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Novel analgesic interventions in cancer-induced bone pain

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DECLARATION

I hereby declare that this thesis and the work presented in it are entirely my own, with the exception of the Chapter 5 pharmacology data carried out by Dr Darren Robertson and Dr Emer Garry, the Chapter 5 immunohistochemical analysis contributed to by Heather Anderson and Dr Ada Delaney and the intrathecal injections and some behavioural work carried out by Dr Ada Delaney. Assistance and advice were given by many individuals who are acknowledged overleaf. Some of the data included in this thesis have been published as poster and oral communications and are included in the appendix.

Gillian Currie

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ABSTRACT

Cancer-induced bone pain (CIBP), due to bony metastases, is a major clinical problem, significantly reducing quality of life in cancer patients. Current therapies often provide inadequate analgesia or unacceptable side effects. The aim of this thesis was to characterise behaviours of a preclinical model of CIBP and test novel analgesic interventions in this model. A secondary aim was to investigate the involvement of the N-methyl-D-Aspartate (NMDA) receptors and TRP channels (TRPM8, TRPV1 and TRPV4) in CIBP. Investigation of CIBP in a preclinical model may lead to better pain management in CIBP patients.

The results presented here demonstrate that this model of CIBP develops behaviours that may be indicative of mechanical allodynia, thermal sensitivity, movement-evoked pain, ongoing pain and spontaneous pain. This suggests that this model reflects the clinical condition of CIBP, where patients suffer from constant background pain with spontaneous and movement-related breakthrough pain.

In this study it was found that radiotherapy significantly attenuated movement-evoked pain and thermal sensitivity to 20°C and 40°C. XRT also significantly reduced anxiety and risk assessment behaviours (grooming behaviour and number of protected stretch attends) compared to untreated CIBP. Duloxetine attenuated CIBP-induced mechanical allodynia, thermal sensitivity to 40°C and movement-evoked pain, whereas S,S-reboxetine attenuated thermal sensitivity to 40°C but did not effect CIBP-induced mechanical allodynia or movement-evoked pain. In addition, CB 65 attenuated movement-evoked pain and thermal sensitivity to 40°C. A single dose of gabapentin did not attenuate CIBP-induced mechanical allodynia, thermal sensitivity to 40°C or movement-evoked pain. These studies confirm that the CIBP model shows characteristics and pharmacological sensitivities consistent with known and predicted mechanisms and validate it as a useful model for assessing potential new treatments proposed for use in patients.

Behavioural results suggest that NMDA receptors containing the NR2A subunit are involved in CIBP-induced movement-evoked pain. This suggests that NR2A antagonists may be useful for treating CIBP-induced movement-evoked pain. Additionally, results show that there is increased expression of NR2A in the laminae I, II and III in the dorsal horn of the spinal cord. XRT treated animals also showed increased expression of NR2A in laminae I and II. The selective involvement of NR2A in CIBP is different to other chronic pain states, for example, neuropathic pain states that appear to involve the NR2B subunit.

The TRPV1 antagonist AMG 9810 did not attenuate mechanical allodynia, thermal sensitivity to 40°C or movement-evoked pain. Interestingly, the TRPM8 agonist icilin attenuated movement-evoked pain, which suggests that icilin might be useful in the treatment of movement-evoked pain. The TRPV4 antagonist RN 1734 attenuated mechanical allodynia, thermal sensitivity to 40°C and movement-evoked pain in CIBP. This suggests RN 1734 may be useful in the treatment of mechanical allodynia, thermal sensitivity to 40°C and movement-evoked pain in CIBP. Results show that the expression of TRPV4 is increased in DRG ipsilateral to the cancer-bearing tibia.

In conclusion, these results show that the preclinical model of CIBP investigated in this thesis is suitable for testing novel analgesic interventions. This thesis identified some useful targets for the analgesic treatment of CIBP and results suggest that many different mechanisms contribute to CIBP. A point to consider is that any robust effective treatment may need to target all (or at least several) of these mechanisms.

ABBREVIATIONS

AMPA	amino-3-hydroxy-5-methyl-4-isoxazole propionate
ASIC	acid-sensing ion channel
ATF3	activating transcription factor-3
ATP	adenosine triphosphate
BDNF	brain-derived neurotrophic factor
CaMK	Ca^{2+}/calmodulin dependent protein kinase
cAMP	cyclic adenosine monophosphate
CBD	cannabidiol
CCD	chronic compression of dorsal root ganglia
CCI	chronic constriction injury
CCR2	chemotactic cytokine receptor
CFA	Complete Freund's Adjuvant
cGMP	cyclic guanosine monophosphate
CGRP	calcitonin gene-related peptide
CIBP	cancer-induced bone pain
CNS	central nervous system
CREB	cAMP response element binding
CRPS	complex regional pain syndrome
DRG	dorsal root ganglion
EPSC	excitatory postsynaptic current
EPSP	excitatory postsynaptic potential
ERK	extracellular signal-regulated kinase
GABA	gamma-aminobutyric acid
GFP	green fluorescent protein
IASP	International Association for the Study of Pain
IL-1β	interleukin-1beta
IL-6	interleukin-6
INF-β	interferon-beta
LTP	long term potentiation
MAGUK	membrane-associated guanylate kinase

mGlu	metabotropic glutamate
Mrgprd	mas-related G-protein-coupled receptors
NA	noradrenaline
NGF	nerve growth factor
NMDA	N-methyl-D-Aspartate
NO	nitric oxide
NS	nociceptive specific
NSAID	non-steroidal anti-inflammatory drug
PAG	periaqueductal grey
PBS	phosphate buffer saline
PDE	phosphodiesterase
PFA	paraformaldehyde
PKA	protein kinase A
PKC	protein kinase C
PoT	posterior triangular nucleus
PSD-93	postsynaptic density-93
PSD-95	postsynaptic density-95
PSNL	partial sciatic nerve ligation
PWT	paw withdrawal threshold
P2X receptor	purinergic receptor subtype X
SAP-97	synapse-associated protein-97
SAP-102	synapse-associated protein-102
SEM	standard error of the mean
siRNA	small interfering RNA
SNI	spinal nerve injury
SNL	spinal nerve ligation
SNRI	serotonin-noradrenaline reuptake inhibitor
SP	Substance P
SSRI	selective serotonin reuptake inhibitor
TCA	tricyclic anti-depressant
THC	tetrahydrocannabinol
TNF-α	tumour necrosis factor-alpha

TRP	transient receptor potential
VDCC	voltage-dependent calcium channels
VEGF	vascular endothelial growth factor
WDR	wide dynamic range
WHO	World Health Organisation
XRT	radiotherapy

1. INTRODUCTION

1.1 An Introduction to Pain Processing

Pain is defined by the International Association for the Study of Pain (IASP) as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage". Nociception is the neural process of encoding noxious input without the emotional input. Noxious stimuli can be mechanical, thermal or chemical that cause, or have the potential to cause, tissue damage. This noxious input is detected in the periphery and is transmitted to the brain via the spinal cord.

Physiological pain is a protective event allowing avoidance of, or withdrawal from, a noxious stimulus. If the stimulus is of short duration or does not cause extensive tissue damage, the pain only lasts for the duration of the stimulus. In chronic pain syndromes, pathological changes occur such that pain no longer acts to protect but persists longer than the noxious stimulus or longer than recovery from initial injury or inflammation (Woolf, 1989). This is because the central nervous system is a plastic system, where changes can be long-lasting. Neuropathic pain is caused by damage or lesion to the peripheral or central nervous system and this type of pain frequently persists. Inflammatory pain is caused by the endogenous immune response to trauma and/or pathogen invasion. The subject of this study is cancer-induced bone pain (CIBP), which can occur in patients with primary bone tumours and more commonly in patients with bone cancers that have metastasized to bone from distant primary sites. Patients with metastatic prostate, breast or lung cancer frequently suffer from CIBP (Mercadante, 1997). CIBP is a major clinical challenge, with limited effective therapies and significantly reduces the quality of life of cancer patients (Weinfurt et al., 2005). CIBP is a unique pain state, with aspects of neuropathic and inflammatory pain (Urch, 2004). This multi-component nature of CIBP means that it is difficult to treat using standard therapies and to allow us to improve the treatment of CIBP we must understand the underlying mechanisms. The focus of this study will be CIBP, however before going on to review the mechanisms of CIBP, the basic neurobiology of pain perception will be reviewed.

1.2 Peripheral Transduction of Sensory Information

Sensory information is transduced in the periphery by receptors present on primary afferents. These receptors are activated by a variety of stimuli including thermal, chemical and mechanical challenges and this sensory information is transduced into action potentials. Primary afferents enter the nervous system and synapse within the dorsal horn of the spinal cord and are termed pseudounipolar because they have an axon with a terminal at the periphery and a central axon with a terminal at the spinal cord (Basbaum et al., 2009). For the main part of the body, primary afferents have their cell bodies located in the dorsal root ganglion (DRG), or for the head, in the corresponding trigeminal ganglia.

Anatomically, there are 2 broad groups of primary afferents: myelinated A-fibres and unmyelinated C-fibres. Primary afferents can be subdivided into three subtypes organised by diameter and conduction properties; myelinated Aβ-fibres and Aδ-fibres as well as unmyelinated C-fibres (Table 1.1). C-fibres can be further divided into two classes dependent on the expression of neuropeptides: peptidergic and non-peptidergic. Peptidergic C-fibres express substance P and calcitonin gene-related peptide (CGRP) and non-peptidergic C-fibres bind the lectin isolectin B4 (IB4) from *Griffonica simplicifolia* and express the ATP receptor $P2X_3$ and Mas-related G-protein-coupled receptors (Mrgprd) (Basbaum et al., 2009). The fibres that detect noxious stimuli are termed nociceptors and generally fall into the C-fibre or Aδ-fibre categories.

Aδ-fibres are classified as either low-threshold D-hair mechanoreceptors that detect innocuous stimuli or mechanonociceptors that detect high intensity, noxious stimuli including thermal stimuli. Aδ-fibres lose their myelin sheath and terminate as free nerve endings in the epidermis (Smith & Lewin, 2009). C-fibres also terminate as free nerve endings in the epidermis and the most common C-fibres observed are described as polymodal because they respond to mechanical, thermal and chemical noxious stimuli (Dubin & Patapoutian, 2010). A small population of C-fibres have been shown to be activated by innocuous stimili (Smith & Lewin, 2009). Aβ-fibres are associated with specialised non-neuronal structures conferring sensitivity to light

touch, stretch, vibration and hair movement such as hair follicles and Meissner corpuscles (Smith & Lewin, 2009).

Fibres	Myelinated/ Unmyelinated	Diameter (μm)	Conduction velocities (m/s)	Function
Aβ-fibres	Myelinated	>10	>10	Innocuous information
Aδ-fibres	Myelinated	2-6	2-10	Innocuous and noxious information
C-fibres	Unmyelinated	0.4-1.2	<1.5	Innocuous and noxious information

Table 1.1 Properties of primary afferents. Diameters from (Millan, 1999) and conduction velocities from (Smith & Lewin, 2009).

1.2.1 Nociceptors

Sherrington was the first to propose, in 1906, the existence of specialised sensory neurons for detecting noxious stimuli (Sherrington, 1906). Many years later, The Gate Control Theory of Pain was proposed by Melzack and Wall. The basic concept of this theory was that incoming painful stimuli can be 'gated' such that activity in other primary afferents such as low threshold touch receptors and descending from supraspinal levels can prevent transmission of pain signals (Melzack & Wall, 1965). This theory remains relevant to our understanding of nociception. In fact, descending pathways from the brainstem can inhibit or facilitate pain signalling. The Gate Control Theory however did presume that specialised neurons for detecting pain did not exist and that there were no specific pain receptors or dedicated central pain pathways. The original theory was challenged by an electrophysiological study carried out by Burgess and Perl who documented fibres responding specifically to noxious stimulation of the skin (Burgess & Perl, 1967).

Nociceptors are now widely accepted as a separate class of primary sensory neurons, which discriminate between noxious and innocuous stimuli.

Of the two main classes of nociceptors, Aδ-nociceptors are thought to be responsible for 'first' pain like the pain of a pinprick whereas C-fibre nociceptors are responsible for 'second' pain such as slow burning pain (Julius & Basbaum, 2001). Nociceptors have higher thresholds to stimuli when compared to low-threshold sensory neurons and detect a wide range of high intensity stimuli of mechanical, thermal and chemical modalities (Bessou et al., 1969). These stimuli are detected by ion channels and receptors present on the free nerve endings innervating the skin at the peripheral terminals of nociceptors. Activation of these ion channels and receptors leads to transduction, where stimuli are encoded in depolarisation, which if sufficient can lead to the generation of an action potential. The action potentials are then conducted along the peripheral axon to the cell body and then along the central axon of nociceptors to the dorsal horn of the spinal cord, where nociceptors synapse with central neurons.

The release of neurotransmitters from nociceptor central terminals activates second order neurons at central synapses. Nociceptors can release many substances potentially involved in central transmission and modulation of nociceptive information. Nociceptors are thought to release glutamate as their primary neurotransmitter; neuropeptides such as substance P and CGRP; adenosine triphosphate (ATP), nitric oxide, prostaglandins and neurotrophins such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF).

Nociceptors (or the ways in which central neurons respond to their activation) do not have fixed properties but display extensive plasticity, demonstrated by sensitisation in chronic pain states. This manifests when 'silent' C-fibres become responsive to noxious stimuli in the setting of injury or inflammation (Julius & Basbaum, 2001). Nociceptive processing also displays plasticity with increased response to sub-threshold stimuli and/or an increased magnitude of response of dorsal horn neurons (discussed in Section 1.7). Sensitisation occurs peripherally

through modulation of ion channels by processes such as intracellular signalling cascades.

1.3 Ion Channels and Transducers of Noxious Stimuli on Primary Afferents

There are many ion channels and receptors expressed on primary afferents (Figure 1.1). The importance of ion channels in neuronal generation of action potentials was originally suggested by Hodgkin and Huxley (Hodgkin & Huxley, 1952a;Hodgkin & Huxley, 1952b), Depolarisation of primary afferent peripheral terminals (which must be of sufficient amplitude and duration) is required to generate and propagate action potentials. The opening of ion channels permeable to Na^+ and Ca^{2+} cause the membrane to depolarise. Any events that facilitate closure of active K^+ channels further depolarise the membrane and increase membrane resistance. The expression of particular ion channels and receptors on primary afferents can change in pathological pain states. The channels and receptors that are of interest in this project will now be discussed.

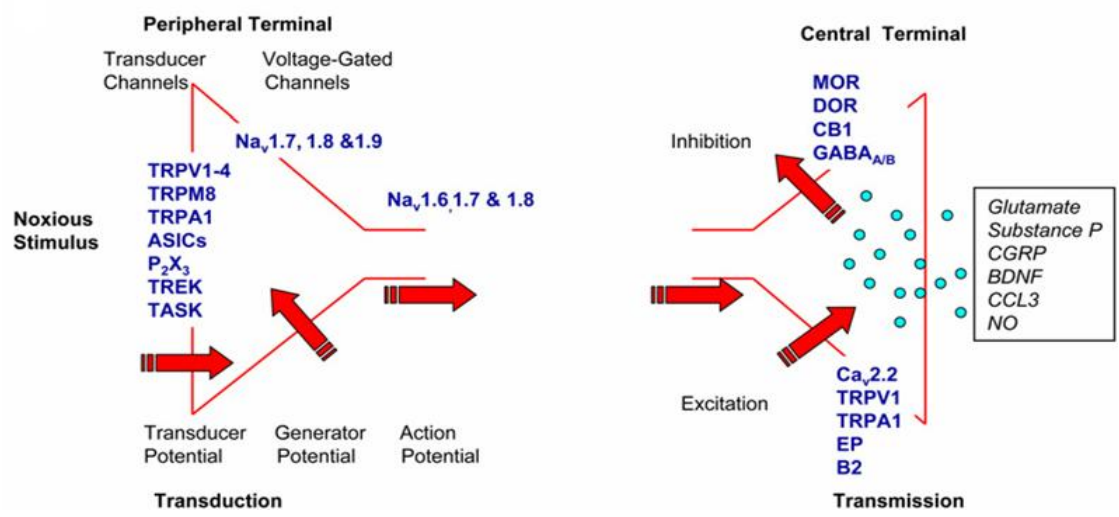


Figure 1.1 Peripheral and central terminals of the sensory neuron figure adapted from (Woolf & Ma, 2007). Noxious stimuli activate sensory neurons and transmit information to the CNS. Peripheral depolarisation may activate Na^+ channels such as Nav1.7 and 1.8. This leads to the generation of action potentials which transmit this information to central terminals. At central terminals, action potentials trigger the

release of neurotransmitters and neuropeptides which act on neurons in the spinal cord.

1.3.1 Voltage-dependent Sodium Channels

Sodium (Na^+) channels are necessary for the generation and propagation of action potentials. Na^+ channels open rapidly when the membrane is depolarised beyond -60 to -40mV. The opening of these channels and the influx of Na^+ rapidly depolarises the membrane. Sodium channels are composed of α subunits associated with auxiliary β-subunits. The α subunits are known as $Na_v1.1$ to $Na_v1.9$. $Na_v1.7$, $Na_v1.8$ and $Na_v1.9$ are expressed only in peripheral neurons (Dib-Hajj et al., 2009). $Na_v1.8$ has been shown to be essential for cold-induced pain (Zimmermann et al., 2007). Sodium channels in the adult CNS are associated with β-subunits β_1 to β_4 (Catterall et al., 2005). Sodium channels are one of the targets for local anaesthetic agents, anticonvulsants, NSAIDs and tricyclic compounds (Dib-Hajj et al., 2009).

1.3.2 Voltage-dependent Calcium Channels

Voltage-dependent calcium (Ca^{2+}) channels (VDCCs) open in response to depolarisation and increase intracellular Ca^{2+} concentration. This increase in intracellular Ca^{2+} concentration influences neurotransmitter release, membrane excitability and gene expression. VDCCs consist of α_1, β, $\alpha_2\delta$ and γ subunits. The α_1 subunit is the pore-forming subunit which is essential for voltage sensing, ion selectivity and permeability (Lee et al., 2005b). Different types of calcium channels are classified according to the α subunit (10 different α subunits exist). These are L-, P/Q-, N-, R- and T-type voltage-dependent calcium channels (Lee et al., 2005b). Voltage-dependent Ca^{2+} channels are important for nociception. N-type channel blockers administered intrathecally attenuates nerve injury-induced allodynia and decreases dorsal horn neuronal responses (Doan, 2010). Gabapentin and pregabalin interact with the $\alpha_2\delta$ subunit of VGCCs, the subunit which influences the function and expression of VGCCs. $\alpha_2\delta$-1 is upregulated in the DRG and spinal cord in animal models of neuropathic pain. Prolonged administration of gabapentin inhibits Ca^{2+} currents and decreases expression of $\alpha_2\delta$ subunits (Hendrich et al., 2008).

1.3.3 Potassium Channels

Potassium (K^+) channels stabilize the membrane potential by producing hyperpolarising outward currents. Opening of K^+ channels therefore results in decreased neuronal excitability. There are 4 different families of K^+ channels; Voltage-dependent K^+ (Kv) channels that open in response to depolarisation and shape the action potential, inwardly-rectifying potassium channels that are involved in cell signalling pathways, Ca^{2+}-activated K^+ channels that are responsible for shaping the after hyperpolarisation of the action potential and finally the two-pore domain K^+ (K2P) channels e.g. TREK1 or TRESK channels, which produce background or leak K^+ currents (Ocana et al., 2004). Kv channel expression is downregulated in a model of neuropathic pain (Kim et al., 2002) and there is a reduction in the density of voltage-dependent K^+ currents in a model of temporomandibular joint inflammation (Takeda et al., 2006). These findings suggest that Kv channels are involved in the development of neuropathic and inflammatory pain (Takeda et al., 2011). Activation of G protein-gated inwardly rectifying K^+ (GIRK) channels has been shown to be important for the analgesic effects induced by intrathecally administered morphine (Marker et al., 2004;Marker et al., 2005). Furthermore, in a mouse model of CIBP, it has been shown that activation of peripheral opioid receptors attenuates thermal hyperalgesia through activation of the nitric oxide/cyclic guanosine monophosphate (cGMP)/ATP-sensitive inwardly-rectifying K^+ channel cascade (Curto-Reyes et al., 2008;Menendez et al., 2007).

1.3.4 Acid-Sensing Ion Channels

Acid-sensing ion channels (ASICs) are activated by extracellular acidity, which can be a significant component of inflammation (Lingueglia, 2007). Activation of ASICs on primary afferents innervating the bone may contribute to CIBP (Yoneda et al., 2011), which is discussed in Section 1.10. The prototypical ASIC structure has two transmembrane domains and a large extracellular loop. In rodents, six subunits exist (ASIC1a, ASIC1b, ASIC2a, AISC2b, ASIC3 and ASIC4) (Deval et al., 2010). Three subunits are necessary to form a functional channel (Deval et al., 2010). Expression of homomeric ASIC1a channels is upregulated by peripheral inflammation (Duan et al., 2007).

1.3.5 P2X Receptors

P2X receptors are non-selective ion channels that are highly permeable to Ca^{2+} and gated by ATP, which in the context of chronic pain states may be released from injured afferents or central glial cells. Seven different subunits exist (P2X1-P2X7), which form homo- and hetero-tetramers (Khakh et al., 2001). P2X3 is highly expressed in nociceptors and can form homomeric or heteromeric channels (Dunn et al., 2001). Administration of P2X3 or P2X2/3 receptor antagonists have been reported to attenuate behavioural hypersensitivity in preclinical models of neuropathic pain, chronic inflammatory pain and CIBP (Kaan et al., 2010;Jarvis et al., 2002;McGaraughty et al., 2003).

1.3.6 TRP Channels

The mammalian Transient Receptor Potential (TRP) family of ion channels consists of around 30 members, which can be divided into at least 6 subfamilies (Wu et al., 2010). In sensory neurons, TRP channels are important for the transduction of thermal, chemical and mechanical signals. TRP proteins have 6 transmembrane domains with the pore region located between the 5th and 6th domains and cytoplasmic N- and C- termini (Wu et al., 2010). TRP channels are homo- or heterotetramers of TRP proteins (Nilius et al., 2007b). All TRP channels are nonselective cation-permeable channels with a high permeability to Ca^{2+} with the exception of TRPM4/5, which is a monovalent cation channel (Gees et al., 2010). Depolarizing currents through TRP channels can lead to the generation of action potentials and therefore the release of neurotransmitters.

The TRP family includes several ion channels that are selectively expressed in primary afferents and respond to temperature stimuli; these are known as thermoTRPs. Activation of these thermoTRPs appears to be the best candidate mechanism for peripheral thermosensation (Ramsey et al., 2006). ThermoTRPs are activated by temperatures ranging from noxious cold to noxious heat and also respond to chemical activators and a number of chemical and environmental irritants summarised below (Table 1.2). ThermoTRPs (discussed further in Section 6.1) are members of the TRPV (Vanilloid), TRPM (Melastatin) and TRPA (Ankyrin)

subfamilies. Temperatures below 10°C or above 52°C are generally considered to be associated with the sensation of pain.

TRP family	TRP channel	Temperature sensitivity	Non-thermal agonists	Locations found
TRPA	TRPA1	< 17°C	Mustard oil, cinnamaldehyde, icilin	Peripheral nervous system, hair cells
TRPV	TRPV1	> 43°C	Capsaicin, various vanilloids compounds, acidic pH, nitric oxide, arachidonic acid metabolites	Peripheral nervous system, brain, spinal cord, skin, tongue, bladder
	TRPV2	> 52°C	growth factors (mouse)	Peripheral nervous system, brain, spinal cord
	TRPV3	33-39°C	Camphor, nitric oxide, arachidonic acid, unsaturated fatty acids	Peripheral nervous system, skin
	TRPV4	27-34°C	Osmolarity, phorbol esters, low pH, citrate, endocannabinoids	Peripheral nervous system, kidney, skin, inner ear, trachea, heart
TRPM	TRPM8	8-28°C	Menthol, icilin, eucalyptol	Peripheral nervous system, bladder, prostate

Table 1.2 Properties of ThermoTRP channels implicated in somatosensory signal transduction. Table adapted from (Dhaka et al., 2006;Wu et al., 2010).

1.4 Dorsal Root Ganglia

The cell bodies of sensory neurons are located in the DRG located close to either side of the spinal cord or in the trigeminal ganglia at the base of the skull. The cell bodies of primary afferents can be characterised by the expression of neurofilament proteins (NF), CGRP, substance P, nitric oxide synthase and IB4 binding sites.

1.5 Central Transduction of Sensory Information

1.5.1 The Spinal Cord

The neuronal grey matter of the spinal cord is organised into ten cell layers known as laminae (Figure 1.2). This anatomical division, proposed by Rexed, is consistent between mammalian species (Rexed, 1952). The dorsal horn of the spinal cord comprises laminae I-VI and receives the majority of primary afferents. The ventral horn comprises laminae VII-X and contains motorneurons as well as a large number of interneurons.

The majority of nociceptors synapse in the superficial laminae I and II with a smaller number of Aδ-nociceptors synapsing in lamina V (Millan, 1999). More specifically, Aδ-nociceptors project to laminae I and V and C-fibre nociceptors project predominantly to laminae I and II (Basbaum et al., 2009). The tactile Aβ-fibres terminate in laminae III, IV and V (Millan, 1999).

In the dorsal horn, primary afferents synapse with projection neurons and these neurons contribute to ascending pathways which transmit signals to higher centres. Projection neurons are classed as; nociceptive specific (NS) cells, which respond selectively to noxious stimuli, wide dynamic range neurons (WDR) neurons, which respond to a full range of stimulation or those which respond only to innocuous mechanical or temperature information (Willis & Coggeshall, 1991). NS cells are mainly found in laminae I-II.

The majority of neurons in the dorsal horn of the spinal cord are interneurons (Willis & Coggeshall, 1991), which receive synaptic connections and synapse with other neurons in the dorsal horn. There are also glial cells known as oligodendrocytes, astrocytes and microglia. Although the normal role of glial cells in the CNS may be to provide support for neurons, following injury to the central nervous system, glial cells can become activated and release a range of chemical signals that play a key role in chronic pain states (Cao & Zhang, 2008).

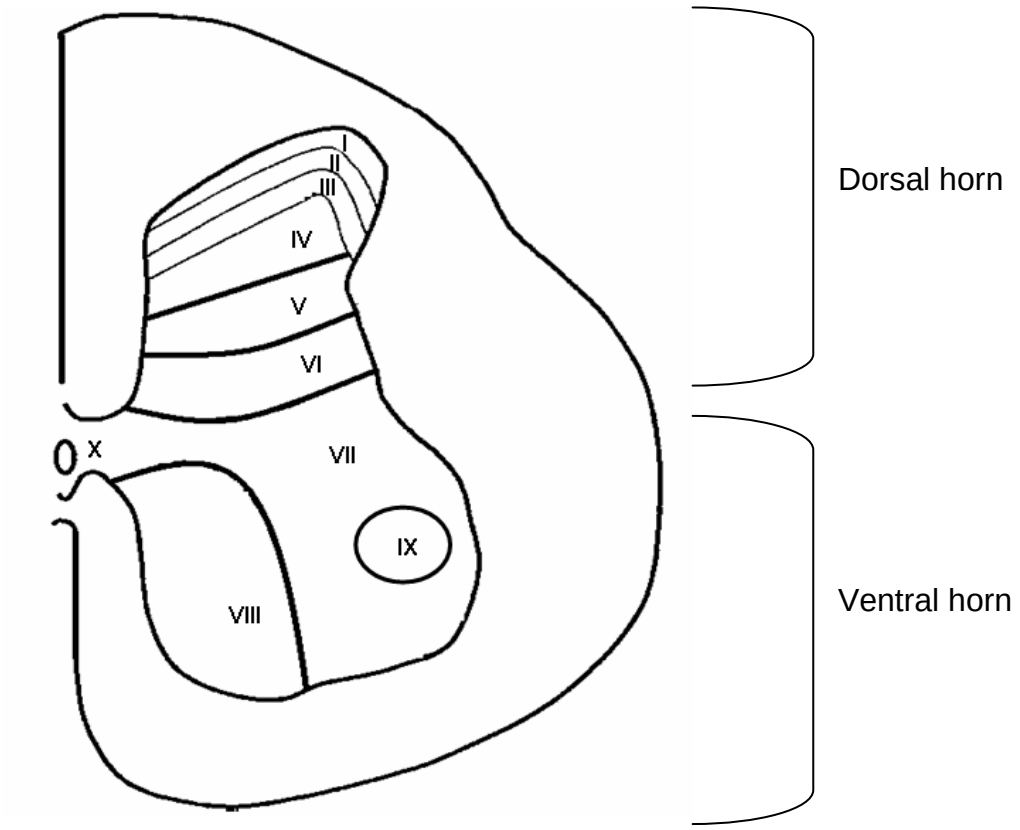


Figure 1.2 Anatomical laminae of the spinal cord according to Rexed (Rexed, 1952).

1.5.2 Ascending Somatosensory Pathways

There are a number of ascending pathways transmitting somatosensory information from the spinal cord to supraspinal levels (Millan, 1999). The major ascending pathways for pain information are the spinothalamic tract and the spinoparabrachial pathway. Additional ascending pathways that can also contribute to pain signalling are the spinoreticulothalamic, spinomesencephalic and dorsal-column medial lemniscal tracts, detailed in Table 1.3.

Tract	Laminae of origin	Projections	Possible roles
Dorsal-column medial lemniscal	III-V (most) VI VII	Decussates in the medulla and projects to the thalamus and the somatosensory cortex	Fine touch, vibration and proprioception
Spinothalamic	I and III-VI	Crosses the midline of the spinal cord and projects to the thalamus	Perception and discriminative aspects of nociception
Spinoparabrachial	I and II (few)	Projects to the parabrachial nucleus to the amygdala and the hypothalamus	Emotional, autonomic and neuroendocrine aspects of nociception
Spinoreticulothalamic	VII and VIII	Projects to the reticular formation of the brainstem and parabrachial internal nucleus	Motor reaction, attention and motivational aspects of nociception
Spinomesencephalic	I-II, V/VI, VII/VIII and X	Projects to the midbrain and periaqueductal grey	Motor reaction, motivational, emotional and autonomic aspects of nociception

Table 1.3 Overview of ascending somatosensory pathways pathways adapted from (Millan, 1999) and (Gauriau & Bernard, 2002).

1.5.3 Ascending Nociceptive Pathways of Different Tissues

Information about noxious stimuli applied to different tissue sources appears to be relayed preferentially through different ascending spinal pathways. Research has suggested that while acute cutaneous nociception is relayed predominantly through the spinothalamic tract, visceral nociception maybe predominantly through dorsal column pathways (Palecek et al., 2002) and acute bone nociception may especially involve the spinoparabrachial pathway (Williams & Ivanusic, 2008).

Evidence on the nociceptive pathways from bone was provided by a study using injections of Fluorogold retrograde tracer and Fos immunohistochemistry to identify the spinal dorsal horn neurons activated by acute noxious mechanical stimulation of bone (Williams & Ivanusic, 2008).

1.5.4 The Pain Matrix

Neuroimaging has allowed investigations into the brain areas activated during pain signalling (Tracey & Mantyh, 2007). Because pain is a complex, subjective experience comprising sensory, cognitive and emotional components, many brain areas are activated during pain processing. Activation of ascending pathways to the brainstem and thalamus transmits pain signalling to various cortical and sub-cortical regions, regions known as 'the pain matrix'. The pain matrix represents brain regions involved in functions such as cognition, emotion, motivation as well as pain. The areas activated include, but are not restricted to, the anterior cingulate cortex (ACC), insula, frontal cortices, primary and secondary somatosensory cortices and the amygdala (Peyron et al., 2000). During each individual experience of pain a unique combination of these areas may be activated. The amygdala plays an important role in emotional responses, stress, depression and anxiety and is believed to be a critical component of the pain matrix (Neugebauer et al., 2004). Human imaging studies suggest that interactions between the prefrontal cortex and the amygdala provide emotional-affective modulation of cognitive function in pain (Neugebauer et al., 2009). Activity in the amygdala is required for the inhibition of pain during stress, but can also facilitate pain behaviours during anxiety and depression (Neugebauer et al., 2004).

1.5.5 Descending Pathways

Descending pathways arise from a number of supraspinal sites including the midbrain periaqueductal grey (PAG) and rostral ventral medulla (RVM). Descending pathways can be facilitatory as well as inhibitory (D'Mello & Dickenson, 2008). The PAG is interconnected with the hypothalamus and the amygdala, and also receives spinomesencephalic input. The PAG projects to the RVM, which sends output to dorsal horn laminae to influence nociceptive processing (Heinricher et al., 2009). In

the RVM there are two classes of neuron that project to the spinal cord; ON-cells which facilitate pain signalling and OFF-cell, which inhibit pain signalling (Fields, 2004). Descending inputs to the superficial dorsal horn predominantly involve the neurotransmitters serotonin (5-HT) and noradrenaline (Millan, 2002). The actions of neurotransmitters released into the dorsal horn of the spinal cord will be detailed in the following section.

The complex ascending and descending pathways and 'the pain matrix' highlight the integrative nature of pain signalling. The ascending and descending pain pathways also display extensive plasticity, which plays a major role in chronic pain states.

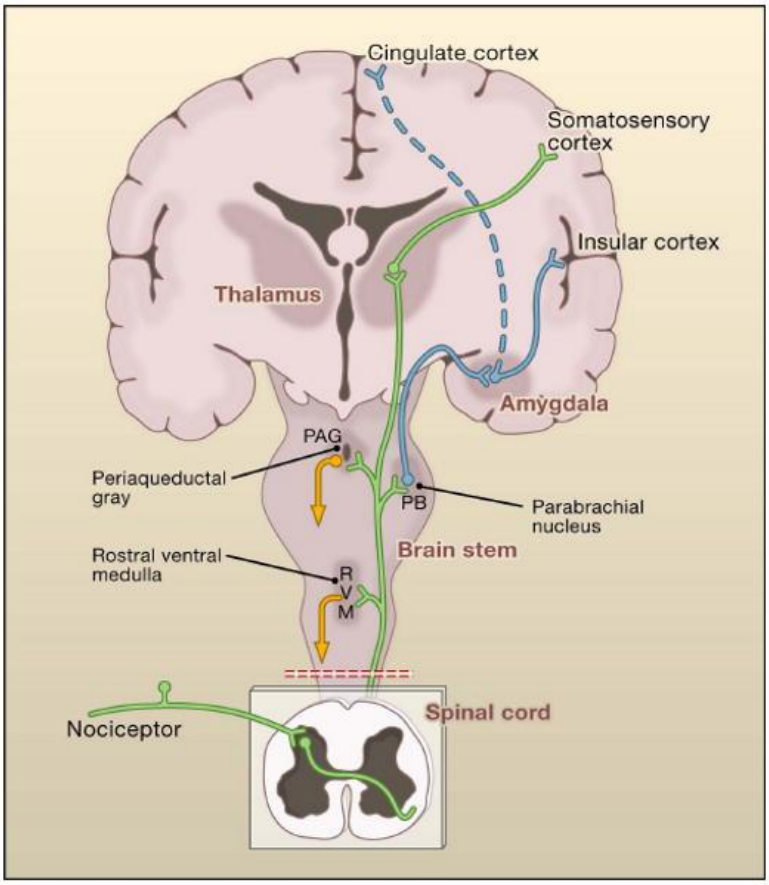


Figure 1.3 Schematic outline of the principle pathways involved in pain processing. Adapted from (Basbaum et al., 2009). Nociceptors convey noxious signals to the dorsal horn of the spinal cord. Projection neurons transmit information to the cortex.

1.6 Neurotransmitters and Peptides Involved in Pain Signalling

Intense mechanical, thermal or chemical stimuli are detected at the peripheral terminals of primary afferents and trigger action potentials that result in the release of a variety of neurotransmitters and neuropeptides. These neurotransmitters and neuropeptides act via postsynaptic receptors on dorsal horn neurons. Excitatory amino acids and several peptides have been implicated in synaptic transmission from nociceptors.

1.6.1 Neuropeptides

Substance P is a neuropeptide which may be co-released with glutamate from a major population of unmyelinated peptidergic neurons. Substance P acts on G protein-coupled receptors of the neurokinin (NK) group, which include subtypes NK1-3, and shows strong selectivity for the NK1 receptor (Quartara & Maggi, 1997). In lamina I, NK1 receptors are expressed over the soma and dendrites of large neurons whose dendrites radiate in lamina I only. Occasional NK1-positive somata are placed in lamina II, with dendrites that enter lamina I. In laminae III-IV NK1 receptor expressing neurones have a group of ascending dendrites that cross lamina II and then branch extensively in lamina I. The majority of NK1-positive neurons have rostrally projecting axons that project to the lateral PB nucleus and lateral reticular formation (Todd et al., 2000).

It has been shown that selective ablation of lamina I/II NK-1 receptor expressing neurones with a substance P-Saporin conjugate reduced pain sensitivity after injury by reducing the excitability of deep dorsal horn neurons (Nichols et al., 1999). A later study showed that this effect was through removal of the influence of NK1-expressing neurons which activate descending facilitatory pathways from the brainstem to the dorsal horn (Suzuki et al., 2002). This study also showed that NK1-expressing cells activate descending inhibitory pathways but to a lesser extent.

NK1 receptors are also present in a number of brain regions involved in the processing of nociception and supraspinal administration of substance P can produce analgesia. A study showed that substance P suppresses GABAergic transmission

onto identified PAG-RVM projection neurons by driving action potential-dependent activation of group I mGluRs and retrograde endocannabinoid signalling (Drew et al., 2009).

Calcitonin gene-related peptide (CGRP) is another neuropeptide and CGRP-positive cells often also express substance P and these two compounds can be co-released (Gibson *et al.*, 1984;Woolf & Wiesenfeld-Hallin, 1986). As well as acting on its own G protein-coupled receptors, CGRP modulates the effect of substance P by inhibiting substance P endopeptidase and therefore potentiating the effects of substance P (Le Greves et al., 1985).

Galanin is a neuropeptide normally expressed in primary afferents and interneurons. Galanin-positive neurons are found in DRG and also contain substance P and CGRP (Ju et al., 1987). Galanin-positive neurons are mainly localized in lamina II where it coexists with GABA, enkephalin and neuropeptide Y (Simmons et al., 1995;Zhang et al., 1995). Three subtypes of galanin receptors have been identified: GalR1, GalR2 and GalR3, these are G protein-coupled receptors. GalR1 is the prominent receptor in the dorsal horn (Brumovsky et al., 2006).

Galanin has both facilitatory and inhibitory effects on nociception, observed after intrathecal administration of galanin at low and high doses, respectively. This inhibitory effect is in part due to postsynaptic blockade of the excitatory effects of substance P and CGRP (Xu et al., 1990;Hua et al., 2005). Galanin may also reduce substance P release (Hua et al., 2005). Under normal conditions galanin plays a small antinociceptive role (Liu & Hokfelt, 2002;Xu *et al.*, 2000) but this role increases after nerve injury. Galanin is upregulated following peripheral nerve injury (Nahin et al., 1994) and it was shown in behavioural studies that reducing the action of galanin leads to increased neuropathic pain behaviours (Verge et al., 1993;Ji et al., 1994).

1.6.2 Glutamate

Glutamate is an excitatory amino acid and is the major excitatory neurotransmitter in the dorsal horn. Glutamate is released from primary afferents and

interneurons onto second order dorsal horn neurons and also from interneurons. Glutamate acts on ionotropic glutamate receptors of the amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA), N-methyl-D-aspartate (NMDA) and kainate subtypes, and also acts on several metabotropic glutamate receptors (Table 1.4). These glutamate receptors are expressed at all levels of the pain pathway; sensory neurons, DRG, spinal cord and brain regions associated with nociception. Most glutamate-mediated excitatory postsynaptic potentials (EPSPs) are composed of a fast AMPA receptor-mediated component that depolarises the cell and a slower NMDA receptor-mediated component. The depolarising effects of AMPA receptor activation and NMDA receptor function in nociceptive pathways contribute to the establishment and maintenance of chronic pain states (Millan, 1999).

AMPA Receptors

AMPA-type glutamate receptors are tetramers composed of a combination of four types of subunit (GluR1-4) (Hollmann & Heinemann, 1994). The AMPA receptor subunits are subject to alternative splicing and RNA editing (Seeburg & Hartner, 2003). The subunit combination, RNA editing and splice variant expression determine the channel's permeability to Ca^{2+} and other cations such as Na^{+} and K^{+} (Hollmann et al., 1991;Sommer et al., 1990;Sommer et al., 1991). GluR1 and GluR3 subunit incorporation into AMPA receptors increases Ca^{2+} permeability, whereas GluR2 incorporation into AMPA receptors strongly reduces permeability to Ca^{2+} (Burnashev et al., 1992). Synapses in the superficial dorsal horn generally contain GluR2, in combination with either GluR1 and GluR3 subunits or both (Polgar et al., 2008). AMPARs are necessary for synaptic plasticity in nociceptive processing (Section 1.7).

NMDA Receptors

NMDA-type glutamate receptors are tetrameric, made up of two obligatory NR1 subunits and two regulatory subunits that can be NR2A-D or NR3A-B to form a non-selective cation-permeable channel (Paoletti & Neyton, 2007). The subunit composition determines the pharmacological and physiological properties of NMDARs (Cull-Candy & Leszkiewicz, 2004). NMDA receptor subunits contain a

long extracellular N-terminal domain, 3 transmembrane domains, a re-entrant pore loop and an intracellular C-terminal domain (Mayer, 2005). NR1/NR2-containing NMDA receptors are normally blocked by Mg^{2+} ions and require depolarization, glutamate and the co-agonist glycine (Johnson & Ascher, 1987) or D-serine (Mothet et al., 2000) acting on the receptor for activation. The co-agonist binds to the NR1 subunit (Lynch et al., 1994), however the NR2 subunit determines the binding and potency of co-agonist. The extracellular amino terminal domain of the NR2 subunits may influence the pharmacological and kinetic properties of the NMDA receptor, including agonist potency, deactivation time course, probability of opening and mean open and shut duration (Yuan et al., 2009). The C-termini of NR1 and NR2 subunits interact with several scaffolding proteins and are subject to phosphorylation and, because of these properties, are involved in the regulation of receptor trafficking and function (Rebola et al., 2010). NMDA receptor activation importantly results in the influx of Ca^{2+} into neurons, which activates downstream signalling molecules known to promote synaptic plasticity (Section 1.73). The most common NMDA complexes in the dorsal horn of the spinal cord contain NR1 in combination with NR2A/B (Mi et al., 2004;Nagy et al., 2004;Petralia et al., 1994).

Kainate Receptors

Kainate-type glutamate receptors are tetramers, which can be homo- or heteromeric receptors made up of various combinations of 5 subunits, known as GluR5-7 and KA1-2 (Stawski et al., 2010). Kainate receptors are present at presynaptic neurons and postsynaptic interneurons. Kainate receptors have been implicated in the control of inhibitory neurotransmission by modulating the release of the inhibitory neurotransmitter GABA (Bleakman et al., 2006).

Metabotropic Glutamate Receptors

Metabotropic glutamate (mGlu) receptors are G protein-coupled receptors that are widely distributed throughout the central nervous system and play an important role in modulating cell excitability and synaptic transmission via second messenger signalling pathways. There are 8 known subtypes; mGluRs 1-8, which are divided into groups I-III based on the receptors' sequence homology, pharmacology

and coupling to second messengers. Group I includes mGluR 1 and 5, group II mGluRs 2 and 3 and group III mGluRs 4, 6, 7 and 8 (Bleakman et al., 2006).

Group I mGlu receptors are often localised postsynaptically and activate signalling pathways such as stimulation of phospholipase C, possibly adenylyl cyclase and phosphorylation of MAP kinase. Activation of Group I mGlu receptors often leads to cell depolarisation and increased neuronal excitability. This modulation of neuronal excitability results from modulation of a number of ion channels for example, group I mGlu receptors are known to increase the activity of NMDA receptors (Niswender & Conn, 2010). Group I mGlu receptors also play important roles in induction of long-lasting forms of synaptic plasticity at glutamatergic synapses. Group II mGlu receptors are located pre- and postsynaptically and Group III mGlu receptors are predominantly localised presynaptically. Both Group II and III mGlu receptors activate signalling pathways such as inhibition of adenylyl cyclase, activation of K^+ channels and inhibition of Ca^{2+} channels. Activation of Group I and Group II mGlu receptors may inhibit neurotransmitter release at synapses (Niswender & Conn, 2010).

Receptor	Structure	Expression in the spinal cord
Ionotropic AMPA	Homo- or heterotetrameric comprised of GluR1-4	Heavily expressed in superficial laminae. Predominantly expressed postsynaptically.
Ionotropic NMDA	Heterotetrameric comprised of NR1, NR2A-D and NR3A-B	Heavily expressed in superficial laminae. Predominantly expressed postsynaptically.
Ionotropic Kainate	Homo- or heterotetrameric comprised of GluR5-7, KA1-2	Expressed pre- and cord.
Metabotropic Glutamate	mGlu1-8	Expressed pre- and postsynaptically in the dorsal horn of the spinal cord.

Table 1.4 Structure and expression of glutamate receptors.

1.6.3 Serotonin

Descending serotonergic pathways can facilitate spinal nociceptive transmission. The medulla, including the RVM and reticular formation, is the major output zone for descending serotonergic pathways originating in the Raphe nuclei. Serotonin (5-hydroxytryptamine, 5-HT) has several G protein-coupled receptor subtypes and one ionotropic receptor (5-HT_3R). There is evidence that serotonin facilitates persistent pain via activation of the 5-HT_3R in particular (Suzuki et al., 2004). This may be due to an increased descending serotonergic drive from higher centres. In addition, serotonin released from activated platelets and enterochromaffin cells may contribute to facilitation of nociceptors. As mentioned above, a study showed that selective ablation of lamina I/II NK1 receptor-expressing neurones reduced the excitability of deep dorsal horn neurons (Nichols et al., 1999). This effect was mimicked by administration of a selective 5-HT_3R antagonist (Suzuki et al., 2002). Descending serotonergic tracts can also contribute to inhibitory influences

in the dorsal horn by means of other 5-HT receptor subtypes (el Yassir & Fleetwood-Walker, 1990). postsynaptically in the dorsal horn of the spinal

1.6.4 Noradrenaline

Descending noradrenergic pathways exert a strong influence over spinal nociceptive transmission that is primarily inhibitory (Millan, 2002). Descending inhibition predominantly involves the release of noradrenaline from brainstem nuclei such as the RVM and locus coerulus. Noradrenaline acts in part at presynaptic α_2-adrenoreceptors to inhibit neurotransmitter release from primary afferents and thereby reduce the firing of projection neurons in the dorsal horn. Noradrenaline also acts at postsynaptic α_2-adrenoreceptors to modulate pain signalling (Pertovaara, 2006). Noradrenaline is also thought to act on α_1-adrenoreceptors present on inhibitory (GABAergic) interneurons, which leads to increased GABA release and enhances inhibition (Gassner et al., 2009).

1.6.5 Gamma-Amino-Butyric Acid (GABA) and Glycine

Gamma-amino-butyric acid (GABA) is the main inhibitory neurotransmitter found throughout the nervous system (Dickenson et al., 1997). A proportion of GABAergic cells contain the co-transmitter glycine (Todd & Sullivan, 1990). In the spinal cord GABA and glycine are concentrated in local interneurones, which exert tonic inhibitory effects on excitatory dorsal horn inputs (Takazawa & MacDermott, 2010). Some important GABAergic neurons originate in the RVM and PAG and form the descending inhibitory pathways mentioned previously (Section 1.1.5.5) (Millan, 2002).

GABA acts on receptors, which are expressed on neurons and glial cells in the superficial dorsal horn and in brain areas important for pain signalling. GABA acts on $GABA_A$ and $GABA_B$ receptors. $GABA_A$ receptors are ionotropic and $GABA_B$ receptors are metabotropic (Gwak & Hulsebosch, 2011). Activation of $GABA_A$ receptors allows Cl^- ions to flow and generally leads to cell hyperpolarisation. Activation of $GABA_B$ receptors causes a decrease in Ca^{2+} and an increase in K^+ currents and this also leads to cell hyperpolarisation. This hyperpolarisation will

contribute to decreasing neurotransmitter release from the hyperpolarised postsynaptic cell (Gwak & Hulsebosch, 2011). Glycine receptors are permeable to Cl^- ions therefore also contribute to the hyperpolarisation of the postsynaptic cell.

GABA and glycine play an important role in the modulation of nociceptive information in the spinal cord, particularly during chronic pain states. During chronic inflammatory and neuropathic pain there is reduced GABAergic and/or glycinergic inhibition in the spinal cord (Sivilotti & Woolf, 1994). During neuropathic pain, $GABA_A$ (and possibly glycine) receptor-mediated inhibition is reduced and this might be partly due to a shift in the neuronal chloride gradient (Coull et al., 2005). Following nerve injury degeneration of GABAergic interneurons, altered storage and/or release of GABA or altered $GABA_A$ receptor subunit expression have also been suggested to contribute to the reduction in inhibitory GABAergic tone. However, these mechanisms have been challenged by other studies and the precise mechanisms are still under debate (Munro et al., 2009).

1.6.6 Neurotrophins

Nerve Growth Factor (NGF), Brain-derived Neurotrophic Factor (BDNF), Neurotrophin-3 and Neurotrophin-4/5 are members of the neurotrophin family of signalling molecules (Bibel & Barde, 2000). The neurotrophins are well-known for their major role in development in regulating survival and differentiation of neuronal populations. Later in life, neurotrophins continue to have a major role by influencing ion channel activity, neurotransmitter release and axonal pathfinding. NGF preferentially binds to TrkA receptors, BDNF preferentially binds to TrkB and NT-3 to Trk-C and all neurotrophins bind to the p75 low affinity neurotrophin receptor. Neurotrophins are synthesised as high-molecular weight precursors (pro-neurotrophins) and interestingly the precursor form of BDNF (Pro-BDNF) is released and preferentially activates the p75 receptor (Lu, 2003).

BDNF is a major regulator of synaptic transmission and plasticity. BDNF action at TrkB, initiates intracellular signalling cascades that lead to transcriptional changes. In this way, BDNF contributes to synaptic plasticity and regulation of

neuronal excitability in nociception (Ren & Dubner, 2007). In the dorsal horn, activation of TrkB receptors by BDNF contributes to hyperalgesia associated with peripheral inflammation (Woolf & Salter, 2000). An early consequence of peripheral nerve injury is the activation of spinal microglia, which leads to an increase in BDNF release. BDNF influences the release of GABA from interneurones which leads to the activation of $GABA_B$ receptors located on the terminals of sensory neurones. This decreases sensory neuron transmission in the dorsal horn (Pezet et al., 2002). However BDNF also acts in GABA-receptive neurons to decrease expression of the KCC2 transporter, which plays an important role normally in establishing that the chloride ion gradient is inward and consequently that $GABA_A$ and glycine receptor activation causes hyperpolarisation (Coull et al., 2005). With reduced KCC2 activity GABA and glycine responses lose their inhibitory impact and may even be excitatory. A recent further study showed that BDNF drives the changes in excitatory synaptic transmission in the rat dorsal horn that follow sciatic nerve injury, where there is an increase in drive to excitatory neurons and a decrease in drive to inhibitory neurons (Lu et al., 2009).

1.7 Consequences of Tissue Damage

After an injury, sensitization of the nociceptive pathways may occur which is reflected in alterations in signs and symptoms of pain (Jensen & Baron, 2003). Thus, there may be spontaneous pain (pain experienced in the absence of any obvious peripheral stimulus), hyperalgesia (increased responsiveness to noxious stimuli) and allodynia (pain in response to normally innocuous stimuli). Pain hypersensitivity occurs in chronic pain states, including neuropathic, inflammatory and CIBP pain. There are two major mechanisms that contribute to pain hypersensitivity: peripheral and central sensitisation (Ji et al., 2003). Such facilitation of nociceptive processing occurs after repeated or intense noxious stimuli and is demonstrated by a lowering of activation thresholds and amplification of responses to subsequent inputs. Peripheral and central sensitisation initially act as a protective mechanism, to prevent further injury, but when peripheral and central sensitisation outlast the initial injury the pain can become chronic and debilitating.

1.7.1 Peripheral Sensitisation

Peripheral sensitisation refers to the reduction in nociceptor activation threshold and increased magnitude of responses evoked in nociceptors (Latremoliere & Woolf, 2009). This occurs in response to tissue damage, inflammation or nerve injury when inflammatory mediators known as the 'inflammatory soup' are released, which includes H^+, K^+, ATP, arachidonic acid and 5-HT that act on receptors and ion channels on nociceptor peripheral terminals to promote nociceptor activation. This inflammatory soup also includes other agents such as prostaglandin E2 (PGE2), bradykinin (BK) and NGF (Julius & Basbaum, 2001). These mediators act on receptors to activate signalling pathways leading to changes in transduction proteins by post-translational processing and altered gene expression, and thereby modifying nociceptor threshold, excitability and transduction properties. Consequently, nociceptors are no longer only activated by noxious stimuli and there is an increase in release of neurotransmitters, neuropeptides, ATP and BDNF into the dorsal horn of the spinal cord, as well as chemokines and cytokines from immune system cells. In nerve injury, microglial cells are activated, and contribute to releasing such mediators (Biggs et al., 2010). Following on from peripheral sensitisation, the increased excitability and spontaneous activity of primary afferents is thought to trigger central sensitisation.

1.7.2 Central Sensitisation

Central sensitisation was first described by Woolf in 1983 (Woolf, 1983) and refers to enhanced excitability of dorsal horn neurons, such that they may fire spontaneously or in an exaggerated manner in response to primary afferent input and there is enlargement of their receptive field size (Cook et al., 1987).

Multiple cellular processes lead to central sensitisation, which is a diffuse heterosynaptic phenomenon, including increased excitatory mechanisms such as enhancement of the activation of NMDA and AMPA receptors and changes in their trafficking to or from the membrane (Latremoliere & Woolf, 2009). Decreased inhibitory mechanisms also contribute, such as reduced release or activity of GABA and glycine (Latremoliere & Woolf, 2009) and this may reveal previously suppressed

inputs from ‘silent nociceptors’ (Torsney & MacDermott, 2006). In central sensitisation there may be recruitment of other neurons, not only nociceptors, to pain pathways. Synapses made by Aβ-fibres may be affected leading to Aβ fibre-derived pain. There are several other types of synaptic plasticity in the spinal cord, such as wind-up. Wind-up is a reversible synaptic plasticity characterised by a progressive increase in action potential output from dorsal horn neurons during a train of repeated low-frequency C-fibre or nociceptor stimuli (Mendell & Wall, 1965). Frequencies of between 0.5Hz and 2Hz are required for the induction of wind-up. It has been shown that NMDA receptor activation is required for windup to occur but not for the baseline activity of dorsal horn neurons (Davies & Lodge, 1987;Woolf & Thompson, 1991). AMPA receptors are also involved in the induction and maintenance of windup (You et al., 2004). Homosynaptic plasticity analogous to classical long-term potentiation (LTP), as first described in the hippocampus, has also been reported in the superficial dorsal horn (Sandkuhler & Liu, 1998). The direction of synaptic plasticity (LTP or long-term depotentiation) may be determined by the NMDA receptor subunit composition and levels of intracellular Ca^{2+} (Liu et al., 2004). Central sensitisation shares some characteristics in common with LTP but there are clearly distinguishing aspects (Ji et al., 2003). Although classical LTP requires high frequency stimulation that may not be readily observed in nociceptive afferents, recent findings indicate that some subgroups of rostrally projecting superficial dorsal horn cells may exhibit LTP at much lower input frequencies (Ikeda et al., 2006)

1.7.3 Mechanisms of Central Sensitisation

In normal nociceptive signalling, AMPA receptor activation sets the baseline depolarisation of dorsal horn neurons. Sustained depolarisation caused by repetitive or high-frequency stimulation of C-fibres leads to the activation of NMDA receptors. As mentioned previously, the activity of NMDA receptors requires depolarisation to release the Mg^{2+} block of the ion channel, and also the agonist glutamate and co-agonist glycine or D-serine. It is the combination of these that allows the NMDA receptor to open and the flow of ions to enter the cell. Ca^{2+} ions are thought to make up a large proportion of the positive ions that flow into the cell upon opening of

NMDA receptors. Ca^{2+} can also enter via some AMPA receptors, voltage-dependent Ca^{2+} channels and be released from intracellular stores after activation of Ca^{2+}-mobilising G protein-coupled receptors, such as mGlu receptors. Ca^{2+} can activate an array of pathways known to promote synaptic plasticity and this can lead to the increased responsiveness and activity of dorsal horn neurons.

One of the major mechanisms for synaptic plasticity is through phosphorylation of AMPA and NMDA receptors. PKC, CaMKII, PKA, tyrosine kinases and ERK can phosphorylate these receptors (Ji et al., 2003). Intracellular pathways involving these molecules are triggered by activation of NMDA, mGlu, TrkB, NK1, CGRP and bradykinin B2 receptors. Phosphorylation of Kv4.2 channels by ERK produces a decrease in K^+ currents and thereby increases general membrane excitability (Ji et al., 2003). Recruitment of AMPA receptors to the membrane leads to an increase AMPA and NMDA receptor mediated currents therefore boosting synaptic efficacy (Shi et al., 1999). These changes are generally short-lasting. Long-lasting changes are dependent on transcriptional regulation. Activation of transcription factors, including cAMP response binding protein (CREB), by kinases, as mentioned above, also leads to modulation of gene expression (Latremoliere & Woolf, 2009). These types of changes can occur in response to both inflammation and peripheral nerve injury. A role of AMPA receptors in neuropathic pain has been demonstrated by intrathecal AMPA/kainate receptor antagonists which blocked thermal hyperalgesia and mechanical allodynia in the sciatic nerve constriction injury (CCI) model (Garry et al., 2003b). The role of NMDA receptors in central sensitisation will be discussed further in Chapter 5 (Section 5.1.3).

1.7.4 Glial Activation

Under certain conditions, including injury to the CNS, infection and peripheral damage, central glial cells can become activated (Mcmahon & Malcangio, 2009). Activation of glial cells is one of the marked features of CIBP (Hald et al., 2009;Medhurst et al., 2002;Schwei et al., 1999;Zhang et al., 2005a). Upon activation, glial cells exhibit many changes in morphology, protein expression and cell proliferation. Additionally, glial cells become more active in modulating neuronal

activity through release of pro-inflammatory cytokines and reactive oxygen and nitrogen species. There is now considerable evidence suggesting a link between glial cell activation and pain facilitation (Gosselin et al., 2010).

Activated microglia exhibit an enlarged cell body, thickening of processes and increased expression of cell surface makers including β2 integrins CD11b and CD11c (recognised by the antibody OX-42), ionized calcium-binding adapter molecule 1 (IBA-1) and major histocompatibility complex (MHC) class II. Activated astrocytes show cell hypertrophy, increased expression of cell surface protein glial fibrillary acidic proteins (GFAP) and release of various neuromodulatory mediators (Gosselin et al., 2010).

The time pattern of glial cell activation in nerve injury has been investigated. Following peripheral nerve injury it appears that microglia are activated quickly but this declines over time. Astrocytes are activated later but this persists beyond microglia activation (Hald et al., 2009). Microglial activation is thought to play a part in pain onset whereas long-lasting activation of astrocytes is thought to be responsible for the maintenance of central sensitisation.

A study using the CCI model of neuropathic pain, showed that BDNF released from microglia in lamina I attenuates inhibitory transmission by down regulating expression of the potassium chloride exporter protein (KCC2) (Coull et al., 2005). This normally maintains an inward gradient of Cl^- ions, ensuring that ligand-gated chloride channels such as $GABA_A$ and glycine receptors are inhibitory when activated but this effect is lost in chronic neuropathic pain. In lamina II, BDNF further acts to increase synaptic drive to excitatory neurons and reduce that to inhibitory neurons. This study suggests that, in this model of neuropathic pain, BDNF may be the key mediator of information transfer between microglia and dorsal horn neurons during the onset of central sensitisation (Biggs et al., 2010).

IL-1β is a pro-inflammatory cytokine involved in a variety of diseases with associated pain and is induced in astrocytes in models including CIBP (Baamonde et

al., 2007;Zhang et al., 2005a). IL-1β may be a messenger between astroglia and neurons through its modulation of the NR1 subunit of NMDA receptors. In a model of CIBP IL-1β has been shown to enhance NR1 subunit phosphorylation (Zhang et al., 2008a). IL-1β enhances NMDA receptor-mediated intracellular Ca^{2+} release (Viviani *et al.*, 2003a). Astrocytes play a key role in maintaining normal synaptic excitability, as they take up glutamate and convert this to inert glutamine (Nakagawa & Kaneko, 2010). Glutamine is then transported back to presynaptic terminals where it is converted to glutamate to replenish the transmitter pool. Down-regulation or functional deficiency of glial glutamate transporters may contribute to chronic pain (Nakagawa & Kaneko, 2010).

1.7.5 The Sympathetic Nervous System

The sympathetic nervous system is part of the autonomic nervous system which regulates the function of all innervated tissues and organs throughout the vertebrate body with the exception of the skeletal muscle fibres (Elenkov et al., 2000). The activities of the autonomic nervous system are largely exempt from direct conscious control and the main function is to maintain homeostasis. The sympathetic nervous system originates in brainstem nuclei and most sympathetic preganglionic fibres terminate in ganglia on either side of the spinal column. From these ganglia postganglionic sympathetic fibres run to the associated tissue and release noradrenaline (Elenkov et al., 2000). The sympathetic nervous system is activated in response to any immune challenge and is responsible for the 'fight or flight' response.

It has been shown that neuropathic and inflammatory pain can be modulated by the sympathetic nervous system (Janig et al., 1996;Raja, 1995). In normal conditions, the sensory neurons in the DRG are not directly innervated by the sympathetic nervous system. McLachlan *et al.* were the first to describe sprouting of sympathetic fibres into the DRG after sciatic nerve transaction (Mclachlan et al., 1993). This study reported basket-like structures formed by sympathetic fibres and suggested these neurons played a role in the abnormal discharge following peripheral nerve injury (Mclachlan et al., 1993). Sprouting of sympathetic nerve fibres has been

shown to occur in many animal pain models (Chung et al., 1993;Lee et al., 1998;Pertin et al., 2007). In complex regional pain syndrome (CRPS), one mechanism that is thought to contribute to the maintenance or exacerbation of hypersensitivity is the sprouting of sympathetic nerve fibres. The release of noradrenaline from these sympathetic nerve fibres can directly activate nociceptors and reduced sympathetic input can attenuate the pain state (Janig & Baron, 2003).

1.8 Endogenous Systems

1.8.1 Endogenous Opioid System

Nociceptive signals can be modulated within the dorsal horn by endogenous opioids such as endorphins, dynorphins, enkephalins and endomorphins (Millan, 2002). Opioid receptors include delta (δ), mu (μ) and kappa (κ) opioid subtypes, which are present in the peripheral and central nervous system. These are G protein-coupled receptors that are negatively coupled to adenylyl cyclase, increase K^+ currents and decrease Ca^{2+} currents leading to decreased membrane excitability (Millan, 2002). In addition, the nociceptin/OFQ receptor (NOP) is a G protein coupled-receptor activated by its endogenous ligand nociceptin/orphanin FQ (N/OFQ) (Meunier et al., 1995;Reinscheid et al., 1995). Activation of NOP similarly inhibits the formation of cyclic AMP, closes Ca^{2+} channels and opens K^+ channels, so consequently reduces neuronal excitability and neurotransmitter release (Lambert, 2008). The original study by Meunier *et al.* demonstrated that intracerebroventricular administration of N/OFQ produced hyperalgesia (Meunier et al., 1995). When delivered supraspinally N/OFQ reverses the effect of exogenous opioids (Heinricher *et al.*, 1997;Pan *et al.*, 2000;Zeilhofer & Calo, 2003). This anti-analgesic effect is thought to be act via the rostral ventromedial medulla (Pan *et al.*, 2010;Zeilhofer & Calo, 2003). In contrast to these effects, spinal administration of N/OFQ produces an anti-nociceptive effect. It is thought that spinal N/OFQ produces anti-nociception by inhibiting neurotransmitter release at primary afferent terminals in the dorsal horn of the spinal cord (Lambert, 2008).

Upon injury endogenous opioids are released and attenuate hyperalgesia. Levels of dynorphin have been shown to increase during peripheral inflammation

(Iadarola et al., 1988), neuropathic pain (Malan et al., 2000) and CIBP (Peters et al., 2005). It is further known that leukocytes containing opioids migrate to the area of inflammation (Stein et al., 1990).

The endogenous opioid system has been shown to play a role in masking early cancer pain in a mouse model of pancreatic cancer. Mantyh *et al.* demonstrated that mice in the early stages of cancer did not exhibit pain behaviours, however after administration of opioid receptor antagonists mice with early stage cancer exhibit significant pain behaviours (Sevcik et al., 2006).

Opioids, such as the μ receptor-selective agonist, morphine, are the most effective analgesics for the treatment of moderate to severe pain. At the dorsal horn of the spinal cord, it has been suggested that μ-opioid receptor agonists bring about analgesia by inhibiting neurotransmitter release from the central terminals of primary afferents and postsynaptic inhibition of dorsal horn neurons (Fields, 2007). Chronic or acute opioid treatment can paradoxically cause hyperalgesia, termed 'opioid-induced hyperalgesia' (Arner et al., 1988;Deconno et al., 1991) which occurs in parallel to opioid tolerance, that is the reduction of opioid analgesic potency. Preclinical studies have shown the development of opioid-induced hyperalgesia following the chronic administration of opioids (Gardell et al., 2006;Mao et al., 1994). A range of mechanisms are thought to contribute to opioid-induced hyperalgesia including NMDA receptor activation, TRPV1 receptor activation and modulation of spinal input by descending pathways (Colvin & Fallon, 2010). Studies suggest that the 5-HT_3 receptors may play a key role, as the selective 5-HT_3 receptor antagonist could prevent and reverse opioid-induced hyperalgesia and tolerance in mice (Liang et al., 2011). The role of opioid receptors in opioid-induced hyperalgesia is a contentious issue, with mixed evidence from studies. It has been suggested that that opioid receptors are not necessary for opioid-induced hyperalgesia, as opioid receptor triple knock-out mice lacking all three genes encoding opioid receptors (mu, delta and kappa) still develop opioid-induced hyperalgesia (Juni et al., 2007). Furthermore, the general opioid receptor antagonist Naltrexone did not prevent the development of opioid-induced hyperalgesia (van Dorp et al., 2009).

Data concerning the effectiveness of opioids for neuropathic pain has generated intense debate. Neuropathic pain was thought to be relatively opioid-resistant as opioids are not effective at tolerable doses in the majority of neuropathic pain patients (Woolf & Mannion, 1999). There is increasing evidence however to suggest that opioids do not lack efficacy in neuropathic pain (Eisenberg et al., 2006). Preclinical studies suggest that the efficacy of opioids in neuropathic pain is variable and seems to depend on several factors including type of injury, behavioural assessment used and route of administration (Dickenson & Suzuki, 2005).

Current treatment for CIBP patients follows the World Health Organisation's ladder approach for relief of cancer pain, which recommends opioids for treatment of moderate to severe cancer pain (for more detail see Chapter 4; Section 4.1). Opioid treatment of CIBP brings about many challenges including those detailed previously, such as opioid-induced hyperalgesia and tolerance, and severe compliance-limiting side-effects such as somnolence and mental confusion.

1.8.2 Endogenous Cannabinoid System

Endocannabinoids are endogenous lipid signalling molecules generated in the cell membrane from phospholipid precursors. Two well studied endocannabinoids are arachidonoylethanolamine (AEA) and 2-arachidonoylglycerol (2-AG). Endocannabinoids bind and activate one or more cannabinoid receptor subtypes; CB1 and CB2, which are G protein-coupled receptors present in the peripheral and central nervous system. Endocannabinoids are involved in many different physiological and pathological functions including analgesia (Guindon & Hohmann, 2009).

CB1 receptors have been found on sensory neurons, interneurons and astrocytes. CB2 receptors are primarily found on cells of the immune system and were thought to exist in the peripheral nervous system only but have been shown in the central nervous system. The signalling mechanisms of cannabinoid receptors appear to have a range of consequences. It has been proposed that activation of

presynaptic CB1 receptors on primary afferents inhibits the release of neurotransmitters by decreasing Ca^{2+} conductance and increasing K^{+} conductance (Guindon & Hohmann, 2009).

It appears that AEA and 2-AG under normal conditions suppress pain through a CB1-dependent mechanism. Studies have demonstrated that endocannabinoids act at the spinal level to modulate pain. Upregulation of cannabinoid receptors and of AEA and 2-AG is observed in the spinal cord following CCI. Studies have shown CB1 and CB2 receptors can modulate inflammatory nociception. The CCI and spinal nerve ligation (SNL) models of neuropathic pain have been used to show that inhibition of endocannabinoid removal processes produces antinociceptive effects through CB1 and CB2 mechanisms (Bridges et al., 2001;Herzberg et al., 1997).

Synthetic cannabinoid receptor agonists targeted at CB1 and/or CB2 receptor show significant analgesic efficacy in acute and neuropathic pain. CB1 agonists have undesirable psychotropic side-effects that prevent their long term use as analgesics. CB2 agonists have been shown to act as analgesics in acute, chronic and neuropathic pain (Ibrahim et al., 2003). A recent study also showed that systemic administration of a CB2 agonist (AM1241) acutely, or for 7 days, significantly attenuated CIBP (Lozano-Ondoua et al., 2010). The mechanism of cannabinoid analgesia could involve glial cells, as a study demonstrated that a CB1/CB2 agonist and a CB2 agonist prevent or reverse glial cell activation in the spinal cord in a model of neuropathic pain (Leichsenring et al., 2009).

1.9 Cancer-Induced Bone Pain

CIBP affects 75-95% of patients with metastatic or advanced-stage cancer (Mercadante & Arcuri, 1998;Portenoy *et al.*, 1999). CIBP is a complex pain syndrome, in which patients suffer from constant background pain with movement-related and spontaneous breakthrough pain components (Mercadante & Arcuri, 1998;Portenoy *et al.*, 1999). Background pain is often described as a dull ache or burning sensation which gets progressively more severe as the tumour grows (Mercadante, 1997). Breakthrough pain is an episode of extreme pain and is the most

difficult component of CIBP to treat (Mercadante & Arcuri, 1998;Portenoy *et al.*, 1999). The multi-component nature of this pain state makes it difficult to manage with analgesics and the current therapeutic regime can leave up to 45% of patients with inadequate pain control (de Wit *et al.*, 2001b;Meuser *et al.*, 2001b). This current regime for CIBP focuses on palliative radiotherapy and opioid analgesics complemented by non-steroidal anti-inflammatory drugs (NSAIDs) and bisphosphonates. Although background pain is usually controlled by opioids, breakthrough pain is more difficult to control due to the quick onset of breakthrough pain when compared to the onset of opioid-induced analgesia and the long duration of action of opioids compared to short-lived breakthrough pain (Delaney et al., 2008). The nature of breakthrough pain, where there are short-lived peaks of pain over and above stable background pain, means that the opioid adverse effects are more likely to be problematic (Delaney et al., 2008). The doses of opioids that are required to control the breakthrough components of CIBP frequently result in unacceptable side effects, in particular, excess sedation and mental confusion. Chronic use of opioids results in severe side-effects including analgesic tolerance, somnolence, constipation and respiratory depression (Vanderah et al., 2000). By investigating the central mechanisms underlying CIBP it may be possible to develop novel, more effective, analgesics. The analgesic efficacy of the therapeutic candidates radiotherapy, gabapentin, duloxetine, S,S-reboxetine and CB 65 will be assessed in a preclinical CIBP model and discussed in Chapter 4.

1.9.1 Animal Models of CIBP

Focal models of CIBP have been developed based on site-specific tumours. These models allow examination of the pain associated with tumour progression without the overall cancer-related sickness that is brought about by metastatic models. A mouse model of bone cancer was developed by Schwei *et al.* where fibrosarcoma cells (NCTC 2472) were implanted directly into the intramedullary space of the femur (Schwei et al., 1999). Mouse models using different tumour cells and rat models have also been established to further investigate the mechanisms that drive CIBP (Medhurst et al., 2002;Urch et al., 2003b). The first rat model was developed by Medhurst *et al.* where MRMT-1 rat mammary gland carcinoma cells

were injected into the tibia (Medhurst et al., 2002). The injection of cancer cells directly into bone allows the location of the tumour to be carefully controlled and site-specific behavioural analysis can be carried out. Neurochemical and neuroanatomical changes that occur at the tumour site, in the DRG and within the spinal cord can all be analyzed.

Animal models of CIBP display pain behaviours such as allodynia and hyperalgesia and have been validated to show that NSAIDs and opioids have an analgesic effect similar to that in humans. Furthermore, pain-related behaviours increase as the tumour develops and correlate with tumour-induced bone destruction, which mirrors the clinical situation. Animal studies indicate that treatments may differentially modify specific components of pain behaviour. One challenge is to ascertain which interventional therapies are most effective for different components of CIBP. Animal studies have shown that CIBP appears to be relatively resistant to opioid analgesia with 10-fold higher doses of morphine required compared to chronic inflammatory pain (Luger et al., 2002). This need for an increased dose may be due to down-regulation of μ opioid receptors in the DRG of CIBP animals compared with inflammatory pain models, which show an increase in μ opioid receptors (Yamamoto et al., 2008). This resistance to morphine treatment in CIBP highlights the importance of alternative analgesics for CIBP treatment. In addition it was shown that sustained morphine treatment may actually accelerate bone destruction when compared to vehicle treated animals (King et al., 2007).

Spinally-mediated reflex responses to noxious stimuli are often used to assess animal pain behaviours. These include von Frey filaments to measure mechanical allodynia or thermal plate to measure thermal sensitivity. Spontaneous pain-like behaviours can also be observed in CIBP animals with spontaneous flicking of the affected limb. In the clinical setting, spontaneous pain is a major symptom in CIBP patients. In addition, abnormal ambulatory movement can be observed. Models of limb bone cancer develop plantar hypersensitivity, this may occur due to the spinal sensitization across a wide area of the spinal cord, and not just the segments in which the central terminals of the afferent fibres innervating the cancer bearing tissues are

located. However, peripheral mechanisms may be involved because the DRG that contain the cell bodies of afferents from the femoral bone also, to a limited extent, contain neurons which make up part of the sciatic nerve.

1.9.2 Affective Component of CIBP

Anxiety and depression are common in patients suffering from cancer pain (Zimmerman et al., 1996). Relatively few animal studies have investigated how prolonged pain influences affective behaviours. However, it has been shown that anxiety-like behaviour in the open field is increased in a model of HIV-associated peripheral neuropathic pain (Wallace et al., 2007). Anxiety-like behaviour on the elevated plus maze has been shown to increase in a chronic constriction injury model of neuropathic pain but not in a partial nerve ligation model of neuropathic pain (Roeska et al., 2008). CIBP is often associated with anxiety and depression. However, no animal study has analysed affective behaviours in CIBP.

1.10 Mechanisms of CIBP

The molecular mechanisms responsible for cancer-induced bone pain are being elucidated. Studies using animal models of CIBP have shown that CIBP is mechanistically distinct compared with neuropathic and inflammatory pain states with both neuropathic and inflammatory components (Honore et al., 2000).

Normal bone undergoes a continuous remodelling cycle of resorption and formation. Osteoclasts are remodelling cells responsible for bone resorption, whereas osteoblasts are support cells responsible for bone formation. In normal bone there is a balance between osteoclast and osteoblast function. This balance is disrupted when tumour cells invade the bone microenvironment. The process of bone metastasis occurs when primary tumour cells invade the surrounding normal tissue by producing proteolytic enzymes, penetrate the walls of small blood vessels and enter the circulation. Cancer cells enter the wide-channelled sinusoids of the bone marrow cavity where they become bone metastases. At this site they stimulate the activity of osteoclasts or osteoblasts (Mundy, 2002).

There is a spectrum of properties in bone metastases where tumours can range from mostly osteoblastic (bone-forming) or osteolytic (destructive). However, evidence indicates that both resorption and formation are activated in most bone metastases (Mundy, 2002). Microfractures resulting from decreased bone density and/or disrupted bone architecture due to increased osteoclastic bone resorption have been implicated in bone pain (Mercadante, 1997).

Primary afferent sensory neurons innervate mineralised bone, bone marrow and the periosteum (the thin fibrous outer covering of the bone highly innervated by sensory fibres). Peptidergic sensory nerves (containing substance P and CGRP) and sympathetic neurons (demonstrated by immunoreactivity for tyrosine hydroxylase, TH, a marker of noradrenergic fibres) innervate the bone marrow (Tabarowski et al., 1996). A recent study indicated that peptidergic C-fibres, but not the non-peptidergic C-fibres, and Aβ/Aδ fibres are the major fibre types signalling pain from the bone (Jimenez-Andrade et al., 2010). The most dense innervation is to the periosteum (Tabarowski et al., 1996). In addition to generating pain through microfractures of the bone, tumour expansion in the bone marrow cavity can stretch the periosteum and activate sensory neurons in CIBP.

A component of CIBP may be due to tumour-induced injury to primary afferent fibres. Peters *et al.* used a mouse model of CIBP with green fluorescent protein (GFP)-expressing tumour cells injected into the femur. This study showed that when tumour cells invade the bone they contact, injure then destroy primary afferents which innervate the bone. Activating transcription factor-3 (ATF3) and galanin are upregulated in these primary afferents. Sensory fibres were observed at the leading edge of the tumour however towards the centre of the tumour these fibres become fragmented then undetectable (Peters et al., 2005).

Sensitisation of nociceptors by production of nociceptive substances in cancer cells and inflammatory cells can also contribute to CIBP. Schwei *et al.* showed sensitisation of primary afferent neurons with an increase in c-fos expression in lamina I and an internalization of substance P receptors in ipsilateral spinal cord after

non-noxious palpatation of the tumour-bearing limb (Schwei et al., 1999). These nociceptive substances include protons, bradykinin, substance P, CGRP, endothelin, histamine, NGF, VEGF, prostaglandins, glutamate and ATP. In fact, osteoclasts, metastatic cancer cells and inflammatory cells all produce protons and therefore make the bone microenvironment acidic. Local acidification has been shown to contribute to CIBP as protons released by osteoclasts, inflammatory cells, immune cells and tumour cells activate TRPV1 and ASIC in afferents (Yoneda et al., 2011). These signals then activate intracellular signalling pathways and transcription factors (Yoneda et al., 2011). TRPV1 is expressed in CGRP-positive sensory neurons (likely nociceptors) that innervate the bone (Wakabayashi et al., 2005). Expression of TRPV1 in DRG neurons and TRPV1/CGRP receptor colocalization have been shown to increase ipsilaterally in a mouse model of CIBP (Niiyama et al., 2007). ASIC3 is co-expressed with CGRP in the sensory neurons innervating the periosteum of bone. A study further found that mRNA expression of ASIC1a, ASIC1b and ASIC3 in DRG was up-regulated ipsilateral to CIBP (Nagae et al., 2007).

In addition to sensitisation of primary afferents, it has been shown that dorsal horn neurons become hyperexcitable in a rat model of CIBP, which is consistent with the establishment of central sensitisation. The firing rate of dorsal horn neurons in response to mechanical, thermal and electrical sensory stimuli was increased and the dorsal horn neurons displayed enlarged receptive fields (Urch et al., 2003b).

A recent study showed that increased excitability of dorsal horn neurons in lamina II exists across a wide area of lumbar segments, not just the segment innervating the cancer-bearing limb (Yanagisawa et al., 2010). This study used patch-clamp recording in spinal cord slices attached to DRG to show that there is an increase in the amplitude of dorsal horn excitatory post-synaptic currents and that this is mediated through C-fibres and Aδ- fibre inputs but not through Aβ- fibres as shown in other pain states (Yanagisawa et al., 2010). These results support the idea that CIBP is a unique pain state with a distinct profile of synaptic changes.

1.10.1 Glial Cell Involvement in CIBP

Glial cell activation in the spinal cord appears to play a critical role in the development and/or maintenance of neuropathic pain (Scholz & Woolf, 2007). As mentioned previously, in neuropathic and inflammatory pain states microglia appear to be involved only in the induction stage (Romero-Sandoval et al., 2008;Schreiber et al., 2008). In CIBP the role of microglia is controversial with different reports indicating involvement in induction, induction and maintenance or neither (Hald et al., 2009;Lan et al., 2010;Zhang et al., 2005a). This is discussed further in Chapter 5 (Section 5.1.4). Lan *et al.* suggest that microglia may be involved in the induction and maintenance of behavioural hypersensitivity in CIBP (Lan et al., 2010). Astrocytes may also be involved in CIBP (Schwei et al., 1999). A study by Hald *et al.* showed that in a preclinical model of CIBP, astrocyte activation spread from the spinal cord segment receiving innervation from the inoculated bone into caudal and rostral spinal cord segments. Hypersensitivity is also shown to spread to distant spinal cord segments, therefore it is possible that both glial cell activation and hypersensitivity may be linked (Hald et al., 2009).

Several pro-inflammatory signals have been proposed to play a role in CIBP, including IL-1β, TNF-α, IL-6 and INF-β. Such pro-inflammatory cytokines can be released via activation of the microglial transmembrane receptor TLR4. Intrathecal administration of TLR4 small interfering RNA (siRNA) in CIBP rats reduced the expression of spinal TLR4 and significantly decreased behavioural hypersensitivity and expression of spinal microglial markers (Lan et al., 2010). In this model of CIBP it has also been shown that there is increased phosphorylation of p38 and increased production of IL-1β and TNF-α. which can be prevented by siRNA against the TLR4 receptor, suggesting that the glial response in CIBP is dependent on TLR4-dependent phosphorylation of p38 (Liu et al., 2010).

1.10.2 NMDA Receptor Involvement in CIBP

NMDA receptors have been implicated in CIBP. A study by Zhang *et al.* suggested that spinal IL-1β enhances NR1 phosphorylation to facilitate bone cancer pain (Zhang et al., 2008a). Another study suggested that there is an increase in the

expression of the NR2B subunit, as seen in neuropathic pain (Gu et al., 2010a). The role that NMDA receptors play in CIBP will be discussed further in Chapter 5.

1.12 Aim

This study uses a rat model of CIBP modified from Medhurst *et al.* (Medhurst et al., 2002). The primary aim of this study was to define the molecular basis for sensitisation in CIBP, through investigating the role of NMDA receptors and the involvement of TRP ion channels. In particular, we investigated whether CIBP caused a change in NMDAR subunit expression in the dorsal horn of the spinal cord. Additionally, we investigated whether CIBP is associated with changes in TRP channel expression in the DRG. The secondary aim was to assess the analgesic efficacy of palliative radiotherapy and the therapeutic candidates, gabapentin, duloxetine, S,S-reboxetine and CB 65. Agents were evaluated for analgesic efficacy against the sensory, movement-related and affective components of CIBP. The analgesic efficacy of a general NMDA receptor antagonist, (R)-CPP, a NR2B subunit-selective antagonist, Ro 25-6981, and a NR2A subunit-selective antagonist, AAM 077, were also evaluated against sensory and movement-related components of CIBP. The analgesic efficacy of a TRPM8 agonist, icilin, a TRPV1 antagonist, AMG 9810, and a TRPV4 antagonist, RN 1734 were also assessed in the sensory and movement-related components of CIBP. Investigation of the roles of these proteins in a CIBP preclinical model by quantified testing techniques could lead to better management in the clinic and insights into new therapeutic targets.

2. MATERIALS AND METHODS

All experiments were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 and were approved by the University of Edinburgh Ethical Review Committee and performed under UK Home Office Licence (Project licence number: 50/3466) and personal licences (PIL number: 60/10911). An *in vivo* model of CIBP was necessary to explore the underlying mechanisms, with the aim of leading to the development of more efficacious analgesics.

2.1 Preclinical Model

This programme of work uses a rat model of CIBP modified from Medhurst *et al.* (2002) and Urch *et al.* (2003). Adult male Sprague Dawley rats (Harlan) were used with an initial weight of 90-100g. All animals were housed in groups of 4-7 per cage in a controlled environment with a 12 hour light/dark cycle, temperature of 21°C ± 1°C and humidity of 50% and were allowed access to food and water *ad libitum.* Animals were acclimatised to housing conditions for at least 1 week prior to pre-surgical behavioural testing/surgery. Animals were randomly allocated to their experimental group and identified by tail mark using a permanent marker.

2.1.1 Cell Culture

MRMT-1 rat mammary gland carcinoma cells (Cell Resource Centre for Biomedical Research, Tohuko University, Japan) were used for surgery at Passage 6-8. MRMT-1 cells were cultured at 37°C in 5% carbon dioxide (CO_2), cultured in RPMI 1640 (GIBCO, UK) medium containing 10% foetal bovine serum (heat-inactivated; Harlan Sera Lab) and 2% penicillin/streptomycin (Gibco). MRMT-1 cells were prepared for intra-tibial injection as follows; the adherent cells were released from the culture flask by brief exposure to 0.1% w/v trypsin and collected by centrifugation for 5 minutes at 800 rpm. The resulting pellet was washed with 10ml of Hank's balanced salt solution (HBSS; GIBCO, UK) and centrifuged for 5 minutes at 800 rpm. The final pellet was re-suspended in 1ml of HBSS and cells were counted using a haemocytometer. Cells were diluted with HBSS to achieve final concentration for injection and kept on ice until 10µl of suspension (containing $6x10^3$cells) of medium was injected into the tibia of an anaesthetised rat.

2.1.2 Surgical Procedure

Surgical procedures were carried out in an aseptic manner. Rats were anaesthetised by inhalation of an isoflurane/O_2 mixture (Zeneca, UK), 4-5% for induction and 2-3% for maintenance, at a flow rate of 2 litres/minute. Adequate depth of anaesthesia was assessed by pinching the front paw to check for absence of a reflex response. Respiratory rate and pattern were monitored throughout the surgery to ensure stability. Following complete induction of anaesthesia the animal was placed abdominal side up, the left hind limb was shaved and the skin was sterilised with 0.5% Hibitane (chlorhexidine gluconate) (Zeneca, UK). A small incision was made in the skin over the tibia, which was then carefully exposed by removing the connective tissue over the bone using a cotton bud (Johnson & Johnson, UK). A dental drill attachment was used to bore a hole through the periosteum of the tibia. Polythene tubing (0.5mm in diameter; Smiths) was fed into the intra-medullary cavity of the tibia and 10µl of medium (containing $6x10^3$cells) was injected using a 1ml micro-syringe (BD Biosciences, UK) and 25-gauge needle (BD Biosciences, UK). The tubing was withdrawn and the hole plugged with dental restorative material (IRM, Dentsply; Henry Schein Minerva), to confine the tumour cells within the marrow and prevent invasion of the adjacent soft tissue. The wound was closed with absorbable subcutaneous suture (5/0 coated vicryl, Ethicon, UK) and sterilised with 0.5 % Hibitane. Animals were placed in a thermoregulated recovery box until they had fully regained consciousness, following which they were returned to their home cages.

Three different Sham models were used throughout this study. These were examined to distinguish between the effect of CIBP and bone damage/injection of MRMT-1 cells on behavioural responses. For the Sham groups, the surgical procedure was the same as CIBP with the following differences; Sham Vehicle (Sham V) received a 10µl intra-tibial injection of HBSS only, Sham Heat-Killed (Sham HK) received an intra-tibial injection of 10µl of medium containing $6x10^3$ heat-killed cells that had been boiled at 100°C for 20 minutes and Sham Exposed (Sham E) received no drill or injection into the tibia, but the bone was exposed in the same way as for the CIBP model.

2.2 Behavioural Analysis

For behavioural analysis, animals were trained to the cages and testing apparatus four days prior to surgery (Day -4) and animals were tested prior to surgery to obtain baseline values (Day 0) for comparison to post-surgical values. Post-surgery animals rested for 2 days (Day 1-2) then behavioural testing resumed from Day 3-4 onwards until Day 18-21 (all animals were culled by Day 21). Behavioural testing was carried out once on each day of testing. The behavioural assessment timeline is shown (Figure 2.1). Behavioural testing was carried out in the same room each time and animals always had a minimum of 20 minutes in their test environment to allow them to become habituated before testing commenced.

CIBP patients suffer from constant background pain and difficult to control movement-evoked and spontaneous pain, therefore it is important to assess a variety of pain behaviours in a preclinical CIBP model. A common comorbidity in CIBP patients is anxiety. To thoroughly assess the rodent CIBP model we investigated several components, which involved measurements of both reflex and non-evoked responses and affective behaviours.

We examined CIBP-induced sensory reflex responses (thermal sensitivity and mechanical allodynia), movement-related behaviours (avoidance of weight bearing on movement in comparison to static weight bearing, distance travelled, speed and rearing), spontaneous pain-like behaviour (spontaneous foot lifting) and affective behaviours (open field and elevated plusmaze). Non-evoked behaviours were analysed by weight bearing difference between hindlimbs and spontaneous foot lifting behaviour. These quantitative tests were utilised to assess the efficacy of focal palliative radiotherapy (XRT; carried out at Day 7 post induction of CIBP; Section 2.7.1) and of the compounds; (R)-CPP, Ro 25-6981, AAM 077, CB 65, gabapentin, icilin, duloxetine and S,S-reboxetine (detailed in Section 2.7.2). The observer was blinded to type of animal and treatment throughout testing. The combination of behavioural tests and pharmacological manipulations was used to attempt to elucidate the mechanisms underlying CIBP.

<table>
<tr><td rowspan="2">Cage testing/ apparatus training</td><td colspan="3" rowspan="2">Baseline behavioural testing</td><td rowspan="2">Surgery: CIBP Model</td><td colspan="2" rowspan="2">Post-surgery recovery</td><td rowspan="2">Post-surgery behavioural testing</td><td rowspan="2">Culled</td></tr>
<tr></tr>
<tr><td>-4</td><td>-3</td><td>-2</td><td>-1</td><td>0</td><td>1</td><td>2</td><td>3 onwards ⟶</td><td>21</td></tr>
<tr><td colspan="4">Pre-surgery (days)</td><td>Surgery</td><td colspan="4">Post-surgery (days)</td></tr>
</table>

Figure 2.1 Behavioural assessment summary timeline.

2.3 Sensory Components

2.3.1 Mechanical Allodynia

To assess the development of mechanical allodynia, each animal was placed in a Perspex chamber on an elevated metal mesh floor allowing the experimenter to reach the plantar surface of the hindpaw from beneath, unobserved by the animal. Following acclimatisation of the animal to the cage, the paw withdrawal threshold (PWT) in response to normally innocuous mechanical stimuli was measured by applying a set of calibrated Semmes-Weinstein von Frey filaments (Stoelting Co., USA) to the plantar surface of the hindpaw of the ipsilateral (injured) hindlimb and the contralateral (non-injured) hindlimb. These filaments exert a fixed bending force ranging from 1.202g to 75.858g (corresponding to a pressure range of 163.68mN/mm^2 to 3327.7mN/mm^2). Each filament was applied perpendicularly to the mid-plantar surface of the foot until the filament flexed/bent. The filaments were applied in ascending order (starting from the weakest) with each force applied 10 times at one-to-two second intervals. The withdrawal response is characterised as a quick paw flick with or without shaking. Threshold was defined as the minimum indentation force (grams) required to elicit a response/paw withdrawal to at least 5 out of 10 applications (i.e. to at least 50% of applications). Data are expressed as the mean PWT (grams) ± standard error of the mean (SEM) for each time point.

2.3.2 Thermal Sensitivity

To test thermal sensitivity of the ipsilateral hindlimb, each animal was placed on a thermal footplate (Incremental Hot/Cold Plate Meter; IITC Life Science Inc., USA), which was set at either 10°C, 20°C, 30°C or 40°C (temperature holding accuracy is ± 0.1°C). The number of times the animal withdrew its ipsilateral hindlimb from the thermal footplate and the latency to the first paw withdrawal over 150 seconds was recorded (from the time of placing the animal on the thermal footplate). If the animal did not flick the latency was recorded as 150 seconds. In addition, the total duration of paw elevation was also noted. Each temperature was tested with a minimum of twenty minutes between temperatures. Data are expressed as the mean ± SEM for each time point for Paw withdrawal/ Latency to paw withdrawal/ Duration of paw elevation to 10°C, 20°C, 30°C or 40°C. These temperatures were chosen to obtain data from a wide range of temperatures and to attempt to elucidate which temperature-sensitive channels might be involved in thermal sensitivity in CIBP.

2.4 Movement-related Components

2.4.1 Movement-evoked Pain

To assess movement-evoked pain, the rotarod (IITC Life Science Inc., USA) was used and set at a constant speed of 5-6 rpm (no ramping of rpm). Goblirsch *et al.* used the rotarod set at a constant speed of 6 rpm to assess paw guarding during forced ambulation in a model of CIBP (Goblirsch et al., 2004b). The number of avoidances of weight bearing on movement of the ipsilateral hindlimb was counted over 30 seconds (from the time of placing the animal on the rotarod). The avoidance of weight bearing on movement can range from a subtle movement, whereby the animal extends/descends the ipsilateral hindlimb (when compared to the contralateral hindlimb) to avoid weight bearing, to a complete avoidance of weight bearing by the animal not using the ipsilateral hindlimb during rotation of the rotarod. Therefore the number of avoidances of weight bearing on movement includes the number of hops, drags or guarding of the ipsilateral hindlimb when compared to the contralateral hindlimb. To use a less subjective measure of avoidance of weight bearing on movement we used this method of counting the total number of avoidances rather

than scoring individual avoidances on a scale system as previously used. Experimenters were blind to the condition of each animal, in addition to the treatment. This test was performed once on each test day by two different experimenters. The figure below illustrates this, showing the presumed line of the average lift position of the ankle on the contralateral hindlimb and the ipsilateral hindlimb in relation to this line when walking on the rotarod (Figure 2.2). Data are expressed as the mean avoidance of weight bearing on movement (number) ± SEM for each time point.

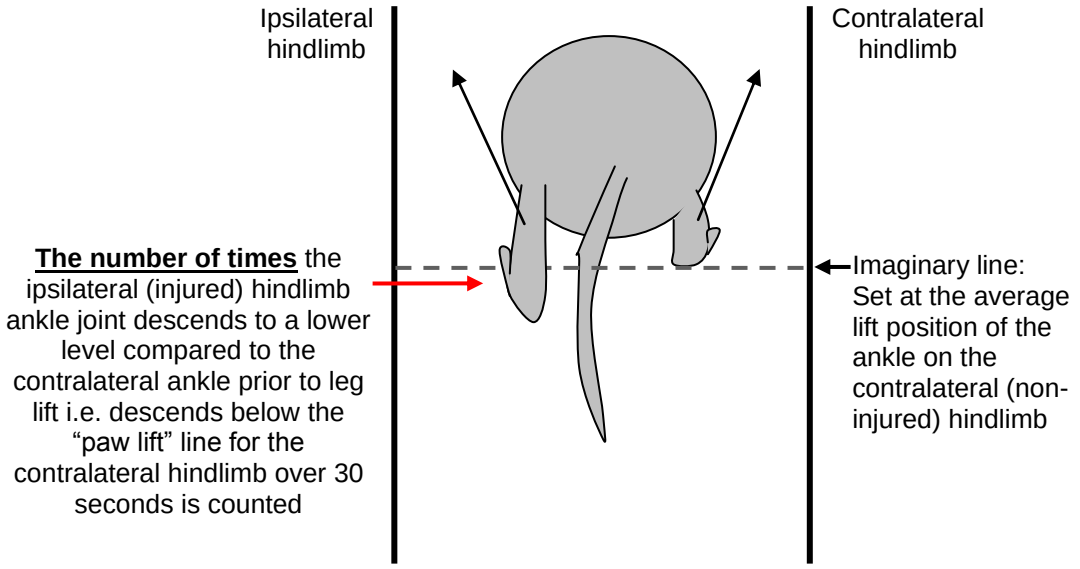
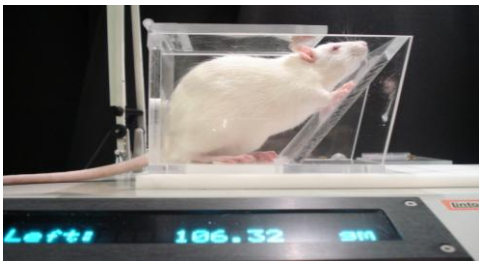


Figure 2.2 Illustration of assessment of movement-evoked pain using the rotarod.

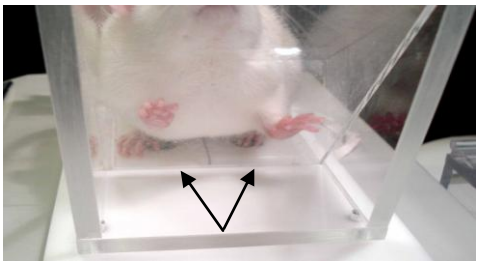
2.4.2 Static Weight Bearing

To assess the static weight bearing difference between ipsilateral and contralateral hindlimbs, the animal was placed in the Linton incapacitance tester chamber and allowed to acclimatise and position itself such that each hindlimb was located on its individual transducer pad and the front paws were supported by the slanted frame of the box (Figure 2.3). When the position of the animal was correct and stable, the static weight bearing value of each hindlimb was measured. The incapacitance tester is set to record the load on each transducer pad over 4 seconds and the two numbers displayed represent the distribution of the rat's weight on each

hindlimb (i.e. right and left). Two to three readings of static weight bearing value for ipsilateral and contralateral hindlimbs were noted and the mean difference for each animal was calculated. Data are expressed as the static weight bearing difference (WBD; grams) ± SEM for each time point. This method has been used by others to obtain an average reading for the distribution of body weight on each hindpaw (Nakazato-Imasato & Kurebayashi, 2009).



(A) Linton incapacitance tester and chamber



(B) Showing the position of each hind-limb on its individual transducer pad

Figure 2.3 Illustration of the Linton Incapacitance tester.

2.4.3 Voluntary Locomotor Activity in the Open Field

Animals were exposed only once to this test as repeated exposure can alter responses, with repeated exposure apparently resulting in decreased exploratory behaviour (van der Staay et al., 2009). The open field apparatus consists of a square arena 40cm by 40cm with 40cm high Perspex walls. The open field is positioned in a quiet light controlled room (with the camera recording from above the open field). Testing is conducted under dim white light (60W bulbs, two floor lamps, 145cm height each positioned over the open field arena with the light directed such that no shadows are cast from the open field walls). The animals were allowed a minimum of 30 minutes to acclimatise to the room/lighting conditions before testing in the open field. The open field is divided into a centre zone and outer zone (illustrated in Figure 2.4). All animals started the test from the same corner in the outer zone of the open field, to ensure consistency between tests, and tested for 5 minutes. To assess locomotion in the open field arena the following parameters were recorded; total distance travelled (metres), average speed (metres/second) and number of rearings. Rearing behaviour has been used in previous studies as a measure of general activity

in the open field and elevated plusmaze (Nosek et al., 2008). ANY-maze software (Version 4.39; Stoetling) was used to track movement and the observer recorded the number of rearings. The open field was cleaned with ethanol (70%) between each animal to remove any olfactory cues. Further measures were also recorded in the open field (see section 2.6.1).

2.4.4 Voluntary Locomotor Activity on the Elevated Plusmaze

Animals were exposed only once to this test as repeated exposure decreases time spent on the open arms in the elevated plusmaze (Rodgers & Dalvi, 1997). The elevated plusmaze has two open arms and two closed arms illustrated in the diagram below (Figure 2.4). The plusmaze is elevated 45cm above the floor (with the camera recording from above the plusmaze) and positioned in a quiet, light controlled room. Testing is conducted under dim red diffused light (60W bulbs, two floor lamps of 145cm height, each positioned over the closed arms of the maze with light directed on the closed arms, the red light is diffused using greaseproof paper). The animals were allowed a minimum of 30 minutes to acclimatise to the room/lighting conditions. The plusmaze is cleaned with ethanol (70%) before and between each animal to remove any olfactory cues. The animals always started the test in the centre square facing one of the open arms, to ensure consistency between tests, and were allowed to explore the plusmaze for 10 minutes. To assess voluntary locomotor activity, the number of rearings on the elevated plusmaze were counted. Further measures were also recorded from the elevated plusmaze (see section 2.6.2).

2.5 Spontaneous Component

To assess the spontaneous component of CIBP, we examined spontaneous foot lifting (SFL), which has been proposed as an indicator of ongoing/spontaneous pain (Djouhri et al., 2001). Each animal was placed in a Perspex chamber at room temperature, allowed to habituate for 5 minutes and the cumulative duration of SFL of the ipsilateral hindlimb over a 5 minute test period was recorded. Foot-lifting associated with walking and grooming was not included. Other studies have quantified spontaneous flinches and flinching/guarding behaviour over a 2 minute period in CIBP model to measure spontaneous pain (King et al., 2007).

2.6 Affective Components of CIBP

Both the elevated plus maze and open field were used to assess anxiety-like behaviours. Both tests are established behavioural tests for the assessment of anxiety-induced modification of exploratory behaviour and have been validated using anxiolytic drugs (for example diazepam)(Pellow *et al.*, 1985;Prut & Belzung, 2003).

2.6.1 Open Field

As detailed above, animals are exposed only once to this test, which is positioned in a quiet light controlled room (testing apparatus, procedure and conditions detailed in section 2.4.3) Animals were allowed at least 30 minutes to acclimatise to the room/lighting conditions. The open field was cleaned with ethanol (70%) before and between each animal to remove any olfactory cues. To assess anxiety-related behaviours, the following parameters were recorded; time spent in the centre zone, number of entries to the centre zone, latency to enter the centre zone, number of groomings and time spent grooming. ANY-maze software (Version 4.39; Stoetling) was used to track movement and the observer recorded the number of groomings.

2.6.2 Elevated Plusmaze

As detailed above, animals were exposed only once to the elevated plusmaze (testing apparatus, procedure and conditions detailed in section 2.4.4) Animals were allowed at least 30 minutes to acclimatise to the room/lighting conditions. The plusmaze was cleaned with ethanol (70%) before and between each animal to remove any olfactory cues. The following parameters were recorded to assess anxiety and risk assessment behaviour; time spent on the open arms, number of groomings and number of protected stretch attends. ANYmaze software was used to track movement and the observer recorded the number of groomings and number of protected stretches.

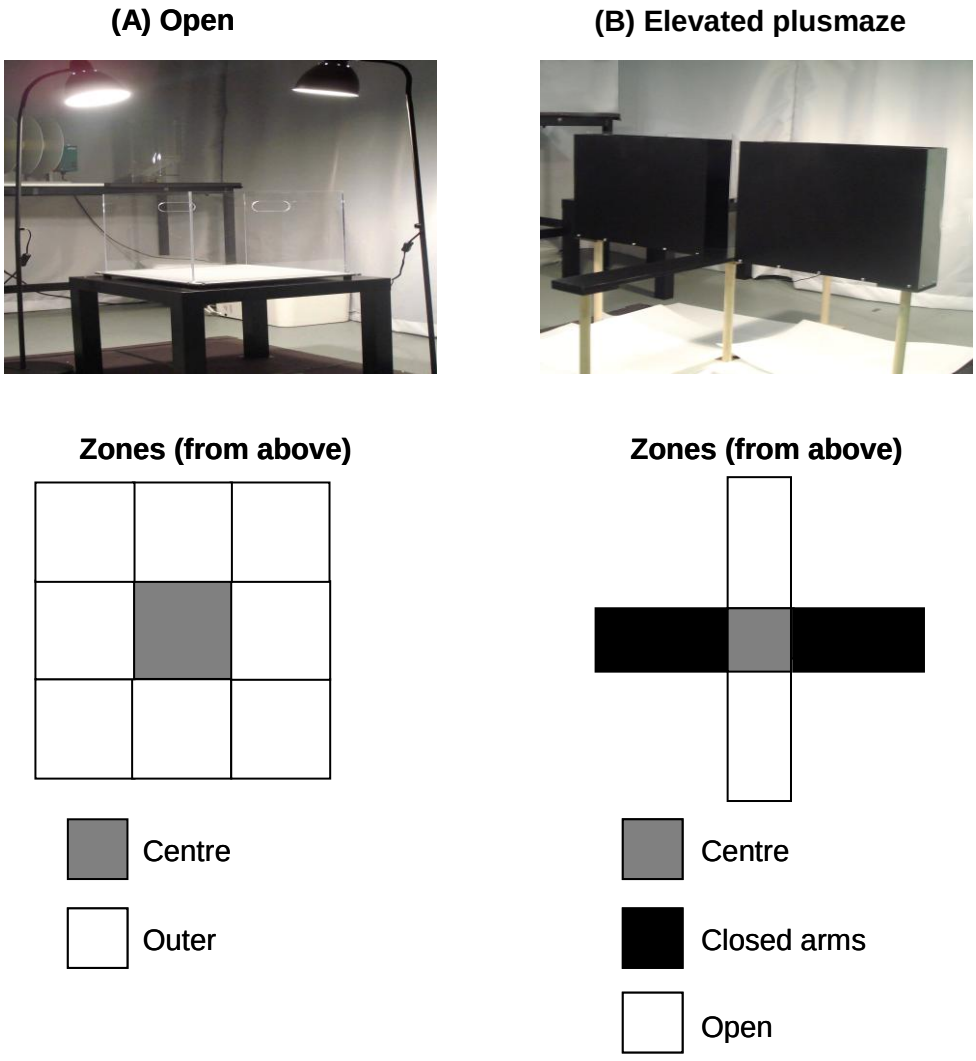


Figure 2.4 Open field and elevated plusmaze testing apparatus. A) Open field is divided into centre zone and outer zones. B) Elevated plusmaze is divided into closed arms (with walls), open arms and centre square.

2.7 Analgesic Interventions

Several interventions were assessed in this thesis, to examine their analgesic efficacy in CIBP across a range of behavioural tests.

2.7.1 Radiotherapy

Focal palliative radiotherapy (XRT), the current clinical gold standard treatment for CIBP, was carried out at Day 7 after induction of CIBP. Animals were anaesthetised with sodium pentobarbitone (50mg/kg, i.p.) and positioned in the chamber, such that irradiation was restricted to the ipsilateral hindlimb using collimators to shield the rest of the animal, thus enabling unattenuated beams of radiation over the exposed area. Animals were exposed to a single dose of 8 Gy (at a dose rate of 1.1 Gy/min) in a Gammacell 40 Extractor (MDS Nordion International Inc.), with a Caesium-137 source above and below the chamber. Animals were allowed to recover for 24 hours before behavioural testing resumed at Day 9. The effect of XRT treatment on sensory, movement-related and affective components of CIBP was assessed. Specifically the analgesic efficacy of XRT on thermal sensitivity to 20°C and 40°C, mechanical allodynia, static weight bearing and movement-related pain was examined. The effect of XRT on affective components of CIBP was also assessed using the open field and elevated plusmaze.

2.7.2 Pharmacological Agents

The following pharmacological agents were tested in CIBP animals in behavioural tests for sensory, movement-related and affective components of CIBP; the voltage-gated calcium channel ligand, gabapentin; the serotonin/noradrenaline reuptake inhibitor, duloxetine; the noradrenaline reuptake inhibitor, S,S-reboxetine; the selective cannabinoid (CB_2) receptor agonist CB 65; the selective NMDA receptor antagonist, 3-(2-carboxypiperazin-4-yl) propyl-1-phosphonic acid ((R)-CPP); the selective antagonist of NMDA receptors containing the NR2B subunit, Ro 25-6981; the selective antagonist of NMDA receptors containing the NR2A subunit, AAM 077; the selective TRPV1 receptor antagonist, AMG 9810; the selective TRPV4 receptor antagonist, RN 1734 and the TRPM8/TRPA1 receptor agonist, icilin. The pharmacological agent target, dose, route of administration and day of administration post CIBP induction of each agent are detailed in the table below (Table 2.1).

Pharmacological agent	Target	Dose	Route	Day	Reference
Gabapentin (Tocris)	Binds to α2δ-1/ α2δ-2 subunits of voltage-gated Ca^{2+} channels	30mg/kg	p.o.	18-21	Dose taken from (1)
Duloxetine (Gift from Wyeth-Pfizer)	5-HT/ NA reuptake inhibitor	30mg/kg	p.o.	16-21	Dose and route taken from (2)
S,S-reboxetine (Gift from Wyeth-Pfizer)	NA reuptake inhibitor	10mg/kg	p.o.	17-19	Dose and route taken from (3)
Oral vehicle control	(2% Tween-80, 0.5% methylcellulose aqueous)		p.o.	14-18	
CB 65 (Tocris)	Selective CB_2 receptor agonist	1mg/kg	i.p.	20	Dose and route based on (4)
(R)-CPP (Tocris)	NMDA receptor antagonist	7.5nmole	i.t.	17	Dose and route based on (5)
Ro 25-6981 (Sigma-Aldrich)	NMDA receptor NR2B-selective antagonist	50nmole	i.t.	15	Route based on (5)
AAM 077 (Gift from Novartis)	NMDA receptor NR2A-selective antagonist	50nmole	i.t.	14	Route based on (5)
Icilin (Tocris)	TRPM8/TRPA1 agonist	100μmole	Topical	14	Dose and route based on (6)
AMG 9810 (Tocris)	TRPV1 antagonist	1nmole	i.t.	18-21	Route based on (7)
RN 1734 (Tocris)	TRPV4 antagonist	5nmole	i.t.	19	Route based on (8)
Intrathecal vehicle control	(0.5% dimethylformamide in saline)		i.t.	20	

Table 2.1 Details of pharmacological agents showing pharmacological agent target, dose, route of administration and day of administration post CIBP induction. (1) Chronic subcutaneous administration of gabapentin (30mg/kg) provided analgesia in a CIBP model (Donovan-Rodriguez et al., 2005). In the present study the oral route of administration was chosen. A Cochrane review showed that a single dose of gabapentin (250mg, p.o.) produces an analgesic effect in the treatment of acute post operative pain (Straube et al., 2010). (2) Duloxetine (30mg/kg, p.o.) provided analgesia in a model of neuropathic pain (Iyengar et al., 2004). A Cochrane review showed that duloxetine (60mg and 120mg, p.o.) are efficacious for treating diabetic peripheral neuropathy and fibromyalgia (Lunn et al., 2009). (3) S,S-reboxetine (30mg/kg, p.o.) provided analgesia in acute and inflammatory pain models (Whiteside et al., 2010). In the present study both 10 and 30mg doses were tested for sedative effects. The 30mg dose had a significant sedative effect in the rotarod sedation ataxia test therefore the 10mg dose was used to test for an anti-nociceptive effect. (4) CB2 agonist AM1241 (1mg/kg, i.p.) provided anti-nociception (PMID: 11514083 Malan et al. 2001). In the present study a different CB2 agonist was used. (5) (R)-CPP (100pmol, i.t.) provided analgesia in a model of peripheral demyelination (Wallace et al., 2003). (6) Icilin (80μM topical) produced analgesia in models of inflammatory and neuropathic pain (Proudfoot et al., 2006). (7) AMG 9810 (5, 15 and 45μg, i.t.) provided analgesia in a model of inflammatory pain (Yu et al., 2008b). In the present study the dose was chosen based on performance on the rotarod in the sedation ataxia test. (8) TRPV4 antagonist ruthenium red (0.1-1 nmol, i.t.) provides analgesia in a model of neuropathic pain (Ding *et al.*, 2010a). In the present study RN 1734, a more selective TRPV4 antagonist, was chosen (Vincent et al., 2009).

2.7.3 Pharmacological Agent Administration

To establish baseline behavioural responses, behavioural tests were carried out before administration of pharmacological agents. Trials were carried out blind to compound identity.

Intrathecal Administration of Pharmacological Agents

For intrathecal administration of pharmacological agents, animals were anaesthetised by inhalation of an isoflurane/O_2 mixture (as detailed above Section 2.1.2). Pharmacological agents were administered into the L5/6 intrathecal space of anaesthetised rats using a 1ml syringe with a 25-gauge needle (BD Biosciences, UK) at a volume of 50µl. To establish the correct site of injection and to ensure the volume of 50µl was sufficient to reach the lumbar enlargements which receive hind limb innervation, experiments with Blue Dye (Merck-BDH, UK) were performed. A tail-flick or movement of the hind limb usually indicated that the needle was correctly positioned prior to injection. Behavioural testing commenced 10-15 minutes post-injection to allow recovery from anaesthesia.

The following pharmacological agents were applied intrathecally: the selective NMDA receptor antagonist, (R)-CPP; the selective antagonist of NMDA receptors containing the NR2B subunit, Ro 25-6981; the selective antagonist of NMDA receptors containing the NR2A subunit, AAM 077; the selective, competitive vanilloid TRPV1 receptor antagonist, AMG 9810 and the selective TRPV4 antagonist, RN 1734 and vehicle controls. The vehicle control (0.5% dimethylformamide in saline) has been shown in previous experiments in the lab to exert no changes in behavioural reflex responses following intrathecal injection.

Oral Administration of Pharmacological Agents

For oral administration of pharmacological agents, all animals were fasted for 12 hours prior to administration of pharmacological agents by oral gavage at a volume of 2ml/kg. For administration, the rat was kept upright with the head immobilised and the feeding tube was directed along the roof of the mouth and toward the right side of the back of the pharynx and then gently passed down into the esophagus. If any resistance was detected the tube was withdrawn and re-inserted. Behavioural testing commenced 1 hour post administration.

The following pharmacological agents were orally administered: the voltage-gated calcium channel ligand, gabapentin; the serotonin/noradrenaline reuptake

inhibitor, duloxetine; the selective noradrenaline reuptake inhibitor, S,S-reboxetine and vehicle control. All pharmacological agents were dissolved in a vehicle consisting of aqueous 2% Tween-80, 0.5% methylcellulose, which has been shown through extensive testing at Wyeth to have no effect alone on behavioural reflex responses.

Topical Application of Pharmacological Agent

For topical application, the pharmacological agent solution was applied to both hindlimbs by placing each animal in a Perspex chamber that had a slanted frame to support the front paws, enabling the solution to cover both hindlimbs. Topical application of pharmacological agent was applied for a 5 minute period. Animals were trained prior to the day of pharmacological agent application to the Perspex chamber and with both hindlimbs covered with water, so as to reduce the impact of stress on the pharmacological agent response. The chamber was designed to allow the animal to be placed in volume of pharmacological agent that covered the hindlimbs to the knee but did not wet the main body of the animal. Behavioural testing commenced 10 minutes after administration. The TRPM8/TRPA1 channel agonist, icilin (dissolved in 0.25% dimethylformamide in water) was applied by this route (Proudfoot et al., 2006).

Intraperitoneal Adminstration of Pharmacological Agents

Animals were restrained to enable injection of pharmacological agents into the peritoneal cavity using a 1ml syringe with a 25-gauge needle (BD Biosciences, UK) to inject in the lateral aspect of the lower left quadrant at a volume of 3ml/kg. The selective cannabinoid (CB_2) receptor agonist, CB 65 was administered by this route.

2.8 Immunohistochemistry

To contribute to assessing the underlying mechanisms of CIBP, the expression of several proteins was assessed by immunohistochemistry and Western blot to determine changes in their expression and localisation in the spinal cord and dorsal root ganglion (DRG).

2.8.1 Spinal Immunohistochemistry

Experimental and naïve animals were terminally anaesthetised with an intraperitoneal injection of sodium pentobarbitone and transcardially perfused with heparinised vascular flush (0.1M phosphate buffer saline pH 7.4 (PBS) containing 0.6mg/ml heparin), followed by fixative solution (4% paraformaldehyde (PFA; Sigma, UK), at a flow rate of 30ml/minute. Post-perfusion, a laminectomy was performed through the lumbar region and segments L3-L6 of the spinal cord were dissected under an operating microscope. A pin was inserted through the ventral horn of the contralateral spinal cord to allow pin-hole identification after immunohistochemistry. Tissue samples were post-fixed with 4% PFA (Sigma, UK) in 0.1 M PBS and transferred through increasing concentrations of sucrose in 0.1M PBS at room temperature (5% for 1 hour, 10% for 3 hours) to a 30% solution overnight at 4°C, then stored in a 0.1M PBS with 0.01% sodium azide (Sigma,UK) at 4°C.

Pins were removed from the spinal cord and tissue was mounted on a freezing microtome (Leitz Kyromat 1700) in 0.2% agar (in 0.1M PBS) and transverse spinal cord sections (40μm) were cut and collected at -15°C and transferred to a 36 well plate containing 0.1M PBS. Sections were washed with 0.1M PBS. Different antigen retrieval techniques were used, depending on the primary antibody (see Table 2.2). Incubation in citrate buffer (pH 6.0) at 90°C for 15 minutes was used for antigen retrieval for NR1 and NR2B subunits. Antigen retrieval for NR2A involved incubating sections for 5 minutes with pepsin (Dako) at 37°C. Sections were washed with 0.1M PBS and blocked with blocking buffer (0.5% blocking reagent (Perkin Elmer, USA) in 0.1M PBS) for 90 minutes at room temperature. Sections were incubated with primary antibodies at the following concentrations; goat-anti-NR1 (Santa Cruz; 1:70 in 0.1M PBS at 4°C overnight), rabbit-anti-NR2B (Santa Cruz; 1:70 in 0.1M PBS at room temperature overnight) or goat-anti-NR2A (Santa Cruz; 1:50 in 0.1M PBS at 4°C for 24 hours). Sections incubated with NR1, NR2B or NR2A antibodies were also incubated with mouse-anti-NeuN (Chemicon; 1:500 in 0.1m PBS at 4°C; a marker of neuronal cells). After primary antibody incubation, sections were washed with 0.1M PBS and incubated

with appropriate secondary antibodies (Table 2.2) in 0.1M PBS for 90 minutes at room temperature. Sections were washed in 0.1M PBS and incubated with the amplification reagent fluorescein tyramide (Perkin Elmer Life Sciences, Inc.) for 25 minutes at room temperature (1:50 in 1x amplification diluent). Sections were washed with deionised water and mounted onto microscope slides pre-coated with poly-L-lysine (Merck-BDH, UK) and cover-slipped with mounting-medium Vecta Shield (Vector Laboratories, USA), sealed with clear varnish and stored at 4°C in a light-sensitive box before confocal microscopy. Control sections were processed as above, omitting the primary antisera.

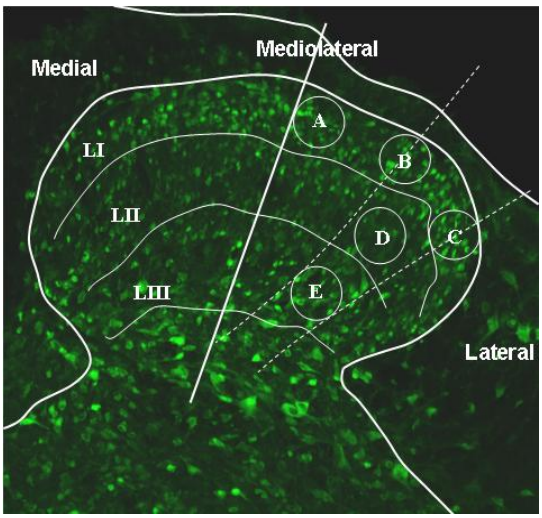


Figure 2.5 Regions of interest in the dorsal horn of the spinal cord. The average fluorescence intensity of the ipsilateral and contralateral dorsal horn of the spinal cord were analysed in regions of interest (ROI). Three ROIs (A, B and C) in lamina I, one ROI (D) in lamina II and one ROI (E) in lamina III were analysed for average fluorescent intensity. Lamina I value was taken as an average of A, B and C.

2.8.2 Confocal Microscopy and Image Analysis

Slides were examined using a Leica TCSNT SP5 confocal microscope (Leica Microsystems GMBH, Germany). To analyse the expression of NMDA receptor subunits, confocal optical sections for ipsilateral and contralateral spinal dorsal horns were acquired using a x20 objective lens. The fluorescence intensity of lamina I, II and III was analysed using ImageJ software (ImageJ version 1.42). The areas analysed are shown in the figure above (Figure 2.5). These areas were chosen to

include lamina I and II, where nociceptive input is integrated and relayed by neurons. Nociceptors terminate mainly in laminae I, II and V of the dorsal horn, on relay and local interneurons (Millan, 1999). The ipsilateral-contralateral difference in fluorescence intensity of each subunit (average ipsilateral-average contralateral fluorescence intensity) was calculated. Such expression of the NMDA receptor subunits was examined in CIBP, Sham E and Naïve animals (six animals per group and six sections per animal). The experimenter was blinded to the treatment group for analysis.

To analyse co-expression of NR2A with the neuronal cell marker NeuN, confocal optical sections were acquired using a x40 oil immersion objective lens. NR2A-immunopositive cells (green) and NeuN-immunopositive cells (red) were counted in laminae I and II of the dorsal horn using Leica LCS Lite software. Co-expression of NR2A with NeuN was compared ipsilateral to contralateral and between CIBP, Sham V and Naïve animals (six animals per group and six sections per animal were analysed). The experimenter was blinded to the treatment group for analysis.

	Antigen retrieval	Primary antibody	Secondary antibody
NR1	Citrate buffer (pH 6.0) at 90°C for 15 minutes	Goat-anti-NR1 (Santa Cruz; 1:70 in 0.1M PBS, overnight at 4°C)	HRP-linked rabbit-anti-goat (1:300 in 0.1M PBS, 90 minutes room temperature)
NR2A	Pepsin at 30 °C for 5 minutes	Goat-anti-NR2A (Santa Cruz; 1:50 in 0.1M PBS, 48 hours at 4°C)	HRP-linked rabbit-anti-goat (1:300 in 0.1M PBS 90 minutes room temperature)
NRB	Citrate buffer (pH 6.0) at 90°C for 15 minutes	Rabbit-anti-NR2B (Santa Cruz; 1:70 in 0.1M PBS overnight at room temperature)	HRP-linked goat-anti-rabbit (1:500 in 0.1M PBS 90 minutes room temperature)
NeuN	Dependent on subunit conditions	Mouse-anti-NeuN (Chemicon; 1:50 in 0.1M PBS at room temperature or 4°C)	Alexa Fluor goat-anti-mouse$_{568}$ (Invitrogen; 1:200 in 0.1M PBS)

Table 2.2 Antigen retrieval techniques and antibodies used for NMDA receptor-related spinal cord immunohistochemistry. Conditions were optimised for each antibody.

2.8.3 Dorsal Root Ganglia Immunohistochemistry

Dorsal root ganglia (DRG) from spinal cord sections L4-L6 were dissected immediately following terminal anaesthesia from CIBP, Sham V and Naive animals ipsilateral and contralateral to injury. DRGs were frozen in optimal cutting temperature medium (OCT; Cell Path Plc., UK) on dry ice. DRG sections were cut on a cryostat at -20°C to a thickness of 15µm and mounted directly onto poly-L-lysine coated slides (Merck-BDH, UK). Sections were encircled with a hydrophobic barrier pen (ImmEdge; Vector Laboratories, USA). For TRPV4 staining, sections were fixed with 4% paraformaldehyde in 0.1M PBS for 5 minutes at room temperature prior to first wash. All sections were washed with 0.1M PBS and then blocked for 2 hours at room temperature with blocking solution (10% normal goat

serum (NGS; Vector Laboratories, USA), 4% fish skin gelatine (FSG; Sigma-Aldrich, UK) and 1% Triton X-100 (Sigma-Aldrich, UK) in 0.1M PBS). Sections were incubated with the following primary antibodies; TRPM8 (Alomone Labs Ltd., 1:150), TRPV1 (Pierce Biotechnology, 1:1000) or TRPV4 (Alomone Labs Ltd., 1:1000), Peripherin (Millipore, 1:500), NF-200 (Sigma-Aldrich, 1:1000) in primary antibody buffer consisting of 4% NGS, 4% FSG and 0.1% Triton X-100 in 0.1M PBS, overnight at room temperature. Antibody conditions are detailed in Table 2.3. Sections were washed in 0.1M PBS then incubated with secondary antibodies; Alexa Fluor goat-anti-rabbit$_{568}$ (Invitrogen, 1:750) and Alexa Fluor goat-anti-mouse$_{488}$ (Invitrogen, 1:300) in secondary antibody buffer of 4% NGS, 4% FSG in 0.1M PBS, for 1 hour at room temperature. Following washing with 0.1M PBS, slides were coverslipped with mounting-medium Vecta Shield, sealed with clear nail varnish and stored at 4°C in a light sensitive box prior to fluorescent microscopy. Control sections were processed as above omitting the primary antisera.

	Antigen retrieval	Primary antibody	Secondary antibody
TRPM8		Rabbit-anti-TRPM8 (Alomone Labs Ltd.; 1:150 in 4% NGS, 4% FSG and 0.1% Triton X-100 in PBS)	Alex Fluor goat-anti-rabbit$_{568}$ (Invitrogen; 1:750 in 4% NGS, 4% FSG in PBS)
TRPV1		Rabbit-anti-TRPV1 (Pierce Biotechnology; 1:1000 in 4% NGS, 4% FSG and 0.1% Triton X-100 in PBS)	Alex Fluor goat-anti-rabbit$_{568}$ (Invitrogen; 1:750 in 4% NGS, 4% FSG in PBS)
TRPV4	2% PFA	Rabbit-anti-TRPV4 (Alomone Labs Ltd.; 1:1000 in 4% NGS, 4% FSG and 0.1% Triton X-100 in PBS)	Alex Fluor goat-anti-rabbit$_{568}$ (Invitrogen; 1:750 in 4% NGS, 4% FSG in PBS)
Peripherin		Mouse-anti-Peripherin (Millipore; 1:500 in 4% NGS, 4% FSG and 0.1% Triton X-100 in PBS)	Alex Fluor goat-anti-mouse$_{488}$ (Invitrogen; 1:300 in 4% NGS, 4% FSG in PBS)
NF200		Mouse-anti-NF200 (Sigma-Aldrich; 1:1000 in 4% NGS, 4% FSG and 0.1% Triton X-100 in PBS)	Alex Fluor goat-anti-mouse$_{488}$ (Invitrogen; 1:300 in 4% NGS, 4% FSG in PBS)

Table 2.3 Antigen retrieval techniques and antibody conditions for TRP channel-related dorsal root ganglia immunohistochemistry. Conditions were optimised for each antibody.

2.8.4 Fluorescence Microscopy and Image Analysis

Images of DRG sections were captured using a Leica DM 2500 fluorescence microscope with Leica DFC 310FX camera and analysis was performed with ImageJ software. To analyse the expression of TRP channels, optical sections for ipsilateral and contralateral DRG were acquired using a x20 objective lens. The number of cells co-expressing TRPM8, TRPV1 or TRPV4 with either NF200 (A-fibre cell body marker; (Whiteside et al., 2010)) or peripherin (C-fibre cell body marker; (Goblirsch

et al., 2004a)) were counted. The number of cells expressing TRPM8, TRPV1 or TRPV4 alone was also counted. Cell counts were performed on 5-6 randomly selected DRG sections from six animals per experimental group (ipsilateral and contralateral DRG of CIBP and Sham V and combined ipsilateral and contralateral Naïve). The experimenter was blinded to the treatment group for analysis.

To count TRPM8-positive cells, the intensity of the brightest cell was assessed using ImageJ Colour Histogram tool and cells with an intensity of over 50% of this maximum were counted as positive. To count TRPV1-positive and TRPV4-positive cells, the background intensity was measured using the same ImageJ Colour Histogram tool and cells with an intensity of twice the background intensity and greater were counted as positive. These two different methods were used in best accordance with the staining profiles achieved by each antibody.

2.9 Western Blotting

2.9.1 Tissue Preparation

DRG were collected immediately following terminal anaesthesia. DRG from L4-6 were dissected both ipsilateral and contralateral to injury. To minimise protein degradation, tissue was collected on ice-cold foil, weighed and rapidly homogenised. DRG tissue was homogenised in 20x volume of Laemmli lysis buffer (5% mercaptoethanol (Sigma, UK), 2% sodium dodecyl sulphate (SDS; Sigma, UK) made up in Tris-hydroxymethylaminoethane buffer (Tris buffer; 50mM, pH 7.4, Sigma, UK)) containing 1% protease inhibitor cocktail III (Calbiochem, UK). DRG homogenates were centrifuged at 10 000 rpm for 10 minutes at 4°C to remove cell debris, aliquoted and stored at -20°C.

2.9.2 Western Blotting Procedure

Proteins were separated by electrophoresis using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) with the NuPage X-Cell Sure Lock™ gel electrophoresis system (Invitrogen, UK). Samples were mixed with 1 µl of loading buffer (0.04% w/v bromophenol in glycerol) and loaded into wells on 4-12% Bis-Tris NuPage gels (Invitrogen, UK). Molecular weight protein standards

were run alongside samples as a molecular weight guide (SeeBlue Plus, Invitrogen, UK). Electrophoresis was carried out using MOPS running buffer (20% in deionised water, NuPage, Invitrogen, UK) at 200V for approximately 50 minutes. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, UK) at 30V for 90 minutes in transfer buffer (5% NuPage transfer buffer (Invitrogen, UK) 10% methanol in double distilled water). Transfer and protein loading was assessed by staining membranes with Coomassie blue (0.1% Coomassie blue in 30% methanol, 10% acetic acid (GE Healthcare Ltd, UK)) and scanned. The membrane was destained using a solution of 50% methanol, 10% acetic acid in distilled water and then rinsed in PBS. The membrane was incubated in blocking buffer (5% Marvel (powdered milk) in PBS or 5% Marvel in PBS-Tween (0.1% Tween-20 in PBS)) overnight at 4°C or from 90 minutes to 2 hours at room temperature and probed for NR2A, TRPM8, TRPV1 and TRPV4 with the following primary antibodies; TRPM8 (rabbit-anti-TRPM8, 1:100, Alomone Labs Ltd. in 5% Marvel/PBS-Tween), TRPV1 (rabbit-anti-TRPV1 1:500, Pierce Biotechnology in 5% Marvel/PBS-Tween) and TRPV4 (rabbit-anti-TRPV4, 1:200, Alomone Labs Ltd. in 5% Marvel/PBS-Tween). Membranes were washed with PBS-Tween and probed with appropriate horseradish peroxidise (HRP)-linked secondary antibody and incubated with enhanced chemiluminescent detection reagent (ECL; Cell Signalling Technology, USA). Antibody conditions are detailed in Table 2.4. Membranes were placed between two transparent sheets before exposure to autoradiography film (GE Healthcare UK Ltd., UK). All membranes were probed for the ubiquitous housekeeping enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH; mouse-anti-GAPDH 1:750; Millipore in 2% BSA/ PBS-Tween overnight at 4°C) for protein level normalization. Sample protein levels were quantified by densitometry using arbitrary grey scale values as a percentage of GAPDH using Adobe Photoshop software (version 7.0). To identify whether anti-TRPM8 antibody (Alomone Labs Ltd., UK) was specific to TRPM8, TRPM8-transfected cells were used as a positive control sample. A line of HEK293 cells stably expressing human TRPM8 were used (generated by R. Mitchell, P. Holland and B. Rosie, Centre for Integrative Physiology).

	Block	Primary antibody	Secondary antibody
TRPM8	5% Marvel/PBS-Tween	Rabbit-anti-TRPM8 (1:100; Alomone Labs Ltd. in 5% Marvel/PBS- Tween-20)	HRP-linked donkey-anti-rabbit (1:7500; Chemicon in 5% Marvel/PBS-Tween-20)
TRPV1	5% Marvel/PBS	Rabbit-anti-TRPV1 (1:500; Pierce Biotechnology in 5% Marvel/PBS-Tween-20)	HRP-linked donkey-anti-rabbit (1:7500; Chemicon in 5% Marvel/PBS-Tween-20)
TRPV4	5% Marvel/PBS	Rabbit-anti-TRPV4 (1:200; Alomone Labs Ltd. in 5% Marvel/PBS-Tween-20)	HRP-linked donkey-anti-rabbit (1:7500; Chemicon in 5% Marvel/PBS-Tween-20)
GAPDH	5% Marvel/PBS	Mouse-anti-GAPDH (1:750; Millipore in 2% BSA/ PBS-Tween-20)	HRP-linked goat-anti-mouse (1:10 000; Chemicon in 2% BSA/ PBS-Tween-20)

Table 2.4 Antibody conditions for Western immunoblots. Conditions were optimised for each antibody.

2.10 Statistical Analysis

2.10.1 Behavioural Assessments

In each behavioural study, data were pooled for each test day, with group mean shown ± SEM. For replicate measures the mean values were determined by calculating the mean for each animal, from which the SEM was calculated. For all analysis significance was set at $p<0.05$. Data were analysed using GraphPad Prism (version 5.0). The Kolmogorov-Smirnov test was used to check that data was normally distributed before statistical analysis was carried out.

Body Weight

The difference in body weight between groups was determined by a repeated measures mixed-model ANOVA followed by Bonferroni's post-hoc analysis.

Mechanical Allodynia

For responses to von Frey filaments, differences between ipsilateral hindlimb to contralateral hindlimb were determined by One-way repeated measures ANOVA on ranks (Friedman's test) followed by Dunn's post-hoc analysis and differences between post-surgical and pre-surgical values were determined by One-way repeated measures ANOVA on ranks (Friedman's test) followed by Dunn's post-hoc analysis (comparing pre-surgical ipsilateral or contralateral values to each time points post-surgical ipsilateral or contralateral values, respectively).

Thermal Sensitivity

For responses to 10°C, 20°C, 30°C or 40°C thermal footplate, differences between ipsilateral post-surgical and pre-surgical values for number of and latency to paw withdrawal and duration of paw elevation were determined by a repeated measures mixed-model ANOVA followed by a Bonferroni's post-hoc analysis (comparing pre-surgical ipsilateral values to each time points post-surgical ipsilateral values).

Movement-evoked Pain

For avoidance of weight bearing on movement, differences between ipsilateral post-surgical and pre-surgical values were determined by a repeated measures mixed-model ANOVA followed by Bonferroni's post-hoc analysis (comparing pre-surgical ipsilateral values to each time points post surgical ipsilateral values).

Static Weight Bearing

For static weight bearing difference (difference in weight bearing between the ipsilateral and contralateral hindlimb), the differences between post-surgical and pre-surgical values were determined by a repeated measures mixed-model ANOVA followed by Bonferroni's post-hoc analysis (comparing pre-surgical ipsilateral values to each time points post-surgical ipsilateral values).

2.10.2 Analysis of Analgesic Interventions

In each behavioural study, data were pooled for each time point, with group mean shown ± SEM. To analyse the effects of XRT or pharmacological agent administration on mechanical allodynia, post-pharmacological agent ipsilateral paw withdrawal thresholds were compared to pre-pharmacological agent baseline using a One-way repeated measures ANOVA on Ranks (Friedman's test) followed by Dunn's post-hoc analysis. Ipsilateral paw withdrawal thresholds were compared to contralateral paw withdrawal thresholds using a One-way repeated measures ANOVA on ranks (Friedman's test) followed by Dunn's post-hoc analysis.

To analyse the effects of XRT on thermal sensitivity, the difference between post-XRT ipsilateral responses and pre-XRT ipsilateral responses and XRT-treated animals were compared to CIBP alone using a repeated measures mixed-model ANOVA followed by Bonferroni's post-hoc analysis. To analyse the effects of pharmacological agent administration on thermal sensitivity the difference between post-pharmacological agent ipsilateral responses and pre-pharmacological agent ipsilateral responses were determined using a One-way repeated measures ANOVA followed by Dunnett's post-hoc analysis.

To analyse the effects of XRT on movement-evoked pain, post-XRT number of avoidances of weight bearing on movement were compared to pre-XRT values and XRT-treated animals were compared to CIBP animals alone using a repeated measures mixed-model ANOVA followed by Dunnett's post-hoc analysis. To analyse the effects of pharmacological agent administration on movement-evoked pain, post-pharmacological agent number of avoidances of weight bearing on movement were compared to pre-pharmacological agent using a One-way repeated measures ANOVA followed by Dunnett's post-hoc analysis.

To analyse the effects of XRT on static weight bearing difference, pos-XRT weight bearing difference was compared to pre-XRT values and XRT-treated animals were compared to CIBP using a repeated measures mixed-model ANOVA.

2.10.3 Analysis of Elevated Plusmaze and Open Field Data

For responses to the elevated plusmaze (for example time spent in the open arms) and the open field (for example time spent in the centre zone) differences between groups (CIBP, CIBP + pharmacological agent, XRT treated, Sham V operated and Naive animals) were determined by a One-way ANOVA followed by Bonferroni's post-hoc analysis. For the elevated plusmaze and open field, there were several separate test sessions and only those run at the same time were compared. Data were not combined for separate test sessions of the same group as these measures of anxiety are subject to variation between tests.

For the elevated plusmaze, the first test session involved Naive, Sham V and CIBP, with Sham V as the comparator for effect of CIBP on behaviours. A separate group of CIBP and XRT were tested together, with CIBP as the comparator for effect of XRT treatment. Gabapentin, duloxetine and vehicle were assessed in parallel, however S,S-reboxetine was run at a separate time to the other pharmacological agents and was therefore not directly compared to the vehicle control. For the open field, the test session involved Naive, Sham V, CIBP and XRT groups, with Sham V as the comparator for the effect of CIBP on behaviours.

2.10.4 Exclusion Criteria

CIBP animals displaying the most CIBP-induced sensitivity on the day of radiotherapy or pharmacological agent administration were included for radiotherapy treatment and pharmacological agent profiling. Animals that fell off the elevated plusmaze were excluded from analysis of the elevated plusmaze data but not from analysis of behavioural tests carried out previously (XRT n=1, CIBP n=2, Vehicle n=1 and S,S-reboxetine n=2).

2.10.5 Spinal Cord Immunohistochemistry

NR1, NR2A and NR2B expression in laminae I, II and III was compared between groups (CIBP Ipsilateral, CIBP Contralateral, Sham E Ipsilateral, Sham E Contralateral and Naive) by a repeated measures mixed-model ANOVA followed by Bonferroni's post-hoc analysis. Differences within groups (e.g. CIBP Ipsilateral versus CIBP Contralateral) were compared by an unpaired two-tailed t-test.

NR2A co-expression with NeuN was compared between groups by a One-way ANOVA followed by Bonferroni's post-hoc analysis.

2.10.6 DRG Immunohistochemistry

Differences between groups (CIBP Ipsilateral, CIBP Contralateral, Sham V Ipsilateral, Sham V Contralateral and Naive) were compared by a One-way ANOVA followed by Bonferroni's post-hoc analysis. Differences within groups (e.g. CIBP Ipsilateral versus CIBP Contralateral) were compared by an unpaired two-tailed t-test.

2.10.7 Western Blot

Differences in relative intensity between groups (CIBP Ipsilateral, CIBP Contralateral, Sham V Ipsilateral, Sham V Contralateral and Naive) were compared by One-way ANOVA followed by Bonferroni's post-hoc analysis. Differences within groups (e.g. CIBP Ipsilateral versus CIBP Contralateral) were detected by an unpaired two-tailed t-test.

3. CHARACTERISATION OF A PRECLINICAL MODEL OF CANCER-INDUCED BONE PAIN (CIBP)

3.1 Introduction

Cancer-induced bone pain (CIBP) is difficult to control using currently available therapeutic regimes, primarily palliative radiotherapy and opioid therapy. Patients suffer from constant background pain with breakthrough pain, which may be movement-evoked or occur spontaneously (Portenoy et al., 1999). The breakthrough pain component is particularly difficult to treat as the doses of opioids required to treat breakthrough pain in CIBP produce unacceptable side-effects (Mercadante et al., 1992). Opioid toxicity is a major problem, where symptoms can range from sedation and poor concentration to hallucination and agitation.

A clinically relevant preclinical animal model is required to elucidate the mechanisms underlying CIBP and facilitate the development of therapies that will enable improved pain management of CIBP patients. The validity of preclinical findings translating to the clinic is dependent on a model that mirrors the clinical condition. Preclinical models of CIBP involving intraosseous injection of carcinoma cells using mice (Schwei et al., 1999) and rats (Dore-Savard et al., 2010;Medhurst et al., 2002) have been developed. One advantage of these models over the earlier metastatic cancer models, which involved systemic (Arguello et al., 1988) or intra muscular injection (Kostenuik et al., 1993), is the confinement of the tumour within the bone, which allows direct assessment of the affected limb without the complication of multiple site metastases. These models have been shown to mirror the clinical development of CIBP including tumour growth, extensive bone destruction and the development of pain-related behaviours (Luger et al., 2005). Anxiety and depression are common comorbidities in patients suffering from CIBP, which can impact significantly on quality of life (Portenoy et al., 1999). In addition these complex behaviours can have considerable impact on the efficacy of analgesic intervention. To date, investigation of anxiety-like behaviours in preclinical CIBP models has not been reported.

Bone is the most common site of metastases in cancer and around 70% of patients dying from breast and prostate cancers have evidence of bone metastases (Coleman, 2006). Bone metastases can potentially occur at any bony site, however most commonly affect the axial skeleton and proximal long bones. Bone metastases can be classified as osteolytic, where there is increased osteoclast activity and net bone destruction, osteoblastic where the tumour-induced lesions are characterised by excess osteoblast activity leading to formation of abnormal new bone or mixed osteolytic/osteoblastic bone lesions. Osteolytic bone lesions are characteristic of breast cancer and osteoblastic lesions are characteristic of prostate cancer (Kingsley et al., 2007). Dore-Savard *et al.* showed X-ray scans of a rat model of cancer pain, where the femur of male rats was implanted with MRMT-1 cells. Results showed that bone density gradually decreased from Day 14 and the cortical line was blurred from Day 18. At Day 21, the bone shape was irregular due to irregular bone formation by osteoblasts. Analysis of these results support the osteolytic character of the MRMT-1 tumour in the femur of male rats (Dore-Savard et al., 2010) (Figure 3.1).

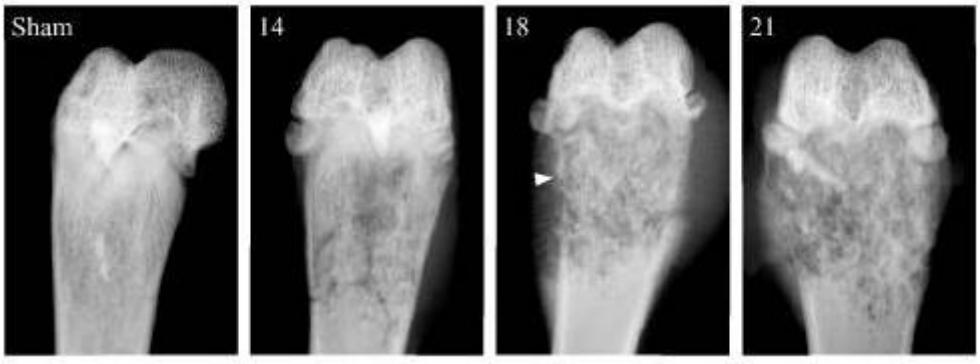


Figure 3.1 Figure adapted from Dore-Savard *et al.* 2010. Figure shows radiographs of sham-operated and CIBP femurs at 14, 18 and 21 days after surgery. At Day 14, decreased bone density is observed in the medullar canal of the bone while cortical integrity is maintained. At Day 18, the cortical line is blurred and cortical integrity is partially compromised. By Day 21 the bone shape is irregular.

This project used the MRMT-1 model of CIBP, which involves the use of rat mammary gland carcinoma cells, to create a clinically relevant model suitable for

studying therapeutic interventions. Previous studies have investigated CIBP using MRMT-1 cells in both male (Donovan-Rodriguez et al., 2004b) and female (Medhurst et al., 2002) rats. Both male and female models, which involve MRMT-1 cell inoculation into the tibia, display the gradual development of mechanical allodynia and progressive bone destruction. In the present study, these cells were implanted in male rats to avoid hormonal influences that are known to affect pain responses (Aloisi & Bonifazi, 2006;Cairns & Gazerani, 2009).

3.2 Aim

The primary aim of this part of the study was to comprehensively characterise the behaviour of a CIBP model adapted from Medhurst *et al.* (Medhurst et al., 2002) and Urch *et al.* (Urch et al., 2003b). Using this rat model of CIBP we investigated pain-related behaviours, pain-related anxiety and risk-assessment behaviours.

3.3 Methods

3.3.1 Surgical Procedure

Experiments were carried out using male Sprague Dawley rats as detailed in Chapter 2; anaesthetised animals received a unilateral intra-tibial injection of MRMT-1 carcinoma cells (Section 2.1.2). Following injection of cells the wound was plugged with dental cement to confine the tumour cells within the tibial marrow and prevent invasion of adjacent soft tissue. Three sham-operated models were used throughout this study; Sham Vehicle (Sham V) model, Sham Heat-killed (Sham HK) and Sham Exposed (Sham E). Surgery for CIBP and all Sham models was the same with the following exceptions; Sham V and Sham HK animals received an intra-tibial injection of vehicle or heat-killed MRMT-1 cells respectively, while the tibia was only exposed in Sham E animals (Section 2.1.2). We also examined all behaviours in Naïve (un-injured) animals. Three sham models were used to allow us to fully characterise the mechanisms behind the pain behaviours observed. This ensured that any identified CIBP behaviours were due to tumour growth and not due to periosteal or bone damage caused by surgery or by the presence of heat-killed cells.

3.3.2 Behavioural Analysis

All animals were behaviourally assessed prior to surgery (to obtain baseline values) and post-surgery from Day 3-4 until Day 18-21. This model has a limited time frame to ensure that the tumour does not grow outside the bone and spontaneous fracture does not occur as a result of bone destruction. Radiographs of a subset of hindlimbs were collected to check this. Histological and radiological analysis of this model has found significant bone destruction from Day 14 onwards, with no evidence of local extra-osseal spread of tumour (Medhurst et al., 2002;Urch et al., 2003b). The behavioural responses of CIBP animals were compared to baseline behaviours (to assess the development of CIBP-induced behavioural changes) and additionally compared to Sham V (to control for the effect of bone damage on behavioural sensitisation) and Naïve animals. To determine the impact of bone damage or the presence of carcinoma cells (without progressive tumour growth) on behavioural sensitisation we compared the responses of each Sham group to Naïve animals.

The development of mechanical allodynia was examined using calibrated von Frey filaments (Section 2.3.1) and signs of movement-evoked pain were evaluated using both the rotarod to assess the avoidance of weight bearing on movement and the open field/elevated plusmaze to determine voluntary locomotor activity (Section 2.4.1, 2.4.3 & 2.4.4). Thermal sensitivity was tested using the thermal footplate, at the temperatures of 10, 20, 30 and 40°C. The number of ipsilateral paw withdrawals, latency to the first paw withdrawal and the duration of paw elevation were recorded (Section 2.3.2). Animals were tested for static pain by assessing difference in weight bearing between hindlimbs using an incapacitance tester (Section 2.4.2) and assessed for the development of spontaneous pain by observation of spontaneous foot lifting (Section 2.5). Pain-related anxiety was analysed in the open field and on the elevated plusmaze. Risk assessment behaviour was analysed on the elevated plusmaze (Section 2.6).

3.3.3 Statistical Analysis

In each behavioural study, data were pooled for each test day, with group

mean shown ± SEM. For replicate measures the mean values were determined by calculating the mean for each animal, from which the SEM was calculated. For all analysis significance was set at $p<0.05$. Data were analysed using GraphPad Prism (version 5.0). The Kolmogorov-Smirnov test was used to check that data was normally distributed before statistical analysis was carried out.

The difference in body weight between groups was determined by a repeated measures mixed-model ANOVA followed by Bonferroni's post-hoc analysis.

For responses to von Frey filaments, differences between ipsilateral hindlimb to contralateral hindlimb were determined by One-way repeated measures ANOVA on ranks (Friedman's test) followed by Dunn's post-hoc analysis and differences between post-surgical and pre-surgical values were determined by One-way repeated measures ANOVA on ranks (Friedman's test) followed by Dunn's post-hoc analysis (comparing pre-surgical ipsilateral or contralateral values to each time points post-surgical ipsilateral or contralateral values, respectively).

For responses to 10°C, 20°C, 30°C or 40°C thermal footplate, differences between ipsilateral post-surgical and pre-surgical values for number of and latency to paw withdrawal and duration of paw elevation were determined by a repeated measures mixed-model ANOVA followed by a Bonferroni's post-hoc analysis (comparing pre-surgical ipsilateral values to each time points post-surgical ipsilateral values).

For avoidance of weight bearing on movement, differences between ipsilateral post-surgical and pre-surgical values were determined by a repeated measures mixed-model ANOVA followed by Bonferroni's post-hoc analysis (comparing pre-surgical ipsilateral values to each time points post surgical ipsilateral values).

For static weight bearing difference (difference in weight bearing between the ipsilateral and contralateral hindlimb), the differences between post-surgical and pre-

surgical values were determined by a repeated measures mixed-model ANOVA followed by Bonferroni's post-hoc analysis (comparing pre-surgical ipsilateral values to each time points post-surgical ipsilateral values).

For responses to the elevated plusmaze and the open field (differences between groups (CIBP, Sham V operated and Naive animals) were determined by a One-way ANOVA followed by Bonferroni's post-hoc analysis. For the elevated plusmaze and open field, there were several separate test sessions and only those run at the same time were compared. Data were not combined for separate test sessions of the same group as these measures of anxiety are subject to variation between tests. For the elevated plusmaze, the first test session involved Naive, Sham V and CIBP, with Sham V as the comparator for effect of CIBP on behaviours. For the open field, the test session involved Naive, Sham V, CIBP, with Sham V as the comparator for the effect of CIBP on behaviours.

3.4 Results

All animals were observed post-surgery to ensure animals maintained a healthy weight and did not show signs of distress.

3.4.1 CIBP animals displayed normal weight gain when compared to Sham V and Naïve controls

Body weight was recorded over the time course of behavioural assessment. CIBP animals maintained a healthy weight that was not significantly different to Sham V and Naïve animals (Figure 3.2).

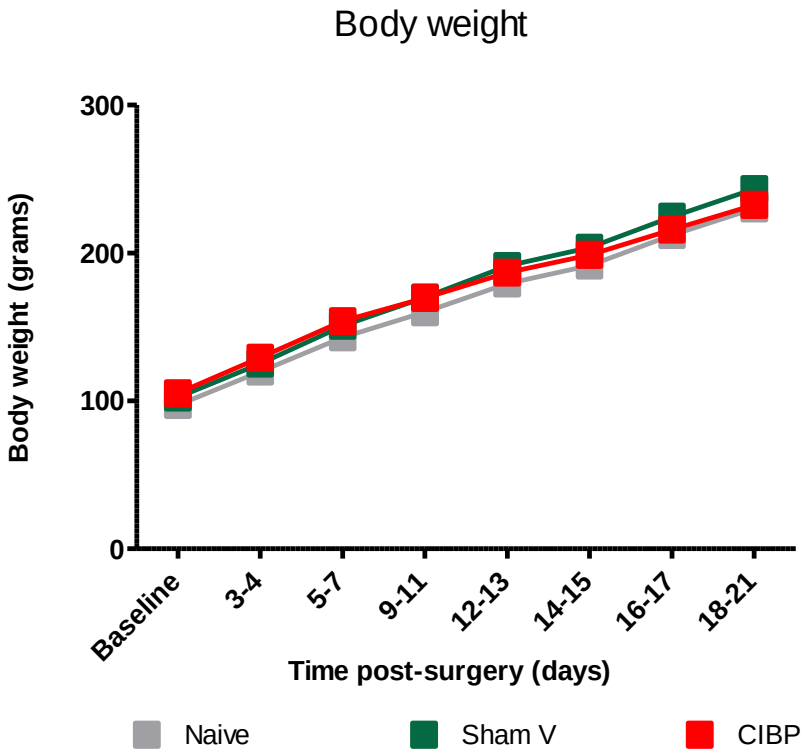


Figure 3.2 Time course of body weight gain. Data shows mean body weight ± SEM of Naive (n=10), Sham V (n=10) and CIBP (n=10) animals. No significant difference in body weight was found in CIBP and Sham V animals when compared to Naïve at any time point.

		Time post-surgery (days)							
Group		Baseline	3-4	5-7	9-11	12-13	14-15	16-17	18-21
CIBP	Weight (grams)	105.1	129.3	154.0	169.7	186.8	198.7	215.9	232.6
	SEM	2.8	3.0	2.9	4.3	3.7	3.6	3.7	3.8
Sham V	Weight (grams)	102.4	125.2	150.8	170.1	191.3	203.6	224.5	243.3
	SEM	2.2	3.0	3.6	3.1	3.0	2.9	2.9	4.7
Naive	Weight (grams)	97.3	119.8	143.0	160.1	179.4	191.7	212.2	230.1
	SEM	3.8	4.6	5.7	6.6	6.5	7.0	7.3	7.2

Table 3.1 Body weight. Data shows mean body weight ± SEM of Naive (n=10), Sham V (n=10) and CIBP (n=10) animals.

3.4.2 Bone density analysed by radiographs of CIBP animals' hindlimbs

Radiographs of the subset of hindlimbs collected showed evidence of bone destruction, with decreased bone density of the ipsilateral (injured) hindlimb when compared to the contralateral (non-injured) hindlimb observed, but no evidence of extra-osseal tumour growth observed (Figure 3.3).

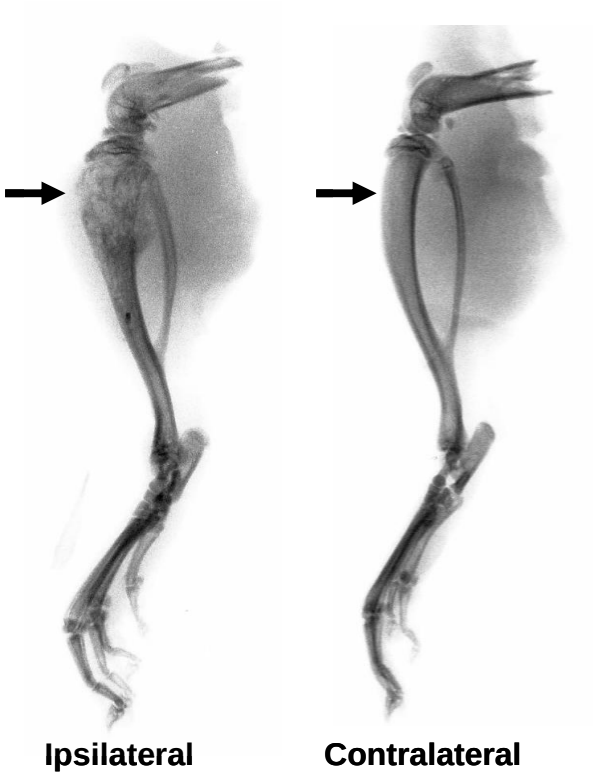


Figure 3.3 Representative radiograph of CIBP hindlimbs, illustrating decreased bone density and increased bone destruction in ipsilateral hindlimb (arrows) compared to contralateral hindlimb.

3.4.3 Development of mechanical allodynia in the CIBP model

The responses to a non-noxious mechanical stimulus (von Frey filaments) were measured over 21 days. CIBP significantly reduced ipsilateral hindlimb paw withdrawal threshold (PWT) in comparison to baseline from Day 9-11 onwards shown by One-way repeated measures ANOVA on ranks (Friedman's test) followed by Dunn's post-hoc analysis, $p<0.05$. CIBP significantly reduced ipsilateral PWT in comparison to contralateral hindlimb values from Day 5-7 onwards shown by One-way repeated measures ANOVA on ranks (Friedman's test) followed by Dunn's post-hoc analysis, $p<0.05$. Sham V and Naive animals did not show a reduction in ipsilateral PWT from baseline or compared to contralateral PWT (Figure 3.4).

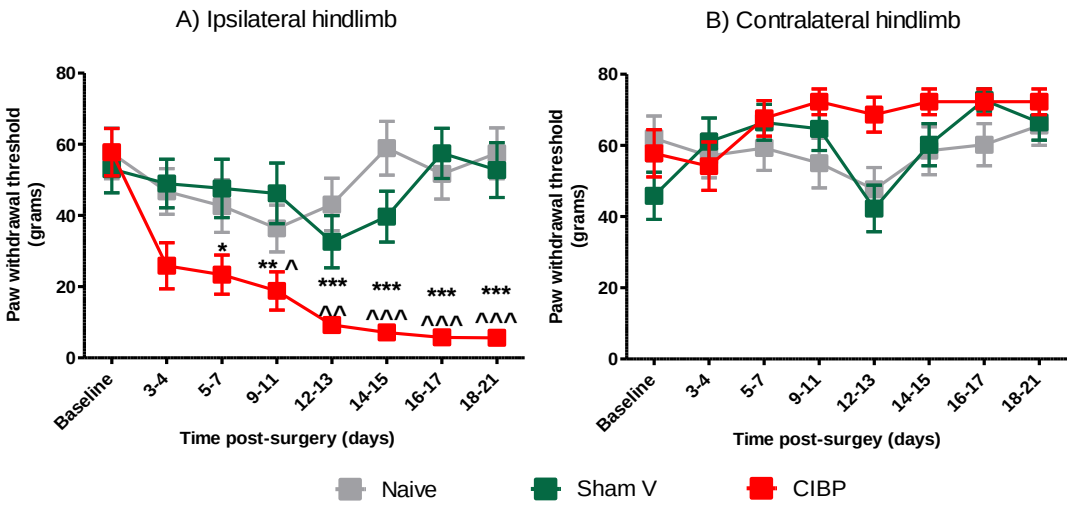


Figure 3.4 Time course of CIBP-induced mechanical allodynia. Data show mean responses ± SEM of Naive (n=15), Sham V (n=15) and CIBP (n=13) animals. CIBP significantly reduced ipsilateral PWT in comparison to baseline from Day 9-11 onwards (^; One-way repeated measures ANOVA on ranks (Friedman's test) followed by Dunn's post-hoc analysis, $p<0.05$). CIBP significantly reduced ipsilateral compared to contralateral PWT from Day 5-7 onwards (*; One-way repeated measures ANOVA on ranks (Friedman's test) followed by Dunn's post-hoc analysis, $p<0.05$). P values ^^^ = <0.001, ^^ = 0.001 to 0.01, ^ = 0.01 to 0.05, *** = <0.001, ** = 0.001 to 0.01 and * = 0.01 to 0.05.

			Time post-surgery (days)							
Group			Baseline	3-4	5-7	9-11	12-13	14-15	16-17	18-21
CIBP	Ipsi	PWT (grams)	57.8	25.9	23.4	18.8	9.2	7.1	5.8	5.6
		SEM	6.6	6.5	5.5	5.4	1.1	0.6	0.6	0.5
	Con	PWT (grams)	57.8	54.2	67.6	72.2	68.6	72.2	72.2	72.2
		SEM	6.6	6.8	5.0	3.6	4.9	3.6	3.6	3.6
Sham V	Ipsi	PWT (grams)	53.0	49.0	47.6	46.2	32.6	39.6	57.4	52.8
		SEM	6.6	6.8	8.2	8.5	7.3	7.1	7.0	7.7
	Con	PWT (grams)	45.8	61.0	66.5	64.6	42.2	60.2	72.7	66.5
		SEM	6.7	6.7	5.0	6.1	6.5	5.9	3.1	5.0
Naïve	Ipsi	PWT (grams)	57.5	46.7	42.6	36.4	43.1	58.9	51.6	57.5
		SEM	7.1	6.4	7.4	6.6	7.4	7.5	7.1	7.1
	Con	PWT (grams)	62.0	57.1	59.3	55.0	47.6	58.5	60.2	65.5
		SEM	6.3	6.2	6.3	6.9	6.2	6.7	5.9	5.6

Table 3.2 CIBP-induced mechanical allodynia. Data show mean responses ± SEM of Naive (n=15), Sham V (n=15) and CIBP (n=13) animals.

3.4.4 Development of thermal sensitivity to 10°C in a CIBP model

At 10°C, CIBP animals showed a significant increase in number of paw withdrawals when compared to baseline from Day 9-11 to Day 18-21. Sham V and Naive animals showed a significant increase in number of paw withdrawals when compared to baseline at Day 14-15. CIBP animals showed increased number of paw withdrawals compared to Sham V at Day 18-21 only. CIBP animals showed decreased latency to paw withdrawal compared to baseline at Day 9-11 and Day 18-21. Only CIBP animals showed a significantly increased duration of paw elevation when compared to baseline at Day 14-15 and Day 18-21. CIBP animals showed increased duration of paw elevation when compared to Sham V at Day 14-15 and

Day 18-21 (Figure 3.5). All shown by repeated measures mixed-model ANOVA followed by Bonferroni's post-hoc analysis, $p<0.05$

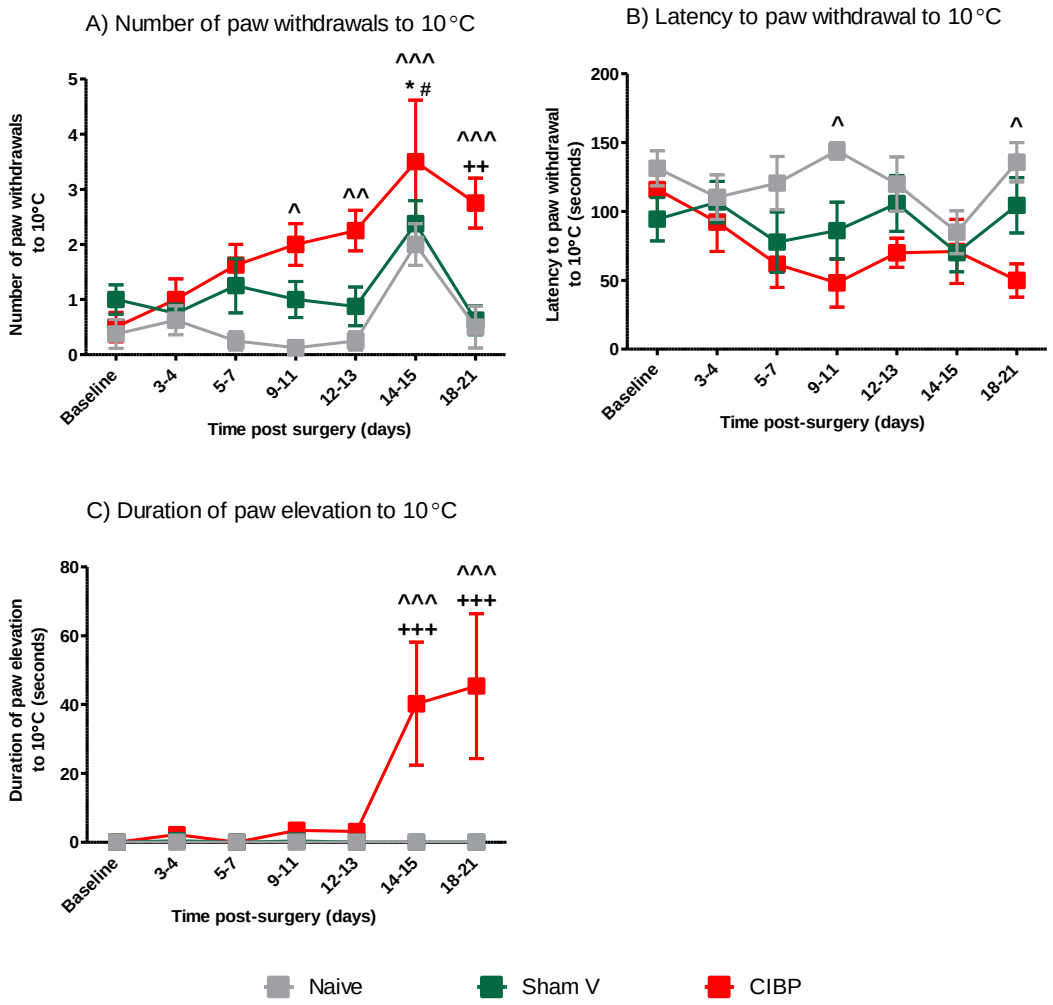


Figure 3.5 Thermal sensitivity to 10°C. Data show mean responses ± SEM of Naive (n=8), Sham V (n=8) and CIBP (n=8) animals. A) CIBP animals showed significantly increased ipsilateral paw withdrawal when compared to baseline from Day 9-11 onwards, Sham V and Naive showed significantly increased ipsilateral paw withdrawal when compared to baseline at Day 14-15 (^, * and #, respectively; repeated measures mixed-model ANOVA followed by Bonferroni's post-hoc analysis, $p<0.05$). CIBP animals showed significantly increased ipsilateral paw withdrawal when compared to Sham V at Day 18-21 only (+; repeated measures mixed-model ANOVA followed by Bonferroni's post-hoc analysis, $p<0.05$). B) CIBP animals showed decreased latency to paw withdrawal when compared to baseline at

Day 9-11 and Day 18-21 (^; repeated measures mixed-model ANOVA followed by Bonferroni's post-hoc analysis, $p<0.05$). CIBP animals did not show significantly altered latency to paw withdrawal when compared to Sham V. C) CIBP animals showed significantly increased duration of paw elevation when compared to baseline at Day 14-15 onwards (^; repeated measures mixed-model ANOVA followed by Bonferroni's post-hoc analysis, $p<0.05$). CIBP showed significantly increased duration of paw elevation at Day 14-15 and Day 18-21 (+; repeated measures mixed-model ANOVA followed by Bonferroni's post-hoc analysis, $p<0.05$). P values ^^^ = <0.001, ^^ = 0.001 to 0.01, ^ = 0.01 to 0.05, * = 0.01 to 0.05 and $^{+++}$ = <0.001.

			Time post-surgery (days)						
	Group		Baseline	3-4	5-7	9-11	12-13	14-15	18-21
Number	CIBP	Number	0.5	1.0	1.6	2.0	2.3	3.5	2.8
		SEM	0.3	0.4	0.4	0.4	0.4	1.1	0.5
	Sham V	Number	1.0	0.8	1.3	1.0	0.9	2.4	0.6
		SEM	0.3	0.2	0.5	0.3	0.4	0.4	0.3
	Naïve	Number	0.4	0.6	0.3	0.1	0.3	2.0	0.5
		SEM	0.3	0.3	0.2	0.1	0.2	0.4	0.4
Latency	CIBP	Latency	116.0	92.3	61.8	48.1	70.0	71.0	49.9
		SEM	17.5	21.3	16.8	17.5	10.6	23.3	12.1
	Sham V	Latency	94.4	106.9	77.8	86.1	105.9	70.1	104.5
		SEM	15.8	15.1	22.0	20.7	20.3	14.0	20.0
	Naïve	Latency	131.4	110.4	120.6	144.0	119.9	85.0	135.8
		SEM	12.7	16.2	19.3	6.0	19.7	15.6	14.3
Duration	CIBP	Duration	0.0	2.3	0.0	3.5	3.1	40.3	45.4
		SEM	0.0	1.5	0.0	2.1	1.6	17.9	21.0
	Sham V	Duration	0.0	0.5	0.0	0.4	0.0	0.0	0.0
		SEM	0.0	0.5	0.0	0.4	0.0	0.0	0.0
	Naive	Duration	0.0	0.0	0.0	0.0	0.0	0.0	0.0
		SEM	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Table 3.3 Thermal sensitivity to 10°C showing number of paw withdrawals, latency to paw withdrawal and duration of paw elevation. Data show mean responses ± SEM of Naive (n=8), Sham V (n=8) and CIBP (n=8) animals.

3.4.5 Development of thermal sensitivity to 20°C in the CIBP model

At 20°C, CIBP animals showed a significant increase in number of paw withdrawals when compared to baseline from Day 12-13 to Day 18-21 and Sham V animals showed an increase in number of paw withdrawals when compared to baseline at Day 3-4 only. CIBP animals showed increased number of paw

withdrawals compared to Sham V from day 12-13. CIBP animals showed decreased latency to paw withdrawal at Day 3-4 and from Day 14-15 to Day 18-21 when compared to baseline and Sham V animals also showed a decrease in latency to paw withdrawal at Day 5-7 and Day 16-17. CIBP animals showed decreased latency to paw withdrawal when compared to Sham V at Day 16-17 and Day 18-21. CIBP animals showed significantly increased duration of paw elevation when compared to baseline from Day 14-15 onwards. CIBP animals showed increased duration of paw elevation when compared to Sham V at Day 16-17 and Day 18-21. All shown by a repeated measures mixed-model ANOVA followed by Bonferroni's post-hoc analysis, $p<0.05$ (Figure 3.6).

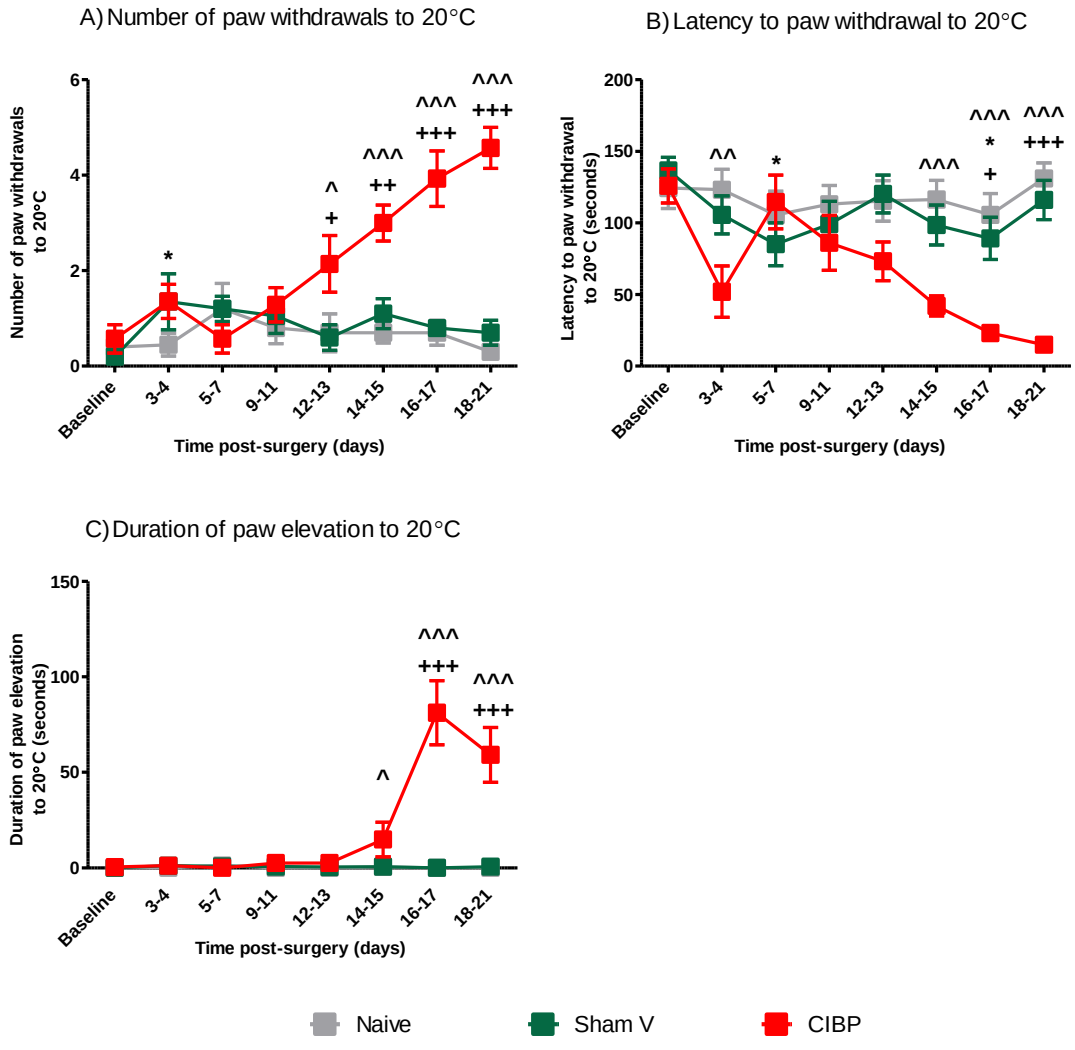


Figure 3.6 Thermal sensitivity to 20°C. Data show mean responses ± SEM of Naive (n=10), Sham V (n=10) and CIBP (n=7) animals. A) CIBP animals showed

significantly increased ipsilateral paw withdrawal when compared to baseline from Day 12-13 onwards, Sham V showed significantly increased ipsilateral paw withdrawal when compared to baseline at Day 3-4 (^ and *, respectively; repeated measures mixed-model ANOVA followed by Bonferroni's post-hoc analysis, $p<0.05$). CIBP animals showed significantly increased ipsilateral paw withdrawal when compared to Sham V from Day 12-13 onwards (+ repeated measures mixed-model ANOVA followed by Bonferroni's post-hoc analysis, $p<0.05$). B) CIBP animals showed decreased latency to paw withdrawal when compared to baseline at Day 3-4 and from Day 14-15 onwards, Sham V showed decreased latency to paw withdrawal when compared to baseline at Day 5-7 and Day 16-17 (^ and *, respectively; repeated measures mixed-model ANOVA followed by Bonferroni's post-hoc analysis, $p<0.05$). CIBP animals showed a significant increase in latency to paw withdrawal when compared to Sham V at Day 16-17 and Day 18-21 (+; repeated measures mixed-model ANOVA followed by Bonferroni's post-hoc analysis, $p<0.05$). C) CIBP animals showed significantly increased duration of paw elevation when compared to baseline at Day 14-15 onwards (^; repeated measures mixed-model ANOVA followed by Bonferroni's post-hoc analysis, $p<0.05$). CIBP animals showed significantly increased duration of paw elevation at Day 16-17 and Day 18-21 (+; repeated measures mixed-model ANOVA followed by Bonferroni's post-hoc analysis, $p<0.05$). P values ^^^ = <0.001, ^^ = 0.001 to 0.01, ^ = 0.01 to 0.05, * = 0.01 to 0.05, $^{+++}$ = <0.001, $^{++}$ = 0.001 to 0.01 and $^{+}$ = 0.01 to 0.05.

			Time post-surgery (days)							
	Group		Baseline	3-4	5-7	9-11	12-13	14-15	16-17	18-21
Number	CIBP		0.6	1.4	0.6	1.3	2.1	3.0	3.9	4.6
		SEM	0.3	0.4	0.3	0.4	0.6	0.4	0.6	0.4
	Sham V		0.2	1.4	1.2	1.1	0.6	1.1	0.8	0.7
		SEM	0.1	0.6	0.3	0.4	0.3	0.3	0.1	0.3
	Naïve		0.4	0.5	1.2	0.8	0.7	0.7	0.7	0.3
		SEM	0.2	0.2	0.5	0.3	0.4	0.2	0.3	0.2
Latency	CIBP		125.9	52.0	114.7	86.1	73.3	42.0	23.1	15.0
		SEM	11.9	18.0	18.8	19.1	13.5	7.0	4.6	4.2
	Sham V		136.8	105.7	85.1	99.2	120.2	98.6	89.2	116.1
		SEM	9.2	13.3	15.0	15.9	13.2	14.0	14.7	13.7
	Naïve		124.4	123.2	105.6	113.1	115.5	116.3	105.7	131.1
		SEM	14.3	14.3	16.5	13.1	14.2	13.4	15.0	10.8
Duration	CIBP		0.4	1.1	0.0	2.6	2.6	14.9	81.3	59.1
		SEM	0.4	0.7	0.0	2.6	2.1	9.1	16.8	14.4
	Sham V		0.0	1.2	0.8	0.7	0.4	0.6	0.0	0.6
		SEM	0.0	1.2	0.8	0.5	0.4	0.6	0.0	0.4
	Naive		0.0	0.0	1.3	0.0	0.0	0.5	0.0	0.0
		SEM	0.0	0.0	1.3	0.0	0.0	0.5	0.0	0.0

Table 3.4 Thermal sensitivity to 20°C showing number of paw withdrawals, latency to paw withdrawal and duration of paw elevation. Data show mean responses ± SEM of Naive (n=10), Sham V (n=10) and CIBP (n=7) animals.

3.4.6 Development of thermal sensitivity to 30°C in the CIBP model

At 30°C, CIBP animals showed a significant increase in number of paw withdrawals when compared to baseline at Day 5-7 and Day 14-15 to Day 18-21, which was also observed in Sham V animals at Day 14-15. CIBP animals showed decreased latency to paw withdrawal when compared to baseline at Day 5-7 and Day

18-21. CIBP animals showed significantly increased duration of paw elevation when compared to baseline at Day 18-21. CIBP animals showed increased duration of paw elevation when compared to Sham V at Day 18-21. All shown by a repeated measures mixed-model ANOVA followed by Bonferroni's post-hoc analysis, $p<0.05$ (Figure 3.7).

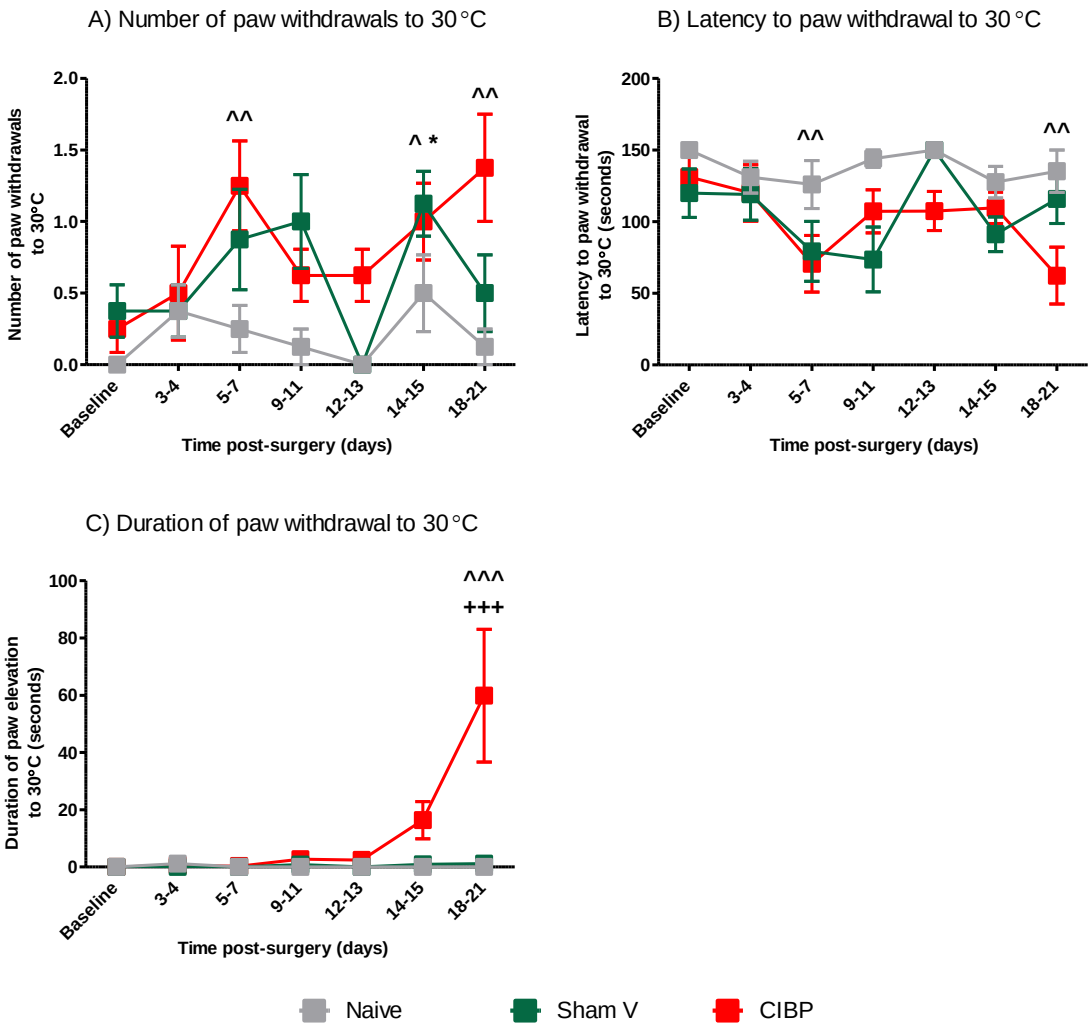


Figure 3.7 Thermal sensitivity to 30°C. Data show mean responses ± SEM of Naive (n=8), Sham V (n=8) and CIBP (n=8) animals. A) CIBP animals showed significantly increased ipsilateral paw withdrawal when compared to baseline at Day 5-7 and from Day 14-15 onwards, Sham V showed significantly increased ipsilateral paw withdrawal when compared to baseline at Day 14-15 (^ and *, respectively; repeated measures mixed-model ANOVA followed by Bonferroni's post-hoc analysis, $p<0.05$). CIBP animals did not show a significant difference in ipsilateral

paw withdrawal when compared to Sham V. B) CIBP animals showed decreased latency to paw withdrawal when compared to baseline at Day 5-7 and Day 18-21 (^; repeated measures mixed-model ANOVA followed by Bonferroni's post-hoc analysis, $p<0.05$). CIBP animals did not show significantly altered latency to paw withdrawal when compared to Sham V. C) CIBP animals showed significantly increased duration of paw elevation when compared to baseline at Day 18-21 (^; repeated measures mixed-model ANOVA followed by Bonferroni's post-hoc analysis, $p<0.05$). CIBP animals showed significantly increased duration of paw elevation at Day 18-21 only (+; repeated measures mixed-model ANOVA followed by Bonferroni's post-hoc analysis, $p<0.05$). P values ^^^ = <0.001, ^^ = 0.001 to 0.01, ^ = 0.01 to 0.05, * = 0.01 to 0.05 and $^{+++}$ = <0.001.

			Time post-surgery (days)						
	Group		Baseline	3-4	5-7	9-11	12-13	14-15	18-21
Number	CIBP	Number	0.3	0.5	1.3	0.6	0.6	1.0	1.4
		SEM	0.2	0.3	0.3	0.2	0.2	0.3	0.4
	Sham V	Number	0.4	0.4	0.9	1.0	0.0	1.1	0.5
		SEM	0.2	0.2	0.4	0.3	0.0	0.2	0.3
	Naïve	Number	0.0	0.4	0.3	0.1	0.0	0.5	0.1
		SEM	0.0	0.2	0.2	0.1	0.0	0.3	0.1
Latency	CIBP	Latency	131.3	120.1	70.6	107.3	107.5	109.8	62.4
		SEM	14.6	19.6	19.8	15.0	13.6	10.9	19.9
	Sham V	Latency	120.0	119.1	79.3	73.6	150.0	91.3	115.9
		SEM	16.9	18.1	21.0	22.7	0.0	12.1	17.1
	Naïve	Latency	150.0	131.1	126.0	144.0	150.0	127.6	135.3
		SEM	0.0	11.2	16.8	6.0	0.0	11.0	14.8
Duration	CIBP	Duration	0.0	0.6	0.4	2.8	2.5	16.4	59.9
		SEM	0.0	0.6	0.4	1.5	1.3	6.5	23.2
	Sham V	Duration	0.0	0.0	0.0	0.9	0.0	1.0	1.3
		SEM	0.0	0.0	0.0	0.9	0.0	1.0	0.8
	Naive	Duration	0.0	1.3	0.0	0.0	0.0	0.0	0.0
		SEM	0.0	1.3	0.0	0.0	0.0	0.0	0.0

Table 3.5 Thermal sensitivity to 30°C showing number of paw withdrawals, latency to paw withdrawal and duration of paw elevation. Data show mean responses ± SEM of Naive (n=8), Sham V (n=8) and CIBP (n=8) animals.

3.4.7 Development of thermal sensitivity to 40°C in a CIBP model

At 40°C, CIBP animals showed a significant increase in number of paw withdrawals when compared to baseline from Day 12-13 to Day 18-21 that was also noted in Sham V animals at Day 3-4 to Day 9-11. CIBP animals showed increased number of paw withdrawals compared to Sham V from Day 12-13 onwards. CIBP

animals showed decreased latency to paw withdrawal when compared to baseline at Day 12-13 and from Day 16-17 onwards. CIBP animals showed decreased latency to paw withdrawal when compared to Sham V at day 16-17 and Day 18-21. CIBP animals showed significantly increased duration of paw elevation when compared to baseline from Day 12-13 onwards and when compared to Sham V from Day 12-13 onwards. CIBP animals showed increased duration of paw elevation when compared to Sham V from Day 12-13 onwards. All shown by a repeated measures mixed-model ANOVA followed by Bonferroni's post-hoc analysis, $p<0.05$ (Figure 3.8).

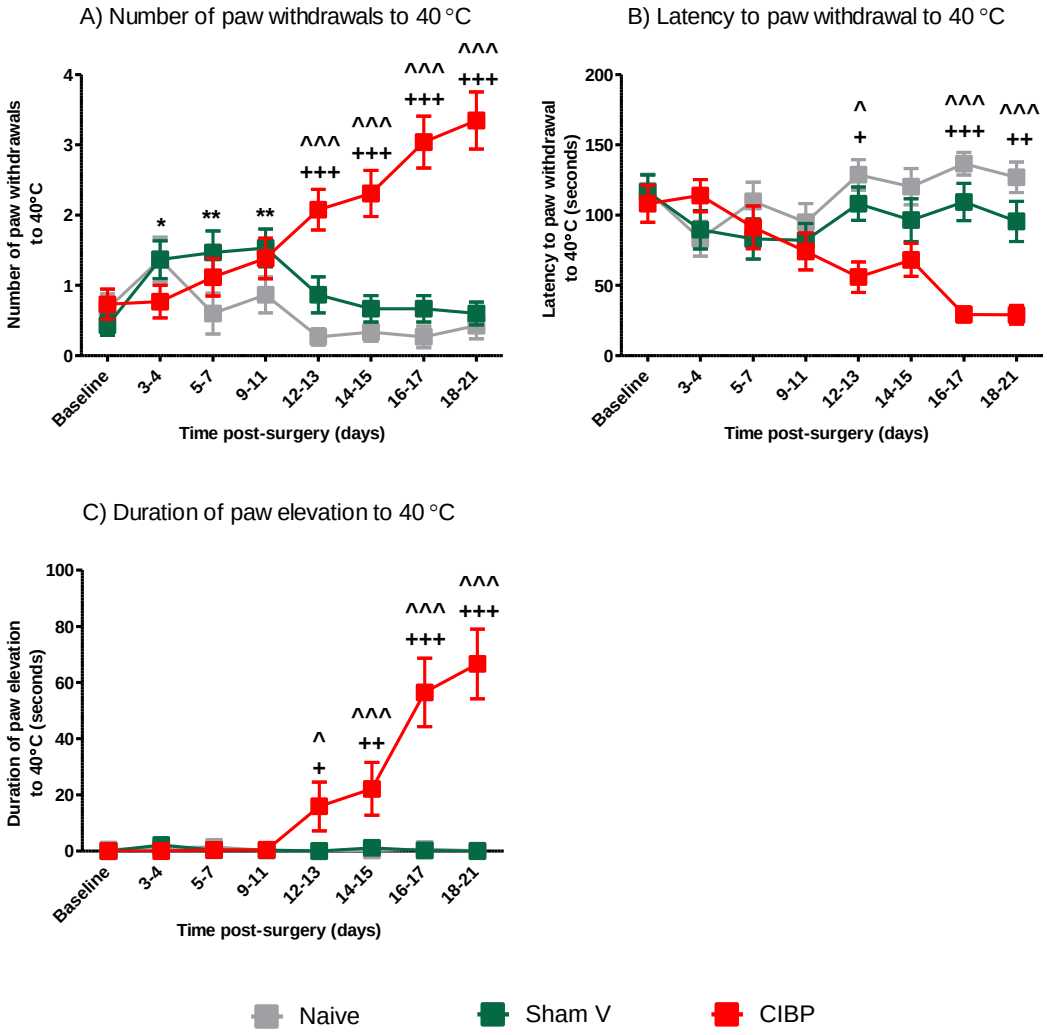


Figure 3.8 Thermal sensitivity to 40°C. Data show mean responses ± SEM of Naive (n=15), Sham V (n=15) and CIBP (n=13) animals. A) CIBP showed significantly increased ipsilateral paw withdrawal when compared to baseline at Day 12-13

onwards, Sham V animals showed significantly increased ipsilateral paw withdrawal at Day 3-4, 5-7 and 9-11 (^ and *, respectively; repeated measures mixed-model ANOVA followed by Bonferroni's post-hoc analysis, $p<0.05$). CIBP animals showed significantly increased ipsilateral paw withdrawal when compared to Sham V at day 12-13 to Day 18-21 (+; repeated measures mixed-model ANOVA followed by Bonferroni's post-hoc analysis, $p<0.05$). B) CIBP animals showed decreased latency to paw withdrawal at Day 12-13, 16-17 and 18-21 (^; repeated measures mixed-model ANOVA followed by Bonferroni's post-hoc analysis, $p<0.05$). CIBP animals showed a significant increase in latency to paw withdrawal when compared to Sham V at Day 12-13, Day 16-17 and Day 18-21 (+; repeated measures mixed-model ANOVA followed by Bonferroni's post-hoc analysis, $p<0.05$). C) CIBP animals showed significantly increased duration of paw elevation when compared to baseline from Day 12-13 onwards (^; repeated measures mixed-model ANOVA followed by Bonferroni's post-hoc analysis, $p<0.05$). CIBP animals showed significantly increased duration of paw elevation when compared to Sham V from Day 12-13 onwards (+; repeated measures mixed-model ANOVA followed by Bonferroni's post-hoc analysis, $p<0.05$). P values ^^^ = <0.001, ^ = 0.01 to 0.05, ** = 0.001 to 0.01, * = 0.01 to 0.05, $^{+++}$ = <0.001, $^{++}$ = 0.001 to 0.01 and $^{+}$ = 0.01 to 0.05.

			Time post-surgery (days)							
	Group		Baseline	3-4	5-7	9-11	12-13	14-15	16-17	18-21
Number	CIBP		0.7	0.8	1.1	1.4	2.1	2.3	3.0	3.3
		SEM	0.2	0.2	0.3	0.3	0.3	0.3	0.4	0.4
	Sham V		0.4	1.4	1.5	1.5	0.9	0.7	0.7	0.6
		SEM	0.1	0.3	0.3	0.3	0.3	0.2	0.2	0.2
	Naïve		0.7	1.4	0.6	0.9	0.3	0.3	0.3	0.4
		SEM	0.2	0.3	0.3	0.3	0.1	0.1	0.2	0.2
Latency	CIBP		108.1	113.9	91.3	74.1	55.9	68.1	29.2	29.0
		SEM	13.2	11.5	15.2	13.1	10.8	11.7	5.4	6.6
	Sham V		116.5	89.6	83.0	82.2	108.1	96.4	109.3	95.5
		SEM	11.9	13.7	14.3	11.8	11.9	15.3	13.3	14.4
	Naïve		117.2	83.1	109.7	94.9	128.7	120.2	136.6	126.9
		SEM	11.9	12.4	13.9	13.2	10.8	12.9	8.0	10.8
Duration	CIBP		0.0	0.0	0.4	0.5	15.9	22.2	56.5	66.6
		SEM	0.0	0.0	0.4	0.3	8.7	9.4	12.2	12.4
	Sham V		0.0	2.1	0.3	0.3	0.0	1.1	0.2	0.0
		SEM	0.0	1.3	0.3	0.3	0.0	0.7	0.2	0.0
	Naive		0.6	1.1	1.5	0.2	0.0	0.2	0.7	0.1
		SEM	0.4	0.5	1.2	0.2	0.0	0.2	0.6	0.1

Table 3.6 Thermal sensitivity to 40°C showing number of paw withdrawals, latency to paw withdrawal and duration of paw elevation. Data show mean responses ± SEM of Naive (n=15), Sham V (n=15) and CIBP (n=13) animals.

3.4.8 Development of avoidance of weight bearing on movement and static weight bearing difference in CIBP animals

Movement-evoked pain was assessed using the rotarod to measure the number of weight bearing (ipsilateral hindlimb only) on movement at a constant speed over a set test period. This method of counting the number of avoidances of weight bearing allowed us to quantify the extent of movement-evoked pain in this model. Due to the repeated testing involved we decided not to measure latency to fall

as an indicator of movement-evoked pain, as repeatedly falling off the rotarod could impact on both the welfare of the animal and the sensitivity of this assay. CIBP animals showed a significant increase in avoidance of weight bearing on movement compared to baseline from Day 5-7 onwards and Sham V animals showed a significant increase in avoidance of weight bearing on movement compared to baseline at Day 9-11 and Day 12-13. CIBP animals showed a significant increase in avoidance of weight bearing on movement when compared to Sham V from Day 12-13. Naïve animals did not display any signs of avoidance of weight bearing on movement at any time point. The static weight bearing of both hindlimbs was assessed and CIBP animals showed a significant increase in static weight bearing difference from Day 12-13 onwards, which was not observed in Sham V or Naïve animals. CIBP animals showed a significant increase in static weight bearing difference when compared to Sham V from Day 12-13 onwards. All shown by repeated measures mixed-model ANOVA followed by Bonferroni's post-hoc analysis, $p<0.05$ (Figure 3.9).

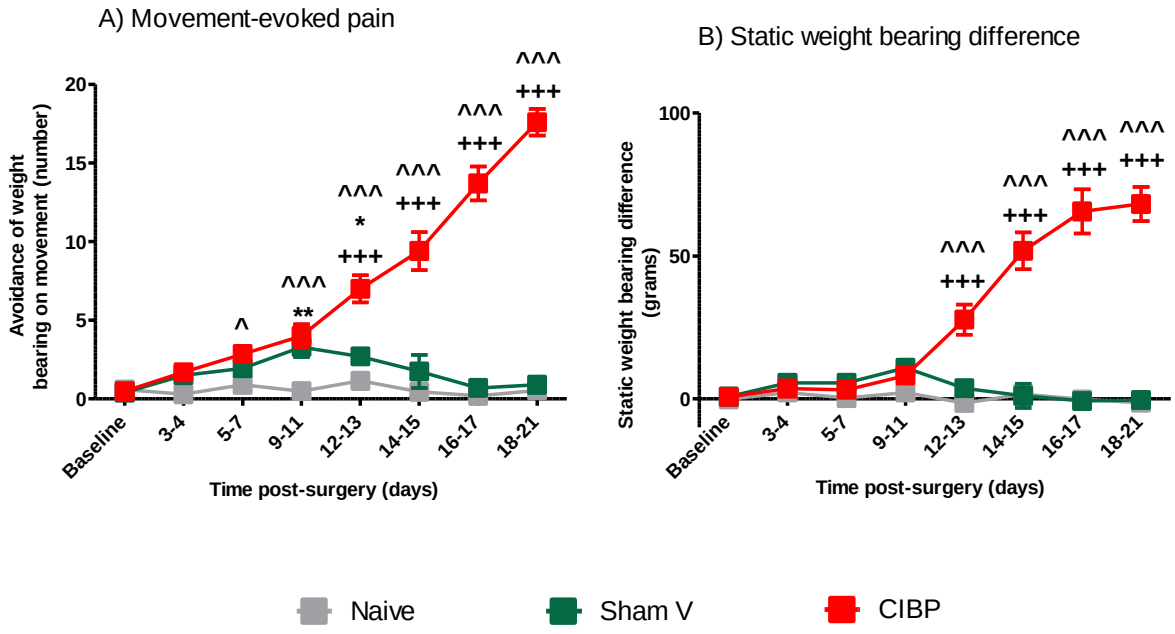


Figure 3.9 A) Movement-evoked pain. Data show mean responses ± SEM of Naive (n=10), Sham V (n=10) and CIBP (n=10) animals. CIBP animals showed significantly increased avoidance of weight bearing on movement in comparison to baseline from Day 5-7 onwards. Sham V animals showed significantly increased

avoidance of weight bearing on movement when compared to baseline at Day 9-11 and 12-13 (^ and *, respectively; repeated measures mixed-model ANOVA followed by Bonferroni's post-hoc analysis, $p<0.05$). CIBP animals showed increased avoidance of weight bearing compared to Sham V at Day 12-13 onwards (+; repeated measures mixed-model ANOVA followed by Bonferroni's post-hoc analysis, $p<0.05$). B) Weight bearing difference between hindlimbs. Data show mean responses ± SEM of Naive (n=14), Sham V (n=15) and CIBP (n=13) animals. CIBP animals showed a significant increase in static weight bearing difference when compared to baseline from Day 12-13 onwards (^; repeated measures mixed-model ANOVA followed by Bonferroni's post-hoc analysis, $p<0.05$). CIBP animals showed a significant increase in static weight bearing difference when compared to Sham V from Day 12-13 onwards (+; repeated measures mixed-model ANOVA followed by Bonferroni's post-hoc analysis, $p<0.05$). P values ^^^ = <0.001, ** = 0.001 to 0.01, * = 0.01 to 0.05, $^{+++}$ = <0.001.

			Time post-surgery (days)							
	Group		Baseline	3-4	5-7	9-11	12-13	14-15	16-17	18-21
Movement-evoked	CIBP	Number	0.5	1.7	2.9	4.0	7.0	9.4	13.7	17.6
		SEM	0.2	0.4	0.5	0.8	0.9	1.2	1.1	0.8
	Sham V	Number	0.4	1.5	2.0	3.3	2.7	1.8	0.7	0.9
		SEM	0.2	0.3	0.5	0.6	0.4	1.1	0.3	0.3
	Naïve	Number	0.6	0.3	0.9	0.5	1.2	0.5	0.2	0.6
		SEM	0.3	0.2	0.5	0.3	0.5	0.3	0.1	0.5
Weight bearing	CIBP	WBD (grams)	0.7	3.6	3.2	8.2	27.8	51.8	65.6	68.2
		SEM	1.0	2.5	2.8	2.7	5.3	6.5	7.7	6.0
	Sham V	WBD (grams)	0.8	5.6	5.6	10.9	3.8	1.1	-0.7	-0.4
		SEM	0.9	2.6	2.4	2.7	2.0	4.2	2.7	2.1
	Naïve	WBD (grams)	-0.2	2.3	0.3	2.3	-1.4	1.7	-0.2	-1.3
		SEM	0.7	1.2	1.3	1.4	2.7	2.6	2.1	2.0

Table 3.7 Movement-evoked pain showing number of avoidances of weight bearing on movement. Data show mean responses ± SEM of Naive (n=10), Sham V (n=10) and CIBP (n=10) animals. Weight bearing difference between hindlimbs showing weight bearing difference between hindlimbs (WBD). Data show mean responses ± SEM of Naive (n=14), Sham V (n=15) and CIBP (n=13) animals.

To further understand the impact of CIBP on movement-related pain we examined the total distance travelled, average speed and number of rearings in the open field and the number of rearings was also measured in the elevated plusmaze. These behaviours were used to assess voluntary locomotor activity in all groups.

3.4.9 Effect of CIBP on voluntary locomotor activity measures in the open field and elevated plusmaze

CIBP (7.3 ± 0.5) and Sham V (6.6 ± 0.7) animals showed a significant reduction in total distance travelled in the open field when compared to Naive

animals (9.5 ± 0.5) and there was no significant difference in total difference travelled between CIBP and Sham V animals. CIBP (0.02 ± 0.001) and Sham V (0.02 ± 0.002) animals also showed a significant reduction in average speed in the open field when compared to Naive animals (0.03 ± 0.002) and there was no significant difference in the average speed of CIBP and Sham V animals. CIBP (18.7 ± 3.4) and Sham V (19.5 ± 2.4) animals showed a significant reduction in the number of rearings in the open field when compared to Naïve (31.7 ± 2.7) animals and there was no significant difference in the number of rearings in the open field between CIBP and Sham V animals. The number of rearings on the elevated plusmaze was significantly decreased in CIBP (14.7 ± 2.9) animals only when compared to Naïve (25.4 ± 2.9) animals and there was no significant difference in the number of rearings in the elevated plusmaze between CIBP and Sham V (21.3 ± 2.5) animals (Figure 3.10). All shown by a One-way ANOVA followed by Bonferroni's post-hoc analysis, $p<0.05$.

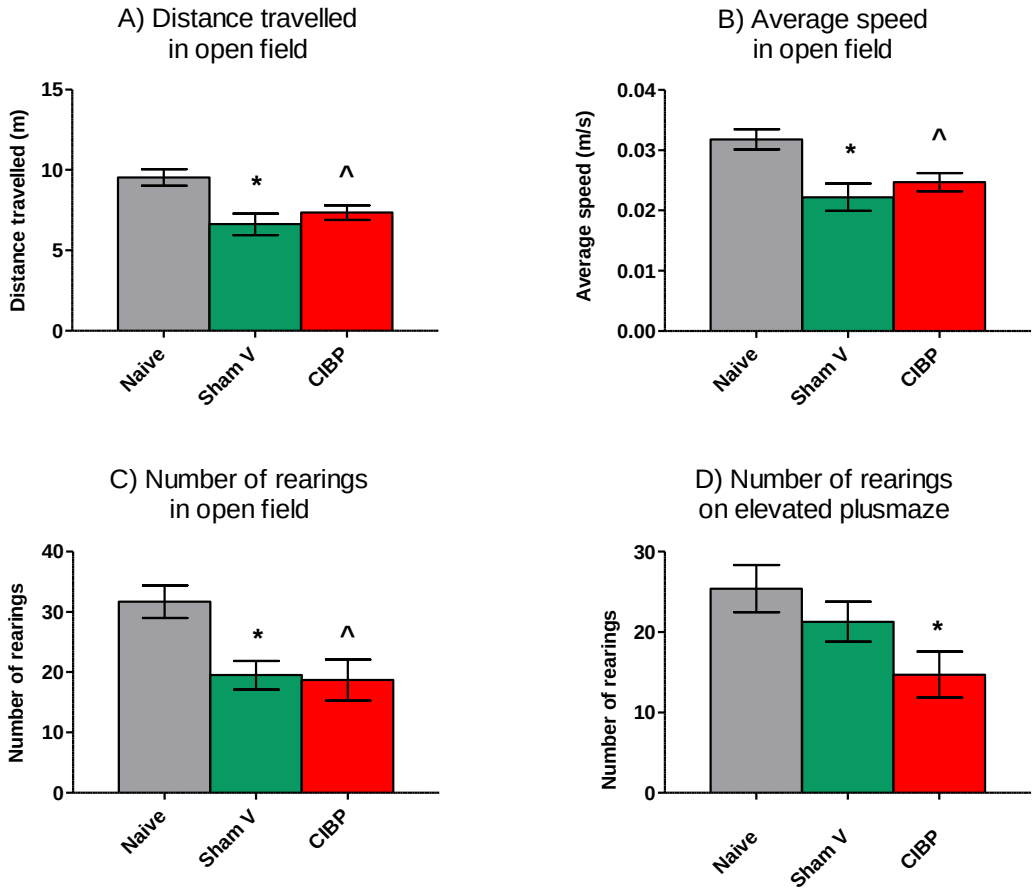


Figure 3.10 Analysis of voluntary locomotor activity measures in the in open field and elevated plusmaze. Data show mean responses ± SEM of Naive (n=10), Sham V (n=10) and CIBP (n=10) animals. A) CIBP and Sham V animals showed a decrease in distance travelled in the open field when compared to Naive (^ and *, respectively; One-way ANOVA followed by Bonferroni's post-hoc analysis, $p<0.05$). CIBP did not alter distance travelled when compared to Sham V. B) CIBP and Sham V animals showed a decrease in average speed in the open field when compared to Naive (^ and *, respectively; One-way ANOVA followed by Bonferroni's post-hoc analysis, $p<0.05$). CIBP did not alter average speed when compared to Sham V. C) CIBP and Sham V animals showed a decrease in number of rearings in the open field when compared to Naive (* and ^, respectively; One-way ANOVA followed by Bonferroni's post-hoc analysis, $p<0.05$, respectively). CIBP did not alter the number of rearings when compared to Sham V. D) CIBP animals showed a significant decrease in number of rearings on the elevated plusmaze when compared to Naive

(*; One-way ANOVA followed by Bonferroni's post-hoc analysis, p<0.05). CIBP did not alter number of rearings when compared to Sham V. P values ^ = 0.01 to 0.05, ** = 0.001 to 0.01 and * = 0.01 to 0.05.

3.4.10 Effect of CIBP on non-evoked spontaneous foot lifting behaviour

Non-evoked spontaneous foot lifting may be a behavioural correlate of spontaneous pain (Djouhri et al., 2006). CIBP animals displayed significantly increased non-evoked spontaneous foot lifting behaviour when compared to baseline at Day 12-13 onwards. CIBP animals showed a significant increase in spontaneous foot lifting when compared to Sham V from day 14-15 onwards. Sham V and Naïve animals did not show significant spontaneous foot lifting when compared to baseline. All shown by a repeated measures mixed-model ANOVA followed by Bonferroni's post-hoc analysis, p<0.05 (Figure 3.11.A). The number of animals displaying spontaneous foot lifting was increased in CIBP animals, when compared to Sham V and Naïve animals (Figure 3.11.B).

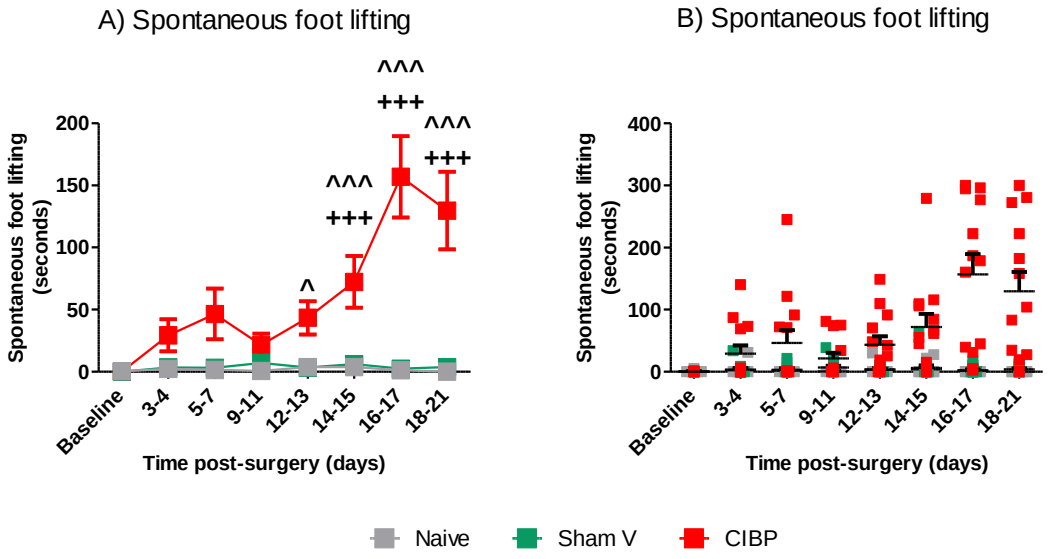


Figure 3.11 Spontaneous foot lifting. Data show mean responses ± SEM of Naive (n=15), Sham V (n=15) and CIBP (n=13) animals. A) Time course of spontaneous foot lifting behaviour. CIBP animals showed a significant increase in spontaneous foot lifting when compared to baseline at Day 12-13 onwards (^; repeated measures

mixed-model ANOVA followed by Bonferroni's post-hoc analysis, $p<0.05$). CIBP animals showed a significant increase in spontaneous foot lifting compared to Sham V at Day 14-15 onwards (+; repeated measures mixed-model ANOVA followed by Bonferroni's post-hoc analysis, $p<0.05$). B) Spontaneous foot lifting of individual animals. P values ^^^ = <0.001, ^ = 0.01 to 0.05, and $^{+++}$ = <0.001.

		Time post-surgery (days)							
Group		Baseline	3-4	5-7	9-11	12-13	14-15	16-17	18-21
CIBP	SFL (seconds)	0.1	29.3	46.5	21.4	43.3	72.3	156.9	129.6
	SEM	0.1	12.9	20.5	9.1	13.5	20.8	32.8	31.3
Sham V	SFL (seconds)	0.1	3.5	3.0	7.0	3.2	6.0	2.4	3.8
	SEM	0.1	2.3	2.1	2.9	1.1	4.3	1.3	1.5
Naive	SFL (seconds)	0.4	2.3	1.3	0.5	3.7	3.7	1.0	0.0
	SEM	0.4	2.1	1.0	0.3	2.0	2.2	0.7	0.0

Table 3.8 Spontaneous foot lifting (SFL). Data show mean responses ± SEM of Naive (n=15), Sham V (n=15) and CIBP (n=13) animals.

The open field is used to assess anxiety in the rodent. The underlying principle of this test is based upon the natural aversion of rodents to unprotected, open spaces. A reduction in time spent in the centre zone of the open field may reflect an anxious-like state, which we used to assess pain-related anxiety in the CIBP model. Grooming behaviour in the open field is also used to assess anxiety. Grooming behaviour is an important part of rodent behaviour and consists of several stages including licking the paws, washing movements over the head, fur licking and tail/genitals cleaning (Berridge & Aldridge, 2000). Mild stress such as exposure to a novel environment is known to induce grooming in rats (Jolles et al., 1979). The elevated plusmaze is based on the natural aversion to open spaces but with the additional element of elevation. The elevated plusmaze has been validated for both

anxiolytic and anxiogenic compounds (Pellow et al., 1985). A reduction of time spent on the open arms of the elevated plusmaze may reflect anxiety and in this CIBP model, pain-related anxiety. The stretch attend posture is an exploratory body posture where the animal stretches without walking forward and is regarded as a risk assessment behaviour. The concept of risk assessment is derived from research on anti-predator defence in rats and refers to a typical pattern of investigation (scanning, stretch attend, flat back approach) that is seen in potentially dangerous situations (Blanchard et al., 1990).

3.4.11 Effect of CIBP on anxiety-like behaviours in the open field

In the open field, the following parameters; time spent in the centre zone, number of entries to the centre zone, latency to enter centre zone, number of groomings and time spent grooming, did not significantly alter in CIBP when compared to Sham V or Naive animals (Figure 3.12).

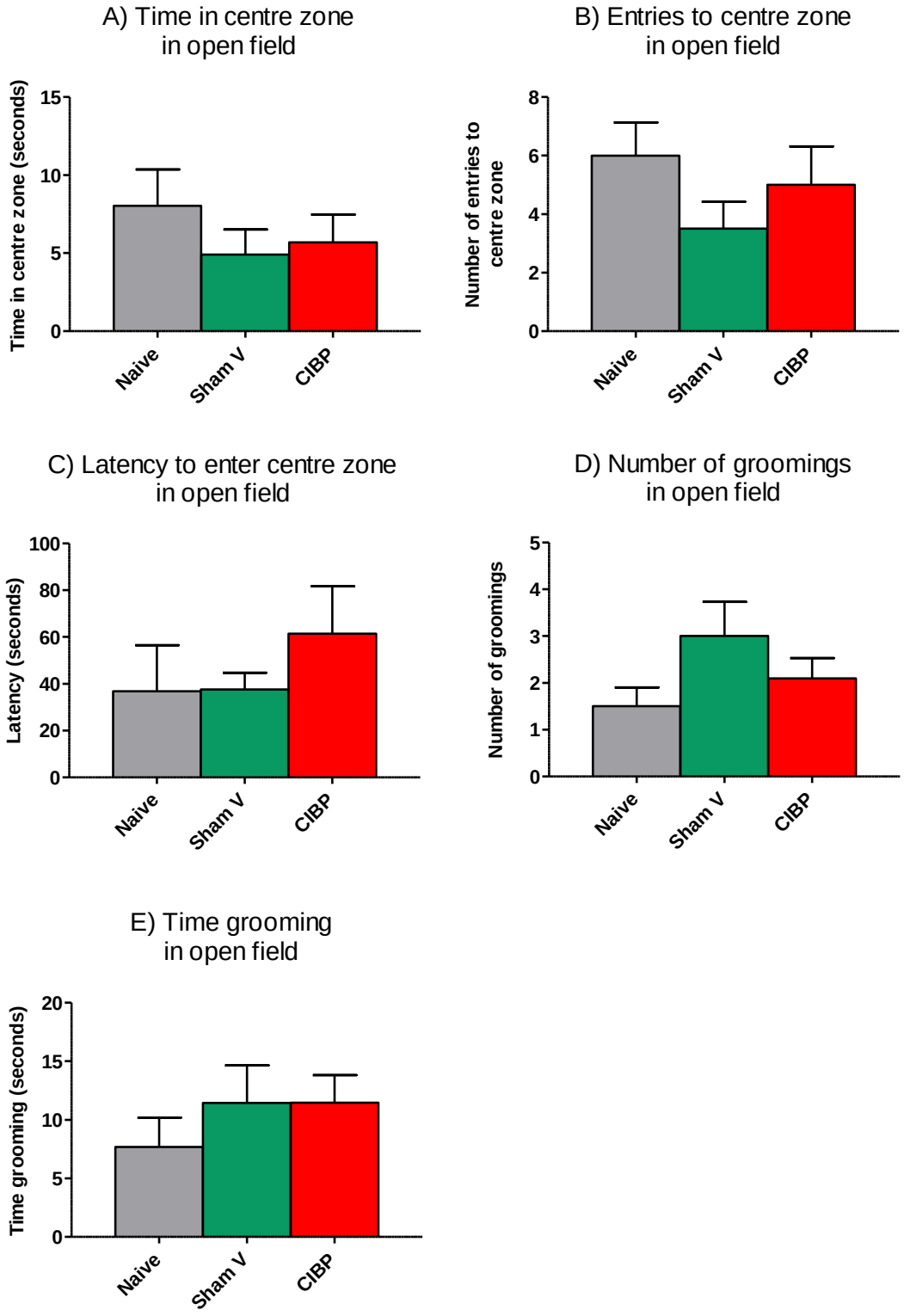


Figure 3.12 Anxiety-like behaviours in open field. Data show mean responses ± SEM of Naive (n=10), Sham V (n=10) and CIBP (n=10) animals. A-E) CIBP animals did not show altered time spent in the centre zone, number of entries to the

centre zone, latency to enter the centre zone, number of groomings or time spent grooming in the open field when compared to Sham V or Naïve animals.

3.4.12 Effect of CIBP on anxiety-like behaviours and risk assessment behaviours on the elevated plusmaze

The elevated plusmaze was used to assess anxiety and risk assessment behaviours. A reduction of time spent on the open arm may reflect anxiety and, in the CIBP model, pain-related anxiety. Grooming behaviour, which may increase during stress, was also analysed (Jolles et al., 1979). The number of protected stretch attends were recorded as risk assessment behaviour. CIBP did not significantly alter time spent on the open arms, grooming behaviour and number of protected stretch attends on the elevated plusmaze when compared to Sham V or Naive animals (Figure 3.13).

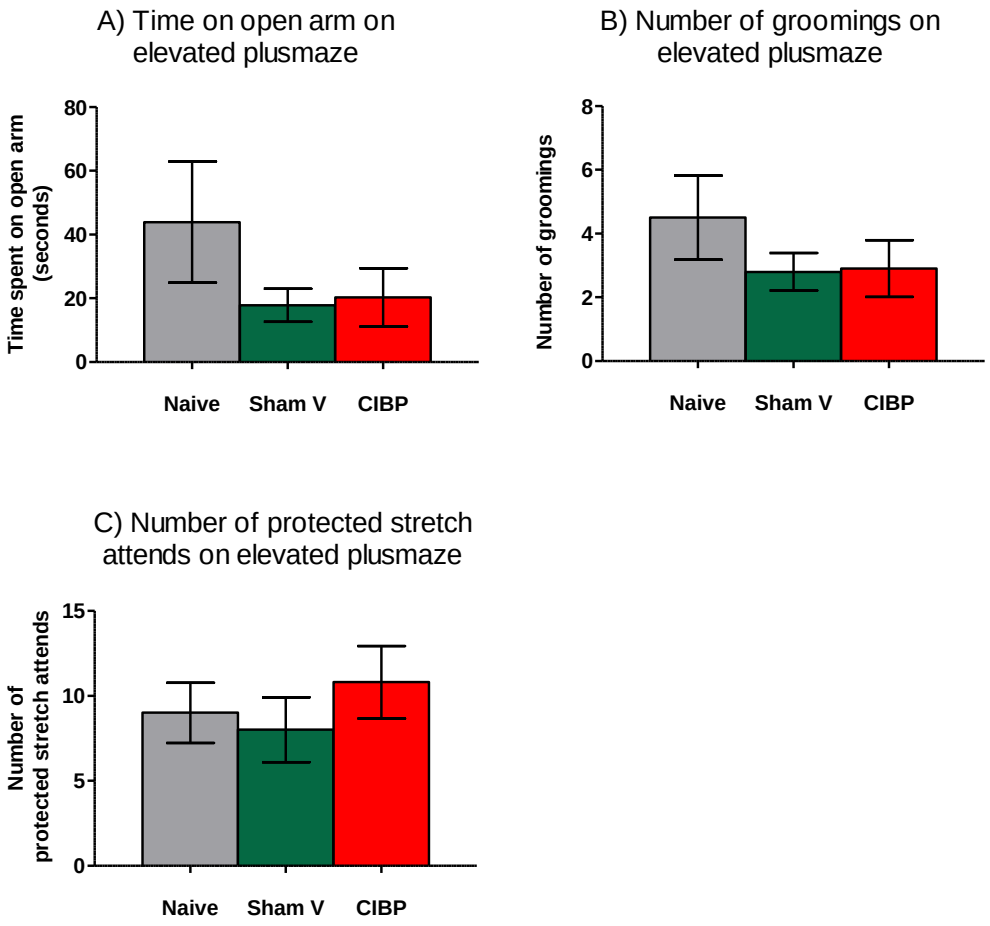


Figure 3.13 Anxiety-like behaviours and risk assessment behaviours on the elevated plusmaze. Data show mean responses ± SEM of Naive (n=10), Sham V (n=10) and

CIBP (n=10) animals. A-C) Time spent on the open arm, number of groomings and number of protected stretch attends was not altered in CIBP when compared to Sham V or Naïve animals.

3.4.13 Comparison of Sham models, Sham V, Sham HK and Sham E behavioural sensitisation to Naïve animals

The development of mechanical allodynia in Sham V, Sham E, Sham HK and Naive animals was not apparent, with no significant reductions in ipsilateral PWT when compared to baseline or contralateral PWT (Figure 3.14.A and 3.14.B). Interestingly Sham V animals showed evidence of movement-evoked pain at Day 9-11 to Day 12-13 with a significant increase in avoidance of weight bearing on movement compared to baseline (0.4 ± 0.2 versus 1.9 ± 0.5, 3.3 ± 0.6 and 2.7 ± 0.4), shown by a repeated measures mixed-model ANOVA followed by Bonferroni's post-hoc analysis, $p<0.05$. Such increases in avoidance of weight bearing on movement were not observed in Naive, Sham HK or Sham E animals when compared to baseline (Figure 3.14.C).

Sham V animals showed an increase in thermal sensitivity to 40°C at Day 3-4 to Day 9-11 with a significant increase in number of paw withdrawals when compared to baseline (0.4 ± 0.1 versus 1.4 ± 0.3, 1.5 ± 0.3 and 1.5 ± 0.3), shown by repeated measures mixed-model ANOVA followed by Bonferroni's post-hoc analysis, $p<0.05$. The number of paw withdrawals to 40°C did not alter in Naive, Sham HK or Sham E animals when compared to baseline (Figure 3.14.D).

Spontaneous foot lifting was only significantly increased in Sham HK at Day 3-4 to Day 9-11 compared to baseline (0 ± 0 versus 27.8 ± 17.4, 17.4 ± 17.4 and 23.2 ± 14.4), shown by repeated measures mixed-model ANOVA followed by Bonferroni's post-hoc analysis, $p<0.05$. Spontaneous foot lifting was not significantly different in Sham V, Sham E or Naive animals when compared to baseline (Figure 3.14.E).

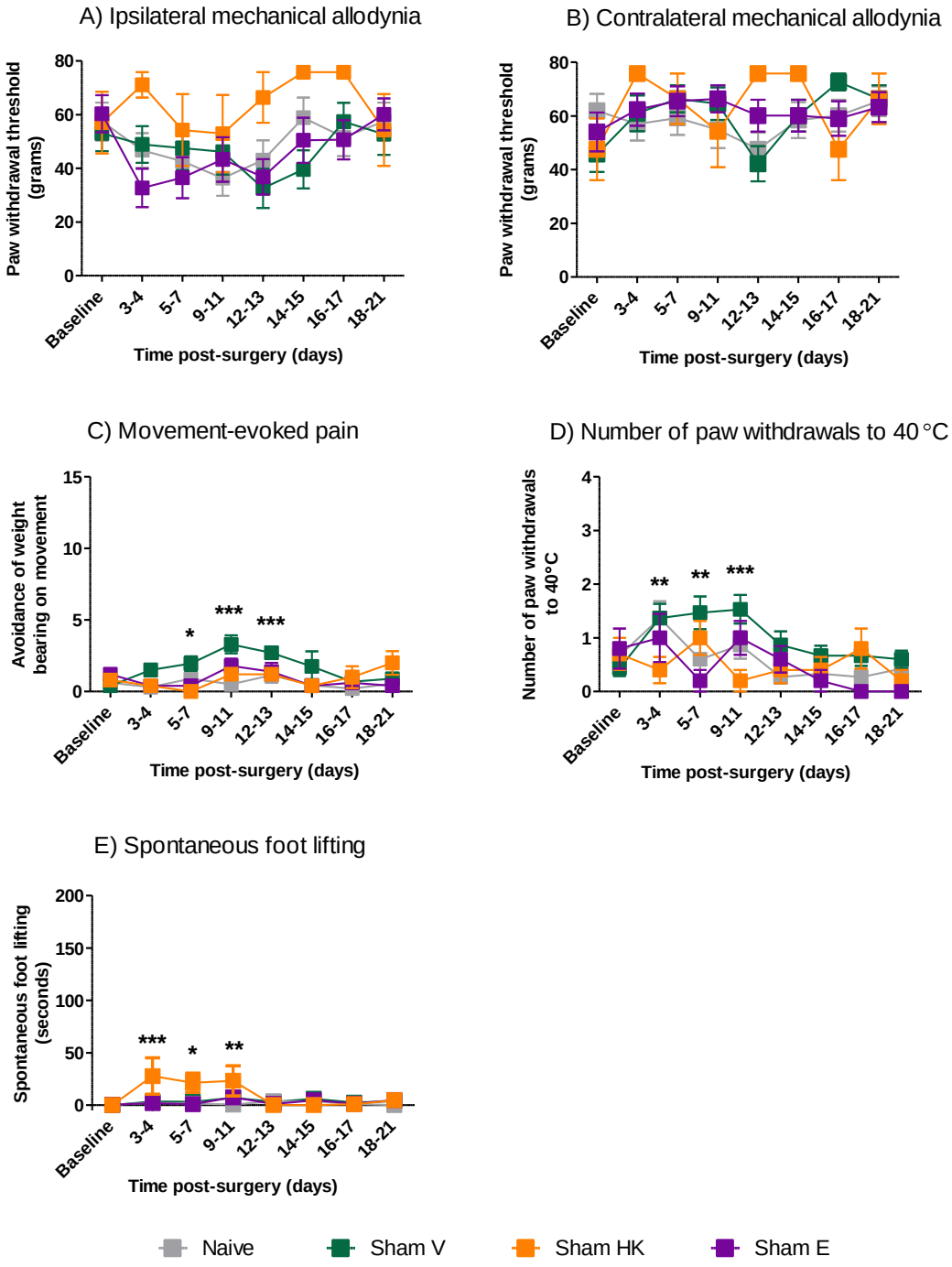


Figure 3.14 Behavioural sensitisation of Sham-operated models. A+B) Mechanical allodynia. Data show mean responses ± SEM of Naive (n=15), Sham V (n=15) Sham E (n=15) and Sham HK (n=5) animals. PWT of the ipsilateral hindlimb was not significantly different when compared to contralateral PWT or when compared to baseline. C) Movement-evoked pain. Data show mean responses ± SEM of Naïve (n=10), Sham V (n=10), Sham HK (n=5) and Sham E (n=5) animals. Avoidance of

weight bearing on movement was increased in Sham V animals at Day 5-7, 9-11 and 12-13 when compared to baseline (*; repeated measures mixed-model ANOVA followed by Bonferroni's post-hoc analysis, $p<0.05$). Avoidance of weight bearing on movement was not changed in Naive, Sham HK or Sham E when compared to baseline. D) Thermal sensitivity to 40°C. Data show mean responses ± SEM of Naïve (n=15), Sham V (n=15), Sham HK (n=5) and Sham E (n=5). Number of paw withdrawals to 40°C is significantly increased at Day 3-4, 5-7 and 9-11 in Sham V animals when compared to baseline (*; repeated measures mixed-model ANOVA followed by Bonferroni's post-hoc analysis, $p<0.05$). Number of paw withdrawals did not alter in Naive, Sham HK or Sham E animals when compared to baseline. E) Spontaneous foot lifting (SFL). Data show mean responses ± SEM of Naïve (n=15), Sham V (n=15), Sham HK (n=5) and Sham E (n=15) animals. SFL was significantly increased in Sham HK animals at Day 3-4, 5-7 and 9-11 when compared to baseline (*; repeated measures mixed-model ANOVA followed by Bonferroni's post-hoc analysis, $p<0.05$). SFL was not altered in Naive, Sham V or Sham E animals when compared to baseline. P values *** = <0.001, ** = 0.001 to 0.01 and * = 0.01 to 0.05.

3.4.14 Behavioural sensitisation is not apparent in the Sham models, Sham V, Sham HK or Sham E at Day 18-21 when compared to Naïve animals

We examined the behavioural sensitisation of the Sham models compared to Naïve and CIBP animals at Day 18-21, when pharmacological agent profiling or anxiety tests in CIBP animals were conducted, to ensure that behavioural responses observed in CIBP animals were a result of tumour growth. Mechanical allodynia, movement-evoked pain, thermal sensitivity to 40°C and spontaneous foot lifting were not altered at Day 18-21 in Sham V, Sham HK or Sham E when compared to Naïve animals. Mechanical allodynia, movement-evoked pain, thermal sensitivity to 40°C and spontaneous foot lifting were significantly increased in CIBP animals when compared to Naive animals (Figure 3.15).

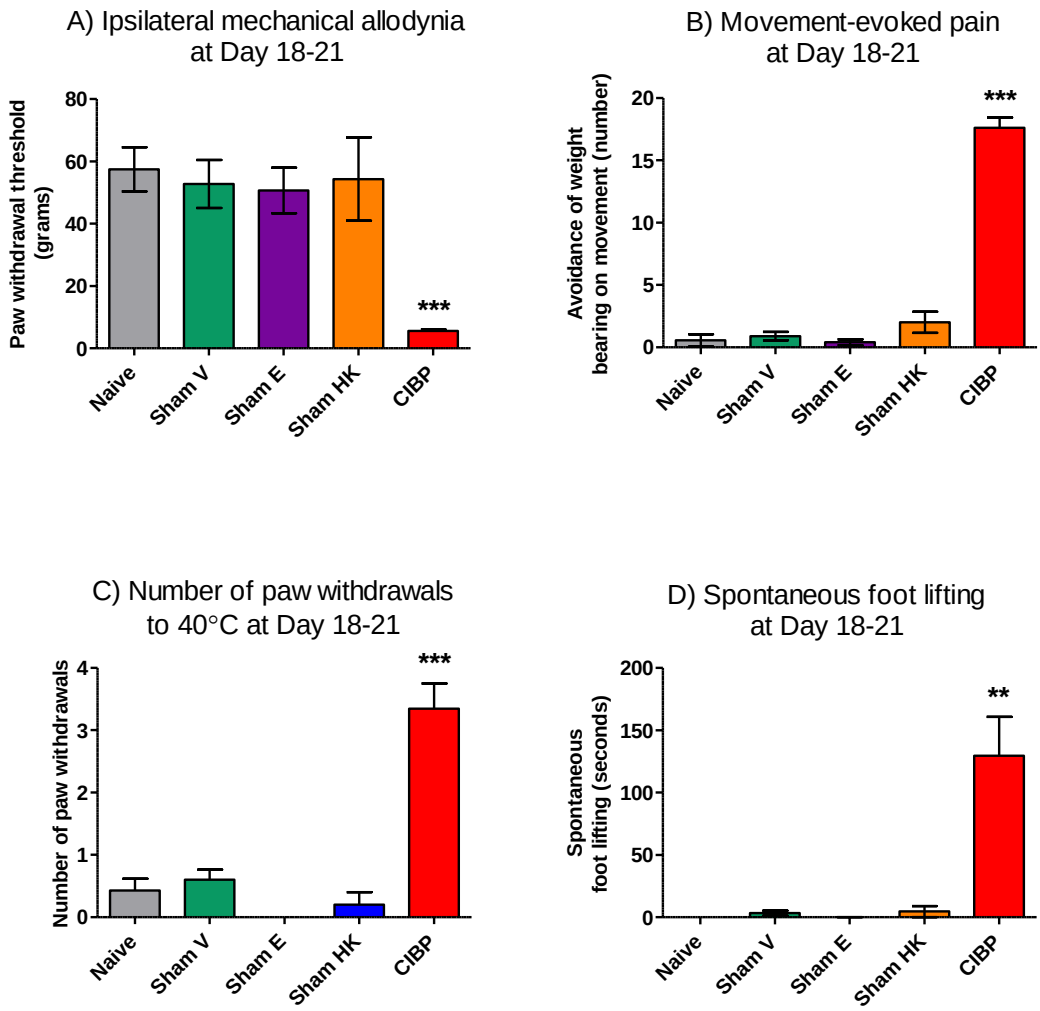


Figure 3.15 Behavioural sensitisation at Day 18-21. A) Mechanical allodynia. Data show mean responses ± SEM of Naïve (n= 15), Sham V (n=15), Sham E (n=15), Sham HK (n=5) and CIBP (n=13) animals. Ipsilateral PWT of Sham V, Sham E and Sham HK animals was not significantly different when compared to Naïve animals. Ipsilateral PWT of CIBP animals was significantly decreased when compared to Naïve animals (***; One-way ANOVA on ranks (Friedman's test) followed by Dunn's post-hoc analysis, $p<0.001$). B) Movement-evoked pain. Data show mean responses ± SEM of Naïve (n=10), Sham V (n=10), Sham E (n=5), Sham HK (n=5) and CIBP (n=10) animals. Avoidance of weight bearing on movement of Sham V, Sham E and Sham HK animals was not significantly different when compared to Naïve animals. Avoidance of weight bearing on movement of CIBP animals was significantly increased when compared to Naïve animals (***; One-way ANOVA

followed by Dunnet's post-hoc analysis, $p<0.001$). C) Thermal sensitivity at 40°C. Data show mean responses ± SEM of Naïve (n=15), Sham V (n=15), Sham E (n=5), Sham HK (n=5) and CIBP (n=13) animals. The number of paw withdrawals of Sham V, Sham E and Sham HK animals was not significantly different when compared to Naïve animals. In CIBP animals, the number of paw withdrawals is significantly increased when compared to Naïve animals (***; One-way ANOVA followed by Dunnett's post-hoc analysis, $p<0.001$). D) Spontaneous foot lifting. Data show mean responses ± SEM of Naïve (n=10), Sham V (n=10), Sham E (n=15), Sham HK (n=5) and CIBP (n=13) animals. Spontaneous foot lifting in Sham V, Sham E and Sham HK animals was not significantly different when compared to Naïve animals. In CIBP animals, spontaneous foot lifting was significantly increased when compared to Naive animals (**; One-way ANOVA followed by Dunnett's post-hoc analysis, $p= 0.001$ to 0.01).

3.5 Discussion

This rat model of CIBP is adapted from the original mouse model by Schwei *et al.* The mouse model resulted in extensive tumour-induced destruction of the bone and as the tumour progressed mice exhibited pain-related behaviour in the form of guarding of the affected limb and severe acute pain to normally non-noxious palpitation of the affected bone (Schwei et al., 1999). These pain-related behaviours, illustrating tactile alloynia and spontaneous pain, are in parallel with the clinical condition (Honore et al., 2000). One advantage of adapting this model to the rat is that the mouse model involves a surgical procedure in which a patellar arthotomia and partial alteration of the knee joint occurs during innoculation of the femur. This may result in damage to the knee and lead to impaired locomotion and therefore biases in the behavioural results in the mouse model. In contrast, during inoculation of the tibial bone in the rat model all joints remain intact. The rat model has been developed as a suitable model for studying therapeutic intervention of CIBP (Medhurst et al., 2002) and therefore should be useful for translating results to the clinical setting.

Medhurst *et al.* measured bone mineral density (BMD) and bone mineral content (BMC) to quantify the extent of bone destruction of the tibia of female rats injected with MRMT-1 cells (Medhurst et al., 2002). Results showed that MRMT-1 cells caused a significant decrease in BMD and BMC in CIBP animals when compared to Sham animals. The authors concluded that MRMT-1 cells produced a mixed type of bone lesion with eroded cortical bone and spiny bone formation around the periosteum (Medhurst et al., 2002). However, another study showed that in the tibia of male rats injected with MRMT-1 there is no alteration in BMC and BMD (Urch et al., 2003b). Dore-Savard *et al.* recently investigated bone destruction using micro X-ray computed tomography in a model using MRMT-1 cells injected into the femur of male rats. These results show a significant decrease in bone density and extra-femoral spiny bone formation was also observed (Dore-Savard et al., 2010).

3.5.1 This CIBP rat model develops sensory hypersensitivity and spontaneous foot lifting

Our observations demonstrate that this model of CIBP develops behaviours that may be indicative of mechanical allodynia, thermal sensitivity, movement-evoked pain, ongoing pain and spontaneous pain. These results are consistent with previous studies in this model where the following pain-related behaviours were observed; weight bearing difference between hindlimbs (Medhurst et al., 2002), mechanical allodynia and ambulatory pain on the rotarod (Donovan-Rodriguez et al., 2004a). The rotarod has been used to assess movement-evoked pain, for example by grading the response or assessing the latency to fall from the rotarod (Urch et al., 2003b). As detailed above, to prevent damage to the injured hindlimb we did not use latency to fall as a measure of movement-evoked pain. We quantified movement-evoked pain by counting the total number of avoidances of weight bearing, rather than the more subjective grading of individual avoidances of weight bearing (rated using a 0 to 3 scale) (Urch et al., 2003b). These present results also expand the range of temperatures at which thermally evoked withdrawal responses are known to be altered in CIBP. The development of thermal sensitivity has been shown in a limited range of temperatures using the acetone cold test (Donovan-Rodriguez et al., 2005)

and Hargreave's radiant heat test (Zhang et al., 2005a). Here we have expanded the range of temperatures, from 10°C to 40°C, to include both noxious and innocuous temperatures.

Evoked reflex behaviours, such as paw withdrawal, are used extensively to assess animal pain behaviours but do not reflect the most prominent pain in the clinic. CIBP patients suffer from uncontrollable movement-evoked, and spontaneous pain (Mercadante et al., 1992). This is why it is important in animal models to assess many different pain behaviours. Additionally, human imaging studies have shown that pain processing involves cortical structures (Tracey & Mantyh, 2007;Vierck *et al.*, 2008), however evoked reflex pain behaviours may not require processing in cortical structures. Vierck *et al.* argue against the use of evoked behaviours and suggest that cortically-dependent operant escape tasks are more useful for translating to the clinical setting (Vierck et al., 2008). Such assessments however may be affected by a number of additional undefined factors therefore could be more difficult to interpret unequivocally.

Spontaneous or breakthrough pain is a particularly difficult component of CIBP to manage. Spontaneous foot lifting has previously been utilised as a measure of spontaneous pain in the rodent, which has previously been utilised in neuropathic pain inflammatory pain and CIBP models (Djouhri *et al.*, 2006;King *et al.*, 2007;Xiao & Bennett, 2007). Indeed high spontaneous firing rates in intact C-fibre nociceptive neurons have been linked with spontaneous foot lifting (Djouhri et al., 2006). Given this linking of C-fibre activity with spontaneous foot lifting, this assessment appears to be a suitable preclinical measure of spontaneous pain in CIBP. It has been questioned whether spontaneous foot lifting reflects spontaneous pain in inflammatory pain models. Xiao *et al.* suggested that evoked hypersensitivity caused by Complete Freund's Adjuvant (CFA) injection might contribute to avoidance of contact with surfaces resulting in foot lifting behaviour (Xiao & Bennett, 2007). A study demonstrated that CFA treatment can induce spontaneous discharge of C-fibres (Xiao & Bennett, 2008) however the frequency and prevalence of such spontaneous

afferent activity is very low making it uncertain whether this level of afferent drive might result in spontaneous pain.

This present study found that CIBP animals display a significant difference in weight bearing between hindlimbs from Day 12-13 onwards whereas Sham V animals do not display any such difference. This is consistent with results from Medhurst *et al.* who showed that CIBP animals display increased weight bearing difference and Sham-operated animals injected with vehicle or heat-killed cells did not (Medhurst et al., 2002).

CIBP results in the development of behavioural sensitisation to both evoked and non-evoked stimuli; these behavioural responses could be generated by many mechanisms. As detailed previously in Chapter 1 (Section 1.10) CIBP may result in tumour cell-induced injury of sensory nerves which innervate the mineralised bone, bone marrow and periosteum. In fact, tumour cells have been shown to injure and destroy the distal processes of sensory nerves that innervate the mineralised bone and bone marrow (Peters et al., 2005). Moreover CIBP results in an acidic microenvironment that acts to sensitise and directly activate peripheral sensory nerves (Nagae et al., 2007). Bone destruction in CIBP can lead to fractures and distortion of the periosteum, which is highly innervated by a subgroup of Aδ- and C-fibres (CGRP-positive fibres) that are likely to be involved in nociceptive transmission (Mach et al., 2002;Jimenez-Andrade et al., 2010). In addition, the immune cells surrounding the tumour release various factors, including prostaglandins and endothelins that can sensitise and/or directly activate sensory neurons (Mantyh et al., 2002). Furthermore, a study by Mantyh *et al.* provided evidence that sensory and sympathetic nerve fibres can undergo reorganisation where there is extensive pathological sprouting and neuroma formation that appears to significantly contribute to cancer pain (Mantyh et al., 2010).

3.5.2 CIBP and Sham V animals show decreased voluntary locomotor activity in the open field

Distance travelled, average speed and rearing behaviour were measured to assess the impact of CIBP on voluntary locomotor activity and were significantly decreased in both CIBP and Sham V animals compared to Naive animals. The lack of difference between Sham V and CIBP animals suggests these behavioural changes may in part be due to the damage to the bone incurred during surgery and not due to progressive tumour growth. Interestingly the Sham V animals did not display behavioural sensitisation at the time point when activity levels were measured, suggesting a long term effect of bone damage that is not detected by sensory testing.

3.5.3 This CIBP model does not show a significant increase in anxiety-related or risk assessment behaviours

Previous studies have shown that decreased time spent in the centre zone of the open field or open arms of the elevated plusmaze is indicative of increased anxiety (Pellow *et al.*, 1985;Prut & Belzung, 2003). The results of this present study show that CIBP did not appear to result in a significant decrease in time spent in the centre zone of the open field or latency to enter the centre zone. Others have shown that the extent of mechanical hypersensitivity is positively correlated with anxiety-like behaviour in the open field (reduced number of entries into the centre zone and reduced time spent in the centre zone) in a rat model of varicella zoster virus-associated pain (Hasnie et al., 2007a). Utilising the elevated plusmaze to further assess anxiety-related behaviours we demonstrated that decreased time spent on the open arms was not observed in CIBP animals. A study found that two different models of neuropathic pain (chronic constriction injury and peripheral nerve injury) developed anxiety-like behaviour measured by decreased time spent on the open arms of the elevated plusmaze. This anxiety-like behaviour was reversed by the analgesics, morphine and gabapentin (Roeska et al., 2008). The trends observed in CIBP animals could suggest the time-point tested may be crucial to identifying the impact of tumour growth on anxiety-related behaviours. There are many other factors that could impact on the results, for example the time of day tested, lighting conditions, environment etc. To attempt to control for these factors standardised

testing procedures were followed when using the open field and elevated plusmaze in this study. Animals were tested in the same temperature and humidity controlled room each time between the daylight hours of 9:00 am and 5:00 pm. Lighting conditions were carefully controlled (see sections 2.4.3 and 2.4.4) and animals were allowed to acclimatise to room/lighting conditions for a minimum of 30 minutes before testing. The open field and elevated plusmaze were cleaned with ethanol (70%) between each animal to remove any olfactory clues.

Rats may demonstrate increased grooming behaviour in response to a novel environment (Jolles et al., 1979). In CIBP animals there was no significant increase in grooming behaviour in the open field when compared to Sham V and Naïve animals. It is possible that we did not observe a significant difference in grooming behaviour because all rats (CIBP, Sham V and Naïve) may have experienced mild stress in this novel environment and therefore all displayed increased grooming behaviour in the open field. Additionally, although rodents show increased grooming behaviour in response to mild stress, rodents also show an increase in grooming when in a 'comfort' situation. Therefore, in rodents, grooming can be increased by both stress and comfort conditions, thus indicating quantitative measures of grooming may not be sufficient for correct interpretation of this behaviour. It is possible to analyse grooming using a grooming analysis algorithm which can discriminate between different levels of anxiety in rats (Kalueff & Tuohimaa, 2005) and future studies could analyse this complex behaviour in more detail.

In the elevated plusmaze increased risk assessment behaviour is reflected by high levels of stretch attend postures (Cole & Rodgers, 1993). Cole and Rodgers revealed the utility of measuring risk assessment even in the absence of convincing effects with the traditional plus-maze parameters (Cole & Rodgers, 1993). However, in the present study CIBP did not result in a significant increase in risk assessment behaviour on the elevated plusmaze.

It is important to note that there have been conflicting reports of anxiety-like behaviours in preclinical pain models. Kontinen *et al*. found that a rat model of

neuropathic pain (spinal nerve ligation) did not alter anxiety-like behaviours in the open field or on the elevated plusmaze (Kontinen et al., 1999). Furthermore, Hasnie *et al.* found that in a mouse model of neuropathic pain (partial sciatic nerve ligation, PSNL) mechanical and cold hypersensitivity is not associated with increased anxiety-like behaviour. In fact, PSNL animals showed increased time spent in the centre zone of the open field and no difference in time spent on the open arms of the elevated plusmaze (Hasnie et al., 2007b). Leite-Almeida *et al.* showed that in a spinal nerve injury (SNI) model of neuropathic pain the percentage of time in the open arms was decreased in SNI young rats (3 months) and old (22 months) rats but not mid-aged (10 months) rats (Leite-Almeida et al., 2009). This study highlights how the influence of pain on affective behaviours is very susceptible to additional factors, for example, age. Therefore in the present study, CIBP animals may not develop anxiety because of the model or the sensitivity of the test or for unknown reasons.

3.5.4 Minor alterations in behavioural responses were observed between the Sham models at early time points post-surgery

Sham V animals developed movement-evoked pain at Day 5-7 when compared to baseline, returning to baseline values by Day 14-15. Sham HK and Sham E animals did not develop movement-evoked pain at any time point tested. Only Sham V animals showed an increase in number of paw withdrawals to 40°C from Day 3-4 to Day 9-11 that returned to baseline values by Day 12-13. Sham HK animals showed an increase in spontaneous foot lifting at Day 3-4 until Day 9-11, that returned to baseline values from Day 12-13 onwards. However Sham V and Sham E animals did not show an increase in spontaneous foot lifting.

Altered behavioural responses in Sham animals have been reported up to Day 3 post-surgery (Gu et al., 2010b). Medhurst *et al.* also demonstrated a slight trend of increased mechanical allodynia in rats treated with heat-killed cells, however there was no statistically significant difference between shams injected with heat-killed cells and shams injected with vehicle. It is important to note that sham behaviours were not compared to naïve behaviours in this particular study (Medhurst et al., 2002). Here we show various alterations in the Sham model responses at early time

points, which may result in post-surgical inflammation and/or damage to the bone during surgery. However the differences observed between Sham V and Sham HK animals suggest the behavioural responses may not be entirely explained by post-surgical inflammation and/or bone damage. The immune responses to heat-killed carcinoma cells may underlie the different behaviours between these models, such as reduced movement-evoked pain and thermal sensitivity to 40°C. Whether this immune response can alter behavioural responses differently, by leading to an increase in spontaneous foot lifting and decrease in thermal sensitivity, needs to be clarified. We did not observe any alterations between the Sham groups (Sham V, E and HK) at Day 18-21, the time point that tissue was taken for *in vitro* analysis. Indeed Sham-operated animals did not show signs of mechanical allodynia, movement-evoked pain, thermal sensitivity to 40°C or spontaneous pain at this time point, suggesting that these Sham-operated animals are suitable controls.

Regardless of the precise mechanism of these minor alterations in behavioural responses of the Sham animals, the results suggest that the increase in pain-related behaviours at early time points in Sham V and Sham HK animals is most likely due in part to post-surgery inflammation, which Sham-operated animals recover from, and the behaviours we observe in CIBP animals at later time points must be a specific consequence of tumour growth.

It should be noted that the model used in this study does have some limitations. The focal tumour model does not mirror the clinical observation that tumours often metastasise to multiple distant sites. Also, due to the fact that the tumour is confined within the bone this implies the animals do not have systemic cancer. This suggests that the animal, apart from the focal tumour site, has normal physiological function which may not be the case in the clinic and the impact of such a systemic condition on pain responses cannot be determined here. However this model was chosen to specifically investigate the effects of tumour within the bone without the variability of multiple metastases models and to allow us to carry out site-specific behavioural tests. In addition, multiple metastases models often result in systemically ill animals. This means that the stress of testing may have had a

negative impact on the animal itself in addition to the outcomes measured. Another limitation of this model is that MRMT-1 rat mammary gland carcinoma cells were injected into male rats. This was to avoid the variation associated with hormonal influences, particularly because MRMT-1 cells are known to express estrogen receptors (Dore-Savard et al., 2010). However, it would have been useful to use both male and female rats in this study or indeed to have conducted a pilot study to identify any sex differences in the behavioural outcomes measured in this thesis.

3.6 Conclusion

The results presented here suggest that this model reflects the clinical condition of CIBP, where patients suffer from constant background pain with spontaneous and movement-related breakthrough pain. This indicates the validity of this model in testing the efficacy of analgesic interventions, where the results of these tests could potentially be translated to the clinic. The study utilises a wide array of behavioural tests that will allow us to identify the efficacy of novel analgesic intervention across several components of the complex pain syndrome. The study identifies some differences from the clinical situation, in that the preclinical rodent model of CIBP does not appear to result in the development of significant pain-related anxiety, as measured in the open field and on the elevated plusmaze (at least under the present conditions). Nevertheless, many aspects of the behavioural profiles point to its validity and likely value in assessing both changes in central somatosensory pathways and the actions of new candidate therapeutic agents.

4. BEHAVIOURAL ASSESSMENT OF ANALGESIC INTERVENTIONS IN A PRECLINICAL MODEL OF CIBP

4.1 Introduction

The CIBP pain state is initiated and maintained by multiple factors, resulting in a unique pain state that includes both neuropathic and inflammatory components making it particularly challenging to treat. The 'gold standard' treatment for CIBP is focal palliative radiotherapy, however radiotherapy treatment may take more than 4 weeks to achieve maximum analgesia with only 50% of patients achieving acceptable analgesia (Li et al., 2008;Hartsell et al., 2005). The current therapeutic regime for CIBP can include combinations of radiotherapy, non-steroidal anti-inflammatory drugs (NSAIDs), bisphosphonates and opioids. This regime follows guidelines proposed by the World Health Organisation (WHO) for the management of cancer pain known as 'the WHO analgesic ladder'. This strategy is a three step programme which relies on non-opioid non-steroidal anti-inflammatory drugs (NSAIDs), such as paracetamol, ibuprofen and COX-2 inhibitors, for weak pain, mild opioids, such as codeine, for moderate pain and strong opioids, such as morphine, for severe pain. At each step drugs can be combined with adjuvant drugs, which may not be primarily analgesic in their mechanism of action but may have a supplementary analgesic effect or control the side-effects of opioid or NSAID treatment. These adjuvant drugs can include antiemetics, laxatives, anti-depressants, anti-convulsants and anxiolytics. This current therapeutic regime used in the clinic can leave up to 45% of patients with inadequate pain control (de Wit *et al.*, 2001a;Meuser *et al.*, 2001a).

Morphine is the most frequently used opioid in the treatment of cancer pain (Mercadante 2005). Oral morphine is an effective analgesic in patients who suffer cancer pain (Wiffen & Mcquay, 2007). Morphine is usually effective at reducing background pain but the doses required to treat breakthrough pain often bring about significant unwanted side-effects, such as sedation, nausea and constipation (Mercadante & Arcuri, 1998;Portenoy & Hagen, 1990;Portenoy *et al.*, 1999). There are conflicting results as to whether morphine enhances or inhibits tumour cell

proliferation. Some studies show that morphine inhibits tumour cell proliferation (Tegeder & Geisslinger, 2004) whereas other studies show that morphine promotes tumour cell proliferation (Farooqui et al., 2007;Gupta et al., 2002). In animal models of CIBP, acute morphine treatment attenuates pain-related behaviours. Morphine has been shown to dose-dependently inhibit CIBP-induced mechanical allodynia, weight bearing difference between hindlimbs (Medhurst et al., 2002) and thermal hyperalgesia, with the greatest analgesic effect achieved through activation of μ opioid receptors (Baamonde et al., 2005;Medhurst et al., 2002). However, it is clear that CIBP is relatively resistant to morphine treatment when compared to inflammatory pain. A preclinical study showed that the doses of morphine required to block CIBP-related behaviours were 10 times those required to block inflammatory pain behaviours (Luger et al., 2002). The expression of peripheral opioid receptors has been shown to decrease in a model of CIBP, which could be a contributory reason for this resistance to morphine treatment (Yamamoto et al., 2008). Preclinical studies have examined whether chronic morphine treatment has any effect on bone destruction. One study found that chronic morphine administration accelerated bone loss and spontaneous fracture and enhanced pain behaviours, including spontaneous and movement-evoked pain behaviours, in a preclinical model of CIBP (King et al., 2007). However, in contrast, another study showed that chronic morphine administration decreased bone loss and produced analgesic effects on spontaneous foot lifting and movement-evoked pain behaviours in a preclinical model (El Mouedden & Meert, 2007b). The dorsal horn pathophysiology of CIBP is unique with increased hyperexcitability of neurons and an increased ratio of wide-dynamic range to nociceptive-specific neurons in the superficial laminae (Donovan-Rodriguez et al., 2004c). A previous study, using the MRMT-1 model of CIBP (as used in this study), showed that chronic morphine treatment significantly attenuated pain behaviour, however the increased ratio of wide dynamic range to nociceptive-specific neurons remained unaltered (Urch et al., 2005).

As mentioned previously, focal palliative radiotherapy is the current ‘gold standard’ treatment for CIBP patients. Studies suggest that single and multiple

fractionation radiotherapy treatments produce equivalent analgesia. A systematic review reported that overall pain reduction responses were 58% for single and 59% for multiple fractionation and the complete pain relief responses were 23% and 24% for single and multiple fractionation, respectively (Chow et al., 2007). However, the re-treatment rate and pathological fracture rate were higher after single fraction radiotherapy (Sze et al., 2003). The mechanisms of the beneficial effect of radiotherapy have not been fully unravelled. Studies in mouse models of CIBP have shown that radiotherapy treatment decreased CIBP-induced behavioural sensitisation, with the mechanism of radiotherapy thought to be through a direct effect on tumour cells resulting in decreased cancer-induced osteolysis and reduced tumour size (Goblirsch et al., 2004b;Goblirsch et al., 2005). However, a more recent study indicated that irradiation did not change tumour size or osteolysis, but did decrease astrocyte and microglial activity in the spinal cord of a mouse model of CIBP as well as expression of pain-associated markers such as dynorphin, COX-2 and chemotactic cytokine receptor-2 (CCR2) (Vit et al., 2006).

A number of co-analgesics/adjuvants are used in the treatment of chronic pain. Gabapentin, an anti-convulsant and analgesic, is successfully used in the treatment of neuropathic pain (Backonja & Glanzman, 2003). Chronically administered gabapentin (30mg/kg) significantly attenuated movement-evoked and spontaneous pain in CIBP and reversed dorsal horn changes associated with CIBP, such as returning the ratio of wide dynamic range to nociceptive specific neurons to normal (Donovan-Rodriguez et al., 2005). However, acute gabapentin administration did not attenuate these behaviours (Donovan-Rodriguez et al., 2005). Another closely related anti-convulsant, pregabalin (Pfizer) is currently being studied in a clinical trial to determine if the addition of pregabalin improves analgesic response to radiotherapy in CIBP. Acute administration of a different type of anti-convulsant, lacosamide, has been shown to inhibit tactile allodynia, thermal hyperalgesia and reduce weight bearing difference in a MRMT-1 rat model of CIBP. This study also showed that morphine was effective at inhibiting mechanical allodynia and reducing weight bearing difference but did not reduce thermal hyperalgesia (Beyreuther et al., 2007).

The usefulness of tricyclic anti-depressants (TCAs) and serotonin-noradrenaline reuptake inhibitor (SNRI) anti-depressants in the treatment of neuropathic pain has been established, in particular for postherpetic neuralgia and diabetic neuropathy (Attal et al., 2010). TCA and SNRI treatments act by increasing the concentration of 5-HT and noradrenaline in synapses by inhibition of noradrenaline and 5-HT transporters. Serotonin and noradrenaline are known to play an important role in the modulation of ascending and descending pain pathways as reviewed in Chapter 1 (Section 1.6.3 and 1.6.4). Duloxetine is a potent and balanced serotonin-noradrenaline reuptake inhibitor, which is effective in the treatment of neuropathic pain caused by fibromyalgia and diabetic neuropathy (Sultan et al., 2008). Duloxetine (following intraperitoneal administration) has been shown to reverse late phase paw licking behaviour in the formalin model of inflammatory pain (Iyengar et al., 2004). In the same study, duloxetine significantly reversed mechanical allodynia in the lumbar L5/L6 spinal nerve ligation model of neuropathic pain after oral administration (Iyengar et al., 2004). A separate study confirmed that duloxetine may be efficacious in the treatment of inflammatory and persistent pain, as identified by reversing acetic acid-induced writhing and carrageenan or capsaicin-induced hyperalgesia and allodynia at doses that had little effect on acute nociception (Jones et al., 2005). A study reported that the TCA desipramine, which selectively inhibits noradrenaline reuptake, and the TCA amitriptyline, which inhibits both serotonin and noradrenaline reuptake, decreased spontaneous pain behaviour in a preclinical CIBP model but only at doses that also caused sedation (El Mouedden & Meert, 2007a). The selective serotonin reuptake inhibitor (SSRI) fluoxetine showed limited efficacy and none of these compounds reversed mechanical allodynia or affected limb use impairment (El Mouedden & Meert, 2007a). Whiteside *et al.* demonstrated that selectively increasing noradrenaline alone is sufficient for analgesic activity in a CIBP preclinical model, as an acute dose of the potent and selective inhibitor of the noradrenaline transporter WAY-318068 reversed mechanical allodynia in a MRMT-1 rat model of CIBP (Whiteside et al., 2010). Another potent and selective noradrenaline reuptake inhibitor, S,S-reboxetine, is investigated here as a comparator against duloxetine.

Several studies have shown that targeting cannabinoids may be effective in the treatment of cancer pain (Alexander et al., 2009;Casanova et al., 2003;Galve-Roperh et al., 2000;Lozano-Ondoua et al., 2010;Saghafi et al., 2011;Sanchez et al., 2001). The classical cannabinoid receptors, CB_1 and CB_2, are G protein-coupled receptors. CB_1 receptors are expressed in the CNS but also in peripheral tissues such as lung, prostate, heart and bone marrow. CB_2 receptors are mostly expressed in cells of the immune system such as B cells, T cells and macrophages but is also known to be expressed in other tissues and some regions of the CNS (Galiegue et al., 1995). A recent study showed that a tetrahydrocannabinol:cannabidiol (THC:CBD) extract from the *Cannabis sativa* plant is efficacious for relief of pain in patients with advanced cancer (Johnson et al., 2010). THC is a partial agonist of CB_1 receptors and CBD is an agonist at both CB_1 and CB_2 receptors (Johnson et al., 2010). A number of studies have shown that cannabinoid receptor agonists inhibit cancer cell proliferation and/or apoptosis *in vitro* (De Petrocellis et al., 1998;Olea-Herrero et al., 2009;Xian et al., 2010) and to cause tumour regression and inhibit metastasis in preclinical models (Xian et al., 2010). Cannabinoids have been shown to reduce cancer cell invasion by inhibition of matrix metalloproteinases (Ramer et al., 2010). CB_2 receptor-selective agonists are not thought to cause centrally mediated side-effects associated with the activation of CB_1 receptors (Hanus et al., 1999;Malan et al., 2001) making the CB_2 receptors an attractive potential analgesic target. This idea is supported by evidence of increased expression of CB_2 receptors in the dorsal horn and sensory afferents in chronic pain models (Anand et al., 2009). CB_2 receptor-selective agonists have recently been shown to reduce pain behaviours in models of acute, inflammatory and nerve injury-induced nociception reviewed by Guindon & Hohmann (Guindon & Hohmann, 2008). Furthermore, the CB_2 receptor-selective agonist AM1241 was shown to reduce pain-related behaviours and tumour-induced bone destruction in preclinical models of CIBP (Curto-Reyes et al., 2010;Lozano-Ondoua et al., 2010). The mechanism of action of AM1241 has been the subject of some controversy. However, *in vivo* the effects of AM1241 are blocked by CB_2 but not CB_1 receptor-selective antagonists and the effects of AM1241 are not seen in CB_2-/- mice. This evidence suggests that AM1241 is a CB_2 receptor agonist (Lozano-

Ondoua et al., 2010). In this thesis, the analgesic efficacy of CB 65, a selective, high affinity CB_2 receptor agonist (Manera et al., 2006), will be investigated in a model of CIBP.

4.2 Aim

The aim of this part of the study was to use pharmacological tools to try and understand the mechanisms of CIBP. The analgesic efficacy of radiotherapy (XRT), which mirrors focal radiotherapy administered to patients, and the effect of the following pharmacological agents; gabapentin, duloxetine, S,S-reboxetine and CB 65 were investigated in our model of CIBP. These agents were chosen based on the current knowledge of what mechanisms might be important in CIBP. We evaluated the efficacy of these potential analgesic interventions in CIBP-induced sensitisation across a range of behavioural assessments. Analysis of the efficacy of these agents in CIBP could throw further light on the mechanisms of CIBP and help improve treatment options for CIBP patients.

4.3 Methods

4.3.1 Surgical Procedure

Experiments were carried out using male Sprague-Dawley rats and, as detailed in Chapter 2, animals underwent CIBP surgery (Section 2.1.2). Rats were anaesthetised by inhalation of an isoflurane/O_2 mixture (Zeneca, UK), 4-5% for induction and 2-3% for maintenance. The carrier gas was compressed oxygen at a flow rate of 2 litres/minute. Following complete induction of anaesthesia the animal was placed abdominal side up, the left hind limb was shaved and the skin was sterilised with 0.5% Hibitane (Zeneca, UK). A small incision was made in the skin over the tibia, which was then carefully exposed by removing the connective tissue over the bone using a cotton bud (Johnson & Johnson, UK). A dental drill was used to bore a hole through the periosteum of the tibia. Polythene tubing (0.5mm in diameter; Smiths) was fed into the intra-medullary cavity of the tibia and 10 µl of medium (containing $6x10^3$ cells) was injected using a 1ml micro-syringe (BD Biosciences, UK) and 25-gauge needle (BD Biosciences, UK). The tubing was withdrawn and the hole plugged with dental restorative material (IRM, Dentsply;

Henry Schien Minerva), to confine the tumour cells within the marrow and prevent invading the adjacent soft tissue. The wound was closed with absorbable subcutaneous suture (5/0 coated vicryl, Ethicon, UK) and sterilised with 0.5% Hibitane. Animals were placed in a thermoregulated recovery box until they had fully regained consciousness, following which they were returned to their home cages.

4.3.2 Analgesic Intervention

Analgesic interventions were administered to CIBP animals, at various time points post surgery (Table 2.1). Focal radiotherapy (XRT) was administered to CIBP animals on Day 7 (Section 2.7.1).

Body weight gain of XRT-treated animals was monitored and compared to CIBP animals. The effect of XRT on CIBP-induced mechanical allodynia, thermal sensitivity to 20°C and 40°C, movement-evoked pain and static weight bearing difference was assessed. In addition the effect of XRT on voluntary locomotor activity, pain-related anxiety and risk assessment behaviour in the open field and on the elevated plusmaze was analysed. CIBP animals that received radiotherapy on Day 7 were tested at the same time as the CIBP, Sham and Naive animals characterised in Chapter 3. Chapter 4 focuses on the XRT behaviours compared to the CIBP animals. Thus the CIBP data in the graphs of Chapter 4 is the same as that shown in Chapter 3.

Gabapentin (30mg/kg), duloxetine (30mg/kg) and S,S-reboxetine (10mg/kg) were administered by oral gavage and CB 65 (1mg/kg) was administered intraperitoneally (Section 2.7.2). The efficacy of these pharmacological agents in attenuating CIBP-induced mechanical allodynia, movement-evoked pain and thermal sensitivity to 40°C was assessed. The effect of these agents on voluntary locomotor activity (as measured by rearing behaviour), pain-related anxiety and risk assessment behaviour on the elevated plusmaze was also analysed (Section 2.10.3).

4.3.3 Statistical Analysis

In each behavioural study, data were pooled for each time point, with group mean shown ± SEM. To analyse the effects of XRT or pharmacological agent administration on mechanical allodynia, post-pharmacological agent ipsilateral paw withdrawal thresholds were compared to pre-pharmacological agent baseline using a One-way repeated measures ANOVA on Ranks (Friedman's test) followed by Dunn's post-hoc analysis. Ipsilateral paw withdrawal thresholds were compared to contralateral paw withdrawal thresholds using a One-way repeated measures ANOVA on ranks (Friedman's test) followed by Dunn's post-hoc analysis.

To analyse the effects of XRT on thermal sensitivity the difference between post-XRT ipsilateral responses and pre-XRT ipsilateral responses and XRT-treated animals were compared to CIBP alone using a repeated measures mixed-model ANOVA followed by Bonferroni's post-hoc analysis. To analyse the effects of pharmacological agent administration on thermal sensitivity the difference between post-pharmacological agent ipsilateral responses and pre-pharmacological agent ipsilateral responses were determined using a One-way repeated measures ANOVA followed by Dunnett's post-hoc analysis.

To analyse the effects of XRT on movement-evoked pain, post-XRT number of avoidances of weight bearing on movement were compared to pre-XRT values and XRT-treated animals were compared to CIBP animals alone using a repeated measures mixed-model ANOVA followed by Dunnett's post-hoc analysis. To analyse the effects of pharmacological agent administration on movement-evoked pain, post-pharmacological agent number of avoidances of weight bearing on movement were compared to pre-pharmacological agent using a One-way repeated measures ANOVA followed by Dunnett's post-hoc analysis.

To analyse the effects of XRT on static weight bearing difference, post-XRT weight bearing difference was compared to pre-XRT values and XRT-treated animals were compared to CIBP using a repeated measures mixed-model ANOVA.

For responses to the elevated plusmaze and the open field (differences between groups (CIBP, CIBP + pharmacological agent, XRT treated, Sham V operated and Naive animals) were determined by a One-way ANOVA followed by Bonferroni's post-hoc analysis. For the elevated plusmaze and open field, there were several separate test sessions and only those run at the same time were compared. Data were not combined for separate test sessions of the same group as these measures of anxiety are subject to variation between tests.

For the elevated plusmaze, the responses of XRT and CIBP animals were compared. The responses of CIBP animals + gabapentin/duloxetine were compared to CIBP + vehicle, however CIBP animals + S,S-reboxetine were run at a separate time to the other pharmacological agents and were therefore not compared to the vehicle control. For the open field, the responses of CIBP and XRT animals were compared.

4.4 Results

All animals were observed post-surgery and treatment to ensure animals maintained a healthy weight and did not show signs of distress. XRT was carried out at Day 7 after induction of CIBP.

4.4.1 The effect of radiotherapy on body weight

XRT-treated animals showed a minor alteration in body weight, as demonstrated by a significant increase in body weight compared to CIBP animals at Day 9-11 only (160.1 ± 6.61 versus 192.8 ± 3.28), indicated by a repeated measures mixed-model ANOVA followed by Bonferroni's post-hoc analysis, $p<0.05$ (Figure 4.1).

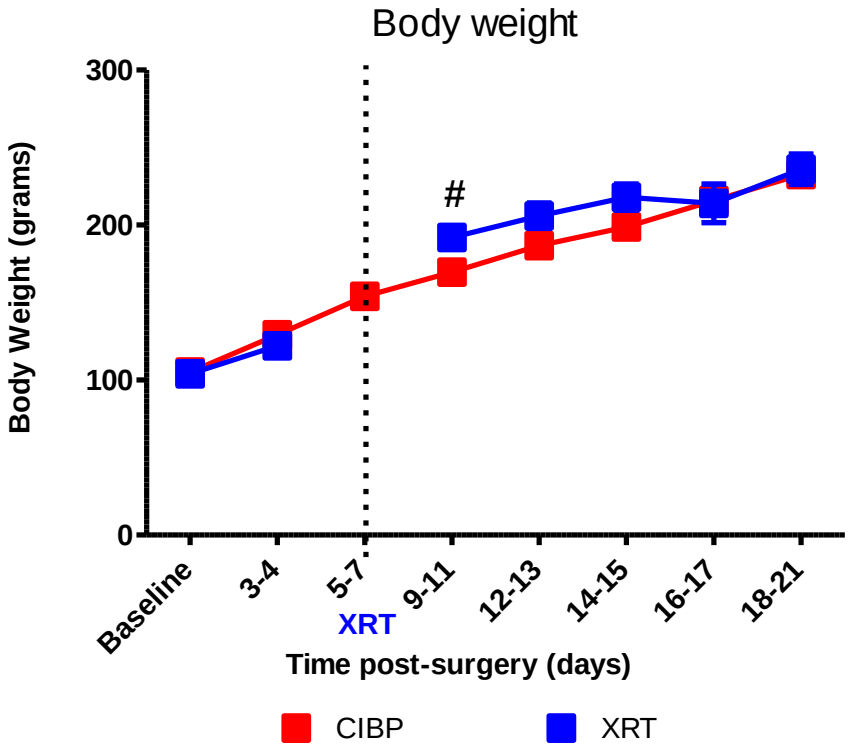


Figure 4.1 Time course of body weight gain. Data show mean body weight ± SEM of CIBP (n=10) and XRT (n=13) animals. XRT animals had significantly increased body weight compared to CIBP at Day 9-11 (#; repeated measures mixed-model ANOVA followed by Bonferroni's post-hoc analysis, p = 0.01 to 0.05).

		Time post-surgery (days)							
Group		Baseline	3-4	5-7	9-11	12-13	14-15	16-17	18-21
CIBP	Weight (grams)	105.1	129.3	154.0	169.7	186.8	198.7	215.9	232.6
	SEM	2.8	3.0	2.9	4.3	3.7	3.6	3.7	3.8
XRT	Weight (grams)	104	122	~	192	206	218	214	236
	SEM	2.95	2.85	~	5.12	8.17	8.5	12.4	9.75

Table 4.1 The effect of XRT treatment on body weight. Data show mean body weight ± SEM of CIBP (n=10) and XRT (n=13) animals. ~ = not recorded.

4.4.2 The effect of XRT on CIBP-induced mechanical allodynia

CIBP animals showed a marked reduction in ipsilateral PWT when compared to baseline from Day 9 onwards, which was also observed in XRT animals at Day 9-11 and from Day 14-15 onwards. CIBP animals showed a significant reduction in ipsilateral PWT when compared to contralateral PWT from Day 5-7 onwards that was also noted in XRT animals at Day 3-4 and from Day 12-13 onwards. CIBP-induced mechanical allodynia was not significantly attenuated by XRT treatment (Figure 4.2). All indicated by a repeated measures One-way ANOVA on ranks (Friedman's test) followed by Dunn's post-hoc analysis, $p<0.05$.

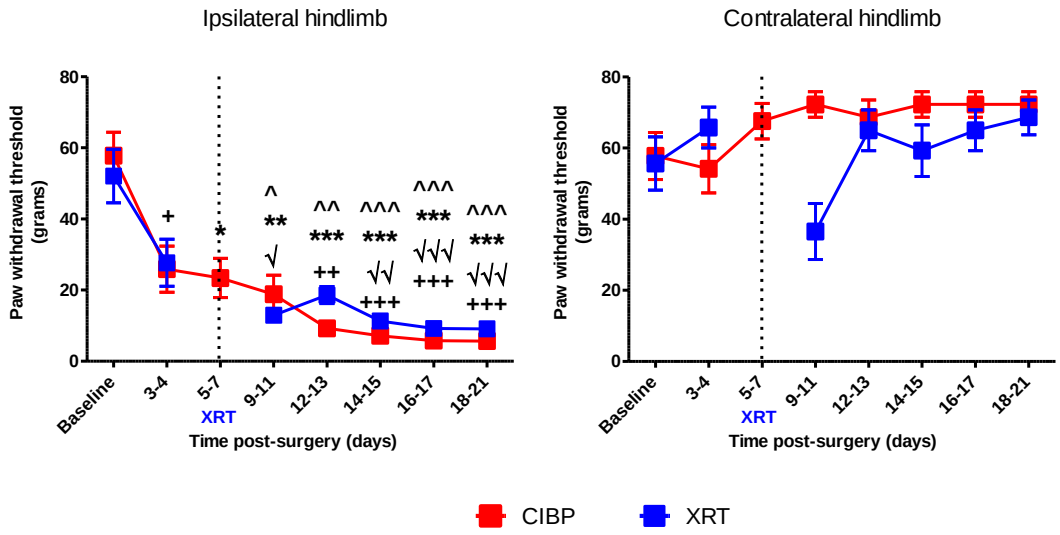


Figure 4.2 Effect of XRT on mechanical allodynia. Data show mean Paw Withdrawal Threshold (PWT) responses ± SEM of CIBP (n=13) and XRT (n=13) animals. A) CIBP and XRT animals showed a significant reduction in PWT when compared to baseline (^ and √, respectively; One-way repeated measures ANOVA on ranks (Friedman's test) followed by Dunn's post-hoc analysis, $p<0.05$). CIBP and XRT animals showed a significant reduction in ipsilateral PWT when compared to contralateral PWT (* and +, respectively; One-way repeated measures ANOVA on ranks (Friedman's test) followed by Dunn's post-hoc analysis, $p<0.05$). XRT did not alter PWT in comparison to CIBP alone. P values ^^^ = <0.001, ^^ = 0.001 to 0.01, ^ = 0.01 to 0.05, *** = <0.001, ** = 0.001 to 0.01, * = 0.01 to 0.05, $^{+++}$ = <0.001, $^{++}$ =

0.001 to 0.01, $^{+}$ = 0.01 to 0.05, √√√ = <0.001, √√ = 0.001 to 0.01 and √ = 0.01 to 0.05.

			Time post-surgery (days)							
Group			Baseline	3-4	5-7	9-11	12-13	14-15	16-17	18-21
CIBP	Ipsi	PWT (grams)	57.8	25.9	23.4	18.8	9.2	7.1	5.8	5.6
		SEM	6.6	6.5	5.5	5.4	1.1	0.6	0.6	0.5
	Con	PWT (grams)	57.8	54.2	67.6	72.2	68.6	72.2	72.2	72.2
		SEM	6.6	6.8	5.0	3.6	4.9	3.6	3.6	3.6
XRT	Ipsi	PWT (grams)	52.0	27.6	~	12.9	18.6	11.3	9.2	9.1
		SEM	7.5	6.6	~	1.6	2.4	1.7	1.0	1.2
	Con	PWT (grams)	55.7	65.8	~	36.5	65.0	59.3	65.0	68.6
		SEM	7.5	5.7	~	7.9	5.7	7.3	5.7	4.9

Table 4.2 The effect of XRT treatment on CIBP-induced mechanical allodynia showing paw withdrawal threshold (PWT). Data show mean PWT responses ± SEM of CIBP (n=13) and XRT (n=13) animals. ~ = not recorded.

4.4.3 The effect of XRT on CIBP-induced thermal sensitivity to 20°C

CIBP animals showed a significant increase in thermal sensitivity to 20°C, with a significant increase in number of paw withdrawals from Day 12-13 onwards, a significant increase in duration of paw elevation from Day 16-17 onwards and significant reductions in latency to paw withdrawal when compared to baseline. XRT animals also showed increased thermal sensitivity to 20°C with an increased number of paw withdrawals at Day 18-21 and reduced latency to paw withdrawal at Day 14-15 and Day 18-21. CIBP-induced thermal sensitivity to 20°C was attenuated by XRT, where the number of paw withdrawals from Day 12-13 onwards and duration of paw elevation from Day 16-17 onwards were decreased. Latency to paw

withdrawal at Day 16-17 was also increased by XRT treatment and at Day 3-4, prior to XRT treatment, latency to paw withdrawal was significantly different between CIBP groups. All shown by repeated measures mixed-model ANOVA followed by Bonferroni's post-hoc analysis, $p<0.05$ (Figure 4.3).

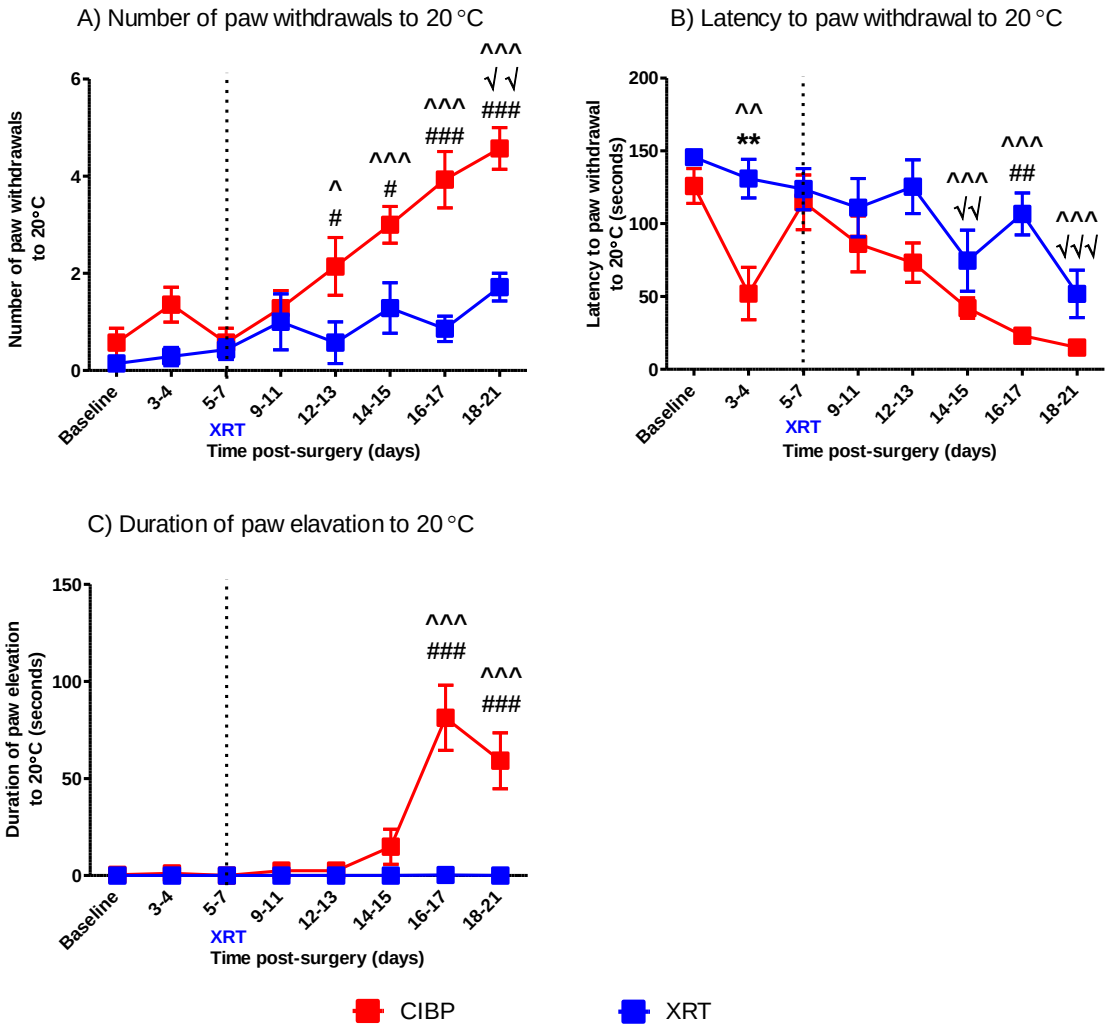


Figure 4.3 Effect of XRT on thermal sensitivity to 20°C. Data show mean responses ± SEM of CIBP (n=7) and XRT (n=7) animals. A) Number of paw withdrawals was significantly increased in CIBP and XRT animals compared to baseline (^ and √, respectively; repeated measures mixed-model ANOVA followed by Bonferroni's post-hoc analysis, $p<0.05$). XRT animals had significantly decreased number of paw withdrawals when compared to CIBP from Day 12-13 onwards (# repeated measures mixed-model ANOVA followed by Bonferroni's post-hoc analysis, $p<0.05$). B)

Latency to paw withdrawal was significantly decreased in CIBP and XRT animals compared to baseline (^ and √, respectively; repeated measures mixed-model ANOVA followed by Bonferroni's post-hoc analysis, $p<0.05$). At Day 3-4 the two groups of CIBP animals showed a significant difference in latency to paw withdrawal and XRT-treated animals had significantly increased latency to paw withdrawal at Day 16-17 when compared to CIBP (* and #, respectively; repeated measures mixed-model ANOVA followed by Bonferroni's post-hoc analysis, $p<0.05$). C) Duration of paw elevation was significantly increased in CIBP animals when compared to baseline (^; repeated measures mixed-model ANOVA followed by Bonferroni's post-hoc analysis, $p<0.05$). XRT animals did not develop any increase in duration of paw elevation, resulting in a significant decrease in duration of paw elevation when compared to CIBP (#; repeated measures mixed-model ANOVA followed by Bonferroni's post-hoc analysis, $p<0.05$). P values ^^^ = <0.001, ^^ = 0.001 to 0.01, ^ = 0.01 to 0.05, ** = 0.001 to 0.01, ### = <0.001, ##= 0.001 to 0.01 and # = 0.01 to 0.05, √√√ = <0.001 and √√ = 0.001 to 0.01.

			Time post-surgery (days)							
	Group		Baseline	3-4	5-7	9-11	12-13	14-15	16-17	18-21
Number	CIBP		0.6	1.4	0.6	1.3	2.1	3.0	3.9	4.6
		SEM	0.3	0.4	0.3	0.4	0.6	0.4	0.6	0.4
	XRT		0.1	0.3	0.4	1.0	0.6	1.3	0.9	1.7
		SEM	0.1	0.2	0.2	0.6	0.4	0.5	0.3	0.3
Latency (seconds)	CIBP		125.9	52.0	114.7	86.1	73.3	42.0	23.1	15.0
		SEM	11.9	18.0	18.8	19.1	13.5	7.0	4.6	4.2
	XRT		145.7	131.0	123.7	111.0	125.4	74.6	106.7	51.9
		SEM	4.3	13.2	14.1	20.0	18.4	21.0	14.4	16.3
Duration (seconds)	CIBP		0.4	1.1	0.0	2.6	2.6	14.9	81.3	59.1
		SEM	0.4	0.7	0.0	2.6	2.1	9.1	16.8	14.4
	XRT		0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.0
		SEM	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.0

Table 4.3 The effect of XRT on thermal sensitivity to 20°C showing number of paw withdrawals, latency to paw withdrawal and duration of paw elevation. Data show mean responses ± SEM of CIBP (n=7) and XRT (n=7) animals.

4.4.4 The effect of XRT on CIBP-induced thermal sensitivity to 40°C

CIBP animals showed a significant increase in number of paw withdrawals from Day 12-13 onwards, a significant increase in duration of paw elevation from Day 14-15 onwards and significant reductions in latency to paw withdrawal from Day 12-13 onwards. XRT animals only showed a reduced latency to paw withdrawal at Day 9-11 and Day 18-21. CIBP-induced thermal sensitivity to 40°C was significantly attenuated by XRT, where the number of paw withdrawals was significantly decreased when compared to CIBP from Day 16-17 onwards, latency to paw withdrawal was significantly increased when compared to CIBP from Day 16-17 onwards and the duration of paw elevation was significantly decreased at Day 16-17 only. All shown by repeated measures mixed-model ANOVA followed by Bonferroni's post-hoc analysis, $p<0.05$ (Figure 4.4).

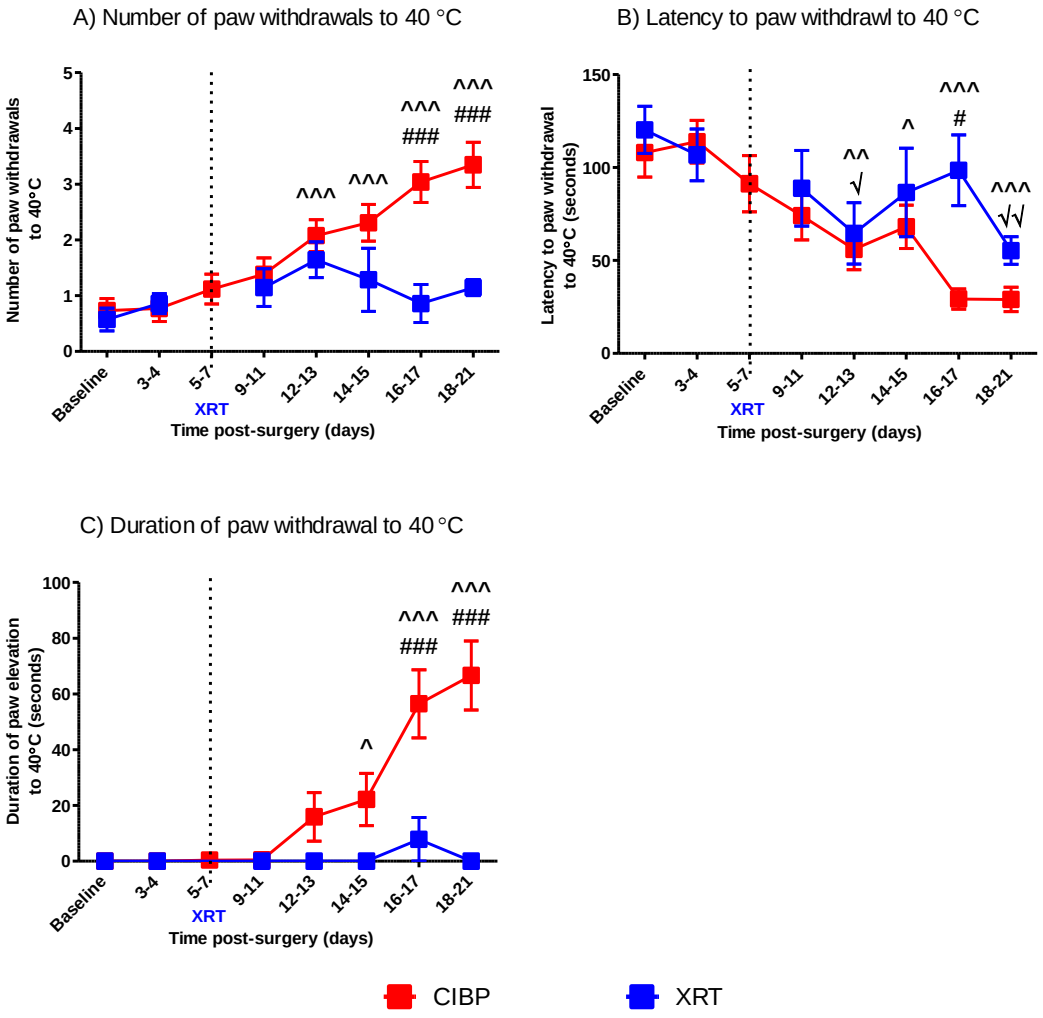


Figure 4.4 Effect of XRT on CIBP-induced thermal sensitivity to 40°C. Data show mean responses ± SEM of CIBP (n=13) and XRT (n=7) animals. A) CIBP animals showed a significant increase in number of paw withdrawals when compared to baseline (^; repeated measures mixed-model ANOVA followed by Bonferroni's post-hoc analysis, $p<0.05$). XRT-treated animals showed a significant decrease in number of paw withdrawals from Day 16-17 onwards when compared to CIBP (#; repeated measures mixed-model ANOVA followed by Bonferroni's post-hoc analysis, $p<0.05$). B) CIBP and XRT-treated animals showed a significant decrease in latency to paw withdrawal when compared to baseline (^ and √, respectively; repeated measures mixed-model ANOVA followed by Bonferroni's post-hoc analysis, $p<0.05$). XRT-treated animals had a significant increase in latency to paw

withdrawal at Day 16-17 when compared to CIBP alone (#; repeated measures mixed-model ANOVA followed by Bonferroni's post-hoc analysis). C) CIBP animals had a significant increase in duration of paw withdrawal when compared to baseline (^; repeated measures mixed-model ANOVA followed by Bonferroni's post-hoc analysis, $p<0.05$). XRT-treated animals showed a significant reduction in duration of paw elevation from Day 16-17 onwards compared to CIBP alone (#; repeated measures mixed-model ANOVA followed by Bonferroni's post-hoc analysis, $p<0.05$). P values ^^^ = <0.001, ^^ = 0.001 to 0.01, ^ = 0.01 to 0.05, ### = <0.001, # = 0.01 to 0.05, √√ = 0.001 to 0.01 and √ = 0.01 to 0.05.

			Time post-surgery (days)							
	Group		Baseline	3-4	5-7	9-11	12-13	14-15	16-17	18-21
Number	CIBP		0.7	0.8	1.1	1.4	2.1	2.3	3.0	3.3
		SEM	0.2	0.2	0.3	0.3	0.3	0.3	0.4	0.4
	XRT		0.6	0.9	~	1.1	1.6	1.3	0.9	1.1
		SEM	0.2	0.2	~	0.3	0.3	0.6	0.3	0.1
Latency (seconds)	CIBP		108.1	113.9	91.3	74.1	55.9	68.1	29.2	29.0
		SEM	13.2	11.5	15.2	13.1	10.8	11.7	5.4	6.6
	XRT		120.3	106.8	~	88.9	64.6	86.6	98.6	55.3
		SEM	12.6	13.9	~	20.4	16.5	23.9	19.1	7.4
Duration (seconds)	CIBP		0.0	0.0	0.4	0.5	15.9	22.2	56.5	66.6
		SEM	0.0	0.0	0.4	0.3	8.7	9.4	12.2	12.4
	XRT		0.0	0.0	~	0.0	0.0	0.0	7.9	0.0
		SEM	0.0	0.0	~	0.0	0.0	0.0	7.9	0.0

Table 4.4 The effect of XRT on thermal sensitivity to 40°C showing number of paw withdrawals, latency to paw withdrawal and duration of paw elevation. Data show mean responses ± SEM of CIBP (n=13) and XRT (n=7) animals. ~ = not recorded.

4.4.5 The effect of XRT on CIBP-induced movement-evoked pain and CIBP-induced ongoing pain

CIBP animals showed a significant increase in avoidance of weight bearing on movement, from Day 5-7 onwards that was also observed in XRT-treated animals from Day 9-11 onwards when compared to baseline. XRT animals show a significant reduction in avoidance of weight bearing on movement when compared to CIBP animals at Day 18-21 only. Static weight bearing difference, as a measure of ongoing pain, was significantly increased in CIBP and XRT animals from Day 12-13. That is, both CIBP and XRT-treated animals displayed significantly less weight bearing on the injured hind limb when compared to baseline; illustrating that CIBP-induced ongoing pain was unaltered by XRT treatment (Figure 4.5). All shown by repeated measures mixed-model ANOVA followed by Bonferroni's post-hoc analysis, $p<0.05$.

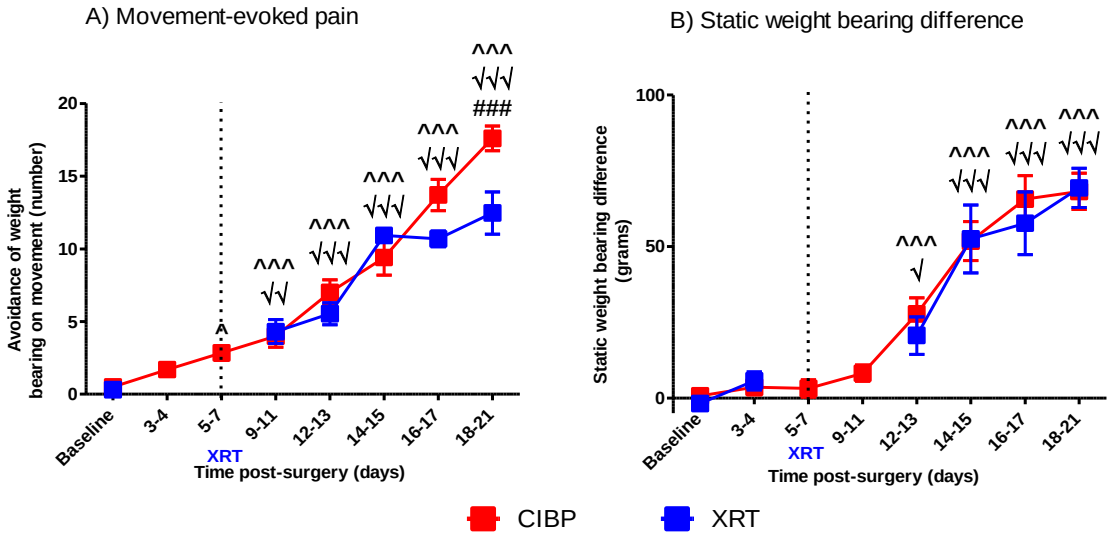


Figure 4.5 Effect of XRT on CIBP-induced avoidance of weight bearing on movement and weight bearing difference between hindlimbs. Data show mean responses ± SEM of CIBP (n=10-13) and XRT (n=13) animals. A) CIBP and XRT animals showed significantly increased avoidance of weight bearing on movement when compared to baseline (^ and √, respectively; repeated measures mixed-model ANOVA followed by Bonferroni's post-hoc analysis, $p<0.05$). XRT significantly decreased avoidance of weight bearing on movement when compared to CIBP at Day 18-21 (#; repeated measures mixed-model ANOVA followed by Bonferroni's

post-hoc analysis, p<0.05). B) CIBP and XRT animals showed significantly increased static weight bearing difference when compared to baseline (^ and √, respectively; repeated measures mixed-model ANOVA followed by Bonferroni's post-hoc analysis, p<0.05). XRT treatment did not affect CIBP-induced increase in static weight bearing difference. P values ^^^ = <0.001, ^ = 0.01 to 0.05, ### = <0.001, √√√ = <0.001, √√ = 0.001 to 0.01 and √ = 0.01 to 0.05.

			Time post-surgery (days)							
	Group		Baseline	3-4	5-7	9-11	12-13	14-15	16-17	18-21
Movement-evoked	CIBP	Number	0.5	1.7	2.9	4.0	7.0	9.4	13.7	17.6
		SEM	0.2	0.4	0.5	0.8	0.9	1.2	1.1	0.8
	XRT	Number	0.3	-	-	4.3	5.5	10.9	10.7	12.5
		SEM	0.1	-	-	0.8	0.8	0.5	0.5	1.5
Weight bearing	CIBP	WBD (grams)	0.7	3.6	3.2	8.2	27.8	51.8	65.6	68.2
		SEM	1.0	2.5	2.8	2.7	5.3	6.5	7.7	6.0
	XRT	WBD (grams)	-1.8	5.7	-	21.3	20.6	52.5	57.7	69.4
		SEM	1.4	3.0	-	4.2	6.2	11.2	10.3	6.5

Table 4.5 The effects of XRT on movement-evoked and static weight bearing difference between hindlimbs showing number of avoidances of weight bearing on movement and weight bearing difference (WBD). Data show mean responses ± SEM of CIBP (n=10-13) and XRT (n=13) animals.

4.4.6 Effect of XRT on measures of voluntary locomotor activity

The following measures of voluntary locomotor activity in the open field were assessed; distance travelled, average speed and number of rearings. XRT treatment did not alter CIBP-induced behavioural responses in the open field. In addition, the number of rearings in the elevated plusmaze was not altered by XRT (Figure 4.6).

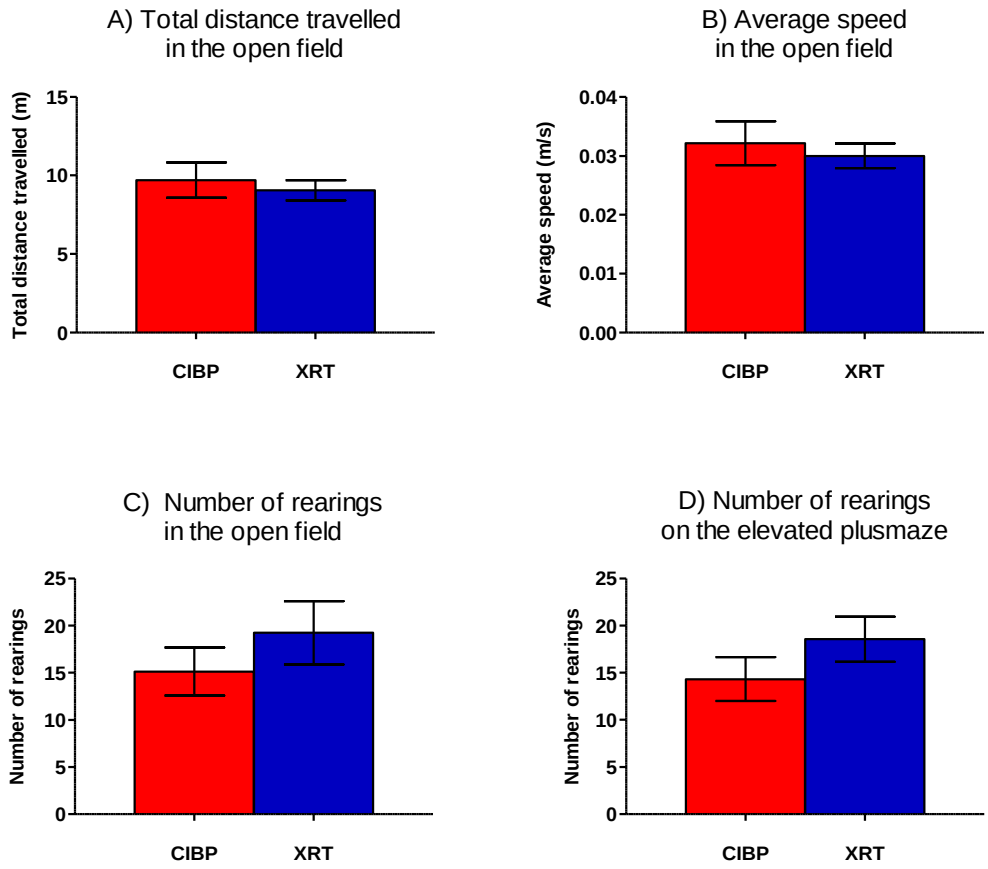


Figure 4.6 Effect of XRT on voluntary locomotor activity in the open field and elevated plusmaze. Data show mean responses ± SEM of CIBP (n=7) and XRT (n=8) animals in the open field and of CIBP (n=9) and XRT (n=14) animals on the elevated plusmaze. A) XRT did not alter the distance travelled in the open field. B) XRT did not alter the average speed in the open field. C) XRT did not alter the number of rearings in the open field. D) XRT did not alter the number of rearings on the elevated plusmaze.

4.4.7 Effect of gabapentin on CIBP-induced mechanical allodynia, movement-evoked pain and thermal sensitivity to 40°C

The anti-convulsant, gabapentin (30mg/kg; p.o. administration), was assessed for reversal of CIBP-induced behavioural sensitisation. Gabapentin (given at Day 18-21 post CIBP induction) did not attenuate CIBP-induced mechanical allodynia as shown by no significant alteration in ipsilateral PWT when compared to pre-

administration values. In addition the pre-administration significant reduction in ipsilateral PWT remained unaltered post pharmacological agent administration, except at 4 hours. All shown by One-way repeated measures ANOVA on ranks (Friedman's test) followed by Dunn's post-hoc analysis, $p<0.05$. Gabapentin did not attenuate CIBP-induced movement-evoked pain or thermal sensitivity to 40°C when compared to pre-administration values, shown by a One-way repeated measures ANOVA followed by Bonferroni's post-hoc analysis, $p<0.05$ (Figure 4.7).

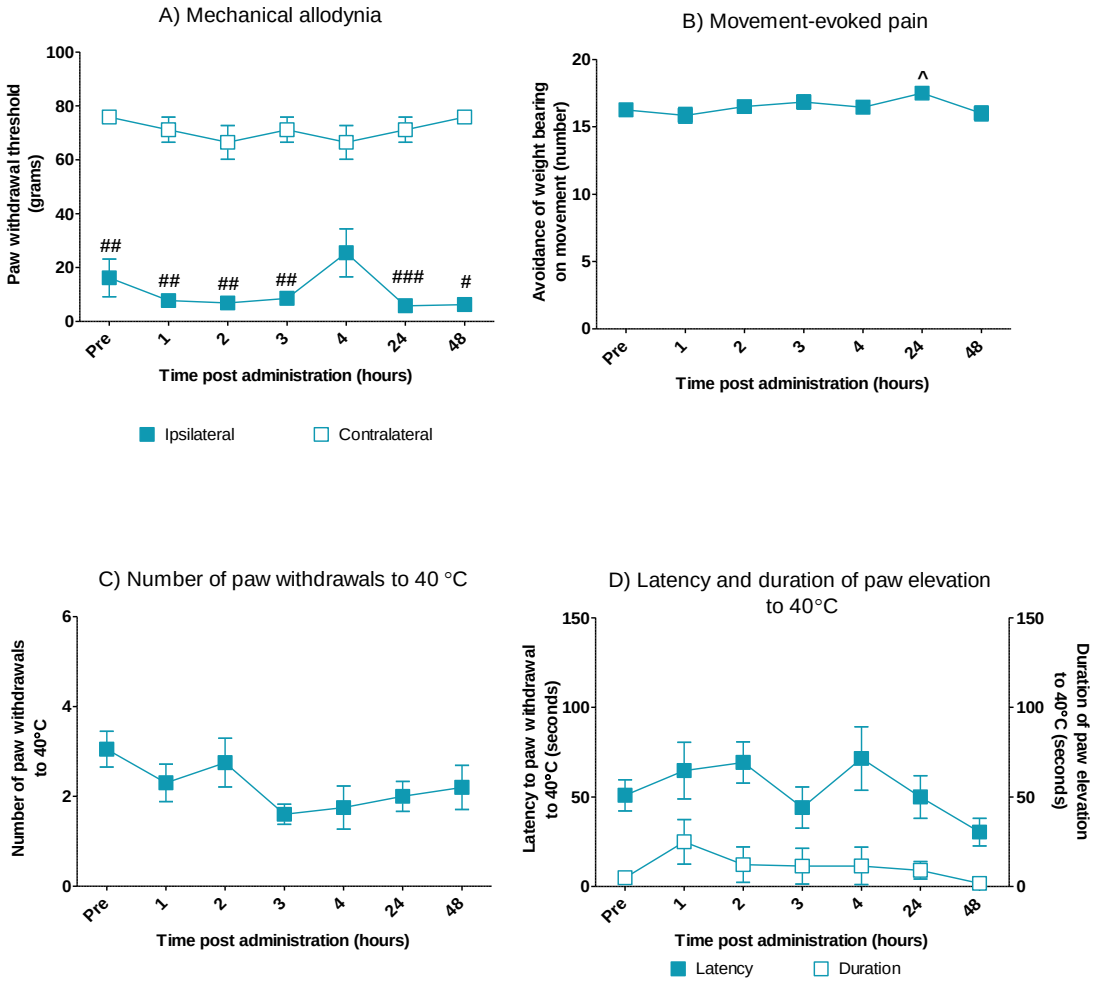


Figure 4.7 Effect of gabapentin (30mg/kg; p.o. administration) on CIBP-induced behavioural sensitisation. Data show mean responses ± SEM (n=10). A) CIBP animals showed a significant reduction in ipsilateral PWT compared to contralateral PWT pre- and post-administration (#; One-way repeated measures ANOVA on ranks

(Friedman’s test) followed by Dunn's post-hoc analysis, $p<0.05$). Gabapentin did not alter ipsilateral PWT when compared to pre-administration values. B) Gabapentin did not attenuate CIBP-induced increased avoidance of weight bearing on movement when compared to pre-administration. A significant increase in avoidance of weight bearing on movement was noted 24 hours post-administration (^; One-way repeated measures ANOVA followed by Bonferroni's post-hoc analysis, $p<0.05$) C) Gabapentin did not alter the number of paw withdrawals to 40°C when compared to pre-administration values. D) Gabapentin did not alter latency and duration of paw elevation to 40°C when compared to pre-administration values. P values ### = <0.001, ## = 0.001 to 0.01, # = 0.01 to 0.05 and ^ = 0.01 to 0.05.

			Time post-administration (hours)						
			Pre	1	2	3	4	24	48
Mechanical allodynia	Ipsi	PWT (grams)	16.2	7.8	6.8	8.6	25.5	5.8	6.2
		SEM	7.0	0.9	0.8	1.0	8.9	0.6	1.7
	Con	PWT (grams)	75.9	71.2	66.5	71.2	66.5	71.2	75.9
		SEM	0.0	4.7	6.3	4.7	6.3	4.7	0.0
Movement-evoked		Number	16.3	15.9	16.5	16.9	16.5	17.5	16.0
		SEM	0.3	0.6	0.4	0.5	0.3	0.5	0.5
Thermal sensitivity to 40°C	Number		3.1	2.3	2.8	1.6	1.8	2.0	2.2
		SEM	0.4	0.4	0.5	0.2	0.5	0.3	0.5
	Latency (seconds)		50.9	64.7	69.3	44.1	71.5	50.0	30.4
		SEM	8.7	15.8	11.6	11.5	17.8	11.9	7.7
	Duration (seconds)		4.9	25.0	12.2	11.4	11.5	9.1	1.8
		SEM	3.5	12.4	9.9	9.9	10.4	4.9	1.8

Table 4.6 The effects of gabapentin on CIBP-induced behavioural sensitisation showing paw withdrawal threshold (PWT), number of avoidances of weight bearing on movement, number of paw withdrawals, latency to paw withdrawal and duration of paw elevation. Data show mean responses ± SEM (n=10).

4.4.8 The effect of duloxetine on CIBP-induced mechanical allodynia, movement-evoked pain and thermal sensitivity to 40°C

A dual reuptake inhibitor of serotonin and noradrenaline, duloxetine (30mg/kg; p.o. administration), which may also act on voltage-dependent sodium channels, was assessed for reversal of CIBP-induced behavioural sensitisation. Duloxetine (given at Day 16-21 after CIBP induction) attenuated mechanical

allodynia at 1 and 2 hours post-administration whereby ipsilateral PWT was significantly increased when compared to the pre-administration values and pre-administration significant reduction in ipsilateral PWT compared to contralateral PWT did not return until 48 hours post-administration, shown by a One-way repeated measures ANOVA on ranks (Friedman's test) followed by Dunn's post-hoc analysis, $p<0.05$. Duloxetine also attenuated CIBP-induced movement-evoked pain when compared to pre-administration values at 1 to 4 hours post-administration and attenuated thermal sensitivity to 40°C, shown by a significant decrease in the number of paw withdrawals up to 24 hours post-administration compared to pre-administration values and a significant increase in latency to paw withdrawal up to 24 hours post-administration. All shown by One-way ANOVA followed by Bonferroni's post-hoc analysis, $p<0.05$. The duration of paw elevation was unaltered post- administration (Figure 4.8).

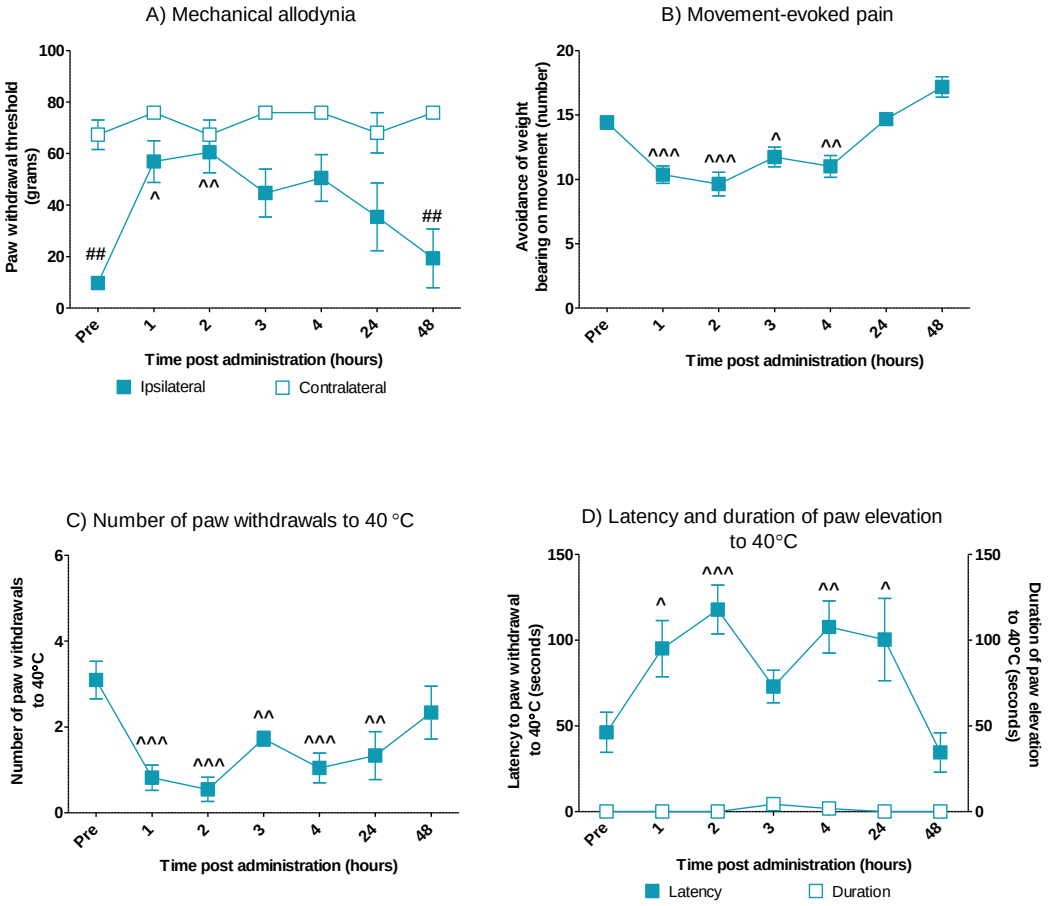


Figure 4.8 Effect of duloxetine (30mg/kg; p.o. administration) on CIBP-induced behavioural sensitisation. Data show mean responses ± SEM (n=11). A) Ipsilateral PWT was significantly decreased (pre-administration and 48 hours post-administration) when compared to contralateral values (#; One-way repeated measures ANOVA on ranks followed by Dunn's post-hoc analysis, $p<0.05$). Duloxetine significantly increased ipsilateral PWT when compared to pre-administration responses (^; One-way repeated measures ANOVA on ranks (Friedman's test) followed by Dunn's post-hoc analysis, $p<0.05$). B) Avoidance of weight bearing on movement was significantly decreased by duloxetine when compared to pre-administration (^; One-way repeated measures ANOVA followed by Bonferroni's post-hoc analysis, $p<0.05$). C) The number of paw withdrawals at 40°C was significantly decreased when compared to pre-administration values (^; One-way repeated measures ANOVA followed by Bonferroni's post-hoc analysis). D) Latency to paw withdrawal was significantly increased when compared to pre-administration values (^; One-way repeated measures ANOVA followed by

Bonferroni's post-hoc analysis, $p<0.05$). The duration of paw elevation did not alter post-administration. P values ^^^ = <0.001, ^^ = 0.001 to 0.01, ^ = 0.01 to 0.05 and ## = 0.001 to 0.01.

			Time post-administration (hours)						
			Pre	1	2	3	4	24	48
Mechanical allodynia	Ipsi	PWT (grams)	9.7	56.9	60.5	44.7	50.5	35.4	19.3
		SEM	1.2	8.1	8.0	9.3	9.1	13.1	11.4
	Con	PWT (grams)	67.3	75.9	67.3	75.9	75.9	68.0	75.9
		SEM	5.7	0.0	5.7	0.0	0.0	7.8	0.0
Movement-evoked		Number	14.4	10.4	9.6	11.7	11.0	14.7	17.2
		SEM	0.5	0.7	0.9	0.8	0.9	0.5	0.8
Thermal sensitivity to 40°C	Number		3.1	0.8	0.5	1.7	1.0	1.3	2.3
		SEM	0.4	0.3	0.3	0.2	0.3	0.6	0.6
	Latency (seconds)		46.3	95.1	117.8	72.9	107.6	100.3	34.5
		SEM	11.7	16.4	14.3	9.5	15.2	24.0	11.6
	Duration (seconds)		0.0	0.0	0.0	4.4	1.8	0.0	0.0
		SEM	0.0	0.0	0.0	2.9	1.8	0.0	0.0

Table 4.7 The effects of duloxetine on CIBP-induced behavioural sensitisation showing paw withdrawal threshold (PWT), number of avoidances of weight bearing on movement, number of paw withdrawals, latency to paw withdrawal and duration of paw elevation. Data show mean responses ± SEM (n=11).

4.4.9 The effect of S,S-reboxetine on CIBP-induced mechanical allodynia, movement-evoked pain and thermal sensitivity to 40°C

A selective noradrenaline reuptake inhibitor, S,S-reboxetine (10mg/kg; po administration) was assessed for reversal of CIBP-induced behavioural sensitisation. S,S-reboxetine (given at Day 17-19 post CIBP induction) attenuated thermal sensitivity to 40°C, where the number of paw withdrawals at 4 hours post-administration were significantly decreased when compared to pre-administration values, shown by One-way ANOVA followed by Bonferroni's post-hoc analysis, $p<0.05$. The latency to withdrawal and duration of paw elevation were unaltered compared to pre-administration values. S,S-reboxetine did not attenuate CIBP-induced mechanical allodynia, where the pre-administration significant reduction of ipsilateral PWT remained unaltered post-administration, shown by a One-way repeated measures ANOVA on ranks (Friedman's test) followed by Dunn's post-hoc analysis, $p<0.05$. Additionally S,S-reboxetine did not attenuate CIBP-induced movement-evoked pain (Figure 4.9).

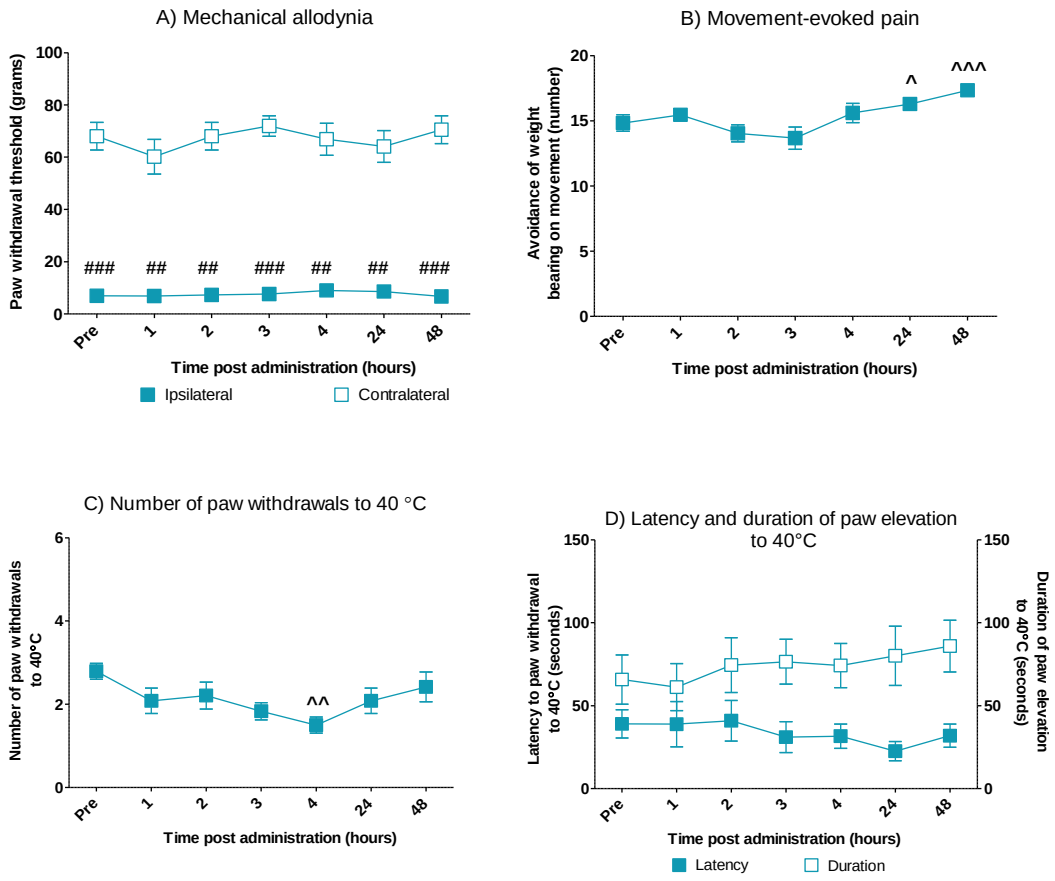


Figure 4.9 Effect of S,S-reboxetine (10mg/kg; p.o. administration) on CIBP-induced behavioural sensitisation. Data show mean responses ± SEM (n= 12). A) Ipsilateral PWT was significantly decreased at all time points when compared to contralateral values (#; One-way repeated measures ANOVA on ranks followed by Dunn's post-hoc analysis, $p<0.05$). Ipsilateral PWT was unaltered by S,S-reboxetine when compared to pre-administration values. B) Avoidance of weight bearing on movement was significantly increased from 24 hours onwards when compared to pre-administration responses (^; One-way repeated measures ANOVA followed by Bonferroni's post-hoc analysis, $p<0.05$). C) The number of paw withdrawals to 40°C was significantly decreased when compared to pre-administration values (^; One-way ANOVA followed by Bonferroni's post-hoc analysis, $p<0.05$). D) Latency to paw withdrawal and duration of paw elevation at 40°C were unaltered compared to pre-administration values. P values ^^^ = <0.001, ^^ = 0.001 to 0.01, ^ = 0.01 to 0.05, ### = <0.001 and ## = 0.001 to 0.01.

			Time post-administration (hours)						
			Pre	1	2	3	4	24	48
Mechanical allodynia	Ipsi	PWT (grams)	6.9	6.8	7.3	7.6	9.0	8.6	6.7
		SEM	1.0	0.9	1.1	1.1	2.1	2.0	0.9
	Con	PWT (grams)	68.0	60.2	68.0	71.9	66.9	64.1	70.5
		SEM	5.3	6.7	5.3	3.9	6.1	6.1	5.3
Movement-evoked		Number	14.6	15.6	14.0	14.4	15.6	16.6	17.4
		SEM	0.7	0.6	0.8	0.8	0.7	0.5	0.4
Thermal sensitivity to 40°C	Number		2.8	2.1	2.2	1.8	1.5	2.1	2.4
		SEM	0.2	0.3	0.3	0.2	0.2	0.3	0.4
	Latency (seconds)		39.0	38.8	40.9	31.0	31.6	22.5	31.9
		SEM	8.6	13.7	12.2	9.3	7.3	5.8	7.0
	Duration (seconds)		65.8	61.2	74.5	76.5	74.2	80.1	85.9
		SEM	14.8	14.2	16.5	13.6	13.4	17.9	15.6

Table 4.8 The effects of S,S-reboxetine on CIBP-induced behavioural sensitisation showing paw withdrawal threshold (PWT), number of avoidances of weight bearing on movement, number of paw withdrawals, latency to paw withdrawal and duration of paw elevation. Data show mean responses ± SEM (n= 12).

4.4.10 The effect of CB 65 on CIBP-induced movement-evoked pain, thermal sensitivity to 40°C and mechanical allodynia

A selective CB_2 receptor agonist, CB 65 (1 mg/kg; i.p. administration), was assessed for reversal of CIBP-induced behavioural sensitisation. CB 65 (given at Day 20 after CIBP induction) did not attenuate CIBP-induced mechanical allodynia where ipsilateral PWT was unaltered post-administration of pharmacological agent

and the pre-administration significant reduction in ipsilateral paw compared to contralateral PWT remained unaltered post-administration, except at 2 to 3 hours, shown by One-way repeated measures ANOVA on ranks (Friedman's test) followed by Dunn's post-hoc analysis, $p<0.05$. CB 65 attenuated CIBP-induced avoidance of weight bearing on movement when compared to pre-administration values at 2 hours post-administration and CB 65 also attenuated the number of paw withdrawals to 40°C at 3 hours post-administration, shown by a One-way ANOVA followed by Bonferroni's post-hoc analysis, $p<0.05$ (Figure 4.10).

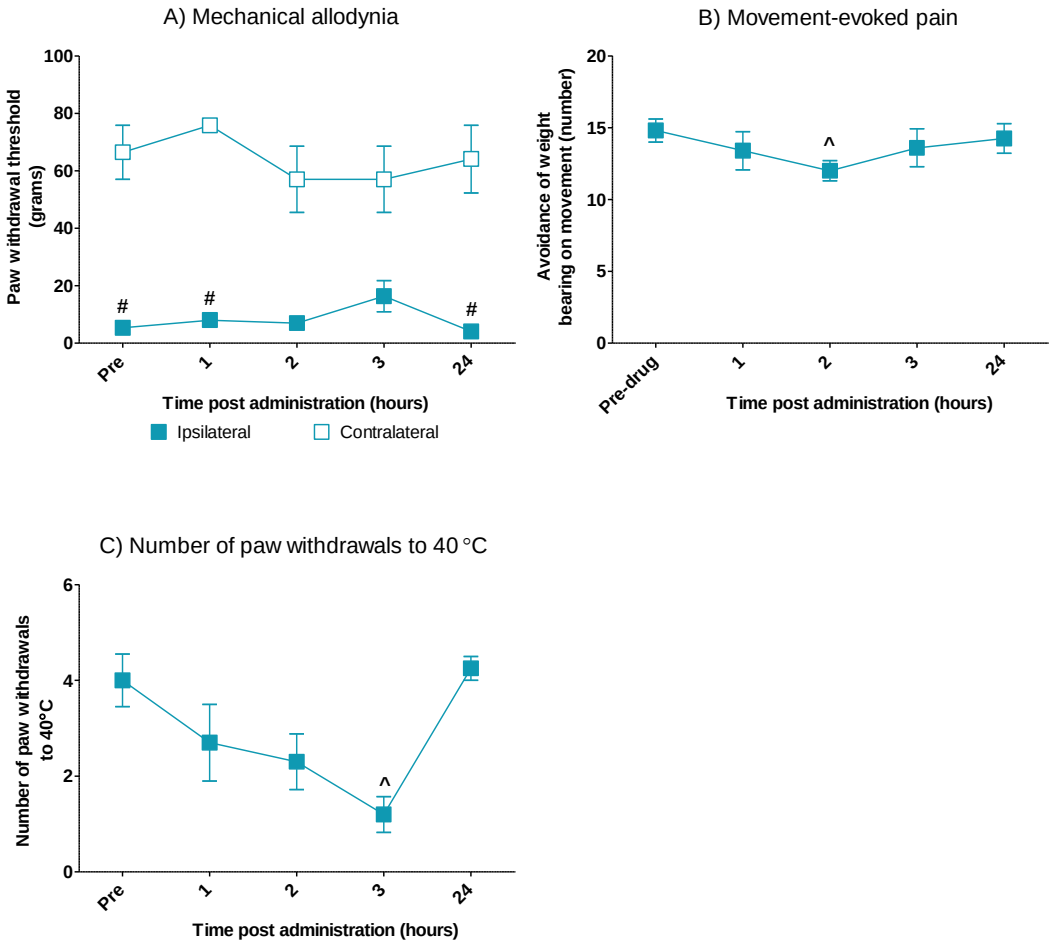


Figure 4.10 Effect of CB 65 (1mg/kg; i.p. administration) on CIBP-induced behavioural sensitisation. Data show mean responses ± SEM (n= 5). A) CIBP resulted in a significant reduction in ipsilateral PWT when compared to contralateral values (#; One-way repeated measures ANOVA on ranks followed by Dunn's post-hoc analysis, $p<0.05$). B) Avoidance of weight bearing on movement was

significantly decreased 2 hours post CB 65 when compared to pre-administration values (^; One-way repeated measures ANOVA followed by Bonferroni's post-hoc analysis, $p<0.05$). C) Number of paw withdrawals to 40°C was significantly decreased 3 hours post-administration when compared to pre-administration values (^; One-way repeated measures ANOVA followed by Bonferroni's post-hoc analysis, $p<0.05$). P values ^ = 0.01 to 0.05 and # = 0.01 to 0.05.

			Time post-administration (hours)				
			Pre	1	2	3	24
Mechanical allodynia	Ipsi	PWT (grams)	5.4	8.0	7.0	16.4	4.1
		SEM	0.9	1.9	1.4	5.4	0.5
	Con	PWT (grams)	66.5	75.9	57.1	57.1	64.1
		SEM	9.4	0.0	11.5	11.5	11.8
Movement-evoked		Number	14.8	13.4	12.0	13.6	14.3
		SEM	0.8	1.3	0.7	1.3	1.0
Thermal sensitivity to 40°C	Number		4.0	2.7	2.3	1.2	4.3
		SEM	0.5	0.8	0.6	0.4	0.3

Table 4.9 The effects of CB 65 on CIBP-induced behavioural sensitisation showing paw withdrawal threshold (PWT), number of avoidances of weight bearing on movement and number of paw withdrawals. Data show mean responses ± SEM (n= 5).

4.4.11 The effect of vehicle control for gabapentin, duloxetine and S,S-reboxetine on CIBP-induced movement-evoked pain, thermal sensitivity to 40°C and mechanical allodynia

CIBP-induced mechanical allodynia, movement-evoked pain and thermal sensitivity to 40°C were not affected by vehicle control for gabapentin, duloxetine and S,S-reboxetine. Vehicle (given at Day 14-18 post CIBP induction) did not alter

CIBP-induced mechanical allodynia when compared to pre-administration values and ipsilateral PWT remained significantly decreased compared to contralateral PWT, shown by a repeated measures ANOVA on ranks (Friedman's test) followed by Dunn's post hoc analysis, $p<0.05$. In addition, vehicle did not alter CIBP-induced movement-evoked pain or thermal sensitivity to 40°C compared to pre-administration values, shown by a repeated measures One-way ANOVA followed by Bonferroni's post-hoc analysis, $p<0.05$ (Figure 4.11).

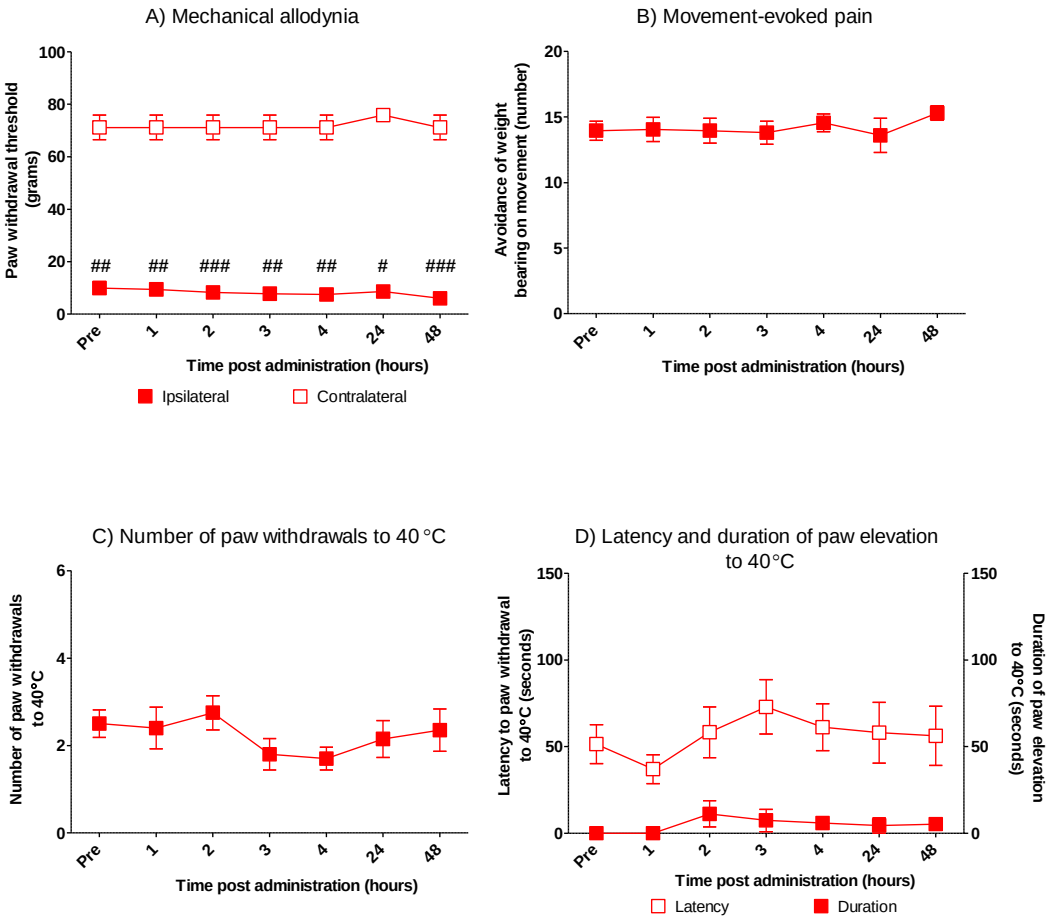


Figure 4.11 Effect of vehicle (p.o. administration) on CIBP-induced behavioural sensitisation. Data show mean responses ± SEM (n=10). A) CIBP resulted in a significant reduction in ipsilateral PWT when compared to contralateral PWT (#; One-way repeated measures ANOVA on ranks followed by Dunn's post-hoc analysis, $p<0.05$), which remained unaltered by vehicle administration. B) Avoidance of weight bearing on movement did not alter post vehicle. C) The number of paw

withdrawals to 40°C did not alter post vehicle. D) Both the latency and duration of paw elevation did not alter post vehicle administration. P values ### = <0.001, ## = 0.001 to 0.01 and # = 0.01 to 0.05.

			Time post-administration (hours)						
			Pre	1	2	3	4	24	48
Mechanical allodynia	Ipsi	PWT (grams)	9.9	9.5	8.3	7.8	7.5	8.7	6.0
		SEM	2.5	2.5	2.4	1.0	1.3	1.3	0.9
	Con	PWT (grams)	71.2	71.2	71.2	71.2	71.2	75.9	71.2
		SEM	4.7	4.7	4.7	4.7	4.7	0.0	4.7
Movement-evoked		Number	14.0	14.1	14.0	13.8	14.6	13.6	15.3
		SEM	0.7	0.9	1.0	0.9	0.7	1.3	0.6
Thermal sensitivity to 40°C	Number		2.5	2.4	2.8	1.8	1.7	2.2	2.4
		SEM	0.3	0.5	0.4	0.4	0.3	0.4	0.5
	Latency (seconds)		51.4	37.0	58.3	72.9	61.2	58.0	56.2
		SEM	11.2	8.3	14.6	15.7	13.5	17.6	17.0
	Duration (seconds)		0.0	0.0	11.2	7.4	5.9	4.5	5.3
		SEM	0.0	0.0	7.5	6.4	2.7	4.5	2.8

Table 4.10 The effects of vehicle on CIBP-induced behavioural sensitisation showing paw withdrawal threshold (PWT), number of avoidances of weight bearing on movement, number of paw withdrawals, latency to paw withdrawal and duration of paw elevation. Data show mean responses ± SEM (n=10).

4.4.12 Effect of gabapentin and duloxetine on number of rearings on the elevated plausmaze

Gabapentin and duloxetine had no effect on voluntary locomotor activity, as measured by number of rearings on the elevated plusmaze (Figure 4.12). The effect

of these pharmacological agents on other measures of voluntary locomotor activity, such as distance travelled or speed in the open field were not assessed in this thesis.

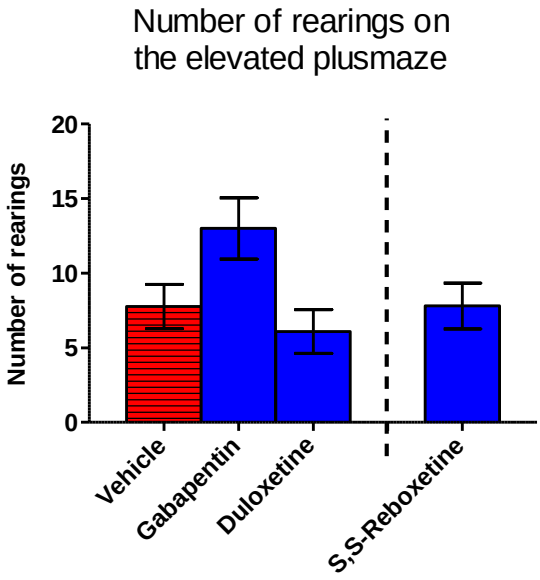


Figure 4.12 Effect of gabapentin, duloxetine and S,S-reboxetine on voluntary locomotor activity. Data show mean responses ± SEM on the elevated plus maze of CIBP + Vehicle (n=9), CIBP + Gabapentin (n=10), CIBP + Duloxetine (n=11) and CIBP + S,S-reboxetine (n=10) animals. Experimental series run at the same time are grouped by a dashed line and statistical analysis was carried out within these groups. S,S-reboxetine was run at a separate time and therefore was not compared to CIBP + Vehicle. Gabapentin and duloxetine did not alter the number of rearings in comparison to CIBP + Vehicle.

4.4.13 Both duloxetine and S,S-reboxetine had no effect on performance in the rotarod test of sedation/ataxia

Both duloxetine (30mg/kg) and S,S-reboxetine (10mg/kg) were tested for potential sedative effects using the rotarod test of sedation/ataxia. This involved an automated accelerating rotarod set to accelerate to 17 rpm in 5 seconds and maintain that speed for 40 seconds (Iyengar et al., 2004). Naïve animals were allowed three training trials on the rotarod 24 hours prior to pharmacological agent testing. Duloxetine (n=5) and S,S-reboxetine (n=5) were administered to naive animals (whose mean scores were 33.6 ± 3.9 and 30.3 ± 4.2 seconds ± SEM pre-duloxetine or

S,S-reboxetine respectively). Both pharmacological agents had no effect on performance in the rotarod test of sedation/ataxia, with no significant reductions in time scored for example at 1 hour post-duloxetine (32.6 ± 4.9 seconds) or post-S,S-reboxetine (27.7 ± 3.8 seconds).

4.4.14 Effect of XRT and pharmacological agent therapy on pain-related anxiety and risk assessment behaviour

Pain-related anxiety (as measured by the time spent on open arms of the elevated plusmaze) was not altered by XRT or pharmacological agent therapy. The number of groomings on the elevated plusmaze was significantly increased by XRT when compared to CIBP (2.33 ± 0.58 versus 4.43 ± 0.45), shown by unpaired two-tailed t-test, $p<0.05$. The number of groomings was unaltered by pharmacological agent therapy. Risk assessment behaviour, as measured by number of protected stretch attends, was significantly decreased by XRT when compared to CIBP (10.89 ± 2.25 versus 4.86 ± 1.03), shown by unpaired two-tailed t-test, $p<0.05$. The number of protected stretch attends was not attenuated by pharmacological agent therapy. The time spent in the centre zone of the open field was not altered by XRT (Figure 4.13).

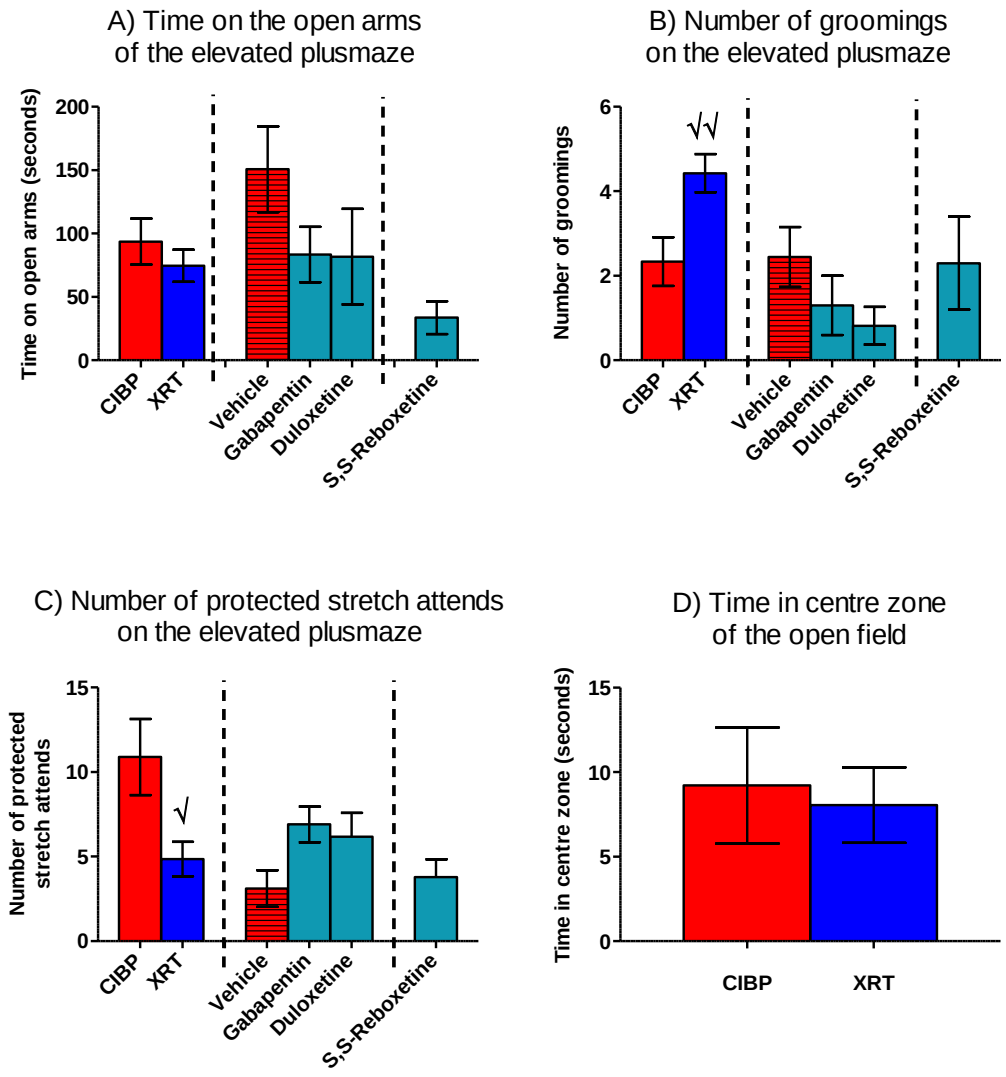


Figure 4.13 Effect of XRT treatment, gabapentin, duloxetine and S,S-reboxetine on affective components of CIBP. Data show mean responses ± SEM on the elevated plus maze in CIBP (n=9), XRT (n=14), CIBP + Vehicle (n=9), CIBP + Gabapentin (n=10), CIBP + Duloxetine (n=11) and CIBP + S,S-reboxetine (n=10) animals and in the open field CIBP (n=7) and CIBP + XRT (n=8) animals. Experimental series run during the same test session are grouped by a dashed line and statistical analysis was carried out within these groups. S,S-reboxetine was run at a separate time and was therefore not compared to CIBP + Vehicle. A) XRT did not alter the time spent on the open arm in comparison to CIBP. Gabapentin and duloxetine did not alter the time spent on the open arm in comparison to CIBP + Vehicle. B) XRT significantly

increased the number of groomings in comparison to CIBP (√√ unpaired two-tailed t-test, p = 0.001 to 0.01). Gabapentin and duloxetine did not alter the number of groomings in comparison to CIBP + Vehicle. C) XRT significantly reduced the number of protected stretch attends in comparison to CIBP (√ unpaired two-tailed t-test, p = 0.01 to 0.05. Gabapentin and duloxetine did not alter the number of rearings in comparison to CIBP + Vehicle. D) XRT did not alter the time spent in the centre zone in the open field.

The effects of acute gabapentin, duloxetine, S,S-reboxetine and CB65 administration on CIBP-induced behavioural sensitisation are summarised in the table below (Table 4.11). Gabapentin did not attenuate CIBP-induced behavioural sensitisation. Duloxetine reversed mechanical allodynia, at 1-2 hours post-administration, reversed thermal sensitivity to 40°C at 1-24 hours post-administration, as measured by number of paw withdrawals, and reversed movement-evoked pain at 1-4 hours post-administration. S,S-reboxetine reversed thermal sensitivity to 40°C, as measured by number of paw withdrawals, at 4 hours only. CB 65 reversed thermal sensitivity to 40°C, as measured by number of paw withdrawals, at 3 hours only, and reversed movement-evoked pain, at 2 hours only. In addition to these behavioural results, gabapentin and duloxetine were found to have no effect on voluntary locomotor or anxiety-related behaviours on the elevated plusmaze.

		Thermal sensitivity to 40°C			
	Mechanical allodynia	Number of paw withdrawals	Latency to paw withdrawal	Duration of paw elevation	Movement-evoked
Gabapentin	X	X	X	X	X
Duloxetine	Reversed at 1-2 hours post-duloxetine	Reversed at 1-24 hours post-duloxetine	Increased at 1-2 and 4-24 hours post-duloxetine	X	Reversed at 1-4 hours post-duloxetine
S,S-reboxetine	X	Reversed at 4 hours post-S,S-reboxetine	X	X	X
CB 65	X	Reversed at 3 hours post-CB 65	~	~	Reversed at 2 hours post-CB 65

Table 4.11 Summary table of the effects of pharmacological agents on CIBP-induced behavioural sensitisation where X = no effect and ~ = not recorded.

4.5 Discussion

4.5.1 Focal radiotherapy carried out at Day 7 after CIBP induction attenuated thermal sensitivity to 20°C and 40°C and movement-evoked pain

These results show that XRT (8 Gy), carried out at Day 7 after CIBP induction, significantly attenuated thermal sensitivity to 20°C and 40°C and also reversed movement-evoked pain on the rotarod (set at a constant speed of 5-6 rpm) at Day 18-21 only. A recent review of clinical guidelines showed pain relief equivalency for the dosing schemes; 30 Gy in 10 fractions, 24 Gy in 6 fractions, 20 Gy in 5 fractions and a single 8 Gy fraction for patients. However, the single dose fraction approach optimises patient and caregiver convenience, and is commonly used in clinical practice (Lutz et al., 2011). One of the most interesting results is that XRT treatment prevented the development of the CIBP-induced increase in duration

of paw elevation, as shown by a significant decrease in the duration of paw elevation at both 20°C and 40°C. XRT did not attenuate CIBP-induced mechanical allodynia or static weight bearing difference between hindlimbs. XRT did not alter the distance travelled or average speed in the open field or number of rearings on the elevated plusmaze, used as measures of voluntary locomotor activity. A previous study found that a single dose (20 Gy) of focal radiotherapy resulted in increased mobility in a mouse model of CIBP with improved spontaneous limb use scores and improved performance on the rotarod noted (Goblirsch et al., 2004b). In that study mice received XRT at Day 7 after inoculation and the rotarod was set at a constant speed of 6 rpm, with the animals also being scored for guarding behaviour on the rotarod. At Day 13 and day 15 (6 and 8 days after XRT) mice with tumour had behavioural scores indistinguishable from mice that received Sham inoculations (Goblirsch et al., 2004b). This decrease in pain behaviour was shown to be due to a decrease in tumour mass and osteoclast activity (Goblirsch et al., 2004b). Another study showed that irradiated CIBP mice showed increased performance on the rotarod and in the forced grip test (Vit et al., 2006). This study administered a single dose of 6 Gy XRT at a later time point (Day 10 after inoculation) than our study (Day 7 after inoculation). The rotarod was set to accelerate from 3.75 rpm with progressive acceleration and the time to fall off was recorded, the performance of mice on the rotarod improved at Day 13 onwards. At Day 17 after inoculation of tumour cells, non-irradiated mice fell after 67+/-16s and irradiated mice fell at 223+/-22s (Vit et al., 2006). The palliative effect of low dose irradiation was not seen to be associated with changes in tumour size or osteolysis in this study (Vit et al., 2006). Results of the present study are fully in agreement, in that XRT successfully reduced movement-induced pain and expand on such findings by showing improvements in thermal sensitivity. In the present study, it is possible that XRT reduced tumour burden, osteoclast activity or pro-inflammatory cytokines and future work could investigate this in the MRMT-1 rat model.

We looked at the efficacy of XRT treatment in attenuating affective components, such as anxiety, a co-morbidity of chronic pain observed in CIBP patients. XRT did not alter time spent in the centre zone of the open field or time

spent on the open arm of the elevated plusmaze (measures of anxiety). However, XRT did significantly increase number of groomings on the elevated plusmaze when compared to CIBP. XRT also significantly decreased risk assessment behaviour, as measured by number of protected stretch attends, on the elevated plusmaze when compared to CIBP. This might indicate that XRT animals show decreases in anxiety as measured by these subtle behaviours. However, as detailed in Chapter 3 (section 3.4.12), these behaviours were not significantly altered in CIBP when compared to Sham V or Naïve groups.

XRT appeared to cause a significant increase in body weight at Day 9-11 when compared to CIBP animals. XRT-treated animals had to be transported to receive radiotherapy (treatment was administered under anaesthesia) therefore this weight increase may have been due to the change in routine. As shown in Chapter 3 (Section 3.4.1), the body weight of CIBP animals was not significantly different from Naïve and Sham animals therefore this increase in body weight is not likely to be due to analgesia from XRT treatment.

4.5.2 Acute gabapentin administered at Day 18-21 did not attenuate CIBP-induced mechanical allodynia, movement-evoked pain or thermal sensitivity to 40°C

Gabapentin is thought to act through voltage-dependent calcium channels expressed in sensory neurons to reduce glutamate release but the precise mechanism of action of gabapentin remains elusive. Gabapentin binds to α2δ-1 and α2δ-2 subunits of voltage-dependent calcium channels. The α2δ protein may be involved in the trafficking of calcium channel complexes to the membrane and gabapentin may act by blocking this trafficking (Bauer et al., 2010). Gabapentin may reduce the stimulated release of transmitters by inhibiting the function of Ca^{2+} channels, however gabapentin does not appear to inhibit the function of recombinant Ca^{2+} channels, although it is possible interacting proteins found at synapses are necessary for this inhibition (Taylor, 2009). Gabapentin does however, cause a slow redistribution of Ca^{2+} channels from plasma membrane to intracellular sites, which would inhibit function in nociceptive afferents (Heblich *et al.*, 2008;Tran-Van-Minh

& Dolphin, 2010). Results of another study suggested that gabapentin produces analgesia through its activation of descending noradrenergic systems and release of noradrenaline in the spinal cord (Hayashida et al., 2007). In addition, a recent study found that thrombospondin (an astrocyte-secreted protein) binds to α2δ-1 to promote synapse formation and that this was inhibited by gabapentin *in vitro* and *in vivo* (Eroglu et al., 2009). The authors proposed that the analgesic action of gabapentin maybe through inhibition of new synapse formation (Eroglu et al., 2009).

A single dose of gabapentin (30mg/kg) given at Day 18-21 after CIBP induction did not attenuate mechanical allodynia, movement-evoked pain or thermal sensitivity to 40°C. Gabapentin did not alter the number of rearings on the elevated plusmaze. A previous study has shown that chronic administration of gabapentin (30mg/kg; given subcutaneously twice daily) attenuated mechanical and cold allodynia, as assessed by the acetone test, in CIBP (Donovan-Rodriguez et al., 2005). However a single systemic dose had no effect on behaviour and chronic gabapentin took 2 days to attenuate behaviours (Donovan-Rodriguez et al., 2005). Therefore our results are consistent with the finding that a single dose of gabapentin did not attenuate CIBP-induced pain behaviours. In addition, gabapentin did not alter any anxiety-like or risk assessment behaviours measured on the elevated plusmaze.

4.5.3 Acute duloxetine given at Day 16-21 after CIBP induction attenuated mechanical allodynia, movement-evoked pain and thermal sensitivity to 40°C, whereas only attenuation of thermal sensitivity was observed following acute S,S-reboxetine administration

Serotonin (5-HT) and noradrenaline are neurotransmitters involved in descending facilitation and inhibition from supraspinal areas onto the dorsal horn of the spinal cord (Millan, 2002). Brainstem and midbrain areas project serotonergic and noradrenergic neurons onto dorsal horn neurons, where noradrenaline and serotonin released from these neurons acts on α2-adrenoreceptors and a variety of 5-HT receptors, respectively, to modulate dorsal horn responses to pain (Bannister et al., 2009). The excitatory influence of the serotonergic system has been shown to be enhanced in a number of models of chronic pain including in a model of CIBP

(Donovan-Rodriguez et al., 2006). Spinally administered ondansetron, a selective 5-HT_3 receptor antagonist, significantly reduced mechanical- and thermal- evoked responses in a MRMT-1 CIBP model (Donovan-Rodriguez et al., 2006). Recently a study in mouse models of inflammatory and neuropathic pain using molecular depletion of a key enzyme in the 5-HT synthesis pathway showed that descending 5-HT from the RVM plays a critical role in the descending facilitation of pain during persistent pain states (Wei et al., 2010).

Duloxetine is a potent and balanced serotonin-noradrenaline reuptake inhibitor and duloxetine is also reported to have some activity at voltage-dependent sodium channels and may block Nav1.7 persistent late neuronal Na^+ currents, which may contribute to its analgesic efficacy (Wang et al., 2010). A single dose of duloxetine (30mg/kg) given at Day 16-21 after CIBP induction attenuated mechanical allodynia, movement-evoked pain and thermal sensitivity to 40°C with significantly decreased number of paw withdrawals and increased latency to paw withdrawal to 40°C. A possible mechanism of duloxetine might be via an enhancement of serotonergic and noradrenergic descending inhibition from the midbrain to the dorsal horn of the spinal cord which thereby decreases transmission of nociceptive information in the dorsal horn and potentially supraspinally as well (Jones et al., 2005). To attempt to elucidate the mechanism of duloxetine analgesia the noradrenaline reuptake inhibitor S,S-reboxetine was tested. A single dose of S,S-reboxetine (10mg/kg) given at Day 17-19 after CIBP induction did not attenuate CIBP-induced mechanical allodynia or movement-evoked pain but did attenuate thermal sensitivity to 40°C. This may suggest that duloxetine attenuates mechanical allodynia and movement-evoked pain by inhibition of serotonin reuptake alone or by inhibition of both noradrenaline and serotonin reuptake. Future studies could test this further and the analgesic efficacy of a selective serotonin reuptake inhibitor, such as fluoxetine, in this MRMT-1 CIBP model could be investigated. In addition, it would be interesting to investigate which serotonin (5-HT) receptors are involved in this observed attenuation of CIBP-induced behavioural sensitisation. By targeting the specific 5-HT receptors involved, it may be possible to improve the efficacy of modulating or mimicking the influence of the descending serotonin pathways. It

should be borne in mind that additional pharmacological actions of any of these compounds may contribute to their analgesic profile.

As mentioned previously, a study by El Mouedden and Meert reported that acute administration of the noradrenaline reuptake inhibitor, desipramine, and the noradrenaline and serotonin reuptake inhibitor, amitriptyline, reduced spontaneous pain behaviour in a preclinical model of CIBP but only at doses that also caused sedation. However, the selective serotonin reuptake inhibitor, fluoxetine, did not attenuate spontaneous pain behaviour. The authors found that these compounds did not affect mechanical hypersensitivity or limb-use impairment (El Mouedden & Meert, 2007a). Whiteside *et al.* demonstrated that the noradrenaline reuptake inhibitor WAY-318068 modestly reversed mechanical allodynia in a CIBP model (Whiteside et al., 2010). These studies suggest that increased noradrenaline, without the effect of serotonin, is sufficient for attenuating some pain-related behaviours in a CIBP model. The effects are not as prominent as with dual serotonin and noradrenaline reuptake inhibitors though, suggesting that there could be some synergy between the noradrenaline and serotonin mechanisms that attenuate pain sensitivity when both are activated simultaneously. A review of preclinical and clinical studies suggests that dual acting serotonin and noradrenaline enhancing drugs produce the greatest analgesic effects in persistent pain states, with smaller effects achieved from enhancing noradrenaline alone (Marks et al., 2009).

4.5.4 CB 65 given at Day 20 after CIBP induction attenuated movement-evoked pain and thermal sensitivity to 40°C but did not attenuate mechanical allodynia

CB 65 is a selective, high affinity CB_2 receptor agonist (Manera et al., 2006). In the present study, a single dose of CB 65 (1mg/kg; i.p.) given at Day 20 after CIBP induction did not attenuate CIBP-induced mechanical allodynia but did attenuate CIBP-induced movement-evoked pain and thermal sensitivity to 40°C. Previous studies have shown that CIBP-induced thermal hyperalgesia and mechanical allodynia are differently affected by the activation of peripheral CB_2 receptors. Curto-Reyes *et al.* found that reversal of thermal hyperalgesia involved both peripheral and spinal CB_2 receptors whereas reversal of mechanical allodynia

relied on activation of spinal CB_2 receptors (Curto-Reyes et al., 2010). These different results may be related to the underlying mechanisms of thermal sensitivity and mechanical allodynia, although it also appears to depend on the pain state, as discussed by Curto-Reyes *et al.* (Curto-Reyes et al., 2010).

Recently, a CB_2 receptor agonist has been shown to provide analgesia and prevent bone loss in a murine model of CIBP (Lozano-Ondoua et al., 2010). Acute administration or 7 days systemic administration of AM1241 (CB_2 receptor agonist) significantly attenuated spontaneous pain, assessed by counting of flinching and guarding behaviour over 2 minutes, and movement-evoked pain, scored during normal ambulation (Lozano-Ondoua et al., 2010). Furthermore, sustained AM1241 significantly reduced bone loss and decreased fractures (Lozano-Ondoua et al., 2010). So, while an acute dose effectively attenuated pain-related behaviours, sustained administration was necessary to reduce bone destruction (Lozano-Ondoua et al., 2010). Another recent study also showed that systemic administration of a non-selective cannabinoid receptor agonist, a CB_1 receptor-selective agonist and a CB_2 receptor-selective agonist significantly attenuated cancer-induced mechanical allodynia in a model of oral cancer pain (Saghafi et al., 2011). Additionally, tumour-growth was significantly attenuated with systemic administration of the CB_2 receptor-selective agonist (AM1241) (Saghafi et al., 2011). These findings suggest activation of cannabinoid receptors in CIBP can have additional benefits, contributing to analgesia via reduction in bone loss/destruction or through direct effects on tumour growth. A study by Cui *et al.* showed that intrathecal administration of WIN 51,212-1, a general cannabinoid receptor agonist, reversed mechanical allodynia in a dose-dependent manner in a MRMT-1 model of CIBP (Cui et al., 2010). This antinociceptive effect was reversed by intrathecal administration of both CB_1 and CB_2 receptor antagonists, suggesting that the antinociceptive effect is mediated through both CB_1 and CB_2 receptors at the spinal level (Cui et al., 2010). Another study showed that activation of spinal CB_1 receptors by intrathecal administration of the CB_1 receptor agonist arachidonyl-2-chloroethylamide reduced spontaneous and movement-evoked pain in a mouse model of CIBP (Furuse et al., 2009). A decrease in endocannabinoid AEA, a partial agonist of CB1 and CB2

receptors, is reported to contribute to the maintenance of CIBP (Khasabova et al., 2008). These results suggest that both CB_1 and CB_2 receptors play a role in CIBP.

Cannabinoids do not only act through the classical CB_1 and CB_2 receptors. The G protein-coupled receptor GPR55 is proposed be a receptor for some cannabinoids (Ryberg et al., 2007). Although several cannabinoid-type compounds modulate GPR55, pharmacological tests of GPR55 activation by different compounds produce inconsistent results (Ross, 2009). A recent study demonstrated that cannabinoids can activate TRPA1, TRPV1, TRPV2 and antagonise TRPM8 receptors (De Petrocellis et al., 2010). Furthermore, cannabinoids have been shown to provide anti-nociception in a rat model of osteoarthritis (Schuelert & McDougall, 2008). This anti-nociception in joints may involve actions on the TRPV1 ion channel (Baker & McDougall, 2004;McDougall *et al.*, 2008;Schuelert & McDougall, 2008). Activation of CB_2 receptors coexpressed with TRPV1 in DRG neurons inhibits responses mediated by TRPV1 (Anand et al., 2008).

Other treatment options for CIBP patients include; radioisotopes with an affinity for bone, nerve blocks and surgical intervention, where surgery is mostly utilised if there is a risk of pathological fracture. Emerging treatments include osteoprotegerin, which is a decoy receptor for the receptor activator of nuclear factor kappa B ligand (RANKL). By binding RANKL, osteoprotegerin can reduce the differentiation of osteoclast precursors into osteoclasts and inhibit bone resorption. An early study has shown that a single dose of osteoprotegerin caused a sustained repression of bone resorption in patients with multiple myeloma or breast carcinoma-related bone metastases (Body et al., 2003). Further studies have investigated antibodies against RANKL, including the antibody densumab, and a recent clinical trial showed that densumab reduced bone resorption in CIBP patients (Body et al., 2006). A recent promising development, comes from the work of Mantyh *et al.* who showed that early sustained administration of anti-NGF, which acts on the TrkA receptor, blocks the sprouting of sensory and sympathetic nerve fibres, formation of neuromas and inhibits the development of CIBP (Mantyh et al., 2010). The anti-NGF

drug tanzemab (Pfizer) is currently undergoing clinical trials for both osteoarthritis and CIBP.

4.6 Conclusion

In this study we found that XRT significantly attenuated movement-evoked pain and thermal sensitivity to 20°C and 40°C. XRT also significantly reduced anxiety and risk assessment behaviours (grooming behaviour and number of protected stretch attend postures) compared to CIBP. It would have been interesting to evaluate the effect of XRT on spontaneous foot lifting in our model. Duloxetine attenuated CIBP-induced mechanical allodynia, thermal sensitivity to 40°C and movement-evoked pain, whereas S,S-reboxetine attenuated thermal sensitivity to 40°C but did not effect CIBP-induced mechanical allodynia or movement-evoked pain. This suggests that duloxetine may attenuate mechanical allodynia and movement-evoked pain by a mechanism involving inhibition of serotonin reuptake in conjunction with inhibition of noradrenaline reuptake. In addition, CB 65 attenuated movement-evoked pain and thermal sensitivity to 40°C. In agreement with previous studies, a single dose of gabapentin did not attenuate CIBP-induced mechanical allodynia, thermal sensitivity to 40°C or movement-evoked pain. These studies confirm that the CIBP model shows characteristics and pharmacological sensitivities consistent with known and predicted mechanisms and validate it as a useful model for assessing potential new treatments proposed for use in patients. Furthermore, these results suggest that inhibition of serotonin and noradrenaline reuptake may be a novel analgesic target for CIBP.

5. THE INVOLVEMENT OF NMDA RECEPTORS IN A PRECLINICAL MODEL OF CIBP

5.1 Introduction

NMDA receptors are cation channels permeable both to monovalent cations and the divalent cation Ca^{2+}. At resting membrane potentials, NMDA receptors are blocked by Mg^{2+} and can only be activated following both depolarisation and agonist binding. The main mechanism of NMDA receptor function is the entry of Ca^{2+} upon channel opening and the subsequent activation of many intracellular pathways by Ca^{2+}.

5.1.1 NMDA Receptor Structure

Functional NMDA receptors are heterotetramers that require a combination of NR1¸ NR2 (NR2A-D) and occasionally NR3 (NR3A-B) subunits. The NR1 and NR3 subunits contain glycine binding sites and the NR2 subunit contains the glutamate binding site. NMDA receptors are most commonly composed of two NR1 subunits and two NR2 subunits (Monyer et al., 1994). The combination of subunits determines the functional properties of NMDA receptors, including sensitisation to the Mg^{2+} block, kinetics of desensitisation and single-channel conductances (Cull-Candy & Leszkiewicz, 2004). For example, the NR2A and NR2B subunits generate high conductance channel openings whereas NR2C- or NR2D- containing receptors give rise to low conductance openings (Misra et al., 2000;Stern et al., 1992;Wyllie et al., 1996). NMDA receptor subunits have an extracellular amino terminal domain, 3 transmembrane domains¸ a re-entrant pore-forming region and a long intracellular cytoplasmic tail (Mayer, 2005). The agonist binding site of an NMDA receptor subunit is located in a region referred to as the S1-S2 domain (Stern-Bach et al., 1994). The amino terminal domain of the NR2 subunits controls agonist potency, channel deactivation time course, open probability and mean open/shut duration (Yuan et al., 2009) (Figure 5.1).

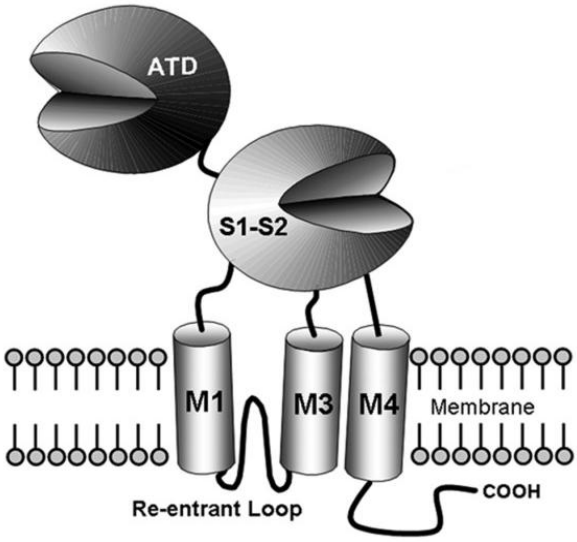


Figure 5.1 Structure of NMDA receptor subunits. Diagram shows the amino-terminal domain (ATD), the S1-S2 domain (S1-S2) and three transmembrane domains (M1, M3 and M4), the re-entrant loop and the intracellular cytoplasmic terminus (-COOH). Agonists bind at the S1-S2 domain. The intracellular C-terminal domain interacts with numerous post-synaptic molecules. Figure adapted from (Ng et al., 2008).

5.1.2 The NMDA Receptor Complex

Synaptic NMDA receptors are localised in the post-synaptic density where they are structurally organised in a large macromolecular complex composed of scaffolding proteins and adaptor proteins that link NMDA receptors to downstream signalling molecules and to other transmembrane proteins (Scannevin & Huganir, 2000). The membrane-associated guanylate kinase (MAGUK) family of proteins are important scaffolding molecules that include postsynaptic density proteins PSD-95, and PSD-93/chapsyn-110 and synapse-associated proteins SAP-97 and SAP102. MAGUK proteins consist of three PDZ domains at the N-terminus, an Src homology region 3 (SH3) domain and a guanylate kinase-like (GK) domain at the C-terminal (Kim & Sheng, 2004). Several studies have shown that NMDA receptor synaptic localisation and binding to scaffolding proteins, such as the MAGUK family, plays a major role in the central downstream signals resulting from receptor activation (Elias & Nicoll, 2007). The last three amino acids of the carboxy- termini of the NR2A and

NR2B subunits have a C-terminal consensus motif, threonine/serine-x-valine (where x is any amino acid) is responsible for efficient binding to PDZ domains of MAGUK members (Niethammer et al., 1996). Two members of the MAGUK family of proteins, PSD-93 and PSD-95, have been shown to be involved in chronic pain states. PSD-95 has been shown to be necessary for the development of neuropathic pain, as PSD-95 mutant mice do not develop pain behaviours following nerve injury but do develop pain behaviours following inflammation, as in wild-type animals (Garry et al., 2003a). PSD-93 appears to be involved in both neuropathic and inflammatory pain as knockdown of PSD-93 reduced both neuropathic and inflammatory pain behaviours (Tao et al., 2003).

5.1.3 Involvement of NMDA Receptors in Central Sensitisation

The activation of postsynaptic NMDA receptors is necessary for the development and maintenance of central sensitisation, which is observed in a number of chronic pain states (Woolf & Thompson, 1991). As detailed previously (Chapter 1, Section 1.7.2), central sensitisation represents the enhancement of nociceptive pathways and is characterised by development of increased spontaneous activity in nociceptive neurons, a reduction in the threshold for activation of nociceptive neurons and enlargement of the receptive fields of nociceptive neurons (Cook et al., 1987). In addition, there is conversion of nociceptive-specific neurons to wide-dynamic range neurons that now respond to both innocuous and noxious stimuli. Central sensitisation also involves wind-up, where neurons show increased responsiveness to further inputs after repeated stimulation. The cellular processes that lead to central sensitisation include an increase in membrane excitability, facilitated synaptic strength and decreased inhibition of dorsal horn neurons (Latremoliere & Woolf, 2009).

The induction of central sensitisation requires intense, repeated and sustained activation of nociceptors. Key mechanisms contributing to activity-dependent central sensitisation brought about by injury are as follows (Latremoliere & Woolf, 2009); tissue injury leads to strong stimulation of nociceptors causing the release of glutamate, substance P and BDNF, from central terminals in the dorsal horn.

Glutamate, substance P and BDNF act on receptors on the postsynaptic neurons to depolarise the postsynaptic neuron. This depolarisation is sufficient to release the Mg^{2+} block of NMDA receptors and agonist binding allows current to flow through the receptor. The concentration of intracellular Ca^{2+} is increased as Ca^{2+} enters through NMDA (and possibly other ionotropic glutamate receptors) and voltage-gated Ca^{2+} channels, with Ca^{2+} also released from internal stores. Elevated Ca^{2+} levels lead to the activation of various down stream signalling molecules including the serine/threonine kinases cAMP-dependent protein kinase A (PKA), protein kinase C (PKC) and CaMKII as well as the tyrosine kinase, Src. These can modulate NMDA and AMPA receptors via phosphorylation, which changes the activity of these receptors as well as their trafficking to or from the membrane, boosting synaptic efficacy. For example, following phosphorylation, NMDA receptors and GluR1-containing AMPA receptors are recruited to the synapse and GluR2-containing receptors are removed from the synapse. Signalling cascades from G-protein-coupled receptors, such as the NK1 receptor, may also activate kinases, converging onto Src-family kinases and contributing to the enhancement of NMDA receptor function (Salter & Kalia, 2004). Downstream of PKA and PKC, Extracellular Signal-Regulated Kinase (ERK) is activated. Many intracellular signalling pathways converge to activate ERK (Figure 5.2). ERK produces diverse effects including an increase in NMDA receptor function, through phosphorylation of the NR1 subunit, and recruitment of AMPA receptors to the membrane. These changes enhance current through these receptors and increases synaptic efficacy. Additionally, ERK also phosphorylates and thereby inhibits the K^+ channel Kv4.2 and therefore reduces K^+ currents, increasing membrane excitability. These are all short-lasting changes, which can be reversed by processes such as dephosphorylation and recycling of receptors. Long-lasting strengthening of the synapse can be produced through transcriptional changes mediated by activation of cyclic adenosine monophosphate (cAMP), cAMP response element-binding protein (CREB) and other transcription factors that drive expression of genes.

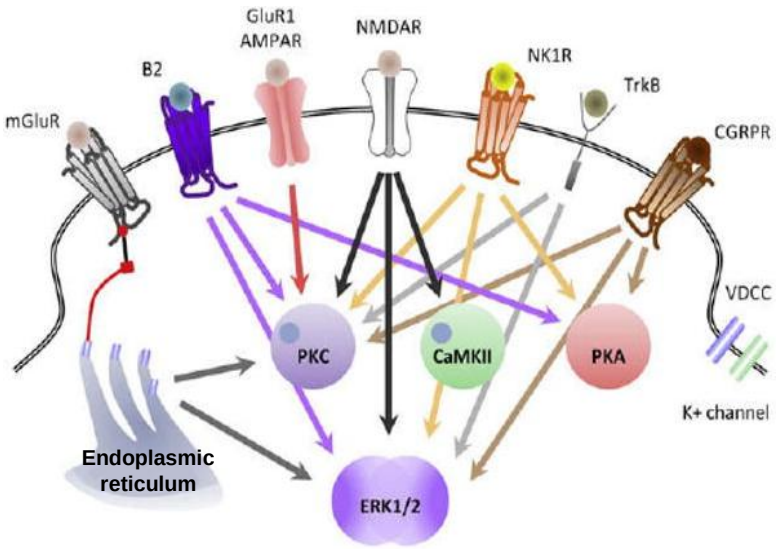


Figure 5.2 Intracellular pathways contributing to the generation of central sensitisation. NMDA receptor activation leads to the activation of PKC, CaMKII and ERK. GluR-1 containing AMPA receptors also activate PKC. Intracellular pathways resulting from the activation of NMDA receptors, mGluR, TrkB, NK1, CGRP1 or B2 receptors converge to activate ERK. Figure adapted from (Latremoliere & Woolf, 2009).

5.1.4 Glial Cells and Central Sensitisation

Activated microglia and astrocytes interact with neurons to influence central sensitisation through the production and release of neurotransmitters, cytokines, and reactive oxygen species which act on neurons. Activated glial cells, as discussed previously (Chapter1 Section 1.7.4) appear to play a role in the development of many pain states. Following nerve injury, central activation of microglia precedes astrocyte activation. In fact, microglia activation is thought to trigger astrocyte reaction (Svensson et al., 1993). There may also be extensive astrocyte and microglial activation in the spinal cord of CIBP animals (Hald et al., 2009;Medhurst et al., 2002;Schwei et al., 1999;Zhang et al., 2005a). Prominent activation of astrocytes, indicated by an increase in GFAP is observed, in the spinal cord ipsilateral to bone cancer, which is uncommon in inflammatory and neuropathic pain conditions (Schwei et al., 1999). Studies investigating the involvement of microglia in CIBP have produced more variable data. Some studies have indicated that activation of

astrocytes occurred ipsilateral to CIBP without activation of microglia, for example, bone cancer resulted in astrocyte activation without microglial reaction in a mouse model of CIBP (Hald et al., 2009). However, a study using a rat model of CIBP showed an increase in GFAP staining and also an increase in the microglial marker OX-42 ipsilateral to inoculation at Day 20 post inoculation (Zhang et al., 2005a). Another study also showed that transcription of the microglial activation markers, CD11b and CD14, was significantly elevated in the initiation phase of CIBP-induced behavioural sensitisation, and moderately increased at the maintenance phase (Lan et al., 2010). Therefore these observations suggest that microglia may be involved in the induction and maintenance of CIBP-induced behavioural sensitisation, which is different from the situation in neuropathic and inflammatory pain, where microglia are only involved in the initiating phase (Lan et al., 2010).

5.1.5 NMDA Receptors in Pain States

General NMDA receptor antagonists have been shown to reduce pain behaviours in models of neuropathic and inflammatory pain. For example, NMDA receptor antagonists, administered intrathecally, reduce spontaneous pain and thermal hyperalgesia in a CCI model of neuropathic pain (Mao et al., 1993). NR2A and NR2B are the most abundant NR2 subunits in adult rat dorsal horn (Nagy et al., 2004;Zhang et al., 2008a). NMDA receptors containing the NR2B subunit located in the spinal cord play an important role in the development of inflammatory and neuropathic pain. NR2B phosphorylation is upregulated during inflammation in a CFA model of inflammatory pain (Guo et al., 2002b;Guo et al., 2004). Expression of spinal NR2B-containing NMDA receptors has been shown to increase in the following models of neuropathic pain; spinal cord injury (Labombarda et al., 2008b), CCI (Wilson et al., 2005) and chronic compression of the DRG (CCD) (Ma et al., 2007;Zhang et al., 2009). The unacceptable side-effect profile of general NMDA receptors antagonists has made subunit-specific antagonists an attractive proposition. Intrathecal administration of the selective NR2B subunit antagonist, Conantokin G (conG), attenuated pain behaviours in formalin and CFA models of inflammatory pain (Malmberg et al., 2003b). Selective NR2B subunit antagonists inhibit mechanical allodynia without causing motor dysfunction in CCI, CCD and spinal

nerve-ligation (SNL) models of neuropathic pain (Boyce et al., 1999;Qu et al., 2009;Wilson et al., 2005;Zhang et al., 2009;Malmberg et al., 2003a). Others have found that in models of inflammatory and neuropathic pain the NR2B subunit is phosphorylated by Src resulting in increased activity of NMDA receptors. Furthermore, uncoupling of Src from the NMDA receptor can suppress pain behaviours (Liu et al., 2008). A recent study showed that perturbing the interaction of PSD-95 with the NR2B subunit of NMDA receptors reduced spinal plasticity and pain behaviours in a model of neuropathic pain (D'Mello et al., 2011).

5.1.6 Central Sensitisation in CIBP

CIBP is associated with spontaneous and evoked behavioural sensitisation partly caused by injury of the primary afferent fibres innervating the tumour-bearing bone (Peters et al., 2005). As well as peripheral sensitisation of primary afferent fibres, changes in responsiveness of dorsal horn neurons may be induced by central sensitisation. Urch *et al.* have shown that in a rat model of CIBP, spinal dorsal horn neurons show increased excitability and there is enlargement of receptive field size. Additonally, there may be an increase in the proportion of WDR neurons in the superficial dorsal horn when compared to those in control rats (Urch et al., 2003a). A recent study by Yanagisawa *et al.* found that in a mouse model of CIBP, with cancer of the femur, mice exhibit unique plastic changes in spinal excitatory synaptic transmission with functional enhancement of excitatory synaptic transmission in lamina II across lumbar levels L2-5. More specifically, they showed the amplitude of monosynaptic C-fibre-evoked excitatory postsynaptic currents (EPSCs) was significantly increased in cancer bearing mice, whereas the monosynaptic Aδ-fibre evoked EPSCs remained unchanged. These plastic changes in the dorsal horn of the spinal cord represent a unique functional alteration in the spinal synaptic transmission when compared to other chronic pain states (Yanagisawa et al., 2010) and, along with extensive glial cell activation, suggest that this distinct central sensitisation may be one of the underlying mechanisms in CIBP. For this reason, it is important to investigate the specific involvement of NMDA receptor subtypes in CIBP.

5.2 Aim

The aim of this part of the study was to investigate the involvement of NMDA receptors and particular subunits in CIBP.

5.3 Methods

5.3.1 Surgical Procedure

Experiments were carried out using male Sprague-Dawley rats. As detailed in Chapter 2; animals underwent CIBP surgery (Section 2.1.2). Rats were anaesthetised by inhalation of an isoflurane/O2 mixture (Zeneca, UK), 4-5% for induction and 2-3% for maintenance. The carrier gas was compressed oxygen at a flow rate of 2 litres/minute. Following complete induction of anaesthesia the animal was placed abdominal side up, the left hind limb was shaved and the skin was sterilised with 0.5% Hibitane (Zeneca, UK). A small incision was made in the skin over the tibia, which was then carefully exposed by removing the connective tissue over the bone using a cotton bud Johnson & Johnson, UK). A dental drill was used to bore a hole through the periosteum of the tibia. Polythene tubing (0.5mm in diameter; Smiths) was fed into the intra-medullary cavity of the tibia and 10 µl of medium (containing 6X103 cells) was injected using a 1ml micro-syringe (BD Biosciences, UK) and 25-gauge needle (BD Biosciences, UK). The tubing was withdrawn and the hole plugged with dental restorative material (IRM, Dentsply; Henry Schien Minerva), to confine the tumour cells within the marrow and prevent invading the adjacent soft tissue. The wound was closed with absorbable subcutaneous suture (5/0 coated vicryl, Ethicon, UK) and sterilised with 0.5% Hibitane. Animals were placed in a thermoregulated recovery box until they had fully regained consciousness, following which they were returned to their home cages.

5.3.2 Analgesic Intervention

The analgesic efficacy of the following drugs was investigated; a general NMDAR antagonist, (R)-CPP, an NR2B subunit selective antagonist, Ro 25-6981, and a NR2A subunit selective antagonist, AAM 077. For analysis of the effects of intrathecal administration of specific NMDAR antagonists on somatosensory behavioural reflexes; the agents were administered by intrathecal injection to CIBP

animals (Section 2.7.3). NMDA receptor antagonists were given when animals were displaying substantial CIBP-induced behavioural sensitisation, that is, sensitisation not displayed by Sham and Naive animals (Section 3.4.13). Prior to intrathecal injection of drugs, response scores for mechanical allodynia (Section 2.3.1) and movement-evoked pain (Section 2.4.1) were recorded to obtain pre-administration values. All animals were briefly anaesthetised by inhalation of an (5%) isoflurane/O_2 mixture and injected intrathecally at the L5/6 level of the spinal cord. Testing continued every 10 minutes thereafter until 40 or 50 minutes post administration. The following drugs were administered at Day 14-17 in CIBP animals (a point where substantial sensitisation of response was evident); a general NMDA receptor antagonist (R)-CPP, a NR2B-selective antagonist, Ro 25-6981, and a NR2A-selective antagonist AAM 077.

5.3.3 Immunohistochemical Analysis

Using NMDA receptor subunit-specific antibodies, the expression of NR1, NR2A and NR2B subunit proteins in the spinal cord was assessed by immunohistochemistry. The co-expression of NR2A with the neuronal cell marker (NeuN) was also investigated.

For analysis of expression of NMDA receptor subunits in whole sections using immunohistochemistry, spinal cord tissue was taken from CIBP, Sham E and Naive animals at Day 18-21 (Section 2.8.1). Animals were terminally anaesthetised and perfused before dissection of the lumbar region. Tissue was transferred through a sucrose gradient and then stored in PBS. Tissue was sectioned on a freezing microtome at 40µM and then placed in PBS. Sections were then processed by subunit-specific antigen retrieval techniques. To perform antigen-retrieval for NR1 and NR2B subunits, sections were incubated in citrate buffer (pH 6.0) at 90°C for 15 minutes. Antigen retrieval for NR2A involved incubating sections for 5 minutes with pepsin (Dako) at 37°C. The optimal antigen-retrieval technique for each antibody was determined by running a trial of different procedures. After antigen-retrieval by the optimised approach, sections were blocked and then probed for anti-NR1, anti-NR2B or anti-NR2A with NeuN and then detected by HRP-linked or fluorescent

secondary antibodies (Section 2.8.1). Sections were washed in 0.1M PBS and incubated with the amplification reagent, fluorescein-tyramide (Perkin Elmer Life Sciences, Inc.) for 25 minutes at room temperature (1:50 in 1x amplification diluent). Dilution trials were run to identify the optimal concentration of each antibody for specific staining and minimal background staining. Control sections were processed without primary antibody to determine specificity of antibodies. The fluorescent secondary antibodies were selected to avoid overlap in excitation/emission wavelengths. Images of sections were captured using a fluorescent microscope at x20 magnification and additionally, for NR2A + NeuN, at x40 magnification. Images captured at x20 were analysed using Image J software where ipsilateral-contralateral differences in fluorescence intensity were calculated for CIBP, Sham E and Naive animals. Images captured at x40 were analysed using Leica LCS Lite software and the number of cells co-expressing NR2A and NeuN were counted and compared ipsilateral to contralateral for CIBP, Sham V and Naïve (Section 2.8.2). Images were numbered to allow analysis to be performed blind.

5.3.4 Statistical Analysis

In each behavioural study, data were pooled for each time point, with group mean shown ± SEM. To analyse the effects of pharmacological agent administration on mechanical allodynia, post-pharmacological agent ipsilateral paw withdrawal thresholds were compared to pre-pharmacological agent baseline using a One-way repeated measures ANOVA on Ranks (Friedman's test) followed by Dunn's post-hoc analysis. Ipsilateral paw withdrawal thresholds were compared to contralateral paw withdrawal thresholds using a One-way repeated measures ANOVA on ranks (Friedman's test) followed by Dunn's post-hoc analysis.

To analyse the effects of pharmacological agent administration on movement-evoked pain, post-pharmacological agent number of avoidances of weight bearing on movement were compared to pre-pharmacological agent using a One-way repeated measures ANOVA followed by Dunnett's post-hoc analysis.

NR1, NR2A and NR2B expression in laminae I, II and III was compared between groups (CIBP Ipsilateral, CIBP Contralateral, Sham E Ipsilateral, Sham E Contralateral and Naive) by a repeated measures mixed-model ANOVA followed by Bonferroni's post-hoc analysis. Differences within groups (e.g. CIBP Ipsilateral versus CIBP Contralateral) were compared by an unpaired two-tailed t-test. NR2A co-expression with NeuN was compared between groups by a One-way ANOVA followed by Bonferroni's post-hoc analysis.

5.4 Results

5.4.1 The involvement of NMDA receptors in CIBP-induced mechanical allodynia

Intrathecal administration of the general NMDA receptor antagonist (R)-CPP 7.5nmole (CIBP Day 17 n=4) attenuated ipsilateral CIBP-induced mechanical allodynia compared to pre-administration values at 10 and 20 minutes post administration, where CIBP resulted in a significant reduction in ipsilateral PWT when compared to contralateral values pre-administration and at 50 minutes post-administration. Intrathecal administration of the NR2B-selective antagonist Ro 25-6981 50nmole (CIBP Day 15 n=4) did not attenuate CIBP-induced mechanical allodynia, where CIBP animals showed a significant reduction in ipsilateral PWT when compared to contralateral values at 30 minutes post-administration only. Intrathecal administration of the NR2A-selective antagonist AAM 077 50nmole (CIBP Day 14 n=4) did not attenuate mechanical allodynia at the doses selected, where CIBP showed a significant reduction in ipsilateral PWT when compared to contralateral values at 40 and 50 minutes post-administration only (Figure 5.3). All data were analysed by One-way repeated measures ANOVA on ranks (Friedman's test) followed by Dunn's post-hoc analysis, $p<0.05$. It may be that the NR2A-selective antagonist did not attenuate mechanical allodynia because there was not such robust mechanical allodynia in this group of CIBP animals pre-administration of AAM 077, shown by no significant difference between ipsilateral and contralateral PWT.

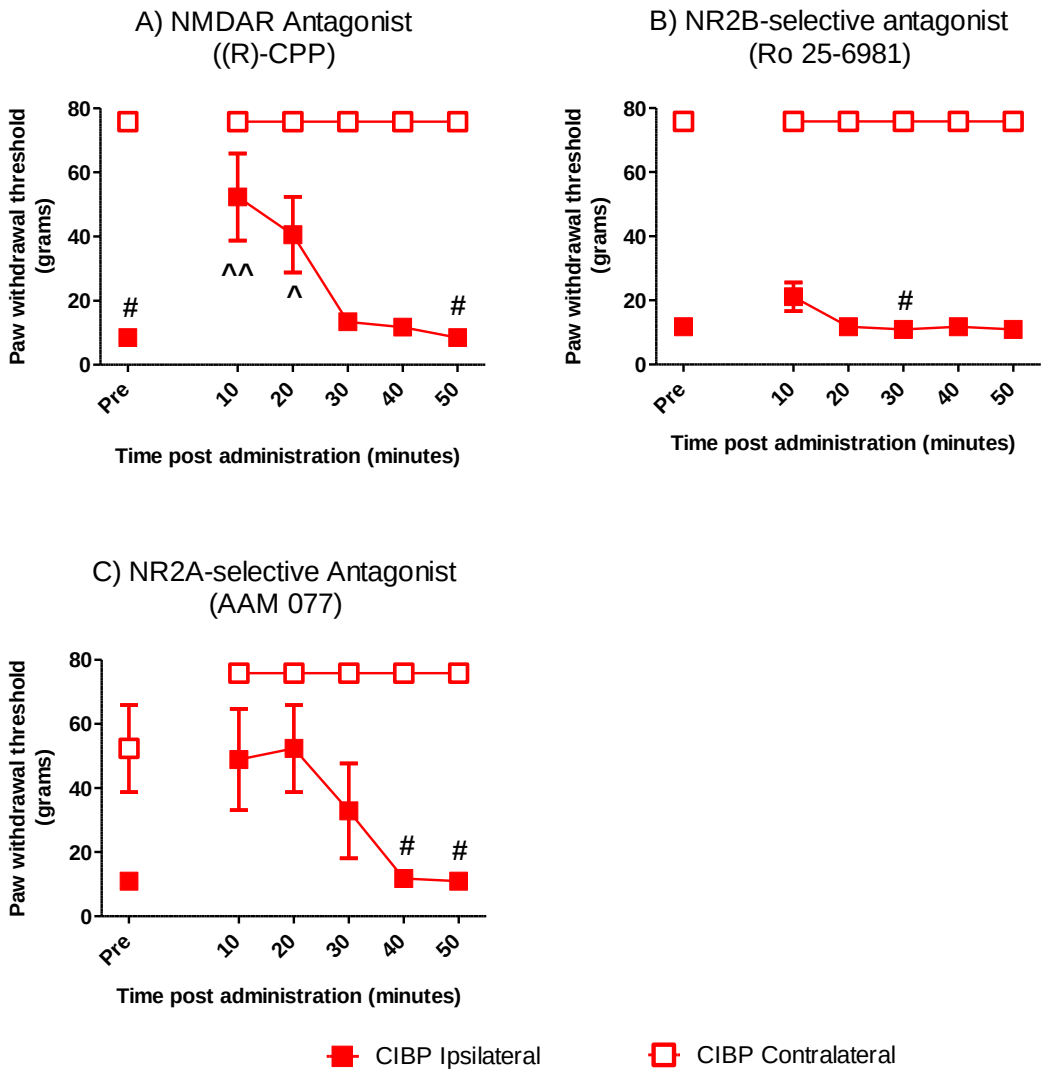


Figure 5.3 Effect of NMDA receptor antagonists on CIBP-induced mechanical allodynia. Data show mean responses ± SEM. A) Effect of the general NMDA receptor antagonist (R)-CPP (n=4). CIBP resulted in a significant reduction in ipsilateral PWT when compared to contralateral values (#; One-way repeated measures ANOVA on ranks followed by Dunn's post-hoc analysis, $p<0.05$). Ipsilateral hindlimb PWT was significantly increased at 10 and 20 minutes post administration when compared to pre-administration values (One-way repeated measures ANOVA on ranks (Friedman's test) followed by Dunn's post-hoc analysis). B) Effect of the NR2B subunit-selective antagonist Ro 25-6981 (n=4). CIBP animals showed a significant reduction in ipsilateral PWT when compared to contralateral values at 30 minutes post-administration only (#; One-way repeated measures ANOVA on ranks followed by Dunn's post-hoc analysis, $p<0.05$). Ipsilateral

hindlimb PWT did not alter when compared to pre-administration values (One-way repeated measures ANOVA on ranks (Friedman's test) followed by Dunn's post-hoc analysis). C) Effect of the NR2A subunit-selective antagonist AAM 077 (n=4). CIBP showed a significant reduction in ipsilateral PWT when compared to contralateral values at 40 and 50 minutes post-administration only (#; One-way repeated measures ANOVA on ranks followed by Dunn's post-hoc analysis, $p<0.05$). Ipsilateral hindlimb PWT did not alter when compared to pre-administration values (One-way repeated measures ANOVA on ranks (Friedman's test) followed by Dunn's post-hoc analysis). P values ^^ = 0.001 to 0.01, ^ = 0.01 to 0.05 and # = 0.01 to 0.05.

				Time post-administration (minutes)					
	Antagonist			Pre	10	20	30	40	50
Mechanical allodynia	(R)-CPP	Ipsi	PWT (grams)	8.5	52.4	40.6	13.4	11.8	8.5
			SEM	0.0	13.6	11.8	1.0	0.0	0.0
		Con	PWT (grams)	75.9	75.9	75.9	75.9	75.9	75.9
			SEM	0.0	0.0	0.0	0.0	0.0	0.0
	Ro 25-6981	Ipsi	PWT (grams)	11.8	21.1	11.8	10.9	11.8	10.9
			SEM	0.0	4.5	1.4	0.8	0.0	0.8
		Con	PWT (grams)	75.9	75.9	75.9	75.9	75.9	75.9
			SEM	0.0	0.0	0.0	0.0	0.0	0.0
	AAM-077	Ipsi	PWT (grams)	11.0	48.9	52.4	32.9	11.8	11.0
			SEM	1.6	15.8	13.6	14.8	1.4	1.6
		Con	PWT (grams)	52.4	75.9	75.9	75.9	75.9	75.9
			SEM	13.6	0.0	0.0	0.0	0.0	0.0

Table 5.1 The effects of (R)-CPP, Ro 25-6981 and AAM-077 on CIBP-induced mechanical allodynia. Data show Paw Withdrawal Threshold (PWT) ± SEM (n=4 per group).

5.4.2 The involvement of NMDA receptors in CIBP-induced movement-evoked pain

The general NMDAR antagonist (R)-CPP and the NR2A subunit-selective antagonist AAM 077 attenuated movement-evoked pain 10 to 30 minutes post-administration. The NR2B subunit-selective antagonist (Ro 25-6981) did not

attenuate movement-evoked pain (Figure 5.4). All data were analysed by One-way repeated measures ANOVA followed by Dunnett's post-hoc analysis, $p<0.05$.

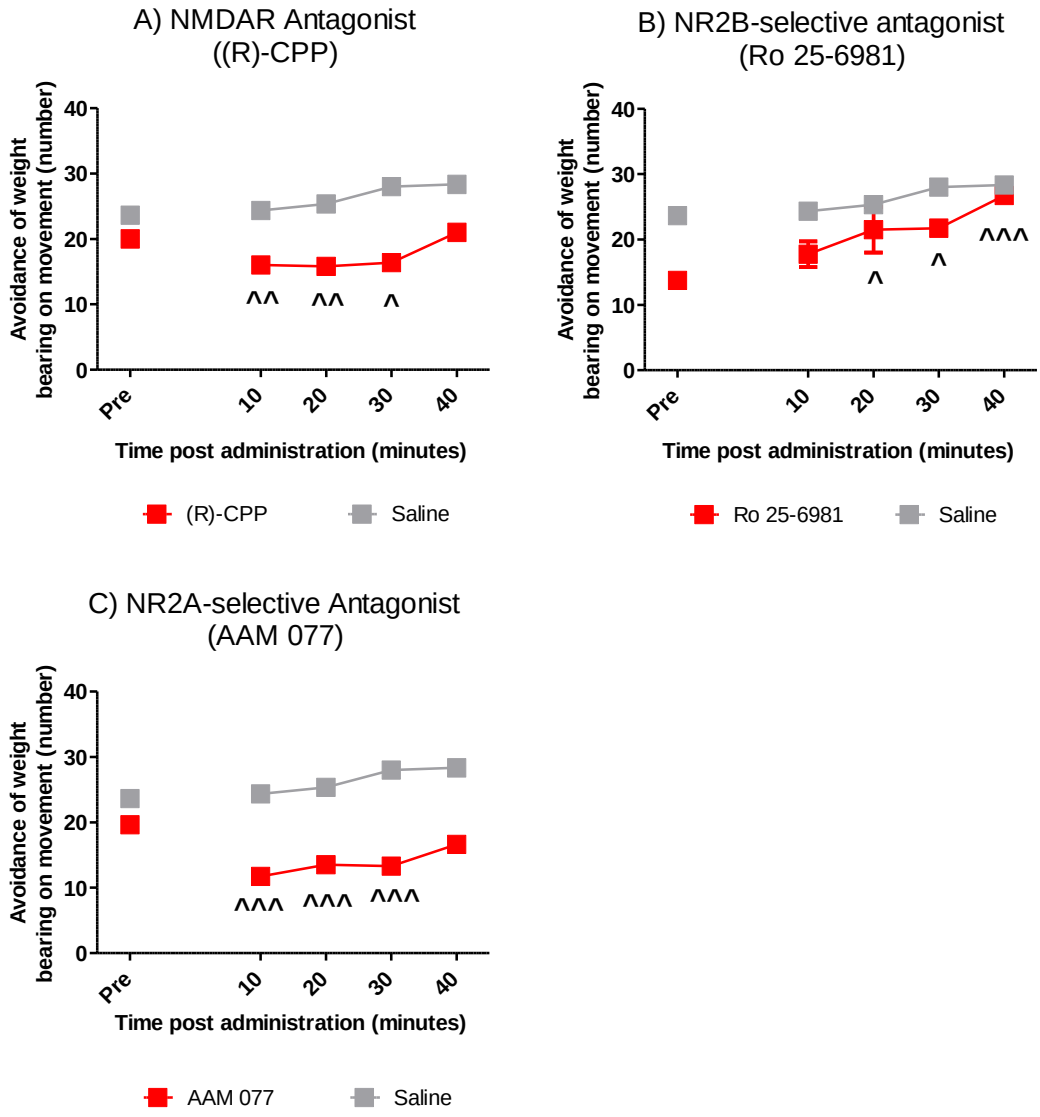


Figure 5.4 Effect of NMDA receptor antagonists on CIBP-induced movement-evoked pain. Data show mean responses ± SEM. A) Effect of general NMDA receptor antagonist (R)-CPP (n= 5) or saline (n=3). Avoidance of weight bearing on movement was significantly decreased when compared to pre-administration values (^, One-way repeated measures ANOVA followed by Dunnett's post-hoc analysis, $p<0.05$). B) Effect of NR2B-selective antagonist Ro 25-6981 (n=4) or saline (n=3). Avoidance of weight bearing on movement was significantly increased when compared to pre-administration values (^, One-way repeated measures ANOVA

followed by Dunnett's post-hoc analysis, $p<0.05$). C) Effect of the NR2A-selective antagonist AAM 077 (n=6) or saline (n=3). Avoidance of weight bearing on movement was significantly decreased when compared to pre-administration values (^, One-way repeated measures ANOVA followed by Dunnett's post-hoc analysis, $p<0.05$). P values ^^^ = <0.001, ^^ = 0.001 to 0.01 and ^ = 0.01 to 0.05.

			Time post administration (minutes)				
	Antagonist		Pre	10	20	30	40
Movement-evoked	(R)-CPP	Number	20.0	16.0	15.8	16.4	21.0
		SEM	0.3	0.9	0.7	0.9	1.3
	Ro 25-6981	Number	1.5	3.9	7.0	2.4	2.4
		SEM	0.8	2.0	3.5	1.2	1.2
	AAM 077	Number	19.7	11.8	13.5	13.3	16.7
		SEM	1.1	0.9	1.3	1.0	0.6

Table 5.2 The effects of (R)-CPP, Ro 25-6981 and AAM 077 on CIBP-induced movement-evoked pain. Data show number of avoidances of weight bearing on movement ± SEM (n=5, 4 and 6, respectively).

5.4.3 NMDA receptor subunit expression in the dorsal horn of the spinal cord in the CIBP model

Spinal cord sections from CIBP and control animals were analysed for NMDA receptor subunit expression using immunohistochemistry. NR2A subunit expression was significantly increased following CIBP in laminae I, II and III, as shown by an increase in ipsilateral-contralateral difference in fluorescence intensity compared to Sham E (Figure 5.5 and 5.6). NR2A subunit expression was still significantly increased in XRT-treated CIBP animals in laminae I and II as shown by a significant increase in ipsilateral-contralateral difference in fluorescence intensity when compared to Sham E (Figure 5.6) All statistical assessments were by mixed-model ANOVA followed by Bonferroni's post-hoc analysis, $p<0.05$. NR1 and NR2B subunits did not show a significant change in ipsilateral-contralateral difference in fluorescence intensity in CIBP animals (Figure 5.6).

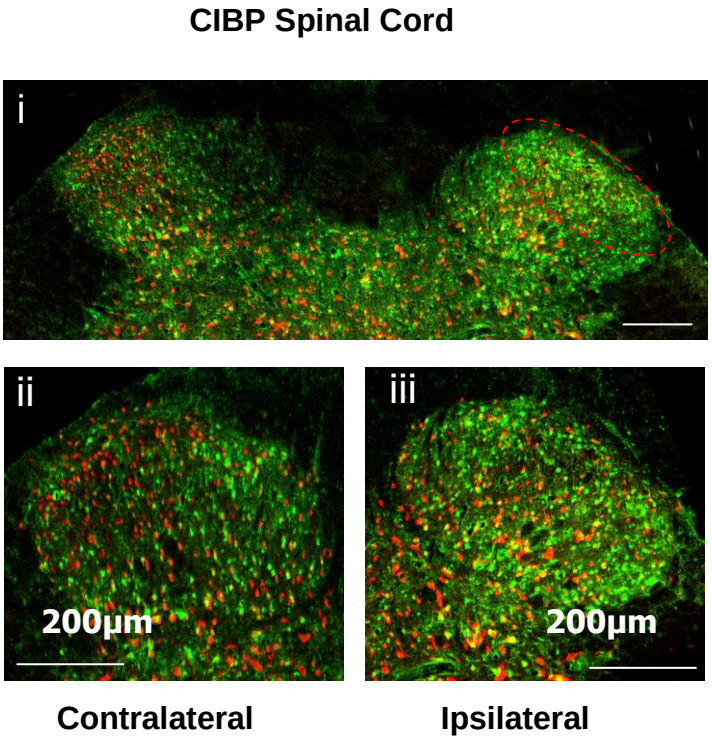


Figure 5.5 Representative images of the contralateral and ipsilateral dorsal horn in CIBP at X5 (i) and X20 (ii and iii) magnification in L4-6 spinal cord. NMDA receptor subunit NR2A (green) showed a significant increase in ipsilateral dorsal horn, when compared to contralateral dorsal horn, following CIBP shown with NeuN (red) to identify neurons. CIBP, Sham E and Naive animals were analysed, n=6 animals per group and n=6 sections per group.

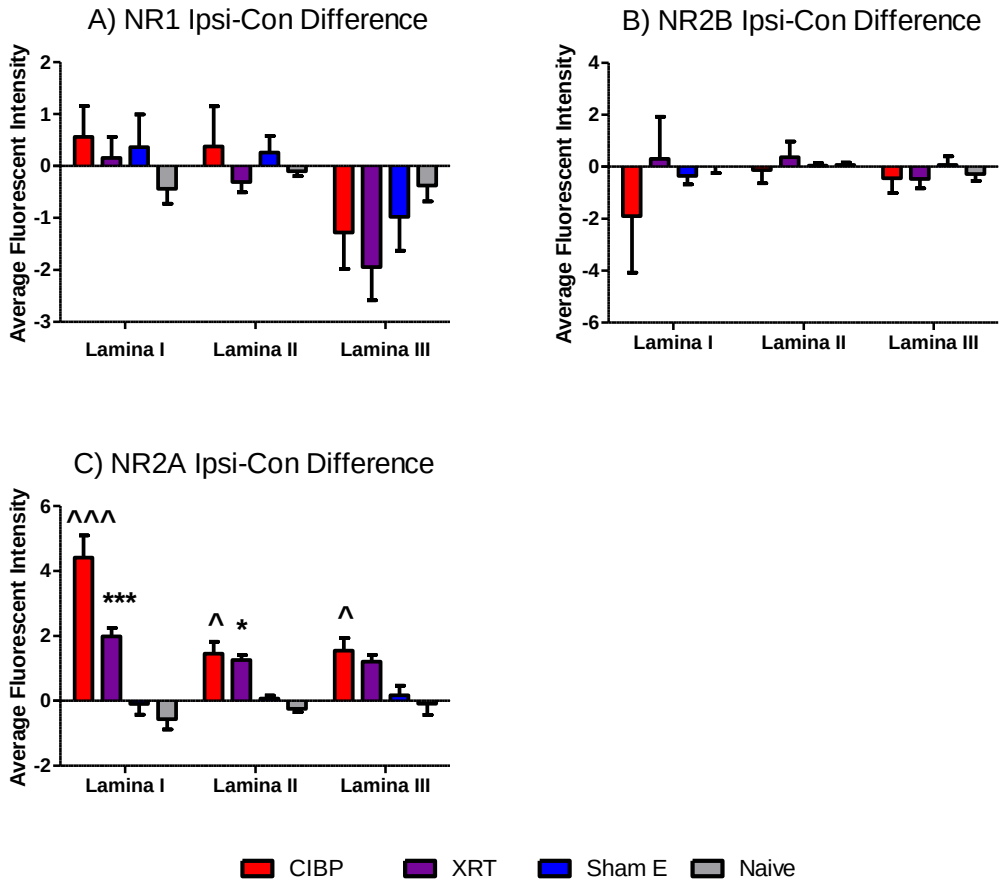


Figure 5.6 NMDA receptor subunit expression in the dorsal horn of the spinal cord. Data show mean Ipsilateral-Contralateral difference in fluorescence intensity (Ipsi-Con difference) ± SEM. A) NR1 Ipsi-Con difference was not altered between groups (mixed-model ANOVA followed by Bonferroni's post-hoc analysis). B) NR2B Ipsi-Con difference was not altered between groups (mixed-model ANOVA followed by Bonferroni's post-hoc analysis). C) NR2A Ipsi-Con difference was significantly increased in CIBP laminae I, II and III when compared to Sham E. NR2A Ipsi-Con difference was still significantly increased in XRT-treated CIBP animals in laminae I and II when compared to Sham E. NR2A Ipsi-Con difference was not altered in Naive when compared to Sham E (^, * mixed-model ANOVA followed by Bonferroni's post-hoc analysis). P values ^^^ = <0.001, ^ = 0.01 to 0.05, *** = <0.001 and * = 0.01 to 0.05.

Group		NR1			NR2B			NR2A		
		Lamina			Lamina			Lamina		
		I	II	III	I	II	III	I	II	III
CIBP	Ipsi-Con	0.6	0.4	-1.3	-1.9	-0.1	-0.5	4.4	1.4	1.5
	SEM	0.6	0.8	0.7	2.2	0.5	0.6	0.7	0.4	0.4
XRT	Ipsi-Con	0.2	-0.3	-1.9	0.3	0.4	-0.5	2.0	1.3	1.2
	SEM	0.4	0.2	0.6	1.6	0.6	0.4	0.3	0.2	0.2
Sham	Ipsi-Con	0.4	0.3	-1.0	-0.4	0.0	0.1	-0.1	0.1	0.2
	SEM	0.6	0.3	0.7	0.3	0.1	0.3	0.3	0.1	0.3
Naïve	Ipsi-Con	-0.4	-0.1	-0.4	0.0	0.1	-0.3	-0.6	-0.2	-0.1
	SEM	0.3	0.1	0.3	0.2	0.1	0.3	0.3	0.1	0.3

Table 5.3 Ipsilateral-Contralateral difference in fluorescence intensity. Data show mean Ipsi-Con difference ± SEM.

5.4.4 Analysis of neuronal NR2A subunit expression

Fluorescent confocal images (Figure 5.7) were analysed to assess the number of cells co-expressing NR2A and NeuN and the number of cells expressing NR2A only in laminae I and II. Results show that the great majority of NR2A is expressed in neuronal cells, shown by NR2A and NeuN co-expression, with relatively few expressing NR2A only. There was no significant difference in the number of cells co-expressing both NR2A and NeuN between CIBP, Sham V and Naïve groups (Figure 5.8). All shown by One-way ANOVA followed by Bonferroni's post-hoc analysis.

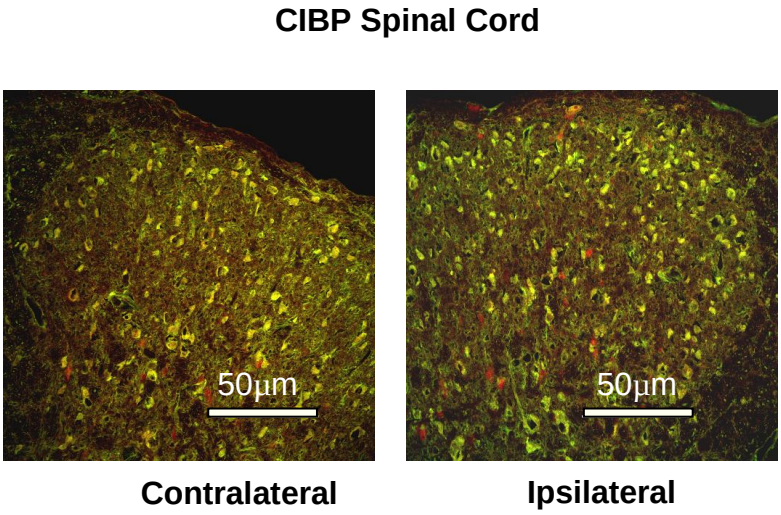


Figure 5.7 Representative images of the contralateral and ipsilateral dorsal horn in CIBP at X40 magnification in L4-6 spinal cord. NMDA receptor subunit NR2A (green) co-expression with NeuN (red) was analysed. CIBP, Sham V and Naive animals were analysed, n=6 animals per group and n=6 sections per group.

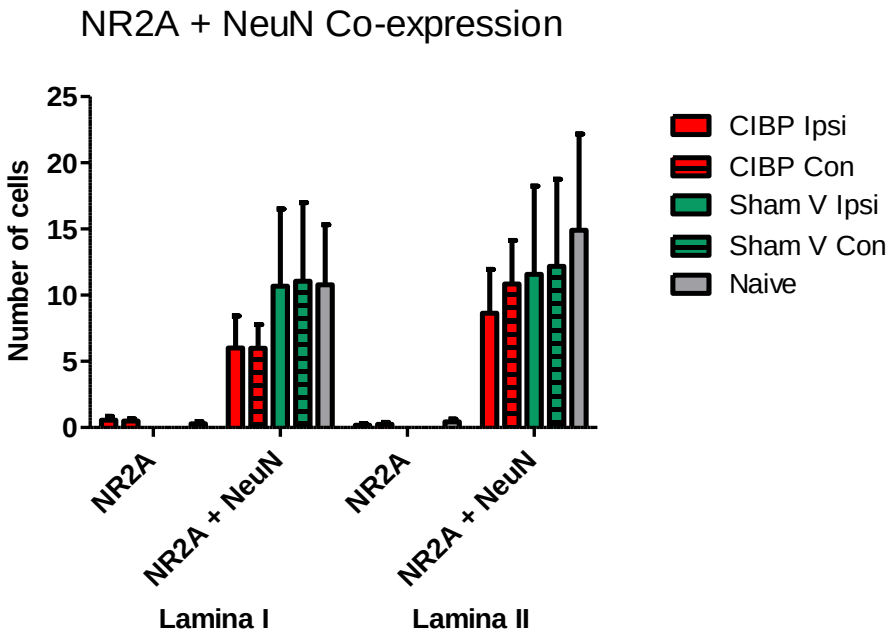


Figure 5.8 Numbers of cells expressing NR2A alone or co-expressing NR2A + NeuN in lamina I and lamina II. Data show mean ± SEM of CIBP (n=3), Sham V (n=3) and Naive (n=3) animals. The number of cells expressing NR2A + NeuN did not alter

significantly in the ipsilateral dorsal horn compared to contralateral dorsal horn or between groups.

			Lamina I		Lamina II	
Group			NR2A	NR2A + NeuN	NR2A	NR2A + NeuN
CIBP	Ipsi	Number	0.5	6.0	0.2	8.6
		SEM	0.3	2.4	0.1	3.3
CIBP	Con	Number	0.5	6.0	0.2	10.8
		SEM	0.2	1.8	0.2	3.3
Sham V	Ipsi	Number	0.0	10.7	0.0	11.6
		SEM	0.0	5.9	0.0	6.7
Sham V	Con	Number	0.0	11.1	0.0	12.2
		SEM	0.0	5.9	0.0	6.6
Naïve		Number	0.3	10.8	0.4	14.9
		SEM	0.2	4.5	0.2	7.3

Table 5.4 Numbers of cells expressing NR2A or NR2A + NeuN in lamina I and lamina II. Data show mean number of cells ± SEM.

5.5 Discussion

5.5.1 The involvement of NR2A subunit-containing NMDA receptors in CIBP-induced behavioural hypersensitivity

The general NMDA receptor antagonist (R)-CPP effectively attenuated mechanical allodynia and movement-evoked pain. A NR2B-selective antagonist Ro 25-6981 did not attenuate mechanical allodynia or movement-evoked pain in CIBP, although this is highly effective in models of neuropathic pain (Boyce et al., 1999). A NR2A specific antagonist AAM 077 did not attenuate mechanical allodynia but effectively attenuated movement-evoked pain. These results suggest that the NR2A subunit may be particularly involved in CIBP-induced movement-evoked pain. The general NMDA receptor antagonist (R)-CPP displays a relative potency order (high to low potency) of NR2A>NR2B>NR2C>NR2D. In addition, (R)-CPP displayed a 50-fold difference in affinity between NR2A and NR2D in one study (Feng et al., 2005). In the present study, (R)-CPP may have attenuated mechanical allodynia through inhibition of both NR2A and NR2B at the same time and this may explain why the

specific NR2A and NR2B antagonists did not significantly attenuate mechanical allodynia alone. It is interesting to note that although the NR2A antagonist did not significantly attenuate mechanical allodynia, there did appear to be a modest increase in PWT when compared to pre-administration values. Further studies with increased animal numbers may potentially allow us to observe a statistically significant attenuation of mechanical allodynia with AAM 077 administration.

5.5.2 NMDA receptor expression in CIBP

Fluorescence immunohistochemistry results show that expression of the NR2A subunit is significantly increased in the ipsilateral dorsal horn of the spinal cord of CIBP animals in laminae I, II and III in L4-L6. These results are significant because laminae I and II of the dorsal horn are known to be important for nociceptive processing (Chapter 1, Section 1.5.1). Lamina I contains many projection neurons which connect to higher centres and the majority of lamina I neurons are nociceptive-specific cells. Unmyelinated nociceptive C-fibres terminate predominantly in lamina II. Lamina III receives primarily large Aβ-fibres and second order neurons from lamina III send dendrites to laminae I and IV and to ascending tracts. Central sensitisation of dorsal horn neurons contributes to behavioural hypersensitivity in models of inflammatory pain, neuropathic pain and CIBP (Hama *et al.*, 2003;Suzuki & Dickenson, 2005;Yanagisawa *et al.*, 2010).

The results of this thesis contrast with a recent study, which suggests that the NR2B subunit plays a role in CIBP (Gu et al., 2010a). Gu *et al.* showed that intrathecal administration of the NR2B subunit-selective antagonist ifenprodil attenuated spontaneous pain, thermal hyperalgesia and mechanical allodynia in a murine model of CIBP at 2 and 12 hours post administration, with ifenprodil most effective at attenuating thermal hyperalgesia. In the current study we did not investigate the effects of NMDA receptor antagonists on thermal nociceptive responses and so therefore cannot directly compare results. Our assessment of Ro 25-6981 on CIBP responses utilises a more selective agent than ifenprodil (Fischer et al., 1997). The study by Gu *et al.* also identified an increase in NMDA receptor subunit NR2B mRNA and protein levels in the spinal cord of CIBP mice when compared to

Sham at Day 14 (Gu et al., 2010a). The disagreement with our results may be due to the different model used. Gu *et al.* used a mouse model of CIBP, whereby C3H/HeJ mice received an injection of osteosarcoma NCTC 2472 cells into the femur. The time frame of behavioural testing post-administration administration is also different, as Gu *et al.* analysed the effects of intrathecal administration of a NR2B antagonist from 2 hours onwards whereas we tested from 10 to 40 minutes post administration.

In this thesis, co-expression analysis showed that the majority of NR2A subunit is expressed in neuronal cells but we found no evidence for an increase in the number of cells expressing NR2A following CIBP. This would suggest that the increase in NR2A is due to an increase in the level of expression of NR2A subunits within each cell rather than an increase in the number of cells expressing NR2A. Further studies could investigate NMDA receptor mRNA expression in the spinal cord of CIBP animals and electrophysiology could be used to characterise the NR2A:NR2B ratio within specific cells through their distinctive influences on channel properties.

It is interesting that NR2A subunit expression is also significantly increased in XRT-treated CIBP animals in laminae I and II. This suggests that the mechanism by which XRT attenuated thermal sensitivity to 20°C and 40°C and also reversed movement-evoked pain at Day 18-21 is not dependent on preventing the increased NR2A expression, which is observed in CIBP. As discussed previously (Section 4.5.1), the mechanism by which XRT attenuates CIBP-induced behavioural hypersensitivity may depend on decreased tumour burden and osteoclast activity (Goblirsch et al., 2004b;Goblirsch et al., 2005). However, another preclinical study showed that tumour burden and osteoclast activity were not altered by XRT (Vit et al., 2006). That study did however show that XRT led to clear differences in the spinal cord including a decrease in glial cell activity, decreased dynorphin, COX-2 and chemotactic cytokine receptor (CCR2) (Vit et al., 2006).

The involvement of NMDA receptors in CIBP is of considerable interest for developing new intervention targets. The proinflammatory cytokine IL-1β has been

shown to be selectively induced in astrocytes in animal models of CIBP (Zhang et al., 2008a). Zhang *et al.* further showed that an IL-1β receptor antagonist attenuated CIBP and inhibited NR1 phosphorylation. The authors suggested that spinal IL-1β facilitates CIBP by enhancing phosphorylation of the NR1 subunit of NMDA receptors (Zhang et al., 2008a). IL-1β has correspondingly been shown to enhance NMDA receptor-mediated intracellular calcium release (Viviani *et al.*, 2003b). In this thesis it would have been interesting to investigate the proteins specifically associated with NR2A in CIBP and this could help elucidate the signalling pathways downstream of NR2A that are activated in CIBP.

5.6 Conclusion

Behavioural results suggest that NMDA receptors containing the NR2A subunit in particular are involved in CIBP-induced movement-evoked pain. This suggests that NR2A subunit-selective antagonists may be useful for treating CIBP-induced movement-evoked pain. Additionally, results show that there is increased expression of the NR2A subunit in the laminae I, II and III in the dorsal horn of the spinal cord, which could provide the basis for particular involvement of NR2A-containing NMDA receptors. XRT-treated animals still showed increased expression of NR2A in laminae I and II, despite improvement of reflex behaviours, suggesting that the analgesic benefit from XRT is not due to its impact on NR2A subunit expression in the dorsal horn.

6. THE INVOLVEMENT OF TRP CHANNELS IN A PRECLINICAL MODEL OF CIBP

6.1 Introduction

Currently 28 mammalian TRP channels have been discovered, which can be subdivided into 6 main subfamilies: TRPC (canonical), TRPV (vanilloid), TRPM (melastatin), TRPP (polycystin), TRPML (mucolipin) and TRPA (ankyrin) subfamilies (Montell, 2005). TRP subunits have a common structure of 6 transmembrane domains (M1-6) with a pore region between the 5th and 6th domains (Figure 6.1). Both the N- and C- termini are located intracellularly. The length of the cytoplasmic domain and the functional and structural domains contained within the cytoplasmic domain vary within subfamilies. A functional TRP channel requires tetrameric assembly of TRP subunits, and these channels can either be homomeric or heteromeric (Cheng et al., 2010).

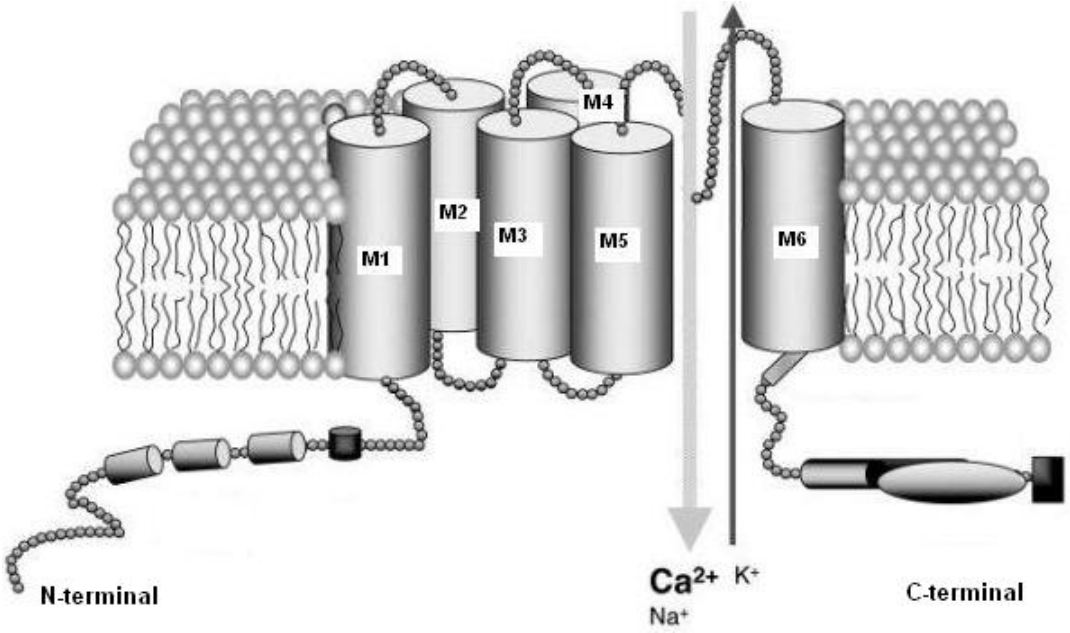


Figure 6.1 Structural organisation of TRP channel subunits. TRP subunits have 6 transmembrane domains (M1-6) with a pore region between the 5th and 6th domains. Functional TRP channels can be homotetrameric or heterotetrameric. Figure adapted from (Planells-Cases & Ferrer-Montiel, 2007).

TRP channels are gated by diverse stimuli that include thermal, chemical and/or mechanical stimuli. When activated, TRP channels conduct cations and

depolarise cells. All functionally characterised TRP channels are permeable to Ca^{2+} with the exceptions of TRPM4 and TRPM5, which only conduct monovalent cations (Nilius et al., 2007a). Ca^{2+} entry through TRP channels (or Ca^{2+} entry mediated by alternative pathways but initiated by TRP channels) causes changes in membrane potential and elevation of cytosolic Ca^{2+} concentrations, both of which may initiate a variety of cellular responses (Wu et al., 2010). TRP channels are expressed in many cell types; however their expression at the peripheral and central terminals of sensory neurons make them particularly interesting targets in pain research.

The temperature-sensitive TRP channels, known as thermoTRPs, have been widely implicated as somatosensory transducers. The family consists of nine members; TRPV (1-4), TRPM (2, 4, 5 and 8) and TRPA1. Each thermoTRP is activated in a specific temperature range and activation of thermoTRPs appears to be the best candidate mechanism for peripheral thermosensation (Ramsey et al., 2006). ThermoTRPs are also activated and modulated by a variety of chemical and other physical stimuli, making them polymodal sensors. ThermoTRPs are expressed in primary sensory neurons, brain areas and a wide array of non-neuronal cells. Several were of particular interest here, TRPM8 and TRPV4 because of their activation at mild cool and warm temperatures respectively, corresponding to the temperatures we showed to elicit enhanced responses in the CIBP model, and TRPV1 as the classic, firmly established mediator of noxious heat responses.

TRPM8

The TRPM8 channel was cloned and characterised in 2002 (Mckemy *et al.*, 2002b;Peier *et al.*, 2002). McKemy *et al.* took an expression-cloning strategy using a cDNA library from rat trigeminal neurons and identified a cDNA clone that encoded TRPM8. Peier *et al.* used a bioinformatics approach to search for sequences that may define thermosensitive channels related to TRPV1 and hence isolated TRPM8 cDNA from DRG. Through these studies, TRPM8 was identified as a non-selective cation channel permeable to both monovalent and divalent cations. TRPM8 is activated by innocuous cool temperatures (8-28°C) and by agents that elicit a sensation of cooling

such as menthol and icilin. Icilin is one of the most potent activators of TRPM8 discovered so far.

It has been proposed that the mechanism of TRPM8 activation by cooling and menthol involves a negative shift of the channel's voltage-dependent opening from very positive non-physiological membrane potentials toward physiological values (Brauchi et al., 2004;Voets et al., 2004). It has been proposed that the C-terminal domain contains the thermal sensor (Brauchi et al., 2006;Vlachova et al., 2003). Icilin can only activate TRPM8 when intracellular Ca^{2+} is elevated, as intracellular Ca^{2+} appears to act as an icilin-selective co-agonist (Chuang et al., 2004). The critical residues for Ca^{2+} acting as a co-agonist appear to be located in the intracellular loop connecting transmembrane domains 2 and 3. The same region is critical for activation of other TRP channels by chemical agonists. Activation of TRPM8 by cold, menthol and icilin is followed by channel desensitisation, which also depends on Ca^{2+} in the extracellular medium (Mckemy et al., 2002a).

TRPM8 is expressed on a subset of small diameter DRG and trigeminal ganglion neurons (Kobayashi et al., 2005) as well as in visceral afferents, most notably in bladder (Lashinger et al., 2008;Stein et al., 2004). In addition it is expressed in non-neuronal cells including prostate gland cells and some primary tumours (Stein et al., 2004;Tsavaler et al., 2001). The expression of TRPM8 in sensory neurons was investigated using mice that express green fluorescent protein (GFP) under the control of the TRPM8 promoter. This study showed that $TRPM8^{GFP}$ mainly marks a unique population of DRG neurons that do not express any nociceptive markers (TRPV1, CGRP or IB4). Only a small percentage of $TRPM8^{GFP}$-expressing neurons (1% of total DRG neurons) expressed CGRP and TRPV1 (Dhaka et al., 2008). This small population could possibly be involved in nociceptive thermosensation, but this may well not be the role of the majority of TRPM8-positive afferents.

Important studies using TRPM8-null mice have established that TRPM8 plays a key role in thermosensation (Bautista et al., 2007;Colburn et al., 2007;Dhaka et al., 2007). The studies confirmed that TRPM8 is an important physiological detector of innocuous cool temperatures and perhaps could also be involved in detecting noxious cold. TRPM8-null mice display severe thermosensory deficits with deficits in avoidance of cold temperatures, tested by temperature-preference chambers, latency on the cold plate at 0°C and 1°C and in paw withdrawal to acetone. Electrophysiological studies of sensory neurons derived from TRPM8-null mice show decreased response to cold and menthol with a small residual population of neurons that do respond to intense cold (Bautista et al., 2007). TRPA1 was proposed as a candidate receptor for these cold responses. However, controversy remains on whether TRPA1 contributes to noxious cold sensing (Kwan & Corey, 2009). There is evidence from recent *in vitro* (Fajardo et al., 2008) and *in vivo* (Dunham et al., 2010) studies that TRPA1 plays a role in cold sensing in visceral sensory neurones but not in somatic sensory neurones. A recent study further provides evidence that TRPA1 plays an important role in cold hypersensitivity rather than cold detection (del Camino et al., 2010).

Clinical models of chronic pain show cold hyperalgesia and cold allodynia in behavioural assays of cold responses. Using the cold plate test, the CCI model of neuropathic pain and the CFA model of inflammatory pain show increased number of paw withdrawals and duration of paw elevation from a cold surface (McCoy et al., 2011). Additionally, the SNL model of neuropathic pain displays a hypersensitivity to normally innocuous evaporative cooling to acetone as well as reduced latencies to paw withdrawal on a cold plate (McCoy et al., 2011). Studies of TRPM8-null mice have shown that TRPM8 is essential for hypersensitivity to cold after injury. In a CCI model of neuropathic pain, injured wild-type mice display flinching behaviour in response to evaporative cooling by acetone application whereas injured TRPM8-null mice do not (Colburn et al., 2007). Similar results were shown with acetone application in the CFA model of inflammatory pain in wild-type and TRPM8-nulls. In the same study, hypersensitivity to heat and mechanical stimuli was unaffected,

indicating that deletion of TRPM8 specifically affected cold hypersensitivity (Colburn et al., 2007).

TRPM8 may also be involved in cooling-induced analgesia. Work from this laboratory showed increased TRPM8 expression in the CCI model of neuropathic pain. Furthermore, activation of TRPM8, using icilin, provides analgesia in this model of neuropathic pain, in a model of inflammatory pain and in the lysolecithin model of demyelination-induced pain (Proudfoot et al., 2006). Moderate cooling also appears to elicit analgesia in the CCI model (Proudfoot *et al.,* 2006) and against the nocifensive responses elicited by intraplantar formalin (Dhaka *et al.,* 2007). In the latter study, moderate cooling reduced formalin responses in wild-type mice whereas in TRPM8-nulls only partial reductions were seen (Dhaka et al., 2007). This suggests that TRPM8 may well be involved in cool-induced analgesia but TRPM8-independent mechanisms could also be involved.

TRPV1

TRPV1 was the first heat-activated TRP channel to be cloned and characterised (Caterina et al., 1997). TRPV1 is activated by noxious heat (>43°C), acidic pH, capsaicin (the pungent component of chilli peppers), resiniferatoxin, voltage and various endogenous lipids, such as anandamide. Other natural compounds such as allicin, present in garlic, and piperine, found in black pepper, have also been shown to activate TRPV1 (Palazzo et al., 2010). TRPV1 stimulation activates Ca^{2+}/calmodulin-dependent protein kinases (CaMK) and protein kinase C (PKC) (Caterina et al., 1997;Tominaga et al., 1998).

TRPV1 is widely expressed in dorsal root and trigeminal ganglia sensory neurons, in C- and Aδ- fibres, as well as in many non-neuronal cells including for example, epithelial keratinocytes. Nociceptors show the most abundant expression of TRPV1 with 30x more than other tissues (Caterina et al., 1997). TRPV1 can also be found in discrete regions of the rat brain, where it may be involved in synaptic plasticity (Eid & Cortright, 2009).

Activation of TRPV1 can be enhanced by inflammatory mediators such as prostaglandins, ATP, NGF and bradykinin released during tissue injury, metabolic stress and inflammation (Szallasi et al., 2007). This enhancement can occur indirectly through phosphorylation and/or increased expression and trafficking of TRPV1 channels. TRPV1 phosphorylation by PKC sensitises the channel by increasing the open probability (Vellani et al., 2001) and phosphorylation by Src mediates recruitment of new channels to the cell surface by exocytosis (Caterina et al., 1997;Zhang et al., 2005b).

TRPV1 has been shown to play a key role in inflammatory pain using studies of TRPV1 knock-out mice and pharmacological agents. TRPV1 knock-out studies have shown that TRPV1 is crucial for thermal hyperalgesia as TRPV1 null mice fail to develop thermal hyperalgesia following a number of inflammatory insults including CFA and carrageenan (Caterina et al., 1997;Davis et al., 2000). Intrathecal AMG 9810, a TRPV1 antagonist, attenuated thermal hyperalgesia and mechanical allodynia induced by CFA (Yu et al., 2008a). Up-regulation and sensitisation of TRPV1 receptors could be important in TRPV1-mediated pain. TRPV1 expression is increased in primary sensory neurons after peripheral inflammation (Carlton & Coggeshall, 2001). TRPV1 expression has further been shown to be up-regulated in animal models of disease including osteoarthritis and CIBP (Fernihough et al., 2005;Ghilardi et al., 2005;Niiyama et al., 2007). More specifically, TRPV1 expression increases within a subpopulation of DRG neurons expressing NF200 and CGRP, but not IB4, in a mouse model of CIBP (Niiyama et al., 2007). Pharmacological blockade of TRPV1 has been shown to reduce pain-related behaviours in models of CIBP (Ghilardi et al., 2005;Honore et al., 2009;Niiyama et al., 2007;Niiyama et al., 2009). Another important aspect of TRPV1 function is that TRPV1 undergoes desensitisation following repeated or prolonged activation. This desensitisation can diminish pain sensation and agonists have been used as analgesics. For example, clinical studies indicate that a new 8% capsaicin patch may be useful in the treatment of neuropathic pain (Backonja et al., 2008;Kennedy et al., 2010).

TRPV4

The TRP vanilloid 4 (TRPV4) channel is activated by a variety of physical and chemical stimuli including warm temperatures (27-34°C), hypotonicity, low pH, endogenous substances such as arachidonic acid and its metabolites and endocannabinoids such as anandamide. TRPV4 is expressed in many tissues including epidermal keratinocytes, osteoblasts and osteoclasts in bone and dorsal root and trigeminal ganglia neurons (Everaerts et al., 2010). A recent study showed that in rat DRG 88.5 ± 4.8% of neurons expressed TRPV4, 33.7 ± 2.4% of neurons expressed TRPV1 and 27.9 ± 2.8% of the neurons co-expressed TRPV4 and TRPV1. This co-expression was mostly observed in small and medium diameter DRG neurons. TRPV1 and TRPV4 were not only expressed in the cell bodies of DRG neurons but also expressed at the central terminals of these sensory nerves, at laminae I and II of the spinal cord dorsal horn (Cao et al., 2009).

A study of TRPV4-null mice by Lee *et al.* showed that TRPV4 is required for normal thermosensation *in vivo*. This was based on the findings that TRPV4-/- mice showed a preference for warmer floor temperatures compared to wild-type mice and TRPV4-/- mice exhibited a strong preference for 34°C (Lee et al., 2005a). Another study using TRPV4-null mice indicated that TRPV4 contributes to thermal hyperalgesia in the hotplate test following inflammatory pain, as TRPV4-null mice exhibited less sensitivity to warmth during carrageenan-induced inflammation (Todaka et al., 2004a). TRPV4 also appears to play a role in chemotherapy-induced neuropathic pain in the rat, shown by spinal administration of antisense oligonucleotide to TRPV4, which reduced expression of TRPV4 in the sensory nerve and attenuated hypersensitive pain behaviours (Alessandri-Haber et al., 2004). Furthermore, TRPV4 may contribute to mechanical allodynia in the chronic compression of DRG (CCD) model of neuropathic pain, where TRPV4 antisense partly reversed mechanical allodynia (Zhang et al., 2008b). In the CCD model, TRPV4 protein and mRNA expression increased significantly 7-28 days post-CCD when compared to sham group. Additionally, TRPV4 has been shown to contribute to thermal hyperalgesia in the same model of neuropathic pain, where administration of antisense TRPV4 or a TRPV4 antagonist attenuated thermal hyperalgesia (Ding *et*

al., 2010b). These data suggest that TRPV4 is important for thermal and mechanical nociception.

TRP Channels in Cancer

Several TRP channels show altered expression in cancer cells. TRPM8, TRPM1 and TRPV6 are highly expressed in cancer cells. Other TRP channels including TRPC1, TRPC6, TRPM5 and TRPV1 are also increased in some cancer tissues. This altered expression does not appear to involve gene mutations of these channels but increased or decreased expression of the wild-type TRP protein (Lehen'kyi & Prevarskaya, 2011). The precise roles that TRP channels play in cancer are still being elucidated (Lehen'kyi & Prevarskaya, 2011). It has been reported that TRPV1-/- mice exhibit increased skin carcinogenesis compared to wild-type mice (Bode et al., 2009). Furthermore, topical application of TRPV1 antagonist AMG 9810 is reported to promote skin tumour development (Li et al., 2011). The authors suggest that TRPV1 could potentially play the role of a tumour suppressor and chronic antagonism of TRPV1 may lead to tumour development (Li et al., 2011), although the mechanistic basis is not firmly established. As mentioned previously, however, thermo-sensitive TRP channels are expressed in a particularly focused profile on sensory neurons and therefore may be important for sensing pain, including that of CIBP.

6.2 Aim

In this thesis the involvement of TRPM8, TRPV1 and TRPV4 in CIBP was investigated. These thermoTRPs were chosen because their activation temperatures cover a range of temperatures; TRPM8 is activated by innocuous cool, TRPV1 by noxious heat and TRPV4 by innocuous warm temperatures. Additionally, these thermoTRPs have been implicated in different pain states. The primary aim of this part of the study was to determine the analgesic efficacy of a TRPM8 agonist (icilin), TRPV1 antagonist (AMG 9810) and TRPV4 antagonist (RN 1734). The analgesic efficacy of these agents on CIBP-induced mechanical allodynia, thermal sensitivity to 40°C and movement-evoked pain was investigated. We also aimed to determine

whether there were any changes in expression of TRPM8, TRPV1 or TRPV4 in the DRG during CIBP.

6.3 Methods

6.3.1 Surgical Procedures

Experiments were carried out using male Sprague-Dawley rats and as detailed in Chapter 2, animals underwent CIBP surgery (Section 2.1.2). Rats were anaesthetised by inhalation of an isoflurane/O2 mixture (Zeneca, UK), 4-5% for induction and 2-3% for maintenance. The carrier gas was compressed oxygen at a flow rate of 2 litres/minute. Following complete induction of anaesthesia, the animal was placed abdominal side up, the left hind limb was shaved and the skin was sterilised with 0.5% Hibitane (Zeneca, UK). A small incision was made in the skin over the tibia, which was then carefully exposed by removing the connective tissue over the bone using a cotton bud Johnson & Johnson, UK). A dental drill was used to bore a hole through the periosteum of the tibia. Polythene tubing (0.5mm in diameter; Smiths) was fed into the intra-medullary cavity of the tibia and 10 µl of medium (containing $6x10^3$ cells) was injected using a 1ml micro-syringe (BD Biosciences, UK) and 25-gauge needle (BD Biosciences, UK). The tubing was withdrawn and the hole plugged with dental restorative material (IRM, Dentsply; Henry Schien Minerva), to confine the tumour cells within the marrow and prevent them invading the adjacent soft tissue. The wound was closed with absorbable subcutaneous suture (5/0 coated vicryl, Ethicon, UK) and sterilised with 0.5% Hibitane. Animals were placed in a thermoregulated recovery box until they had fully regained consciousness, following which they were returned to their home cages.

6.3.2 Analgesic Intervention

For analysis of the effect of topically applied or intrathecally applied pharmacological agents on somatosensory behavioural reflexes; pharmacological agents were administrated when animals were displaying CIBP-induced sensitisation. CIBP-induced sensitisation was defined by decreased ipsilateral PWT to von Frey filaments, increased ipsilateral avoidance of weight bearing on movement on the

rotarod and increased number of ipsilateral paw withdrawals on the thermal footplate to 40°C when compared to pre-surgery. Prior to topical or intrathecal administration of agents, measurement of mechanical allodynia (Section 2.3.1), movement-evoked pain (Section 2.4.1) and thermal sensitivity to 40°C were recorded to obtain pre-administration values. Mechanical allodynia was assessed by placing the animals in a Perspex chamber on an elevated metal mesh floor; the experimenter could then reach the plantar surface of the hind paw from beneath, unobserved by the animal. Following acclimatisation of the animal to the cage, the PWT in response to normally innocuous mechanical stimuli was measured by applying a set of calibrated Semmes-Weinstein von Frey filaments (Stoelting co., USA) to the plantar surface of the hindpaw of the ipsialteral and contralateral hindlimbs. Each filament was applied perpendicularly to the mid-plantar surface of the foot until the filament flexed/bent. Threshold was defined as the minimum indentation force (grams) required to elicit a response/paw withdrawal to at least 5 out of 10 applications (i.e. to at least 50% of applications). Data are expressed as the mean PWT (grams) ± standard error of the mean (SEM) for each time point.

To test thermal sensitivity of the ipsilateral hindlimb, each animal was placed on a thermal footplate (IITC Incremental Hot/Cold Plate Meter), which was set at 40°C (temperature holding accuracy is ± 0.1°C). The number of times the animal withdrew its ipsilateral hindlimb from the thermal footplate and the latency to the first paw withdrawal over 150 seconds was recorded (from the time of placing the animal on the thermal footplate). If the animal did not flick its paw, the latency was recorded as 150 seconds. In addition, the total duration of paw elevation was also noted. Data are expressed as the mean ± SEM for Paw Withdrawal Threshold/Latency to paw withdrawal/ Duration of Paw elevation to 40°C for each time point.

To assess movement-evoked pain, the rotarod (IITC) was used and set at a constant speed of 5-6 rpm (no ramping of rpm). The avoidance of weight bearing on movement of the ipsilateral hindlimb only was measured (number of times) over a 30 second test period (from the time of placing the animal on the rotarod). Data are

expressed as the mean avoidance of weight bearing on movement (number) ± SEM for each time point.

For topical application of the TRPM8/TRPA1 agonist icilin (100μM), icilin solution was applied to both hindlimbs by placing each animal in a Perspex chamber that had a slanted frame to support the front paws, enabling the solution to cover both hindlimbs (Section 2.7.3). Topical application of pharmacological agent was maintained for a 5 minute period. Behavioural testing commenced at 10 minutes post-administration until 80 minutes post-administration. For intrathecal administration of the TRPV1 antagonist AMG 9810 (1nmole), TRPV4 antagonist RN 1734 (5nmole) and vehicle control (0.5% dimethylformamide in saline), all animals were briefly anaesthetised by inhalation of an isoflurane/O_2 mixture and injected intrathecally at the L5/6 level of the spinal cord (Section 2.7.3). Behavioural testing continued every 15 minutes post-administration until 85 minutes post-administration. A TRPM8 agonist, icilin (100μM), was administrated by topical application to CIBP animals at Day 14. The following pharmacological agents were administrated to CIBP animals by intrathecal administration; a TRPV1 antagonist, AMG 9810 (1nmole), at Day 18-21, a TRPV4 antagonist, RN 1734 (5nmole), at Day 19 and vehicle control, 0.5% dimethylformamide in saline, at Day 20. RN 1734 has been shown to be selective for TRPV4 over closely related TRP channels including TRPV1, TRPV3 and TRPM8. RN 1734 has been shown to antagonise both ligand-gated activation and hypotonicity-induced opening of TRPV4 (Vincent et al., 2009). Icilin activates TRPM8 at low micromolar concentrations and activates TRPA1 at high micromolar concentrations (Mckemy *et al.*, 2002c;Story *et al.*, 2003). Icilin can also elicit mechanisms of desensitisation/shut down of TRPM8 channels (Kuhn et al., 2009). AMG 9810 is a TRPV1 antagonist with high selectivity and blocks all known modes of TRPV1 activation by protons, heat and endogenous ligands (Gavva et al., 2005).

6.3.3 Immunohistochemical Analysis

Using specific antibodies, the expression of TRPM8, TRPV1 and TRPV4 channel proteins in the DRG was assessed. For analysis of expression of TRP

channels in DRG sections using immunohistochemistry, L4-6 DRG were taken from CIBP, Sham V and Naive animals at Day 18-21 (Section 2.8.3). Animals were terminally anaesthetised and DRG were frozen in optimal cutting medium on dry ice. Tissue was sectioned on the cryostat to obtain 15µm sections and then mounted onto poly-L-lysine slides. Sections were prepared by particular antigen retrieval techniques (Table 2.8.2), blocked and then probed with anti-TRPM8, anti-TRPV1 or anti-TRPV4 together with a marker of myelinated neurons (anti-NF200) and a marker of unmyelinated neurons (anti-peripherin). The primary antibodies were then detected by fluorescent secondary antibodies. Sections were then captured using a fluorescence microscope at x20 magnification. Images were then analysed using Image J (Section 2.8.4) where the numbers of myelinated and unmyelinated neurons expressing each of the TRP channels were counted. The percentage of cells co-expressing NF200 or peripherin with TRPM8/TRPV1/TRPV4 in ipsilateral and contralateral DRG in CIBP, Sham V and Naive animals were compared. The total number of cells expressing TRPM8/TRPV1/TRPV4 in ipsilateral and contralateral DRG in CIBP, Sham V and Naive animals were also compared.

For analysis of total TRP channel protein using Western blot, DRG tissue was taken from CIBP, Sham V and Naive animals at Day 18-21. Animals were terminally anaesthetised and the DRG were dissected from L4-L6, both ipsilateral and contralateral to injury. DRG were then homogenised in Laemmli lysis buffer containing 1% protease inhibitor cocktail III and centrifuged at 10 000 rpm at 4°C to remove cell debris (Section 2.9.2). Proteins in DRG extracts were then separated by electrophoresis and transferred to PVDF membranes, blocked and probed with anti-TRPM8, anti-TRPV1 and anti-TRPV4 before detection using peroxidase-linked secondary antibodies and enhanced chemiluminescence (Section 2.9.1). Densitometry was performed to quantify the grey levels of positive protein bands, to give a ratio of protein of interest to total GAPDH (Section 2.9.1).

6.3.4 Statistical Analysis

In each behavioural study, data were pooled for each time point, with group mean shown ± SEM. For all analysis significance was set at $p<0.05$. Data were tested for normal distribution with the Kolmogorov-Smirnov test.

To analyse the effects of pharmacological agent administration on mechanical allodynia, post-pharmacological agent ipsilateral paw withdrawal thresholds were compared to pre-pharmacological agent baseline using a One-way repeated measures ANOVA on ranks (Friedman's test) followed by Dunn's post-hoc analysis. Differences between ipsilateral and contralateral hindlimb pre- and post-pharmacological agent were determined by One-way repeated measures ANOVA on ranks (Friedman's test) followed by Dunn's post-hoc analysis.

To analyse the effects of pharmacological agent administration on thermal sensitivity, post-pharmacological agent ipsilateral responses were compared to pre-pharmacological agent baseline using a One-way repeated measures ANOVA followed by Dunnett's post-hoc analysis.

To analyse the effect of pharmacological agent administration on movement-evoked pain, the number of avoidances of weight bearing on movement following drug administration were compared to pre-pharmacological agent using a One-way repeated measures ANOVA followed by Dunnett's post-hoc analysis.

For DRG immunohistochemistry differences between groups (CIBP Ipsilateral, CIBP Contralateral, Sham V Ipsilateral, Sham V Contralateral and Naive) were compared by One-way ANOVA followed by Bonferroni's post-hoc analysis. Differences within groups (e.g. CIBP Ipsilateral versus CIBP Contralateral) were detected by an unpaired two-tailed t-test.

For Western blot analysis, differences in relative densitometric intensity between groups (CIBP Ipsilateral, CIBP Contralateral, Sham V Ipsilateral, Sham V Contralateral and Naive) were compared by One-way ANOVA followed by

Bonferroni's post-hoc analysis. Differences within groups (e.g. CIBP Ipsilateral versus CIBP Contralateral) were detected by an unpaired two-tailed t-test.

6.4 Results

6.4.1 Effect of the TRPM8/TRPA1 agonist, icilin, on CIBP-induced mechanical allodynia, movement-evoked pain and thermal sensitivity

Topical application of the TRPM8/TRPA1 agonist icilin 100μM (CIBP Day 14 n=4-5) did not attenuate mechanical allodynia when compared to pre-administration values. Reflecting this allodynia, ipsilateral PWT was significantly reduced compared to contralateral PWT pre-administration and at 70 and 80 minutes post-administration shown by One-way ANOVA on ranks (Friedman's test) followed by Dunn's post-hoc analysis, $p<0.05$, (but not at 10-60 minutes post-icilin) (Figure 6.2.A). However icilin successfully attenuated movement-evoked pain (avoidance of weight-bearing on the rotarod) at 20-60 minutes post-administration, shown by One-way repeated measures ANOVA followed by Dunnett's post-hoc analysis, $p<0.05$ (Figure 6.2.B). Icilin had no effect on number of paw withdrawals or latency to paw elevation to 40°C, but the duration of paw elevation to 40°C was significantly increased at 70 minutes post-administration (Figures 6.2.C-E). All were evaluated by One-way repeated measures ANOVA followed by Dunnett's post-hoc analysis, $p<0.05$.

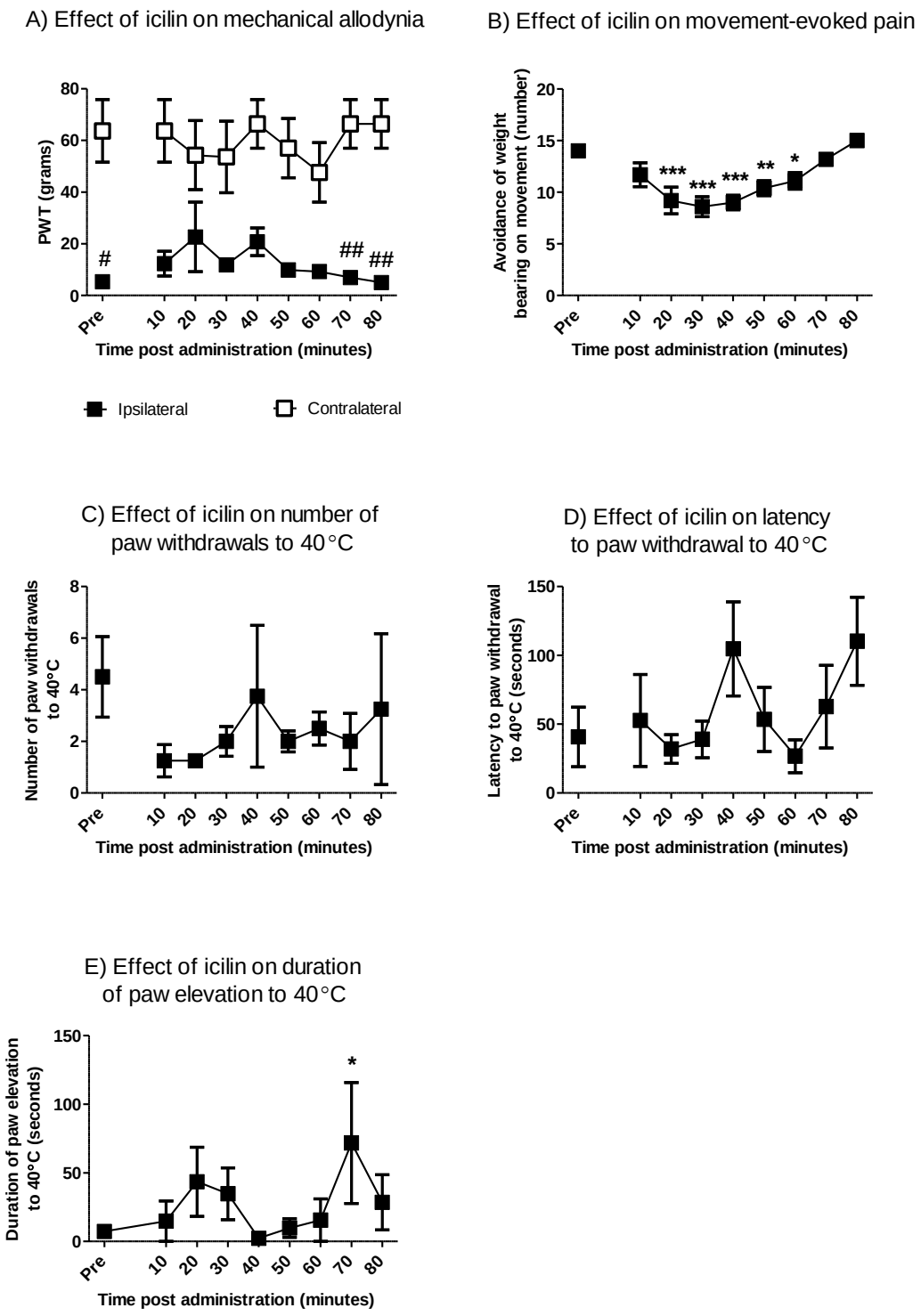


Figure 6.2 Effect of the TRPM8/TRPA1 agonist icilin (100µM, topical application) on CIBP-induced behavioural sensitisation. Data show mean responses ± SEM of CIBP animals (n=4-5). A) Icilin did not significantly alter post-administration ipsilateral PWT to von Frey filaments compared to that pre-administration. Ipsilateral

PWT was significantly different when compared to contralateral PWT pre and at 70 to 80 minutes post-administration (#; One-way repeated measures ANOVA on ranks (Friedman's test) followed by Dunn's post-hoc analysis, $p<0.05$). B) Icilin significantly attenuated movement-evoked pain at 20 to 60 minutes post-administration (*; One-way repeated measures ANOVA followed by Dunnett's post-hoc analysis, $p<0.05$). C) Icilin did not alter number of paw withdrawals to 40°C when compared to pre-administration. D) Icilin did not alter latency to paw elevation to 40^{o}C compared to pre-administration. E) Duration of paw elevation to 40^{o}C was significantly increased at 70 minutes post icilin (*; One-way repeated measures ANOVA followed by Dunnett's post-hoc analysis, $p<0.05$). P values ## = 0.001 to 0.01, # = 0.01 to 0.05, *** = <0.001, ** = 0.001 to 0.01 and * = 0.01 to 0.05.

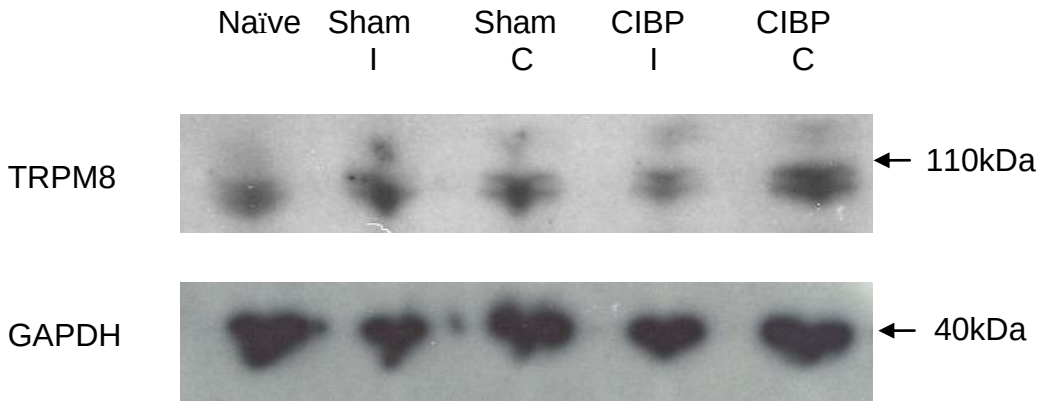
		Time post icilin administration (minutes)								
Test		Pre	10	20	30	40	50	60	70	80
Mechanical allodynia	Ipsi PWT (grams)	5.3	12.3	22.7	11.8	20.7	9.8	9.3	6.9	5.0
	SEM	2.5	4.8	13.5	2.5	5.4	2.2	2.6	2.0	1.8
	Con PWT (grams	63.7	63.7	54.3	53.6	66.5	57.1	47.7	66.5	66.5
	SEM	12.1	12.1	13.4	13.9	9.4	11.5	11.5	9.4	9.4
Rotarod	Number	14.0	11.7	9.2	8.6	9.0	10.4	11.1	13.2	15.0
	SEM	0.7	1.2	1.3	1.0	0.7	0.7	0.8	0.5	0.6
Thermal sensitivity to 40°C	Number	4.5	1.3	1.3	2.0	3.8	2.0	2.5	2.0	3.3
	SEM	1.6	0.6	0.3	0.6	2.8	0.4	0.6	1.1	2.9
	Latency	40.8	52.8	32.0	39.0	104.8	53.5	26.8	62.8	110.3
	SEM	21.5	33.5	10.4	13.3	34.2	23.3	11.9	30.1	32.0
	Duration	7.3	14.8	43.5	34.8	2.3	9.8	15.5	71.8	28.5
	SEM	2.9	14.8	25.2	18.9	2.3	6.8	15.5	44.0	20.0

Table 6.1 The effects of icilin on CIBP-induced behavioural sensitisation showing paw withdrawal threshold (PWT), number of avoidances of weight bearing on movement, number of paw withdrawals, latency to paw withdrawal and duration of paw elevation. Data show mean responses ± SEM of CIBP animals (n=4-5).

6.4.2 The effect of CIBP on levels of TRPM8 expression in DRG

TRPM8 protein was identified by Western blot, where two bands were identified at around 110kDa. TRPM8-transfected cells were used as a positive control sample. TRPM8 protein expression was quantified by determining relative intensity normalised to GAPDH (Figure 6.3). TRPM8 expression did not alter in CIBP Ipsilateral (0.62 ± 0.06) or CIBP Contralateral (0.73 ± 0.05) when compared to Sham V Ipsilateral (0.76 ± 0.04), Sham V Contralateral (0.72 ± 0.04) or Naïve (0.66 ± 0.07), shown by a One-way ANOVA followed by Bonferroni's post-hoc analysis.

TRPM8 expression also did not alter between ipsilateral and contralateral sides within groups, as determined by an unpaired two-tailed t-test.



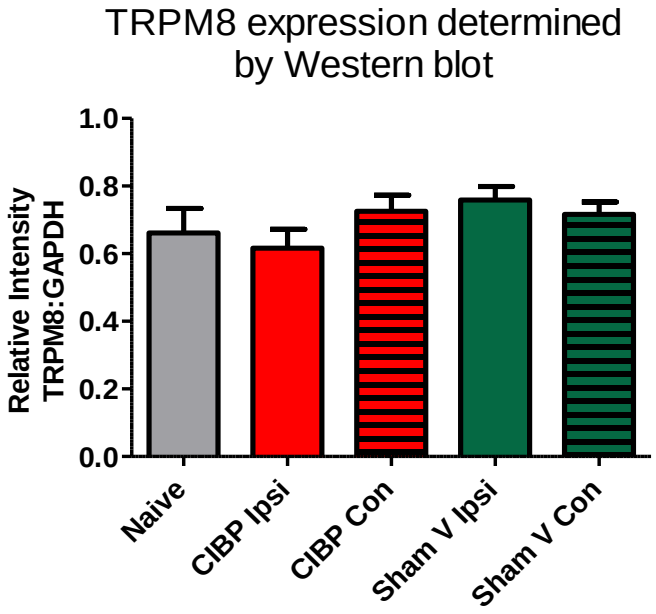


Figure 6.3 TRPM8 expression in CIBP as determined by Western blot. Data from densitometry of film images, as illustrated above, show mean relative intensity of TRPM8 normalised to GAPDH ± SEM in CIBP (n=6), Sham V (n=6) and Naive (n=6) animals. TRPM8 expression did not alter in CIBP when compared to Naïve or Sham V (One-way ANOVA followed by Bonferroni's post-hoc analysis) or between ipsilateral/contralateral sides (unpaired two-tailed t-test). The two bands identified around 110kDa were analysed together. To quantify in Photo Shop, the image was inverted and a box was drawn around the largest band. The mean density was measured (taken as the average of 3 measurements). The same box was dragged over other bands. Mean background density was subtracted from the density of each band.

Corresponding GAPDH bands were measured in the same way. The mean of each band was normalised to GAPDH.

6.4.3 Effect of CIBP on TRPM8 expression within individual DRG cells

The percentage of TRPM8-positive myelinated and unmyelinated cells was determined immunohistochemically by analysing co-expression of TRPM8 with NF200 or peripherin, respectively (Figure 6.4). The percentage of NF200-positive that were TRPM8-positive was not altered in CIBP Ipsilateral (4.09 ± 1.13) or CIBP Contralateral (4.64 ± 1.62) when compared to Sham V Ipsilateral (1.94 ± 0.63), Sham V Contralateral (2.35 ± 0.49) or Naïve (2.12 ± 0.31), shown by One-way ANOVA followed by Bonferroni's post-hoc analysis. The percentage of peripherin-positive cells that were TRPM8-positive was not altered in CIBP Ipsilateral (5.06 ± 1.32) or CIBP Contralateral (5.55 ± 2.34) when compared to Sham V Ipsilateral (2.63 ± 0.63) or Sham V Contralateral (2.86 ± 0.67) or Naïve (5.6 ± 1.23), shown by One-way ANOVA followed by Bonferroni's post-hoc analysis. The total number of TRPM8-positive cells per section was not altered in CIBP Ipsilateral (5.78 ± 0.5) or CIBP Contralateral (5.19 ± 0.91) when compared to Sham V Ipsilateral (6.42 ± 0.64) or Sham V Contralateral (5.47 ± 0.76) or Naïve (7.63 ± 0.89), shown by a One-way ANOVA followed by Bonferroni's post-hoc analysis. No differences in percentage of NF200-positive or peripherin-positive cells that were TRPM8-positive were detected between ipsilateral/contralateral sides within groups, as shown by an unpaired two-tailed t-test (Figure 6.5).

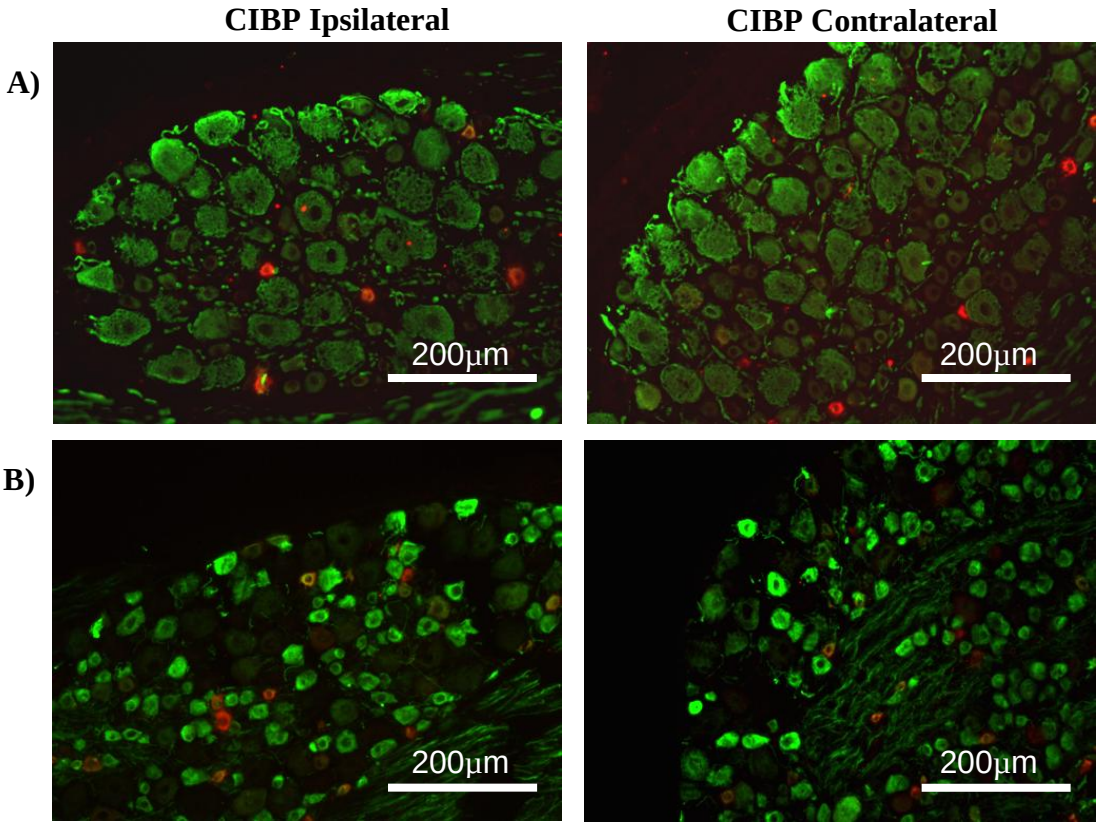


Figure 6.4 Representative immunofluorescence images of CIBP DRG, showing TRPM8 co-expression with markers of myelinated or unmyelinated afferents, NF200 and peripherin, respectively. A) TRPM8 (red) co-expression with NF200 (green) ipsilateral and contralateral to CIBP. B) TRPM8 (red) co-expression with peripherin (green) ipsilateral and contralateral to CIBP.

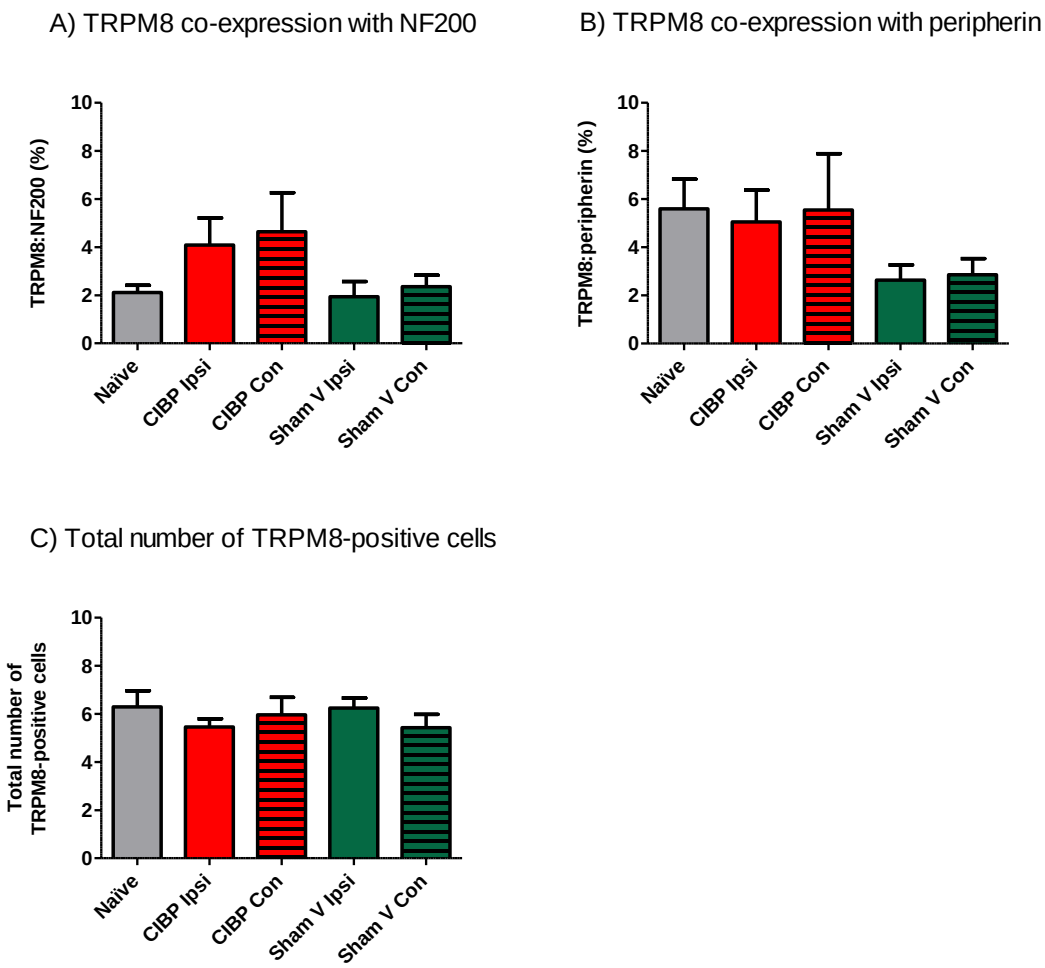


Figure 6.5 Quantification of TRPM8 co-expression with markers of myelinated or unmyelinated afferents as determined by immunofluorescence histochemistry. Data show the mean percentage of cells or mean number of cells expressing TRPM8 ± SEM in CIBP (n=6), Sham V (n=6) and Naive (n=6) animals. A) The percentage of NF200-positive cells co-expressing TRPM8 did not alter in CIBP or between ipsilateral/contralateral sides (One-way ANOVA followed by Bonferroni's post-hoc analysis and unpaired two-tailed t-test, respectively). B) The percentage of peripherin-positive cells co-expressing TRPM8 did not alter in CIBP or between ipsilateral/contralateral sides (One-way ANOVA followed by Bonferroni's post-hoc analysis and unpaired two-tailed t-test, respectively). C) The total number of TRPM8-positive cells did not alter in CIBP or between ipsilateral/contralateral sides (One-way ANOVA followed by Bonferroni's post-hoc test and unpaired two-tailed t-test, respectively).

6.4.4 Effect of the selective TRPV1 antagonist, AMG 9810, on CIBP-induced behavioural sensitivity

Intrathecal administration of the potent and selective TRPV1 antagonist AMG 9810 1nmole (CIBP Day 18-21 n=9) did not attenuate mechanical allodynia, movement-evoked pain or thermal sensitivity to 40°C (Figure 6.6). AMG 9810 did not alter PWT from von Frey filament testing compared to pre-administration values. Ipsilateral PWT threshold was significantly different to contralateral values at all time points shown by a One-way ANOVA on ranks (Friedman's test) followed by Dunn's post-hoc analysis, $p<0.05$. Avoidance of weight bearing on movement did not alter compared to values pre-administration. Number of paw withdrawals to 40°C was significantly decreased at 15 minutes post-administration only, although this was also observed after vehicle control (Figure 6.14). This suggests that at 15 minutes post-administration there may be a residual anaesthetic effect. In fact, intrathecal administration of vehicle alone appeared to significantly decrease avoidance of weight bearing on movement, significantly decrease number of paw withdrawals to 40°C and significantly increase latency to paw withdrawal at 40°C at 15 minutes post-administration (Figure 6.14). Latency to paw withdrawal to 40°C did not alter following AMG 9810 administration. Surprisingly, duration of paw elevation was significantly increased at 45 and 75 minutes post-administration of AMG 9810 compared to pre-administration shown by One-way ANOVA followed by Dunnett's post-hoc analysis, $p<0.05$ (Figure 6.6).

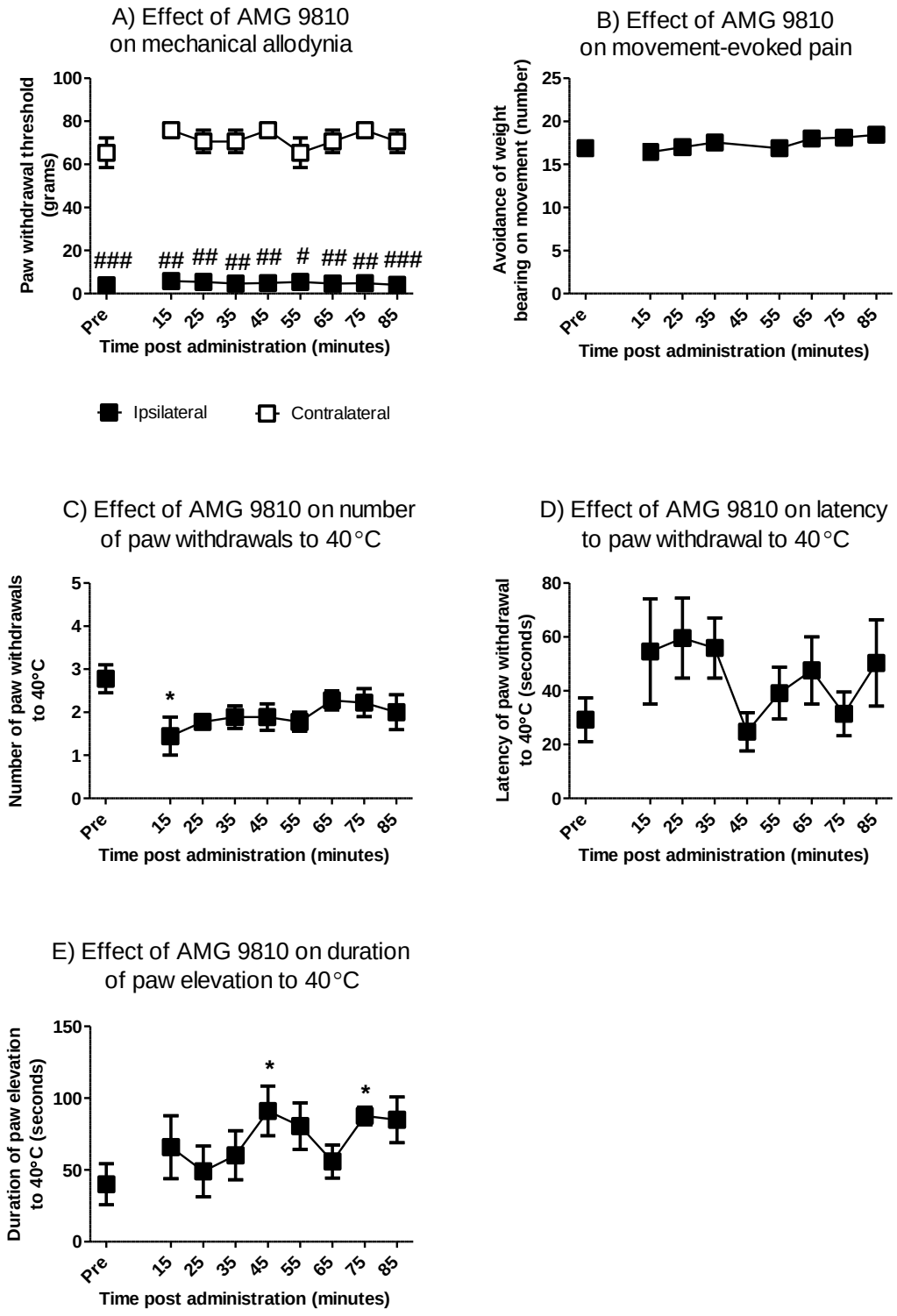


Figure 6.6 Effect of the selective TRPV1 antagonist AMG 9810 (1nmole, i.t. administration) on CIBP-induced behavioural sensitisation. Data show mean responses ± SEM of CIBP animals (n=9). A) PWT to von Frey filaments did not show significantly altered responses compared to values pre-administration.

Ipsilateral PWT was significantly decreased compared to contralateral values at all time points (#; One-way repeated measures ANOVA on ranks (Friedman's test) followed by Dunn's post-hoc analysis, $p<0.05$). B) Avoidance of weight bearing on movement was not altered compared to pre-administration values. C) Number of paw withdrawals to 40°C was significantly decreased 15 minutes post-administration when compared to pre-administration values (*; One-way repeated measures ANOVA followed by Dunnett's post-hoc analysis, $p<0.05$). D) Latency to paw withdrawal to 40°C did not alter post-administration (One-way repeated measures ANOVA followed by Dunnett's post-hoc analysis). E) Duration of paw elevation at 40°C is significantly increased at 45 and 75 minutes post-administration (*; One-way repeated measures ANOVA followed by Dunnett's post-hoc analysis, $p<0.05$). P values ### = <0.001, ## = 0.001 to 0.01, # = 0.01 to 0.05 and * = 0.01 to 0.05.

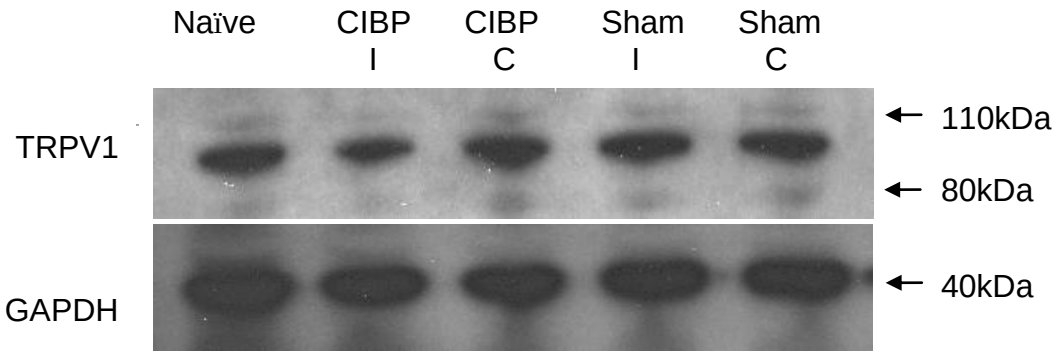
		Time post AMG 9810 administration (minutes)								
Test		Pre	15	25	35	45	55	65	75	85
Mechanical allodynia	Ipsi PWT (grams)	3.9	5.8	5.4	4.7	4.9	5.3	4.7	4.8	4.0
	SEM	0.4	0.8	0.9	0.3	0.3	0.7	0.3	0.6	0.3
	Con PWT (grams)	65.4	75.9	70.6	70.6	75.9	65.4	70.6	75.9	70.6
	SEM	6.9	0.0	5.2	5.2	0.0	6.9	5.2	0.0	5.2
Rotarod	Number	16.9	16.4	17.0	17.6	~	16.9	18.0	18.1	18.4
	SEM	0.8	0.8	0.8	0.7	~	0.8	0.6	0.4	0.4
Thermal sensitivity to 40°C	Number	2.8	1.4	1.8	1.9	1.9	1.8	2.3	2.2	2.0
	SEM	0.3	0.4	0.1	0.3	0.3	0.2	0.2	0.3	0.4
	Latency	29.2	54.6	59.6	55.9	24.8	39.1	47.6	31.4	50.3
	SEM	8.1	19.5	14.9	11.2	7.1	9.6	12.5	8.1	16.0
	Duration	40.0	65.9	49.0	60.2	91.1	80.6	55.9	87.7	84.9
	SEM	14.3	21.9	17.6	17.0	17.3	16.2	11.5	6.2	15.9

Table 6.2 The effects of the TRPV1 antagonist AMG 9810 on CIBP-induced behavioural sensitisation showing paw withdrawal threshold (PWT), number of avoidances of weight bearing on movement, number of paw withdrawals, latency to paw withdrawal and duration of paw elevation.. Data show mean responses ± SEM of CIBP animals (n=9). ~ = not recorded.

6.4.5 Effect of CIBP on levels of TRPV1 expression in DRG

TRPV1 protein was identified by Western blotting with a band at the predicted molecular size of 95kDa. TRPV1 protein expression was quantified by determining relative intensity normalised to GAPDH (Figure 6.7). TRPV1 expression did not alter in CIBP Ipsilateral (0.41 ± 0.03) or CIBP Contralateral (0.47 ± 0.06) when compared to Sham V Ipsilateral (0.53 ± 0.03), Sham V Contralateral (0.48 ± 0.03) or Naïve (0.44 ± 0.03), shown by a One-way ANOVA followed by Bonferroni's post-hoc analysis. TRPV1 expression also did not alter between

ipsilateral/contralateral sides within groups, as determined by an unpaired two-tailed t-test.



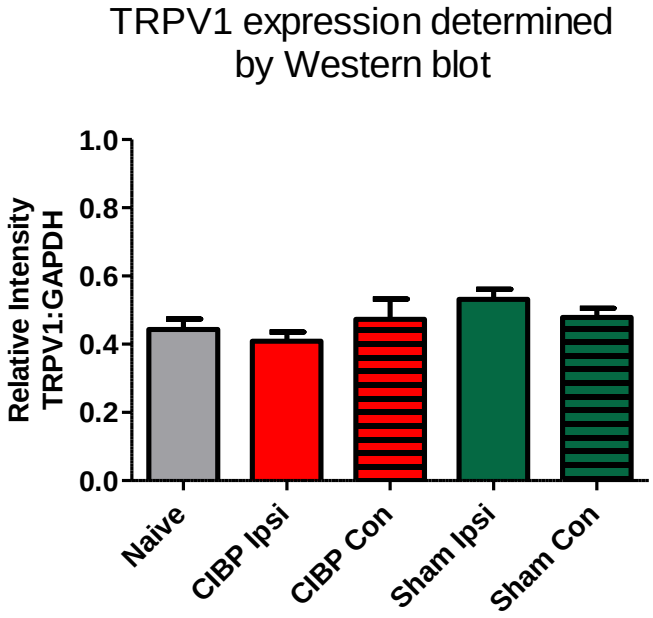


Figure 6.7 TRPV1 expression as determined by Western blot. Data from densitometry of film images as illustrated above show mean relative intensity of TRPV1 normalised to GAPDH ± SEM of CIBP (n=6), Sham V (n=6) and Naive (n=6) animals. TRPV1 expression did not alter in CIBP when compared to Naïve or Sham V or between ipsilateral/contralateral sides. The band at 95kDa was analysed. To quantify in Photo Shop, the image was inverted and a box was drawn around the largest band. The mean density was measured (taken as the average of 3 measurements). The same box was dragged over other bands. Mean background was

subtracted from the density of each band. Corresponding GAPDH bands were measured in the same way. The mean of each band was normalised to GAPDH.

6.4.6 Effect of CIBP on TRPV1 expression within individual DRG cells

The percentage of TRPV1-positive myelinated and unmyelinated cells was determined immunohistochemically by analysing co-expression of TRPV1 with NF200 or peripherin, respectively (Figure 6.8). The percentage of NF200-positive cells co-expressing TRPV1 was not altered in CIBP Ipsilateral (9.06 ± 0.49) or CIBP Contralateral (11.68 ± 1.83) when compared to Sham V Ipsilateral (11.65 ± 1.69) or Sham V Contralateral (9.54 ± 1.56) or Naïve (11.51 ± 1.42) shown by One-way ANOVA followed by Bonferroni's post-hoc analysis. The percentage of NF200-positive cells co-expressing TRPV1 was not altered between ipsilateral/contralateral sides within groups, as shown by unpaired two-tailed t-test. The percentage of peripherin-positive cells co-expressing TRPV1 appeared to be significantly decreased in CIBP Ipsilateral (64.87 ± 3.6) and Sham V Ipsilateral (66.96 ± 2.42) when compared to Naïve (82.02 ± 2.54), shown by One-way ANOVA followed by Bonferroni's post-hoc analysis, $p<0.05$. The percentage of peripherin-positive cells co-expressing TRPV1 was not altered between ipsilateral/contralateral sides within groups as shown by unpaired two-tailed t-test. The total number of TRPV1-positive cells was not altered in CIBP Ipsilateral (31.87 ± 2.97) or CIBP Contralateral (34.19 ± 3.30) when compared to Sham V Ipsilateral (33.61 ± 2.96), Sham V Contralateral (29.49 ± 2.85) or Naïve (33.61 ± 4.21), as shown by One-way ANOVA followed by Bonferroni's post-hoc analysis or between ipsilateral/contralateral sides within groups, as shown by unpaired two-tailed t-test (Figure 6.9).

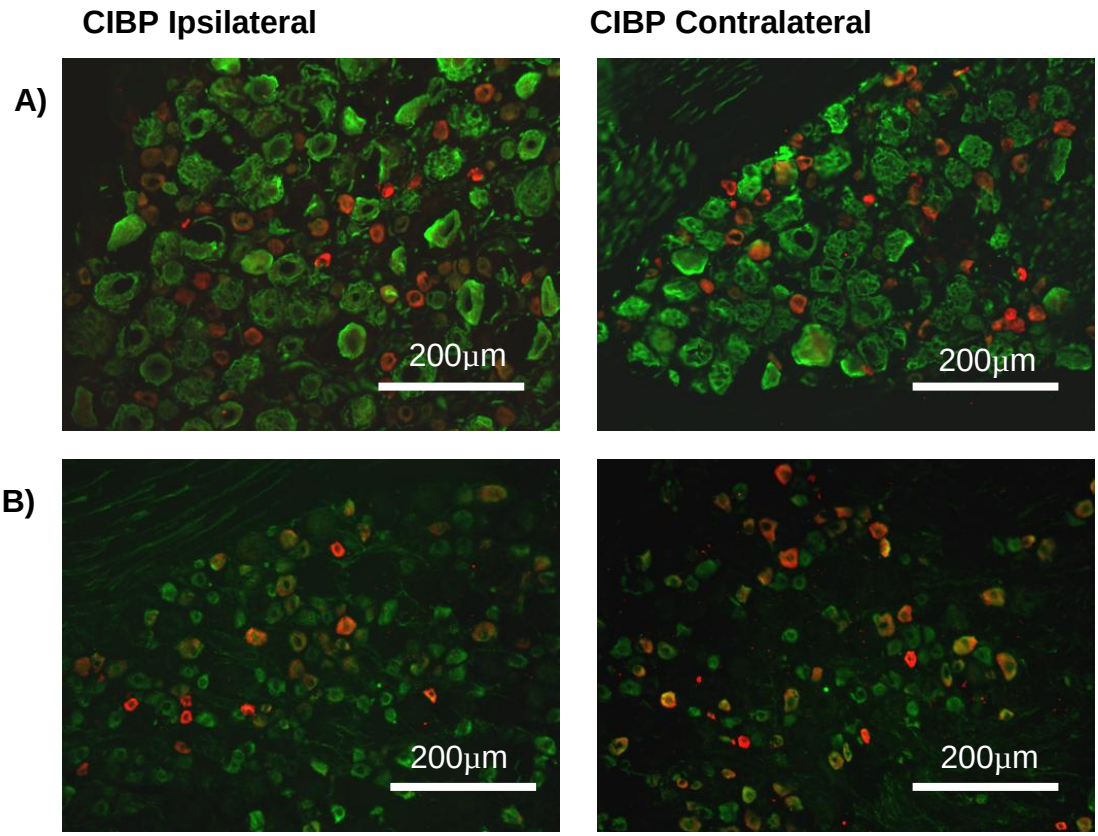


Figure 6.8 Representative immunofluorescence images of CIBP DRG showing TRPV1 co-expression with markers of myelinated and unmyelinated afferents, NF200 and peripherin, respectively. A) TRPV1 (red) co-expression with NF200 (green) ipsilateral and contralateral to CIBP. B) TRPV1 (red) co-expression with peripherin (green) ipsilateral and contralateral to CIBP.

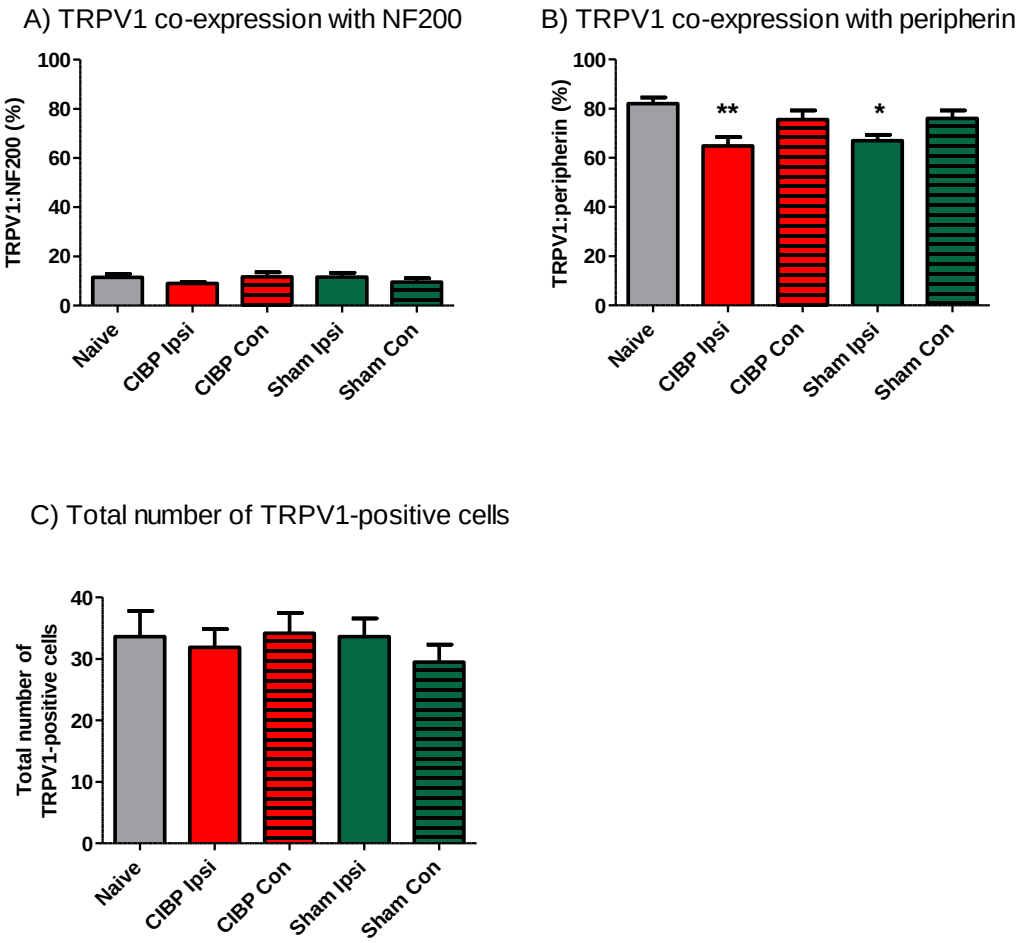


Figure 6.9 Quantification of TRPV1 co-expression with markers of myelinated or unmyelinated afferents as determined by immunofluorescence histochemistry. Data show the mean percentage of cells or mean number of cells expressing TRPV1 ± SEM of CIBP (n=6), Sham V (n=6) and Naive (n=6) animals. A) The percentage of NF200-positive cells co-expressing TRPV1 did not alter in CIBP or between ipsilateral/contralateral sides. B) The percentage of peripherin-positive cells co-expressing TRPV1 is significantly decreased in CIBP Ipsilateral (**) and Sham V Ipsilateral (*) when compared to Naive (One-way ANOVA followed by Bonferroni's post-hoc analysis, where ** p = 0.001 to 0.01 and * p = 0.01 to 0.05). Ipsilateral/contralateral sides however were not significantly different. C) The total number of TRPV1 cells did not alter in CIBP or between Ipsilateral/contralateral sides.

6.4.7 Effect of the selective TRPV4 antagonist RN 1734 on CIBP-induced behavioural sensitisation

The selective TRPV4 antagonist RN 1734 attenuated mechanical allodynia, movement-evoked pain and thermal sensitivity to 40°C (Figure 6.10). Ipsilateral PWT from von Frey filament testing threshold was significantly different from contralateral values pre- and at 25 minutes and 45-85 minutes post-RN 1734 administration as shown by One-way repeated measures ANOVA on ranks (Friedman's test) followed by Dunn's post-hoc analysis, $p<0.05$. RN 1734 significantly altered PWT compared to pre-administration values at 15 and 35 minutes post-administration shown by One-way ANOVA followed by Dunn's post-hoc analysis, $p<0.05$. Avoidance of weight bearing on movement was significantly attenuated at 15 to 35 minutes post-administration when compared to pre-administration, as shown by One-way repeated measures ANOVA followed by Dunnett's post-hoc analysis, $p<0.05$. The number of paw withdrawals to 40°C was significantly decreased at 35 and 85 minutes post-administration only when compared to pre-administration, shown by a One-way ANOVA followed by Dunnett's post-hoc analysis, $p<0.05$. Latency to paw withdrawal to 40°C did not alter post-administration, whereas duration of paw elevation appeared to be significantly increased at 45 to 85 minutes post-administration, shown by One-way ANOVA followed by Dunnett's post-hoc analysis, $p<0.05$.

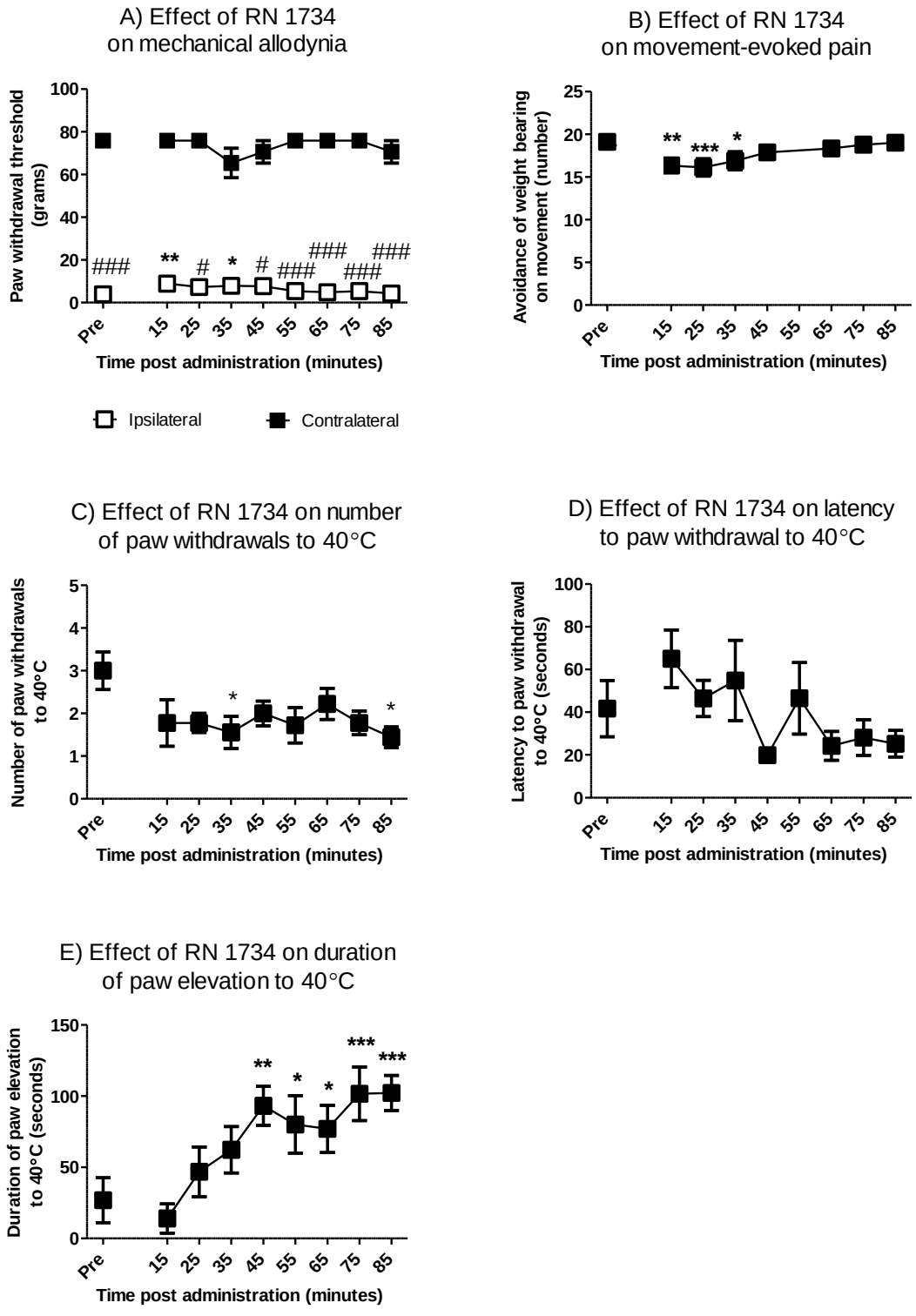


Figure 6.10 Effect of the selective TRPV4 antagonist RN 1734 (5nmole, i.t. administration) on CIBP-induced behavioural sensitisation. Data show mean responses ± SEM of CIBP animals (n=9). A) Ipsilateral PWT was significantly less than contralateral pre-administration and at all later time points except 15 and 35

minutes, at which time it was significantly increased when compared to pre-administration values (# and *, respectively; One-way repeated measures ANOVA on ranks (Friedman's test) followed by Dunn's post-hoc analysis, $p<0.05$). B) Avoidance of weight bearing on movement was significantly decreased at 15 to 35 minutes post-administration compared to pre-administration values (*; One-way repeated measures ANOVA followed by Dunnett's post-hoc analysis, $p<0.05$). C) Number of paw withdrawals to 40°C was significantly decreased at 35 and 85 minutes post-administration (*; One-way ANOVA followed by Dunnett's post-hoc analysis, $p<0.05$). D) Latency to paw withdrawal to 40°C did not alter post-administration compared to pre-administration. E) Duration of paw elevation to 40°C was significantly increased at 45 to 85 minutes post-administration compared to pre-administration values (*; One-way ANOVA followed by Dunnett's post-hoc analysis, $p<0.05$). P values ### = <0.001, # = 0.01 to 0.05, * = 0.01 to 0.05, ** = 0.001 to 0.01 and * = 0.01 to 0.05.

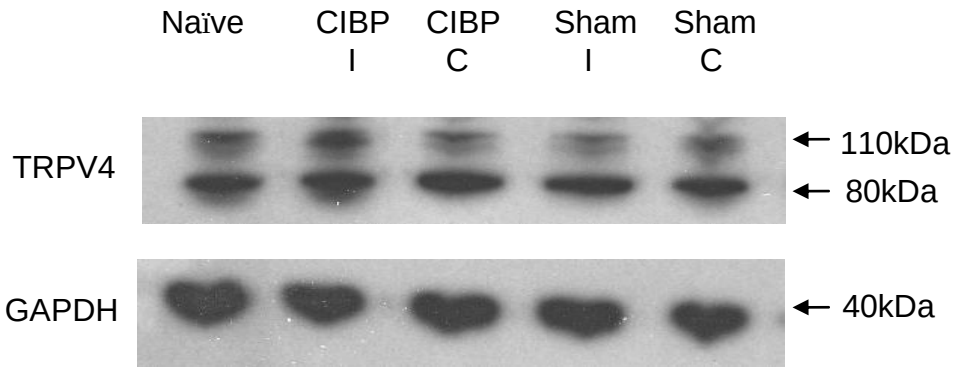
		Time post RN 1734 administration (minutes)								
Test		Pre	15	25	35	45	55	65	75	85
Mechanical allodynia	Ipsi PWT (grams)	3.9	8.9	7.3	7.9	7.7	5.4	4.9	5.3	4.3
	SEM	0.5	0.6	0.8	0.9	1.0	0.9	0.3	0.7	0.3
	Con PWT (grams)	75.9	75.9	75.9	65.4	70.6	75.9	75.9	75.9	70.6
	SEM	0.0	0.0	0.0	6.9	5.2	0.0	0.0	0.0	5.2
Rotarod	Number	19.1	16.3	16.1	16.9	-	17.9	18.4	18.7	19.1
	SEM	0.4	0.6	1.0	1.1	-	0.6	0.5	0.4	0.3
Thermal sensitivity to 40°C	Number	3.0	1.8	1.8	1.6	2.0	1.7	2.2	1.8	1.4
	SEM	0.4	0.5	0.2	0.4	0.3	0.4	0.4	0.3	0.2
	Latency	41.7	65.0	46.4	54.9	19.9	46.6	24.2	28.1	25.2
	SEM	13.2	13.5	8.5	18.8	3.2	16.8	6.8	8.3	6.2
	Duration	26.9	14.0	46.9	62.3	93.2	80.1	77.0	101.7	102.2
	SEM	15.7	10.3	17.5	16.3	13.6	20.2	16.6	18.8	12.3

Table 6.3 The effect of the selective TRPV4 antagonist RN 1734 on CIBP-induced behavioural sensitisation showing paw withdrawal threshold (PWT), number of avoidances of weight bearing on movement, number of paw withdrawals, latency to paw withdrawal and duration of paw elevation. Data show mean responses ± SEM of CIBP animals (n=9).

6.4.8 Effect of CIBP on levels of TRPV4 expression in DRG

TRPV4 protein was identified by Western blotting with two bands at the predicted molecular size of 98-107kDa. TRPV4 protein expression was quantified by determining relative intensity normalised to GAPDH (Figure 6.11). TRPV4 expression did not alter in CIBP Ipsilateral (3.29 ± 0.36) or CIBP Contralateral (2.39 ± 0.08) when compared to Sham V Ipsilateral (2.84 ± 0.65), Sham V Contralateral (2.45 ± 0.36) or Naïve (2.13 ± 0.22), shown by a One-way ANOVA followed by

Bonferroni's post-hoc analysis. TRPV4 expression was significantly increased in CIBP ipsilateral DRG (3.29 ± 0.36) when compared to CIBP contralateral DRG (2.39 ± 0.08), as determined by an unpaired two-tailed t-test, $p<0.05$.



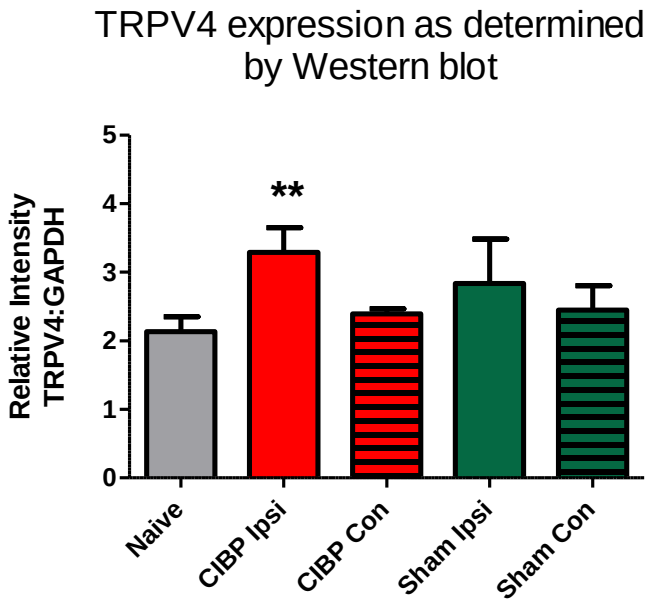


Figure 6.11 TRPV4 expression as determined by Western blot. Data from densitometry of film images as illustrated above show mean relative intensity of TRPV4 normalised to GAPDH ± SEM of CIBP (n=6), Sham V (n=6) and Naive (n=6) animals. TRPV4 expression was not significantly altered between treatment groups but was significantly increased in CIBP Ipsilateral when compared to CIBP Contralateral (**; unpaired two-tailed t-test, p=0.001 to 0.01). TRPV4 expression was not significantly altered between Sham Ipsilateral and Sham Contralateral. The two bands at around 85kDa and 110kDa were analysed. To quantify in Photo Shop,

the image was inverted and a box was drawn around the largest band. The mean density was measured (taken as the average of 3 measurements). The same box was dragged over other bands. Mean background density was subtracted from the density of each band. Corresponding GAPDH bands were measured in the same way. The mean of each band was normalised to GAPDH.

6.4.9 Effect of CIBP on TRPV4 expression within individual DRG cells

The percentage of TRPV4-positive myelinated and unmyelinated cells was determined immunohistochemically by analysing co-expression of TRPV4 with NF200 or peripherin, respectively (Figure 6.12). The percentage of NF200-positive cells that were TRPV4-positive was not altered in CIBP Ipsilateral (60.97 ± 6.04) or CIBP Contralateral (69.96 ± 3.47) when compared to Sham V Ipsilateral (63.11 ± 3.05), Sham V Contralateral (55.48 ± 2.03) or Naïve (58.37 ± 6.39). The percentage of peripherin-positive cells that were TRPV4-positive was not altered in CIBP Ipsilateral (72.62 ± 5.46) or CIBP Contralateral (73.77 ± 3.27) when compared to Sham V Ipsilateral (59.47 ± 5.89), Sham V Contralateral (59.21 ± 3.46) or Naïve (65.42 ± 7.86). The total number of TRPV4-positive cells per section was not altered in CIBP Ipsilateral (66.85 ± 6.09) or CIBP Contralateral (68.84 ± 5.41) when compared to Sham V Ipsilateral (62.26 ± 3.77), Sham V Contralateral (55.19 ± 3.30) or Naïve (53.59 ± 4.15), shown by a One-way ANOVA followed by Bonferroni's post-hoc analysis (Figure 6.13). There were no differences in the percentage of NF200-positive or peripherin-positive cells that were TRPV4-positive between ipsilateral/contralateral sides within groups, as shown by an unpaired two-tailed t-test.

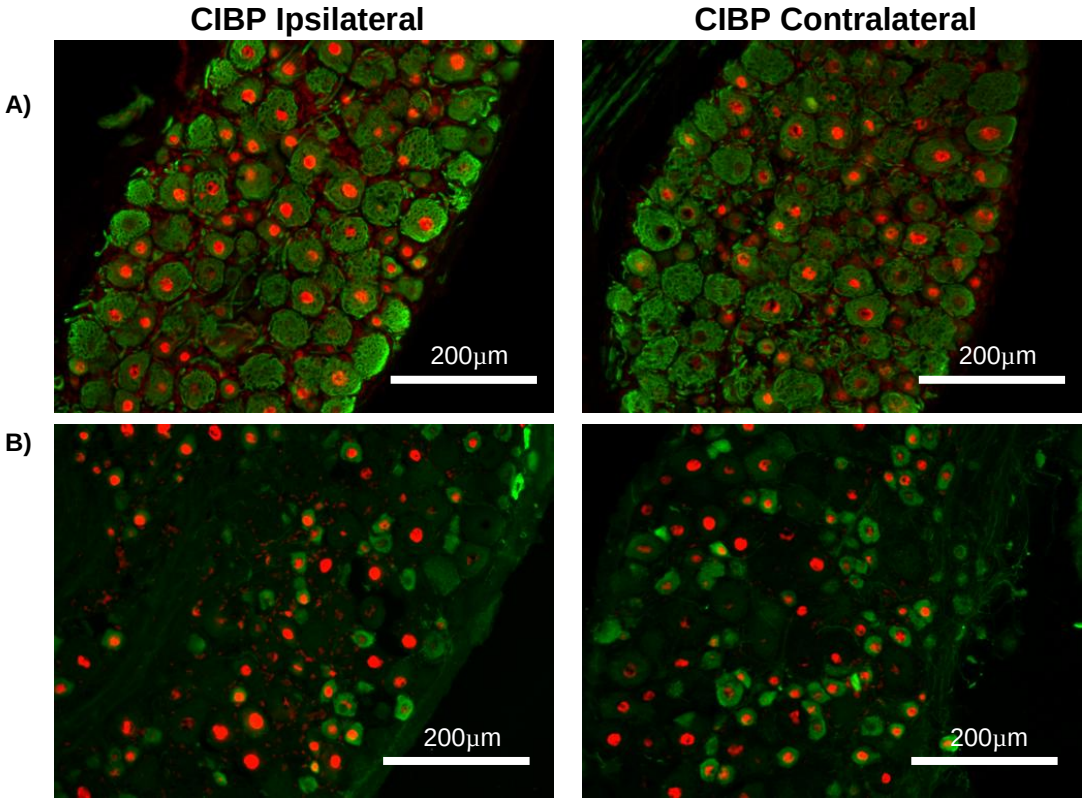


Figure 6.12 Representative immunofluorescence images of CIBP DRG, showing TRPV4 co-expression with markers of myelinated and unmyelinated afferents, NF200 and peripherin, respectively. A) TRPV4 (red) and NF200 (green) co-expression ipsilateral and contralateral to CIBP. B) TRPV4 (red) and peripherin (green) co-expression in CIBP ipsilateral and contralateral.

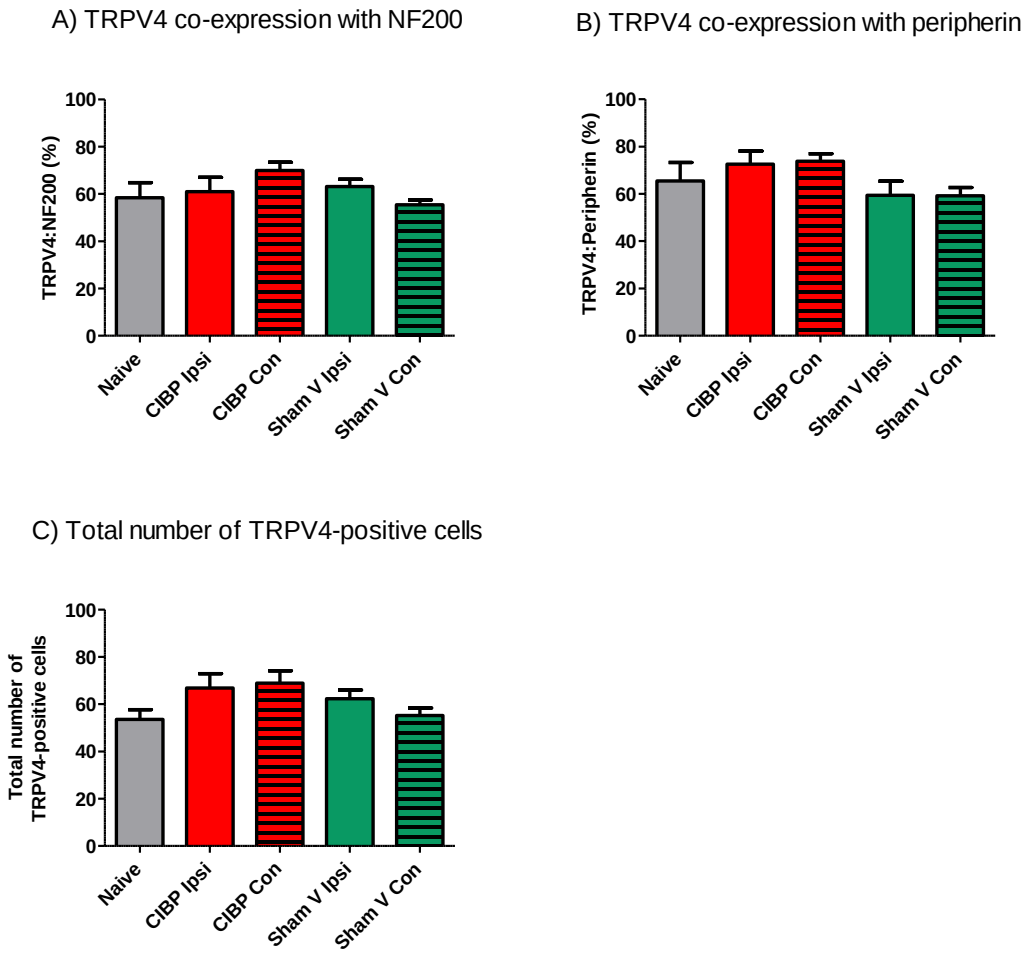


Figure 6.13 Quantification of TRPV4 expression with markers of myelinated or unmyelinated afferents as determined by immunofluorescence histochemistry. Data show the mean percentage of cells or number of cells expressing TRPV4 ± SEM of CIBP (n=6), Sham V (n=6) and Naive (n=6) animals. A) The percentage of NF200-positive cells co-expressing TRPV4 did not alter in CIBP or between ipsilateral/contralateral sides within groups. B) The percentage of peripherin-positive cells co-expressing TRPV4 did not alter in CIBP or between ipsilateral/contralateral sides within groups. C) The total number of TRPV4-positive cells did not alter in CIBP or between ipsilateral/contralateral sides within groups.

6.4.10 The effect of vehicle control for AMG 9810 and RN 1734 on CIBP-induced behavioural sensitisation

Vehicle (0.5% dimethylformamide in saline) control for AMG 9810 and RN 1734 did not significantly attenuate mechanical allodynia. Ipsilateral PWT from von Frey filaments testing was significantly different when compared to contralateral values pre-administration and at 25 – 85 minutes post-administration but not at 15 minutes shown by One-way repeated measures ANOVA on ranks (Friedman's test) followed by Dunn's post-hoc analysis, $p<0.05$. Avoidance of weight bearing on movement was attenuated at 15 minutes post-administration only, shown by a One-way repeated measures ANOVA followed by Dunnett's post-hoc analysis, $p<0.05$. Number of paw withdrawals to 40°C appeared to be significantly reduced at 15 and 65-85 minutes post-administration when compared to pre-administration. Latency to paw withdrawal to 40°C was significantly increased only at 15 minutes post-administration and duration of paw elevation to 40°C appeared to be significantly increased only at 75 minutes post-administration (Figure 6.14); shown in each case by One-way repeated measures ANOVA followed by Dunnett's post-hoc analysis, $p<0.05$. The effect of vehicle control on CIBP-induced behavioural sensitivity at 15 minutes post-administration may be attributed to a residual anaesthetic effect. The decrease in number of paw withdrawals to 40°C at 65-85 minutes post-administration may correspond to increased duration of individual paw elevation events.

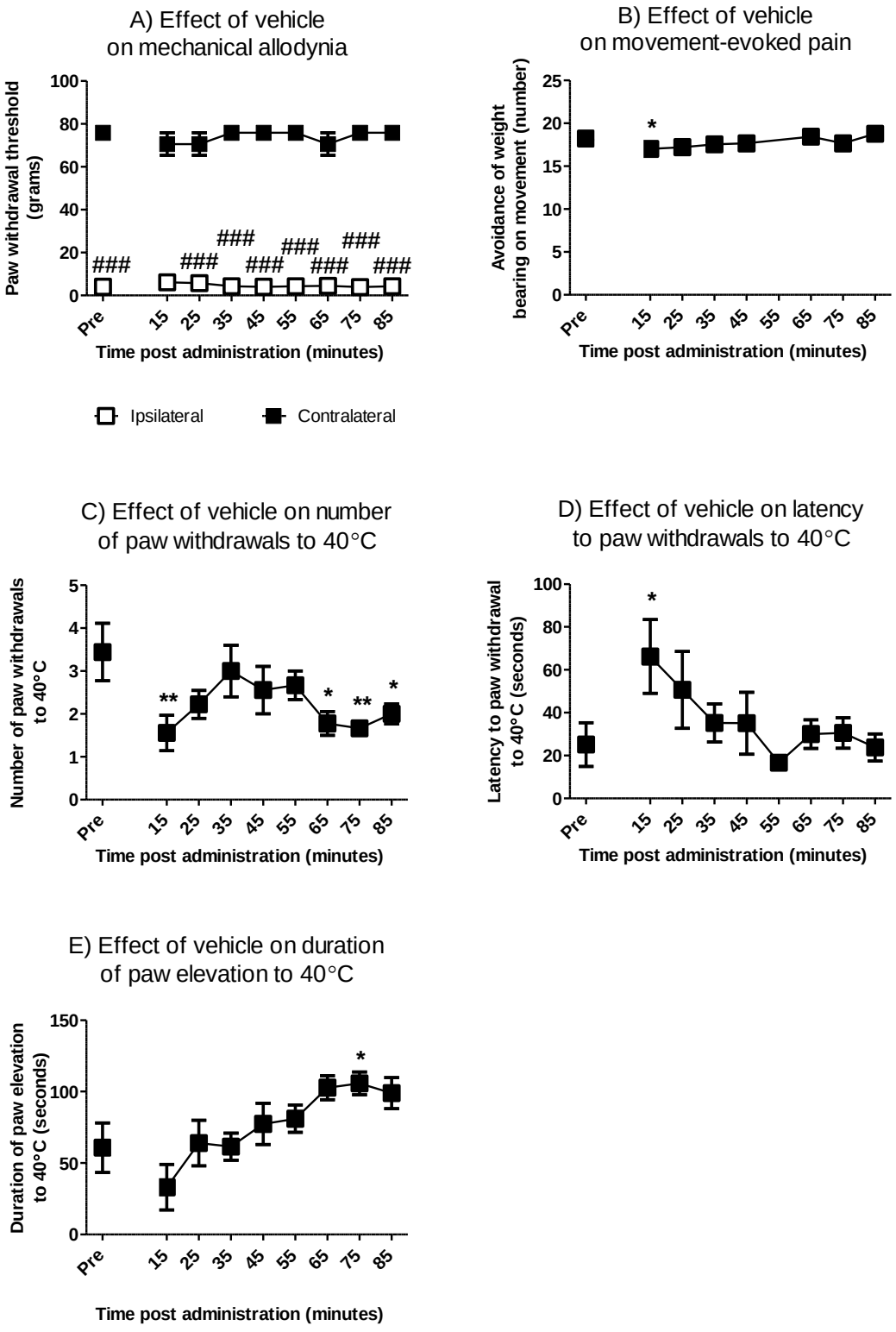


Figure 6.14 Effect of vehicle (0.5% dimethylformamide in saline, i.t. administration) on CIBP-induced behavioural sensitisation. Data shows mean responses ± SEM of CIBP animals (n=9). A) Ipsilateral PWT to von Frey filaments was not significantly

altered when compared to pre-administration values. PWT ipsilateral to CIBP was significantly decreased compared to contralateral PWT except at 15 minutes post-administration (#; One-way ANOVA on ranks (Friedman's test) followed by Dunn's post-hoc analysis, $p<0.05$). B) Avoidance of weight bearing on movement was significantly decreased at 15 minutes post-administration compared to pre-administration (*; One-way repeated measures ANOVA followed by Dunnett's post-hoc analysis, $p<0.05$). C) Number of paw withdrawals to 40℃ was significantly decreased at 15 and 65 to 85 minutes post-administration (*; One-way repeated measures ANOVA followed by Dunnett's post-hoc analysis, $p<0.05$). D) Latency of paw withdrawal to 40℃ was significantly increased at 15 minutes post-administration compared to pre-administration (*; One-way repeated measures ANOVA followed by Dunnett's post-hoc analysis, $p<0.05$). E) Duration of paw elevation to 40℃ was significantly increased at 75 minutes post-administration compared to pre-administration (*; One-way repeated measures ANOVA followed by Dunnett's post-hoc analysis, $p<0.05$).

		Time post vehicle administration (minutes)								
Test		Pre	15	25	35	45	55	65	75	85
Mechanical allodynia	Ipsi PWT (grams)	4.0	6.1	5.7	4.3	4.0	4.3	4.5	3.9	4.3
	SEM	0.3	0.8	0.8	0.3	0.3	0.3	0.4	0.4	0.3
	Con PWT (grams)	75.9	70.6	70.6	75.9	75.9	75.9	70.6	75.9	75.9
	SEM	0.0	5.2	5.2	0.0	0.0	0.0	5.2	0.0	0.0
Rotarod	Number	18.2	17.0	17.2	17.6	17.7	-	18.4	17.7	18.8
	SEM	0.6	0.7	0.5	0.7	0.6	-	0.6	0.7	0.5
Thermal sensitivity to 40°C	Number	3.4	1.6	2.2	3.0	2.6	2.7	1.8	1.7	2.0
	SEM	0.7	0.4	0.3	0.6	0.6	0.3	0.3	0.2	0.2
	Latency	25.1	66.2	50.7	35.2	35.1	16.7	30.0	30.6	23.8
	SEM	10.2	17.2	18.0	8.9	14.5	2.8	6.7	7.1	6.3
	Duration	60.8	33.1	64.0	61.6	77.4	81.1	102.8	105.8	98.9
	SEM	17.3	16.0	15.9	9.4	14.3	9.6	8.4	8.0	10.9

Table 6.4 The effect of vehicle on CIBP-induced behavioural sensitisation showing paw withdrawal threshold (PWT), number of avoidances of weight bearing on movement, number of paw withdrawals, latency to paw withdrawal and duration of paw elevation. Data shows mean responses ± SEM of CIBP animals (n=9).

6.5 Discussion

The TRPM8/TRPA1 agonist, icilin, attenuated movement-evoked pain in this preclinical model of CIBP, which suggests that icilin might be useful in the treatment of movement-evoked pain. The selective TRPV1 antagonist AMG 9810 did not attenuate mechanical allodynia, thermal sensitivity to 40°C or movement-evoked pain. However, the selective TRPV4 antagonist RN 1734 attenuated mechanical allodynia, thermal sensitivity to 40°C and movement-evoked pain in CIBP. Expression of TRPM8 and TRPV1 in the DRG was not altered in this preclinical

model of CIBP. Notably, the expression of TRPV4 in DRG ipsilateral to CIBP was increased, which may play a part in its increased role in CIBP. These results suggest that TRPM8 agonists and TRPV4 antagonists may be useful in the treatment of behavioural sensitisation in this model of CIBP, while no direct evidence to support a role of TRPV1 was obtained in the present study.

6.5.1 The involvement of TRPM8 in CIBP

The TRPM8/TRPA1 agonist icilin (topically applied at 100μM) attenuated movement-evoked pain but did not attenuate mechanical allodynia or thermal sensitivity to 40°C. The attenuation of movement-evoked pain by icilin could be of great significance in the clinic because movement-evoked pain is one of the most difficult components of CIBP to control (Zeppetella, 2009). A previous study from our laboratory showed that topically applied icilin (80μM) reversed mechanical allodynia, (demonstrated using von Frey filaments), and thermal sensitisation to noxious heat, (using the Hargreaves' test), in the following rat models; the CCI model of neuropathic pain, the CFA model of inflammatory pain and the lysolecithin model of demyelination-induced pain (Proudfoot et al., 2006). The mechanism of icilin-induced analgesia is thought to be largely centrally-mediated (Proudfoot et al., 2006). The same study also found an increase in TRPM8 expression in the DRG of CCI rats ipsilateral to nerve injury, occurring in both myelinated Aδ-fibres and unmyelinated C fibres. It appears that in CIBP, there was no increase in TRPM8 expression. In principle, this may contribute to the finding that TRPM8 activation did not reverse mechanical allodynia or thermal sensitivity to 40°C in the CIBP model. The mechanism by which icilin achieves a degree of selectivity in attenuating movement-evoked pain in CIBP would be worth exploring in further studies.

6.5.2 The involvement of TRPV1 in CIBP

The selective TRPV1 antagonist AMG 9810 did not attenuate mechanical allodynia, movement-evoked pain or thermal sensitivity to 40°C in our preclinical model of CIBP. These behavioural results are in contrast with other studies showing that administration of TRPV1 antagonists can effectively attenuate CIBP. Administration of the TRPV1 antagonist JNJ-17203212 injected subcutaneously into

a mouse model of CIBP reduced both ongoing pain and movement-evoked pain as measured by spontaneous flinching and guarding induced by (normally non-noxious) palpation of the distal femur, respectively (Ghilardi et al., 2005). Another study also showed in a murine model of CIBP that systemic administration of the selective TRPV1 antagonist, 5'-iodoresiniferatoxin reduced both movement-evoked and ongoing pain as measured by limb use during spontaneous ambulation, spontaneous flinching and weight bearing (Niiyama et al., 2007). However, systemic administration of another, more potent, TRPV1 antagonist SB366791 reduced the number of spontaneous flinches but did not improve ambulation and weight bearing, suggesting that the precise impact of TRPV1 blockade may be to reduce ongoing but not movement-evoked pain (Niiyama et al., 2009). The authors suggested that these differences in behavioural results may be due to the different pharmacological profiles of TRPV1 antagonists. The TRPV1 antagonist ABT-102 also has been shown to decrease spontaneous pain behaviours and those evoked by thermal and mechanical stimuli in a murine model of CIBP (Honore et al., 2009). Furthermore, this study found that repeated dosing of ABT-102 enhanced its analgesic activity. In view of these results, the current finding that AMG 9810 was without effect is surprising since it is a highly selective and potent TRPV1 antagonist (Gavva et al., 2005) and it was tested at a dose shown to be effective for agents with comparable affinity for other receptors. Nevertheless, results from the literature suggest that other TRPV1 antagonists should be tested for analgesic activity in our preclinical rat model of CIBP and repeated dosing might be more likely to achieve analgesic efficacy.

A study showed that TRPV1 is expressed in sensory neurons innervating mineralised bone and bone marrow (Ghilardi et al., 2005). In this thesis, results showed that TRPV1 expression was not altered in DRG ipsilateral to CIBP at Day 18-21, as determined by Western blot and immunohistochemistry. This result is consistent with a study carried out by Nagae *et al*., which demonstrated in a rat model of CIBP that there was no change in expression of TRPV1 mRNA in DRG ipsilateral to CIBP. Their study used female rats and MRMT-1 cells injected into the tibia to model CIBP (Nagae et al., 2007). However, other studies have demonstrated

increased TRPV1 expression in CIBP models (Ghilardi et al., 2005;Niiyama et al., 2007;Pan et al., 2010). For example, TRPV1 expression was reported to be increased in DRG ipsilateral to CIBP shown by Western blot and real time-PCR (Ghilardi et al., 2005). A recent study showed that in a CIBP model where female Sprague-Dawley rats received an intratibial injection of Walker 256 rat mammary gland carcinoma cells, there was an increase of TRPV1 expression in DRG at Day 14, as determined by Western blotting (Pan et al., 2010). This suggests that an increase in TRPV1 expression might be detected at an earlier time point in our model, although such extrapolation is not necessarily secure and it should be borne in mind that different CIBP models could have quite different characteristics. Studies of TRPV1-deficient mice have also shown that in a CIBP model these mice exhibit reduced hypersensitivty, with no significant increase in flinching behaviour in the cancer-bearing tibia of TRPV1-deficient mice (Ghilardi et al., 2005).

Overall, our study did not find pharmacological evidence for a role of TRPV1 in CIBP hypersensitivity or altered TRPV1 expression at Day 18-21. However, it would be interesting to investigate whether other TRPV1 antagonists showed analgesic efficacy (especially after repeated dosing) and explore whether TRPV1 expression might be increased at different time points in this model.

Analysis of the expression of TRPV1 channels in the present study indicates that TRPV1 shows low expression in myelinated (NF200-positive) cells, although TRPV1 shows high expression in unmyelinated (peripherin-positive) cells. TRPV1 was expressed in around 12% of NF200-positive cells in Naive DRG and around 9% in CIBP Ipsilateral DRG. TRPV1 was expressed in around 82% of peripherin-positive cells in Naïve DRG and around 65% in CIBP Ipsilateral DRG. This is in contrast to TRPM8 expression, which is in only a small number of both NF200-positive and peripherin-positive cells. We showed that TRPM8 was expressed in around 2% of NF200-positive cells in Naïve DRG and 4% of NF200-postive cells in CIBP DRG. TRPM8 was expressed in around 6% of peripherin-positive cells in Naïve DRG and around 5% of peripherin-positive cells in CIBP Ipsilateral DRG.

6.5.3 The involvement of TRPV4 in CIBP

The selective TRPV4 antagonist RN 1734 showed modest but statistically significant attenuation of mechanical allodynia, movement-evoked pain and thermal sensitivity to 40°C at particular time points in this preclinical model of CIBP. These results suggest that TRPV4 may contribute in part to establishing the hypersensitive pain state. Furthermore, Western blot results indicate that TRPV4 expression was significantly increased in ipsilateral DRG in the CIBP model when compared to contralateral or Naïve DRG (unpaired two-tailed t-test). However, TRPV4 co-expression with either NF200 or peripherin was not discernibly altered. Analysis of the expression of TRPV4 channels in the present study indicates that TRPV4 shows high expression in myelinated (NF200-positive) and unmyelinated (peripherin-positive) cells. TRPV4 was expressed in around 58% of NF200-positive cells in Naïve DRG and 61% of NF200 positive-cells in CIBP Ipsilateral DRG. TRPV4 was expressed in around 65% of peripherin-positive cells in Naïve DRG and TRPV4 was expressed in around 72% of peripherin-positive cells.

In future work it would be interesting to investigate the analgesic efficacy of different TRPV4 antagonists, doses and routes of administration as well as the impact of TRPV4 knockout on CIBP-induced behavioural sensitisation.

6.5.4 Effect of vehicle control on CIBP-induced behavioural sensitisation

Vehicle (0.5% dimethylformamide) control administrated intrathecally under isoflurane/O_2 anaesthetic attenuated movement-evoked pain and number of paw withdrawals to 40°C at 15 minutes post-administration. Animals also showed an increased latency to paw elevation to 40°C at 15 minutes post-administration. These results suggest that the anaesthetic may still have an effect at 15 minutes post-administration. Any results with pharmacological agents will therefore be questionable at the 15 minute time point. The number of paw withdrawals to 40°C is also reduced at later time points but it was observed that repeated testing on the thermal footplate often led to a reduction in number of paw withdrawals but an increase in the duration of paw elevation. This may be due to increased avoidance of

ipsilateral hindpaw contact with the thermal footplate, perhaps through an element of behavioural learning.

These results suggest that the anaesthetic effect is a major limitation to this study, at early time points post-pharmacological agent administration. It would be interesting to test the efficacy of topical application of TRPV1 and TRPM8 antagonists. However, as discussed previously (Section 6.1) it has been shown that topical application of a TRPV1 antagonist led to an increase in skin cancer (Li et al., 2011). This might suggest that topical application of TRPV1 antagonists would not be a useful analgesic intervention.

Another limitation in the present study was the small numbers used when testing the effect of topical icilin application on behavioural sensitisation, in particular thermal sensitivity to 40°C (n=4). It would have been interesting to carry out these behavioural tests on a larger sample size. Additionally, because icilin has some agonist activity at TRPA1, it would have been interesting to investigate whether TRPA1 expression is altered in CIBP.

6.6 Conclusion

The current findings suggest that TRPM8 activation delivers an analgesic effect in this model of CIBP whereas TRPV4, but apparently not TRPV1, contributes to the hypersensitive pain state. The present data highlight the potential utility of TRPM8 agonists and TRPV4 antagonists as analgesics in CIBP. These results suggest that TRP channels are potentially useful analgesic targets in CIBP.

7. SUMMARY AND CONCLUSIONS

The primary aim of this study was to define the molecular basis for sensitisation in CIBP to gain a better understanding of the CIBP pain state, which could lead to improved management in the clinic and insights into new therapeutic targets. This study comprehensively characterised behavioural changes in a preclinical model of CIBP. The results show that this CIBP preclinical model elicits sensitisation in evoked pain behaviours; mechanical allodynia and thermal sensitivity and in ongoing pain behaviour; weight bearing difference between hindlimbs. This model also elicits movement-evoked pain and spontaneous foot lifting. The behavioural characteristics seen in this model closely parallel the clinical condition, where breakthrough pain, that may be movement-evoked or occur spontaneously, is the most difficult component to treat. This suggests that the present CIBP preclinical model is a useful model for testing the effectiveness of novel analgesic interventions against breakthrough pain. This model is particularly useful because it allows examination of the effects of isolated bone metastases on pain and nociception, without the effects of widespread cancer.

A further aim of this study was to determine the analgesic efficacy of XRT treatment (the clinical 'gold standard') and therapeutic candidates in the model. A single dose of 8 Gy XRT attenuated thermal sensitivity to 20°C and 40°C and movement-evoked pain in this CIBP model. We found that a single dose of the dual noradrenaline and serotonin reuptake inhibitor duloxetine effectively attenuated mechanical allodynia, movement-evoked pain and thermal sensitivity to 40°C. In comparison, the selective noradrenaline reuptake inhibitor S,S-reboxetine only attenuated thermal sensitivity to 40°C. These results may suggest that duloxetine attenuates movement-evoked pain through inhibition of serotonin reuptake alone, whereas mechanical allodynia and thermal sensitivity may be attenuated through inhibition of noradrenaline reuptake in synergy with inhibition of serotonin reuptake. However, these agents may produce analgesic effects through different mechanisms. Our findings support the idea that duloxetine could be useful in the treatment of CIBP patients in the clinic. In conclusion, this thesis shows that the preclinical model

of CIBP investigated is suitable for testing novel analgesic interventions. This thesis established that radiotherapy and duloxetine act as effective analgesics in this model of CIBP.

The general NMDA receptor antagonist (R)-CPP and a NR2A-selective antagonist were effective at attenuating CIBP-induced behavioural sensitisation. There was also an increased expression the NR2A subunit of NMDA receptors in the dorsal horn of the spinal cord. The specific involvement of NR2A in CIBP appears to be a unique characteristic of this model.

The TRPM8 channel agonist, icilin, attenuated movement-evoked pain but did not attenuate mechanical allodynia or thermal sensitivity to 40°C. TRPM8 expression was not increased in CIBP DRG, although this does not rule out an increase in peripheral afferents not detected at the DRG level. Surprisingly, the TRPV1 antagonist AMG 9810 did not attenuate mechanical allodynia, thermal sensitivity to 40°C or movement-evoked pain. However, interestingly the TRPV4 antagonist RN 1734 attenuated mechanical allodynia, thermal sensitivity to 40°C and movement-evoked pain. Additionally, TRPV4 expression was shown to increase in the ipsilateral DRG in CIBP. This result is particularly interesting because it may suggest that one of the mechanisms of CIBP involves increased expression of TRPV4 in sensory neurons. These results show, for the first time, that TRPM8 and TRPV4 may be a useful analgesic targets in CIBP.

The characterisation of this preclinical model of CIBP shows that this model develops behavioural sensitisation such as mechanical allodynia and weight bearing difference between hindlimbs similar to other preclinical models (Donovan-Rodriguez et al., 2004a;Medhurst et al., 2002;Urch et al., 2003b). These results correspond with a recent clinical study from our group, which found that CIBP patients demonstrate altered thresholds to mechanical and thermal stimuli with increased activation, heightened responsiveness and plasticity of primary afferents (Scott et al., 2011). Furthermore, as shown in some components of CIBP-induced

behavioural sensitisation in the present preclinical model, radiotherapy treatment reversed allodynia, hyperalgesia and the size of the sensitive area (Scott et al., 2011).

The finding that duloxetine effectively attenuated CIBP-induced behavioural sensitisation corresponds with preclinical and clinical studies of osteoarthritis. In a preclinical model of osteoarthritis duloxetine was moderately efficacious in reversing osteoarthritis-induced reduction in grip force (a measure of movement-evoked pain) (Chandran et al., 2009). In a clinical study duloxetine reduced pain and improved function in patients with osteoarthritis of the knee (Chappell et al., 2011).

The present study showed that the general NMDA receptor antagonist (R)-CPP attenuated mechanical allodynia and movement-evoked pain and the NR2A subunit-selective antagonist AAM 077 attenuated movement-evoked pain. However, the NR2B subunit-selective antagonist Ro 25-6981 did not attenuate mechanical allodynia or movement-evoked pain. These results are in contrast with results from neuropathic and inflammatory pain which are reported to involve the NR2B subunit of NMDA receptors, where NR2B-selective antagonists produce analgesia in preclinical models of neuropathic and inflammatory pain (Malmberg et al., 2003a;Qu et al., 2009;Zhang et al., 2009). Furthermore, spinal NR2B expression is increased in neuropathic pain (Labombarda et al., 2008a) and NR2B phosphorylation is upregulated during inflammation (Guo et al., 2002a). Additionally, these results are in contrast with findings from a mouse model of CIBP where the NR2B antagonist was shown to alleviate pain behaviours and NR2B mRNA was increased in the spinal cord (Gu et al., 2010a). The clinical implications of increased NR2A expression in the spinal cord in this preclinical model of CIBP are that CIBP may involve unique downstream signalling pathways compared to inflammatory and neuropathic pain states.

The present study showed that the TRPV1 antagonist AMG 9810 did not attenuate CIBP-induced mechanical allodynia, thermal sensitivity to 40°C or movement-evoked pain. These results contrast with some other studies which show that TRPV1 antagonists can attenuate CIBP-induced behavioural sensitisation

(Ghilardi et al., 2005;Honore et al., 2009;Niiyama et al., 2007). The results of the present study may be different because of the preclinical model used, the dose of TRPV1 antagonist or method of administration of TRPV1 antagonist.

The present study shows that the TRPM8 agonist icilin may be effective in the treatment of movement-evoked pain. A study showed that icilin provided analgesia in the CCI model of neuropathic pain and there was a significant increase of TRPM8 expression in the DRG ipsilateral to injury (Proudfoot et al., 2006). The present results suggest that TRPM8 attenuates movement-evoked pain in CIBP through activation of the native population of TRPM8 channels.

The present study identifies TRPV4 as a potential analgesic target in CIBP, with both behavioural and immunohistochemical evidence that TRPV4 has a role in CIBP. TRPV4 has been shown to be important for thermal and mechanical nociception in preclinical models of neuropathic pain (Alessandri-Haber *et al.*, 2004;Ding *et al.*, 2010a;Zhang *et al.*, 2008b) and thermal nociception in a preclinical model of inflammatory pain (Todaka et al., 2004b). This is the first time that TRPV4 has been shown to be involved in CIBP. It may be that TRPV4 is involved in neuropathic, inflammatory and CIBP pain states and TRPV4 antagonists could be widely applicable analgesic agents.

This thesis has found that different treatments are often effective against different components of cancer-induced bone pain. This suggests that combinatorial studies should be investigated in future work. Such studies would also identify if the analgesic effect of one target would not be hindered/lost when combined with another drug. One of the current clinical studies within our research programme is studying the combined effect of pregabalin and XRT. From my current work there is some evidence of the potential efficacy of duloxetine in CIBP. Future work could examine the combination of radiotherapy + duloxetine in attenuating CIBP.

Single-dose studies were used in this thesis. These doses and routes of administration were chosen based on previous studies (see Table 2.1) to attempt to

identify novel analgesic targets in cancer-induced bone pain. The major limitations of single-dose studies are that higher doses or repeated dosing may have been effective and useful analgesic targets could be missed. For example, Donovan-Rodriguez *et al.* investigated the effect of both acute and chronic administration of gabapentin in a CIBP model and showed that an acute dose of gabapentin had no effect, whereas chronic administration of gabapentin attenuated pain-related behaviours (Donovan-Rodriguez et al., 2005).

Future Directions

Characterisation of this preclinical model of CIBP confirmed that CIBP is a multi-component pain state, as shown in CIBP patients. The model shows evoked pain, ongoing pain and movement-evoked or spontaneous breakthrough pain. This suggests that an effective treatment might be a combination of treatments against these different components.

The results from testing of analgesic interventions indicate that many different mechanisms contribute to CIBP. It is possible that duloxetine produces its marked analgesia in this model through inhibition of both noradrenaline and serotonin reuptake. The impact of a resulting monoaminergic inhibitory influence may affect synapses with increased expression of the NR2A subunit of NMDA receptors. It may be that primary afferents expressing TRPV4 transmit noxious information in CIBP. A point to consider is that any robust effective treatment may need to target all (or at least several) of these mechanisms. It also appears that the different pain-related behaviours observed in this thesis respond in varying degrees to different analgesic interventions.

This thesis also identified some novel targets for the analgesic treatment of CIBP. It would be interesting to ascertain the signalling molecules that interact with the NR2A subunit and the downstream signalling pathways involved in CIBP. For example, this could involve investigating the interactions of the NR2A subunit with PSD-95. In this way, it might be possible to target downstream pathways and target

CIBP in a more specific way. It would also be interesting to investigate whether repeated dosing of a TRPV4 antagonist can attenuate CIBP to a greater extent.

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Appendix: Publications arising from research

Winter Scientific Meeting of the Anaesthetic Research Society, London, December 2009

Oral communication: Central sensitisation in a model of cancer-induced bone pain (CIBP) is dependent on NMDA receptors containing the NR2A subunit

Abstract published in the British Journal of Anaesthesia.
Awarded the Annual Anaesthetic Research Society student prize, 2009.

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CIBP, due to bony metastases, is a major clinical problem, significantly reducing quality of life in cancer patients. Current therapies often provide inadequate analgesia or unacceptable side effects. The spontaneous and movement-induced components of CIBP are particularly challenging. Animal models of CIBP have demonstrated that the underlying neurobiology is unique and different from other chronic pain states[1]. We have investigated the involvement of specific subunits of the NMDA receptor (NMDAR), which is a strong candidate for a role in the spinal plasticity and sensory hypersensitivity in this condition.
Syngeneic MRMT-1 rat mammary gland carcinoma cells were introduced into the intramedullary canal of one tibial bone in anaesthetised Sprague-Dawley rats[2] and analysis of sensory hypersensitivity carried out over 21 days in CIBP, sham and naïve rats measuring: 1. Movement-related pain (abnormal paw movements on a rotating cylinder; weight bearing difference between hindpaws); 2. Mechanical allodynia (withdrawal threshold to von Frey hairs); 3. Thermal sensitivity (paw withdrawals at 40°C); 4. Spontaneous pain (spontaneous foot lifts). Intrathecal NMDAR antagonist (R)-CPP, selective NR2A antagonist (AAM 1077) and selective NR2B antagonist (Ro 25-6981) effects were measured when hypersensitivity was

established. Alterations in neuronal expression levels of the NMDAR subunits NR1, NR2A and NR2B in the dorsal spinal cord were identified by immunofluorescent quantification.

CIBP (n=27-31; mean (SD)) was associated with the development of movement-related pain (15.67 (2.1) versus baseline 0.63 (1.0)); mechanical allodynia (ipsilateral threshold= 643.1(557.5) compared to contralateral threshold = 3179.5 (463.1) mN/mm^2); thermal sensitivity (number of ipsilateral paw withdrawals = 2.5 (1.0) versus baseline 0.92 (0.8); naives 0 (0)); increased weight bearing difference (72.51 (30.3) CIBP versus prior baseline 0.00 (7.1); naïves 1.71 (6.5) gms); and spontaneous pain (spontaneous foot-lifting 77.97 (77.8) seconds) ipsilateral to CIBP. Intrathecal administration of the NR2A selective antagonist attenuated movement-evoked pain (n=4; abnormal paw movements 22 (2.8) decreased to 17.13 (4.6)) and mechanical allodynia (n=4; ipsilateral threshold: 783.6 (158.6) increased to 2285.6 (1174.9) mN/mm^2) in CIBP animals. (R)-CPP attenuated movement-related pain (n=4; abnormal paw movements 20 (0.7) decreased to 16 (2)) and mechanical allodynia (n=4; ipsilateral threshold of 654.18 (0) increased to 2468.61 (992.0) mN/mm^2) in CIBP animals. The NR2B antagonist had no effect. Immunohistochemical analysis found that NR2A but not NR1 and NR2B increased significantly in ipsilateral spinal cord in laminae I and II (n=3; 9.9 (3.4) fluorescence intensity) compared to contralateral (5.4 (3.3)).

The sensory hyperresponsiveness that developed appears to involve the NR2A subunit of the NMDAR. This is a unique finding and distinguishes CIBP from other forms of chronic pain. This has clear implications for developing future targeted therapies.

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North British Pain Association Spring Scientific Meeting, Edinburgh, May 2010 and Neuroscience Day, University of Edinburgh, Edinburgh, May 2010

Poster presentation: Investigating the TRPM8 ion channel as a potential analgesic target for cancer-induced bone pain.

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Aim: Cancer-induced bone pain (CIBP), due to bony metastases, is a major clinical problem. Current therapies often provide inadequate analgesia, particularly for spontaneous and movement-evoked components of CIBP. TRPM8 activation has been shown to produce effective analgesia in chronic pain of neuropathic origin. We used an established rat model of CIBP to investigate the efficacy of topical administration of a TRPM8 agonist icilin on sensory, movement-evoked and affective components of CIBP. We also analysed TRPM8 expression in the dorsal root ganglion (DRG) of CIBP rats.

Methods: In this CIBP model, MRMT-1 rat mammary carcinoma cells were injected into the intramedullary canal of one tibial bone in anaesthetised rats. Ipsilateral sensitisation to mechanical and thermal stimuli was assessed by measuring paw withdrawal from von Frey filaments and 40°C stimuli. Movement-evoked pain was evaluated by measuring rotarod-induced avoidance of weight bearing on movement. Anxiety was assessed by the elevated plus maze. Following topical administration of the TRPM8 agonist, icilin (100μM) we evaluated analgesic efficacy on these components of CIBP. TRPM8 expression in the DRG of CIBP rats was also investigated.

Results and conclusions: Administration of icilin did not reduce CIBP-induced mechanical or thermal (40°C) sensitivity or the anxiety-related component of CIBP. However, icilin did significantly reduce avoidance of weight bearing on movement up to 50 minutes post icilin. Western blot and immunohistochemistry analysis showed no significant change in TRPM8 expression in the DRG of CIBP rats. These results suggest TRPM8 is a potential analgesic target for CIBP-induced movement-evoked pain.

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International Association for the Study of Pain, 13th World Congress on Pain, Montreal, August 2010 and Scottish Neuroscience Group Meeting, Strathclyde University, Glasgow, August 2010

Poster presentation: A comparative analysis of gabapentin and duloxetine as novel analgesic treatments for cancer-induced bone pain.

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Cancer-induced bone pain (CIBP), due to bony metastases, is a major clinical problem, significantly reducing quality of life in cancer patients. Current therapies often provide inadequate analgesia, particularly for spontaneous and movement related components of CIBP, often with unacceptable side effects. Pre-clinical models have shown the underlying neurobiology of CIBP is unique and different from other chronic pain states. Chronic administration of gabapentin can reduce CIBP-induced dorsal horn neuronal responses and pain-related behaviour. Duloxetine, a selective serotonin-norepinephrine reuptake inhibitor, has recently been approved for the treatment of neuropathic pain. Here we investigated the efficacy of acute administration of duloxetine or gabapentin on sensory, movement-evoked and affective components of CIBP.

We used a laboratory model of CIBP, in which MRMT-1 rat mammary carcinoma cells are injected into the intramedullary canal of one tibial bone in anaesthetised rats. We assessed the development of ipsilateral sensitisation to mechanical and thermal stimuli, by measuring paw withdrawal from von Frey filaments and 40°C stimuli. We evaluated movement-evoked pain by measuring rotarod-induced

avoidance of weight bearing on movement. Anxiety was assessed by the elevated plus maze. Following acute administration of gabapentin, duloxetine (both 30mg/kg po) or vehicle from 16 days post-surgery, we evaluated their analgesic efficacy on sensory, movement-evoked and anxiety-related components of CIBP. This study reveals duloxetine to be highly efficacious in attenuating both sensory and movement related components of CIBP.

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Scottish Pain Research Community Launch Event, March 2011

Oral communication: Analysis of gabapentin and duloxetine as novel analgesic treatments for cancer-induced bone pain

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Background: Cancer-induced bone pain (CIBP), due to bony metastases, is a major clinical problem, significantly reducing quality of life in cancer patients. Current therapies often provide inadequate analgesia with unacceptable side effects. Pre-clinical models have shown the underlying neurobiology of CIBP is unique and different from other chronic pain states.

Objective(s): To investigate the efficacy of acute administration of duloxetine or gabapentin on sensory, movement-evoked and anxiety-related components of CIBP.

Methods: We used a laboratory model of CIBP, in which MRMT-1 rat mammary carcinoma cells are injected into the intramedullary canal of one tibial bone in anaesthetised rats. Following acute administration of gabapentin, duloxetine (both 30mg/kg po) or vehicle from 16 days post-surgery, we evaluated their analgesic efficacy.

Results: Acute administration of duloxetine attenuated CIBP-induced ipsilateral sensitivity to thermal (40°C) stimulus up to 24 hours post-injection (from 3.0 ± 0.4 to 0.8 ± 0.2 (at 1hour) and 1.3 ± 0.5 (at 24 hours) mean paw withdrawal ± SEM; n=11) and to mechanical stimuli up to 4 hours post-injection. Gabapentin reversed thermal sensitivity at 3 hours post-injection only (from 3.0 ± 0.3 to 1.6 ± 0.2 mean paw withdrawal ± SEM; n=10) and had no effect on CIBP-induced sensitivity to mechanical stimuli. CIBP-induced movement-evoked pain behaviour was attenuated

up to 4 hours by duloxetine only (from 14.4 ± 0.5 to 10.3 ± 0.7 (at 1hour) mean avoidance of weight bearing on movement ± SEM; n=10). Administration of vehicle had no effect on CIBP-induced ipsilateral sensitivity to either sensory (mechanical and thermal) stimuli or movement-evoked pain behaviour at all time points. Additionally gabapentin or duloxetine treatment did not alter anxiety levels, measured by open-arm time on the elevated plus maze, when compared to CIBP (no treatment) animals.

Conclusions: This study reveals acute duloxetine treatment to be highly efficacious in attenuating both sensory and movement-evoked components of CIBP.