed onset of pain-like behaviour at 21 days after infant SNI is marked by changes in the late expression of cytokines and chemokines, a screening cytokine array (blot) on ipsilateral dorsal horn mouse tissue was completed and compared to contralateral dorsal horn tissue from the same animal (n=5). Each membrane contained40 different cytokine and chemokine antibodies (Fig 4.2 A) and was incubated with either ipsilateral or contralateral dorsal horn tissue (Fig 4.2B).

A

	1,2	3,4	5,6	7,8	9,10	11,12	13,14	15,16	17,18	19,20	21,22	23,24
A	**Control**											
B	BLC	C5a	G-CSF	GM-CSF	CCL1	CCL11	ICAM-1	IFN-γ	IL-1α	IL-1β	IL-1ra	IL-2
C	IL-3	IL-4	IL-5	IL-6	IL-7	IL-10	IL-13	1L-12P70	IL-16	IL-17	IL-23	IL-27
D	IP-10	I-TAC	KC	M-CSF	MCP-1	MCP-5	CXCL9	CCL3	CCL4	MIP-2	RANTES	SDF-1
E	CCL17	TIMP-1	TNF-α	Trem-1								
F	**Control**											

B C

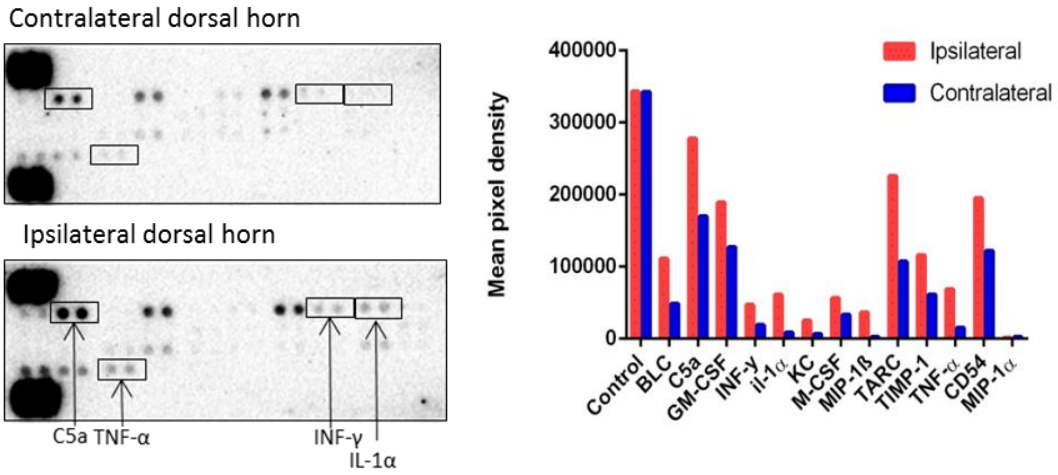


Fig 4.2 Cytokine array reveals an up-regulation of inflammatory mediators in infant mice 21 days after infant SNI in the ipsilateral dorsal horn.

(A)Illustration of the cytokine array containing 40 different antibodies with duplicates as well as positive control proteins (controls). Highlighted mediators identify mediators that were upregulated in the ipsilateral dorsal horn compared to the contralateral dorsal horn.(B) Membranes were incubated with ipsilateral or contralateral dorsal horn spinal cords (400µg overnight at 4^0C) from mice 21 days after P10 SNI.(C) Bar graph showing the mean signal (pixel density) of the pair of duplicate spots (minus an averaged background), indicating an increase in the expression of inflammatory mediators in the ipsilateral dorsal horn (highlighted in green in A): BLC, CXCL13, C5a, GM-CSF, INF- γ, IL-1α, KC, M-CSF,MIP-1β, CCL3 , TARC, CCL17, CCL2, TNF, CD54, ICAM-1, MIP-1α, CCL3. Average data from two replicates of one pooled sample from 5 animals. Note the control protein levels in the two arrays are the same.

The mean signal (pixel density) of each pair of duplicate spots representing each cytokine from each membrane was quantified and plotted (Fig 4.2C). There was an increase in the level of several proteins in the ipsilateral dorsal horn compared to the contralateral dorsal horn including IL-1α (7.0 fold), IFN-γ (4.7 fold), MIP-1β (13.01fold), TNF (4.5 fold) and KC (3.7 fold). Notably, the expression of positive controls was the same on the ipsilateral and contralateral sides.

4.4.2 Age dependent expression of pro-inflammatory mediators following SNI.

After this initial screening, the expression of immune cell markers and pro-inflammatory mediators in the ipsilateral dorsal horn was characterised using real time qPCR, at three different ages;

1) Infant mice, 1 week after SNI (infant+7d), which do not exhibit behavioural mechanical hypersensitivity.
2) Infant mice, 21 days after SNI (infant+21d), at the onset of behavioural mechanical hypersensitivity.
3) Adult mice, 1 week after SNI (adult+7d). As it is well documented that adult rodents exhibit robust mechanical hypersensitivity, increases in glial cell markers and pro-inflammatory mediators in their ipsilateral dorsal horn, this age group was used as a positive control.

Expression levels in SNI animals were compared to ipsilateral dorsal horn tissue taken from age matched, sham controls.

At 21 days after infant SNI, there is a profound increase in the expression of pro-inflammatory mediators and immune cell markers in the ipsilateral dorsal horn compared to sham controls including microglia marker IBA1 (Fig 4.3A; Student's *t*-test, P =0.004), T-cell/natural killer cell marker CD2 (Fig 4.3B; Student's *t*-test, P =0.02), growth factor BDNF (Fig 4.3C; Student's *t*-test, P =0.004) and the pro-inflammatory cytokine TNF (Fig 4.3D; Student's *t*-test, P= 0.00028). Although IFN- γ protein increased in the ipsilateral compared to contralateral dorsal horn spinal cord, this was not significant (Fig 4.3F).

In the adult ipsilateral dorsal horn, expression of these immune cells and pro-inflammatory mediators was also significantly increased 7 days after adult SNI (Calvo et al., 2010; Tsuda et al., 2004; Xu et al., 2006). (Fig4.3A-D, IBA1 P=0.0012, CD2 P=0.049, BDNF P=0.032, TNF P=0.03, Student t-test) However, adult mice also exhibited an increase in the expression of astrocyte marker GFAP (Fig 4.3E; Student's *t*-test, P=0.02), pro-inflammatory mediator IFN- γ (Fig 4.3F; Student's *t*-test, P=0.023) and T box transcription factor (T-bet) (Fig 4.3G; Student's *t*-test, P=0.042).

In contrast, there was no significant difference in the expression of pro-inflammatory mediators or immune cell markers compared to sham controls in infants 7 days post SNI.

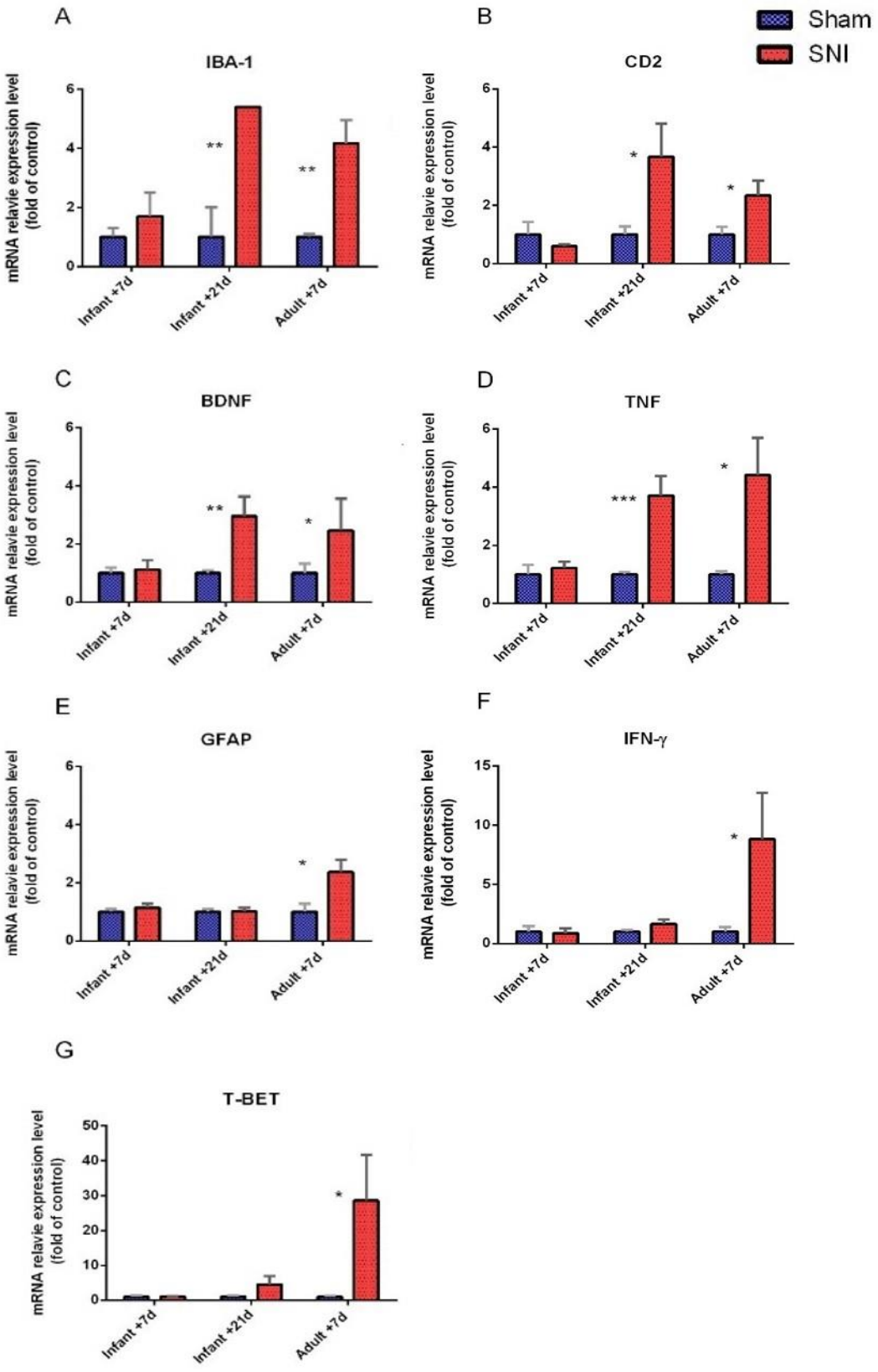


Fig. 4.3 A pro-inflammatory immune responses emerges 21 days after infant SNI.

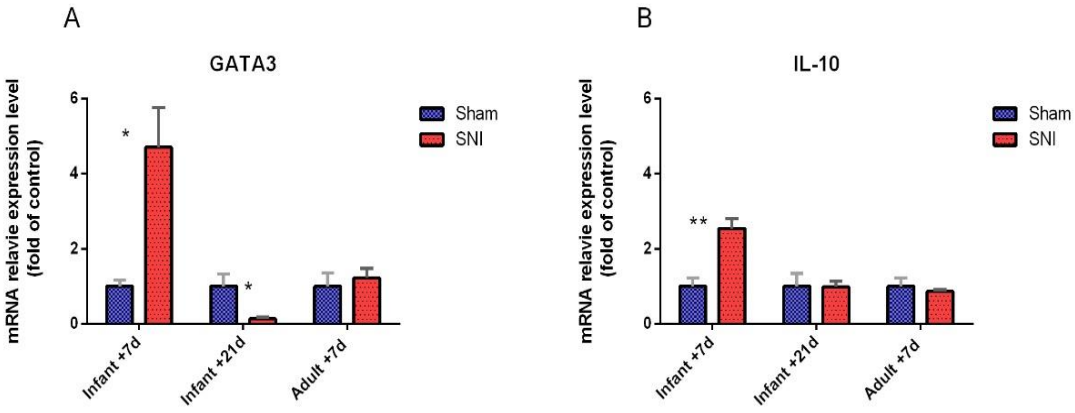
Real time qPCR analysis shows no changes in the expression of immune cell markers or pro-inflammatory mediators in infants 7 days after SNI compared to sham controls. There is a significant increase in the expression of (A) IBA-1 (n=6) (B) CD2 (n=4) (C) BDNF (n=8) and (D) TNF (n=8) in mice 21 days after infant nerve injury compared to sham controls. An increase in these markers is also observed in adults 7 days after SNI (IBA-1, n=6, CD2, n=8, BDNF,

n=4, TNF, n=5), In SNI adult mice (E) GFAP (n=6) (E), IFN-γ (n=4) (G) and T-bet (n=8) are also upregulated. Student's *t*-test, * P <0.05, ** P <0.01 *** P <0.001. Data expressed as fold of sham controls ±standard error of the mean.

4.4.3 Age dependent expression of anti-inflammatory mediators following nerve injury.

Figure 4.4 shows the expression of anti-inflammatory mediators in the dorsal horn spinal cord at the three different age groups following nerve injury. 7 days after infant SNI there is a significant increase in the expression of transcription factor GATA-3 (Fig 4.4A; Student's *t*-test, P=0.026). GATA-3 is required for the differentiation of CD4+ T cells into a Th2 anti-inflammatory subset, and the subsequent transcription of all anti-inflammatory Th2 cytokine genes including the powerful anti-inflammatory cytokines IL-10 and 4 IL-4 (Shoemaker et al., 2006; Zheng and Flavell, 1997). As further confirmation, a significant increase in the expression of both IL-10 (Fig 4.4B; Student's *t*-test, P=0.004) and IL-4 (Fig 4.4C; Student's *t*-test, P=0.028) also occurred 7 days after infant SNI.

The expression of IL-10 and IL-4 in the ipsilateral dorsal horn return to control levels 21 days after infant SNI and expression of GATA-3 in SNI treated mice decreases below sham control levels (Fig 4.4A; Student's *t*-test, $p<0.05$). In marked contrast to infants, was no change in the expression of any anti-inflammatory mediator tested 7 days after adult SNI.



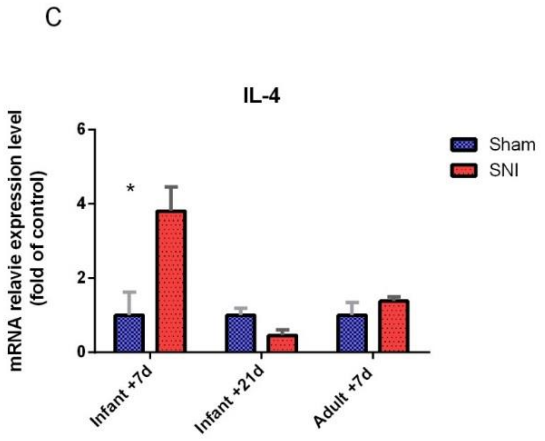


Fig 4.4 Infant nerve injury is characterised by an increase in the expression of anti-inflammatory immune markers.

(A)Real-time qPCR analysis in infant mice 7 days after SNI indicates a significant increase in the expression of transcription factor GATA-3 (n=4) and (B) anti-inflammatory markers IL-10 (n=4) and (C) IL-4 (n=4) compared to sham controls. However, 21 days after infant SNI, the expression level of IL10 (n=6) and IL4 (n=4) returned to baseline while the expression of GATA3 (n=5) was significantly less than sham controls. In adults SNI did not alter the expression levels of GATA-3 (n=6), IL-10 (n=4) or IL-4 (n=6). Student's *t*-test, * P<0.05 ** P<0.01. Data expressed as fold of sham controls ±standard error of the mean.

4.4.4 Infant C-fibre stimulation induces an increase in the expression of anti-inflammatory mediators

Previous studies show that direct non-damaging stimulation of C-fibres in the sciatic nerve of uninjured adult rats induces mechanical hypersensitivity and an increase in the expression of microglia markers and expression of pro-inflammatory mediators in the dorsal horn and central sensitization (Hathway et al, 2009). Electrical stimulation of the sciatic nerve in infant mice at 10Hz for 5 min at intensities sufficient to activate C-fibres (6mA, 500µs) had no significant effect upon mechanical thresholds of the ipsilateral paw compared to sham controls (Fig 4.5A). Identical C-fibre stimulation of the sciatic nerve in adults caused a significant decrease in mechanical thresholds 3 and 24 hours after C-fibre stimulation (Fig 4.5B; 2 -way ANOVA indicated a significant main effect of time P=0.0001, stimulation P=0.0002 and interaction P=0.0002) at 3 hours and 12 hours post stimulation (P<0.0001 Bonferroni post-test at 3 and 12 hours, sham n=7, stimulation n=9). Real time qPCR analysis of anti-inflammatory mediator expression in the ipsilateral tissue of adult and infant mice 24 hours after C-fibre stimulation shows that in infants the expression of both IL-4 (Student's *t*-test, P=0.01) and IL-10 (Student's *t*-test, P=0.04) in the

ipsilateral dorsal horn is increased (Fig 4.5C). However the expression of IL-13 and TGF-β did not alter in infants after C-fibre stimulation. In contrast, in adults the expression of anti-inflammatory mediators in the dorsal horn spinal cord following C-fibre stimulation was not significantly different from sham controls.

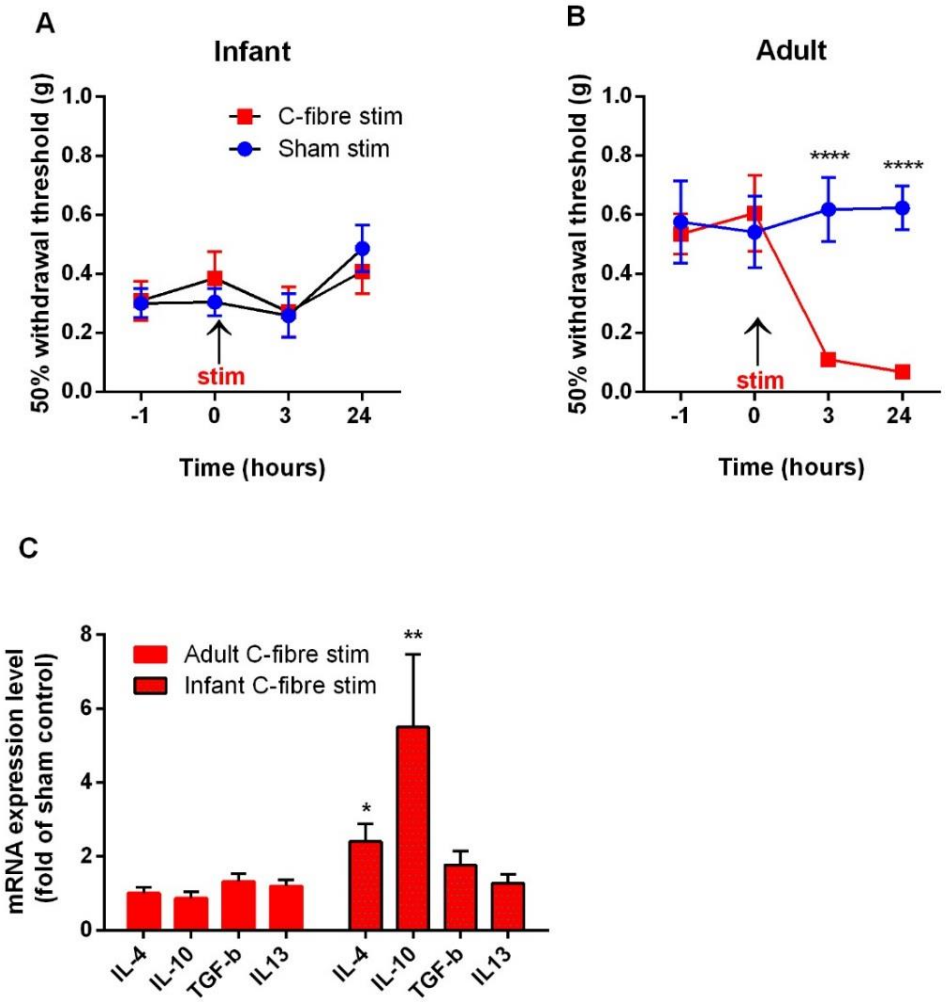


Fig 4.5 C-fibre stimulation in infants is characterised by an increase in the expression of anti-inflammatory immune markers.

(A)In infants, C-fibre stimulation did not alter mechanical thresholds of the ipsilateral paw compared to sham controls(n=8/treatment group) (B) Adult C-fibre stimulation (6mA, 500µs) at 10Hz for 5 min reduced mechanical thresholds significantly in the ipsilateral paw of adult mice compared to sham animals(2-way ANOVA, stimulation $F(1,14)=24.35$, $P=0.0002$, time $F(3,42)8.287$, $P<0.0001$, interaction $F(3,42)=14.38$, $P=0.0001$; Bonferroni post-test, ****$P<0.0001$; sham n=7, stimulation n=9). (C) Real-time qPCR analysis revealed an increased expression of IL-10 and IL-4 in infants 24 hours after C-fibre stimulation compared to sham controls, that is not present in adults (IL-4 adult n=6, infant n=5; IL-10 adult n=5, infant n=5; TGFβ adult n=4, infant n=5; IL-13 adult n=6, infant n=6). (Student's t-test, *$P<0.05$, **$P<0.01$, n=4-8 animals per group). Real-time qPCR data expressed as fold of sham controls ±standard error of the mean.

4.4.5 Intrathecal administration of anti-IL-10 unmasks hypersensitivity in infants after SNI.

Having established the presence of an anti-inflammatory profile in the dorsal horn of infant mice 7 days after SNI, the functional importance of the anti-inflammatory mediator IL-10 was examined. Anti-IL-10 (10ug, 3.5 µl) was intrathecally administered to the lumbar cord of mice 7 days after infant SNI for 3 consecutive days (days 7-9 post surgery) and mechanical thresholds of the ipsilateral paw were tested 1 hour after each injection. Before intrathecal injections, all groups exhibited comparable baseline thresholds (Fig 4.6). Administration of intrathecal anti-IL10 reduced the mechanical thresholds of infant mice on day 2 and 3 of injections in SNI treated mice, but not sham controls (Fig 4.6, 2 -way ANOVA indicated a significant main effect of time P=0.0007, treatment P=0.0007 and interaction P<0.0001, n=6/treatment group). Thresholds recovered on day 4, 1 day after the last intrathecal anti-IL10 injection. Importantly, mechanical thresholds of SNI infant mice treated with isotype-control antibody remained constant.

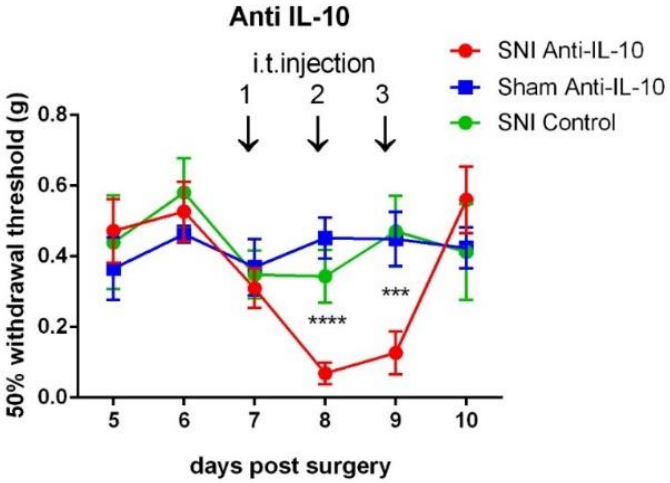


Fig. 4.6 Blocking anti-inflammatory cytokine IL-10 unmasks hypersensitivity in infants after SNI.

Intrathecal administration of anti-IL-10 for 3 consecutive days after infant SNI (days 7-9) reduced mechanical thresholds one hour after the second and third injection, but recovered one day later (2-way ANOVA, treatment F(2,90)=6.685, P=0.0020, time F(5.90)=4.746, P=0.0007, interaction F(10,90)=4.326, P<0.0001; Bonferroni post-test, ***P<0.001, ****p<0.0001. SNI mice treated with control antibody or sham mice treated with anti-IL-10 displayed no change in mechanical thresholds. n=6/treatment group. Line with error bars represent mean±standard error of the mean

4.4.6 Intrathecal administration of TNF-alpha induces hypersensitivity in infants after SNI.

In adults intrathecal administration of the pro-inflammatory cytokine TNF can induce hypersensitivity (Zhang et al., 2010). Intrathecal administration of TNF (20ng, 3.5µl) to mice 7 days after infant SNI induced a marked reduction in mechanical thresholds 30 and 180 minutes after injection which was the same in both SNI and sham treated animals (Fig 4.7; 2 -way ANOVA indicated a significant main effect of time P<0.0001, treatment P=0.0026 and interaction P<0.0001, n=7/treatment group). Mechanical thresholds in both groups recovered 1 day later. Mechanical thresholds of SNI mice treated with vehicle remained unchanged for the duration of testing.

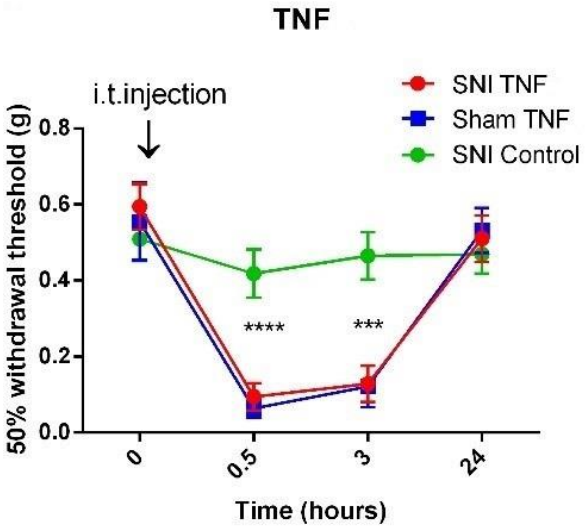


Fig. 4.7 Administration of pro-inflammatory TNF-α induced hypersensitivity in infants after SNI and sham surgery

Intrathecal administration of TNF (20ng) 7 days after infant SNI or sham surgery reduced mechanical thresholds 30 to 180 minutes after injection (2-way ANOVA, treatment $F(2,18)=8.470$ $P=0.0026$, time $F(3.54)=35.12$, $P<0.0001$, interaction $F(6,54)=6.899$, $P<0.0001$; Bonferroni post-test, ***$P<0.001$, ****$p<0.0001$, n=7 per group. Both SNI TNF and sham TNF treated groups had the same significance values at 0.5 and 3 hours post injection). Mechanical thresholds recovered to base line 1 day later. Intrathecal injection of vehicle control in mice 7 days after SNI did not change mechanical thresholds. Line with error bars represent mean±standard error of the mean.

4.4.7 Intrathecal administration of LPS-activated microglia induced hypersensitivity in infants after SNI.

Intrathecal administration of LPS-activated microglia 7 days after infant SNI significantly reduced mechanical thresholds 1 hour after injection (Fig 4.8) compared to SNI infants administered with non-activated microglia, and sham infants administered with LPS-activated microglia (2 -way ANOVA indicated a significant main effect of treatment only, P=0.0003) This was maintained from 1 hour to 5 hours post injection (Bonferroni post-test, * P <0.05, ** P<0.01, *** P <0.001, n=5/group). At 6 hours post intrathecal injection, there was a small but significant difference in mechanical threshold between SNI treated animals administered with LPS-activated microglia and non-activated microglia, but not compared to sham animals administered with LPS-activated microglia (P<0.05). Intrathecal injection of LPS-activated microglia into sham treated mice failed to induce mechanical hypersensitivity at any time point post injection. There was also a non-significant decrease in baseline thresholds of control animals, presumably arising from the repeated testing of animals.

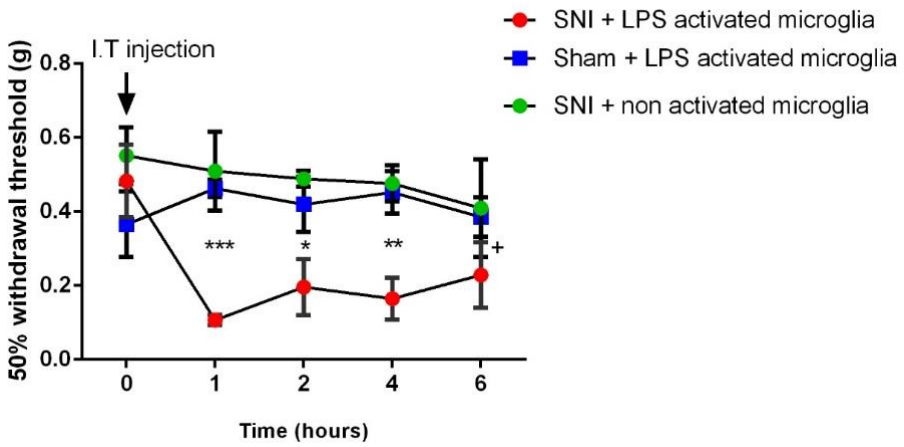


Fig. 4.8 Administration of LPS activated microglia unmasks hypersensitivity in infants after nerve injury

Seven days after infant SNI or sham surgery mice received one 3.5µl intrathecal injection of LPS activated microglia or PBS non–activated microglia control (1000 cells/10µl). SNI, but not sham treated mice displayed a decrease in mechanical threshold following administration of LPS activated microglia (2-way ANOVA, F (2, 12)-10.68, P=0.002, n=5.group) one to 4 hours later (Bonferroni post-test *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 and SNI+LPS activated microglia and non-activated microglia only +p<0.05). Thresholds of control animals show a non-significant decrease in baseline, arising presumably from repeated testing of animals. Line with error bars represent mean±standard error

4.5 Discussion

In adults the role of the immune system in the development and maintenance of neuropathic pain is well documented and is dominated by a pro-inflammatory response that leads to the sensitization of neurons in the dorsal horn and pain-like hypersensitivity (Costigan et al., 2009; Coull et al., 2005; Gao et al., 2009; Ji and Suter, 2007). In contrast, nerve injuries that lead to neuropathic pain in adults have little effect in human or rodent infants which has been associated with a weak pro-inflammatory immune response (Costigan et al., 2009; Moss et al., 2007; Vega-Avelaira et al., 2007; Walco et al., 2010).

When animals that have undergone SNI at infancy reach adolescence, hind-paw hypersensitivity develops. Data presented in Chapter 3 indicated a profound alteration in dorsal horn processing of sensory information occurs, at this time point. The aim of this study was to examine the inflammatory profile in the ipsilateral dorsal horn spinal cord in mice 7 and 21 days after infant SNI, in the absence and onset of pain-like behaviour respectively, and compare this to age matched sham controls to establish if the different pain states have underlying differential dorsal horn immune profiles (Costigan et al., 2009; Coull et al., 2005; Moss et al., 2007; Tsuda et al., 2009; Vega-Avelaira et al., 2009, 2007). SNI in infants induced a striking increase in the expression of anti-inflammatory mediators 7 days after infant SNI, which when blocked unmasked mechanical hypersensitivity. Notably, the delayed onset of mechanical hypersensitivity that occurs when animals are older coincides with a switch in the immune response to a characteristic pro-inflammatory profile, similar to that observed in nerve injured adults.

4.5.1 The immune profile in the infant dorsal horn 7 days after infant SNI is associated with an anti-inflammatory response

In infant mice, SNI surgery failed to increase the expression of either microglia (IBA-1) or astrocyte (GFAP) markers in the dorsal horn spinal cord 7 days after infant SNI. In previous studies microglia (MHC-II DMα, MHC-II DMβ, integrin-αM, CD68, IBA-1) and astrocyte (GFAP) markers did significantly increase in infant rats 7 days after P10 SNI but to a substantially lower magnitude than

observed in adults, leading to the suggestion that the immune response in infants is weak (Costigan et al., 2009; Moss et al., 2007; Vega-Avelaira et al., 2009, 2007). However, an increase in expression of immune cell markers such as IBA-1, is not necessarily a reflection of the responsiveness of a cell or indicative of a so called activated state (Beggs and Salter, 2010; Ji et al., 2013; McMahon and Malcangio, 2009; Zhuang et al., 2006). For example, P2X4R null mutant mice which fail to develop mechanical hypersensitivity after peripheral nerve injury coincides with significant increases in IBA-1 (McMahon and Malcangio, 2009; Ulmann et al., 2008). Therefore the presence of microglia and T-cell are not necessarily correlate to pain-like behaviour, while the up-regulation of inflammatory mediators (ATP, BDNF, TNF) that modulate neuronal activity and pain sensitivity are better associated with pain states (Ji et al., 2013; McMahon and Malcangio, 2009).

In infants, SNI failed to increase the expression of any pro-inflammatory mediator tested (including TNF, BDNF or IFN-γ) in the dorsal horn 7 days after surgery, that is a dominant feature in adults following SNI and can lead to changes in excitability or synaptic transmission, and ultimately sensitization (Coull et al., 2005; Marchand et al., 2005). This complements previous studies indicating that neither TNF nor IFN-γ increase in expression in the infant dorsal horn following SNI (Costigan et al., 2009; Li et al., 2009). This blunted response is also observed in infants following other insults, including bacterial infection and C-fibre stimulation where immune cells mount a weak inflammatory cytokine production associated with fewer immune cells compared to adults (Levy, 2007; Moss et al., 2007; Prescott et al., 1998).

However, it is suggested that the 'default' response in neonates is skewed towards an anti-inflammatory response (high IL-10) (Elahi et al., 2013; Moss et al., 2007; PrabhuDas et al., 2011; Shigemoto-Mogami et al., 2014). Examples of these age dependent differences are identified in residing immune cells both in the peripheral and central nervous systems where T-cells and microglia exhibit a skewed Th2 and M2 profile respectively. For example, under basal and LPS-stimulated conditions, postnatal mouse microglia up-regulate genes associated with an M2 orientation expression including arginase and CCL22, whereas more M1 associated gene expression such as

CXCL10 are down regulated (Scheffel et al., 2012). In addition, neonatal T-cells activated with anti CD3, show little proliferation and are deficient in the secretion of IL-2 and IFN-γ and instead secrete Th2 cytokines (Adkins et al., 1994, 1993). Neonatal responses to some vaccines such as hepatitis B virus are diminished in Th1 activity and biased towards Th2 function (Rose et al., 2007) and suggests that under normal conditions the default neonatal response is the Th2/M2 pathway (Adkins, 2000).

Data presented in this chapter shows that SNI at infancy does initiate an immune response, but one that is characterised by an increase in the expression of transcription factor GATA-3, IL-10 and IL-4, associated with an anti-inflammatory not pro-inflammatory response. The transcription factor GATA-3 is an important regulator of T-cell development and promotes the differentiation of CD4+ T-cells into a Th2 cell lineage, promoting the secretion of anti-inflammatory cytokines including IL-4, IL-10, and IL-13 as well as inhibiting Th1 cell differentiation and IFN-γ production (Ferber et al., 1999; Ouyang et al., 1998). Both IL-10 and IL-4 are released by a number of cells but are mainly associated with T-cell signalling, although IL-10 is also released by macrophages and microglia (Aloisi et al., 1999; Durafourt et al., 2012; Lord and Lamb, 1996; Milligan and Watkins, 2009; Ponomarev et al., 2005; Poole et al., 1995; Üçeyler et al., 2009). IL-4 signalling induces T-cell proliferation and differentiation into a Th2 phenotype, activating GATA3 and suppressing macrophage and microglia M1 phenotypes and pro-inflammatory mediator expression (Chao et al., 1993; Durafourt et al., 2012; Kopf et al., 1993; Kuhn et al., 1991). Like IL-4, IL-10 also acts to inhibit pro-inflammatory mediator release and reduces the recruitment of immune related glia cells in the spinal cord (Frei et al., 1994; Hu et al., 1999; Ledeboer et al., 2000; Poole et al., 1995). IL-10 can also inhibit the expression of major histocompatibility complex (MHC) class II on microglia (that serve an antigen presenting cell to T-cells) but not astrocytes (Frei et al., 1994; Sweitzer et al., 2002).

Although the origin of IL-10, IL4 and GATA-3 cannot be confirmed by this study, these markers are associated with T-cell signalling. As T-cells originate from hematopoietic stem cells in bone marrow, and only divide in the thymus, increased levels of these cells must be due to an infiltration from the blood via

leukocyte extravasation (Engelhardt, 2006). Both infant and adult rodents have CD2+ T-cells residing in their spinal cord which provide a surveillance role in the CNS (Costigan et al., 2009). Further investigation into whether infant SNI disrupts the integrity of the blood brain barrier, as observed in adults following peripheral nerve injury, as well as examination of other T-cell and monocyte markers would clarify if infiltration of immune cells occurs in infants (Beggs et al., 2010; Echeverry et al., 2011; Zhang et al., 2007).

Regardless of the origin of anti-inflammatory mediators in the infant dorsal horn following SNI, it is clear that nerve injury induces a differential age dependent immune response. Importantly, the data shows that the expression of IL-10 following nerve injury is functionally essential for the absence of mechanical hypersensitivity in this early period following P10 SNI as blocking spinal IL-10 unmasked pain-like behaviour causing infant mice to develop mechanical hypersensitivity. In line with this, evidence in adult neuropathic models suggests that both IL10 and IL-4 are anti-nociceptive, suppressing pro-inflammatory cytokines, microglia responses and pain behaviour (see introduction) (Hao et al., 2006; Milligan et al., 2005a, 2005b).

The function of a predominant anti-inflammatory response observed during this postnatal period in response to nerve injury and other insults may act to stop excessive inflammation that may occur with the transition from a sterile in utero setting to colonization with commensal microbes (Maynard et al., 2012). In addition, studies in the healthy brain indicate a dual role for microglia during postnatal development associated with the substantial changes that occur in the connectivity of the CNS that occur over this period. This includes their ability to remove cellular debris and dead cells via phagocytosis activity and secondly, to establish contacts with synapses and regulate the size of dendritic spines during critical periods (Arnoux et al., 2013; Marın-Teva et al., 2004; Schafer et al., 2012; Tremblay et al., 2010). For example, microglia within the juvenile visual cortex can modify their association with dendritic spines in response to changes in visual sensory experience and actively engulf synaptic structures and exert a major role in controlling the number of synapses through synaptic pruning (Tremblay et al., 2010). This is confirmed by the disruption in microglia function such as depletion of microglia CX3CL1 receptor or

administration of a microglia inhibitor that results in the delayed synaptic pruning and maturation of hippocampal synaptic circuits during development (Paolicelli et al., 2011; Schafer et al., 2012). As substantial rearrangement and refining of synaptic connections occur at P10 in the dorsal horn it is possible that dorsal horn microglia are also providing similar functions at this time to enable the refinement and rearrangement of synaptic connections and pruning of axonal projections which would temporarily increase the production of damage associated molecular patterns (DAMP) during the postnatal period. As a result, a peripheral nerve injury at P10, in the context of dorsal horn postnatal rearrangements and colonization with commensal microbes in the periphery, that leads to an increase in DAMP and other mediators instigates signal pathways associated with a default anti-inflammatory immune responses in both the periphery (T-cell) and centrally in the dorsal horn spinal cord (microglia) and the absence of pain like behaviour (Bianchi, 2007; Elahi et al., 2013; Gordon and Taylor, 2005; Levy, 2007, p. 200; Moss et al., 2007; Mosser and Edwards, 2008; Scheffel et al., 2012). This would enable normal postnatal development in the dorsal horn to occur through phagocytic activity, without the initiation of an extensive and damaging inflammatory response resulting in the absence of hypersensitivity.

Interestingly, despite the age-dependent difference in the immune profile following nerve injury, immature dorsal horn neurons are capable of responding to pro-inflammatory mediators although the degree of responsiveness of neonatal immune cells varies markedly with the stimulation conditions (Adkins et al., 1994; Moss et al., 2007). The data presented in this chapter indicates that intrathecal application of TNF induces mechanical hypersensitivity in mice 7 days after P10 SNI. A study by Li et al., showed that this is the result of an increase in the efficacy of glutamatergic synapses and intrinsic excitability of neonatal dorsal horn neurones following SNI (Li et al., 2009). In addition, intrathecal administration of LPS activated microglia also induce mechanical hypersensitivity in SNI but not sham treated infant mice. LPS is a bacterial endotoxin that causes the activation of numerous signalling pathways (PKC, MAPK and NF-ƙB) in microglia that are involved in the release of immune-related cytotoxic factors including nitrous oxide (NO), pro-

inflammatory cytokines including TNF and IL-6 LPS and reactive oxygen species (Bhat et al., 1998; Chao et al., 1992; Lee et al., 1993; Sawada et al., 1989), that also efficiently down regulates IL-10 (Mizuno et al., 1994). LPS activated microglia release TNF at lower concentrations than the single TNF injection administered here (Berta et al., 2014; Welser-Alves and Milner, 2013). The results show that the lower levels of TNF sensitized infant SNI mice, while leaving sham animals of the same age unaffected and suggests that although infants can mount pro-inflammatory immune response that lead to pain behaviour to various insults, peripheral nerve injury in infants induces a response that is skewed towards a Th2/M2 response characterised by the release of anti-inflammatory mediators. As a result in infants anti-nociception naturally dominates following nerve injury.

4.5.2 In infants, C-fibre stimulation induces the release of anti-inflammatory mediators in the dorsal horn spinal cord.

In adults, primary afferent input and C-fibre electrical stimulation of the dorsal root or sciatic nerve, at intensities known to cause central sensitization, result in a 'microglial pain-related enhanced response state' and behavioural hypersensitivity 24 hours after stimulation (Beggs et al., 2010; Hathway et al., 2009; McMahon and Malcangio, 2009; Wen et al., 2007; Xie et al., 2009). In adults such stimulation releases cathepsin S from microglia which liberates CX3CL1 from dorsal horn neurons contributing to amplification and maintenance of chronic pain (Clark et al., 2009). This response is attenuated with pre-treatment with minocycline, an inhibitor of pro-inflammatory polarised microglia (Hathway et al., 2009; Kobayashi et al., 2013; Taves et al., 2013).

It has been confirmed in adults that C-fibre stimulation also increases the permeability of the blood brain barrier 24 hours after stimulation (Beggs et al., 2010) and pain-like behaviour may also be linked to T-cell infiltration. Notably, mustard oil irritant does not induce IBA-1 or GFAP immunoreactivity in the dorsal horn which suggests that glial responses are selective to different forms of primary afferent input (Molander et al., 1997). Remarkably the absence of hypersensitivity in infants following stimulation is accompanied by an increase in the expression of anti-inflammatory IL-10 and IL-4 which is not observed in adults. This suggests that IL-10 and IL-4 release, in the absence of pathology

is directly activated by neurotransmitter release from C-fibre terminals in the dorsal horn and the response could be a direct result of activity in damaged C-fibre afferent terminals. Interestingly, IL-10 release from neonatal spinal cord microglia is potentiated by the excitatory neurotransmitter glutamate (Werry et al., 2011). Importantly, the result was not a global increase in anti-inflammatory mediators as other anti-inflammatory mediators remained unaltered following stimulation.

4.5.3 Hypersensitivity onset at adolescence is characterised by a pro-inflammatory response in the ipsilateral dorsal horn

When SNI treated infants reach adolescence a delayed mechanical hypersensitivity develops that coincides with an increase in the expression of immune cell markers (microglia and T-cells) and pro- inflammatory mediators including TNF and BDNF that can alter synaptic transmission in the dorsal horn and are associated with pain sensitization (see introduction) (Coull et al., 2005; Marchand et al., 2005; Taves et al., 2013). In addition, anti-inflammatory mediators (IL-10 and IL-4) that can act in a regulatory capacity to control inflammation are now reduced to sham control levels and maybe insufficient to control the increase in expression of pro-inflammatory mediators. The development of a delayed pro-inflammatory response was also confirmed at a protein level by a cytokine array which indicated an increase in protein expression of pro-inflammatory mediators in the ipsilateral compared with the contralateral dorsal horn.

Although real time qPCR showed that IFN-γ expression was not increased in mice 21 days after P10 SNI, IFN-γ protein levels were higher in the ipsilateral compared to contralateral dorsal horn. In addition although was no increase in GFAP 21 days after P10 SNI, which is in contrast to a previous study in rats 21 days after P10 SNI where an increase in GFAP was observed (Vega-Avelaira et al., 2012). As this analysis was undertaken at the onset of mechanical hypersensitivity in mice, an increase in astrocyte makers may occur at a later time point (Zhuang et al., 2006).

4.5.4 Postnatal development of the immune profile

It is increasingly recognised that the immune profile in both peripheral (T-cells) and central residing immune cells (microglia) undergo a developmental shift with age from an anti-inflammatory skewed response (such as high IL-10) to pro-inflammatory in both rodents and humans (Elahi et al., 2013; Moss et al., 2007; PrabhuDas et al., 2011; Shigemoto-Mogami et al., 2014). For example cytokine analysis of LPS-activated microglia between P0 to P49 mice reveal a gradual developmental shift to a defence-orientated M1 phenotype, with an increasing induction of TNF, IL-1β, IL-6 and a decline in arginase with age (Scheffel et al., 2012). In addition, following both LPS and non-TLR mediated challenges such as experimental autoimmune encephalomyelitis, with increasing age discrete subsets of microglia secrete TNF indicating a shift towards microglia response heterogeneity (Scheffell et al., 2012). T-cell responses also show an age dependent shift and at P4, neonatal T-cells produce high levels of IL-4, that diminishes by P6 and from the first week of life onwards, there is a gradual and continual increase in the capacity of T cells to mount Th1 responses (Adkins et al., 1993; Adkins and Hamilton, 1992).

Furthermore, vaccinations in infants are associated with a skewed Th2 response and secretes IL-4 and IL-10 and lower T-cell mediated IFN-γ/IL-12 responses in the first 3-6 weeks in mice or 12-18 months in humans that gradually increase (Adkins, 2000, 1999; Barrios et al., 1996; Mosmann and Coffman, 1989; Powell and Streilein, 1990). In humans, TLR stimulation of whole blood, induces the production of anti-inflammatory innate cytokines (IL10) that dominates in preterm infants and declines over the first few years of life while the production of TNF increases (Corbett et al., 2010; Kollmann et al., 2009; Lavoie et al., 2010) and one of the last cytokines to reach adult-level, around the age of 2, is IL-12p70 which promotes the development of Th1 cell immune responses (Corbett et al., 2010). This is despite TLR sensor function and downstream signalling molecules being developed in new born infants (Strunk et al., 2011; Tulic et al., 2011) and indicates that the neonatal environment may be responsible for promoting the skew towards a Th2 lineage.

The mechanisms underlying the developmental switch from immune suppression in the neonate to pro-inflammatory responses in the adult, whether intrinsic or external to immune cells, is an understudied area of research, particularly in microglia, but is probably multifactorial (PrabhuDas et al., 2011). Lewis and colleagues showed that during human infancy recent thymic emigrants (T-cells recently produced by the thymus) are present in large proportions in the periphery and are impaired in the acquisition of Th1 function (Haines et al., 2009). These cells also predominate in infant mice (Opiela et al., 2009). In addition the anti-inflammatory cytokine profile in infants is induced during foetal life by regulatory T-cells which dominate in the foetal circulation suppressing reactivity to non-inherited antigens and promote a suppressive environment (Mold et al., 2008). These remain during postnatal development and maybe an important source of Th2-type cytokines in early life in mice and humans (Adkins, 2003; Prescott et al., 1998).

Another suggested mechanism is that Th1 cells in neonates, but not Th2 cells undergo apoptosis in response to re-exposure to antigens and so provide a potential mechanism for the Th2 biased secondary responses (Li et al., 2004). In addition, studies in humans and mice indicate distinct epigenetic mechanisms may also have a major role. Hypomethylation of cytokine loci contributes to the expression of cytokine genes and hypermethalation contributes to their silencing. The Th2 locus is hypomethylated in infant humans and mice relative to adults that corresponds to the Th2-polarizing cytokine responses in infants (Rose et al., 2007; Webster et al., 2007). In addition skewed responses maybe due to quantitative differences in T-cells and, due to low numbers, when animals are challenged the antigen load may affect the response. For example Sarzotti et al, demonstrated that if neonatal mice were infected with a high dose of virus a Th1 response was mounted but a low does initiated a Th2 response and subsequent protection (Sarzotti et al., 1996).

In summary both quantitative and qualitative properties of neonatal CD4+ cells are probably factors in the plasticity of neonatal Th1/Th2-cell responses. Given the central role of CD4+ Th cells in influencing the responses of many other immune cell types, variation in Th-cell responses in neonates may contribute

substantially to variability in the responses of other cell populations. However, the mechanisms underlying postnatal changes of microglia are yet to be studied.

4.6 Conclusion

Depending on the developmental status of the animal, nerve injury will trigger differential immune responses in the dorsal horn spinal cord. In infants an anti-inflammatory immune response dominates, characterised by the release of anti-inflammatory mediators IL10 and IL4 which can also be induced at this age by C-fibre stimulation. As an anti-inflammatory cascade in the dorsal horn suppresses neuronal sensitization anti-nociception naturally dominates in the early period following infant nerve injury. At a later period, a gradual developmental change occurs in the immune response tipping the balance to pro-inflammatory response which dominates and the development of pain like behaviour. This data suggests that nerve injury in early life may affect pain processing later in life and is associated with the dysfunction of the immune system and may underlie the onset of complex pain behaviours that also emerge at adolescence.

Chapter Five

General Discussion

5. 1 Introduction

The absence of pain following nerve injury in infant mammals is of particular clinical interest as pain behaviour to noxious stimuli and inflammation is present, even before birth (Andrews and Fitzgerald, 1994; Fabrizi et al., 2011; Fitzgerald, 1991; Rokyta and Fricová, 2012; Walker et al., 2007). However, longitudinal studies in rats and humans have revealed that following a nerve injury sustained at infancy, mechanical hypersensitivity does eventually emerge, but much later (Melzack et al., 1997; Vega-Avelaira et al., 2012).

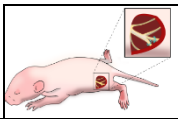
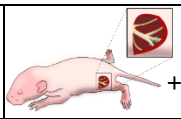
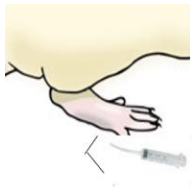
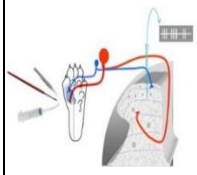
As the dorsal horn is the first point in sensory integration where information from the periphery is modulated locally by spinal interneurons before being relayed to higher centres, alterations in dorsal horn neuronal activity are fundamental to the processing and perception of sensory information following nerve injury. While it is clear that in adult rodents the hypersensitivities arising following nerve injury coincide with alterations in both neuronal and immune systems in the dorsal horn (Beggs and Salter, 2007; Cao and DeLeo, 2008; Chapman et al., 1998b; Clark et al., 2009, 2007; Costigan et al., 2009; Gao et al., 2005, 2010b; Griffin et al., 2007; Hao et al., 2007; Laird and Bennett, 1993; Marchand et al., 2009; Milligan et al., 2005b; Moore et al., 2002; Palecek et al., 1992; Polgár et al., 2005; Suzuki and Dickenson, 2006; Takaishi et al., 1996; Taves et al., 2013; Tsuda et al., 2013, 2003; Zhuang et al., 2005) the mechanism underlying the absence and late onset of hypersensitivities in the early and late period following infant nerve injury had not been investigated before the work presented in this thesis (Moss et al., 2007; Vega-Avelaira et al., 2012)

In the last 30 years research into the postnatal development of pain circuitry indicates extensive alterations occur in the first few weeks of life which can be altered by both noxious and non-noxious sensory inputs (Albuquerque et al., 1999; Andrews and Fitzgerald, 1994; Baccei and Fitzgerald, 2004; Beggs et al., 2002; Fitzgerald, 1985a; Fitzgerald and Jennings, 1999; Fitzgerald and Swett, 1983; Hathway et al., 2006; Koch et al., 2012; Watanabe et al., 1994). Expression studies in the dorsal horn show that the most differentially

regulated genes in the dorsal horn between infant and adult nerve injured rodents are immune related (Costigan et al., 2009). A study by Vega-Avelaira et al., revealed that early life nerve injury leads to alterations in behavioural hypersensitivity that correlate with alterations in the expression of microglia and astrocyte markers in the dorsal horn spinal cord (Vega-Avelaira et al., 2012)

5.2 Summary of findings

The aim of this thesis was to further our understanding into the mechanisms that underlie the absence and delayed onset of pain-like behaviour following infant nerve injury and elucidate immune profile in the dorsal horn at these time points. A summary of the major findings are presented in Table 5.1.

	 + 1 week	 + 3-4 weeks	 + 1 week
Chapter Two: 	• Pain-like behaviour to a range of hind paw tests is absent in rats • Mechanical hypersensitivity is also absent in mice • Spinal cord neuronal degeneration is absent	• Pain-like behaviour to a range of hind paw tests is present in rats. Thermal hypersensitivity is absent • Mechanical hypersensitivity is present in mice	• Pain-like behaviour to a range of hind paw tests is present in rats. Thermal hypersensitivity is absent • Mechanical hypersensitivity is present in mice
Chapter Three: 	Compared to sham controls, dorsal horn cells in SNI animals exhibit: • a decrease in activity to threshold and 6.3g Von Frey hairs • a decrease in both brush and pinch receptive fields	Compared to sham controls, dorsal horn cells in SNI animals exhibit: • an increase in spontaneous activity • increase in activity evoked by brush. noxious and non-noxious cold • increase in brush receptive field	Compared to sham controls, dorsal horn cells in SNI animals exhibit: • an increase in spontaneous activity • increase in activity evoked by non-noxious cold

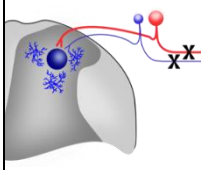
Chapter Four:  	Compared to sham controls, the dorsal horn of SNI animals: • have an increase in the expression of anti-inflammatory mediators • C-fibre nerve stimulation does not cause mechanical hypersensitivity but induces an anti-inflammatory response • blockade of spinal anti-inflammatory IL-10 reveals mechanical hypersensitivity • spinal administration of TNF induces mechanical hypersensitivity in SNI and sham animals • spinal administration of LPS activated microglia induces mechanical hypersensitivity	Compared to sham controls, the dorsal horn of SNI animals have: • an increase in the expression of microglia and T-cell markers but not astrocytes • an increase in the expression of pro-inflammatory mediators	Compared to sham controls, the dorsal horn of SNI animals have: • an increase in the expression of microglia, astrocyte and T-cell markers • an increase in the expression of pro-inflammatory mediators • C-fibre nerve stimulation induces mechanical hypersensitivity and an absence of anti-inflammatory dorsal horn response

Table 5.1 Summary of the major findings in this thesis

Upper panel denotes the three different age ranges used in experiments i) 1 week after infant SNI, ii) 3-4 weeks after infant SNI and iii) 1 week after adult SNI. The column on the left illustrates the main methods utilised in each chapter; hind paw sensory testing, in vivo electrophysiology in the dorsal horn and real time qPCR of inflammatory mediators in the dorsal horn and spinal application of pharmacological agents.

5.3 Technical considerations

5.3.1 Animals

P10 animals were chosen to represent infants in this thesis for three reasons i) The rat is born at an immature stage and the first postnatal week in the rat pup and P0 on the rat put corresponds to 24 to 40 post-conception weeks and pups are considered infants by P7 (Fitzgerald, 1991) ii) between P0-P5 transection or crush of the sciatic nerve causes cell death in the DRG and

collateral sprouting of primary afferent central terminals in the dorsal horn, but does not occur if the surgery is performed at P10 (Fitzgerald et al., 1990, 1985b) and iii) ensures consistency with previous studies investigating mechanisms underlying the age dependent differences in pain behaviour following nerve injury all of which have been carried out in P10 rodents (Costigan et al., 2009; Moss et al., 2007; Vega-Avelaira et al., 2006, 2012). P33 was used for all investigations in the young adult as postnatal changes to the dorsal horn spinal cord are complete at this time and ensures the consistency of ages with previous studies (Costigan et al., 2009; Fitzgerald, 2005a; Moss et al., 2007; Vega-Avelaira et al., 2012, 2007).

All studies were carried out in male rodents. In recent years the study of pain-related sex differences has received greater attention (Aloisi et al., 1994; Craft et al., 2004; Greenspan et al., 2007; Mogil, 2012; Wiesenfeld-Hallin, 2005). These studies suggest that females (both humans and non-human) exhibit lower thresholds to noxious stimuli and have a greater pain sensitivity and prevalence of chronic pain compared to men (Aloisi et al., 1999; Wiesenfeld-Hallin, 2005) and that chronic neuropathic pain is more prevalent in females than in males (Torrance et al., 2006; Vacca et al., 2014) (although see Bouhassira et al., 2008). Studies into the mechanism underlying these sex differences suggest that gonadal steroid hormones play an important but complex role (Berkley, 1997; Craft et al., 2004; Fillingim and Ness, 2000). One recent study by Vecca et al., shows that following nerve injury, female mice have a higher pain perception and a show a delay of recovery from neuropathic pain compared to adults (Vacca et al., 2014). This study suggested that male mice exhibited faster nerve regeneration and reduction in both microglial and astrocytic activity compared to female mice (Vacca et al., 2014).

In rodents although sex differences are present, they are unlikely to exist to the same extent in neonatal animals (Banik et al., 2006; Beggs et al., 2012). For example, tail flick latencies in hot plate tests are shorter in infant female rats although in the incision pain model, mechanical sensitivity did not differ between males and females (Beggs et al., 2012). However, sex differences in the long term effects of early life pain have been reported in the animal literature (LaPrairie and Murphy, 2007). In human clinical studies the long-term

effects of early pain on sex differences have rarely been studied and demonstrate contradictory results (Grunau et al., 2006; McGrath et al., 2013). As the experiments presented in this thesis are longitudinal, starting at infancy until the adolescent period and after careful consideration, it was decided that experiments in this thesis should be performed in male animals.

The choice to use rats or mice in a study was to ensure the mechanisms underlying the delayed onset of pain behaviour could be elucidated in the most efficient way and keep animal use to a minimum. For sensory behavioural tests, immunohistochemistry, staining and *in vivo* electrophysiology studies, rats were utilised over mice due to a the following reasons i) the outcomes of behavioural assessment following SNI have been extensively documented in the rat compared to mouse models ii) rats are more resilient to surgery under anaesthesia which is particularly important during *in vivo* electrophysiology experiments where the stability of the animal is essential for effective recordings and iii) tissue preparation and anti-bodies used and protocols were already published and optimised for use with rat tissue (Bourquin et al., 2006, 2006; Decosterd and Woolf, 2000; Schmued et al., 2005; Walker et al., 2010).

Before carrying out any experiment in mice, the delayed pain-like behaviour following infant nerve injury model was replicated and confirmed in mice that were subsequently used for cytokine arrays and real time qPCR analysis due to i) the availability of high quality reagents such as the cytokine array being available in mice as opposed to rats and ii) to enable the use of genetically modified mouse lines that will enable future investigations into mechanisms underlying delayed pain behaviour following infant nerve injury to be used.

5.3.2 Sensory hind-paw behavioural tests

Reflex responses such as those evoked by thermal, mechanical (Vfh) or innocuous cold (acetone) stimuli are widely used in pain research as a tool to assess hypersensitivity (Decosterd and Woolf, 2000; Flatters and Bennett, 2004). These reflex responses reflect the excitability of spinal circuits and has led to our current understanding of pain processing. Furthermore, newborn mammals display clear reflex withdrawal behaviour from noxious stimulation at birth and this has been used to demonstrate pain behaviour following

noxious stimulation and inflammatory injury in both the rodent pups and humans (Cornelissen et al., 2013; Fitzgerald et al., 1989, 1988; Walker et al., 2007). However, it is accepted that measures of spinal reflexes are not measures of pain 'perception' as such but act as a surrogate measure. Measures of evoked reflexes are justified as i) they are practical to obtain ii) they can be repeated and quantified, and iii) they are clinically relevant as humans do suffer these hypersensitivities (Mogil and Crager, 2004; Tétreault et al., 2011).

In addition to the use of evoked reflex responses, weight bearing in rodents was also measured following infant nerve injury. Although a test most frequently used to assess pain behaviour in joint pain animal models (Schött et al., 1994; Tétreault et al., 2011), previous studies show that the measure of contralateral weight bearing is different from that observed with reflex withdrawal testing as in adult rodents they have different time courses with respect to both onset and maintenance of hypersensitivity (Mogil et al., 2010). In addition, while both SNI and CCI in mice induce changes in mechanical thresholds, measured using vFh, changes in contralateral weight bearing is only present in SNI treated mice, supporting the argument for independence of these behaviours (Mogil et al., 2010).

It should be noted that within this thesis the increased pain-like behaviour to a particular stimuli is described as hypersensitivity as opposed to allodynia (increased pain in response to normally innocuous stimuli) or hyperalgesia (increased response to noxious stimuli) which are human interpretations of stimuli and therefore cannot be terms allocated to responses of rodents to a stimuli (Sandkühler, 2009).

5.3.3 Anaesthesia

It is known that anaesthesia can affect sensory processing in the spinal dorsal horn (Devonshire et al., 2010) altering firing patterns of neurons (Haseneder et al., 2004; Leite and Cascio, 2001) and it is therefore essential to take anaesthetic into account in the analysis, particularly during *in vivo* recordings as it can alter neuronal firing patterns. However, although the equilibration times changed depending on the size of the animal, in all studies the same

inhalation anaesthetic was used and the final anaesthetic depth was held in all animals. For example, during *in vivo* electrophysiology recording a depth of 1.8% was used as previously documented for all recordings, regardless of the age of animal (Hathway et al., 2009; Koch et al., 2012). Furthermore, the heart rate, body temperature and perfusion of the animal were continuously assessed and together this ensured valid comparisons between groups. These potential effects of anaesthesia on spinal neurons during *in vivo* electrophysiology studies can by eliminated through decerebration and withdrawal of anaesthesia. However, the surgery can be very severe, particularly when working with young animals and can result in cranial bleeding and a high mortality rate and so was not performed in this thesis (Coderre and Wall, 1987; Swett and Woolf, 1985).

5.3.4 *In vivo* electrophysiology

In vivo electrophysiology enables detailed recordings to be made from individual spinal neurones in response to specific peripheral input. This is advantageous over measurements of reflexes, such as those analysed in behavioural or electromyographical (EMG) studies which require sensory and motor integration. This is of particular importance when investigating age-dependent changes as recordings from dorsal horn neurones are not susceptible to changes in the postnatal development of motor output. Furthermore, in contrast to *in vitro* slice preparations the animal is intact, enabling investigations into neuronal activity in the context of a functioning animal system. However, there are a few disadvantages of an *in vivo* preparation i) performing electrophysiology preparations, particularly in infant animals, is challenging so the n numbers in some experiments are low and ii) unlike behavioural or EMG studies, recordings from spinal neurones are varied, as they are a heterogeneous population and so the responses are unlikely to be uniform from one cell to the next and iii) the spinal cord is heterogeneous in composition so identification of the phenotype of a recorded cell is limited. As a result recordings of activity from a projection neuron, excitatory or inhibitory interneuron would have different outcomes. This disadvantage was overcome by ensuring the cells analysed in each study were firstly characterised and confirmed as WDR neurones (responding to both

innocuous and noxious stimuli) with cutaneous receptive fields in the sural nerve territory, thus limiting both the variation of responses and laminar distribution of the cells.

5.3.5 Real time qPCR

The real time qPCR method has been extensively used to quantify expressional changes of inflammatory mediator transcript numbers amplified from nucleic acids in the dorsal horn spinal cord (Berta et al., 2014; Costigan et al., 2009; Moss et al., 2007; Zhang et al., 2011). This technique is fast and effective as, in addition to the processing being short, multiple genes can be run at the same time. However, this technique can be influenced by a number of confounding factors including i) nucleic acid extraction efficiencies are variable between samples that are processed at different times ii) SYBR green, used for detection binds to all double-stranded DNA and if a primer is not specific to the target gene a non-specific product will be formed iii) quantification of the initial target sequence of an unknown concentration is determined from the cycle threshold and is expressed relative to a co-amplified steady state (housekeeping gene) and any variation in the expression of the housekeeping gene can mask or indicate artificial changes in the gene of interest. These factors were addressed by i) ensuring that the samples within an age group were subject to the same extraction procedure at the same time ii) analysing the post-PCR dissociation curve following each experiment to confirm that the fluorescence signal was generated from the target template and not that of a non-specific product, thus ensuring that the primers used were specific (Smith and Osborn, 2009) and experiments whereby the dissociation curve varied were discarded, iii) ensuring that quantification of target genes were completed using the comparative cycle threshold method (Schmittgen and Livak, 2008) and using GAPDH as the control transcript (housekeeping gene) for comparisons to transcripts from other genes expressed in the dorsal horn spinal cord. GAPDH is an important enzyme in glycolysis and is frequently used as a control in models of nerve injury and is not affected by age dependent changes (Barber et al., 2005; Berta et al., 2014; Raghavendra et al., 2003; Scheffel et al., 2012; Vasudeva et al., 2014) iv) in all experiments amplicons were short (between 50-100 base pairs) to enhance

the specificity of the products. It should also be noted that real time qPCR is unaffected by differences in spinal cord size as the same amount of RNA material was used and so the amount of DNA material at each age was also identical.

5.3.6 Volumes of drug

In this thesis intrathecal injections were carried out in P10 mice under anaesthesia using a 1 ml insulin syringe with a 30-gauge needle perpendicular to the skin at the low lumbar level (L5-L6). Although some studies use intrathecal injection volumes of up to 10µl of solution (Xu et al., 2013; Zhang et al., 2011) a study by Westin et al., 2010 identified that the volume of intrathecal injectate required to produce spread across lumbar and low thoracic segments was 0.5 mcl per gram bodyweight (Walker et al., 2010; Westin et al., 2010). In line with these studies, this thesis used an injection volumes of 3.5µl to ensure spread across the lumbar spinal cord in P10 pups.

5.4 Interpreting the results in this thesis

To date substantial progress has been made into elucidating the postnatal changes occurring in the neuronal circuity associated with pain processing, with an aim of improving our understanding of both the mechanisms underlying neonatal pain and its treatment. However, little work into the postnatal development of immune function within the CNS has been completed. This is of particular interest in the context of nerve injury as expression studies indicate that in nerve injured animals the most prevalent functional class of differentially regulated genes in the dorsal horn spinal cord are from the immune system (Costigan et al., 2009; Griffin et al., 2007). The data presented in this thesis offers a mechanistic explanation for the observed clinical findings.

5.4.1 Infants do not display pain-like behaviour following nerve injury due to an anti-inflammatory response in the dorsal horn

While nerve injury in adults stimulates spinal glial cells and recruits T-cells which powerfully contribute to the induction of pain facilitation (due to the release of pro-inflammatory mediators), this thesis indicates that the absence of pain behaviour following infant nerve injury is characterised by an anti-

inflammatory response in the dorsal horn spinal cord typified by an increase in expression of transcription factor GATA-3 and cytokines IL-10 and IL-4 (Fig 5.1).

Following infant nerve injury spontaneous dorsal horn neuronal activity was absent, which is in contrast to the increase in spontaneous dorsal horn activity consistently observed in adult neuropathic pain models (Chapman et al., 1998b; Laird and Bennett, 1993). Although evoked-dorsal horn activity remained equivalent to sham controls for most sensory modalities, activity to Von Frey hair stimulation and receptive field areas were reduced in SNI animals. As a generalised reduction in activity to all sensory modalities was not observed in infants following SNI activity, this illustrates that there is not a global hyposensitivity of dorsal horn neurons. This is supported by behavioural studies showing that seven days after infant SNI and sham surgery animals have similar responses and thresholds.

It is well documented that infant receptive fields are larger than adults and are subsequently refined and reduced over postnatal development by activity dependent mechanisms that can be by prevented by spinal cord application of NMDA blockers (Beggs et al., 2002; Fitzgerald and Jennings, 1999; Fitzgerald and Koltzenburg, 1986; Torsney and Fitzgerald, 2002; Koch et al., 2012). It is possible that injury induced activity at infancy eliminated the excitatory synaptic connections while strengthening inhibitory connections during the postnatal period so that receptive fields were reduced to those observed in a more mature rodent.

The anti-inflammatory response in the dorsal horn is consistent with the 'default' immune response in neonates which is skewed in an anti-inflammatory direction where responses of T-cells and microglia exhibit Th2 and M2 responses respectively (Adkins, 2000; Elahi et al., 2013; PrabhuDas et al., 2011; Scheffel et al., 2012). GATA-3 is an important regulator of T-cell development and promotes the differentiation of CD4 T-cells into a Th2 cell lineage and the secretion of anti-inflammatory cytokines including IL-4, IL-10 and IL-13 while inhibiting Th1 cell differentiation and IFN-γ production (Ferber et al., 1999; Ouyang et al., 1998). IL-4 signalling induces T-cell proliferation and differentiation into a Th1 phenotype and suppresses macrophage and

microglia M1 phenotypes and pro-inflammatory mediator expression (Stein et al., 1992). IL-10 inhibits pro-inflammatory mediator release and reduces the recruitment of immune related glia cells in the dorsal horn spinal cord (Gordon, 2003; Ponomarev et al., 2005). Interestingly IL-10 supresses the pro-inflammatory functions of antigen presenting cells by antagonizing the expression of co-stimulatory molecules, the release of pro-inflammatory cytokines and maturation thus hindering their ability to stimulate adaptive immune effector cells (Fiorentino et al., 1991).

The infant anti-inflammatory response does not require nerve damage as stimulation of intact afferent C fibre nociceptors stimulates the expression of anti-inflammatory IL-10 and IL-4 in infants, but not adults. This suggests that their release is directly activated by neurotransmitter release from C fibre terminals in the dorsal horn and that the response following infant nerve injury could be a direct result of activity in damaged C fibre afferent terminals. This is supported by the fact that IL-10 release from microglia is potentiated by glutamate (Werry et al., 2011). Anti-inflammatory cytokine IL-13 and TGF-β were not up-regulated in the spinal cord following C-fibre stimulation in infants indicating that there is not a general increase in anti-inflammatory mediator expression but one that is specific to IL-10 and IL-4.

This thesis did not elucidate the origin of IL-10 and IL-4. Although both IL-10 and IL-4 are usually associated with T-cells, studies also suggest they may be released from microglia (Chabot et al., 1999; Milligan and Watkins, 2009; Park et al., 2005; Ponomarev et al., 2005). Furthermore, resident CD2+ T-cells are found in both infant and adult dorsal horn spinal cords but in the infant these markers (CD2+ and CD3+) are not up-regulated following nerve injury (Costigan et al., 2009).

Importantly blocking IL-10 following infant SNI mice unmasks pain like behaviour indicating that the expression of IL-10 is functionally essential for the absence of mechanical hypersensitivity. It also indicates that the signalling of pain-like behaviour is not absent rather it is actively suppressed and can be unmasked if the anti-inflammatory response is unbalanced.

5.4.2 Nerve injured and sham treated infants develop pain-like behaviour following administration of pro-inflammatory mediators

Despite the dominant anti-inflammatory response observed in infants following SNI, infant pain circuits are capable of responding to pro-inflammatory mediators. For example, nerve injury sensitizes neonatal lamina I neurones to TNF *in vitro* (Li and Baccei, 2011) and naïve rodents can develop pain-like behaviour in response to intrathecal administration of LPS and ATP-activated microglia, but to a significantly lesser extent than observed in adults (Moss et al., 2007). The application of intrathecal TNF and LPS-activated microglia can also induce mechanical hypersensitivity in mice seven days after SNI. Results presented in this thesis suggest that in these studies, the anti-inflammatory activity is overcome by tipping the balance to a pro-inflammatory response in infants. While administration of TNF induced mechanical hypersensitivity in both sham and SNI treated infants, administration of LPS-activated microglia evoked hypersensitivity in SNI but not sham treated infants. LPS is a bacterial endotoxin that causes the activation of TLR4 and the subsequent activation of the MAPK and NF-ƙB signalling pathways in microglia involved in the release of cytotoxic factors including TNF and IL-6 that can also efficiently down regulate IL-10 (Harry, 2013).

Although LPS-activated microglia also produces TNF, an explanation for this inconsistency in behaviour maybe because LPS- activated microglia would not produce as much TNF as administration compared to the application of TNF directly to the spinal cord. For example, LPS administration to neonatal spinal cord cultures *in vitro* does not increase the secretion of pro-inflammatory mediators or phagocytosis activity (Baskar et al., 2014; Werry et al., 2011) and LPS application to microglia derived from infant spinal cords significantly increases IL-10 release and IL-10 mRNA expression (Baskar et al., 2014; Werry et al., 2011). This suggests that although neonatal preparations of LPS-activated microglia may not secrete TNF to the same extent as adults and that TNF is released at lower concentrations than the single TNF injection administered (Berta et al., 2014; Welser-Alves and Milner, 2013). As a result the lower levels of TNF can sensitized young nerve injured mice, while leaving sham animals of the same age unaffected. This is consistent with the finding

that the amount and type of effector released by microglia vary with the nature of the activating stimulus and age of animal (Lai and Todd, 2008).

The results presented in this thesis indicate that despite infant nerve injured mice being able to respond to stressors such as TNF and LPS-activated microglia, infant mice do not normally produce TNF and BDNF following nerve injury due to the predominant anti-inflammatory response.

5.4.3 Nerve injured infants eventually develop pain-like behaviour and a pro-inflammatory response in the dorsal horn spinal cord.

When nerve injured infant rodents reach adolescence there is a switch in the immune response from anti to pro-inflammatory that coincides with a delayed onset of mechanical hypersensitivity and increases in spontaneous and activity-evoked responses in dorsal horn neurones similar to that seen in adult nerve injury models as summarised in Fig 5.1 (Laird and Bennett, 1993). This timing is consistent with postnatal changes in TLR inducible cytokine and chemokine release from microglia which is at its lowest at P21 and after which it rises considerably towards adolescence (Scheffel et al., 2012). Therefore despite the fact that nerve injury was performed in infancy, pain behaviour emerges at adolescence, at least in part as a result of a change in neuroimmune activity.

This is consistent with previous studies suggesting that the onset of pain behaviour in rats following infant SNI coincides with an increase in IBA-1 immunofluorescence in the ipsilateral dorsal horn (Vega-Avelaira et al., 2012). Interestingly pre-emptive administration of minocycline, which selectively inhibits M1 polarization of microglia (and release of TNF, IL-1β and IFN-γ), failed to prevent the onset of pain hypersensitivity at adolescence (Vega-Avelaira et al., 2012). This maybe because other studies administrated the drug via an intrathecal route, not subcutaneously and also minocycline decreases IL-10 expression in the dorsal horn spinal cord so the overall response may still have been pro-inflammatory (Ledeboer et al., 2005). Furthermore, at the onset of pain behaviour, the significant increase in T-cell marker CD2 and other proteins involved in T-cell chemo-attraction and signalling (including IFN-γ and CXCL9) suggests that the infiltration of T-cells

in the dorsal horn spinal cord may occur at this age, although this needs to be verified.

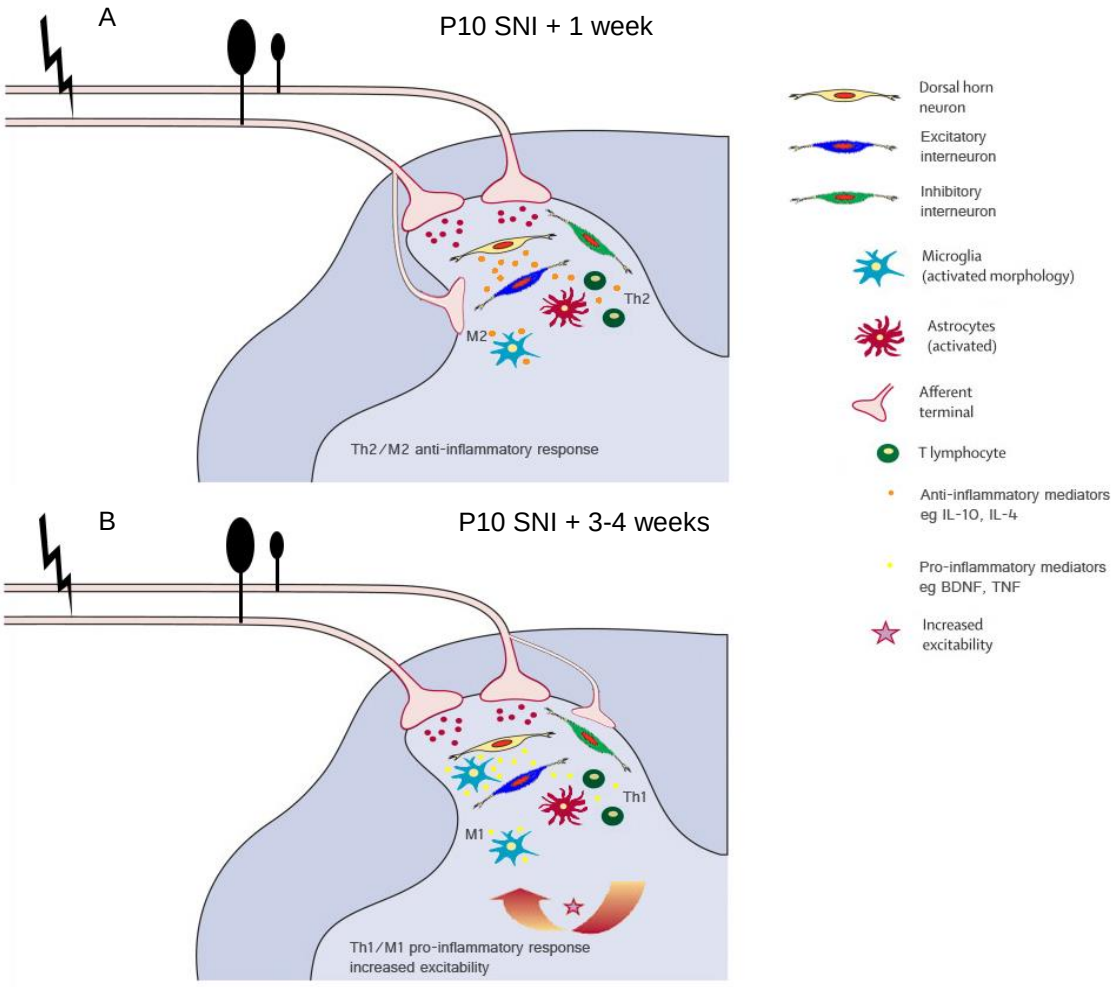


Fig. 5.1 Summary diagram showing alterations of the neuro-immune system in the dorsal horn following infant nerve injury

(A) Infant (P10) SNI triggers an anti-inflammatory response in the dorsal horn including the release of anti-inflammatory mediators IL-4 and IL-10 and transcription factor GATA3. This is characteristic of anti-inflammatory microglial activity (M2) and T helper cell activity (Th2). Spontaneous and evoked dorsal horn activity is not enhanced and pain-like behaviour is absent (B) 3-4 weeks after P10 SNI IL-10, IL-4 and GATA3 return to control levels. A pro-inflammatory response arises due to a switch in microglial and T-cell activity to M1 and Th2 respectively and is associated with the release of TNF and BDNF. These mediators excite dorsal horn neurons (both spontaneous and evoked activity) and trigger pain-like behaviour. Reprinted from The Lancet, 11, Calvo, Daves and Bennett, The role of the immune system in the generation of neuropathic pain, page 629-642., Copyright (2012), with permission from Elsevier.

T-cells are known to participate in pain sensitization by releasing pro-inflammatory cytokines in the adult dorsal horn spinal cord and, remarkably, nerve injured animals lacking T cells show attenuation of pain behaviour (Cao

and DeLeo, 2008; Costigan et al., 2009; Moalem et al., 2004; Sarah M Sweitzer et al., 2002). As the astrocyte marker GFAP did not increase at this time, astrocyte activation is unlikely to drive the delayed onset of hypersensitivity but maybe associated with its maintenance (Tsuda et al., 2011).These findings show that neuropathic pain following early life nerve injury is not absent but suppressed by neuroimmune activity and that 'latent' pain can still emerge at adolescence, when the neuroimmune profile changes. This data may explain why neuropathic pain is rare in young children and also why it can emerge for no observable reason, in adolescent patients (McKelvey et al., 2015).

5.5 Wider implications of research

5.5.1 The absence of neuropathic pain in infants

As observed in rodent neonates, it is well documented that even premature human infants show strong spinal nociceptive reflex activity and distinct cortical potentials following skin breaking and other noxious procedures (Cornelissen et al., 2013; Fabrizi et al., 2011; Slater et al., 2010). Children are also capable of developing, and do report, chronic pain associated with pathologies such as arthritis and cancer (McGrath et al., 1990; Palermo, 2000). In contrast neuropathic pain in human infants, occurring acutely or as a feature of post-surgical pain (Kehlet and Rathmell, 2010), is rare under the age of 5 and increases in likelihood at older ages (particularly in adolescence) with a median age of onset in paediatric patients of 13 years of age (Anand and Birch, 2002; Atherton et al., 2008; Walco et al., 2010).

There are many examples to support this observation. Although chronic neuropathic pain is exhibited by adults after brachial plexus injuries (such as following spinal root avulsion) infants do not show evidence of chronic pain behaviour or neuropathic syndromes (Anand and Birch, 2002). Similarly following limb amputation adult patients may develop phantom limb pain and, although there are some reports in young children, there is a negative correlation between age and onset of phantom pain (i.e. older age, shorter time of phantom onset) with no reference to chronic neuropathic pain in infants in the majority of these studies (Hall et al., 2006; Krane and Heller, 1995;

Kristensen et al., 2010; McGrath and Hillier, 1992; Melzack et al., 1997; Poeck, 1964; Weinstein et al., 1964). Furthermore, distal nerve injury does lead to neuropathic pain in children, but only at 5 years of age or older, with an increase in severity in teenagers (Atherton et al., 2008; Hwang et al., 2008). One study by Atherton et al. (Atherton et al., 2008) studied 49 children with distal upper limb nerve injury at 1 month to 18 years of age at injury and followed them up at a mean of 2 years and 3 months later. Patients younger than 5 years did not report chronic neuropathic pain or allodynia (0/15). Those older than 5 had an increased sensitivity to pinprick or thermal stimuli and children which presented chronic neuropathic pain were all older than 12 years of age at the time of surgery. This explains why preadolescents with neuropathic pain are 'infrequent visitors to paediatric pain clinics' and why all reported cases of complex regional pain syndromes (CRPS) in children are older than 8 years of age (Walco et al., 2010). Therefore laboratory and clinical studies show that infant nerve injury presents very differently in comparison to adults where neuropathic pain is one of the most challenging aspects of pain management (Dworkin et al., 2007; Woolf and Mannion, 1999).

It is important to reiterate that the response of infants to nerve injury is unique in comparison to other insults such as inflammatory and incisional pain which are prevalent in young infants. This thesis suggests that the immune system may underlie the difference in pain behaviour observed in infants that show a predominant anti-inflammatory response in the dorsal horn spinal cord following nerve injury. The function of a predominant anti-inflammatory response in the infant dorsal horn maybe to prevent excessive responses to microbes following the transition from a sterile environment in utero (Maynard et al., 2012) and also facilitate the role of microglia in regulating CNS synaptic development by synaptic stripping involving the removal damaged cells and dysfunctional synapses (Kettenmann et al., 2013) which if were not contained, would produce wide spread and damaging inflammation.

In the infant dorsal horn IL-10 may act to reduce antigen presenting cell maturation, thus preventing T-cell activation, a notion supported by a study by Costigan and colleagues where nerve injured adults exhibited T-cell infiltration into the dorsal horn spinal cord and the up-regulation of IFN-γ that was absent

in infants (Costigan et al., 2009; Moss et al., 2007). Therefore the anti-inflammatory response to nerve injury in infants maybe an indirect consequence of the requirements for normal postnatal development in the dorsal horn, while in an adult this balance changes towards a protective pro-inflammatory response (Beggs et al., 2002; Bremner and Fitzgerald, 2008; Koch et al., 2012).

These experiments highlight the differences in the maturing immune processes that can alter nociception and, as there is increased recognition that the immune system is intimately involved in pain, any new avenues of treatment which target this system warrants caution when extrapolating data on pain management from adults to infants.

5.5.2 The onset of neuropathic pain in adolescence

The advances in medical care have led to a profound increase in the survival of infants but the implication is that infants exposed to procedural and/or post-surgical pain may display alterations in pain sensitivity later in life. Animal studies support this and show that stimulation of peripheral nociceptors at infancy does cause long term alterations in the circuitry of the CNS (see Chapter One).

To understated these long term changes in humans, studies into the pain sensitivity of children and adolescents who had early life pain experiences as infants including surgery, burns and circumcision have been conducted and provide evidence for long term alterations in pain sensitivity which vary depending on the type of surgery and the area tested. Following chest surgery in early life, the skin sensitivity of children aged 9-12 years of age showed hyposensitivity to touch, cold and heat in the area of surgery in addition to dysaesthesia (Schmelzle-Lubiecki et al., 2007). In a separate study infants who had surgery in the first 3 months of life and follow up surgery in the same dermatome, had higher pain sensitivity and required more postoperative morphine compared to infants with no prior surgery (Peters et al., 2005) and children who had suffered moderate burns at infancy (in the first 24 months of life) showed greater pain and sensitisation to noxious stimulation but a decrease in general mechanical and thermal sensitivity (at 16 years of age)

(Wollgarten-Hadamekl et al., 2011). Finally, boys who had been circumcised early in life display greater pain behaviour to immunization at age 4 compared to uncircumcised boys (Taddio et al., 1995).

The aforementioned clinical studies indicate that painful experiences at infancy do alter pain processing later in life. However, although studies show that the prevalence of neuropathic pain increases with age, the long term effect of infant nerve injury are relatively sparse and it has been recognised that neuropathic pain in adolescence has been under recognized (Howard, 2003) and the lack of longitudinal studies into the long term effects of infant nerve injury warrants further investigation (Jones, 2011). However, the discovery that delayed onset of pain hypersensitivity emerges at adolescence in animal models is consistent with the observation that infant phantom limb pain does appear for many years after the original loss of limbs during infants, in some cases up to 3-15 years later (Melzack et al., 1997). This data may also explain the peak onset of complex pain syndromes such as CRPS type 2 occurs in adolescent patients (Walco et al., 2010). Interestingly one study has shown that patients with long standing CRPS exhibit significant increases in microglia and astrocyte immunoreactivity in their spinal cord compared to controls (Del Valle et al., 2009).

As with other types of injury long term outcomes following infant nerve injury does vary with type of nerve injury and brachial plexus injuries and repair in neonates show that no patient of 5 years of age or less at the time of nerve injury developed chronic neuropathic pain later in life (Anand and Birch, 2002). It is increasingly recognised that adolescents show a relatively high prevalence of chronic idiopathic pain-like syndromes (Hoftun et al., 2011; Howard, 2003). For example, sensory polyneuropathy syndromes that appear at adolescence such as non-length dependent small fibre polyneuropathy that is often iodiopathic with symptoms presenting similar to those observed in neuropathic pain patients including burning and pin prick pain, and can occur at adolescence (Khan and Zhou, 2012). This syndrome is usually considered a pschogenic disorder due to a lack of awareness of its status as a distinct disorder but it has recently been associated with immune disorders (Khan and Zhou, 2012).

Although some patients do respond to treatment and reassurance with simple analgesics, a significant proportion go on to develop various degrees of chronicity that is difficult to treat and perplexing. Although under reported iodiopathic functional pain syndromes, CRPS type II and juvenile idiopathic arthritis which have an onset at adolescence are increasingly recognised and, although the underlying mechanisms and causes remain to be elucidated, some studies suggest that dysfunctional inflammatory processes may underlie these pain syndromes (Ham et al., 2009; Lahdenne et al., 2003; Low et al., 2007; Pharoah et al., 2006; Prakken et al., 2011; Sethna et al., 2007).

The experiments presented in this thesis suggest that a shift in the balance of anti- to pro inflammatory activity at adolescence may reveal a latent neuropathic hypersensitivity and could reveal new therapeutic avenues. Multiple rodent studies have shown that exogenous IL-10 and IL-4 are anti-nociceptive in adult neuropathic models and suppress pro-inflammatory cytokines, microglia responses and pain behaviour, and in infants this anti-nociceptive response naturally dominates over the pro-inflammatory response following nerve injury (Hao et al., 2006; Ledeboer et al., 2007; Milligan et al., 2005a, b).

Clinically, treatment using IL-10 remains insufficient because of difficulties in the route of IL-10 administration and its biological half-life (Milligan et al., 2005a). Recently Soderquist and colleagues produced a plasmid DNA encoding IL-10 which is slowly released from biodegradable microparticles and provides long term pain relief for 70 days in an animal model of neuropathic pain, suggesting a potential use for intrathecal gene therapy (Soderquist et al., 2010). In addition Van Montfran and colleagues presented a novel method of IL-10 delivery by utilising transduced T cells (Van Montfrans et al., 2002). One advantage of using T-cells as delivery vehicles is the likelihood of IL-10 being released only on activation in local sites of inflammation/injury and secondly this would prevent systemic exposure to the retro viral vector while ensuring sustained gene expression. However, this technology still needs to be developed and tested in humans. One alternative is to utilise substances that are already used to treat other diseases that enhance the expression of anti-inflammatory mediators. A recent study by Ji and colleagues has illustrated

that intrathecal administration of Resolvin E1 in a nerve injured rat prevented the development of hypersensitivity, up-regulation of microglial markers and TNF in the dorsal horn spinal cord and could also transiently reduce pre-established hypersensitivity (Xu et al., 2013). However, this has the disadvantage of having to be intrathecal administered. One reagent of particular interest is Glatiramer acetate, used to alleviate the symptoms of multiple sclerosis and acts to induce regulatory Th2 cell responses (Dhib-Jalbut, 2003). Oral administration of Glatiramer acetate in a rat neuropathic pain model reduced microglia markers and TNF while increasing the invasion of CD3+ and CD4+ T-cells and expression of IL-4 and IL-10 in the dorsal horn while attenuating neuropathic hypersensitivity (Leger et al., 2011). Future studies investigating the ability of anti-inflammatory reagents to treat complex pain syndromes that arise at adolescence warrants further study.

5.6 Further work

The experiments presented in this thesis have raised additional questions which could be addressed to better understand the changing pain profile following infant nerve injury. These include, but are not limited to;

1. In infants seven days after SNI, what alterations occur in the in dorsal horn circuitry that underlie the observed reduction in the receptive fields and response to Von Frey hairs? How does the blockade anti-inflammatory or application of pro-inflammatory mediators alter activity of dorsal horn neurones of nerve injured infants?
2. What is the origin of infant SNI induced IL-10 and IL-4 in the dorsal horn? What mechanisms underlie the switch from anti to pro-inflammatory responses in the dorsal horn at the time of pain-like behaviour onset following infant nerve injury? Can the immune balance in the dorsal horn spinal cord of animals prior to the onset of pain-like behaviour be manipulated to further delay the onset of pain behaviour? Do mice lacking in key pro-inflammatory mediators develop pain-like behaviour following infant nerve injury?

5.7 Conclusion

The experiments presented in this thesis have examined the long term consequences of infant peripheral nerve injury to show a delay in the onset of pain-like behaviour does occur in response to stimulation with a range of stimuli. The alterations in dorsal horn processing and the immune profile in the dorsal horn have also been characterised in infants in the early and late period following infant nerve injury and, for the first time, responses of dorsal horn neurons in SNI treated adult rats have also been examined using *in vivo* electrophysiological recordings. These studies indicate that neuropathic pain behaviour following early life peripheral nerve injury is not absent, but suppressed by neuro-immune activity. This is characterised by an anti-inflammatory profile in the infant dorsal horn spinal cord that can be evoked by brief C-fibre stimulation. This suggests that the release of anti-inflammatory mediators at this age is directly activated by neurotransmitter release from C-fibre terminals in the dorsal horn upon nerve injury. Infant nerve injury may have consequences later in life resulting in alterations that occur centrally in the dorsal horn spinal cord characterised by an increase in dorsal horn neuronal activity and a shift in the balance from anti- to pro inflammatory activity at adolescence to reveal a latent neuropathic pain hypersensitivity, similar to that observed in the adult. The data therefore provides a mechanistic explanation for the currently poorly understood paediatric chronic pain states that emerge at adolescence that may be the result of an earlier trauma or injury whose effects have been masked until later in life by the local CNS immune suppression

References

Abbadie, C., Lindia, J.A., Cumiskey, A.M., Peterson, L.B., Mudgett, J.S., Bayne, E.K., DeMartino, J.A., MacIntyre, D.E., Forrest, M.J., 2003. Impaired neuropathic pain responses in mice lacking the chemokine receptor CCR2.PNAS. 13, 7947-7952.

Adkins, B., 1999. T-cell function in newborn mice and humans. Immunol. 20, 330–335

Adkins, B., 2000. Development of Neonatal Th1/Th2 Function. Int. Rev. Immunol. 19, 157–171.

Adkins, B., 2003. Peripheral CD4+ lymphocytes derived from fetal versus adult thymic precursors differ phenotypically and functionally. J. Immunol. 171, 5157–5164.

Adkins, B., Bu, Y., Cepero, E., Perez, R., 2000. Exclusive Th2 primary effector function in spleens but mixed Th1/Th2 function in lymph nodes of murine neonates. J. Immunol. 164, 2347–2353.

Adkins, B., Hamilton, K., 1992. Freshly isolated, murine neonatal T cells produce IL-4 in response to anti-CD3 stimulation. J. Immunol. 149, 3448–3455.

Adkins, B., Ghanei, A., Hamilton, K., 1993. Developmental regulation of IL-4, IL-2, and IFN-gamma production by murine peripheral T lymphocytes. J. Immunol. 151, 6617–6626.

Adkins, B., Ghanei, A., Hamilton, K., 1994. Up-regulation of murine neonatal T helper cell responses by accessory cell factors. J. Immunol. 153, 3378–3385.

Akopian, A.N., Souslova, V., England, S., Okuse, K., Ogata, N., Ure, J., Smith, A., Kerr, B.J., McMahon, S.B., Boyce, S., Hill, R., Stanfa, L.C., Dickenson, A.H., Wood, J.N., 1999. The tetrodotoxin-resistant sodium channel SNS has a specialized function in pain pathways. Nat. Neurosci. 2, 541–548.

Albuquerque, C., Lee, C.J., Jackson, A.C., MacDermott, A.B., 1999. Subpopulations of GABAergic and non-GABAergic rat dorsal horn neurons express Ca2+-permeable AMPA receptors. Eur. J. Neurosci. 11, 2758–2766.

Al-Chaer, E.D., Traub, R.J., 2002. Biological basis of visceral pain: recent developments. Pain 96, 221–225.

Aldskogius, H., Risling, M., 1981. Effect of sciatic neurectomy on neuronal number and size distribution in the L7 ganglion of kittens. Exp. Neurol. 74, 597–604.

Alliot, F., Godin, I., Pessac, B., 1999. Microglia derive from progenitors, originating from the yolk sac, and which proliferate in the brain. Dev. Brain Res. 117, 145–152.

Aloisi, A.M., Albonetti, M.E., Carli, G., 1994. Sex differences in the behavioural response to persistent pain in rats. Neurosci. Lett. 179, 79–82.

Aloisi, F., Simone, R.D., Columba-Cabezas, S., Levi, G., 1999. Opposite effects of interferon-γ and prostaglandin E2 on tumor necrosis factor and interleukin-10 production in microglia: A regulatory loop controlling microglia pro- and anti-inflammatory activities. J. Neurosci. Res. 56, 571–580.

Amir, R., Kocsis, J.D., Devor, M., 2005. Multiple Interacting Sites of Ectopic Spike Electrogenesis in Primary Sensory Neurons. J. Neurosci. 25, 2576–2585.

Anand, P., Birch, R., 2002. Restoration of sensory function and lack of long-term chronic pain syndromes after brachial plexus injury in human neonates. Brain 125, 113–122.

Andrews, K., Fitzgerald, M., 1994. The cutaneous withdrawal reflex in human neonates: sensitization, receptive fields, and the effects of contralateral stimulation. Pain 56, 95–101.

Andrews, K., Fitzgerald, M., 1999. Cutaneous flexion reflex in human neonates: a quantitative study of threshold and stimulus-response characteristics after single and repeated stimuli. Dev. Med. Child Neurol. 41, 696–703.

Andrews, K.A., Desai, D., Dhillon, H.K., Wilcox, D.T., Fitzgerald, M., 2002. Abdominal sensitivity in the first year of life: comparison of infants with and without prenatally diagnosed unilateral hydronephrosis. Pain 100, 35–46.

Anseloni, V.C.Z., He, F., Novikova, S.I., Turnbach Robbins, M., Lidow, I.A., Ennis, M., Lidow, M.S., 2005. Alterations in stress-associated behaviors and neurochemical markers in adult rats after neonatal short-lasting local inflammatory insult. Neuroscience 131, 635–645.

Apkarian, A.V., Bushnell, M.C., Treede, R.-D., Zubieta, J.-K., 2005. Human brain mechanisms of pain perception and regulation in health and disease. Eur. J. Pain 9, 463–463.

Arnoux, I., Hoshiko, M., Mandavy, L., Avignone, E., Yamamoto, N., Audinat, E., 2013. Adaptive phenotype of microglial cells during the normal postnatal development of the somatosensory “Barrel” cortex. Glia 61, 1582–1594.

Atherton, D.D., Taherzadeh, O., Elliot, D., Anand, P., 2008. Age-Dependent Development Of Chronic Neuropathic Pain, Allodynia and Sensory Recovery after Upper Limb Nerve Injury in Children. J. Hand Surg. Eur. Vol. 33, 186–191.

Attal, N., Cruccu, G., Haanpää, M., Hansson, P., Jensen, T.S., Nurmikko, T., Sampaio, C., Sindrup, S., Wiffen, P., 2006. EFNS guidelines on pharmacological treatment of neuropathic pain. Eur. J. Neurol. 13, 1153–1169.

Austin, P.J., Moalem-Taylor, G., 2010. The neuro-immune balance in neuropathic pain: involvement of inflammatory immune cells, immune-like glial cells and cytokines. J. Neuroimmunol. 229, 26–50.

Austin, P.J., Kim, C.F., Perera, C.J., Moalem-Taylor, G., 2012. Regulatory T cells attenuate neuropathic pain following peripheral nerve injury and experimental autoimmune neuritis. PAIN 153, 1916–1931.

Azkue, J.J., Zimmermann, M., Hsieh, T.-F., Herdegen, T., 1998. Peripheral nerve insult induces NMDA receptor-mediated, delayed degeneration in spinal neurons. Eur. J. Neurosci. 10, 2204–2206.

Baba, H., Ji, R.-R., Kohno, T., Moore, K.A., Ataka, T., Wakai, A., Okamoto, M., Woolf, C.J., 2003. Removal of GABAergic inhibition facilitates polysynaptic A fiber-mediated excitatory transmission to the superficial spinal dorsal horn. Mol. Cell. Neurosci. 24, 818–830.

Baccei, M.L., Bardoni, R., Fitzgerald, M., 2003. Development of nociceptive synaptic inputs to the neonatal rat dorsal horn: glutamate release by capsaicin and menthol. J. Physiol. 549, 231–242.

Baccei, M.L., Fitzgerald, M., 2004. Development of GABAergic and Glycinergic Transmission in the Neonatal Rat Dorsal Horn. J. Neurosci. 24, 4749–4757.

Backonja, M.-M., Stacey, B., 2004. Neuropathic pain symptoms relative to overall pain rating. J. Pain 5, 491–497.

Banik, R.K., Woo, Y.C., Park, S.S., Brennan, T.J., 2006. Strain and sex influence on pain sensitivity after plantar incision in the mouse. Anesthesiology 105, 1246–1253.

Barbaresi, P., 2010. Postnatal development of GABA-immunoreactive neurons and terminals in rat periaqueductal gray matter: A light and electron microscopic study. J. Comp. Neurol. 518, 2240–2260.

Barber, R.D., Harmer, D.W., Coleman, R.A., Clark, B.J., 2005. GAPDH as a housekeeping gene: analysis of GAPDH mRNA expression in a panel of 72 human tissues. Physiol. Genomics 21, 389–395.

Bardin, L., 2011. The complex role of serotonin and 5-HT receptors in chronic pain: Behav. Pharmacol. 22, 390–404.

Baron, R., 2006. Mechanisms of Disease: neuropathic pain—a clinical perspective. Nat. Clin. Pract. Neurol. 2, 95–106.

Baron, R., Binder, A., Wasner, G., 2010. Neuropathic pain: diagnosis, pathophysiological mechanisms, and treatment. Lancet Neurol. 9, 807–819.

Barr, G.A., 1998. Maturation of the Biphasic Behavioral and Heart Rate Response in the Formalin Test. Pharmacol. Biochem. Behav. 60, 329–335.

Barrios, C., Brawand, P., Berney, M., Brandt, C., Lambert, P.-H., Siegrist, C.-A., 1996. Neonatal and early life immune responses to various forms of vaccine antigens qualitatively differ from adult responses: predominance of a Th2-biased pattern which persists after adult boosting. Eur. J. Immunol. 26, 1489–1496.

Basbaum, A.I., Marley, N.J.E., O'Keefe, J., Clanton, C.H., 1977. Reversal of morphine and stimulus-produced analgesia by subtotal spinal cord lesions. Pain 3, 43–56.

Basbaum, A.I., Gautron, M., Jazat, F., Mayes, M., Guilbaud, G., 1991. The spectrum of fiber loss in a model of neuropathic pain in the rat: an electron microscopic study. Pain 47, 359–367.

Basbaum, A.I., Jessell, T.M., 2000. The perception of pain. Princ. Neural Sci. 4, 472–491.

Basbaum, A.I., Bautista, D.M., Scherrer, G., Julius, D., 2009. Cellular and Molecular Mechanisms of Pain. Cell 139, 267–284.

Baskar Jesudasan, S.J., Todd, K.G., Winship, I.R., 2014. Reduced Inflammatory Phenotype in Microglia Derived from Neonatal Rat Spinal Cord versus Brain. PLoS ONE 9, e99443.

Baumgärtner, U., Magerl, W., Klein, T., Hopf, H.C., Treede, R.-D., 2002. Neurogenic hyperalgesia versus painful hypoalgesia: two distinct mechanisms of neuropathic pain. Pain 96, 141–151.

Bautista, D.M., Jordt, S.-E., Nikai, T., Tsuruda, P.R., Read, A.J., Poblete, J., Yamoah, E.N., Basbaum, A.I., Julius, D., 2006. TRPA1 mediates the inflammatory actions of environmental irritants and proalgesic agents. Cell 124, 1269–1282.

Bee, L.A., Dickenson, A.H., 2008. Descending facilitation from the brainstem determines behavioural and neuronal hypersensitivity following nerve injury and efficacy of pregabalin. PAIN 140, 209–223.

Beggs, S. 2000. Plasticity of primary sensory neurons and their central connections: normal` development and the effects of neonatal axotomy. Thesis (Ph.D.), UCL.

Beggs, S., Torsney, C., Drew, L.J., Fitzgerald, M., 2002. The postnatal reorganization of primary afferent input and dorsal horn cell receptive fields in the rat spinal cord is an activity-dependent process. Eur. J. Neurosci. 16, 1249–1258.

Beggs, S., Salter, M.W., 2010. Microglia–neuronal signalling in neuropathic pain hypersensitivity 2.0. Curr. Opin. Neurobiol., Signalling mechanisms 20, 474–480.

Beggs, S., Salter, M.W., 2007. Stereological and somatotopic analysis of the spinal microglial response to peripheral nerve injury. Brain. Behav. Immun. 21, 624–633.

Beggs, S., Liu, X.J., Kwan, C., Salter, M.W., 2010. Peripheral nerve injury and TRPV1-expressing primary afferent C-fibers cause opening of the blood-brain barrier. Mol. Pain 6, 74.

Beggs, S., Currie, G., Salter, M.W., Fitzgerald, M., Walker, S.M., 2012. Priming of adult pain responses by neonatal pain experience: maintenance by central neuroimmune activity. Brain 135, 404–417.

Benarroch, E.E., 2007. Sodium channels and pain. Neurology 68, 233–236.

Bennett, G.J., Xie, Y.-K., 1988. A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. Pain 33, 87–107.

Bennett, G.J., Kajander, K.C., Sahara, Y., Iadarola, M.J., Sugimoto, T., 1989. Neurochemical and Anatomical Changes in the Dorsal Horn of Rats with an Experimental Painful Peripheral Neuropathy, in: Cervero, F., Bennett, G.J., Headley, P.M. (Eds.), Processing of Sensory Information in the Superficial Dorsal Horn of the Spinal Cord. Springer US, pp. 463–471.

Benn, S.C., Costigan, M., Tate, S., Fitzgerald, M., Woolf, C.J., 2001. Developmental expression of the TTX-resistant voltage-gated sodium channels Nav1. 8 (SNS) and Nav1. 9 (SNS2) in primary sensory neurons. J. Neurosci. 21, 6077–6085.

Berkley, K.J., 1997. Sex differences in pain. Behav. Brain Sci. 20, 371–380.

Berta, T., Park, C.-K., Xu, Z.-Z., Xie, R.-G., Liu, T., Lu, N., Liu, Y.-C., Ji, R.-R., 2014. Extracellular caspase-6 drives murine inflammatory pain via microglial TNF-? secretion. J. Clin. Invest. 124, 1173–1186.

Berthele, A., Boxall, S.J., Urban, A., Anneser, J.M.H., Zieglgänsberger, W., Urban, L., Tölle, T.R., 1999. Distribution and developmental changes in metabotropic glutamate receptor messenger RNA expression in the rat lumbar spinal cord. Dev. Brain Res. 112, 39–53.

Bessou, P., Perl, E.R., 1969. Response of cutaneous sensory units with unmyelinated fibers to noxious stimuli. J Neurophysiol 32, 1025–1043.

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

Bhat, N.R., Zhang, P., Lee, J.C., Hogan, E.L., 1998. Extracellular Signal-Regulated Kinase and p38 Subgroups of Mitogen-Activated Protein Kinases Regulate Inducible Nitric Oxide Synthase and Tumor Necrosis Factor-α Gene Expression in Endotoxin-Stimulated Primary Glial Cultures. J. Neurosci. 18, 1633–1641.

Bianchi, M.E., 2007. DAMPs, PAMPs and alarmins: all we need to know about danger. J. Leukoc. Biol. 81, 1–5.

Bian, G.-L., Wei, L.-C., Shi, M., Wang, Y.-Q., Cao, R., Chen, L.-W., 2007. Fluoro-Jade C can specifically stain the degenerative neurons in the substantia nigra of the 1-methy-4-phenyl-1,2,3,6-tetrahydro pyrindine-treated C57BL/6 mice. Brain Res. 1150, 55–61.

Binshtok, A.M., Wang, H., Zimmermann, K., Amaya, F., Vardeh, D., Shi, L., Brenner, G.J., Ji, R.-R., Bean, B.P., Woolf, C.J., others, 2008. Nociceptors are interleukin-1β sensors. J. Neurosci. 28, 14062–14073.

Boada, M.D., Houle, T.T., Eisenach, J.C., Ririe, D.G., 2010. Differing Neurophysiologic Mechanosensory Input From Glabrous and Hairy Skin in Juvenile Rats. J. Neurophysiol. 104, 3568–3575.

Bondok, A.A., Sansone, F.M., 1984. Retrograde and transganglionic degeneration of sensory neurons after a peripheral nerve lesion at birth. Exp. Neurol. 86, 322–330.

Boucher, T., Jennings, E., Fitzgerald, M., 1998. The onset of diffuse noxious inhibitory controls in postnatal rat pups: a C-Fos study. Neurosci. Lett. 257, 9–12.

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

Bouhassira, D., Lantéri-Minet, M., Attal, N., Laurent, B., Touboul, C., 2008. Prevalence of chronic pain with neuropathic characteristics in the general population. Pain 136, 380–387.

Bourgeais, L., Gauriau, C., Monconduit, L., Villanueva, L., Bernard, J.-F., 2003. Dendritic domains of nociceptive-responsive parabrachial neurons match terminal fields of lamina I neurons in the rat. J. Comp. Neurol. 464, 238–256.

Bourquin, A.-F., Süveges, M., Pertin, M., Gilliard, N., Sardy, S., Davison, A.C., Spahn, D.R., Decosterd, I., 2006. Assessment and analysis of mechanical allodynia-like behavior induced by spared nerve injury (SNI) in the mouse. PAIN 122, 14.e1–14.e14.

Boyd, I.A., Kalu, K.U., 1979. Scaling factor relating conduction velocity and diameter for myelinated afferent nerve fibres in the cat hind limb. J. Physiol. 289, 277–297.

Bráz, J.M., Sharif-Naeini, R., Vogt, D., Kriegstein, A., Alvarez-Buylla, A., Rubenstein, J.L., Basbaum, A.I., 2012. Forebrain GABAergic Neuron Precursors Integrate into Adult Spinal Cord and Reduce Injury-Induced Neuropathic Pain. Neuron 74, 663–675.

Bremner, L.R., Fitzgerald, M., 2008. Postnatal tuning of cutaneous inhibitory receptive fields in the rat. J. Physiol. 586, 1529–1537.

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

Brown, A.G., Fyffe, R.E., 1981. Form and function of dorsal horn neurones with axons ascending the dorsal columns in cat. J. Physiol. 321, 31–47.

Burgess, P.R., Perl, Er., 1967. Myelinated afferent fibres responding specifically to noxious stimulation of the skin. J. Physiol. 190, 541–562.

Burgess, P.R., Petit, D., Warren, R.M., 1968. Receptor types in cat hairy skin supplied by myelinated fibers. J. Neurophysiol. 31, 833–848.

Burgess, S.E., Gardell, L.R., Ossipov, M.H., Malan, T.P., Vanderah, T.W., Lai, J., Porreca, F., 2002. Time-Dependent Descending Facilitation from the Rostral Ventromedial Medulla Maintains, But Does Not Initiate, Neuropathic Pain. J. Neurosci. 22, 5129–5136.

Cain, D.M., Khasabov, S.G., Simone, D.A., 2001. Response Properties of Mechanoreceptors and Nociceptors in Mouse Glabrous Skin: An In Vivo Study. J. Neurophysiol. 85, 1561–1574.

Calvo, M., Zhu, N., Tsantoulas, C., Ma, Z., Grist, J., Loeb, J.A., Bennett, D.L.H., 2010. Neuregulin-ErbB Signaling Promotes Microglial Proliferation and Chemotaxis Contributing to Microgliosis and Pain after Peripheral Nerve Injury. J. Neurosci. 30, 5437–5450.

Cameron, A.A., Cliffer, K.D., Dougherty, P.M., Willis, W.D., Carlton, S.M., 1991. Changes in lectin, GAP-43 and neuropeptide staining in the rat superficial dorsal horn following experimental peripheral neuropathy. Neurosci. Lett. 131, 249–252.

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

Campbell, J.N., Meyer, R.A., 2006. Mechanisms of Neuropathic Pain. Neuron 52, 77–92.

Campero, M., Serra, J., Ochoa, J.L., 1996. C-polymodal nociceptors activated by noxious low temperature in human skin. J. Physiol. 497, 565–572.

Cao, L., DeLeo, J.A., 2008. CNS-infiltrating CD4+ T lymphocytes contribute to murine spinal nerve transection-induced neuropathic pain. Eur. J. Immunol. 38, 448–458.

Cao, L., Palmer, C.D., Malon, J.T., De Leo, J.A., 2009. Critical role of microglial CD40 in the maintenance of mechanical hypersensitivity in a murine model of neuropathic pain. Eur. J. Immunol. 39, 3562–3569.

Carlton, S.M., Dougherty, P.M., Pover, C.M., Coggeshall, R.E., 1991. Neuroma formation and numbers of axons in a rat model of experimental peripheral neuropathy. Neurosci. Lett. 131, 88–92.

Carlton, S.M., Hargett, G.L., 1995. Treatment with the NMDA antagonist memantine attenuates nociceptive responses to mechanical stimulation in neuropathic rats. Neurosci. Lett. 198, 115–118

Carvalho, A.L., Duarte, C.B., Carvalho, A.P., 2000. Regulation of AMPA Receptors by Phosphorylation. Neurochem. Res. 25, 1245–1255

Casals-Díaz, L., Vivó, M., Navarro, X., 2009. Nociceptive responses and spinal plastic changes of afferent C-fibers in three neuropathic pain models induced by sciatic nerve injury in the rat. Exp. Neurol. 217, 84–95.

Cata, J.P., Weng, H.-R., Chen, J.-H., Dougherty, P.M., 2006. Altered discharges of spinal wide dynamic range neurons and down-regulation of glutamate transporter expression in rats with paclitaxel-induced hyperalgesia. Neuroscience 138, 329–338.

Caterina, M.J., Rosen, T.A., Tominaga, M., Brake, A.J., Julius, D., 1999. A capsaicin-receptor homologue with a high threshold for noxious heat. Nature 398, 436–441.

Caterina, M.J., Schumacher, M.A., Tominaga, M., Rosen, T.A., Levine, J.D., Julius, D., 1997. The capsaicin receptor: a heat-activated ion channel in the pain pathway. Nature 389, 816–824.

Cavallaro, M., Mariani, J., Lancini, C., Latorre, E., Caccia, R., Gullo, F., Valotta, M., DeBiasi, S., Spinardi, L., Ronchi, A., Wanke, E., Brunelli, S., Favaro, R., Ottolenghi, S., Nicolis, S.K., 2008. Impaired generation of mature neurons by neural stem cells from hypomorphic Sox2 mutants. Development 135, 541–557.

Chabot, S., Williams, G., Hamilton, M., Sutherland, G., Yong, V.W., 1999. Mechanisms of IL-10 production in human microglia-T cell interaction. J. Immunol. 162, 6819–6828.

Chao, C.C., Hu, S., Molitor, T.W., Shaskan, E.G., Peterson, P.K., 1992. Activated microglia mediate neuronal cell injury via a nitric oxide mechanism. J. Immunol. 149, 2736–2741.

Chao, C.C., Molitor, T.W., Hu, S., 1993. Neuroprotective role of IL-4 against activated microglia. J. Immunol. 151, 1473–1481.

Chaplan, S.R., Bach, F.W., Pogrel, J.W., Chung, J.M., Yaksh, T.L., 1994. Quantitative assessment of tactile allodynia in the rat paw. J. Neurosci. Methods 53, 55–63.

Chaplan, S.R., Malmberg, A.B., Yaksh, T.L., 1997. Efficacy of spinal NMDA receptor antagonism in formalin hyperalgesia and nerve injury evoked allodynia in the rat. J. Pharmacol. Exp. Ther. 280, 829–838.

Chapman, V., Suzuki, R., Chamarette, H.L.C., Rygh, L.J., Dickenson, A.H., 1998a. Effects of systemic carbamazepine and gabapentin on spinal neuronal responses in spinal nerve ligated rats. Pain 75, 261–272.

Chapman, V., Suzuki, R., Dickenson, A.H., 1998b. Electrophysiological characterization of spinal neuronal response properties in anaesthetized rats after ligation of spinal nerves L5-L6. J. Physiol. 507, 881–894.

Cheema, S.S., Rustioni, A., Whitsel, B.L., 1984. Light and electron microscopic evidence for a direct corticospinal projection to superficial laminae of the dorsal horn in cats and monkeys. J. Comp. Neurol. 225, 276–290.

Chessell, I.P., Hatcher, J.P., Bountra, C., Michel, A.D., Hughes, J.P., Green, P., Egerton, J., Murfin, M., Richardson, J., Peck, W.L., Grahames, C.B.A., Casula, M.A., Yiangou, Y., Birch, R., Anand, P., Buell, G.N., 2005. Disruption of the P2X7 purinoceptor gene abolishes chronic inflammatory and neuropathic pain. Pain 114, 386–396.

Chiu, I.M., von Hehn, C.A., Woolf, C.J., 2012. Neurogenic inflammation and the peripheral nervous system in host defense and immunopathology. Nat. Neurosci. 15, 1063–1067.

Cho, I.-H., Lee, M.J., Jang, M., Gwak, N.G., Lee, K.Y., Jung, H.-S., 2012. Minocycline markedly reduces acute visceral nociception via inhibiting neuronal ERK phosphorylation. Mol. Pain 8, 1–13.

Christensen, D., Gautron, M., Guilbaud, G., Kayser, V., 1999. Combined systemic administration of the glycine/NMDA receptor antagonist, (+)-HA966 and morphine attenuates pain-related behaviour in a rat model of trigeminal neuropathic pain. Pain 83, 433–440.

Christianson, J.A., Bielefeldt, K., Malin, S.A., Davis, B.M., 2010. Neonatal colon insult alters growth factor expression and TRPA1 responses in adult mice. PAIN® 151, 540–549.

Chul Han, H., Hyun Lee, D., Mo Chung, J., 2000. Characteristics of ectopic discharges in a rat neuropathic pain model. Pain 84, 253–261.

Chu, Y.-C., Chan, K.-H., Tsou, M.-Y., Lin, S.-M., Hsieh, Y.-C., Tao, Y.-X., 2007. Mechanical Pain Hypersensitivity after Incisional Surgery Is Enhanced in Rats Subjected to

Neonatal Peripheral Inflammation: Effects of N-Methyl-d-aspartate Receptor Antagonists. Anesthesiology 106, 1204–1212.

Clark, A.K., Yip, P.K., Grist, J., Gentry, C., Staniland, A.A., Marchand, F., Dehvari, M., Wotherspoon, G., Winter, J., Ullah, J., 2007. Inhibition of spinal microglial cathepsin S for the reversal of neuropathic pain. Proc. Natl. Acad. Sci. 104, 10655–10660.

Clark, A.K., Yip, P.K., Malcangio, M., 2009. The liberation of fractalkine in the dorsal horn requires microglial cathepsin S. J. Neurosci. 29, 6945–6954.

Clark, A.K., Staniland, A.A., Marchand, F., Kaan, T.K., McMahon, S.B., Malcangio, M., 2010. P2X7-dependent release of interleukin-1β and nociception in the spinal cord following lipopolysaccharide. J. Neurosci. 30, 573–582.

Clark, A.K., Grist, J., Al-Kashi, A., Perretti, M., Malcangio, M., 2012. Spinal cathepsin S and fractalkine contribute to chronic pain in the collagen-induced arthritis model. Arthritis Rheum. 64, 2038–2047.

Clark, A.K., Malcangio, M., 2012. Microglial signalling mechanisms: Cathepsin S and Fractalkine. Exp. Neurol. 234, 283–292.

Coderre, T.J., Wall, P.D., 1987. Ankle joint urate arthritis (AJUA) in rats: an alternative animal model of arthritis to that produced by Freund's adjuvant. Pain 28, 379–393.

Coggeshall, R.E., 1992. A consideration of neural counting methods. Trends Neurosci. 15, 9–13.

Coghill, R.C., Mayer, D.J., Price, D.D., 1993. Wide dynamic range but not nociceptive-specific neurons encode multidimensional features of prolonged repetitive heat pain. J. Neurophysiol. 69, 703–716.

Coggeshall, R.E., Pover, C.M., Fitzgerald, M., 1994. Dorsal root ganglion cell death and surviving cell numbers in relation to the development of sensory innervation in the rat hindlimb. Dev. Brain Res. 82, 193–212.

Coggeshall, R.E., Jennings, E.A., Fitzgerald, M., 1996. Evidence that large myelinated primary afferent fibers make synaptic contacts in lamina II of neonatal rats. Dev. Brain Res. 92, 81–90.

Coggeshall, R.E., Carlton, S.M., 1997. Receptor localization in the mammalian dorsal horn and primary afferent neurons. Brain Res. Rev. 24, 28–66.

Coggeshall, R.E., Lekan, H.A., Doubell, T.P., Allchorne, A., Woolf, C.J., 1997. Central changes in primary afferent fibers following peripheral nerve lesions. Neuroscience 77, 1115–1122.

Colburn, R.W., Rickman, A.J., DeLeo, J.A., 1999. The Effect of Site and Type of Nerve Injury on Spinal Glial Activation and Neuropathic Pain Behavior. Exp. Neurol. 157, 289–304.

Cook, A.J., Woolf, C.J., Wall, P.D., McMahon, S.B., 1987. Dynamic receptive field plasticity in rat spinal cord dorsal horn following C-primary afferent input. Nature 325, 151–153.

Corbett, N.P., Blimkie, D., Ho, K.C., Cai, B., Sutherland, D.P., Kallos, A., Crabtree, J., Rein-Weston, A., Lavoie, P.M., Turvey, S.E., 2010. Ontogeny of Toll-like receptor mediated cytokine responses of human blood mononuclear cells. PloS One 5, e15041.

Cordero-Erausquin, M., Allard, S., Dolique, T., Bachand, K., Ribeiro-da-Silva, A., De Koninck, Y., 2009. Dorsal horn neurons presynaptic to lamina I spinoparabrachial neurons revealed by transynaptic labeling. J. Comp. Neurol. 517, 601–615.

Cornelissen, L., Fabrizi, L., Patten, D., Worley, A., Meek, J., Boyd, S., Slater, R., Fitzgerald, M., 2013. Postnatal Temporal, Spatial and Modality Tuning of Nociceptive Cutaneous Flexion Reflexes in Human Infants. PLoS ONE 8, e76470.

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

Costigan, M., Moss, A., Latremoliere, A., Johnston, C., Verma-Gandhu, M., Herbert, T.A., Barrett, L., Brenner, G.J., Vardeh, D., Woolf, C.J., Fitzgerald, M., 2009. T-Cell Infiltration and Signaling in the Adult Dorsal Spinal Cord Is a Major Contributor to Neuropathic Pain-Like Hypersensitivity. J. Neurosci. 29, 14415–14422.

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

Coyle, D.E., 1998. Partial peripheral nerve injury leads to activation of astroglia and microglia which parallels the development of allodynic behavior. Glia 23, 75–83.

Craft, R.M., Mogil, J.S., Aloisi, A.M., 2004. Sex differences in pain and analgesia: the role of gonadal hormones. Eur. J. Pain 8, 397–411.

Daulhac, L., Mallet, C., Courteix, C., Etienne, M., Duroux, E., Privat, A.-M., Eschalier, A., Fialip, J., 2006. Diabetes-Induced Mechanical Hyperalgesia Involves Spinal Mitogen-Activated Protein Kinase Activation in Neurons and Microglia via N-Methyl-D-aspartate-Dependent Mechanisms. Mol. Pharmacol. 70, 1246–1254.

David, S., Kroner, A., 2011. Repertoire of microglial and macrophage responses after spinal cord injury. Nat. Rev. Neurosci. 12, 388–399.

DeBerry, J., Randich, A., Shaffer, A.D., Robbins, M.T., Ness, T.J., 2010. Neonatal bladder inflammation produces functional changes and alters neuropeptide content in bladders of adult female rats. J. Pain 11, 247–255.

Decosterd, I., Woolf, C.J., 2000. Spared nerve injury: an animal model of persistent peripheral neuropathic pain. Pain 87, 149–158.

Degterev, A., Boyce, M., Yuan, J., 2003. A decade of caspases. Oncogene 22, 8543–8567.

Del Valle, L., Schwartzman, R.J., Alexander, G., 2009. Spinal cord histopathological alterations in a patient with longstanding complex regional pain syndrome. Brain. Behav. Immun. 23, 85–91.

Deval, E., Gasull, X., Noël, J., Salinas, M., Baron, A., Diochot, S., Lingueglia, E., 2010. Acid-Sensing Ion Channels (ASICs): Pharmacology and implication in pain. Pharmacol. Ther. 128, 549–558.

Devonshire, I.M., Grandy, T.H., Dommett, E.J., Greenfield, S.A., 2010. Effects of urethane anaesthesia on sensory processing in the rat barrel cortex revealed by combined optical imaging and electrophysiology. Eur. J. Neurosci. 32, 786–797.

Devor, M., Schonfeld, D., Seltzer, Z., Wall, P.D., 1979. Two modes of cutaneous reinnervation following peripheral nerve injury. J. Comp. Neurol. 185, 211–220.

Devor, M., Wall, P.D., 1981. Plasticity in the spinal cord sensory map following peripheral nerve injury in rats. J. Neurosci. 1, 679–684.

Devor, M., 1991. Neuropathic pain and injured nerve: peripheral mechanisms. Br. Med. Bull. 47, 619–630.

De Vry, J., Kuhl, E., Franken-Kunkel, P., Eckel, G., 2004. Pharmacological characterization of the chronic constriction injury model of neuropathic pain. Eur. J. Pharmacol. 491, 137–148.

Dhaka, A., Murray, A.N., Mathur, J., Earley, T.J., Petrus, M.J., Patapoutian, A., 2007. TRPM8 Is Required for Cold Sensation in Mice. Neuron 54, 371–378.

Dhib-Jalbut, S., 2003. Glatiramer acetate (Copaxone®) therapy for multiple sclerosis. Pharmacol. Ther. 98, 245–255.

Dib-Hajj, S.D., Yang, Y., Waxman, S.G., 2008. Genetics and Molecular Pathophysiology of Na< sub> v</sub> 1.7-Related Pain Syndromes. Adv. Genet. 63, 85–110.

Djouhri, L., Lawson, S.N., 2004. Aβ-fiber nociceptive primary afferent neurons: a review of incidence and properties in relation to other afferent A-fiber neurons in mammals. Brain Res. Rev., Nociception in Vertebrates 46, 131–145.

Dong, Y., Benveniste, E.N., 2001. Immune function of astrocytes. Glia 36, 180–190.

Dougherty, K.J., Sawchuk, M.A., Hochman, S., 2009. Phenotypic diversity and expression of GABAergic inhibitory interneurons during postnatal development in lumbar spinal cord of glutamic acid decarboxylase 67–green fluorescent protein mice. Neuroscience 163, 909–919.

Drew, L.J., Rohrer, D.K., Price, M.P., Blaver, K.E., Cockayne, D.A., Cesare, P., Wood, J.N., 2004. Acid-sensing ion channels ASIC2 and ASIC3 do not contribute to mechanically activated currents in mammalian sensory neurones. J. Physiol. 556, 691–710.

Driessler, F., Venstrom, K., Sabat, R., Asadullah, K., Schottelius, A.J., 2004. Molecular mechanisms of interleukin-10-mediated inhibition of NF-κB activity: a role for p50. Clin. Exp. Immunol. 135, 64–73.

Duan, S., Anderson, C.M., Keung, E.C., Chen, Y., Chen, Y., Swanson, R.A., 2003. P2X7 receptor-mediated release of excitatory amino acids from astrocytes. J. Neurosci. 23, 1320–1328.

Dubner, R., Bennett, G.J., 1983. Spinal and Trigeminal Mechanisms of Nociception. Annu. Rev. Neurosci. 6, 381–418.

Dubner, R., Kenshalo, D.R., Maixner, W., Bushnell, M.C., Oliveras, J.L., 1989. The correlation of monkey medullary dorsal horn neuronal activity and the perceived intensity of noxious heat stimuli. J. Neurophysiol. 62, 450–457.

Dumontier, C., Gilbert, A., 1990. Traumatic brachial plexus palsy in children. Ann. Chir. Main Memb. Supér. 9, 351–357.

Durafourt, B.A., Moore, C.S., Zammit, D.A., Johnson, T.A., Zaguia, F., Guiot, M.-C., Bar-Or, A., Antel, J.P., 2012. Comparison of polarization properties of human adult microglia and blood-derived macrophages. Glia 60, 717–727.

Dworkin, R.H., Backonja, M., Rowbotham, M.C., Allen, R.R., Argoff, C.R., Bennett, G.J., Bushnell, M.C., Farrar, J.T., Galer, B.S., Haythornthwaite, J.A., others, 2003. Advances in neuropathic pain: diagnosis, mechanisms, and treatment recommendations. Arch. Neurol. 60, 1524–1534.

Dworkin, R.H., O'Connor, A.B., Backonja, M., Farrar, J.T., Finnerup, N.B., Jensen, T.S., Kalso, E.A., Loeser, J.D., Miaskowski, C., Nurmikko, T.J., Portenoy, R.K., Rice, A.S.C., Stacey, B.R., Treede, R.-D., Turk, D.C., Wallace, M.S., 2007. Pharmacologic

management of neuropathic pain: Evidence-based recommendations. PAIN 132, 237–251.

Echeverry, S., Shi, X.Q., Rivest, S., Zhang, J., 2011. Peripheral Nerve Injury Alters Blood–Spinal Cord Barrier Functional and Molecular Integrity through a Selective Inflammatory Pathway. J. Neurosci. 31, 10819–10828.

Eijkelkamp, N., Linley, J.E., Torres, J.M., Bee, L., Dickenson, A.H., Gringhuis, M., Minett, M.S., Hong, G.S., Lee, E., Oh, U., Ishikawa, Y., Zwartkuis, F.J., Cox, J.J., Wood, J.N., 2013. A role for Piezo2 in EPAC1-dependent mechanical allodynia. Nat. Commun. 4, 1682.

Eippert, F., Finsterbusch, J., Bingel, U., Büchel, C., 2009. Direct Evidence for Spinal Cord Involvement in Placebo Analgesia. Science 326, 404–404.

Ekholm, J., 1967. Postnatal changes in cutaneous reflexes and in the discharge pattern of cutaneous and articular sense organs. A morphological and physiological study in the cat. Acta Physiol. Scand. Suppl. 297, 1–130.

Elahi, S., Ertelt, J.M., Kinder, J.M., Jiang, T.T., Zhang, X., Xin, L., Chaturvedi, V., Strong, B.S., Qualls, J.E., Steinbrecher, K.A., Kalfa, T.A., Shaaban, A.F., Way, S.S., 2013. Immunosuppressive CD71+ erythroid cells compromise neonatal host defence against infection. Nature 504, 158–162.

El-Gammal, T.A., El-Sayed, A., Kotb, M.M., 2003. Surgical treatment of brachial plexus traction injuries in children, excluding obstetric palsy. Microsurgery 23, 14–17.

Engelhardt, B., 2006. Molecular mechanisms involved in T cell migration across the blood–brain barrier. J. Neural Transm. 113, 477–485.

Engelhardt, B., Ransohoff, R.M., 2005. The ins and outs of T-lymphocyte trafficking to the CNS: anatomical sites and molecular mechanisms. Trends Immunol. 26, 485–495.

Erichsen, H.K., Blackburn-Munro, G., 2002. Pharmacological characterisation of the spared nerve injury model of neuropathic pain. Pain 98, 151–161.

Estacion, M., Dib-Hajj, S.D., Benke, P.J., te Morsche, R.H., Eastman, E.M., Macala, L.J., Drenth, J.P.H., Waxman, S.G., 2008. NaV1. 7 gain-of-function mutations as a continuum: A1632E displays physiological changes associated with erythromelalgia and paroxysmal extreme pain disorder mutations and produces symptoms of both disorders. J. Neurosci. 28, 11079–11088.

Fabrizi, L., Slater, R., Worley, A., Meek, J., Boyd, S., Olhede, S., Fitzgerald, M., 2011. A shift in sensory processing that enables the developing human brain to discriminate touch from pain. Curr. Biol. 21, 1552–1558.

Falcon, M., Guendellman, D., Stolberg, A., Frenk, H., Urca, G., 1996. Development of thermal nociception in rats. PAIN 67, 203–208.

Fang, X., Djouhri, L., McMullan, S., Berry, C., Waxman, S.G., Okuse, K., Lawson, S.N., 2006. Intense Isolectin-B4 Binding in Rat Dorsal Root Ganglion Neurons Distinguishes C-Fiber Nociceptors with Broad Action Potentials and High Nav1.9 Expression. J. Neurosci. 26, 7281–7292.

Ferber, I.A., Lee, H.-J., Zonin, F., Heath, V., Mui, A., Arai, N., O'Garra, A., 1999. GATA-3 significantly downregulates IFN-γ production from developing Th1 cells in addition to inducing IL-4 and IL-5 levels. Clin. Immunol. 91, 134–144.

Ferrini, F., De Koninck, Y., 2013. Microglia control neuronal network excitability via BDNF signalling. Neural Plast. 2013.

Fields, H.L., Basbaum, A.I., Clanton, C.H., Anderson, S.D., 1977. Nucleus raphe magnus inhibition of spinal cord dorsal horn neurons. Brain Res. 126, 441–453.

Fields, H.L., Heinricher, M.M., 1985. Anatomy and Physiology of a Nociceptive Modulatory System. Philos. Trans. R. Soc. Lond. B Biol. Sci. 308, 361–374.

Fields, H.L., Heinricher, M.M., Mason, P., 1991. Neurotransmitters in Nociceptive Modulatory Circuits. Annu. Rev. Neurosci. 14, 219–245.

Fields, H.L., Malick, A., Burstein, R., 1995. Dorsal horn projection targets of ON and OFF cells in the rostral ventromedial medulla. J. Neurophysiol. 74, 1742–1759.

Fillingim, R.B., Ness, T.J., 2000. Sex-related hormonal influences on pain and analgesic responses. Neurosci. Biobehav. Rev. 24, 485–501.

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

Fiorentino, D.F., Bond, M.W., Mosmann, T.R., 1989. Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. J. Exp. Med. 170, 2081–2095.

Fiorentino, D.F., Zlotnik, A., Vieira, P., Mosmann, T.R., Howard, M., Moore, K.W., O'Garra, A., 1991. IL-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells. J. Immunol. 146, 3444–3451.

Fitzgerald, M., Swett, J., 1983. The termination pattern of sciatic nerve afferents in the substantia gelatinosa of neonatal rats. Neurosci. Lett. 43, 149–154.

Fitzgerald, M., 1985a. The post-natal development of cutaneous afferent fibre input and receptive field organization in the rat dorsal horn. J. Physiol. 364, 1–18.

Fitzgerald, M., 1985b. The sprouting of saphenous nerve terminals in the spinal cord following early postnatal sciatic nerve section in the rat. J. Comp. Neurol. 240, 407–413.

Fitzgerald, M., Koltzenburg, M., 1986. The functional development of descending inhibitory pathways in the dorsolateral funiculus of the newborn rat spinal cord. Dev. Brain Res. 24, 261–270.

Fitzgerald, M., 1987a. Prenatal growth of fine-diameter primary afferents into the rat spinal cord: A transganglionic tracer study. J. Comp. Neurol. 261, 98–104.

Fitzgerald, M., 1987b. Cutaneous primary afferent properties in the hind limb of the neonatal rat. J. Physiol. 383, 79–92.

Fitzgerald, M., 1987c. Spontaneous and evoked activity of fetal primary afferents in vivo.

Fitzgerald, M., 1988. The development of activity evoked by fine diameter cutaneous fibres in the spinal cord of the newborn rat. Neurosci. Lett. 86, 161–166.

Fitzgerald, M., Millard, C., Macintosh, N., 1988. Hyperalgesia in premature infants. The Lancet 331, 292.

Fitzgerald, M., Shortland, P., 1988. The effect of neonatal peripheral nerve section on the somadendritic growth of sensory projection cells in the rat spinal cord. Dev. Brain Res. 42, 129–136.

Fitzgerald, M., Millard, C., McIntosh, N., 1989. Cutaneous hypersensitivity following peripheral tissue damage in newborn infants and its reversal with topical anaesthesia. Pain 39, 31–36.

Fitzgerald, M., Woolf, C.J., Shortland, P., 1990. Collateral sprouting of the central terminals of cutaneous primary afferent neurons in the rat spinal cord: Pattern, morphology, and influence of targets. J. Comp. Neurol. 300, 370–385.

Fitzgerald, M., 1991. A physiological study of the prenatal development of cutaneous sensory inputs to dorsal horn cells in the rat. J. Physiol. 432, 473–482.

Fitzgerald, M., Reynolds, M.L., Benowitz, L.I., 1991. GAP-43 expression in the developing rat lumbar spinal cord. Neuroscience 41, 187–199.

Fitzgerald, M., Butcher, T., Shortland, P., 1994. Developmental changes in the laminar termination of A fibre cutaneous sensory afferents in the rat spinal cord dorsal horn. J. Comp. Neurol. 348, 225–233.

Fitzgerald, M., Jennings, E., 1999. The postnatal development of spinal sensory processing. Proc. Natl. Acad. Sci. 96, 7719–7722.

Fitzgerald, M., 2005. The development of nociceptive circuits. Nat. Rev. Neurosci. 6, 507–520.

Fitzgerald, M., Walker, S.M., 2009. Infant pain management: a developmental neurobiological approach. Nat. Clin. Pract. Neurol. 5, 35–50.

Flatters, S.J.L., Bennett, G.J., 2004. Ethosuximide reverses paclitaxel- and vincristine-induced painful peripheral neuropathy. Pain 109, 150–161.

Forsthuber, T., Yip, H.C., Lehmann, P.V., 1996. Induction of TH1 and TH2 Immunity in Neonatal Mice. Science 271, 1728–1730.

Frei, K., Lins, H., Schwerdel, C., Fontana, A., 1994. Antigen presentation in the central nervous system. The inhibitory effect of IL-10 on MHC class II expression and production of cytokines depends on the inducing signals and the type of cell analyzed. J. Immunol. 152, 2720–2728.

Fukuoka, T., Kondo, E., Dai, Y., Hashimoto, N., Noguchi, K., 2001. Brain-Derived Neurotrophic Factor Increases in the Uninjured Dorsal Root Ganglion Neurons in Selective Spinal Nerve Ligation Model. J. Neurosci. 21, 4891–4900.

Gadani, S.P., Cronk, J.C., Norris, G.T., Kipnis, J., 2012. IL-4 in the Brain: A Cytokine To Remember. J. Immunol. 189, 4213–4219.

Gammon, G., Dunn, K., Shastri, N., Oki, A., Wilbur, S., Sercarz, E.E., 1986. Neonatal T-cell tolerance to minimal immunogenic peptides is caused by clonal inactivation.

Gao, X., Kim, H.K., Chung, J.M., Chung, K., 2005. Enhancement of NMDA receptor phosphorylation of the spinal dorsal horn and nucleus gracilis neurons in neuropathic rats. Pain 116, 62–72.

Gao, Y.-J., Ji, R.-R., 2010. Chemokines, neuronal–glial interactions, and central processing of neuropathic pain. Pharmacol. Ther. 126, 56–68.

Gao, Y.-J., Xu, Z.-Z., Liu, Y.-C., Wen, Y.-R., Decosterd, I., Ji, R.-R., 2010a. The c-Jun N-terminal kinase 1 (JNK1) in spinal astrocytes is required for the maintenance of bilateral mechanical allodynia under a persistent inflammatory pain condition. Pain 148, 309–319.

Gao, Y.-J., Zhang, L., Ji, R.-R., 2010b. Spinal injection of TNF-α-activated astrocytes produces persistent pain symptom mechanical allodynia by releasing monocyte chemoattractant protein-1. Glia 58, 1871–1880.

Gao, Y.-J., Zhang, L., Samad, O.A., Suter, M.R., Yasuhiko, K., Xu, Z.-Z., Park, J.-Y., Lind, A.-L., Ma, Q., Ji, R.-R., 2009. JNK-induced MCP-1 production in spinal cord astrocytes contributes to central sensitization and neuropathic pain. J. Neurosci. 29, 4096–4108.

Gauriau, C., Bernard, J.-F., 2002. Pain pathways and parabrachial circuits in the rat. Exp. Physiol. null, 251–258.

Gauriau, C., Bernard, J.-F., 2004. A comparative reappraisal of projections from the superficial laminae of the dorsal horn in the rat: The forebrain. J. Comp. Neurol. 468, 24–56.

Gavrieli, Y., Sherman, Y., Ben-Sasson, S.A., 1992. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. J. Cell Biol. 119, 493–501.

Gehrmann, J., Banati, R. b., Wiessnert, C., Hossmann, K.-A., Kreutzberg, G. w., 1995. Reactive microglia in cerebral ischaemia: an early mediator of tissue damage? Neuropathol. Appl. Neurobiol. 21, 277–289.

Géranton, S.M., Jiménez-Díaz, L., Torsney, C., Tochiki, K.K., Stuart, S.A., Leith, J.L., Lumb, B.M., Hunt, S.P., 2009. A Rapamycin-Sensitive Signaling Pathway Is Essential for the Full Expression of Persistent Pain States. J. Neurosci. 29, 15017–15027.

Gillespie, P.G., Walker, R.G., 2001. Molecular basis of mechanosensory transduction. Nature 413, 194–202.

Gold, M.S., Gebhart, G.F., 2010. Nociceptor sensitization in pain pathogenesis. Nat. Med. 16, 1248–1257.

Gonzalez, D.L., Fuchs, J.L., Droge, M.H., 1993. Distribution of NMDA receptor binding in developing mouse spinal cord. Neurosci. Lett. 151, 134–137.

González, Y., Angulo, A.W., 1932. The prenatal growth of the albino rat. Anat. Rec. 52, 117–138.

Gordon, S., 2003. Alternative activation of macrophages. Nat. Rev. Immunol. 3, 23–35.

Gordon, S., Taylor, P.R., 2005. Monocyte and macrophage heterogeneity. Nat. Rev. Immunol. 5, 953–964.

Gosselin, R.D., Varela, C., Banisadr, G., Mechighel, P., Rostene, W., Kitabgi, P., Melik-Parsadaniantz, S., 2005. Constitutive expression of CCR2 chemokine receptor and inhibition by MCP-1/CCL2 of GABA-induced currents in spinal cord neurones. J. Neurochem. 95, 1023–1034.

Grace, P.M., Rolan, P.E., Hutchinson, M.R., 2011. Peripheral immune contributions to the maintenance of central glial activation underlying neuropathic pain. Brain. Behav. Immun. 25, 1322–1332.

Granmo, M., Petersson, P., Schouenborg, J., 2008. Action-based body maps in the spinal cord emerge from a transitory floating organization. J. Neurosci. 28, 5494–5503.

Green, G.M., Gibb, A.J., 2001. Characterization of the single-channel properties of NMDA receptors in laminae I and II of the dorsal horn of neonatal rat spinal cord. Eur. J. Neurosci. 14, 1590–1602.

Greenspan, J.D., Craft, R.M., LeResche, L., Arendt-Nielsen, L., Berkley, K.J., Fillingim, R.B., Gold, M.S., Holdcroft, A., Lautenbacher, S., Mayer, E.A., others, 2007. Studying sex and gender differences in pain and analgesia: a consensus report. Pain 132, S26–S45.

Griffin, R.S., Costigan, M., Brenner, G.J., Ma, C.H.E., Scholz, J., Moss, A., Allchorne, A.J., Stahl, G.L., Woolf, C.J., 2007. Complement induction in spinal cord microglia results in anaphylatoxin C5a-mediated pain hypersensitivity. J. Neurosci. 27, 8699–8708.

Gruber-Schoffnegger, D., Drdla-Schutting, R., Hönigsperger, C., Wunderbaldinger, G., Gassner, M., Sandkühler, J., 2013. Induction of thermal hyperalgesia and synaptic long-term potentiation in the spinal cord lamina I by TNF-α and IL-1β is mediated by glial cells. J. Neurosci. 33, 6540–6551.

Grunau, R.E., Holsti, L., Peters, J.W., 2006. Long-term consequences of pain in human neonates, in: Seminars in Fetal and Neonatal Medicine. Elsevier, pp. 268–275.

Güler, A.D., Lee, H., Iida, T., Shimizu, I., Tominaga, M., Caterina, M., 2002. Heat-evoked activation of the ion channel, TRPV4. J. Neurosci. 22, 6408–6414.

Gu, X., Spitzer, N.C., 1995. Distinct aspects of neuronal differentiation encoded by frequency of spontaneous Ca2+ transients. Nature 375, 784–787.

Haines, C.J., Giffon, T.D., Lu, L.-S., Lu, X., Tessier-Lavigne, M., Ross, D.T., Lewis, D.B., 2009. Human CD4+ T cell recent thymic emigrants are identified by protein tyrosine kinase 7 and have reduced immune function. J. Exp. Med. 206, 275–285.

Hall, G.C., Carroll, D., Parry, D., McQuay, H.J., 2006. Epidemiology and treatment of neuropathic pain: The UK primary care perspective. PAIN 122, 156–162.

Hamburger, V., 1958. Regression versus peripheral control of differentiation in motor hypoplasia. Am. J. Anat. 102, 365–409.

Hamburger, V., Oppenheim, R.W., 1990. Naturally Occurring Neuronal Death in Vertebrates, in: Neuroembryology. Birkhäuser Boston, pp. 126–142.

Hamilton, N.B., Attwell, D., 2010. Do astrocytes really exocytose neurotransmitters? Nat. Rev. Neurosci. 11, 227–238.

Ham, H.-J. van den, Jager, W. de, Bijlsma, J.W.J., Prakken, B.J., Boer, R.J. de, 2009. Differential cytokine profiles in juvenile idiopathic arthritis subtypes revealed by cluster analysis. Rheumatology 48, 899–905.

Handwerker, H.O., Anton, F., Reeh, P.W., 1987. Discharge patterns of afferent cutaneous nerve fibers from the rat's tail during prolonged noxious mechanical stimulation. Exp. Brain Res. 65, 493–504.

Handwerker, H.O., Iggo, A., Zimmermann, M., 1975. Segmental and supraspinal actions on dorsal horn neurons responding to noxious and non-noxious skin stimuli. PAIN 1, 147–165.

Handwerker, H.O., Kobal, G., 1993. Psychophysiology of experimentally induced pain. Physiol. Rev. 73, 639–671.

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

Han, L., Ma, C., Liu, Q., Weng, H.-J., Cui, Y., Tang, Z., Kim, Y., Nie, H., Qu, L., Patel, K.N., Li, Z., McNeil, B., He, S., Guan, Y., Xiao, B., Lamotte, R.H., Dong, X., 2013. A subpopulation of nociceptors specifically linked to itch. Nat. Neurosci. 16, 174–182.

Hao, S., Mata, M., Glorioso, J.C., Fink, D.J., 2007. Gene transfer to interfere with TNFα signaling in neuropathic pain. Gene Ther. 14, 1010–1016.

Hao, S., Mata, M., Glorioso, J.C., Fink, D.J., 2006. HSV-mediated expression of interleukin-4 in dorsal root ganglion neurons reduces neuropathic pain. Mol Pain 2.

Hargreaves, K., Dubner, R., Brown, F., Flores, C., Joris, J., 1988. A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. Pain 32, 77–88.

Harper, A.A., Lawson, S.N., 1985. Electrical properties of rat dorsal root ganglion neurones with different peripheral nerve conduction velocities. J. Physiol. 359, 47–63.

Harris, A.J., McCaig, C.D., 1984. Motoneuron death and motor unit size during embryonic development of the rat. J. Neurosci. 4, 13–24.

Harry, G.J., 2013. Microglia during development and aging. Pharmacol. Ther. 139, 313–326.

Hartmann, B., Ahmadi, S., Heppenstall, P.A., Lewin, G.R., Schott, C., Borchardt, T., Seeburg, P.H., Zeilhofer, H.U., Sprengel, R., Kuner, R., 2004. The AMPA Receptor Subunits GluR-A and GluR-B Reciprocally Modulate Spinal Synaptic Plasticity and Inflammatory Pain. Neuron 44, 637–650.

Haseneder, R., Kurz, J., Dodt, H.-U.U., Kochs, E., Zieglgnsberger, W., Scheller, M., Rammes, G., Hapfelmeier, G., 2004. Isoflurane Reduces Glutamatergic Transmission in Neurons in the Spinal Cord Superficial Dorsal Horn: Evidence for a Presynaptic Site of an Analgesic Action: Anesth. Analg. 1718–1723.

Hathway, G.J., Vega-Avelaira, D., Fitzgerald, M., 2012. A critical period in the supraspinal control of pain: opioid-dependent changes in brainstem rostroventral medulla function in preadolescence. PAIN® 153, 775–783.

Hathway, G., Harrop, E., Baccei, M., Walker, S., Moss, A., Fitzgerald, M., 2006. A postnatal switch in GABAergic control of spinal cutaneous reflexes. Eur. J. Neurosci. 23, 112–118.

Hathway, G.J., Vega-Avelaira, D., Moss, A., Ingram, R., Fitzgerald, M., 2009. Brief, low frequency stimulation of rat peripheral C-fibres evokes prolonged microglial-induced central sensitization in adults but not in neonates. PAINÂ® 144, 110–118.

Hedo, G., Laird, J.M.A., Lopez-Garcia, J.A., 1999. Time-course of spinal sensitization following carrageenan-induced inflammation in the young rat: a comparative electrophysiological and behavioural study in vitro and in vivo. Neuroscience 92, 309–318.

Heinricher, M.M., Barbaro, N.M., Fields, H.L., 1989. Putative Nociceptive Modulating Neurons in the Rostral Ventromedial Medulla of the Rat: Firing of On- and Off-Cells Is Related to Nociceptive Responsiveness. Somatosens. Mot. Res. 6, 427–439.

Hickey, W.F., 2001. Basic principles of immunological surveillance of the normal central nervous system. Glia 36, 118–124.

Hide, I., Tanaka, M., Inoue, A., Nakajima, K., Kohsaka, S., Inoue, K., Nakata, Y., 2000. Extracellular ATP Triggers Tumor Necrosis Factor-α Release from Rat Microglia. J. Neurochem. 75, 965–972.

Himes, B. t., Tessler, A., 1989. Death of some dorsal root ganglion neurons and plasticity of others following sciatic nerve section in adult and neonatal rats. J. Comp. Neurol. 284, 215–230.

Hjerling-Leffler, J., AlQatari, M., Ernfors, P., Koltzenburg, M., 2007. Emergence of Functional Sensory Subtypes as Defined by Transient Receptor Potential Channel Expression. J. Neurosci. 27, 2435–2443.

Hoftun, G.B., Romundstad, P.R., Zwart, J.-A., Rygg, M., 2011. Chronic idiopathic pain in adolescence – high prevalence and disability: The young HUNT study 2008. PAIN® 152, 2259–2266.

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

Hori, Y., Kanda, K., 1994. Developmental alterations in NMDA receptor-mediated [Ca2+]i elevation in substantia gelatinosa neurons of neonatal rat spinal cord. Dev. Brain Res. 80, 141–148.

Hossaini, M., Goos, J.A.C., Kohli, S.K., Holstege, J.C., 2012. Distribution of Glycine/GABA Neurons in the Ventromedial Medulla with Descending Spinal Projections and Evidence for an Ascending Glycine/GABA Projection. PLoS ONE 7, e35293.

Howard, R.F., 2003. Current status of pain management in children. Jama 290, 2464–2469.

Howard, R.F., Walker, S.M., Michael Mota, P., Fitzgerald, M., 2005. The ontogeny of neuropathic pain: Postnatal onset of mechanical allodynia in rat spared nerve injury (SNI) and chronic constriction injury (CCI) models. Pain 115, 382–389.

Howard, R.F., Wiener, S., Walker, S.M., 2014. Neuropathic pain in children. Arch. Dis. Child. 99, 84–89.

Huang, S., Gu, Y., Quinlan, E.M., Kirkwood, A., 2010. A Refractory Period for Rejuvenating GABAergic Synaptic Transmission and Ocular Dominance Plasticity with Dark Exposure. J. Neurosci. 30, 16636–16642.

Hu, H.-J., Glauner, K.S., Gereau, R.W., 2003. ERK Integrates PKA and PKC Signaling in Superficial Dorsal Horn Neurons. I. Modulation of A-Type K+ Currents. J. Neurophysiol. 90, 1671–1679.

Hunt, S.P., Rossi, J., 1985. Peptide- and Non-Peptide-Containing Unmyelinated Primary Afferents: The Parallel Processing of Nociceptive Information. Philos. Trans. R. Soc. Lond. B Biol. Sci. 308, 283–289.

Hunt, S., Koltzenburg, M. (Eds.), 2005. The Neurobiology of Pain: (Molecular and Cellular Neurobiology). Molecular and Cellular Neurobiology Series.

Hu, P., Bembrick, A.L., Keay, K.A., McLachlan, E.M., 2007. Immune cell involvement in dorsal root ganglia and spinal cord after chronic constriction or transection of the rat sciatic nerve. Brain. Behav. Immun. 21, 599–616.

Hu, S., Chao, C.C., Ehrlich, L.C., Sheng, W.S., Sutton, R.L., Rockswold, G.L., Peterson, P.K., 1999. Inhibition of microglial cell RANTES production by IL-10 and TGF-beta. J. Leukoc. Biol. 65, 815–821.

Hwang, R.W., Bae, D.S., Waters, P.M., 2008. Brachial Plexus Palsy Following Proximal Humerus Fracture in Patients Who Are Skeletally Immature: J. Orthop. Trauma 22, 286–290.

Hylden, J.L., Nahin, R.L., Traub, R.J., Dubner, R., 1989. Expansion of receptive fields of spinal lamina I projection neurons in rats with unilateral adjuvant-induced inflammation: the contribution of dorsal horn mechanisms. Pain 37, 229–243.

Iannetti, G.D., Mouraux, A., 2010. From the neuromatrix to the pain matrix (and back). Exp. Brain Res. 205, 1–12.

Inagaki-Ohara, K., Hanada, T., Yoshimura, A., 2003. Negative regulation of cytokine signaling and inflammatory diseases. Curr. Opin. Pharmacol. 3, 435–442.

Isaev, D., Gerber, G., Park, S.K., Chung, J.M., Randic, M., 2000. Facilitation of NMDA-induced currents and Ca2+ transients in the rat substantia gelatinosa neurons after ligation of L5-L6 spinal nerves. Neuroreport 11, 4055–4061.

Iwata, H., Takasusuki, T., Yamaguchi, S., Hori, Y., 2007. NMDA receptor 2B subunit-mediated synaptic transmission in the superficial dorsal horn of peripheral nerve-injured neuropathic mice. Brain Res. 1135, 92–101.

Jackman, A., Fitzgerald, M., 2000. Development of peripheral hindlimb and central spinal cord innervation by subpopulations of dorsal root ganglion cells in the embryonic rat. J. Comp. Neurol. 418, 281–298.

Jaggi, A.S., Jain, V., Singh, N., 2011. Animal models of neuropathic pain. Fundam. Clin. Pharmacol. 25, 1–28.

Jakowec, M.W., Fox, A.J., Martin, L.J., Kalb, R.G., 1995. Quantitative and qualitative changes in AMPA receptor expression during spinal cord development. Neuroscience 67, 893–907.

Jankowska, E., Lindström, S., 1972. Morphology of interneurones mediating Ia reciprocal inhibition of motoneurones in the spinal cord of the cat. J. Physiol. 226, 805–823.

Jennings, E., Fitzgerald, M., 1996. C-fos can be induced in the neonatal rat spinal cord by both noxious and innocuous peripheral stimulation. Pain 68, 301–306.

Jennings, E., Fitzgerald, M., 1998. Postnatal changes in responses of rat dorsal horn cells to afferent stimulation: a fibre-induced sensitization. J. Physiol. 509, 859–868.

Jin, S.-X., Zhuang, Z.-Y., Woolf, C.J., Ji, R.-R., 2003. p38 mitogen-activated protein kinase is activated after a spinal nerve ligation in spinal cord microglia and dorsal root ganglion neurons and contributes to the generation of neuropathic pain. J. Neurosci. 23, 4017–4022.

Ji, R.-R., Baba, H., Brenner, G.J., Woolf, C.J., 1999. Nociceptive-specific activation of ERK in spinal neurons contributes to pain hypersensitivity. Nat. Neurosci. 2, 1114–1119.

Ji, R.-R., Samad, T.A., Jin, S.-X., Schmoll, R., Woolf, C.J., 2002. p38 MAPK Activation by NGF in Primary Sensory Neurons after Inflammation Increases TRPV1 Levels and Maintains Heat Hyperalgesia. Neuron 36, 57–68.

Ji, R.-R., Kohno, T., Moore, K.A., Woolf, C.J., 2003. Central sensitization and LTP: do pain and memory share similar mechanisms? Trends Neurosci. 26, 696–705.

Ji, R.-R., Suter, M.R., 2007. p38 MAPK, microglial signaling, and neuropathic pain. Mol Pain 3, 1–9.

Ji, R.-R., Gereau IV, R.W., Malcangio, M., Strichartz, G.R., 2009. MAP kinase and pain. Brain Res. Rev., A Decade of Pain Research: New Approaches, New Targets 60, 135–148.

Ji, R.-R., Berta, T., Nedergaard, M., 2013. Glia and pain: Is chronic pain a gliopathy? PAIN®, Biennial Review of Pain 154, Supplement 1, S10–S28.

Jones, G.T., 2011. Pain in children – a call for more longitudinal research. PAIN® 152, 2202–2203.

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

Jr, W.D.W., Coggeshall, R.E., 2004. Sensory Mechanisms of the Spinal Cord: Volume 1 Primary Afferent Neurons and the Spinal Dorsal Horn. Springer Science & Business Media.

Julius, D., Basbaum, A.I., 2001. Molecular mechanisms of nociception. Nature 413, 203–210.

Jupelli, M., Selby, D.M., Guentzel, M.N., Chambers, J.P., Forsthuber, T.G., Zhong, G., Murthy, A.K., Arulanandam, B.P., 2010. The Contribution of Interleukin-12/Interferon-γ Axis in Protection Against Neonatal Pulmonary Chlamydia muridarum Challenge. J. Interferon Cytokine Res. 30, 407–415.

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

Kajander, K.C., Sahara, Y., Iadarola, M.J., Bennett, G.J., 1990. Dynorphin increases in the dorsal spinal cord in rats with a painful peripheral neuropathy. Peptides 11, 719–728.

Kajander, K.C., Wakisaka, S., Bennett, G.J., 1992. Spontaneous discharge originates in the dorsal root ganglion at the onset of a painful peripheral neuropathy in the rat. Neurosci. Lett. 138, 225–228.

Kalyuzhny, A.E., Wessendorf, M.W., 1998. Relationship of μ- and δ-opioid receptors to GABAergic neurons in the central nervous system, including antinociceptive brainstem circuits. J. Comp. Neurol. 392, 528–547.

Kaplan, D.R., Martin-Zanca, D., Parada, L.F., 1991. Tyrosine phosphorylation and tyrosine kinase activity of the trk proto-oncogene product induced by NGF. Nature 350, 158–160.

Kashihara, Y., Kuno, M., Miyata, Y., 1987. Cell death of axotomized motoneurones in neonatal rats, and its prevention by peripheral reinnervation. J. Physiol. 386, 135–148.

Karashima, Y., Talavera, K., Everaerts, W., Janssens, A., Kwan, K.Y., Vennekens, R., Nilius, B., Voets, T., 2009. TRPA1 acts as a cold sensor in vitro and in vivo. Proc. Natl. Acad. Sci. 106, 1273–1278.

Kato, G., Yasaka, T., Katafuchi, T., Furue, H., Mizuno, M., Iwamoto, Y., Yoshimura, M., 2006. Direct GABAergic and Glycinergic Inhibition of the Substantia Gelatinosa from the Rostral Ventromedial Medulla Revealed by In Vivo Patch-Clamp Analysis in Rats. J. Neurosci. 26, 1787–1794.

Katsura, H., Obata, K., Mizushima, T., Sakurai, J., Kobayashi, K., Yamanaka, H., Dai, Y., Fukuoka, T., Sakagami, M., Noguchi, K., 2006. Activation of Src-family kinases in spinal microglia contributes to mechanical hypersensitivity after nerve injury. J. Neurosci. 26, 8680–8690.

Kawasaki, Y., Zhang, L., Cheng, J.-K., Ji, R.-R., 2008. Cytokine Mechanisms of Central Sensitization: Distinct and Overlapping Role of Interleukin-1β, Interleukin-6, and Tumor Necrosis Factor-α in Regulating Synaptic and Neuronal Activity in the Superficial Spinal Cord. J. Neurosci. 28, 5189–5194.

Kazansky, D.B., 2008. MHC restriction and allogeneic immune responses. J. Immunotoxicol. 5, 369–384.

Kehlet, H., Rathmell, J.P., 2010. Persistent Postsurgical Pain: The Path Forward through Better Design of Clinical Studies. Anesthesiology 112, 514–515.

Keller, A.F., Beggs, S., Salter, M.W., De Koninck, Y., 2007. Transformation of the output of spinal lamina I neurons after nerve injury and microglia stimulation underlying neuropathic pain. Mol Pain 3, 27.

Keller, A.F., Coull, J.A.M., Chéry, N., Poisbeau, P., Koninck, Y.D., 2001. Region-Specific Developmental Specialization of GABA–Glycine Cosynapses in Laminas I–II of the Rat Spinal Dorsal Horn. J. Neurosci. 21, 7871–7880.

Kettenmann, H., Kirchhoff, F., Verkhratsky, A., 2013. Microglia: New Roles for the Synaptic Stripper. Neuron 77, 10–18.

Khan, S., Zhou, L., 2012. Characterization of non–length-dependent small-fiber sensory neuropathy. Muscle Nerve 45, 86–91.

Kierdorf, K., Erny, D., Goldmann, T., Sander, V., Schulz, C., Perdiguero, E.G., Wieghofer, P., Heinrich, A., Riemke, P., Hölscher, C., Müller, D.N., Luckow, B., Brocker, T., Debowski, K., Fritz, G., Opdenakker, G., Diefenbach, A., Biber, K., Heikenwalder, M., Geissmann, F., Rosenbauer, F., Prinz, M., 2013. Microglia emerge from erythromyeloid precursors via Pu.1- and Irf8-dependent pathways. Nat. Neurosci. 16, 273–280.

Kigerl, K.A., Gensel, J.C., Ankeny, D.P., Alexander, J.K., Donnelly, D.J., Popovich, P.G., 2009. Identification of Two Distinct Macrophage Subsets with Divergent Effects Causing either Neurotoxicity or Regeneration in the Injured Mouse Spinal Cord. J. Neurosci. 29, 13435–13444.

Kiguchi, N., Maeda, T., Kobayashi, Y., Fukazawa, Y., Kishioka, S., 2010. Macrophage inflammatory protein-1α mediates the development of neuropathic pain following peripheral nerve injury through interleukin-1β up-regulation. PAIN® 149, 305–315.

Kim, D., Kim, M.A., Cho, I.-H., Kim, M.S., Lee, S., Jo, E.-K., Choi, S.-Y., Park, K., Kim, J.S., Akira, S., others, 2007. A critical role of toll-like receptor 2 in nerve injury-induced spinal cord glial cell activation and pain hypersensitivity. J. Biol. Chem. 282, 14975–14983.

Kim, H.Y., Park, C.-K., Cho, I.-H., Jung, S.J., Kim, J.S., Oh, S.B., 2008. Differential Changes in TRPV1 expression after trigeminal sensory nerve injury. J. Pain 9, 280–288.

Kim, D., Figueroa, K.W., Li, K.-W., Boroujerdi, A., Yolo, T., David Luo, Z., 2009. Profiling of dynamically changed gene expression in dorsal root ganglia post peripheral nerve injury and a critical role of injury-induced glial fibrillary acetic protein in maintenance of pain behaviors. Pain 143, 114–122.

Kirschstein, T., Greffrath, W., Büsselberg, D., Treede, R.-D., 1999. Inhibition of Rapid Heat Responses in Nociceptive Primary Sensory Neurons of Rats by Vanilloid Receptor Antagonists. J. Neurophysiol. 82, 2853–2860.

Kleinschnitz, C., Hofstetter, H.H., Meuth, S.G., Braeuninger, S., Sommer, C., Stoll, G., 2006. T cell infiltration after chronic constriction injury of mouse sciatic nerve is associated with interleukin-17 expression. Exp. Neurol. 200, 480–485.

Kobayashi, K., Imagama, S., Ohgomori, T., Hirano, K., Uchimura, K., Sakamoto, K., Hirakawa, A., Takeuchi, H., Suzumura, A., Ishiguro, N., Kadomatsu, K., 2013. Minocycline selectively inhibits M1 polarization of microglia. Cell Death Dis. 4, e525.

Koch, S.C., Tochiki, K.K., Hirschberg, S., Fitzgerald, M., 2012. C-fiber activity-dependent maturation of glycinergic inhibition in the spinal dorsal horn of the postnatal rat. Proc. Natl. Acad. Sci. 109, 12201–12206.

Koch, S.C., Fitzgerald, M., 2013. Activity-dependent development of tactile and nociceptive spinal cord circuits. Ann. N. Y. Acad. Sci. 1279, 97–102.

Koch, S.C., Fitzgerald, M., 2014. The selectivity of rostroventral medulla descending control of spinal sensory inputs shifts postnatally from A fibre to C fibre evoked activity. J. Physiol. 592, 1535–1544.

Kohama, I., Ishikawa, K., Kocsis, J.D., 2000. Synaptic reorganization in the substantia gelatinosa after peripheral nerve neuroma formation: aberrant innervation of lamina II neurons by Aβ afferents. J. Neurosci. 20, 1538–1549.

Kohno, T., Moore, K.A., Baba, H., Woolf, C.J., 2003. Peripheral nerve injury alters excitatory synaptic transmission in lamina II of the rat dorsal horn. J. Physiol. 548, 131–138.

Kollmann, T.R., Crabtree, J., Rein-Weston, A., Blimkie, D., Thommai, F., Wang, X.Y., Lavoie, P.M., Furlong, J., Fortuno, E.S., Hajjar, A.M., 2009. Neonatal innate TLR-mediated responses are distinct from those of adults. J. Immunol. 183, 7150–7160.

Koltzenburg, M., 1998. Painful neuropathies. Curr. Opin. Neurol. Oct. 1998 11, 515–521.

Kopf, M., Le Gros, G., Bachmann, M., Lamers, M.C., Bluethmann, H., Köhler, G., 1993. Disruption of the murine IL-4 gene blocks Th2 cytokine responses. Nature. 362. 245-248.

Krane, E.J., Heller, L.B., 1995. The prevalence of phantom sensation and pain in pediatric amputees. J. Pain Symptom Manage. 10, 21–29.

Kristensen, A.D., Pedersen, T. a. L., Hjortdal, V.E., Jensen, T.S., Nikolajsen, L., 2010. Chronic pain in adults after thoracotomy in childhood or youth. BJA.104, 75-79.

Kuhn, R., Rajewsky, K., Muller, W., 1991. Generation and analysis of interleukin-4 deficient mice. Science 254, 707–710.

Lahdenne, P., Vähäsalo, P., Honkanen, V., 2003. Infliximab or etanercept in the treatment of children with refractory juvenile idiopathic arthritis: an open label study. Ann. Rheum. Dis. 62, 245–247.

Lai, A.Y., Dibal, C.D., Armitage, G.A., Winship, I.R., Todd, K.G., 2013. Distinct activation profiles in microglia of different ages: A systematic study in isolated embryonic to aged microglial cultures. Neuroscience 254, 185–195.

Lai, A.Y., Todd, K.G., 2008. Differential regulation of trophic and proinflammatory microglial effectors is dependent on severity of neuronal injury. Glia 56, 259–270.

Laird, J.M., Bennett, G.J., 1993. An electrophysiological study of dorsal horn neurons in the spinal cord of rats with an experimental peripheral neuropathy. J. Neurophysiol. 69, 2072–2085.

Lamotte, C., 1977. Distribution of the tract of lissauer and the dorsal root fibers in the primate spinal cord. J. Comp. Neurol. 172, 529–561.

LaMotte, R.H., Thalhammer, J.G., Torebjork, H.E., Robinson, C.J., 1982. Peripheral neural mechanisms of cutaneous hyperalgesia following mild injury by heat. J. Neurosci. 2, 765–781.

LaPrairie, J.L., Murphy, A.Z., 2007. Female rats are more vulnerable to the long-term consequences of neonatal inflammatory injury. PAIN, Pain in Women 132, Supplement 1, S124–S133.

LaPrairie, J.L., Murphy, A.Z., 2009. Neonatal Injury Alters Adult Pain Sensitivity by Increasing Opioid Tone in the Periaqueductal Gray. Front. Behav. Neurosci. 3.

Latremoliere, A., Woolf, C.J., 2009. Central sensitization: a generator of pain hypersensitivity by central neural plasticity. J. Pain 10, 895–926.

Lau, C.G., Zukin, R.S., 2007. NMDA receptor trafficking in synaptic plasticity and neuropsychiatric disorders. Nat. Rev. Neurosci. 8, 413–426.

Lavoie, P.M., Huang, Q., Jolette, E., Whalen, M., Nuyt, A.M., Audibert, F., Speert, D.P., Lacaze-Masmonteil, T., Soudeyns, H., Kollmann, T.R., 2010. Profound lack of interleukin (IL)-12/IL-23p40 in neonates born early in gestation is associated with an increased risk of sepsis. J. Infect. Dis. 202, 1754–1763.

Lawn, J.E., Cousens, S., Zupan, J., 2005. 4 million neonatal deaths: When? Where? Why? The Lancet 365, 891–900.

Lawson, S.J., Davies, H.J., Bennett, J.P., Lowrie, M.B., 1997. Evidence That Spinal Interneurons Undergo Programmed Cell Death Postnatally in the Rat. Eur. J. Neurosci. 9, 794–799.

Lawson, S.N., Waddell, P.J., 1991. Soma neurofilament immunoreactivity is related to cell size and fibre conduction velocity in rat primary sensory neurons. J. Physiol. 435, 41–63.

Lawson, S.J., Lowrie, M.B., 1998. The role of apoptosis and excitotoxicity in the death of spinal motoneurons and interneurons after neonatal nerve injury. Neuroscience 87, 337–348.

Lawson, S.N., 2002. Phenotype and function of somatic primary afferent nociceptive neurones with C-, Adelta- or Aalpha/beta-fibres. Exp. Physiol. 87, 239–244.

Ledeboer, A., Breve, J.J., Poole, S., Tilders, F.J., Van Dam, A.-M., 2000. Interleukin-10, interleukin-4, and transforming growth factor-β differentially regulate lipopolysaccharide-induced production of pro-inflammatory cytokines and nitric oxide in co-cultures of rat astroglial and microglial cells. Glia 30, 134–142.

Ledeboer, A., Sloane, E.M., Milligan, E.D., Frank, M.G., Mahony, J.H., Maier, S.F., Watkins, L.R., 2005. Minocycline attenuates mechanical allodynia and proinflammatory cytokine expression in rat models of pain facilitation. Pain 115, 71–83.

Ledeboer, A., Jekich, B.M., Sloane, E.M., Mahoney, J.H., Langer, S.J., Milligan, E.D., Martin, D., Maier, S.F., Johnson, K.W., Leinwand, L.A., 2007. Intrathecal interleukin-10 gene therapy attenuates paclitaxel-induced mechanical allodynia and proinflammatory cytokine expression in dorsal root ganglia in rats. Brain. Behav. Immun. 21, 686–698.

Lee, D.H., Chung, J.M., 1996. Neuropathic pain in neonatal rats. Neurosci. Lett. 209, 140–142.

Lee, H., Caterina, M.J., 2005. TRPV channels as thermosensory receptors in epithelial cells. Pflüg. Arch. 451, 160–167.

Leem, J.W., Park, E.S., Paik, K.S., 1995. Electrophysiological evidence for the antinociceptive effect of transcutaneous electrical stimulation on mechanically evoked responsiveness of dorsal horn neurons in neuropathic rats. Neurosci. Lett. 192, 197–200.

Lee, Sc., Liu, W., Dickson, D.W., Brosnan, C.F., Berman, J.W., 1993. Cytokine production by human fetal microglia and astrocytes. Differential induction by lipopolysaccharide and IL-1 beta. J. Immunol. 150, 2659–2667.

Leger, T., Grist, J., D'Acquisto, F., Clark, A.K., Malcangio, M., 2011. Glatiramer acetate attenuates neuropathic allodynia through modulation of adaptive immune cells. J. Neuroimmunol. 234, 19–26.

Leite, J.F., Cascio, M., 2001. Structure of Ligand-Gated Ion Channels: Critical Assessment of Biochemical Data Supports Novel Topology. Mol. Cell. Neurosci. 17, 777–792.

Leith, J.L., Koutsikou, S., Lumb, B.M., Apps, R., 2010. Spinal Processing of Noxious and Innocuous Cold Information: Differential Modulation by the Periaqueductal Gray. J. Neurosci. 30, 4933–4942.

Levy, O., 2007. Innate immunity of the newborn: basic mechanisms and clinical correlates. Nat. Rev. Immunol. 7, 379–390.

Lewis, S.E., Mannion, R.J., White, F.A., Coggeshall, R.E., Beggs, S., Costigan, M., Martin, J.L., Dillmann, W.H., Woolf, C.J., 1999. A Role for HSP27 in Sensory Neuron Survival. J. Neurosci. 19, 8945–8953.

Liao, B., Zhao, W., Beers, D.R., Henkel, J.S., Appel, S.H., 2012. Transformation from a neuroprotective to a neurotoxic microglial phenotype in a mouse model of ALS. Exp. Neurol. 237, 147–152.

Lidow, M.S., Song, Z.M., Ren, K., 2001. Long-term effects of short-lasting early local inflammatory insult. Neuroreport 12, 399–403.

Light, A.R., Perl, E.R., 1979. Spinal termination of functionally identified primary afferent neurons with slowly conducting myelinated fibers. J. Comp. Neurol. 186, 133–150.

Li, J., Xie, W., Zhang, J.-M., Baccei, M.L., 2009. Peripheral nerve injury sensitizes neonatal dorsal horn neurons to tumor necrosis factor-alpha. Mol Pain 5.

Li, J., Baccei, M.L., 2011. Pacemaker Neurons within Newborn Spinal Pain Circuits. J. Neurosci. 31, 9010–9022.

Li, J., Blankenship, M.L., Baccei, M.L., 2013. Inward-Rectifying Potassium (Kir) Channels Regulate Pacemaker Activity in Spinal Nociceptive Circuits during Early Life. J. Neurosci. 33, 3352–3362.

Li, L., Openheim, R.W., Lei, M., Houenou, L.J., 1994. Neurotrophic agents prevent motoneuron death following sciatic nerve section in the neonatal mouse. J. Neurobiol. 25, 759–766.

Li, L., Lee, H.-H., Bell, J.J., Gregg, R.K., Ellis, J.S., Gessner, A., Zaghouani, H., 2004. IL-4 utilizes an alternative receptor to drive apoptosis of Th1 cells and skews neonatal immunity toward Th2. Immunity 20, 429–440.

Lindia, J.A., Köhler, M.G., Martin, W.J., Abbadie, C., 2005. Relationship between sodium channel Na< sub> V</sub> 1.3 expression and neuropathic pain behavior in rats. Pain 117, 145–153.

Linley, J.E., Rose, K., Ooi, L., Gamper, N., 2010. Understanding inflammatory pain: ion channels contributing to acute and chronic nociception. Pflüg. Arch. - Eur. J. Physiol. 459, 657–669.

Liu, L., Rudin, M., Kozlova, E.N., 2000. Glial cell proliferation in the spinal cord after dorsal rhizotomy or sciatic nerve transection in the adult rat. Exp. Brain Res. 131, 64–73.

Lord, C.J.M., Lamb, J.R., 1996. TH2 cells in allergic inflammation: a target of immunotherapy. Clin. Exp. Allergy 26, 756–765.

Low, A.K., Ward, K., Wines, A.P., 2007. Pediatric Complex Regional Pain Syndrome: J. Pediatr. Orthop. 27, 567–572.

Lowrie, M.B., Lawson, S.J., 2000. Cell death of spinal interneurones. Prog. Neurobiol. 61, 543–555.

Lu, Y., Dong, H., Gao, Y., Gong, Y., Ren, Y., Gu, N., Zhou, S., Xia, N., Sun, Y.-Y., Ji, R.-R., others, 2013. A feed-forward spinal cord glycinergic neural circuit gates mechanical allodynia. J. Clin. Invest. 123, 4050–4062.

Ma, C., Shu, Y., Zheng, Z., Chen, Y., Yao, H., Greenquist, K.W., White, F.A., LaMotte, R.H., 2003. Similar Electrophysiological Changes in Axotomized and Neighboring Intact Dorsal Root Ganglion Neurons. J. Neurophysiol. 89, 1588–1602.

Ma, W., Behar, T., Barker, J.L., 1992. Transient expression of GABA immunoreactivity in the developing rat spinal cord. J. Comp. Neurol. 325, 271–290.

Ma, W., Quirion, R., 2002. Partial sciatic nerve ligation induces increase in the phosphorylation of extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) in astrocytes in the lumbar spinal dorsal horn and the gracile nucleus. Pain 99, 175–184.

Magerl, W., Fuchs, P.N., Meyer, R.A., Treede, R.-D., 2001. Roles of capsaicin-insensitive nociceptors in cutaneous pain and secondary hyperalgesia. Brain 124, 1754–1764.

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

Malan Jr, T.P., Mata, H.P., Porreca, F., 2002. Spinal GABAA and GABAB receptor pharmacology in a rat model of neuropathic pain. Anesthesiology 96, 1161–1167.

Malcangio, M., Bowery, N.G., Flower, R.J., Perretti, M., 1996. Effect of interleukin-1β on the release of substance P from rat isolated spinal cord. Eur. J. Pharmacol. 299, 113–118.

Malmberg, A.B., Chen, C., Tonegawa, S., Basbaum, A.I., 1997. Preserved Acute Pain and Reduced Neuropathic Pain in Mice Lacking PKCγ. Science 278, 279–283.

Mao, J., Price, D.D., Hayes, R.L., Lu, J., Mayer, D.J., Frenk, H., 1993. Intrathecal treatment with dextrorphan or ketamine potently reduces pain-related behaviors in a rat model of peripheral mononeuropathy. Brain Res. 605, 164–168.

Mao, J., Price, D.D., Phillips, L.L., Lu, J., Mayer, D.J., 1995. Increases in protein kinase C gamma immunoreactivity in the spinal cord dorsal horn of rats with painful mononeuropathy. Neurosci. Lett. 198, 75–78.

Ma, Q.-P., Woolf, C.J., 1995. Noxious stimuli induce an< i> N</i>-methyl-d-aspartate receptor-dependent hypersensitivity of the flexion withdrawal reflex to touch: implications for the treatment of mechanical allodynia. Pain 61, 383–390.

Marchand, F., Perretti, M., McMahon, S.B., 2005. Role of the Immune system in chronic pain. Nat. Rev. Neurosci. 6, 521–532.

Marchand, F., Tsantoulas, C., Singh, D., Grist, J., Clark, A.K., Bradbury, E.J., McMahon, S.B., 2009. Effects of Etanercept and Minocycline in a rat model of spinal cord injury. Eur. J. Pain 13, 673–681.

Marinelli, S., Vaughan, C.W., Schnell, S.A., Wessendorf, M.W., Christie, M.J., 2002. Rostral Ventromedial Medulla Neurons That Project to the Spinal Cord Express Multiple Opioid Receptor Phenotypes. J. Neurosci. 22, 10847–10855.

Marın-Teva, J.L., Dusart, I., Colin, C., Gervais, A., van Rooijen, N., Mallat, M., 2004. Microglia promote the death of developing Purkinje cells. Neuron 41, 535–547.

Marsh, D., Dickenson, A., Hatch, D., Fitzgerald, M., 1999. Epidural opioid analgesia in infant rats II: responses to carrageenan and capsaicin. Pain 82, 33–38.

Matsumoto, M., Xie, W., Ma, L., Ueda, H., 2008. Pharmacological switch in Abeta-fiber stimulation-induced spinal transmission in mice with partial sciatic nerve injury. Mol Pain 4, 8069–4.

Mayer, D.J., Wolfle, T.L., Akil, H., Carder, B., Liebeskind, J.C., 1971. Analgesia from Electrical Stimulation in the Brainstem of the Rat. Science 174, 1351–1354.

Mayer, M.L., Westbrook, G.L., Guthrie, P.B., 1984. Voltage-dependent block by Mg2+ of NMDA responses in spinal cord neurones.

Maynard, C.L., Elson, C.O., Hatton, R.D., Weaver, C.T., 2012. Reciprocal interactions of the intestinal microbiota and immune system. Nature 489, 231–241.

McCutcheon, J.E., Marinelli, M., 2009. Age matters. Eur. J. Neurosci. 29, 997–1014.

McGrath, P.A., Hillier, L.M., 1992. Phantom limb sensations in adolescents: A case study to illustrate the utility of sensation and pain logs in pediatric clinical practice. J. Pain Symptom Manage. 7, 46–53.

McGrath, P.J., Hsu, E., Cappelli, M., Luke, B., Goodman, J.T., Dunn-Geier, J., 1990. Pain from Pediatric Cancer. J. Psychosoc. Oncol. 8, 109–124.

McGrath, P.J., Stevens, B.J., Walker, S.M., Zempsky, W.T. (Eds.), 2013. Oxford Textbook of Paediatric Pain. Also available as: eBook | Online.

McKay, S.E., Oppenheim, R.W., 1991. Lack of evidence for cell death among avian spinal cord interneurons during normal development and following removal of targets and afferents. J. Neurobiol. 22, 721–733.

McKelvey, R., Berta, T., Old, E., Ji, R.-R., Fitzgerald, M., 2015. Neuropathic Pain Is Constitutively Suppressed in Early Life by Anti-Inflammatory Neuroimmune Regulation. J. Neurosci. 35, 457–466.

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

McMahon, S.B., Malcangio, M., 2009. Current Challenges in Glia-Pain Biology. Neuron 64, 46–54.

Melzack, R., Israel, R., Lacroix, R., Schultz, G., 1997. Phantom limbs in people with congenital limb deficiency or amputation in early childhood. Brain 120, 1603–1620.

Menétrey, D., Jr, G.J.G., Besson, J.M., 1977. An analysis of response properties of spinal cord dorsal horn neurones to nonnoxious and noxious stimuli in the spinal rat. Exp. Brain Res. 27, 15–33.

Mesulam, M.-M., Brushart, T.M., 1979. Transganglionic and anterograde transport of horseradish peroxidase across dorsal root ganglia: A tetramethylbenzidine method for tracing central sensory connections of muscles and peripheral nerves. Neuroscience 4, 1107–1117.

Michelucci, A., Heurtaux, T., Grandbarbe, L., Morga, E., Heuschling, P., 2009. Characterization of the microglial phenotype under specific pro-inflammatory and anti-

inflammatory conditions: Effects of oligomeric and fibrillary amyloid-β. J. Neuroimmunol. 210, 3–12.

Mika, J., Osikowicz, M., Rojewska, E., Korostynski, M., Wawrzczak-Bargiela, A., Przewlocki, R., Przewlocka, B., 2009. Differential activation of spinal microglial and astroglial cells in a mouse model of peripheral neuropathic pain. Eur. J. Pharmacol. 623, 65–72.

Millan, M.J., 1999. The induction of pain: an integrative review. Prog. Neurobiol. 57, 1–164.

Milligan, E.D., O'Connor, K.A., Nguyen, K.T., Armstrong, C.B., Twining, C., Gaykema, R.P.A., Holguin, A., Martin, D., Maier, S.F., Watkins, L.R., 2001. Intrathecal HIV-1 Envelope Glycoprotein gp120 Induces Enhanced Pain States Mediated by Spinal Cord Proinflammatory Cytokines. J. Neurosci. 21, 2808–2819.

Milligan, E.D., Twining, C., Chacur, M., Biedenkapp, J., O'Connor, K., Poole, S., Tracey, K., Martin, D., Maier, S.F., Watkins, L.R., 2003. Spinal glia and proinflammatory cytokines mediate mirror-image neuropathic pain in rats. J. Neurosci. 23, 1026–1040.

Milligan, E.D., Langer, S.J., Sloane, E.M., He, L., Wieseler-Frank, J., O'Connor, K., Martin, D., Forsayeth, J.R., Maier, S.F., Johnson, K., Chavez, R.A., Leinwand, L.A., Watkins, L.R., 2005a. Controlling pathological pain by adenovirally driven spinal production of the anti-inflammatory cytokine, interleukin-10. Eur. J. Neurosci. 21, 2136–2148.

Milligan, E.D., Sloane, E.M., Langer, S.J., Cruz, P.E., Chacur, M., Spataro, L., Wieseler-Frank, J., Hammack, S.E., Maier, S.F., Flotte, T.R., 2005b. Controlling neuropathic pain by adeno-associated virus driven production of the anti-inflammatory cytokine, interleukin-10. Mol Pain 1.

Milligan, E.D., Watkins, L.R., 2009. Pathological and protective roles of glia in chronic pain. Nat. Rev. Neurosci. 10, 23–36.

Min, B., Legge, K.L., Li, L., Caprio, J.C., Pack, C.D., Gregg, R., McGavin, D., Slauson, D., Zaghouani, H., 2000. Neonatal Tolerant Immunity for Vaccination against Autoimmunity. Int. Rev. Immunol. 19, 247–264.

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

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

Mirnics, K., Koerher, H.R., 1995. Prenatal development of rat primary afferent fibers: II. Central projections. J. Comp. Neurol. 355, 601–614.

Miyabe, T., Miletic, G., Miletic, V., 2006. Loose ligation of the sciatic nerve in rats elicits transient up-regulation of Homer1a gene expression in the spinal dorsal horn. Neurosci. Lett. 398, 296–299.

Mizuno, T., Sawada, M., Marunouchi, T., Suzumura, A., 1994. Production of interleukin-10 by mouse glial cells in culture. Biochem. Biophys. Res. Commun. 205, 1907–1915.

Moalem, G., Xu, K., Yu, L., 2004. T lymphocytes play a role in neuropathic pain following peripheral nerve injury in rats. Neuroscience 129, 767–777.

Mogil, J.S., Crager, S.E., 2004. What should we be measuring in behavioral studies of chronic pain in animals? Pain 112, 12–15.

Mogil, J.S., Ritchie, J., Sotocinal, S.G., Smith, S.B., Croteau, S., Levitin, D.J., Naumova, A.K., 2006. Screening for pain phenotypes: analysis of three congenic mouse strains on a battery of nine nociceptive assays. Pain 126, 24–34.

Mogil, J.S., 2009. Animal models of pain: progress and challenges. Nat. Rev. Neurosci. 10, 283–294.

Mogil, J.S., Graham, A.C., Ritchie, J., Hughes, S.F., Austin, J.-S., Schorscher-Petcu, A., Langford, D.J., Bennett, G.J., 2010. Hypolocomotion, asymmetrically directed behaviors (licking, lifting, flinching, and shaking) and dynamic weight bearing (gait) changes are not measures of neuropathic pain in mice. Mol. Pain 6, 34.

Mogil, J.S., 2012. Sex differences in pain and pain inhibition: multiple explanations of a controversial phenomenon. Nat. Rev. Neurosci. 13, 859–866.

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

Molander, C., Grant, G., 1985. Cutaneous projections from the rat hindlimb foot to the substantia gelatinosa of the spinal cord studied by transganglionic transport of WGA-HRP conjugate. J. Comp. Neurol. 237, 476–484.

Molander, C., Grant, G., 1986. Laminar distribution and somatotopic organization of primary afferent fibers from hindlimb nerves in the dorsal horn. A study by transganglionic transport of horseradish peroxidase in the rat. Neuroscience 19, 297–312.

Molander, C., Xu, Q., Rivero-Melian, C., Grant, G., 1989. Cytoarchitectonic organization of the spinal cord in the rat: II. The cervical and upper thoracic cord. J. Comp. Neurol. 289, 375–385.

Molander, C., Wang, H.F., Rivero-Melián, C., Grant, G., 1996. Early decline and late restoration of spinal cord binding and transganglionic transport of isolectin B4 from Griffonia simplicifolia I after peripheral nerve transection or crush. Restor. Neurol. Neurosci. 10, 123–133.

Molander, C., Hongpaisan, J., Svensson, M., Aldskogius, H. akan, 1997. Glial cell reactions in the spinal cord after sensory nerve stimulation are associated with axonal injury. Brain Res. 747, 122–129.

Mold, J.E., Michaëlsson, J., Burt, T.D., Muench, M.O., Beckerman, K.P., Busch, M.P., Lee, T.-H., Nixon, D.F., McCune, J.M., 2008. Maternal alloantigens promote the development of tolerogenic fetal regulatory T cells in utero. Science 322, 1562–1565.

Molliver, D.C., Wright, D.E., Leitner, M.L., Parsadanian, A.S., Doster, K., Wen, D., Yan, Q., Snider, W.D., 1997. IB4-binding DRG neurons switch from NGF to GDNF dependence in early postnatal life. Neuron 19, 849–861.

Moore, K.A., Kohno, T., Karchewski, L.A., Scholz, J., Baba, H., Woolf, C.J., 2002. Partial Peripheral Nerve Injury Promotes a Selective Loss of GABAergic Inhibition in the Superficial Dorsal Horn of the Spinal Cord. J. Neurosci. 22, 6724–6731.

Mosmann, T.R., Coffman, R.L., 1989. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Annu. Rev. Immunol. 7, 145–173.

Mosmann, T.R., Sad, S., 1996. The expanding universe of T-cell subsets: Th1, Th2 and more. Immunol. Today 17, 138–146.

Mosmann, T.R., Schumacher, J.H., Fiorentino, D.F., Leverah, J., Moore, K.W., Bond, M.W., 1990. Isolation of monoclonal antibodies specific for IL-4, IL-5, IL-6, and a new Th2-

specific cytokine (IL-10), cytokine synthesis inhibitory factor, by using a solid phase radioimmunoadsorbent assay. J. Immunol. 145, 2938–2945.

Moss, A., Beggs, S., Vega-Avelaira, D., Costigan, M., Hathway, G.J., Salter, M.W., Fitzgerald, M., 2007. Spinal microglia and neuropathic pain in young rats. PAIN 128, 215–224.

Mosser, D.M., Edwards, J.P., 2008. Exploring the full spectrum of macrophage activation. Nat. Rev. Immunol. 8, 958–969.

Mosser, D.M., Zhang, X., 2008. Interleukin-10: new perspectives on an old cytokine. Immunol. Rev. 226, 205–218.

Muller, W.A., 2009. Mechanisms of Transendothelial Migration of Leukocytes. Circ. Res. 105, 223–230.

Munger, B.L., Bennett, G.J., Kajander, K.C., 1992. An experimental painful peripheral neuropathy due to nerve constriction: I. Axonal pathology in the sciatic nerve. Exp. Neurol. 118, 204–214.

Nackley, A.G., Zvonok, A.M., Makriyannis, A., Hohmann, A.G., 2004. Activation of cannabinoid CB2 receptors suppresses C-fiber responses and windup in spinal wide dynamic range neurons in the absence and presence of inflammation. J. Neurophysiol. 92, 3562–3574.

Nagy, J.I., Hunt, S.P., 1982. Fluoride-resistant acid phosphatase-containing neurones in dorsal root ganglia are separate from those containing substance P or somatostatin. Neuroscience 7, 89–97.

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

Nakatsuka, T., Ataka, T., Kumamoto, E., Tamaki, T., Yoshimura, M., 2000. Alteration in synaptic inputs through C-afferent fibers to substantia gelatinosa neurons of the rat spinal dorsal horn during postnatal development. Neuroscience 99, 549–556.

Nassar, M.A., Stirling, L.C., Forlani, G., Baker, M.D., Matthews, E.A., Dickenson, A.H., Wood, J.N., 2004. Nociceptor-specific gene deletion reveals a major role for Nav1. 7 (PN1) in acute and inflammatory pain. Proc. Natl. Acad. Sci. U. S. A. 101, 12706–12711.

Noguchi, K., Kawai, Y., Fukuoka, T., Senba, E., Miki, K., 1995. Substance P induced by peripheral nerve injury in primary afferent sensory neurons and its effect on dorsal column nucleus neurons. J. Neurosci. 15, 7633–7643.

Ochoa, J.L., Yarnitsky, D., 1994. The triple cold syndrome Cold hyperalgesia, cold hypoaesthesia and cold skin in peripheral nerve disease. Brain 117, 185–197.

Ogawa, Y., Duru, E.A., Ameredes, B.T., 2008. Role of IL-10 in the resolution of airway inflammation. Curr. Mol. Med. 8, 437–445.

Okamoto, M., Baba, H., Goldstein, P.A., Higashi, H., Shimoji, K., Yoshimura, M., 2001. Functional reorganization of sensory pathways in the rat spinal dorsal horn following peripheral nerve injury. J. Physiol. 532, 241–250.

Olah, M., Biber, K., Vinet, J., W.G.M. Boddeke, H., 2011. Microglia Phenotype Diversity. CNS Neurol. Disord. - Drug Targets Former. Curr. Drug Targets 10, 108–118.

Olausson, H., Cole, J., Rylander, K., McGlone, F., Lamarre, Y., Wallin, B.G., Krämer, H., Wessberg, J., Elam, M., Bushnell, M.C., Vallbo, Å., 2007. Functional role of

unmyelinated tactile afferents in human hairy skin: sympathetic response and perceptual localization. Exp. Brain Res. 184, 135–140.

Oliveira, A, Risling M, Deckner, M, Lindholm, T, Langone, F, Cullheim, S, 1997. Neonatal sciatic nerve transection induces TUNEL labeling of neurons in the rat spinal cord and DRG. Neuroreport 8, 2837–2840.

Opiela, S.J., Koru-Sengul, T., Adkins, B., 2009. Murine neonatal recent thymic emigrants are phenotypically and functionally distinct from adult recent thymic emigrants. Blood 113, 5635–5643.

Oppenheim, R.W., 1991. Cell Death During Development of the Nervous System. Annu. Rev. Neurosci. 14, 453–501. doi:10.1146/annurev.ne.14.030191.002321

Ossipov, M.H., Dussor, G.O., Porreca, F., 2010. Central modulation of pain. J. Clin. Invest. 120, 3779–3787.

Ouyang, W., Ranganath, S.H., Weindel, K., Bhattacharya, D., Murphy, T.L., Sha, W.C., Murphy, K.M., 1998. Inhibition of Th1 development mediated by GATA-3 through an IL-4-independent mechanism. Immunity 9, 745–755.

Palecek, J., Dougherty, P.M., Kim, S.H., Paleckova, V., Lekan, H., Chung, J.M., Carlton, S.M., Willis, W.D., 1992. Responses of spinothalamic tract neurons to mechanical and thermal stimuli in an experimental model of peripheral neuropathy in primates. J. Neurophysiol. 68, 1951–1966.

Palermo, T.M., 2000. Impact of Recurrent and Chronic Pain on Child and Family Daily Functioning: A Critical Review of the Literature. [Review]. J. Dev. 21, 58–69.

Palmer, M.T., Weaver, C.T., 2010. Autoimmunity: increasing suspects in the CD4+ T cell lineup. Nat. Immunol. 11, 36–40.

Paolicelli, R.C., Bolasco, G., Pagani, F., Maggi, L., Scianni, M., Panzanelli, P., Giustetto, M., Ferreira, T.A., Guiducci, E., Dumas, L., Ragozzino, D., Gross, C.T., 2011. Synaptic Pruning by Microglia Is Necessary for Normal Brain Development. Science 333, 1456–1458.

Park, J.-S., Nakatsuka, T., Nagata, K., Higashi, H., Yoshimura, M., 1999. Reorganization of the primary afferent termination in the rat spinal dorsal horn during post-natal development. Dev. Brain Res. 113, 29–36.

Park, K. w., Lee, D. y., Joe, E. h., Kim, S. u., Jin, B. k., 2005. Neuroprotective role of microglia expressing interleukin-4. J. Neurosci. Res. 81, 397–402.

Parrilla-Reverter, G., Agudo, M., Nadal-Nicolás, F., Alarcón-Martínez, L., Jiménez-López, M., Salinas-Navarro, M., Sobrado-Calvo, P., Bernal-Garro, J.M., Villegas-Pérez, M.P., Vidal-Sanz, M., 2009. Time-course of the retinal nerve fibre layer degeneration after complete intra-orbital optic nerve transection or crush: A comparative study. Vision Res. 49, 2808–2825.

Pattinson, D., Fitzgerald, M., 2004. The neurobiology of infant pain: development of excitatory and inhibitory neurotransmission in the spinal dorsal horn. Reg. Anesth. Pain Med. 29, 36–44.

Peier, A.M., Moqrich, A., Hergarden, A.C., Reeve, A.J., Andersson, D.A., Story, G.M., Earley, T.J., Dragoni, I., McIntyre, P., Bevan, S., Patapoutian, A., 2002. A TRP Channel that Senses Cold Stimuli and Menthol. Cell 108, 705–715.

Perl, E.R., 2007. Ideas about pain, a historical view. Nat. Rev. Neurosci. 8, 71–80.

Pertin, M., Gosselin, R.-D., Decosterd, I., 2012. The Spared Nerve Injury Model of Neuropathic Pain, in: Luo, Z.D. (Ed.), Pain Research, Methods in Molecular Biology. Humana Press, pp. 205–212.

Pertovaara, A., Wei, H., Hämäläinen, M.M., 1996. Lidocaine in the rostroventromedial medulla and the periaqueductal gray attenuates allodynia in neuropathic rats. Neurosci. Lett. 218, 127–130.

Peschanski, M., Guilbaud, G., Gautron, M., 1981. Posterior intralaminar region in rat: Neuronal responses to noxious and nonnoxious cutaneous stimuli. Exp. Neurol. 72, 226–238.

Petersen, M., Zhang, J., Zhang, J.-M., LaMotte, R.H., 1996. Abnormal spontaneous activity and responses to norepinephrine in dissociated dorsal root ganglion cells after chronic nerve constriction. Pain 67, 391–397.

Peters, J.W.B., Schouw, R., Anand, K.J.S., van Dijk, M., Duivenvoorden, H.J., Tibboel, D., 2005. Does neonatal surgery lead to increased pain sensitivity in later childhood? Pain 114, 444–454.

Pfau, D.B., Klein, T., Putzer, D., Pogatzki-Zahn, E.M., Treede, R.-D., Magerl, W., 2011. Analysis of hyperalgesia time courses in humans after painful electrical high-frequency stimulation identifies a possible transition from early to late LTP-like pain plasticity. PAIN® 152, 1532–1539.

Pharoah, D.S., Varsani, H., Tatham, R.W., Newton, K.R., de Jager, W., Prakken, B.J., Klein, N., Wedderburn, L.R., 2006. Expression of the inflammatory chemokines CCL5, CCL3 and CXCL10 in juvenile idiopathic arthritis, and demonstration of CCL5 production by an atypical subset of CD8+ T cells. Arthritis Res. Ther. 8, R50.

Plenderleith, M.B., Wright, L.L., Snow, P.J., 1992. Expression of lectin binding in the superficial dorsal horn of the rat spinal cord during pre- and postnatal development. Dev. Brain Res. 68, 103–109.

Poeck, K., 1964. Phantoms Following Amputation in Early Childhood and in Congenital Absence of Limbs. Cortex 1, 269–275.

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

Polgár, E., Hughes, D.I., Arham, A.Z., Todd, A.J., 2005. Loss of Neurons from Laminas I-III of the Spinal Dorsal Horn Is Not Required for Development of Tactile Allodynia in the Spared Nerve Injury Model of Neuropathic Pain. J. Neurosci. 25, 6658–6666.

Pomeroy, S.L., Behbehani, M.M., 1979. Physiologic evidence for a projection from periaqueductal gray to nucleus raphe magnus in the rat. Brain Res. 176, 143–147.

Ponomarev, E.D., Shriver, L.P., Maresz, K., Dittel, B.N., 2005. Microglial cell activation and proliferation precedes the onset of CNS autoimmunity. J. Neurosci. Res. 81, 374–389.

Ponomarev, E.D., Maresz, K., Tan, Y., Dittel, B.N., 2007. CNS-derived interleukin-4 is essential for the regulation of autoimmune inflammation and induces a state of alternative activation in microglial cells. J. Neurosci. 27, 10714–10721.

Poole, S., Cunha, F.Q., Selkirk, S., Lorenzetti, B.B., Ferreira, S.H., 1995. Cytokine-mediated inflammatory hyperalgesia limited by interleukin-10. Br. J. Pharmacol. 115, 684–688.

Porreca, F., Ossipov, M.H., Gebhart, G.F., 2002. Chronic pain and medullary descending facilitation. Trends Neurosci. 25, 319–325.

Powell, T.J., Streilein, J.W., 1990. Neonatal tolerance induction by class II alloantigens activates IL-4-secreting, tolerogen-responsive T cells. J. Immunol. 144, 854–859.

PrabhuDas, M., Adkins, B., Gans, H., King, C., Levy, O., Ramilo, O., Siegrist, C.-A., 2011. Challenges in infant immunity: implications for responses to infection and vaccines. Nat. Immunol. 12, 189.

Pradhan, A.A.A., Yu, X.H., Laird, J.M.A., 2010. Modality of hyperalgesia tested, not type of nerve damage, predicts pharmacological sensitivity in rat models of neuropathic pain. Eur. J. Pain 14, 503–509.

Prakken, B., Albani, S., Martini, A., 2011. Juvenile idiopathic arthritis. The Lancet 377, 2138–2149. doi:10.1016/S0140-6736(11)60244-4

Prescott, S.L., Macaubas, C., Holt, B.J., Smallacombe, T.B., Loh, R., Sly, P.D., Holt, P.G., 1998. Transplacental Priming of the Human Immune System to Environmental Allergens: Universal Skewing of Initial T Cell Responses Toward the Th2 Cytokine Profile. J. Immunol. 160, 4730–4737.

Qian, J., Brown, S.D., Carlton, S.M., 1996. Systemic ketamine attenuates nociceptive behaviors in a rat model of peripheral neuropathy. Brain Res. 715, 51–62.

Queiroz, G., Gebicke-Haerter, P.J., Schobert, A., Starke, K., Von Kügelgen, I., 1997. Release of ATP from cultured rat astrocytes elicited by glutamate receptor activation. Neuroscience 78, 1203–1208.

Racz, I., Nadal, X., Alferink, J., Banos, J.E., Rehnelt, J., Martín, M., Pintado, B., Gutierrez-Adan, A., Sanguino, E., Bellora, N., 2008. Interferon-γ is a critical modulator of CB2 cannabinoid receptor signaling during neuropathic pain. J. Neurosci. 28, 12136–12145.

Raghavendra, V., Tanga, F., DeLeo, J.A., 2003. Inhibition of microglial activation attenuates the development but not existing hypersensitivity in a rat model of neuropathy. J. Pharmacol. Exp. Ther. 306, 624–630.

Raghavendra, V., Tanga, F.Y., DeLeo, J.A., 2004. Complete Freunds adjuvant-induced peripheral inflammation evokes glial activation and proinflammatory cytokine expression in the CNS. Eur. J. Neurosci. 20, 467–473.

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

Ramsey, I.S., Delling, M., Clapham, D.E., 2006. An Introduction to Trp Channels. Annu. Rev. Physiol. 68, 619–647.

Randich, A., Uzzell, T., DeBerry, J.J., Ness, T.J., 2006. Neonatal urinary bladder inflammation produces adult bladder hypersensitivity. J. Pain 7, 469–479.

Ransohoff, R.M., Kivisäkk, P., Kidd, G., 2003. Three or more routes for leukocyte migration into the central nervous system. Nat. Rev. Immunol. 3, 569–581.

Raouf, R., Quick, K., Wood, J.N., 2010. Pain as a channelopathy. J. Clin. Invest. 120, 3745–3752.

Rasmussen, P.V., Sindrup, S.H., Jensen, T.S., Bach, F.W., 2004. Symptoms and signs in patients with suspected neuropathic pain. Pain 110, 461–469.

Ren, K., Anseloni, V., Zou, S.-P., Wade, E.B., Novikova, S.I., Ennis, M., Traub, R.J., Gold, M.S., Dubner, R., Lidow, M.S., 2004. Characterization of basal and re-inflammation-

associated long-term alteration in pain responsivity following short-lasting neonatal local inflamatory insult. Pain 110, 588–596.

Rexed, B., 1952. The cytoarchitectonic organization of the spinal cord in the cat. J. Comp. Neurol. 96, 415–495.

Rexed, B., 1954. A cytoarchitectonic atlas of the spinal coed in the cat. J. Comp. Neurol. 100, 297–379.

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

Ridge, J.P., Fuchs, E.J., Matzinger, P., 1996. Neonatal Tolerance Revisited: Turning on Newborn T Cells with Dendritic Cells. Science 271, 1723–1726.

Ririe, D.G., Bremner, L.R., Fitzgerald, M., 2008. Comparison of the Immediate Effects of Surgical Incision on Dorsal Horn Neuronal Receptive Field Size and Responses during Postnatal Development: Anesthesiology 109, 698–706.

Ririe, D.G., Eisenach, J.C., 2006. Age-dependent Responses to Nerve Injury–induced Mechanical Allodynia. Anesthesiology 104, 344–350.

Rokyta, R., Fricová, J., 2012. Neurostimulation methods in the treatment of chronic pain. Physiol Res 61, S23–S31.

Rose, K., Ooi, L., Dalle, C., Robertson, B., Wood, I.C., Gamper, N., 2011. Transcriptional repression of the M channel subunit Kv7. 2 in chronic nerve injury. Pain 152, 742–754.

Rose, S., Lichtenheld, M., Foote, M.R., Adkins, B., 2007. Murine Neonatal CD4+ Cells Are Poised for Rapid Th2 Effector-Like Function. J. Immunol. 178, 2667–2678.

Roza, C., Laird, J.M., Souslova, V., Wood, J.N., Cervero, F., 2003. The Tetrodotoxin-Resistant Na+ Channel Nav1.8 is Essential for the Expression of Spontaneous Activity in Damaged Sensory Axons of Mice. J. Physiol. 550, 921–926.

Ruda, M.A., Ling, Q.-D., Hohmann, A.G., Peng, Y.B., Tachibana, T., 2000. Altered nociceptive neuronal circuits after neonatal peripheral inflammation. Science 289, 628–630.

Ruscheweyh, R., Wilder-Smith, O., Drdla, R., Liu, X.-G., Sandkuhler, J., 2011. Long-term potentiation in spinal nociceptive pathways as a novel target for pain therapy. Mol Pain 7, 20.

Samad, T.A., Moore, K.A., Sapirstein, A., Billet, S., Allchorne, A., Poole, S., Bonventre, J.V., Woolf, C.J., 2001. Interleukin-1β-mediated induction of Cox-2 in the CNS contributes to inflammatory pain hypersensitivity. Nature 410, 471–475.

Sandkühler, J., 2009. Models and Mechanisms of Hyperalgesia and Allodynia. Physiol. Rev. 89, 707–758.

Sarnat, H.B., Nochlin, D., Born, D.E., 1998. Neuronal nuclear antigen (NeuN): a marker of neuronal maturation in the early human fetal nervous system. Brain Dev. 20, 88–94.

Sarzotti, M., Robbins, D.S., Hoffman, P.M., 1996. Induction of protective CTL responses in newborn mice by a murine retrovirus. Science 271, 1726–1728.

Sawada, M., Kondo, N., Suzumura, A., Marunouchi, T., 1989. Production of tumor necrosis factor-alpha by microglia and astrocytes in culture. Brain Res. 491, 394–397.

Schafer, D.P., Lehrman, E.K., Kautzman, A.G., Koyama, R., Mardinly, A.R., Yamasaki, R., Ransohoff, R.M., Greenberg, M.E., Barres, B.A., Stevens, B., 2012. Microglia Sculpt Postnatal Neural Circuits in an Activity and Complement-Dependent Manner. Neuron

Schaffner, A.E., Behar, T., Nadi, S., Barker, J.L., 1993. Quantitative analysis of transient GABA expression in embryonic and early postnatal rat spinal cord neurons. Dev. Brain Res. 72, 265–276.

Scheffel, J., Regen, T., Van Rossum, D., Seifert, S., Ribes, S., Nau, R., Parsa, R., Harris, R.A., Boddeke, H.W.G.M., Chuang, H.-N., Pukrop, T., Wessels, J.T., Jürgens, T., Merkler, D., Brück, W., Schnaars, M., Simons, M., Kettenmann, H., Hanisch, U.-K., 2012. Toll-like receptor activation reveals developmental reorganization and unmasks responder subsets of microglia. Glia 60, 1930–1943.

Schlegelmilch, T., Henke, K., Peri, F., 2011. Microglia in the developing brain: from immunity to behaviour. Curr. Opin. Neurobiol., Developmental neuroscience 21, 5–10.

Schmalbruch, H., 1984. Motoneuron death after sciatic nerve section in newborn rats. J. Comp. Neurol. 224, 252–258.

Schmelzle-Lubiecki, B. m., Campbell, K.A.A., Howard, R. h., Franck, L., Fitzgerald, M., 2007. Long-term consequences of early infant injury and trauma upon somatosensory processing. Eur. J. Pain 11, 799–809.

Schmelz, M., Schmidt, R., Bickel, A., Handwerker, H.O., Torebjörk, H.E., 1997. Specific C-receptors for itch in human skin. J. Neurosci. Off. J. Soc. Neurosci. 17, 8003–8008.

Schmittgen, T.D., Livak, K.J., 2008. Analyzing real-time PCR data by the comparative CT method. Nat. Protoc. 3, 1101–1108.

Schmued, L.C., Hopkins, K.J., 2000. Fluoro-Jade B: a high affinity fluorescent marker for the localization of neuronal degeneration. Brain Res. 874, 123–130.

Schmued, L.C., Stowers, C.C., Scallet, A.C., Xu, L., 2005. Fluoro-Jade C results in ultra high resolution and contrast labeling of degenerating neurons. Brain Res. 1035, 24–31.

Scholz, J., Woolf, C.J., 2002. Can we conquer pain? Nat. Neurosci. 5, 1062–1067.

Scholz, J., Broom, D.C., Youn, D.-H., Mills, C.D., Kohno, T., Suter, M.R., Moore, K.A., Decosterd, I., Coggeshall, R.E., Woolf, C.J., 2005. Blocking Caspase Activity Prevents Transsynaptic Neuronal Apoptosis and the Loss of Inhibition in Lamina II of the Dorsal Horn after Peripheral Nerve Injury. J. Neurosci. 25, 7317–7323.

Scholz, J., Woolf, C.J., 2007. The neuropathic pain triad: neurons, immune cells and glia. Nat. Neurosci. 10, 1361–1368.

Schött, E., Berge, O.-G., Ängeby-Möller, K., Hammarström, G., Dalsgaard, C.-J., Brodin, E., 1994. Weight bearing as an objective measure of arthritic pain in the rat. J. Pharmacol. Toxicol. Methods 31, 79–83.

Seal, R.P., Wang, X., Guan, Y., Raja, S.N., Woodbury, C.J., Basbaum, A.I., Edwards, R.H., 2009. Injury-induced mechanical hypersensitivity requires C-low threshold mechanoreceptors. Nature 462, 651–655.

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

Sens, E., Teschner, U., Meissner, W., Preul, C., Huonker, R., Witte, O.W., Miltner, W.H.R., Weiss, T., 2012. Effects of Temporary Functional Deafferentation on the Brain, Sensation, and Behavior of Stroke Patients. J. Neurosci. 32, 11773–11779.

Sethna, N.F., Meier, P.M., Zurakowski, D., Berde, C.B., 2007. Cutaneous sensory abnormalities in children and adolescents with complex regional pain syndromes. Pain 131, 153–161.

Sheen, K., Chung, J.M., 1993. Signs of neuropathic pain depend on signals from injured nerve fibers in a rat model. Brain Res. 610, 62–68.

Sherrington, C.S., 1910. Flexion-reflex of the limb, crossed extension-reflex, and reflex stepping and standing. J. Physiol. 40, 28–121.

Sherrington, C.S., 1906. The Integrative Action of the Nervous System Scribner. N. Y.

Shields, S.D., Eckert III, W.A., Basbaum, A.I., 2003. Spared nerve injury model of neuropathic pain in the mouse: a behavioral and anatomic analysis. J. Pain 4, 465–470.

Shigemoto-Mogami, Y., Hoshikawa, K., Goldman, J.E., Sekino, Y., Sato, K., 2014. Microglia Enhance Neurogenesis and Oligodendrogenesis in the Early Postnatal Subventricular Zone. J. Neurosci. 34, 2231–2243.

Shoemaker, J., Saraiva, M., O'Garra, A., 2006. GATA-3 Directly Remodels the IL-10 Locus Independently of IL-4 in CD4+ T Cells. J. Immunol. 176, 3470–3479.

Shortland, P., Fitzgerald, M., 1994. Neonatal Sciatic Nerve Section Results in a Rearrangement of the Central Terminals of Saphenous and Axotomized Sciatic Nerve Afferents in the Dorsal Horn of the Spinal Cord of the Adult Rat. Eur. J. Neurosci. 6, 75–86.

Shortland, P., Fitzgerald, M., 1991. Functional Connections Formed by Saphenous Nerve Terminal Sprouts in the Dorsal Horn Following Neonatal Sciatic Nerve Section. Eur. J. Neurosci. 3, 383–396.

Simone, D.A., Kajander, K.C., 1997. Responses of Cutaneous A-Fiber Nociceptors to Noxious Cold. J. Neurophysiol. 77, 2049–2060.

Singh, R.R., Hahn, B.H., Sercarz, E.E., 1996. Neonatal peptide exposure can prime T cells and, upon subsequent immunization, induce their immune deviation: implications for antibody vs. T cell-mediated autoimmunity. J. Exp. Med. 183, 1613–1621.

Sivilotti, L., Woolf, C.J., 1994. The contribution of GABAA and glycine receptors to central sensitization: disinhibition and touch-evoked allodynia in the spinal cord. J. Neurophysiol. 72, 169–179.

Slater, R., Fabrizi, L., Worley, A., Meek, J., Boyd, S., Fitzgerald, M., 2010. Premature infants display increased noxious-evoked neuronal activity in the brain compared to healthy age-matched term-born infants. NeuroImage 52, 583–589.

Slater, R., Fitzgerald, M., Meek, J., 2007. Can cortical responses following noxious stimulation inform us about pain processing in neonates?, in: Seminars in Perinatology. Elsevier, pp. 298–302.

Smith, C.J., Osborn, A.M., 2009. Advantages and limitations of quantitative PCR (Q-PCR)-based approaches in microbial ecology. FEMS Microbiol. Ecol. 67, 6–20.

Snider, W.D., McMahon, S.B., 1998. Tackling pain at the source: new ideas about nociceptors. Neuron 20, 629–632.

Soderquist, R.G., Sloane, E.M., Loram, L.C., Harrison, J.A., Dengler, E.C., Johnson, S.M., Amer, L.D., Young, C.S., Lewis, M.T., Poole, S., Frank, M.G., Watkins, L.R., Milligan, E.D., Mahoney, M.J., 2010. Release of Plasmid DNA-Encoding IL-10 from PLGA

Microparticles Facilitates Long-Term Reversal of Neuropathic Pain Following a Single Intrathecal Administration. Pharm. Res. 27, 841–854.

Sommer, C., Kress, M., 2004. Recent findings on how proinflammatory cytokines cause pain: peripheral mechanisms in inflammatory and neuropathic hyperalgesia. Neurosci. Lett. 361, 184–187.

Sorkin, L.S., Yaksh, T.L., 2009. Behavioral Models of Pain States Evoked by Physical Injury to the Peripheral Nerve. Neurotherapeutics, Development of Analgesics for the Control and Prevention of Neuropathic Pain 6, 609–619.

Spike, R.C., Puskár, Z., Andrew, D., Todd, A.J., 2003. A quantitative and morphological study of projection neurons in lamina I of the rat lumbar spinal cord. Eur. J. Neurosci. 18, 2433–2448.

Stein, M., Keshav, S., Harris, N., Gordon, S., 1992. Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation. J. Exp. Med. 176, 287–292.

Sternberg, W.F., Scorr, L., Smith, L.D., Ridgway, C.G., Stout, M., 2005. Long-term effects of neonatal surgery on adulthood pain behavior. Pain 113, 347–353.

Story, G.M., Peier, A.M., Reeve, A.J., Eid, S.R., Mosbacher, J., Hricik, T.R., Earley, T.J., Hergarden, A.C., Andersson, D.A., Hwang, S.W., McIntyre, P., Jegla, T., Bevan, S., Patapoutian, A., 2003. ANKTM1, a TRP-like Channel Expressed in Nociceptive Neurons, Is Activated by Cold Temperatures. Cell 112, 819–829.

Strunk, T., Currie, A., Richmond, P., Simmer, K., Burgner, D., 2011. Innate immunity in human newborn infants: prematurity means more than immaturity. J. Matern. Fetal Neonatal Med. 24, 25–31.

Study, R.E., Kral, M.G., 1996. Spontaneous action potential activity in isolated dorsal root ganglion neurons from rats with a painful neuropathy. Pain 65, 235–242.

Sukhotinsky, I., Ben-Dor, E., Raber, P., Devor, M., 2004. Key role of the dorsal root ganglion in neuropathic tactile hypersensibility. Eur. J. Pain 8, 135–143.

Sung, B., Lim, G., Mao, J., 2003. Altered Expression and Uptake Activity of Spinal Glutamate Transporters after Nerve Injury Contribute to the Pathogenesis of Neuropathic Pain in Rats. J. Neurosci. 23, 2899–2910.

Sun, Q., Tu, H., Xing, G.-G., Han, J.-S., Wan, Y., 2005. Ectopic discharges from injured nerve fibers are highly correlated with tactile allodynia only in early, but not late, stage in rats with spinal nerve ligation. Exp. Neurol. 191, 128–136.

Suter, M.R., Papaloïzos, M., Berde, C.B., Woolf, C.J., Gilliard, N., Spahn, D.R., Decosterd, I., 2003. Development of neuropathic pain in the rat spared nerve injury model is not prevented by a peripheral nerve block. Anesthesiology 99, 1402–1408.

Suter, M.R., Wen, Y.-R., Decosterd, I., Ji, R.-R., 2007. Do glial cells control pain? Neuron Glia Biol. 3, 255–268.

Suter, M.R., Berta, T., Gao, Y.-J., Decosterd, I., Ji, R.-R., 2009. Large A-fiber activity is required for microglial proliferation and p38 MAPK activation in the spinal cord: different effects of resiniferatoxin and bupivacaine on spinal microglial changes after spared nerve injury. Mol Pain 5, 1–14.

Suzuki, R., Kontinen, V., Matthews, E., Williams, E., Dickenson, A., 2000. Enlargement of the Receptive Field Size to Low Intensity Mechanical Stimulation in the Rat Spinal Nerve Ligation Model of Neuropathy. J. Peripher. Nerv. Syst. 5, 248–248.

Suzuki, R., Matthews, E.A., Dickenson, A.H., 2001. Comparison of the effects of MK-801, ketamine and memantine on responses of spinal dorsal horn neurones in a rat model of mononeuropathy. Pain 91, 101–109.

Suzuki, R., Dickenson, A.H., 2006. Differential pharmacological modulation of the spontaneous stimulus-independent activity in the rat spinal cord following peripheral nerve injury. Exp. Neurol. 198, 72–80.

Svensson, C.I., Schäfers, M., Jones, T.L., Powell, H., Sorkin, L.S., 2005. Spinal blockade of TNF blocks spinal nerve ligation-induced increases in spinal P-p38. Neurosci. Lett. 379, 209–213.

Sweitzer, S., Martin, D., DeLeo, J.A., 2001. Intrathecal interleukin-1 receptor antagonist in combination with soluble tumor necrosis factor receptor exhibits an anti-allodynic action in a rat model of neuropathic pain. Neuroscience 103, 529–539.

Sweitzer, S.M., Hickey, W.F., Rutkowski, M.D., Pahl, J.L., DeLeo, J.A., 2002. Focal peripheral nerve injury induces leukocyte trafficking into the central nervous system: potential relationship to neuropathic pain. Pain 100, 163–170.

Sweitzer, S.M., White, K.A., Dutta, C., DeLeo, J.A., 2002. The differential role of spinal MHC class II and cellular adhesion molecules in peripheral inflammatory versus neuropathic pain in rodents. J. Neuroimmunol. 125, 82–93.

Swett, J.E., Woolf, C.J., 1985. The somatotopic organization of primary afferent terminals in the superficial laminae of the dorsal horn of the rat spinal cord. J. Comp. Neurol. 231, 66–77.

Taddio, A., Goldbach, M., Ipp, M., Stevens, B., Koren, G., 1995. Effect of neonatal circumcision on pain responses during vaccination in boys. The Lancet 345, 291–292.

Takaishi, K., Eisele Jr., J.H., Carstens, E., 1996. Behavioral and electrophysiological assessment of hyperalgesia and changes in dorsal horn responses following partial sciatic nerve ligation in rats. Pain 66, 297–306.

Takasusuki, T., Fujiwara, T., Yamaguchi, S., Fukushima, T., Akagawa, K., Hori, Y., 2007. Enhancement of synaptic transmission and nociceptive behaviour in HPC-1/syntaxin 1A knockout mice following peripheral nerve injury. Eur. J. Neurosci. 26, 2179–2187.

Tal, M., Eliav, E., 1996. Abnormal discharge originates at the site of nerve injury in experimental constriction neuropathy (CCI) in the rat. Pain 64, 511–518.

Tandrup, T., Woolf, C.J., Coggeshall, R.E., 2000. Delayed loss of small dorsal root ganglion cells after transection of the rat sciatic nerve. J. Comp. Neurol. 422, 172–180.

Tanga, F.Y., Raghavendra, V., DeLeo, J.A., 2004. Quantitative real-time RT-PCR assessment of spinal microglial and astrocytic activation markers in a rat model of neuropathic pain. Neurochem. Int., Glial Biology: Functional Interactions Among Glia and Neurons 45, 397–407.

Tanga, F.Y., Nutile-McMenemy, N., DeLeo, J.A., 2005. The CNS role of Toll-like receptor 4 in innate neuroimmunity and painful neuropathy. Proc. Natl. Acad. Sci. U. S. A. 102, 5856–5861.

Tao, Y.-X., Rumbaugh, G., Wang, G.-D., Petralia, R.S., Zhao, C., Kauer, F.W., Tao, F., Zhuo, M., Wenthold, R.J., Raja, S.N., Huganir, R.L., Bredt, D.S., Johns, R.A., 2003. Impaired NMDA Receptor-Mediated Postsynaptic Function and Blunted NMDA Receptor-Dependent Persistent Pain in Mice Lacking Postsynaptic Density-93 Protein. J. Neurosci. 23, 6703–6712.

Tappe-Theodor, A., Kuner, R., 2014. Studying ongoing and spontaneous pain in rodents–challenges and opportunities. Eur. J. Neurosci.

Taves, S., Berta, T., Chen, G., Ji, R.-R., 2013. Microglia and Spinal Cord Synaptic Plasticity in Persistent Pain. Neural Plast. 2013, e753656.

Teng, C.J., Abbott, F.V., 1998. The formalin test: a dose–response analysis at three developmental stages. Pain 76, 337–347.

Terman, G.W., Shavit, Y., Lewis, J.W., Cannon, J.T., Liebeskind, J.C., 1984. Intrinsic mechanisms of pain inhibition: activation by stress. Science 226, 1270–1277.

Tétreault, P., Dansereau, M.-A., Doré-Savard, L., Beaudet, N., Sarret, P., 2011. Weight bearing evaluation in inflammatory, neuropathic and cancer chronic pain in freely moving rats. Physiol. Behav. 104, 495–502.

Thompson, S.W.N., Dray, A., McCarson, K.E., Krause, J.E., Urban, L., 1995. Nerve growth factor induces mechanical allodynia associated with novel A fibre-evoked spinal reflex activity and enhanced neurokinin-1 receptor activation in the rat. Pain 62, 219–231.

Todd, A.J., 2010. Neuronal circuitry for pain processing in the dorsal horn. Nat. Rev. Neurosci. 11, 823–836.

Todd, A.J., 2002. Anatomy of primary afferents and projection neurones in the rat spinal dorsal horn with particular emphasis on substance P and the neurokinin 1 receptor. Exp. Physiol. null, 245–249.

Todd, A.J., Spike, R.C., Price, R.F., Neilson, M., 1994. Immunocytochemical evidence that neurotensin is present in glutamatergic neurons in the superficial dorsal horn of the rat. J. Neurosci. 14, 774–784.

Torebjörk, H.E., 1974. Afferent G Units Responding to Mechanical, Thermal and Chemical Stimuli in Human Non-Glabrous Skin. Acta Physiol. Scand. 92, 374–390.

Torebjörk, H.E., Hallin, R.G., 1974. Responses in human A and C fibres to repeated electrical intradermal stimulation. J. Neurol. Neurosurg. Psychiatry 37, 653–664.

Torebjörk, H.E., Hallin, R.G., 1973. Perceptual changes accompanying controlled preferential blocking of A and C fibre responses in intact human skin nerves. Exp. Brain Res. 16, 321–332.

Torrance, N., Smith, B.H., Bennett, M.I., Lee, A.J., 2006. The Epidemiology of Chronic Pain of Predominantly Neuropathic Origin. Results From a General Population Survey. J. Pain 7, 281–289.

Torsney, C., Meredith-Middleton, J., Fitzgerald, M., 2000. Neonatal capsaicin treatment prevents the normal postnatal withdrawal of A fibres from lamina II without affecting Fos responses to innocuous peripheral stimulation. Dev. Brain Res. 121, 55–65.

Torsney, C., Fitzgerald, M., 2002. Age-dependent effects of peripheral inflammation on the electrophysiological properties of neonatal rat dorsal horn neurons. J. Neurophysiol. 87, 1311–1317.

Torsney, C., Fitzgerald, M., 2003. Spinal dorsal horn cell receptive field size is increased in adult rats following neonatal hindpaw skin injury. J. Physiol. 550, 255–261.

Torsney, C., MacDermott, A.B., 2006. Disinhibition opens the gate to pathological pain signaling in superficial neurokinin 1 receptor-expressing neurons in rat spinal cord. J. Neurosci. 26, 1833–1843.

Toth, C., Lander, J., Wiebe, S., 2009. The Prevalence and Impact of Chronic Pain with Neuropathic Pain Symptoms in the General Population. Pain Med. 10, 918–929.

Tracey, I., Mantyh, P.W., 2007. The Cerebral Signature for Pain Perception and Its Modulation. Neuron 55, 377–391.

Trang, T., Beggs, S., Wan, X., Salter, M.W., 2009. P2X4-receptor-mediated synthesis and release of brain-derived neurotrophic factor in microglia is dependent on calcium and p38-mitogen-activated protein kinase activation. J. Neurosci. 29, 3518–3528.

Treede, R.D., Meyer, R.A., Campbell, J.N., 1998. Myelinated mechanically insensitive afferents from monkey hairy skin: heat-response properties. J. Neurophysiol. 80, 1082–1093.

Treede, R.D., Meyer, R.A., Raja, S.N., Campbell, J.N., 1995. Evidence for two different heat transduction mechanisms in nociceptive primary afferents innervating monkey skin. J. Physiol. 483, 747–758.

Treede, R.-D., Meyer, R.A., Raja, S.N., Campbell, J.N., 1992. Peripheral and central mechanisms of cutaneous hyperalgesia. Prog. Neurobiol. 38, 397–421.

Tremblay, M.-È., Lowery, R.L., Majewska, A.K., 2010. Microglial interactions with synapses are modulated by visual experience. PLoS Biol. 8, e1000527.

Tsuda, M., Shigemoto-Mogami, Y., Koizumi, S., Mizokoshi, A., Kohsaka, S., Salter, M.W., Inoue, K., 2003. P2X4 receptors induced in spinal microglia gate tactile allodynia after nerve injury. Nature 424, 778–783.

Tsuda, M., Mizokoshi, A., Shigemoto-Mogami, Y., Koizumi, S., Inoue, K., 2004. Activation of p38 mitogen-activated protein kinase in spinal hyperactive microglia contributes to pain hypersensitivity following peripheral nerve injury. Glia 45, 89–95.

Tsuda, M., Kohro, Y., Yano, T., Tsujikawa, T., Kitano, J., Tozaki-Saitoh, H., Koyanagi, S., Ohdo, S., Ji, R.-R., Salter, M.W., Inoue, K., 2011. JAK-STAT3 pathway regulates spinal astrocyte proliferation and neuropathic pain maintenance in rats. Brain 134, 1127–1139.

Tsuda, M., Masuda, T., Kitano, J., Shimoyama, H., Tozaki-Saitoh, H., Inoue, K., 2009. IFN-γ receptor signaling mediates spinal microglia activation driving neuropathic pain. Proc. Natl. Acad. Sci. 106, 8032–8037.

Tsuda, M., Beggs, S., Salter, M.W., Inoue, K., 2013. Microglia and intractable chronic pain. Glia 61, 55–61.

Tulic, M.K., Hodder, M., Forsberg, A., McCarthy, S., Richman, T., D'Vaz, N., van den Biggelaar, A.H., Thornton, C.A., Prescott, S.L., 2011. Differences in innate immune function between allergic and nonallergic children: new insights into immune ontogeny. J. Allergy Clin. Immunol. 127, 470–478.

Üçeyler, N., Schäfers, M., Sommer, C., 2009. Mode of action of cytokines on nociceptive neurons. Exp. Brain Res. 196, 67–78.

Ulmann, L., Hatcher, J.P., Hughes, J.P., Chaumont, S., Green, P.J., Conquet, F., Buell, G.N., Reeve, A.J., Chessell, I.P., Rassendren, F., 2008. Up-regulation of P2X4 receptors in

spinal microglia after peripheral nerve injury mediates BDNF release and neuropathic pain. J. Neurosci. 28, 11263–11268.

Urban, M.O., Gebhart, G.F., 1999. Supraspinal contributions to hyperalgesia. Proc. Natl. Acad. Sci. 96, 7687–7692.

Urch, C.E., Dickenson, A.H., 2003. In vivo single unit extracellular recordings from spinal cord neurones of rats. Brain Res. Protoc. 12, 26–34.

Vacca, V., Marinelli, S., Pieroni, L., Urbani, A., Luvisetto, S., Pavone, F., 2014. Higher pain perception and lack of recovery from neuropathic pain in females: A behavioural, immunohistochemical, and proteomic investigation on sex-related differences in mice. PAIN® 155, 388–402.

Van Montfrans, C., Hooijberg‡, E., Rodriguez Pena, M.S., de Jong, E.C., Spits, H., te Velde, A.A., van Deventer, S.J.H., 2002. Generation of regulatory gut-homing human T lymphocytes using ex vivo interleukin 10 gene transfer. Gastroenterology 123, 1877–1888.

Van Praag, H., Frenk, H., 1991. The development of stimulation-produced analgesia (SPA) in the rat. Dev. Brain Res. 64, 71–76.

Varrassi, G., Müller-Schwefe, G., Pergolizzi, J., Orónska, A., Morlion, B., Mavrocordatos, P., Margarit, C., Mangas, C., Jaksch, W., Huygen, F., Collett, B., Berti, M., Aldington, D., Ahlbeck, K., 2010. Pharmacological treatment of chronic pain – the need for CHANGE. Curr. Med. Res. Opin. 26, 1231–1245.

Vasudeva, K., Andersen, K., Zeyzus-Johns, B., Hitchens, T.K., Patel, S.K., Balducci, A., Janjic, J.M., Pollock, J.A., 2014. Imaging neuroinflammation in vivo in a neuropathic pain rat model with near-infrared fluorescence and 19F magnetic resonance. PloS One 9, e90589.

Vega-Avelaira, D., Moss, A., Fitzgerald, M., 2007. Age-related changes in the spinal cord microglial and astrocytic response profile to nerve injury. Brain. Behav. Immun. 21, 617–623.

Vega-Avelaira, D., Géranton, S.M., Fitzgerald, M., 2009. Differential regulation of immune responses and macrophage/neuron interactions in the dorsal root ganglion in young and adult rats following nerve injury. Mol Pain 5, 70.

Vega-Avelaira, D., McKelvey, R., Hathway, G., Fitzgerald, M., 2012. The emergence of adolescent onset pain hypersensitivity following neonatal nerve injury. Mol. Pain 8, 30.

Vera-Portocarrero, L.P., Zhang, E.-T., Ossipov, M.H., Xie, J.Y., King, T., Lai, J., Porreca, F., 2006. Descending facilitation from the rostral ventromedial medulla maintains nerve injury-induced central sensitization. Neuroscience 140, 1311–1320.

Verge, G.M., Milligan, E.D., Maier, S.F., Watkins, L.R., Naeve, G.S., Foster, A.C., 2004. Fractalkine (CX3CL1) and fractalkine receptor (CX3CR1) distribution in spinal cord and dorsal root ganglia under basal and neuropathic pain conditions. Eur. J. Neurosci. 20, 1150–1160.

Vikman, K.S., Hill, R.H., Backström, E., Robertson, B., Kristensson, K., 2003. Interferon-γ induces characteristics of central sensitization in spinal dorsal horn neurons in vitro. PAINÂ® 106, 241–251.

Vikman, K.S., Siddall, P.J., Duggan, A.W., 2005. Increased responsiveness of rat dorsal horn neurons< i> in vivo</i> following prolonged intrathecal exposure to interferon-γ. Neuroscience 135, 969–977.

Von Hehn, C.A., Baron, R., Woolf, C.J., 2012. Deconstructing the Neuropathic Pain Phenotype to Reveal Neural Mechanisms. Neuron 73, 638–652.

Wagner, R., Myers, R.R., 1996. Schwann cells produce tumor necrosis factor alpha: expression in injured and non-injured nerves. Neuroscience 73, 625–629.

Walco, G.A., Dworkin, R.H., Krane, E.J., LeBel, A.A., Treede, R.-D., 2010. Neuropathic Pain in Children: Special Considerations. Mayo Clin. Proc. 85, S33–S41.

Walczak, J.-S., Pichette, V., Leblond, F., Desbiens, K., Beaulieu, P., 2006. Characterization of chronic constriction of the saphenous nerve, a model of neuropathic pain in mice showing rapid molecular and electrophysiological changes. J. Neurosci. Res. 83, 1310–1322.

Waldenström, A., Thelin, J., Thimansson, E., Levinsson, A., Schouenborg, J., 2003. Developmental Learning in a Pain-Related System: Evidence for a Cross-Modality Mechanism. J. Neurosci. 23, 7719–7725.

Walker, S.M., Meredith-Middleton, J., Cooke-Yarborough, C., Fitzgerald, M., 2003. Neonatal inflammation and primary afferent terminal plasticity in the rat dorsal horn. Pain 105, 185–195.

Walker, S.M., Meredith-Middleton, J., Lickiss, T., Moss, A., Fitzgerald, M., 2007. Primary and secondary hyperalgesia can be differentiated by postnatal age and ERK activation in the spinal dorsal horn of the rat pup. Pain 128, 157–168.

Walker, S.M., Franck, L.S., Fitzgerald, M., Myles, J., Stocks, J., Marlow, N., 2009. Long-term impact of neonatal intensive care and surgery on somatosensory perception in children born extremely preterm. Pain 141, 79–87.

Walker, S.M., Westin, B.D., Deumens, R., Grafe, M., Yaksh, T.L., 2010. Effects of Intrathecal Ketamine in the Neonatal Rat: Evaluation of Apoptosis and Long-term Functional Outcome. Anesthesiology 113, 147–159.

Wall, P.D., Devor, M., Inbal, R., Scadding, J.W., Schonfeld, D., Seltzer, Z., Tomkiewicz, M.M., 1979. Autotomy following peripheral nerve lesions: experimental anesthesia dolorosa. Pain 7, 103–113.

Wall, P.D., Devor, M., 1983. Sensory afferent impulses originate from dorsal root ganglia as well as from the periphery in normal and nerve injured rats. Pain 17, 321–339.

Walsh, M.A., Graham, B.A., Brichta, A.M., Callister, R.J., 2009. Evidence for a critical period in the development of excitability and potassium currents in mouse lumbar superficial dorsal horn neurons. J. Neurophysiol. 101, 1800–1812.

Wang, F., Smith, N.A., Xu, Q., Fujita, T., Baba, A., Matsuda, T., Takano, T., Bekar, L., Nedergaard, M., 2012. Astrocytes modulate neural network activity by Ca2+-dependent uptake of extracellular K+. Sci. Signal. 5, ra26.

Wang, G., Ji, Y., Lidow, M.S., Traub, R.J., 2004. Neonatal hind paw injury alters processing of visceral and somatic nociceptive stimuli in the adult rat. J. Pain 5, 440–449.

Wang, H., Rivero-Melián, C., Robertson, B., Grant, G., 1994. transganglionic transport and binding of the isolectin B4 from Griffonia simplicifolia I in rat primary sensory neurons. Neuroscience 62, 539–551.

Wang, J., Gu, C., Al-Chaer, E.D., 2008. Altered behavior and digestive outcomes in adult male rats primed with minimal colon pain as neonates. Behav. Brain Funct. 4, 28.

Wang, W., Mei, X.-P., Wei, Y.-Y., Zhang, M.-M., Zhang, T., Wang, W., Xu, L.-X., Wu, S.-X., Li, Y.-Q., 2011. Neuronal NR2B-containing NMDA receptor mediates spinal astrocytic c-Jun N-terminal kinase activation in a rat model of neuropathic pain. Brain. Behav. Immun. 25, 1355–1366.

Wang, Z., Gardell, L.R., Ossipov, M.H., Vanderah, T.W., Brennan, M.B., Hochgeschwender, U., Hruby, V.J., Malan, T.P., Lai, J., Porreca, F., 2001. Pronociceptive Actions of Dynorphin Maintain Chronic Neuropathic Pain. J. Neurosci. 21, 1779–1786.

Watanabe, M., Mishina, M., Inoue, Y., 1994. Distinct spatiotemporal distributions of the N-Methyl-D-aspartate receptor channel subunit mRNAs in the mouse cervical cord. J. Comp. Neurol. 345, 314–319.

Webster, R.B., Rodriguez, Y., Klimecki, W.T., Vercelli, D., 2007. The human IL-13 locus in neonatal CD4+ T cells is refractory to the acquisition of a repressive chromatin architecture. J. Biol. Chem. 282, 700–709.

Weinstein, S., Sersen, E.A., Vetter, R.J., 1964. Phantoms and Somatic Sensation in Cases of Congenital Aplasia. Cortex 1, 276–290.

Welser-Alves, J.V., Milner, R., 2013. Microglia are the major source of TNF-α and TGF-β1 in postnatal glial cultures; regulation by cytokines, lipopolysaccharide, and vitronectin. Neurochem. Int. 63, 47–53.

Wen, Y.-R., Suter, M.R., Kawasaki, Y., Huang, J., Pertin, M., Kohno, T., Berde, C.B., Decosterd, I., Ji, R.-R., 2007. Nerve conduction blockade in the sciatic nerve prevents but does not reverse the activation of p38 mitogen-activated protein kinase in spinal microglia in the rat spared nerve injury model. Anesthesiology 107, 312–321.

Werry, E.L., Liu, G.J., Lovelace, M.D., Nagarajah, R., Hickie, I.B., Bennett, M.R., 2011. Lipopolysaccharide-stimulated interleukin-10 release from neonatal spinal cord microglia is potentiated by glutamate. Neuroscience 175, 93–103.

Westin, B.D., Walker, S.M., Deumens, R., Grafe, M., Yaksh, T.L., 2010. Validation of a Preclinical Spinal Safety Model: Effects of Intrathecal Morphine in the Neonatal Rat. Anesthesiology 113, 183–199.

White, F.A., Bhangoo, S.K., Miller, R.J., 2005. Chemokines: integrators of pain and inflammation. Nat. Rev. Drug Discov. 4, 834–844.

Whiteside, G., Doyle, C.A., Hunt, S.P., Munglani, R., 1998. Differential time course of neuronal and glial apoptosis in neonatal rat dorsal root ganglia after sciatic nerve axotomy. Eur. J. Neurosci. 10, 3400–3408.

Whiteside, G. t., Munglani, R., 2001. Cell death in the superficial dorsal horn in a model of neuropathic pain. J. Neurosci. Res. 64, 168–173.

Wiesenfeld-Hallin, Z., 2005. Sex differences in pain perception. Gend. Med. 2, 137–145.

Williams, S., Evants, G., Hunt, P. 1990. Spinal c-fos induction by sensory stimulation in neonatal rats. Neuroscience letters. 109, 309-314.

Willis, W.D., Trevino, D.L., Coulter, J.D., Maunz, R.A., 1974. Responses of primate spinothalamic tract neurons to natural stimulation of hindlimb. J. Neurophysiol. 37, 358–372.

Wilson, P., Kitchener, P.D., 1996. Plasticity of cutaneous primary afferent projections to the spinal dorsal horn. Prog. Neurobiol. 48, 105–129.

Wollgarten-Hadamekl, I., Hohmeisterl, J., Zohsell, K., Florl, H., Hermannl, C., 2011. Do school-aged children with burn injuries during infancy show stress-induced activation of pain inhibitory mechanisms? Eur. J. Pain 15, 423–e1.

Woodbury, C.J., Koerber, H.R., 2003. Widespread Projections from Myelinated Nociceptors throughout the Substantia Gelatinosa Provide Novel Insights into Neonatal Hypersensitivity. J. Neurosci. 23, 601–610.

Woodbury, C.J., Kullmann, F.A., McIlwrath, S.L., Koerber, H.R., 2008. Identity of myelinated cutaneous sensory neurons projecting to nocireceptive laminae following nerve injury in adult mice. J. Comp. Neurol. 508, 500–509.

Woolf, C.J., 1983. Evidence for a central component of post-injury pain hypersensitivity. Nature 306, 686–688. doi:10.1038/306686a0

Woolf, C.J., Fitzgerald, M., 1983. The properties of neurones recorded in the superficial dorsal horn of the rat spinal cord. J. Comp. Neurol. 221, 313–328.

Woolf, C.J., Fitzgerald, M., 1986. Somatotopic organization of cutaneous afferent terminals and dorsal horn neuronal receptive fields in the superficial and deep laminae of the rat lumbar spinal cord. J. Comp. Neurol. 251, 517–531.

Woolf, C.J., Wall, P.D., 1986. Relative effectiveness of C primary afferent fibers of different origins in evoking a prolonged facilitation of the flexor reflex in the rat. J. Neurosci. 6, 1433–1442.

Woolf, C.J., King, A.E., 1990. Dynamic alterations in the cutaneous mechanoreceptive fields of dorsal horn neurons in the rat spinal cord. J. Neurosci. 10, 2717–2726.

Woolf, C.J., Thompson, S.W.N., 1991. The induction and maintenance of central sensitization is dependent on N-methyl-d-aspartic acid receptor activation; implications for the treatment of post-injury pain hypersensitivity states. Pain 44, 293–299.

Woolf, C.J., Walters, E.T., 1991. Common patterns of plasticity contributing to nociceptive sensitization in mammals and Aplysia. Trends Neurosci. 14, 74–78.

Woolf, C.J., Safieh-Garabedian, B., Ma, Q.-P., Crilly, P., Winter, J., 1994. Nerve growth factor contributes to the generation of inflammatory sensory hypersensitivity. Neuroscience 62, 327–331.

Woolf, C.J., Allchorne, A., Safieh-Garabedian, B., Poole, S., 1997. Cytokines, nerve growth factor and inflammatory hyperalgesia: the contribution of tumour necrosis factor α. Br. J. Pharmacol. 121, 417–424.

Woolf, C.J., Mannion, R.J., 1999. Neuropathic pain: aetiology, symptoms, mechanisms, and management. Lancet 353, 1959–1964.

Woolf, C.J., Salter, M.W., 2000. Neuronal plasticity: increasing the gain in pain. Science 288, 1765–1768.

Wood, J.N., Boorman, J.P., Okuse, K., Baker, M.D., 2004. Voltage-gated sodium channels and pain pathways. J. Neurobiol. 61, 55–71.

Woolf, C.J., Ma, Q., 2007. Nociceptors--noxious stimulus detectors. Neuron 55, 353–364. doi:10.1016/j.neuron.2007.07.016

Woolf, C.J., 2011. Central sensitization: implications for the diagnosis and treatment of pain. Pain 152, S2–S15.

Wu, G., Ringkamp, M., Hartke, T.V., Murinson, B.B., Campbell, J.N., Griffin, J.W., Meyer, R.A., 2001. Early onset of spontaneous activity in uninjured C-fiber nociceptors after injury to neighboring nerve fibers. J. Neurosci. Off. J. Soc. Neurosci. 21, RC140–RC140.

Wu, J., Fang, L., Lin, Q., Willis, W.D., 2001. Nitric oxide synthase in spinal cord central sensitization following intradermal injection of capsaicin. Pain 94, 47–58.

Xie, W., Strong, J.A., Zhang, J.-M., 2009. Early blockade of injured primary sensory afferents reduces glial cell activation in two rat neuropathic pain models. Neuroscience 160, 847–857.

Xie, Y.K., Xiao, W.H., 1990. Electrophysiological evidence for hyperalgesia in the peripheral neuropathy. Sci. China B 33, 663–672.

Xu, J.-T., Xin, W.-J., Zang, Y., Wu, C.-Y., Liu, X.-G., 2006. The role of tumor necrosis factor-alpha in the neuropathic pain induced by Lumbar 5 ventral root transection in rat. PAIN 123, 306–321.

Xu, Z.-Z., Zhang, L., Liu, T., Park, J.Y., Berta, T., Yang, R., Serhan, C.N., Ji, R.-R., 2010. Resolvins RvE1 and RvD1 attenuate inflammatory pain via central and peripheral actions. Nat. Med. 16, 592–597.

Xu, Z.-Z., Berta, T., Ji, R.-R., 2013. Resolvin E1 inhibits neuropathic pain and spinal cord microglial activation following peripheral nerve injury. J. Neuroimmune Pharmacol. Off. J. Soc. NeuroImmune Pharmacol. 8, 37–41.

Yip, H.K., Rich, K.M., Lampe, P.A., Johnson, E.M., 1984. The effects of nerve growth factor and its antiserum on the postnatal development and survival after injury of sensory neurons in rat dorsal root ganglia. J. Neurosci. 4, 2986–2992.

Yoon, Y.W., Na, H.S., Chung, J.M., 1996. Contributions of injured and intact afferents to neuropathic pain in an experimental rat model. Pain 64, 27–36.

Zeilhofer, H.U., 2008. Loss of glycinergic and GABAergic inhibition in chronic pain—contributions of inflammation and microglia. Int. Immunopharmacol. 8, 182–187.

Zhang, H., Nei, H., Dougherty, P.M., 2010. A p38 Mitogen-Activated Protein Kinase-Dependent Mechanism of Disinhibition in Spinal Synaptic Transmission Induced by Tumor Necrosis Factor-α. J. Neurosci. 30, 12844–12855.

Zhang, J., Shi, X.Q., Echeverry, S., Mogil, J.S., De Koninck, Y., Rivest, S., 2007. Expression of CCR2 in both resident and bone marrow-derived microglia plays a critical role in neuropathic pain. J. Neurosci. 27, 12396–12406.

Zhang, L., Berta, T., Xu, Z.-Z., Liu, T., Park, J.Y., Ji, R.-R., 2011. TNF-alpha contributes to spinal cord synaptic plasticity and inflammatory pain: Distinct role of TNF receptor subtypes 1 and 2. PAIN® 152, 419–427.

Zhang, W., Gardell, S., Zhang, D., Xie, J.Y., Agnes, R.S., Badghisi, H., Hruby, V.J., Rance, N., Ossipov, M.H., Vanderah, T.W., Porreca, F., Lai, J., 2009. Neuropathic pain is maintained by brainstem neurons co-expressing opioid and cholecystokinin receptors. Brain 132, 778–787.

Zhang, X., Huang, J., McNaughton, P.A., 2005. NGF rapidly increases membrane expression of TRPV1 heat-gated ion channels. EMBO J. 24, 4211–4223.

Zhang, Y.-H., Wang, X.-M., Ennis, M., 2010. Effects of neonatal inflammation on descending modulation from the rostroventromedial medulla. Brain Res. Bull. 83, 16–22.

Zheng, W., Flavell, R.A., 1997. The Transcription Factor GATA-3 Is Necessary and Sufficient for Th2 Cytokine Gene Expression in CD4 T Cells. Cell 89, 587–596.

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

Zhuang, Z.-Y., Wen, Y.-R., Zhang, D.-R., Borsello, T., Bonny, C., Strichartz, G.R., Decosterd, I., Ji, R.-R., 2006. A peptide c-Jun N-terminal kinase (JNK) inhibitor blocks mechanical allodynia after spinal nerve ligation: respective roles of JNK activation in primary sensory neurons and spinal astrocytes for neuropathic pain development and maintenance. J. Neurosci. 26, 3551–3560.

Zhuo, M., Gebhart, G.F., 1997. Biphasic Modulation of Spinal Nociceptive Transmission From the Medullary Raphe Nuclei in the Rat. J. Neurophysiol. 78, 746–758.

Zhuo, M., Gebhart, G.F., 1992. Characterization of descending facilitation and inhibition of spinal nociceptive transmission from the nuclei reticularis gigantocellularis and gigantocellularis pars alpha in the rat. J. Neurophysiol. 67, 1599–1614.

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