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Studies in a rat model of

cancer-induced bone pain:

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Abstract

Cancer-induced bone pain (CIBP), resulting from primary sarcoma in the bone or metastatic spread to bone, is a common complaint among cancer patients and a major clinical problem. Recent innovations in rodent models of CIBP have facilitated investigation of novel drug treatments and of the mechanisms underlying this condition. The aim of this project was to characterize behavioural and dorsal horn neuronal alterations in a rat model of CIBP, and to study the effects of these alterations on different pharmacological systems.

Following intratibial injection of MRMT1 mammary carcinoma cells to induce CIBP, behavioural testing revealed that cancer animals developed significant mechanical and cold allodynia, as well as ambulatory-evoked pain, commencing on post-operative day 9. Sham-operated animals (injection of cell medium alone) showed no behavioural alterations. *In vivo* electrophysiological characterization of superficial dorsal horn (SDH) neuronal responses to natural (mechanical, heat, brush and cold) and electrical stimuli in the halothane anaesthetized rat revealed neuropathological alterations correlating temporally with the behavioural alterations. There was a marked shift in SDH neuronal populations such that in shams the ratio of wide dynamic range (WDR) to nociceptive specific (NS) cells was 26%:74% whereas in animals with CIBP it was 47%:53%. Furthermore, WDR cells in animals with CIBP had significantly increased responses to mechanical, thermal, and electrical-evoked stimuli compared to shams.

These first studies show that the spinal cord is hyperexcitable in CIBP, probably driven by changes in populations of SDH neurons, and that there is a clear temporal link between behavioural and neuronal alterations proving the latter to be a viable substrate for pharmacological testing in this pain model.

Acute electrophysiological study using the selective 5-HT$_3$ receptor antagonist ondansetron showed that descending facilitatory serotonergic pathways are enhanced in CIBP. Gabapentin and morphine worked both acutely and chronically to normalise the evoked dorsal horn neuronal responses in rats with CIBP. Chronic behavioural studies showed that these drugs also significantly reduced pain behaviour to the pre-operative baseline response and, furthermore, gabapentin shifted the abnormal WDR:NS SDH cell ratio back towards the sham ratio. These investigations therefore confirmed that pain behaviour in CIBP is strongly linked to changes in populations and excitability of SDH neurones, and highlighted gabapentin as a possible novel treatment for CIBP in the clinic.

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Chapter 1.

INTRODUCTION

1.1 Pain

"All pain is one malady with many names." - Antiphanes 414 – *c.* 369 BC

"An unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" – standard definition given by the International Association for the Study of Pain (IASP).

A definition of pain may seem unnecessary as it is a sensation that the vast majority of people will experience. This very fact suggests, quite rightly, that some pains serve an important purpose, but people suffering from intractable persistent pain would argue very strongly against any benefit from these sensations. Clearly there are many types of pain, but whatever the cause it is always an unpleasant experience and in this sense Antiphanes was right.

The modern understanding of pain involves a much more complex system than the first description of a 'pain pathway' by French philosopher René Descartes (Figure 1.1) who, in 1644, used the example of "fire particles" coming into contact with the foot setting in motion a single pathway to the brain.

"If for example fire (A) comes near the foot (B), the minute particules of this fire, which as you know move with great velocity, have the power to set in motion the spot of the skin of the foot which they touch, and by this means pulling upon the delicate thread (cc) which is attached to the spot of skin they open up at the same instance the pore (d.e.) against which the delicate thread ends, just as by pulling at one end of a rope makes to strike at the same instance a bell which hangs at the other end."

(From Melzack and Wall, 1965)
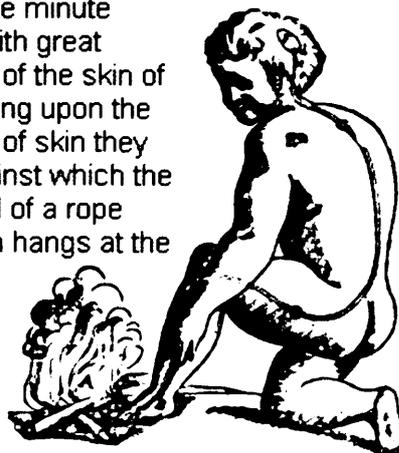
Figure 1.1 The Descartesian model of pain. A single pain pathway that, when triggered, results in a unified proportional response. This has been replaced by a much more complex system which can be modulated at various levels.

We now know that pain perception is multifaceted with sensory-discriminative, affective-motivational, and cognitive-evaluative components (Melzack 1999), involving numerous pathways at the level of the periphery, spinal cord, and higher brain centres, which can be modulated by excitatory and inhibitory systems.

Pain can be divided broadly into two categories according to its duration: acute and chronic. Acute pain, which does not outlast the causal injury, serves as a warning for potential tissue damage and thus has an important physiological purpose. The adaptive response to acute pain is motor withdrawal and/or 'flight' reaction such that there is no longer exposure to the potentially damaging (noxious) stimulus (Millan 1999). The importance of acute pain is demonstrated by the morbidity of conditions resulting in insensitivity to pain, with sufferers tending to die in childhood as a consequence of failure to notice tissue injury or illness (Nagasako et al. 2003). Chronic pain, which outlasts tissue or nerve damage, or where damage is unresolved due to ongoing disease, has no obvious physiological value and thus no adaptive value. Furthermore, chronic pain is often accompanied by hyperalgesia (increased pain sensation to a noxious stimulus), allodynia (pain sensation to a normally innocuous stimulus), paresthesias (abnormal sensations, both evoked and spontaneous, that are not unpleasant), and dysthesias (abnormal sensations, both evoked and spontaneous, that are unpleasant). The characteristics of these two major classes of pain are summarized in Table 1.1

A systematic review has found severe chronic pain to have a prevalence of around 8% in children and 11% in adults (Harstall and Ospina 2003) representing an immense number of people worldwide. It is clear that improved understanding of processes underlying chronic pain states, and subsequently better management in the clinic, is an area of utmost importance. The introductory chapter of this thesis gives a general discussion of nociceptive processing, that is to say the processes by which pain information is transmitted

from the periphery to the central nervous system (CNS), as a preface to the studies which will be described on cancer-induced bone pain, a type of chronic pain of major clinical importance.

Table 1.1 Classification of major pain types.

Type	Duration	Temporal features in relation to cause	Major characteristics	Adaptive response	Examples
Acute	Seconds	Instantaneous Simultaneous	Proportional to cause	Withdrawal 'flight' Escape	Contact with hot surface
Chronic	Months to years	Persistent Long-term disease May exceed tissue damage	Hyperalgesia Allodynia Spontaneous Paresthesias Dysthesias	Psychological and cognitive	CNS injury, arthritis, metastatic disease.

Adapted from (Millan 1999)

1.1.1 Cancer-Induced Bone Pain (CIBP)   Pain from metastatic disease, as listed in the table above, represents one of the major classes of chronic pain which, in the presence of ongoing illness, is very difficult to treat effectively with the drugs currently available. It is estimated that up to 75% of cancer patients in the advanced stages of disease have severe pain (Foley 2004). There are many types of cancer pain which can be sub-classified according to cause:

- Pain from treatment – chemotherapy-induced neuropathy and the associated pain, for example, present a problem in treatment of cancer.
- Pain from procedures – there are a number of potentially painful procedures that might be employed, including lumbar punctures and bone marrow aspirations.

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- Pain from disease – many metastatic diseases are associated with ongoing pain, and CIBP represents the most common cancer-related pain (Mercadante 1997).

CIBP, which can affect patients with primary sarcomas of the bone or metastatic invasion of bone, consists of a triad of pain states: tonic pain (this background pain, often described as a 'dull ache', increases in intensity with disease progression), spontaneous pain, and movement-evoked pain (Mercadante 1997; Portenoy et al. 1999). The movement-evoked pains, also known as incident pain, are acute severe episodes of pain and pose the main clinical problem in treatment of CIBP as doses of analgesics, such as opioids, which would control these episodes result in unacceptable side-effects (Bruera et al. 1995; Mercadante et al. 1992). Hence, this type of pain is sometimes referred to as 'breakthrough pain', due to the fact that it breaks through the analgesic barrier.

There has been a paucity of information surrounding the neurobiology of CIBP due to a lack of suitable and reliable animal models. Previously, the models entailed systemic injection of carcinoma cells, resulting in systemically unwell animals with multiple randomly sited metastases (Kostenuik et al. 1993; Sasaki et al. 1998) thus precluding systematic investigation of any neuronal and pharmacological alterations. Recently, however, a murine model of CIBP was described that mimicked the clinical condition in terms of bone destruction and development of pain, but where the tumour was confined to a single bone (Schwei et al. 1999). The model involves injection of a sarcoma cell line into the femur and characteristically shows progressive bone destruction, elevated osteoclast activity (see 1.6 Structure and innervation of bone), and progressive behaviour indicative of pain (both spontaneous and evoked). Development and alteration of the model by other groups include using different species (rats), cancer lines (fibrosarcoma, melanoma, adenocarcinoma), and bones (humerus, calcaneus) (Medhurst et al. 2002; Sabino et al. 2003; Wacnik et al. 2001; Wacnik et al. 2003). All of these investigations have begun to shed light on the mechanisms underlying CIBP and have shown that, although sharing some

features with neuropathic and inflammatory pain, it is in fact a unique pain state. Studies in this area will hopefully provide better drug targets for use in the clinic.

1.2 Primary afferent fibres

The primary afferent fibres are the specialized fibres responsible for conveying sensory information from the periphery to the CNS. Their cell bodies are located in the dorsal root ganglia (DRG) and they synapse within the CNS at the level of the dorsal horn of the spinal cord, entering via the dorsal root (see 1.3 Dorsal horn architecture, and Figure 1.3). The fibres that selectively respond to noxious stimuli and are thus responsible for transmitting pain information are termed *nociceptors*.

1.2.1 Classification of primary afferent fibres The cutaneous primary afferents have been grouped into three main types according to their structure, diameter, and conduction velocity:

Aβ fibres thickly myelinated, large diameter (>10μm), fast conduction (30-100 m s^{-1})

Aδ fibres thinly myelinated, medium diameter (2-6μm) and conduction (12-30 m s^{-1})

C fibres unmyelinated, small diameter (0.4-1.2μm), slow conduction (0.5-2 m s^{-1})

Normally the low threshold Aβ fibres transmit only information regarding innocuous stimuli to the brain, but it is thought that under pathological conditions they are responsible for mediating mechanical allodynia (Ma and Woolf 1996).

The nociceptors have been sub-classified according to their response characteristics. Many nociceptors respond to a variety of stimulus modalities, including mechanical, thermal, chemical, and cold for example. However, mainly heat and mechanical stimuli have been used in their classification and as a result the terms CMH and AMH have been adopted to refer to the C-fibre mechano-heat sensitive nociceptors and A-fibre mechano-heat sensitive

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nociceptors respectively (Meyer et al. 1994). Table 1.2 summarizes the main characteristics of the primary afferent fibres.

Table 1.2 Characteristics of primary afferent fibres.

Fibre class	Activation threshold	Sensation mediated	Diameter (μm)	Myelination	Conduction Velocity (ms^{-1})
Aβ	low	innocuous	>10	thick	30 – 100
Aδ	high	noxious	2 – 6	thin	12 – 30
C	high	noxious	0.4 – 1.2	none	0.5 – 2

Two subtypes of AMH nociceptors have been identified. Type I AMH nociceptors have high heat thresholds (>50°C) and low conduction velocities of between 30 - 55 m s^{-1} putting them somewhere between typical Aβ and Aδ fibre categories. Type II AMH nociceptors have lower heat thresholds and lower conduction velocities of around 15 m s^{-1}. The activation of type II AMH nociceptors is responsible for the 'sharp', rapid, first phase of pain, whilst activation of the CMH nociceptors, whose responses increase monotonically with stimulus intensity, is responsible for the 'dull' second wave of pain (Millan 1999).

A third type of 'silent' nociceptors has been identified. These small diameter primary afferents are, under normal conditions, irresponsive to noxious stimuli but under pathological conditions such as nerve injury or inflammation they may become sensitive to a variety of chemical mediators (Millan 1999).

1.2.2 Inflammatory mediators   There is a plethora of substances produced and released during inflammation and tissue damage that can activate and, moreover, sensitize primary afferent fibres. Although a full account of these

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mediators is beyond the scope of this introduction they cannot go without mention as cancer cells produce an inflammatory infiltrate and release a cocktail of growth factors, cytokines, chemokines, prostanoids and endothelins leading to a reduction of pH to acidic levels. The results of this are activation, sensitization and direct deformation of primary afferents (Griffiths 1991; Safieh-Garabedian et al. 1995; Sorkin et al. 1997; Woolf et al. 1997). Figure 1.2 gives a pictorial summary of the inflammatory mediators acting on nociceptors. For more detailed accounts of the roles of these mediators the reader is directed to the following reviews: (Apfel 2000; Dray 1995; Dray 1997; Funk 2001; McMahon 1996; Ueno and Oh-ishi 2002).

1.2.3 Ion channels The excitability of nerve cells that allows them to transmit information throughout the body is a result of electrical potentials across the cell membrane maintained by ion pumps and ion channels contained therein, the principal ions being Na^+, K^+, Ca^{2+}, and Cl^-. The resting membrane potential is negative as a result of the ion distribution maintained by the pumps and channels: low Na^+ and Cl^- and high K^+ inside the cell, and high Na^+ and Cl^- and low K^+ outside the cell. An action potential, which is basically a brief wave of reversal of the membrane potential propagated along the axon away from the cell body, occurs when Na^+ channels open and allow Na^+ ions to flow along the concentration gradient into the cell. Ca^{2+} channels then open allowing Ca^{2+} to enter the cell initiating neurotransmitter release and K^+ outflow which eventually brings the action potential to an end. As excitable membranes, and thus neuronal activity, are dependent on ion channels, it is obvious that they should have a major role in pain processing.

Figure 1.2 Schematic representation of the influence of inflammatory mediators on primary afferent fibre. Following injury and inflammation a cocktail of mediators is secreted by blood vessels and immune cells activating primary afferent terminals by increasing conductance of sodium (gNa^+) and calcium (gCa^{2+}) channels, increasing intracellular calcium, decreasing conductance of potassium channels (gK^+) and activation of second messenger systems (adenylate cyclase (**AC**), phospholipase C (**PLC**)). This peripheral sensitization results in increased neurotransmitter release and neuronal excitability which in turn causes central sensitization leading to hyperalgesia and allodynia.

Hist. – histamine, **NGF** – nerve growth factor, **PG** – prostaglandins, **NO** – nitric oxide, **ATP** – adenosine triphosphate, **SP** – substance P, **CGRP** – calcitonin gene-related peptide, **EAA** – excitatory amino acid.

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1.2.3 (i) Sodium channels   All voltage-gated sodium channels
(VGSCs) have a similar structure consisting of a large pore-forming
functional α-subunit, comprised of four domains of six transmembrane
segments, and two auxiliary β-subunits - β1, β1A, β2 and/or β3 (Catterall
2000a). VGSCs are responsible, as described above, for the depolarization
phase of the action potential and local anaesthetics act by blocking these
channels. Sodium currents in sensory neurones can be differentiated by
their gating kinetics, voltage dependence, and sensitivity to the non-
competitive inhibitor tetrodotoxin (TTX). Table 1.3 details the nine α-subunits
expressed by sensory neurones.

The sodium channel subunits expressed almost exclusively by small
diameter sensory neurones are the subjects of much interest with respect to
nociceptive processing and they have been shown to have important roles in
both inflammatory and neuropathic pain states as the expression and
function of the channels changes in these pathologies (Benham 2001; Lai et
al. 2003). The $Na_v1.8$ channel is of particular interest, its level of expression
and its TTX-r sodium current are increased in models of inflammation, an
increase which persists for months and is consistent with a role for this
channel in the maintenance of hyperalgesia (Gold 1999; Khasar et al. 1998).
Furthermore, knockdown of $Na_v1.8$ does not alter baseline sensitivity to
noxious thermal stimuli of otherwise normal rats, but in a model of
mononeuropathy reverses neuropathic pain normalising responses to
thermal and mechanical stimuli thus providing direct evidence of a role for
this channel in neuropathic pain (Lai et al. 2002). This brief discussion of
sodium channels is enough to make clear that selective blockers of TTX-r
sodium currents, in particular $Na_v1.8$, could provide very useful tools for the
treatment of pain. For detailed discussions of the role of sodium channels in
pain processing see the following reviews: (Benham 2001; Cummins et al.
2000; Gold 1999; Lai et al. 2003; Wood et al. 2002).

Table 1.3 Mammalian cloned voltage-gated sodium channel α-subunits of sensory neurones. Adapted from (Baker and Wood 2001) and (Lai et al. 2003).

Channel	TTX-sensitivity	Tissue localization	Null phenotype	Abundance in DRG
$Na_v1.1$	TTX-s	Mainly CNS but also DRG and motorneurones.	Epilepsy, seizures	Present
$Na_v1.2$	TTX-s	Predominantly CNS	Lethal	Present
$Na_v1.3$	TTX-s	Embryonic nervous system, adult CNS, injured DRG		Upregulated in axotomy
$Na_v1.4$	TTX-s	Skeletal muscle		Absent
$Na_v1.5$	TTX-r	Heart		Absent
$Na_v1.6$	TTX-s	DRG and motorneurones, also CNS	Multiple neurological and neuromuscular dysfunctions	Abundant
$Na_v1.7$	TTX-s	Predominantly DRG, also CNS		Abundant
$Na_v1.8$	TTX-r	DRG (80% small diameter, 20% large diameter)	Moderate analgesia, may be due to $Na_v1.7$ upregulation.	Abundant
$Na_v1.9$	TTX-r	DRG (small diameter), also in hippocampus		Abundant

TTX-r – TTX resistant, TTX-s – TTX sensitive

1.2.3 (ii) Acid-Sensing Ion Channels (ASICs) ASICs are H^+-gated receptor channels that were first cloned based on their homology to ENaC/degenerin (ENaC/DEG) channels, a family of epithelial sodium channels identified from a genetic screen of the mechanosensory pathway of the nematode worm *Caenorhabditis elegans* (Kellenberger and Schild 2002). There are four ASIC genes (ASIC1-4) with two isoforms of ASIC1 (ASIC1a and ASIC1b) and two isoforms of ASIC2 (ASIC2a and ASIC2b) (Krishtal

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2003). ASICs have widespread distribution in the mammalian nervous system, both central and peripheral (Kellenberger and Schild 2002; Krishtal 2003). In the PNS ASICs are found mainly in small diameter primary afferents (Chen et al. 1998) suggesting a role in pain processing. Electrophysiological studies show a role for ASIC3 in pain from cardiac ischemia and transient global ischemia induces expression of ASIC2a protein in neurones that survive the event (Kellenberger and Schild 2002). ASIC expression has also been shown to be facilitated by inflammation and in addition pro-inflammatory mediators such as NGF, 5-HT, interleukin-1 and bradykinin have been shown to excite primary afferents via expression of ASICs (Mamet et al. 2002; Voilley et al. 2001)

As these channels belong to the same family as mechanosensitive channels it follows that they might also have a role in mechanotransduction. ASIC distribution studies have indeed shown that they are expressed in sensory terminals, consistent with a possible role in mechanosensation (Price et al. 2001). Also, in ASIC2 knockouts Aβ fibres have reduced firing rates in response to suprathreshold stimulation whilst other fibre types maintain normal responses suggesting that ASIC2 plays a role in setting the sensitivity of low threshold mechanoreceptors (Price et al. 2000). Furthermore, ASIC3 null mutant mice have deficits in neuronal response to mechanical stimulation as well as protons (Price et al. 2001). These studies clearly show a role for ASICs in mechanotransduction and nociception, although the mechanisms by which they perform these roles are still unclear. For reviews of the involvement of ASICs in pain see (Kellenberger and Schild 2002; Krishtal 2003; Waldmann et al. 1999).

1.2.3 (iii) Calcium channels

Calcium entry into cells through voltage-gated calcium channels (VGCCs) mediates a plethora of responses including muscle contraction, gene transcription, neurotransmitter release and regulation of neuronal excitability (Snutch et al. 2001). VGCCs are made up of a number of subunits; the α_1-subunit forms the basic structure of the channel containing amino acids that form the pore and voltage sensor, and

there are also auxiliary subunits - β, $\alpha_2\delta$, and γ - which modulate the function of the α_1-subunit (Catterall 2000b) which itself determines the major functional properties of the channel and defines its subtype. A number of VGCC subtypes have been identified and classified according to their electrophysiological and pharmacological properties into T-, N-, L-, P/Q- and R-types, also referred to according to the order in which the mRNA was cloned (A-I) and gene homology (Catterall 2000b):

- L-type (skeletal muscle) α_{1S} $Ca_v1.1$
- L-type (cardiac muscle) α_{1C} $Ca_v1.2$
- L-type (endocrine) α_{1D} $Ca_v1.3$
- L-type (retina) α_{1F} $Ca_v1.4$
- P/Q-type α_{1A} $Ca_v2.1$
- N-type α_{1B} $Ca_v2.2$
- R-type α_{1E} $Ca_v2.3$
- T-type α_{1G} $Ca_v3.1$
- T-type α_{1H} $Ca_v3.2$
- T-type α_{1I} $Ca_v3.3$

The Ca_v2 channels are those of interest with respect to nociceptive processing as they are primary neuronal, found in the soma, dendrites, and nerve terminals (Elmslie 2004). The $Ca_v2.2$ channel has been the focus of particular attention as it seems to have a pathological role in pain conditions, selective blockade of the channel through intrathecal administration of ω-conotoxin GVIA or ω-conotoxin MVIIA having been shown to depress thermal hyperalgesia, mechanical allodynia, and post-surgical pain (Snutch et al. 2001). Furthermore, the $Ca_v2.3$ channel, which is expressed at high levels in the superficial dorsal horn as well as primary afferent neurones of the DRG, has been suggested to have a role in inflammatory pain as α_{1E} mutant mice show a lowered formalin phase 2 response (Saegusa et al. 2000).

As with the VGSCs discussed above, selective blockers for the calcium channels implicated in pain states provide an attractive target for pharmacotherapy but at present specificity and pharmacodynamics are

problematic (Elmslie 2003). However, there are a couple of $Ca_v2.2$ blockers that show promise: ziconotide which has antinociceptive actions in animal models of persistent, post-operative, and neuropathic pain with potency several orders of magnitude higher than morphine after intrathecal administration (Snutch et al. 2001), is now under consideration by the FDA as a treatment for neuropathic pain, and AM336 which after intrathecal dosing in rats inhibited substance P release and produced potent antinociception (Scott et al. 2002; Smith et al. 2002) is currently in phase I clinical trials (Elmslie 2003). Furthermore, Gabapentin, an anticonvulsant drug which binds to the $\alpha_2\delta$ subunit of VGCCs, selectively inhibits noxious-evoked responses of dorsal horn neurones in models of neuropathy and after inflammation (Chapman et al. 1998a; Matthews and Dickenson 2002; Stanfa et al. 1997) and is used widely in clinical neuropathies. The actions of this drug with respect to pain processing will be discussed further in chapter 5, whilst detailed reviews of the roles of VGCCs in pain can be found in the following: (Elmslie 2004; Kochegarov 2003; McGivern and McDonough 2004; Snutch et al. 2001; Wallace 2000).

1.3 Dorsal horn architecture

The grey matter of the spinal cord is organized into 10 layers, or laminae, according to the functional characteristics of the cells found therein (Sorkin and Carlton 1997). The dorsal horn of the spinal cord is made up of laminae I – VI and receives sensory input whilst laminae VII – IX make up the ventral horn which contains motor neurones. Lamina X surrounds the central canal.

1.3.1 Termination pattern of primary afferent fibres   Primary
afferent fibres carrying sensory information from the periphery to the CNS enter the dorsal horn via dorsal roots, terminating in the segment of entry or sending rostro-caudal projections out of this segment. The thickly myelinated Aβ fibres, carrying non-noxious information, terminate mainly in laminae III-V onto non-nociceptive neurones which project into spinal tracts – SCT and

STT – and the postsynaptic dorsal column (PSDC). Terminations in lamina V are also made onto WDR neurones receiving C-fibre input via interneurones. The thinly myelinated $A\delta$ fibres, mediating transmission of noxious information, terminate ipsilaterally in laminae I and II, and contralaterally in lamina V on WDR neurones projecting to spinal tracts and the PBN. Finally, unmyelinated C fibres carrying noxious information terminate superficially in laminae I and II of the dorsal horn, with very weak input to lamina V and possibly lamina X (see Millan (1999) and Sorkin & Carlton (1997)). The laminar organization of the dorsal horn and primary afferent termination pattern as discussed above is shown in Figure 1.3.

1.3.2 Lamina I – the marginal layer Lamina I is the area where the first synapses occur in the nociceptive pathway. It is the main region of the dorsal horn receiving monosynaptic input from $A\delta$- and C fibres, and the main source of output from the superficial dorsal horn (SDH). Lamina I is therefore of intrinsic importance in pain processing. The cells found within lamina I have been classified into three major morphological types: pyramidal, fusiform and multipolar (Lima and Coimbra 1986). Most of the cells found in the marginal layer are nociceptive specific (NS) responding only to noxious pinch and/or heat with a smaller population of polymodal nociceptive (HPC) cells also responding to cold (Andrew and Craig 2002; Bester et al. 2000a; Bester et al. 2000b; Craig and Andrew 2002). The cutaneous receptive fields of lamina I neurones are usually restricted over a small area and thus suitable for signaling localized pain (Gauriau and Bernard 2002). Interestingly, there are subpopulations of lamina I neurones that specifically encode innocuous thermal stimuli (Light et al. 1993) and itch-inducing stimuli (Andrew and Craig 2001). Recently, a small population of wide dynamic range (WDR) cells has been identified in the SDH, which respond from the innocuous through to the noxious range to mechanical and thermal stimuli, as well as cold (Seagrove et al. 2004).

A
B
Spinal tracts
Dorsal Root
DRG
Ventral Root
Thickly-myelinated non-nociceptive processing
A-beta
C-fibre
A-delta
Unmyelinated nociceptive processing
Thinly-myelinated nociceptive processing
I
II
III
IV
V
VI
VII
VIII
IX
X
B
Interneurone
NS neurone
WDR neurone
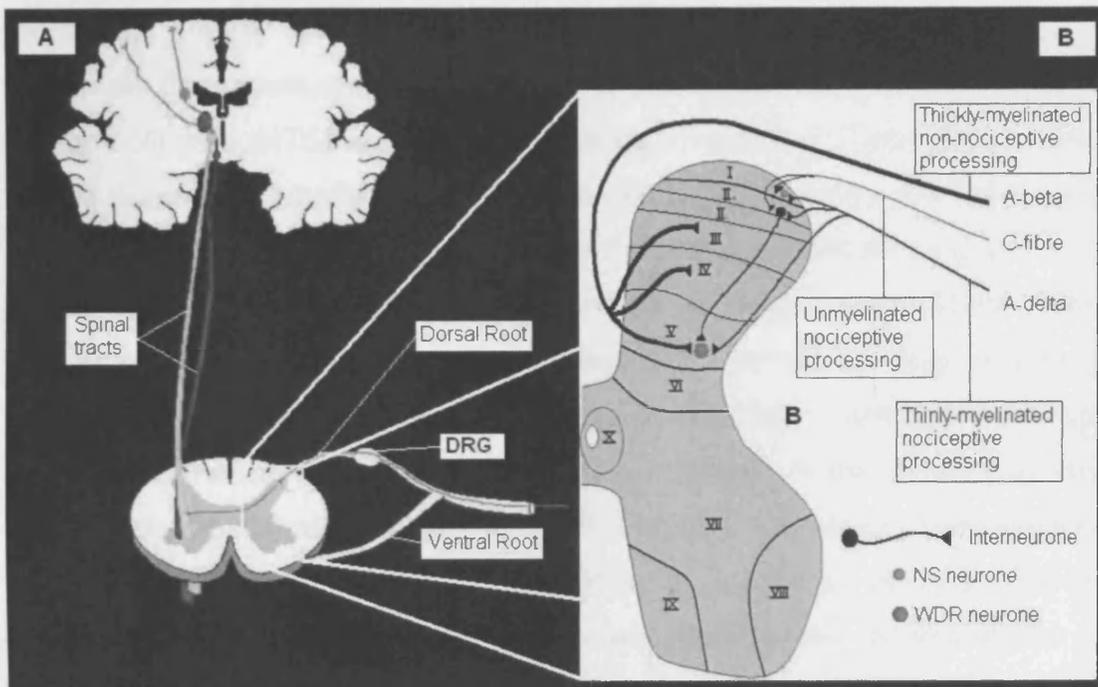
Figure 1.3 (A) Primary afferent entry to the dorsal horn of the spinal cord and connection via spinal tracts to supraspinal pain centres. Adapted from (Snyder 1996).

Figure 1.3 (B) Laminar organization of the spinal cord and termination pattern of primary afferent fibres. Adapted with permission from (Matthews 2001).

This same study showed that lamina I cells lack typical NMDA-mediated wind-up showing instead a slow incremental potentiation (SIP) to repeated C-fibre stimulation, and suggested a vital role for AMPA receptors in the neuronal responses, as well as GABAergic control of Aδ fibre and mechanical-evoked responses.

The injection of retrograde tracers into target nuclei has been used to study the projection of Lamina I neurones which have been shown to send processes, mainly contralaterally, to various central sites including the thalamus, periaqueductal grey (PAG), lateral parabrachial area, nucleus tractus solitarius (NTS) and medullary reticular formation (Todd 2002). The largest number of labelled cells was observed after injection into the region between the lateral reticular nucleus and the spinal trigeminal nucleus in the caudal ventrolateral medulla (CVLM), and in the lateral parabrachial area. The majority of lamina I projection neurones (up to 80%) have been shown to express the neurokinin receptor NK_1 (Todd et al. 2000). Furthermore, it has been found that ablation of NK_1-containing neurones in the dorsal horn by intrathecal administration of substance P conjugated to saporin (see section *1.4.2 Substance P*) results in a significant reduction of hyperalgesia suggesting that these lamina I cells are vital to the maintenance of hyperalgesia (Mantyh et al. 1997). It is these same neurones that form the origin of the spinal-supraspinal loop regulating spinal excitability through a descending serotonergic pathway (Suzuki et al. 2002). Serotonergic innervation is in fact highest in lamina I compared to any other layer of the dorsal horn, and neurones expressing the NK_1 receptor receive significantly more contacts (Polgar et al. 2002).

Comparatively little is known about the population of lamina I neurones that do not express the NK_1 receptor. However, a group of non-NK_1-expressing giant cells projecting to the parabrachial area and showing high levels of the glycine receptor-associated protein gephyrin have been characterized (Puskar et al. 2001). The cells express c-fos following intradermal injection of formalin to the ipsilateral hindpaw showing that they respond to acute noxious stimuli. Most of the gephyrin sites on the cells are adjacent to axons

containing glutamate decarboxylase implying that they are GABAergic inhibitory cells (glutamate decarboxylase catalyses the removal of the carboxyl group adjacent to the α-amino group from L-glutamic acid to produce GABA and carbon dioxide).

1.3.3 Lamina II – The Substantia Gelatinosa

Lamina II, also known as the substantia gelatinosa (SG) owing to its gelatinous appearance, is divided into an outer (II_o) and an inner (II_i) layer. The SG is comprised of two main types of cells: stalk and islet (Sorkin and Carlton 1997). Stalk cells are so called due to their short stalk-like spines and are predominately located in II_o near the border with II_i. The axons of stalk cells project mainly to lamina I although some ramifications have been observed in deeper layers. Islet cells are found predominately in II_i and their dendrites extend longitudinally within lamina II. These are thought to be inhibitory cells. Their dendrites contain vesicles and are capable of forming dendroaxonic and dendrodendritic synapses. The outer layer of lamina II contains neurones that respond to noxious stimulation whereas the inner layer contains neurones responding mainly to non-noxious inputs (Li et al. 1999).

1.3.4 The Deep Dorsal Horn (DDH)

Laminae III-V make up the nucleus proprius, whilst laminae V and VI (defined only in lumbo-sacral and cervical region) together are the deep dorsal horn (DDH) layers (Millan 1999). Most DDH neurones are WDR and have peripheral fields much larger than those in the SDH (Gauriau and Bernard 2002). These cells signal the intensity of pain as well as a variety of mechanical stimuli (Besson and Caouch 1987). Anterograde tracing studies have shown dense projections from the DDH to the caudal reticular nuclei (Raboisson et al. 1996): the Lateral reticular nucleus (LRN) and the subnucleus reticularis dorsalis (SRD). As the LRN is a motor area closely linked to the cerebellum it suggests that DDH neurones play a role in the motor response to noxious stimuli (Gauriau and Bernard 2002). The majority of SRD neurones are nociceptive and their main thalamic target is the ventromedial thalamic nucleus (Villanueva et al. 1998) which in turn projects to the dorsolateral prefrontal cortex (Desbois and Villanueva 2001) as well as the ventral horn and motor nuclei of the brain

stem (Villanueva et al. 1995). Labelling studies have also identified the gigantocellular/lateral paragigantocellular reticular nuclei (NGc) and the parabrachial internal lateral subnucleus (PBil) as areas receiving projections from DDH neurones (Bernard et al. 1995). NGc neurones respond to noxious stimuli and evoke escape behaviour and PBil neurones have marked wind-up and post-discharge after noxious stimulation (Gauriau and Bernard 2002).

1.4 Neurotransmitters and neuromodulators of nociception

The classification of primary afferent fibres according to the physiological characteristics of their axons has been outlined; however they can also be distinguished according to the neurotransmitters that they release. The neurochemical composition of primary afferents may vary according to tissue type, normal state versus inflammation or nerve injury, and also between the different fibre types (Millan 1999). In addition, there may be variation within a fibre type where, for example, peptidergic and non-peptidergic subpopulations exist. Of the many neurotransmitters and neuromodulators involved in nociceptive transmission a few of the main players are detailed below.

1.4.1 Glutamate   Glutamate, released by both large and small diameter primary afferents, is the main excitatory neurotransmitter in the mammalian nervous system. Receptors mediating its effects are located throughout the CNS (Ozawa et al. 1998) as well as on peripheral sensory terminals (Carlton 2001) and can be divided into two main categories: fast ligand-gated ion channels (ionotropic receptors) and slow G-protein coupled metabotropic glutamate receptors (mGluRs). Three subtypes of ionotropic glutamate receptors have been identified:

- α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)
- *N*-methyl-D-aspartic acid (NMDA)
- Kainate

AMPA receptors located postsynaptically in the spinal cord (Ye and Westlund 1996) set the baseline level of nociception transmitting the intensity and duration of the peripheral stimulus (Dickenson et al. 1997). Kainate receptors play a facilitatory role and are located both pre- and postsynaptically, their presynaptic role being upgraded after inflammation, possibly as a result of increased glutamate release (Stanfa and Dickenson 1999). The AMPA receptors are made up of four subunits, GluR1-4, whilst the kainate receptors are made up of subunits GluR5-7 and KA1-2, and are arranged as either homo- or heteromeric pentamers (Hollmann and Heinemann 1994). Most AMPA receptors are impermeable to Ca^{2+} due to the presence of the GluR2 subunit (Burnashev et al. 1995), absence of this subunit resulting in permeability to Ca^{2+}. AMPA receptors permeable to Ca2+ have been reported in the superficial dorsal horn suggesting a role in nociceptive transmission and it is also suggested that there is a link between these receptors and inhibitory systems of the dorsal horn of the spinal cord (Kerr et al. 1998; Stanfa et al. 2000a).

The NMDA receptor has been the most widely studied glutamate receptor due to availability of pharmacological tools, and a significant role for NMDA receptors in nociceptive transmission has been revealed. The receptors are heterogeneous structures capable of responding to selected agonists and antagonists in multiple ways. This diversity in functional response suggests that these channels are comprised of unique combinations of receptor subunits which determine a cell's functional NMDA signature (Kutsuwada et al. 1992). There are three subunit families which (in rat and human) are termed NR1-3. Within the NR2 subunit family there are four types - NR2A, NR2B, NR2C and NR2D whilst NR3 has two subtypes – NR3A and NR3B. A tetrameric structure comprising two NR1 subunits and any two NR2 subunits has been proposed for functional NMDA receptors (Laube et al. 1998).

The channel has high Na^+ and Ca^{2+} permeability and, along with glutamate, binding of glycine is necessary for the channel to open (Johnson and Ascher 1987), which under normal conditions is plugged by a magnesium ion

(Johnson and Ascher 1990) so that, initially, noxious inputs to the CNS are *not* transmitted via the NMDA receptor. With repeated stimulation, however, peptidergic-mediated depolarizations (discussed below) remove the magnesium block thus recruiting the NMDA channels. The result of this is hugely increased neuronal depolarizations leading to increased excitability of the neurone, such that its response to subsequent stimuli is enhanced - a phenomenon called 'wind-up' (Mendell 1966). Electrophysiology studies using AP5, a selective NMDA receptor antagonist, to drastically reduce wind-up have confirmed the pivotal role of NMDA receptors in this event (Dickenson and Sullivan 1987), which contributes to 'central sensitization', a class of mechanisms fundamental to chronic pain (Melzack et al. 2001). Furthermore, the physiological role of NMDA receptors has been highlighted in a number of pathological states such as inflammation, peripheral and central ischemia, and nerve injury, where NMDA receptor antagonists have been shown to reduce neuronal hypersensitivity and/or behavioural responses indicative of allodynia (Coderre and Melzack 1992; Dickenson and Aydar 1991; Eisenberg et al. 1994; Seltzer et al. 1991; Sher and Mitchell 1990).

These findings highlight the NMDA receptor as an ideal target for the pharmacological control of pain. However, NMDA receptors are important for normal CNS functioning and the use of antagonists is therefore limited by side effects including memory impairment, psychotomimetic effects, and ataxia (Petrenko et al. 2003). Despite this, NMDA antagonism still represents an area of ongoing interest as NR2B antagonists are better tolerated and have high efficacy. More selective antagonists of this subunit will hopefully provide a new class of drug for the treatment of pain.

Eight mGluR receptor subtypes have been identified and classed into three groups based on sequence homology, signal transduction mechanisms, and pharmacological profile (Neugebauer 2002): group I (mGluRs 1 and 5), group II (mGluRs 2 and 3) and group III (mGluRs 4, 6, 7 and 8). A major difference between the groups is their signal transduction pathways. Group I couples through $G_{q/11}$ proteins activating phospholipase C (PLC) leading to hydrolysis

of phosphoinositide (PI), protein kinase C (PKC) activation, and release of Ca^{2+} from intracellular stores. Groups II and III, negatively coupled to adenylyl cyclase via G_i/G_o proteins, inhibit cyclic AMP (cAMP) formation and cAMP-dependent protein kinase (PKA) activation. Furthermore, through a number of intracellular effector systems, mGluRs can regulate phosphorylation of various kinases, receptors, and ion channels (Neugebauer 2002). At the cellular level activation of mGluRs modulates high voltage-activated (HVA) Ca^{2+} channels, K^+ channels, and non-selective cation channels. The overall effect of group I mGluR activation is enhanced neuronal excitability, whilst activation of group II and III mGluRs produces inhibitory effects, although agonists of group III mGluRs do not distinguish too well between nociceptive and antinociceptive processing (Neugebauer 2002). Thus, selective antagonists of group I mGluRs and agonists of group II mGluRs provide attractive targets for pharmacotherapy.

1.4.2 Substance P The neuropeptide Substance P (SP) belongs to the same tachykinin family as neurokinins A and B (NKA and NKB), and is released from central and peripheral endings of small diameter primary afferents (Otsuka and Yoshioka 1993) to mediate its actions via the G-protein-coupled NK receptors. It has been shown that the effects of glutamate and SP on dorsal horn neurones may result from co-release from the same afferent terminals (De Biasi and Rustioni 1988).

There are three subtypes of NK receptors: NK_1 which exhibits preference for SP, NK_2 exhibiting preference for NKA, and NK_3 with preference for NKB (Regoli et al. 1994). In the CNS NK_1 receptors are expressed at high levels in the olfactory bulb, medial amygdala, dentate gyrus, superior colliculus, dorsal parabrachial nucleus and locus coeruleus, with moderate densities being found in the nucleus accumbens, striatum, periaqueductal grey, caudate-putamen and superior coliculus, and in the dorsal horn of the spinal cord (Quirion et al. 1983; Shults et al. 1984). In the peripheral nervous system (PNS) NK_1 receptors have been located in rat and mouse DRGs, and

unmyelinated axons in glabrous skin (Andoh et al. 1996; Carlton et al. 1996; Li and Zhao 1998).

As mentioned previously, the peptidergic transmitters are involved in removing the magnesium block of the NMDA receptors. This is by virtue of co-release of the tachykinins with glutamate from the small diameter primary afferent fibres. The activation of the NK receptors produces a slow summating depolarization the result of which, in addition to the AMPA-mediated effects, is a depolarization sufficiently large to remove the magnesium block (Dickenson 1995; Urban et al. 1994).

Activation of the NK_1 receptor by SP leads to internalization of the receptor and investigations into the role of NK_1 receptors in pain have made use of this by conjugating the neurotoxin saporin to SP in order to ablate neurones expressing the receptor (Mantyh et al. 1997). It has been shown that NK_1 receptor deletion blocks wind-up and central sensitization (De Felipe et al. 1998; Khasabov et al. 2002; Laird et al. 2000; Suzuki et al. 2002), and knock-out studies have shown the NK_1 receptor to have a role in the maintenance of hyperalgesia in severe inflammatory pain (Kidd et al. 2003). Furthermore, it has been demonstrated that NK_1 receptors are vital for normal neuronal coding of high threshold mechanical and thermal stimuli (Suzuki et al. 2002). The role of SP in pain processing has also been established in behavioural studies where intrathecal injection of SP results in nocifensive behaviour, such as scratching and biting, which is resolved by NK_1 receptor antagonists (Gamse and Saria 1986; Hylden and Wilcox 1981). Also, noxious stimulation results in increased NK_1 receptor protein and the receptor seems to have a role in inflammatory pain and nerve injury (Abbadie et al. 1996; De Felipe et al. 1998; Honore et al. 1999). However, despite the significant role of NK_1 receptors in nociceptive processing highlighted by these animal studies, there has been no clinical success with NK_1 receptor antagonists (Hill 2000), which is difficult to reconcile with species differences as the same models have been reliably predictive of analgesia in humans for other drug classes such as cyclooxygenase 2 (COX2) inhibitors. It may be that the clinical and laboratory studies are not in alignment in terms of the stimulus intensities and

modalities of painful stimuli, as mentioned above NK$_1$ receptors seem to be particularly important in coding suprathreshold mechanical and thermal stimuli (Suzuki et al. 2002).

1.4.3 Calcitonin Gene-Related Peptide (CGRP)   Another

important neuropeptide in pain transmission is CGRP, although comparatively little is known about it due to a lack of agonists. CGRP is released by small diameter primary afferent neurones and activation of CGRP receptors is thought to enhance the actions of SP. As with SP, the activation of the neuropeptide receptors produces a slow depolarizing response increasing Ca^{2+} levels through influx and release from internal stores (Wimalawansa 1996).

1.4.4 Serotonin / 5-hydroxytryptamine (5-HT)   5-HT is a monoamine

neurotransmitter that produces antinociceptive and pronociceptive effects depending on which receptors it is acting on; more than 15 receptor subtypes have been identified so far, this fact in itself suggests a diverse role of 5-HT systems in pain processing (Suzuki et al. 2004b). Recently a spinal 5-HT pathway has been shown to play a role in mediating excitations from the brainstem to the dorsal horn of the spinal cord via 5-HT$_3$ receptors (Suzuki et al. 2002). These excitatory ionotropic receptors are located on the terminals of a subset of small diameter primary afferents (Zeitz et al. 2002) and their activation enhances neurotransmitter release in the dorsal horn of the spinal cord (Farber et al. 2004). The involvement of 5-HT systems in nociceptive processing is discussed further in section 1.5, and in chapter 4.

1.4.5 γ-aminobutyric acid (GABA)   This is the major inhibitory

neurotransmitter in the mammalian CNS, ubiquitously distributed and present in more than 50% of central synapses (Ashton and Young 2003). GABA is essential for limiting excitation through modulation of the release of excitatory transmitters, including glutamate and the monoamines. It exerts its effects via three subtypes of receptors, GABA$_A$ and GABA$_C$ which are ligand-gated chloride channels, and GABA$_B$ which is a G-protein-coupled receptor. Thus, activation of the ionotropic GABA receptors causes a chloride-driven

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hyperpolarization of the cell and activation of $GABA_B$ receptors causes hyperpolarization via an efflux of potassium ions. The highest concentration of GABA receptors in the spinal cord is found in the superficial dorsal horn where they are co-localized with a number of transmitter receptors including glycine, 5-HT, neuropeptide Y (NPY) and NK_1 (Bohlhalter et al. 1996; Castro et al. 2004; Keller et al. 2001; Li et al. 2000; Maxwell et al. 1996; Parker et al. 1998; Yang et al. 2002; Zheng et al. 2003). GABAergic terminals also make reciprocal synapses with primary afferent fibre terminals which provide an important drive for GABAergic neurones – GABA inhibitions control small diameter fibre inputs, blockade of the receptors resulting in nociceptive responses to what would normally be innocuous stimulation (Dickenson et al. 1997). Evidence of GABAergic roles in pain transmission has also come from behavioural studies where intrathecal injection of $GABA_A$ receptor antagonist bicuculline produces tactile allodynia (Yaksh 1989) and electrophysiological studies have shown that loss of GABAergic control may be important in neuropathic pain states (Kontinen and Dickenson 2000; Kontinen et al. 2001). GABA is also involved in nociceptive processing supraspinally – a significant proportion of fibres descending from the rostroventromedial medulla (RVM), a brain area of major importance in descending control of pain, are GABAergic (Antal et al. 1996).

1.4.6 Opioids   The fact that opioids are effective in many pain states demonstrates the importance of the inhibitory opioid system in nociceptive processing. The main groups of endogenous opioid peptides are endorphins, enkephalins and dynorphins which act on the μ-opioid receptor (MOR), δ-opioid receptor (DOR), and κ-opioid receptor (KOR) respectively. These represent the three classic types of opioid receptors belonging to the G-protein-coupled receptor family, all sharing extensive structural homology, and were cloned in the early 1990s (Chen et al. 1993; Fukuda et al. 1993; Minami et al. 1993). The receptors couple via PTX-sensitive G_i/G_o proteins to inhibit adenylate cyclase, activate inwardly rectifying K^+ channels, and inhibit voltage-gated calcium channels (VGCC – N, P, Q and R-type). Recently an orphan opioid-like receptor (ORL1) was cloned having close to 70% sequence homology with the classic opioid receptors and the

endogenous ligand was identified and named orphanin FQ (OFQ), or nociceptin (Meunier 1997). Table 1.4 lists the opioid receptor families along with their ligands.

Table 1.4 Opioid receptor families and their ligands.

Receptor type	μ (Mu)	δ (Delta)	κ (Kappa)	ORL1
Agonists	Endorphins	Enkephalins	Dynorphins	Nociceptin/OFQ
	Morphine	DPDPE	U-50488	
	DAMGO	SNC 80	U-69593	
Antagonists	Naloxone	Naloxone	Naloxone	Ac-Arg-D-Cha-
	CTAP	Naltrindole	Nor-binaltorphimine	Qaa-D-Arg-D-p-
				ClPhe-NH(2) 4

Endogenous opioid peptide-containing neurones are highly represented in regions involved in nociceptive processing such as the thalamus, periaqueductal grey (PAG), limbic system, cortex, and spinal cord (Przewlocki and Przewlocka 2001). In the spinal cord the classic opioid receptors are expressed mainly in the superficial layers of the dorsal horn with lower levels in deeper layers (Besse et al. 1990; Rahman et al. 1998), MOR accounting for 70% of total receptors, DOR 24% and KOR 6% (Besse et al. 1990). Opioid receptors have also been found in the PNS (Wittert et al. 1996) and are expressed by a variety of immune cells (Carr et al. 1988; Sibinga and Goldstein 1988).

Most of the receptors are located presynaptically on C- and Aδ-fibre afferents, thus opioids selectively inhibit nociceptive transmission whilst the innocuous-evoked activity in Aβ-fibres is not significantly affected (Dickenson 1990). ORL1 receptors are expressed in the amygdala septum, hypothalamus and thalamus, in the perikarya in DRG, and in the grey matter of the spinal cord (Monteillet-Agius et al. 1998), thus they are located in some neurones in which the classic opioid receptors are not expressed.

The effects of opioids in persistent pain states have been studied widely and will be discussed in more detail in chapter 6.

1.5 Descending pain pathways

Experiments carried out in the 1960s showed that electrical stimulation of the PAG provides sufficient analgesia to carry out abdominal surgery in the rat in the absence of general anesthesia (Reynolds 1969). We now know that descending pathways from the brain to the DH play vital roles to either increase (descending facilitation) or decrease (descending inhibition) the passage of nociceptive information, and this descending control of pain has been an area of much investigation and recent review (Gebhart 2004; Millan 2002; Suzuki et al. 2004b). Serotonergic and noradrenergic pathways comprise the major components of descending control, although there is a host of other transmitters that are delivered to the spinal cord by descending pathways including histamine, vasopressin, oxytocin, acetylcholine (ACh), GABA, opioid peptides, nociceptin, galanin, glutamate, and substance P (Millan 2002). Electrophysiological, anatomical, and pharmacological studies of descending pathways have highlighted areas of the medulla, particularly the rostroventral medulla (RVM), as the major output for descending influences (Gebhart 2004).

1.5.1 Descending Inhibition Up until recently most of the work on descending control of pain has concentrated on inhibitory pathways which are often tonically active, and descend the spinal cord in the dorsolateral funiculi (Gebhart 2004). Some investigations have suggested that descending inhibition preferentially targets excitation of WDR neurones by noxious rather than innocuous stimuli, which may be explained by presynaptic inhibition of release of neurotransmitters from nociceptive fibres and not Aβ fibres, indirect inhibition of nociceptors via inhibitory interneurones, inhibition of excitatory interneurones selectively targeting nociceptors, or effects on second messenger systems selectively interfering with actions mediated by NMDA or other receptors coupled to nociceptors

(Millan 1999). On the other hand, electrophysiological studies have suggested that descending inhibitory actions on WDR neurones in the DDH are non-selective between noxious and innocuous inputs, which could imply an antinociceptive role for descending inhibitory pathways against $A\beta$ fibre-mediated mechanical allodynia in pathological states (Millan 1999).

1.5.2 Descending Facilitation As discussed previously (section 1.4.4 Serotonin), an individual transmitter may have multiple actions depending on the receptor that it is acting on, thus it is clear that descending pathways cannot only exert inhibitory effects. There is in fact a growing body of evidence to highlight a vital role for descending excitations in spinal pain processing. Facilitatory pathways descend from the RVM in the ventral/ventrolateral spinal cord (Gebhart 2004). Inhibitory and excitatory pathways may have the same origin; it has been shown that both pathways run from the nucleus reticularlis gigantocellularis pars alpha of the medulla to WDR laminae IV-V and NS lamina I neurones respectively (Monhemius et al. 1997). It is demonstrated that descending excitatory pathways can act on terminals of primary afferent fibres as well as intrinsic DH neurones. The intrinsic DH neurones may be inhibitory interneurones, excitatory interneurones, or projection neurones, and the effects of descending facilitation on the latter will be enhancement of nociceptive transmission (Millan 1999).

Recently a 5-HT$_3$-mediated descending facilitatory pathway acting on and originating from NK1-expressing lamina I DH neurones, and relaying in the RVM, has been found to be involved in the modulation of mechanical and thermal nociceptive transmission, and is also necessary for full coding of inputs by deep DH neurones (Suzuki et al. 2002; Suzuki et al. 2004a; Suzuki et al. 2004b). As this pathway links the spinal cord to higher centres involved in mood and emotion it is thought that this serotonergic system may be important in the affective component of pain. Figure 1.4 summarises the influences of descending pathways on the DH. Descending facilitatory controls, in particular this 5-HT$_3$-mediated pathway will be discussed in more detail in chapter 4.

BRAIN

Cortex, limbic system

PAIN

Thalamus,
hypothalamus,
NTS, PBN,
PAG,
amygdala

± PAG, amygdala

Relay — ± Modulation

+

PBN, NTS,
hypothalamus, RVM,
DRT, cortex

±

DP

PAF: C, Aδ, Aβ
PN: NS, WDR
IN: inhibitory, excitatory
DP: inhibitory, facilitatory

±

DRG

IN

±

PAF   Noxious
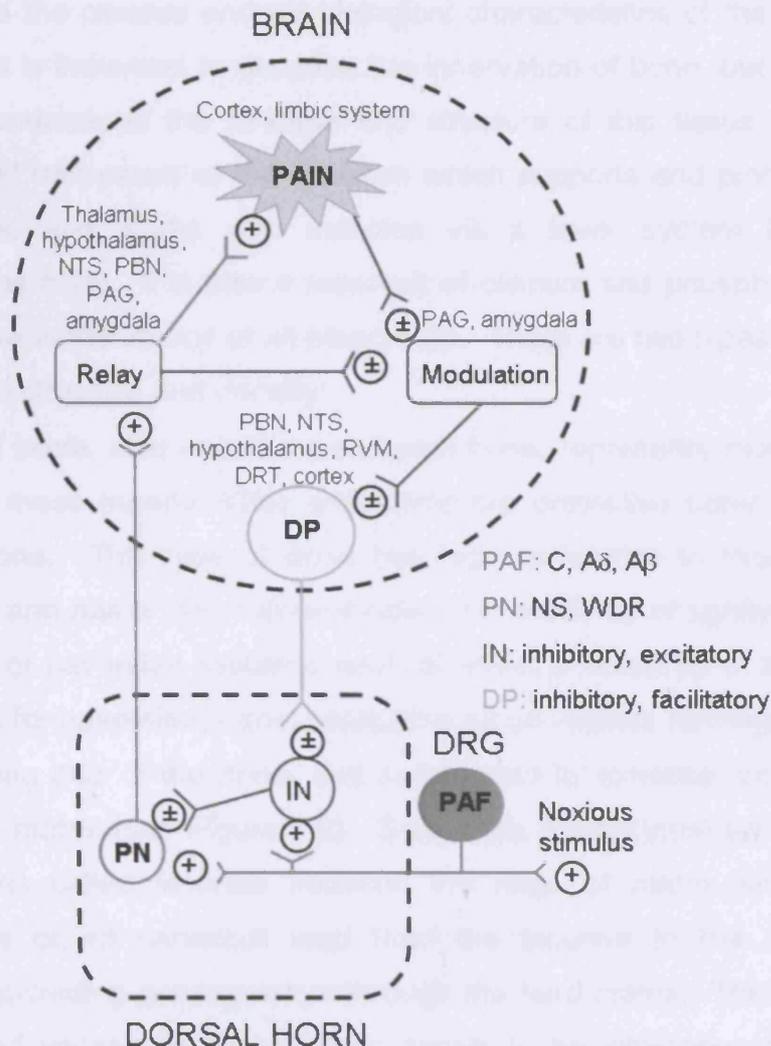stimulus

PN +

+

DORSAL HORN

Figure 1.4 Schematic representation of descending pathways to the dorsal horn.
Adapted from (Millan 2002). Descending pathways (DP) originating from the rostroventral medulla (RVM), nucleus tractus solitarius (NTS), parabrachial nucleus (PBN), dorsal reticular nucleus (DRT), the hypothalamus and the cortex, interact with interneurones (IN), primary afferent fibres (PAF) and projection neurones (PN, which may be nociceptive specific (NS) or wide dynamic range (WDR)) in the dorsal horn. These interactions serve either to facilitate or inhibit nociceptive information to the periaqueductal grey (PAG), thalamus, hypothalamus, PBN, NTS, amygdala and other higher centres.

1.6 Structure & innervation of bone

Having outlined the classes and physiological characteristics of the primary afferent fibres it is important to describe the innervation of bone, but first it is necessary to understand the function and structure of this tissue. Bones provide the rigid framework of the skeleton which supports and protects the internal organs, and works with muscles via a lever system to allow movement of the body. It is also a reservoir of calcium and phosphate, and the bone marrow is the source of all blood cells. There are two types of bone tissue based on structure and density:

- **Cortical bone**, also known as compact bone, represents most of the skeletal mass (nearly 80%) and forms the protective outer layer of every bone. This type of bone has high resistance to torsion and bending and has a slow turnover rate. It is made up of tightly packed osteons or haversian systems, each of which is made up of a central osteonic (or haversian) canal containing blood vessels running parallel to the long axis of the bone, and surrounded by lamellae (concentric rings) of matrix (see Figure 1.2). Bone cells (osteocytes) are located in spaces called lacunae between the rings of matrix and small channels called canaliculi lead from the lacunae to the osteonic canals, providing passageways through the hard matrix. These allow the blood vessels in the osteonic canals to be interconnected with those on the surface of the bone.

- **Trabecular bone**, also known as spongy or cancellous bone, represents the remaining 20% of skeletal tissue but 80% of bone surface. It has a much higher turnover rate than cortical bone and is less dense and more elastic due to its intricate structural mesh consisting of plates (trabeculae) and bars of bone adjacent to irregular cavities containing red bone marrow. Canaliculi connect to the adjacent cavities (rather than osteonic canals as in cortical bone) to receive blood supply. Trabecular bone is found in the epipheseal regions of long bones and constitutes most of the bone tissue of the skull, ribs and spine.

Depending on their shape, bones can be placed into one of four categories:

- **Long bones** – these structures are longer than they are wide consisting of a long shaft (diaphysis) with bulkier extremities (epiphyses). They are composed mainly of cortical bone with trabecular bone at the extremities. Examples of long bones are the femur, tibia, humerus, radius and ulna. (See Figure 1.2)

- **Short bones** – roughly cube-like in shape with equal vertical and horizontal dimensions, these bones are largely trabecular with a just a thin outer layer of cortical bone. Examples of short bones include the carpals and tarsals.

- **Flat bones** – thin, flattened and usually curved, examples include most bones of the cranium.

- **Irregular bones** – this group consists of bones not belonging to any of the three categories listed above and examples include the spinal vertebrae.

Bones undergo constant remodelling in response to mechanical stress, a process which is dependent on a dynamic equilibrium between two types of cells; osteoclasts and osteoblasts (Blair 1998; Mackie 2003). Osteoblasts secrete a substance rich in collagen which is essential for mineralization of hydroxyapatite and other crystals. The collagen forms spiral fibres of bone matrix called osteoids. Osteoblasts cause calcium salts and phosphorus to precipitate from the blood, and these minerals bond with osteoids to mineralize the bone tissue. Osteoblasts that have been trapped in the osteoids are called osteocytes, the aforementioned bone cells. These play a role in controlling the extracellular concentration of calcium and phosphate. Osteoclasts derive from bone marrow mononuclear cells and have ruffled edges where active resorption takes place. The osteoclasts secrete bone-reabsorbing enzymes, which digest bone matrix.
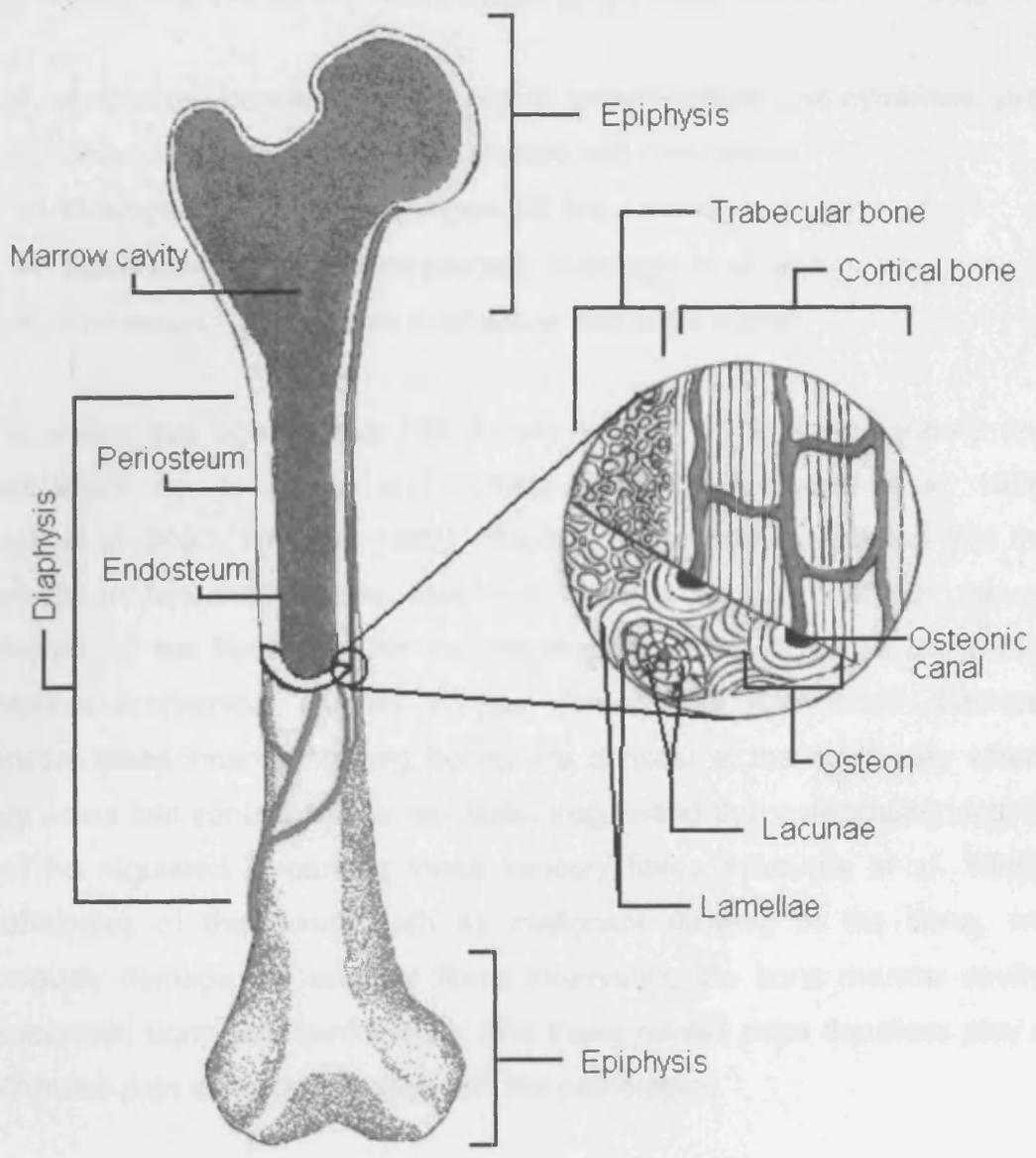
Figure 1.5 Physiology of bone – showing typical structure of a long bone (left) and detail of trabecular and cortical bone (right).   Adapted from (Warwick and Williams 1973).

The normal process of bone remodelling can be divided into four stages:

- Activation – under the influence of growth factors and cytokines, pre-osteoclasts are stimulated to mature into osteoclasts.
- Resorption – osteoclasts digest old bone matrix.
- Reversal – the resorption process is brought to an end.
- Formation – osteoblasts synthesize new bone matrix.

It is known that bone tissue has a very rich supply of both sensory and sympathetic nerves (Calvo and Forteza-Vila 1969; Hara-Irie et al. 1996; Mach et al. 2002; Thurston 1982). Studies in the rat have shown that the periosteum has the densest supply of sensory and sympathetic nerves followed by the bone marrow and mineralized bone (Mach et al. 2002). Immunohistochemical studies in rats demonstrate that small diameter sensory fibres innervating long bones are densest at the epiphyses where they come into contact with osteoclasts, suggesting that osteoclastic activity may be regulated in part by these sensory fibres (Hara-Irie et al. 1996). Pathologies of the bone, such as malignant disease of the bone, will obviously damage the sensory fibres innervating the bone marrow cavity, mineralized bone, and periosteum, and these nerves must therefore play a role in the pain states associated with the pathologies.

1.7 Aims of this study

The studies mentioned in this introductory chapter have shown that pain processing involves a complex network of pathways at the level of the periphery, spinal cord, and higher brain centres, and highlight the dorsal horn of the spinal cord as a particularly important junction in this network. The pathways are under dynamic control from excitatory and inhibitory systems, and in pathological states this control is disrupted. The development of more effective pharmacological tools for the treatment of pain is reliant on better understanding of these pathways in the normal state *and* of the changes occurring in chronic pain states.

The aims of this thesis were to characterise the behavioural changes and to attempt to correlate these to potential electrophysiological alterations of dorsal horn neurones in a rat model of cancer-induced bone pain. Furthermore, to investigate the influence of selected pharmacological systems on CIBP-induced behaviour and neuronal alterations through use of a number of compounds:

- Ondansetron – the selective 5-HT$_3$ receptor antagonist, to study the role of descending excitatory pathways in CIBP.

- Gabapentin – a novel inhibitory drug acting on VGCCs.

- Morphine – to investigate the effects of activation of an inhibitory system using a 'gold-standard' analgesic.

The overall approach is to characterise the model, to relate neuronal activity to behaviour and to attempt to define possible future clinical treatments for CIBP.

Chapter 2.

METHODS

2.1 Animals

All experiments were carried out on Sprague-Dawley rats, obtained from University College London Biological Services, weighing approximately 170g at the time of surgery to induce CIBP and approximately 300g at the time of electrophysiological testing. Animals were housed maximum 5 per cage on a 12h day – 12h night cycle (lights on 07:30, lights off 19:30) for at least one week prior to the start of experiments. Food and water were available *ad libitum*. Experimental methods used were approved by the Home Office, performed under valid Project and Personal licenses, and according to ethical guidelines of the IASP (Zimmermann 1983).

2.2 Behavioural Testing

In general, the most reliable signs of pain are physical. The study of behavioural reactions in animals to a stimulus therefore provides an indicator of the perceived disagreeable sensation (Le Bars et al. 2001). Behavioural testing prior to induction of the model, and at regular intervals (every other day) throughout the post-operative period, was carried out to obtain information on normal baseline responses to stimuli, and to monitor development of hyperalgesia and allodynia evidenced by progressive changes in the baseline response. All animals were habituated to the behavioural room for at least 30 minutes before testing.

2.2.1. Von Frey Test   Allodynia to tactile stimuli is one of the most common symptoms for a number of chronic pain states including neuropathy, inflammation, and CIBP (Chaplan et al. 1994a; Ma and Woolf 1996; Schwei et al. 1999), thus assessment of mechanical allodynia often goes hand-in-hand with *in vivo* pain studies whether it is the principle method or a tool used to verify the model. For mechanical stimulation von Frey filaments (North Coast Medical Inc., USA), also known as Semmes-Weinstein filaments, were used. These are calibrated plastic filaments of varying thickness that, when applied with sufficient force to make the filament

bend, exert a known pressure usually expressed in grams. This type of mechanical testing has been used, both clinically and in animal experimentation, for over a century and is the most common method of assessing responses to mechanical stimuli in animal pain models. It is therefore unsurprising that there are various methods used for von Frey testing. The basic setup however is similar for most methods with the animals placed on metal mesh flooring, probing of the plantar surface of the hind paw with the von Frey providing the stimulus, and withdrawal of the paw upon probing or immediately afterwards considered a positive response. Von Frey testing has however been performed over other areas such as the dorsal surface of the hind paw.

As well as various methods of carrying out the test, there are a number of paradigms for determining the withdrawal threshold. One of the more commonly used is the up-down method (Dixon 1980). In this test the middle filament of a range of forces is used first, and depending on whether there is a negative or positive response, a higher or lower filament is applied respectively. From the pattern of negative and positive responses a 50% withdrawal threshold can be calculated. Another common paradigm, on which the tests employed in these investigations are based, is testing filaments in ascending order of force and basing the threshold on the smallest fibre required to elicit a positive response (Sato et al. 1999) or the fibre giving a certain response rate, varying between 40 and 100% (Kauppila et al. 1998; Ren 1999).

For the tests used in these investigations, rats were placed in transparent plastic chambers on a wire mesh floor and left to acclimatize for at least 10 minutes (see Figure 2.1). Four von Frey filaments (bending forces of 1, 5, 9 and 15g) were applied for 2 -3 seconds to the plantar surface of the hind paw in ascending order alternating between left (ipsilateral side to surgery) and right (contralateral side to surgery) hind paws. There was a minimum of 5 minutes separating testing of ascending von Frey filaments for any one rat. The number of positive responses (taken as a brisk withdrawal of the hind paw during or immediately after the stimulus) observed out of the maximum

total of 10 was expressed as percentage response. This method has been used reliably and reproducibly in our laboratory for a number of years (Chapman et al. 1998a; Flatters et al. 2002; Maie and Dickenson 2004; Suzuki et al. 1999).
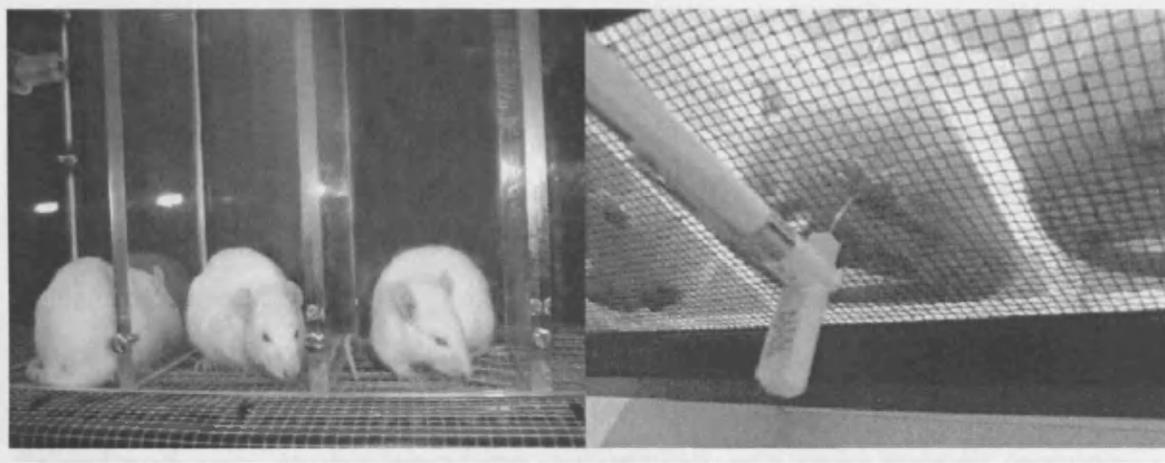

Figure 2.1 von Frey Testing. The left hand panel shows rats acclimatizing to the apparatus whilst the right hand panel shows the process of von Frey testing at the plantar surface of the hind paw.

2.2.2. Acetone Test   Allodynia to cold stimuli is another symptom of chronic pain states, particularly neuropathic pain, and behavioural responses to cooling stimuli are therefore often employed in studies on pain models (Zimmermann 2001). In these studies acetone was used as the cooling stimulus and the protocol, also having been used reliably and reproducibly in our laboratory for a number of years, was similar to that for the von Frey test. A drop of acetone was applied to the plantar surface of the hind paw via a syringe, again alternating between ipsilateral and contralateral sides. A total of 5 applications were made with at least 5 minutes between successive acetone tests. The number of lifts observed out of the maximum total of 5 was expressed as percentage response.

2.2.3. Rotorod Test   As discussed in chapter 1, the main clinical problem in treating CIBP is breakthrough pain. Thus it was necessary to test

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for the presence of this type of pain in order to validate the model. For this the rotorod was used. This apparatus, usually employed to assess motor performance, has more recently been used to study ambulatory-evoked pain (Honore et al. 2000; Luger et al. 2002). The rotorod (Ugo Basile model 7750, Linton Instruments, UK – see figure 2.2) consists of a revolving rod with plastic discs providing compartments such that four animals can be tested at a time. When the animals are placed on the rotorod they are forced to walk in the forward direction to prevent falling off the rod. For these tests the apparatus was set to accelerate from 0 to 20 revolutions per minute (rpm) over 60 seconds. The time in seconds maintained on the beam before the rat fell was recorded with a maximum cut-off of 150 seconds. Ambulation was also scored as follows: 0 – normal, 1 – slight limping, 2 – marked limping, 3 – avoidance of use of limb. Rats received two training sessions on the rotorod prior to beginning an experiment and only animals scoring between 90 and 120 seconds at each training session were used for subsequent studies.
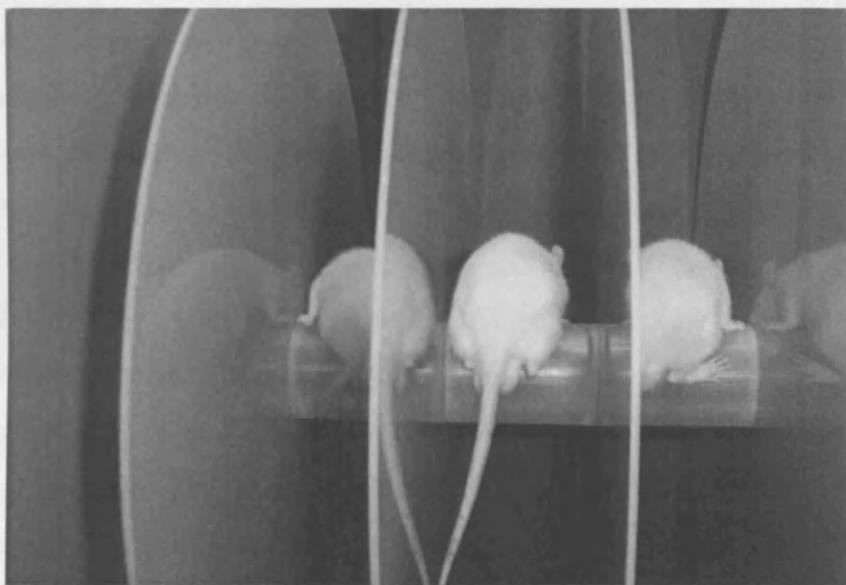

Figure 2.2 Rotorod Testing. Animals placed on the rotorod are forced to walk in the forward direction. Latency to fall and quality of ambulation are scored.

2.3 Anaesthetic Agents

Surgery and electrophysiological experimentation were carried out under general anaesthesia using the volatile anaesthetic halothane in a 66%:33% mixture of N_2O and O_2. This is an ideal agent due to ease of administration and adjustment of depth of anaesthesia whilst maintaining spontaneous respiration. Also, N_2O is claimed to antagonise the depressant effect of halothane such that when used in combination the second phase of the formalin response, depressed by halothane alone, is restored to the control baseline level (Goto et al. 1994; O'Connor and Abram 1995).

2.4 Cancer cell line

Syngeneic mammary gland carcinoma cells, kindly donated by the Novartis Institute (London), were prepared for injection as described in a previously published rat model of CIBP (Medhurst et al. 2002). The cells were cultured in RPMI 1640 (Gibco) with 10% foetal bovine serum (FBS), 1% L-glutamine and 2% penicillin/streptomycin (Gibco). Adherent cells were released by brief exposure (no more than 2 minutes) to 0.1% w/v trypsin and then quenched with an equal volume of FBS. The resulting volume was centrifuged for 3 minutes at 1200rpm, the supernatant tipped away and the pellet dissolved in 1ml of Hanks-Hepes solution (Hanks-10mM hepes: 1.19g hepes/500ml Hanks, pH 7.4 with NaOH). Ten µl trypan blue dye was mixed with 10µl cells and carefully added to a haemocytometer chamber. The number of viable cells (i.e. all those not taking up the dye) was counted and the cells were diluted as necessary to achieve a final injection concentration of 3×10^3 cells in a volume of 10µl.

2.5 Induction of CIBP – Osteotomy and Injection of Cells

The methods used here were adapted from previous reports (Medhurst et al. 2002). Anaesthesia was induced by placing the animal in a sealed box with an inflow of halothane (3-5%) in a mixture of N_2O and O_2 (66:33) and maintained via a nose cone with halothane reduced to 1.5 - 2%. Noxious pinch of the hind paw was used to confirm areflexia. The left leg was shaved and the skin disinfected with chlorhexidine (Animalcare Ltd, UK). A small incision was made over the anterio-medial surface of the distal end of the tibia to expose the bone. A 0.7mm dental drill attachment was used to bore a hole through the periosteum and a 2cm length of polythene tubing was fed 1cm into the intra-medullar cavity of the tibia. Ten µl of cells were injected into the tibia via a Hamilton syringe and the tubing withdrawn. The hole was plugged with bone restorative material (IRM, Dentsply USA), the area was irrigated with 0.5mls 0.9% saline, and the wound was closed with a metal clip. The procedure was identical for sham-operated animals except these were injected with Hanks media alone. Heat-killed cells were not used in a separate control group in order to reduce the numbers of animals used as a previous publication has shown no behavioural alterations in such a control (Medhurst et al. 2002). After surgery rats were placed in a thermo-regulated recovery box until they had fully regained consciousness, upon which they were returned to their home cages. The whole procedure lasts an average of 10 minutes, with rats returning to full consciousness within a further 5 to 10 minutes.

2.6 *In vivo* Electrophysiology

In vivo electrophysiology was used to characterize the electrical- and natural-evoked responses of dorsal horn neurones in CIBP and sham-operated rats, and to establish the effects of pharmacological agents on these responses.

2.6.1 Surgery   Anaesthesia was induced as described above. The animal was removed from the sealed box and placed on a heating blanket (a rectal probe connected to a feedback unit maintaining a core body temperature of 36-36.5°C) with anaesthesia maintained (2.5-2.8% halothane) via a nose cone. Upon confirmation of areflexia a tracheotomy was performed and anaesthesia was thereafter delivered via a tracheal cannula (1.8-2.2% halothane). The animal was then placed in a stereotaxic frame, the head secured with ear bars and a tooth bar. The location of lumbar vertebrae L1-3 was determined in relation to the position of the ribs and a clamp was placed rostral to this area and secured tightly to the side bar of the frame. A laminectomy was performed to remove some of the dorsal arch of L1-3 thus exposing the dorsal surface of spinal segments L4-5. The dura mater, when not having been removed with the bone, was carefully peeled away with sharp forceps. A second clamp was then placed caudal to the laminectomy and secured to the opposite side bar of the frame to the first clamp. Anaesthesia was reduced to maintenance level of 1-1.2% halothane, sufficiently deep to maintain areflexia with animals still breathing spontaneously.

2.6.2 Neuronal Isolation   Parylene-coated tungsten electrodes were used to make recordings. The electrode was lowered into the superficial spinal cord close to the central vessel using a manual micromanipulator and descended vertically through the cord in 10μm increments using an Epson Digital Stepper SCAT microdrive. Simultaneous with lowering of the electrode tapping of the ipsilateral hind paw, and noxious pinch to confirm C-fibre inputs, was used as the search stimulus to isolate a single neurone over background activity. Only single neurones were studied so that a unit was isolated by movement of the electrode and the signal to noise ratio was at least 4. Single neurones could be monitored, characterized and tested for many hours without change in the form or height of the action potential. SDH and DDH neurones were identified as such according to depth from the surface of the spinal cord (<290μm superficial, >500μm deep) as well as characteristic responses to electrical-evoked stimuli; lamina I neurones have

been shown to be mainly high threshold and do not show true wind-up but rather a slow incremental potentiation in response to repeated stimulation (Seagrove et al. 2004). The depths of all of the SDH neurones in these studies are within the mean ± standard error depth of lamina I projection neurones identified by histology and antidromic stimulation (Bester et al. 2000b).

2.6.3 Data Capture As mentioned above, parylene-coated tungsten electrodes were used to make single unit extracellular recordings. A Neurolog (Digitimer) system was used to amplify, filter, record, and analyze these signals. The electrode is held in a headstage which not only receives signal from the electrode (A) but also electrical activity from the rat and surroundings (B) via a clip attached to the rat and earthed to the stereotaxic frame. Signal B is therefore subtracted from signal A in order to minimize interference. The resulting signal (A-B) is amplified, filtered, and fed through an audio speaker and oscilloscope so that neuronal activity can be monitored aurally and visually (see figure 2.3). Upon isolation of a single neurone the window discriminator is set so that only action potentials from that neurone would be counted against the background activity; for this to be achievable for the duration of an experiment a signal to noise ratio of at least 4:1 was chosen. Action potentials above the set window then trigger a counting pulse which is relayed to the oscilloscope (appearing as a dot over the 'spike' – action potential), the latch counter (displaying a cumulative count), and to the CED1401 for analysis. Spike 2 software, installed on a Pentium computer, is used to interpret CED output.
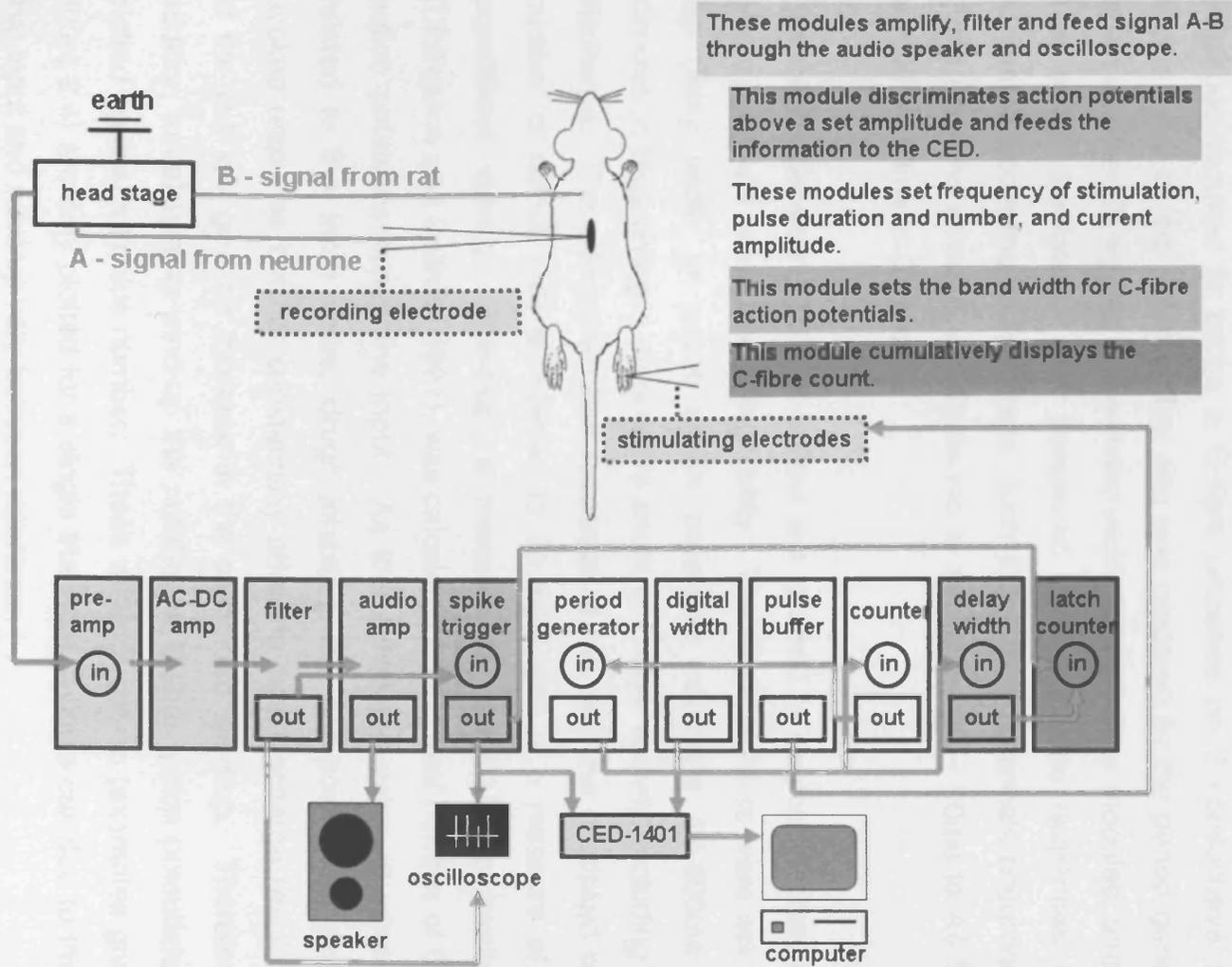
These modules amplify, filter and feed signal A-B through the audio speaker and oscilloscope.

This module discriminates action potentials above a set amplitude and feeds the information to the CED.

These modules set frequency of stimulation, pulse duration and number, and current amplitude.

This module sets the band width for C-fibre action potentials.

This module cumulatively displays the C-fibre count.

earth

head stage

B - signal from rat

A - signal from neurone

recording electrode

stimulating electrodes

pre-amp | AC-DC amp | filter | audio amp | spike trigger | period generator | digital width | pulse buffer | counter | delay width | latch counter

in | | | | in | in | | | in | in | in

| | out | out | out | out | out | out | out | out |

speaker

oscilloscope

CED-1401

computer

Figure 2.3 Schematic representation of data capture. Adapted from (Urch and Dickenson 2003)

2.6.4 Electrical Stimuli   Trains of transcutaneous electrical stimuli were applied via stimulating electrodes placed in the centre of the peripheral receptive field of an isolated dorsal horn neurone. A train consisted of 16 stimuli (frequency 0.5Hz – set by the period generator, 2ms pulse width – set by the digital width) at three times C-fibre threshold (the lowest possible amplitude required to evoke a C-fibre response on 3 consecutive single pulses – set by the pulse buffer) and was triggered by the period generator which also sends signals to the delay width, latch counter modules, and CED from which the latency was measured. This allowed responses to be separated according to fibre type, such that action potentials occurring 0 – 20ms after the stimulus are attributed to Aβ fibres, 20 – 90ms to Aδ fibres, and 90 – 350ms to C fibres.

Responses between 350 and 800ms are referred to as post-discharge and give a measure of neuronal excitability. The latch counters were set using the delay width to count action potentials occurring 90-800ms after stimulation thus giving a cumulative count of C-fibre activity, including post-discharge. The primary-evoked response of the neurone multiplied by the number of stimuli (16) is referred to as input and is a measure of non-potentiated activity. Wind-up, a measure of neuronal hyperexcitability (Dickenson and Sullivan 1987), was calculated as the total number of C-fibre action potentials minus the input. As this calculated value of wind-up is related to the input value, drugs inhibiting the non-potentiated primary-evoked response but not substantially affecting post-discharge (excitability) of the cell will give an increase in the calculated wind-up. Therefore, in addition to calculating wind-up the number of C-fibre action potentials was plotted against stimulus number. These wind-up curves (examples given in figure 2.4) are only plotted for a single trial from a single cell due to the fact that input and wind-up vary between neurones.
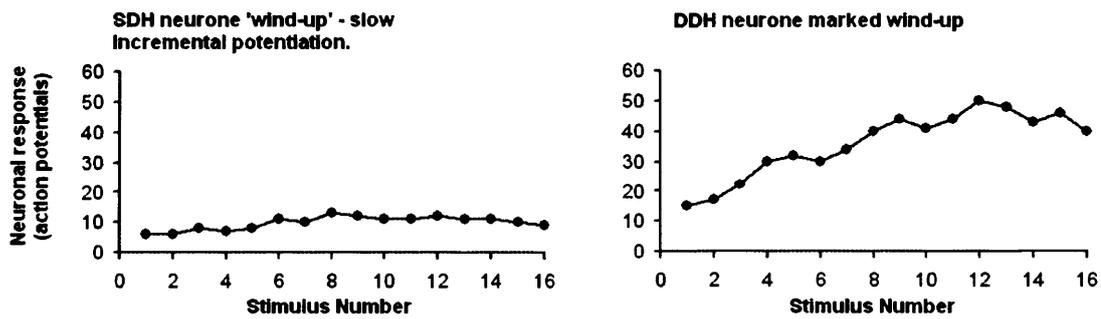
SDH neurone 'wind-up' - slow incremental potentiation.

DDH neurone marked wind-up

Neuronal response (action potentials)

Stimulus Number

Figure 2.4 Examples of typical wind-up curves for SDH and DDH neurones. Note that the primary-evoked response of the SDH neurone is typically lower than that of the DDH neurone and that the increase in activity over the train of activity is small compared to the DDH neurone.

The neuronal response to the train of 16 stimuli is captured on the computer by the spike 2 software and presented as a post-stimulus time histogram (PSTH) – figure 2.5.

A. Superficial Dorsal Horn Neurone PSTH

Aβ-fibre evoked responses
Aδ-fibre evoked responses
C-fibre evoked responses

B. Deep Dorsal Horn Neurone PSTH

Aβ-fibre evoked responses
Aδ-fibre evoked responses
C-fibre evoked responses

Action potentials per second

Time after stimulus (ms)
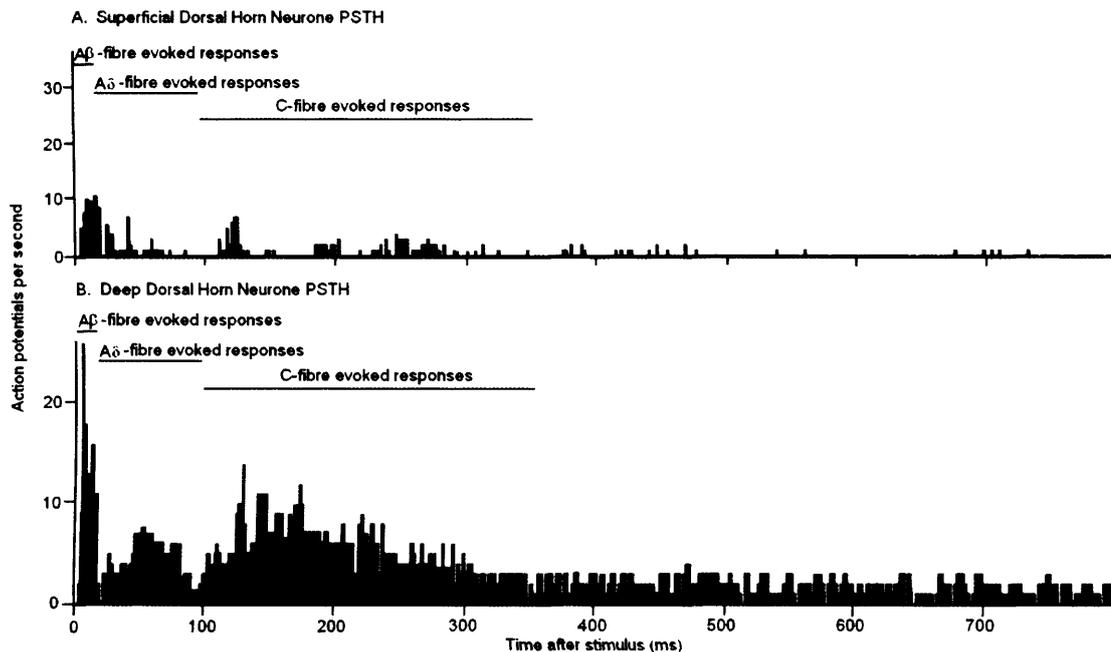
Figure 2.5 Example of PSTHs from SDH neurone (A) and DDH neurone (B). Note that the SDH neurone has much a lower response compared to the DDH neurone which shows large A- and C-fibre bands, and sustained post-discharge.

2.6.5 Natural Stimuli

Neuronal responses to a range of natural stimuli were characterized. All stimuli were applied to the peripheral receptive field for 10 seconds and a rate programme in Spike 2 was used to record the total number of action potentials occurring during each of the stimuli. A range of von Frey filaments (1, 5, 9, 15, 30, and 75g) was used for punctate mechanical stimuli, a standard soft-bristle paint brush (Pro Arte No. 8) for non-noxious brush, and constant water jet at a set temperature, delivered via a syringe, for thermal (32, 35, 38, 40, 42, 45, 48 and 50°C) and cold (4°C) stimuli. Any neuronal response from 32°C was used as a baseline and subtracted from subsequent temperatures to take into account the mechanical response to the water jet. Figure 2.6 shows typical examples of thermal-evoked responses of SDH and DDH neurones.
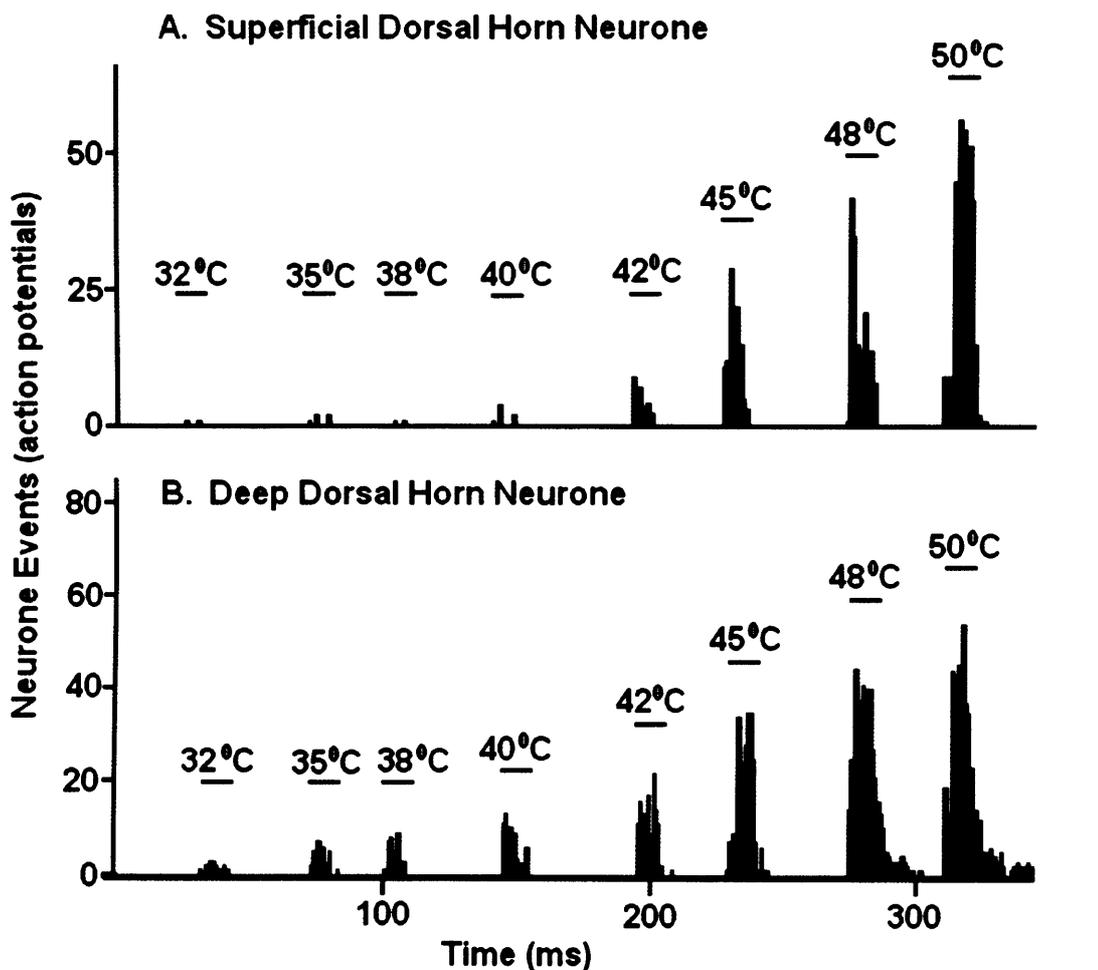
Figure 2.6 Typical thermal-evoked responses of SDH (A) and DDH (B) neurones. Panel A shows typical responses of a NS SDH neurone to thermal stimuli. Panel B shows typical responses of a WDR DDH neurone to thermal stimuli, with the cell coding throughout the range of temperatures showing increasing responses from innocuous to noxious.

2.6.6 Neuronal classification   Neurones were classified as NS or WDR according to their responses to thermal and mechanical punctate stimuli.   Responses to brush were not taken into account for this classification as many superficial cells not responding to innocuous von Frey filaments have a paradoxical response to non-noxious brush (Seagrove et al. 2004).   NS cells were therefore taken as those responding (with more than 10 action potentials per stimulus) at von Frey 9g and above and 42°C and above.

2.6.7 Quantification of peripheral receptive field   The   peripheral receptive fields of the neurones characterized were mapped on diagrams of the plantar surface of the hind paw.   For DDH neurones this was done using three von Frey filaments (9, 15 and 75g) and for SDH neurones noxious pinch was applied with forceps since many of these cells are high threshold. The diagrams were copied to plain paper ($80g/m^2$) and the mapped areas were cut out and weighed.   Receptive field areas were expressed as a percentage of the mean weight of the total area.

2.6.8 Testing protocol   Electrical followed by natural stimuli (brush, punctate mechanical, thermal, cold) were repeated at 20 minute intervals. For characterization studies the full range of von Frey filaments and temperatures described above were used.   For pharmacology experiments fewer thermal stimuli were given (35, 40, 45, 48 and 50°C) in order to allow approximately 10 minutes between giving the last natural stimulus and carrying out the next electrical test so that the cell had time to recover. Pharmacological testing began on confirmation of stable neuronal responses (three consecutive trials with less than 10% variation).   These responses were averaged to give pre-drug control values to which subsequent responses were compared.   Drugs were administered either spinally or systemically.

Spinal administration was made directly onto the spinal cord, in a volume of 50μl, via a Hamilton syringe.   The drug is kept in contact with the cord due to

the well that is formed by the muscle tissue around the laminectomy. The integrity of this well was checked prior to searching for a neurone by delivering 50μl of saline ensuring that this volume would be held without any leakage. Drug was left in contact with the cord for 1 hour, testing at 20 minute intervals, and absorbent tissue paper was used to remove it before administering the next dose.

Systemic administration of drug was via subcutaneous injection of a volume of 250μl into the scruff of the back of the neck. Testing, as before, was carried out at 20 minute intervals, following each dose of drug for 1 hour.

2.7 Analysis of results

Data are presented as mean ± standard error of mean (sem), unless stated otherwise. All statistical analyses were performed using Prism 3.0 software and significance was set at $P<0.05$.

For data from behavioural testing, the non-parametric Mann-Whitney U test was used due to its suitability for comparing sets of ordinal data.

Electrical-evoked neuronal responses obtained post-drug administration were compared to pre-drug controls using one-way analysis of variance (ANOVA). Where a significant effect was observed with increasing doses, post-hoc analysis was performed using the Dunnett's test to assess individual dose effects. Natural-evoked neuronal responses, where the outcome is affected both by dose of drug and intensity of stimulus, were analyzed using two-way ANOVA and the Bonferroni test for post-hoc analysis where appropriate.

PART ONE: BEHAVIOURAL AND DORSAL HORN NEURONAL CHARACTERISATIONS.

3.1 Introduction

Improvements in the detection and treatment of cancers have resulted in sufferers surviving longer, but severe pain is often a major contributor to the decreased quality of life endured by many of these patients (Portenoy et al. 1999). Bone metastases are frequently predictive of pain and CIBP is in fact the most common cancer-related pain, with metastatic cancers invading the skeleton in 60-84% of cases (Mercadante 1997). Interestingly, the presence and intensity of CIBP do not correlate with the size or number of active malignancies. Although the majority (up to 80%) of patients with breast cancer have metastatic spread to the bone, about 66% of the metastatic sites are painless but, on the other hand, there may be severe pain reported from a single bone metastasis in the absence of fracture (Mercadante 1997).

As mentioned in the introductory chapter, patients experience a triad of pain states consisting of background pain, spontaneous pain, and incident pain. The intermittent nature of spontaneous and incident pains makes them hard to treat pharmacologically, and it is very difficult to attain freedom from pain on movement in patients with bone metastases (Mercadante 1997). Skeletal metastases are poor prognostic factors, thus current therapies are palliative rather than curative. A better understanding of the processes underlying CIBP is required to provide more effective pharmacological agents to treat it, and thereby improve the quality of life of many cancer patients.

3.1.1 Factors contributing to CIBP

Section 1.6 on the structure and innervation of bone described how a dynamic equilibrium between osteoclastic resorption of bone and osteoblastic deposition of new bone is necessary for normal remodelling. With osteoclastic tumour cell lines this equilibrium is disrupted in favour of excessive osteoclastic activity, resulting in decreased bone density and, ultimately, destruction of bone architecture. As described in the section mentioned above, bones have a rich supply of sensory nerves to the periosteum, bone marrow, and mineralized bone (Mach et al. 2002). Osteoclastic destruction of bone architecture in regions

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with sensory innervation will clearly result in direct mechanical damage of these afferent fibres and thus lead to pain. Furthermore, stretching of the periosteum due to growth of tumour within the bone, or nerve entrapment after pathological fracture or by the invading tumour itself may also result in pain (Mercadante 1997).

As well as mechanical damage or distension of primary afferents by tumours invading the bone, pain may arise as a result of stimulation of nociceptors by factors released by tumour cells and the accompanying inflammatory infiltrate. In addition to cancer cells, tumours are made up of a number of cell types including macrophages, neutrophils, and T-cells (Mantyh et al. 2002), and release a plethora of growth factors, cytokines, interleukins, chemokines, prostanoids and endothelins (Safieh-Garabedian et al. 1995; Sorkin et al. 1997; Suzuki and Yamada 1994) resulting in activation of primary afferents which express receptors for many of these factors (see 1.2.2 Inflammatory mediators, and Figure 1.2). Furthermore, the local environment of the tumour is made more acidic (Griffiths 1991) and this decrease in pH can activate ASICs (see 1.2.3 (ii) Acid Sensing Ion Channels – ASICs) expressed by primary afferents. In addition, the vanilloid receptor VR1, a cation channel activated by noxious heat and capsaicin and expressed by nociceptors, is also activated by decreases in pH and, furthermore, protons decrease the temperature threshold for VR1 activation (Tominaga et al. 1998). The local acidosis induced by cancers not only causes activation of primary afferents, but also facilitates osteoclastic bone destruction; osteoclast formation and activation are reliant on macrophage colony stimulating factor (M-CSF), and the interaction between the receptor activator for nuclear factor κB (RANK - expressed on osteoclast precursors) with the RANK ligand (RANK-L - expressed on various cell types including osteoblasts), in an acidic environment (Urch 2004). Osteoblasts secrete a soluble protein called osteoprotegrin (OPG) which acts as a decoy receptor for RANK-L preventing RANK and thus osteoclast activation (Lacey et al. 1998). It is this interaction between RANK – RANK-L that is disrupted by tumours invading the bone as cancer cells and invading T-cells secrete RANK-L and sequester OPG (Urch

2004). Figure 3.1 summarises the factors contributing to CIBP discussed above.

3.1.2 Current drug treatments

The treatment of CIBP involves a number of approaches including radiotherapy, chemotherapy, surgical intervention, and pharmacotherapy. In terms of pharmacotherapy, the main classes of drugs used are bisphosphonates, non-steroidal anti-inflammatory drugs (NSAIDs), and opioids.

Bisphosphonates bind strongly to hydroxyapatite crystals in bone and inhibit osteoclastic bone resorption (Mercadante 1997). These drugs therefore reduce pain in patients by decreasing destruction of the bone. Bisphosphonates have been reported to potentiate the effects of analgesics in treating CIBP, although the effect is modest and does not allow for reduction of the dose of analgesic (Mercadante 1997). Side effects associated with bisphosphonates include gastrointestinal tract toxicity, fever, and electrolyte abnormalities (Mantyh et al. 2002).

NSAIDs are the first step on the analgesic ladder for cancer pain relief and even where the severity of pain requires progression to other analgesics, administration of NSAIDs may continue to take advantage of possible additive effects (Mercadante 1997). NSAIDs inhibit the cyclo-oxygenase pathway of arachadonic acid breakdown to reduce the formation of prostaglandins. The rationale for their use in CIBP is based on preventing activation and sensitization of primary afferents by prostaglandins, as described in sections 1.2.2 and 3.1.1. However, trials of NSAIDs in cancer pain which include bone metastases do not specify effects on incident pain and there is a general lack of clinical evidence to support a significant analgesic effect of NSAIDs in CIBP (Urch 2004).
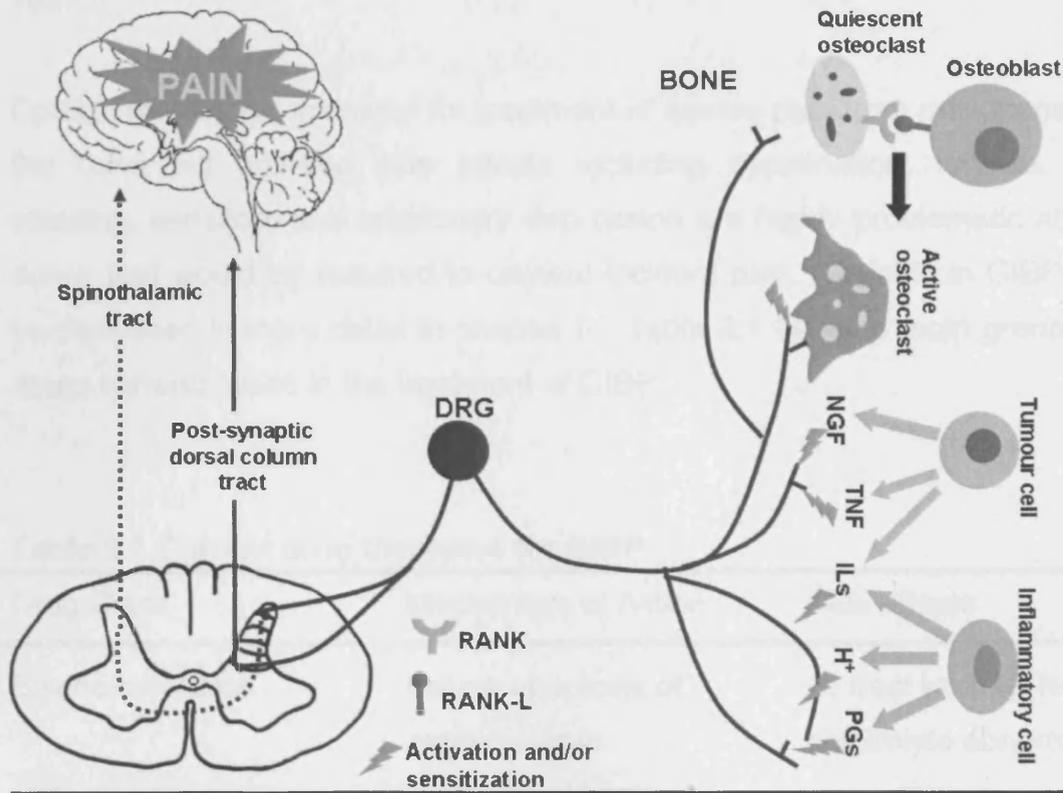
Figure 3.1 Factors contributing to CIBP. Tumours invading bone alter the osteoclast/osteoblast balance in favour of osteoclast activity, leading to bone destruction. Entrapment, distension, or direct mechanical damage of nerves innervating the destroyed area of bone will result in pain. Furthermore, tumour cells and inflammatory cells in the local area release a cocktail of factors including nerve growth factor (**NGF**), tumour necrosis factor (**TNF**), interleukins (**ILs**), protons, and prostaglandins (**PGs**) which can activate and/or sensitize the primary afferents. The net result of this would be increased neurotransmitter release into the dorsal horn of the spinal cord and increased signalling via the spinothalamic tract and post-synaptic dorsal column to higher centres in the brain where pain is perceived. Adapted from (Mantyh et al. 2002).

Side effects associated with NSAIDs include bleeding, gastrointestinal ulceration and renal toxicity (Mantyh et al. 2002). The new class of COX-2 selective antagonists may provide better pharmacological tools for treating CIBP as they have been reported to inhibit cancer cell growth (Sheng et al. 1997b).

Opioids remain the mainstay for treatment of severe pain from malignancy in the bone but adverse side effects including constipation, nausea and vomiting, sedation, and respiratory depression are highly problematic at the doses that would be required to combat incident pain. Opioids in CIBP will be discussed in more detail in chapter 6. Table 3.1 lists the main groups of drugs currently used in the treatment of CIBP.

Table 3.1 Current drug therapies for CIBP

Drug Class	Mechanism of Action	Side effects
Bisphosphonates	Cause apoptosis of osteoclasts in mineralized bone.	GI tract toxicity, fever, electrolyte abnormalities.
NSAIDs	Block synthesis of COX I and COX-2.	Bleeding, GI ulceration, renal toxicity.
Opioids	Agonists at central and peripheral mu-opioid receptors.	Constipation, nausea and vomiting, sedation, respiratory depression.

COX, cyclooxygenase; NSAIDs, non-steroidal anti-inflammatory drugs; GI, gastrointestinal. Adapted from (Mantyh et al. 2002).

3.1.3 Animal models of CIBP

As mentioned in the introduction, earlier animal models of CIBP involved systemic injection of carcinoma cells resulting in systemically unwell animals with multiple randomly sited metastases (Kostenuik et al. 1993; Sasaki et al. 1998). This precluded

systematic investigation of mechanisms underlying this pain state. Recently however, more suitable models have been developed.

In 1999, (Schwei et al. 1999) described a murine model of bone cancer that closely resembled the clinical condition but in which the tumour was confined to the femur. The cancer was induced by injecting sarcoma cells (NCTC 2472), which had previously been shown to induce lytic lesions in bone after intramedullary injection (Clohisy et al. 1996; Clohisy et al. 1995), into the femur of adult mice. The injection site was then plugged with a bone restorative material confining the tumour to the femur for the duration of the experiment. Over the next 21 days mice showed behavioural signs indicative of pain progressing at the same time as osteoclastic destruction of the bone. In addition, the study showed unique neurochemical and cellular alterations in segments of spinal cord receiving afferent input from the damaged bone: massive astrocyte hypertrophy and elevated expression of dynorphin (pro-hyperalgesic peptide) in the dorsal horn of the spinal cord – changes which were almost exclusive to the side of the spinal cord ipsilateral to the tumour-laden femur. Furthermore, non-noxious palpation of the bone resulted in nocifensive behaviours as well as SP receptor internalisation and increased c-Fos protein expression in lamina I neurones, providing evidence that primary afferent fibres are sensitised after malignant destruction of the bone.

Hypertrophy of astrocytes, increased expression of dynorphin and c-Fos, and internalisation of SP-R have all been reported in models of neuropathic and/or inflammatory pain (Abbadie et al. 1996; Draisci et al. 1991; Honore et al. 1995; Nichols et al. 1997; Wagner et al. 1993). Conversely, whilst SP levels in primary afferent neurones are elevated in models of inflammation and decreased in models of neuropathy, they are not altered in the murine bone cancer model (Schwei et al. 1999). Thus, the neurochemical alterations occurring in this model are unique, reflecting a unique pain state but one that shares some features of neuropathy and inflammation.

Subsequent studies in this same model have further suggested CIBP to be distinct as efficacy of systemic morphine at reducing pain-related behaviour

was far lower than in a chronic inflammatory model (Luger et al. 2002). This, and other investigations in CIBP models, has shown that with acute opioid administration high doses are necessary to significantly reduce pain behaviour (Menendez et al. 2003a; Vermeirsch et al. 2004). This is consistent with the clinical problem of high doses of opioids being necessary to combat incident pain as discussed previously, and thus further validation of this as a suitable animal model of CIBP closely mimicking the clinical condition.

Other pharmacological studies in the 2472 murine model have demonstrated that chronic bisphosphonate treatment reduces destruction of bone and sensory nerves innervating the bone, as well as movement-evoked bone pain (Sevcik et al. 2004). In addition, selective inhibition of COX-2 has been shown to reduce pain, bone destruction, and tumour growth in the model (Sabino et al. 2002) supporting the previously reported evidence for anti-tumorigenic properties of this class of drug (Sheng et al. 1997a; Sheng et al. 1997b) and the involvement of prostaglandin sensitisation of primary afferents in this pain state. Chronic administration of OPG also diminished movement-evoked pain and bone destruction as well as spinal neurochemical markers of sensitisation, showing reductions in palpation-induced SP-R internalisation, c-Fos expression, and dynorphin expression (Luger et al. 2001). This study highlights the pivotal role of osteoclastic destruction of bone in the 2472 murine model of CIBP.

The original protocol has been further developed to involve different bones such as calcaneus (Wacnik et al. 2001), humerus (Wacnik et al. 2003), and tibia (Menendez et al. 2003a), as well as different cell lines – fibrosarcoma (Wacnik et al. 2003), melanoma and adenocarcinoma (Sabino et al. 2003). The latter study showed that the different cell lines caused distinct localisation and extent of bone destruction, type of pain behaviour, and neurochemical reorganisation of the spinal cord demonstrating that multiple mechanisms are involved in generating and maintaining CIBP.

A recent development has been the description of a rat model of CIBP where injection of syngeneic MRMT-1 mammary gland carcinoma cells into the tibia of Sprague-Dawley rats resulted in astrocyte hypertrophy, progressive bone destruction, and pain behaviour thus having parallels to the murine models (Medhurst et al. 2002). This study showed acute treatment with morphine to reduce pain behaviour and subsequent investigation demonstrated, again similar to findings in the 2472 murine model, that bisphosphonate treatment attenuated pain behaviour whilst inhibiting tumour proliferation and preserving bone architecture (Walker et al. 2002). Although the initial rat study showed acute dosing with COX-2 antagonist celebrex to be ineffective against CIBP (Medhurst et al. 2002), a subsequent pharmacological investigation in the same model showed chronic administration of COX-2 antagonists lumiracoxib and valdecoxib to significantly reduce pain behaviour (Fox et al. 2004). This discrepancy could be due to acute versus chronic administration regimes or perhaps differing selectivity for COX-2. Regardless, it is clear that further investigation is necessary to evaluate the use of this class of drug in CIBP.

Translational research is a key to gain knowledge of mechanisms contributing to chronic pain states and improving clinical treatments. The models described above have begun to shed light on neurochemical and cellular changes occurring in CIBP. The aim of the following study was to use *in vivo* electrophysiology to characterise superficial and deep dorsal horn neuronal responses in a rat model of CIBP, in order to provide a description of potential neuronal correlates of the behavioural alterations.

3.2 Methods

Behavioural tests were carried out pre-operatively (to establish the baseline) and on post-operative days 2, 4, 7, 9, 11, 14, 16 and 18 as described in chapter 2, testing for ambulatory-evoked pain using the rotorod, for mechanical-evoked pain using von Frey filaments, and for cold-evoked pain using acetone. Preparation of MRMT-1 cells, surgery to induce CIBP, sham

surgery, behavioural testing, and dorsal horn neurone characterisations were also carried out as described previously. Dorsal horn neuronal responses to repetitive electrical stimuli and natural stimuli (punctate mechanical with von Frey, innocuous brush with artist's paintbrush, thermal and cold with water jet at set temperature) were recorded between post-operative days 15-17 at the time of maximum behavioural changes, and the peripheral receptive fields were mapped and quantified. Neurones were classed as superficial or deep based on depth from the surface of the cord and characteristic responses, and as either NS or WDR based on responses to punctate mechanical and thermal stimuli with neurones only responding at von Frey 9g and above and 42°C plus being NS. No more than six neurones were characterised in a single rat. Neuronal responses characterised in MRMT-1-injected rats were compared to those in sham-operated animals.

3.3 Results

3.3.1 Behaviour

All rats showed normal grooming behaviour and weight gain (weight at time of electrophysiology for sham-operated rats was 311 ± 40g and 302 ± 31g for MRMT-1-injected rats) throughout the post-operative period. Sham-operated rats showed no change to the baseline behavioural responses to von Frey or acetone at any point of the post-operative period whilst there was a significant increase in time maintained on the rotorod from day 7 onwards (Figure 3.2).
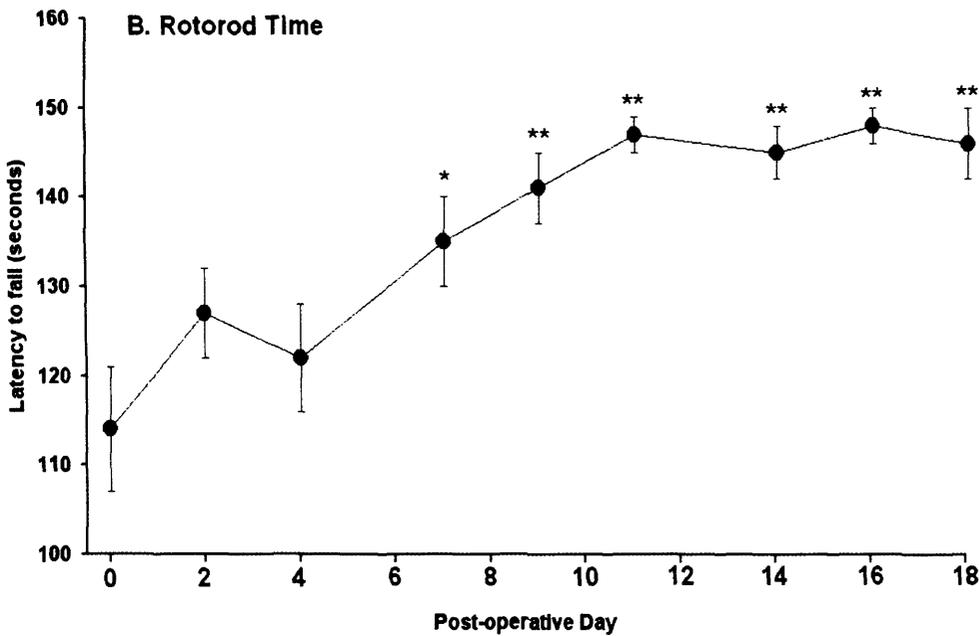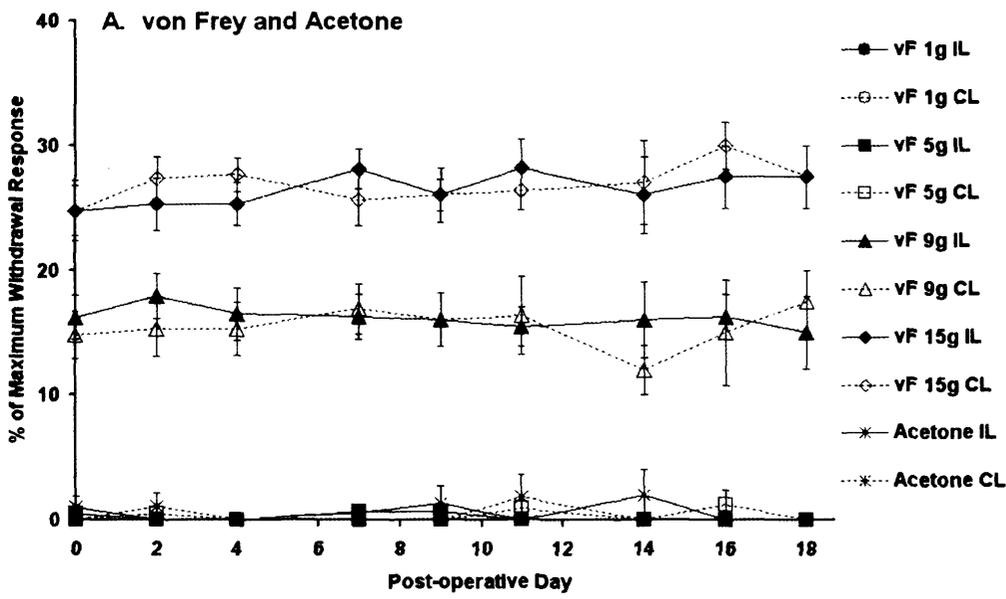
Figure 3.2   Behavioural responses in sham operated rats.   (A) Sham-operated rats show no alteration in the baseline response to mechanical punctate or cooling stimuli at any point of the post-operative period. **(B)** There is a significant improvement in performance on the rotorod in sham-operated rats from post-operative day 7 onwards.   Each point represents mean ± SEM, n ≥ 7.   *P<0.05, **P<0.01.

However, the tests revealed progressive development of hyperalgesia and allodynia in the MRMT-1-injected rats as von Frey filaments 9g and 15g induced withdrawal responses significantly higher (P<0.05) on the ipsilateral side compared to the contralateral side from day 9 onwards (Figure 3.3A), and to von Frey 1g and 5g from day 11 onwards (Figure 3.3B). The withdrawal responses to acetone were also significantly increased from day 11 onwards (data not shown). Furthermore, the rats showed increasing ambulatory-evoked pain throughout the post-operative period evidenced by a significant decrease in latency to fall from the rotorod by day 18 (Figure 3.3C), and increased ambulatory-evoked pain score from day 9 onwards (Figure 3.3D).
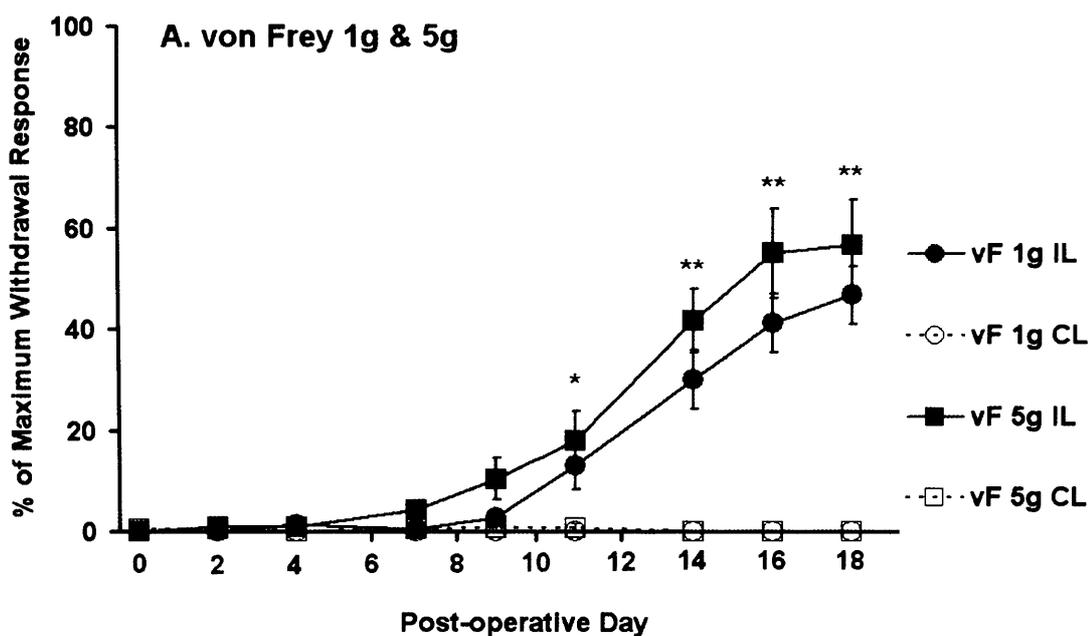
Figure 3.3 A. Withdrawal responses to von Frey 1g and 5g in MRMT-1-injected rats. Rats receiving intratibial injection of MRMT-1 show increasing responses to von Frey 1g and 5g, significant from post-operative day 11 onwards. Each point represents mean ± SEM, n ≥ 7. *P<0.05, **P<0.01.
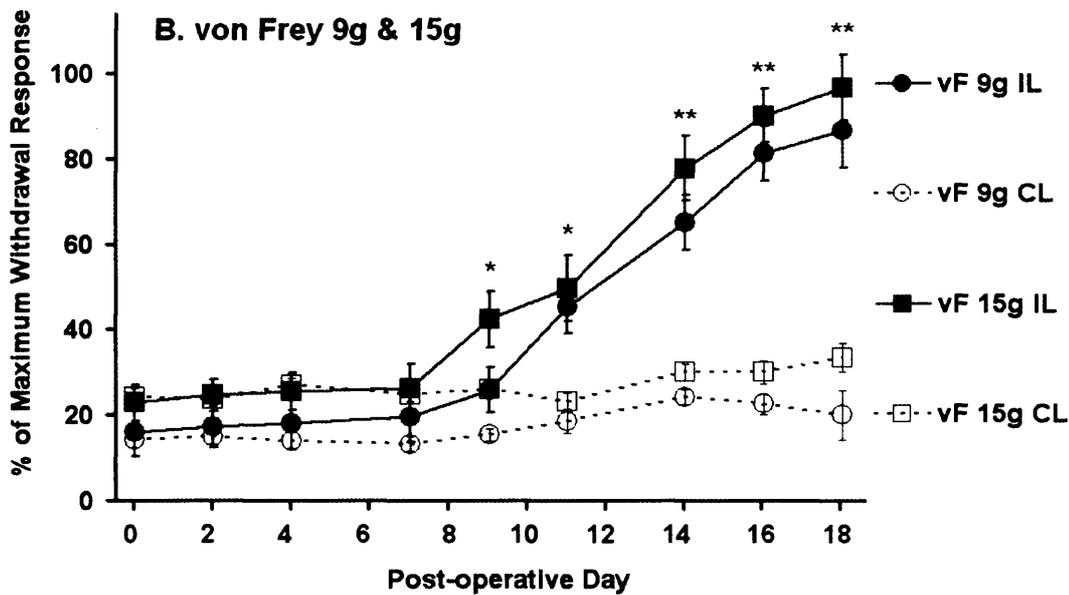
Figure 3.3 B. Withdrawal responses to von Frey 9g and 15g in MRMT-1-injected rats. Rats receiving intratibial injection of MRMT-1 show increasing responses to von Frey 9g and 15g, significant from post-operative day 9 onwards. Each point represents mean ± SEM, n ≥ 7. $*P<0.05$, $**P<0.01$.
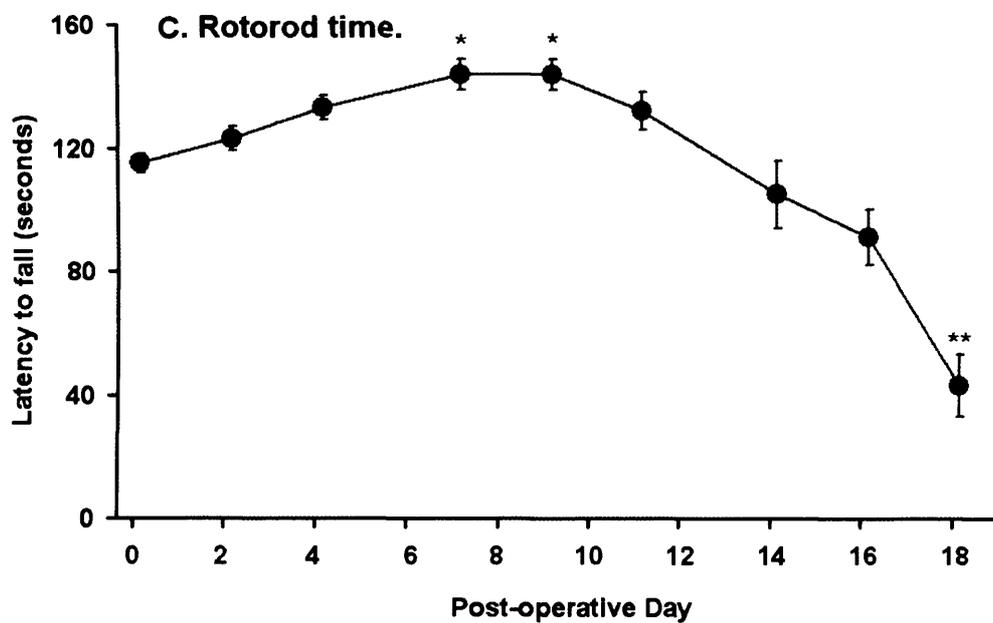
Figure 3.3 C. Latency to fall from the rotorod in MRMT-1-injected rats. Rats receiving intratibial injection of MRMT-1 show significant improvement on the rotorod by days 7 to 9, but a significant decrease in time maintained on the rotorod by day 18. Each point represents mean ± SEM, n ≥ 7. $*P<0.05$, $**P<0.01$.
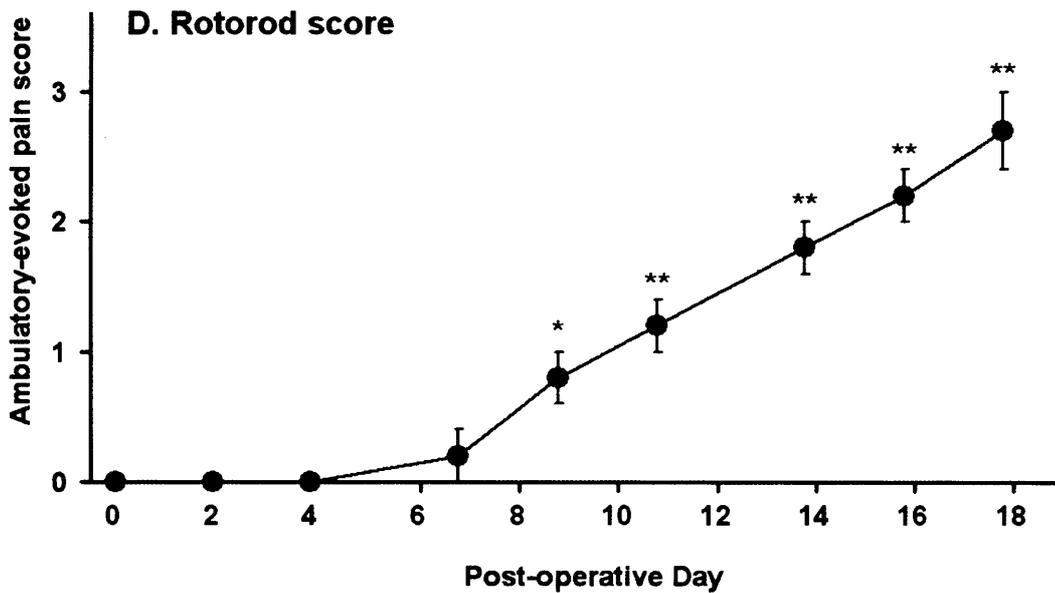
Figure 3.3 D. Ambulatory-evoked pain score in MRMT-1-injected rats. Rats receiving intratibial injection of MRMT-1 show significant ambulatory-evoked pain from day 7 onwards. Each point represents mean ± SEM, n ≥ 7. *$P<0.05$, **$P<0.01$.

3.3.2 Electrophysiology

Cells were divided into groups based on surgical procedure and location in the dorsal horn. A total of 67 neurones in sham-operated rats and 58 in MRMT-1-injected rats were characterised.

(i) Deep dorsal horn neurones

A total of 33 neurones in sham-operated rats and 28 in MRMT-1-injected rats were characterised with mean depths of 685 ± 34μm and 675 ± 32μm, respectively. All neurones in this population were WDR. Fifteen percent of cells in the sham-operated group and 18% in the MRMT-1-injected group showed low levels of spontaneous activity (<1.5Hz). All of the cells had peripheral receptive fields over the plantar surface of the hind paw, typically located centrally over the toes. Table 3.2 shows the mean areas of peripheral receptive fields for the two groups.

Table 3.2 Peripheral receptive field (% of total hind paw area) of DDH neurones in MRMT-1-injected and sham-operated rats.

Group	Von Frey 9g	Von Frey 15g	Von Frey 75g
MRMT-1	16 ± 3	17 ± 3	30 ± 6
Sham	13 ± 2	16 ± 3	25 ± 5

There was no significant difference in receptive field size between MRMT-1-injected and sham-operated rats at any of the von Frey forces.

The mean thresholds for C-fibre activation by electrical stimulation were also similar for the two groups, 1.5 ± 0.1mA and 1.3 ± 0.1mA for sham and MRMT-1 animals respectively. However, the C-fibre-evoked responses (including input and post-discharge) were significantly greater ($P<0.05$) in the MRMT-1-injected group (Table 3.3).

Table 3.3 Comparison of mean electrical-evoked responses of DDH neurones in MRMT-1-injected and sham-operated rats.

	Sham	MRMT-1
Input	322 ± 40	433 ± 35 *
A-beta	113 ± 7	116 ± 7
A-delta	89 ± 8	91 ± 8
C-fibre	359 ± 23	430 ± 28 *
Post-discharge	191 ± 16	272 ± 27 *
Wind-up	295 ± 29	376 ± 57

Values presented as mean action potentials ± SEM.

The post-discharge responses are indicators of neuronal excitability, as are wind-up values, however, the wind-up of neurones in MRMT-1-injected

animals only showed a tendency to be increased compared to sham animals, whereas for post-discharge the difference was significant. Observation of individual wind-up curves (Figure 3.4) revealed large increases in the initial responses of the neurones (reflected in the increased input value for the population). The shift in the baseline accounts for the apparent lack of significant increase in calculated wind-up.
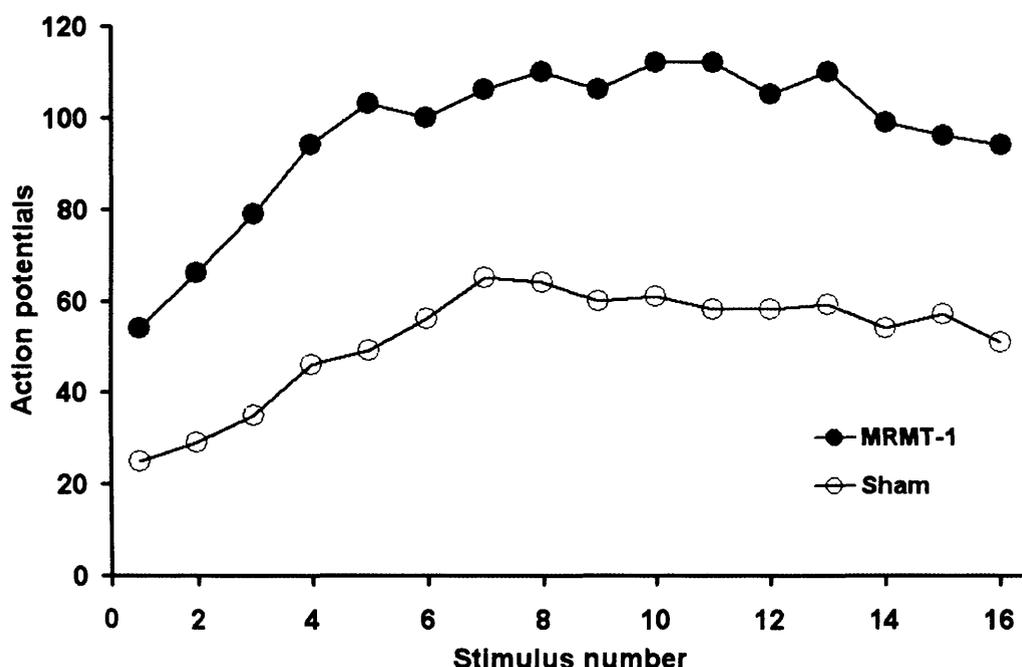
Figure 3.4 Wind-up curves for individual DDH neurones in MRMT-1-injected and sham-operated rats. An increase in the primary-evoked response results in a shift of the wind-up curve in the positive direction of the Y-axis but with the general shape and slope of the plot remaining the same. This may explain why notable changes in calculated wind-up were not significantly different.

The response to brush and noxious cold was not significantly different between the groups. In the sham-operated group 94% responded to brush and 91% responded to noxious cold, compared to 96% and 89% respectively in MRMT-1-injected rats. All of the cells characterised responded to mechanical and thermal stimuli with increasing responses throughout the

innocuous to the noxious range (thus they were all classified as WDR). The mean response to thermal stimulation was significantly higher (P<0.05) in the MRMT-1 group compared to shams at 35, 38, 40 and 48°C (Figure 3.5). The mechanical-evoked responses also showed a tendency to be higher in the MRMT-1 group but the difference was not significant (Figure 3.6).
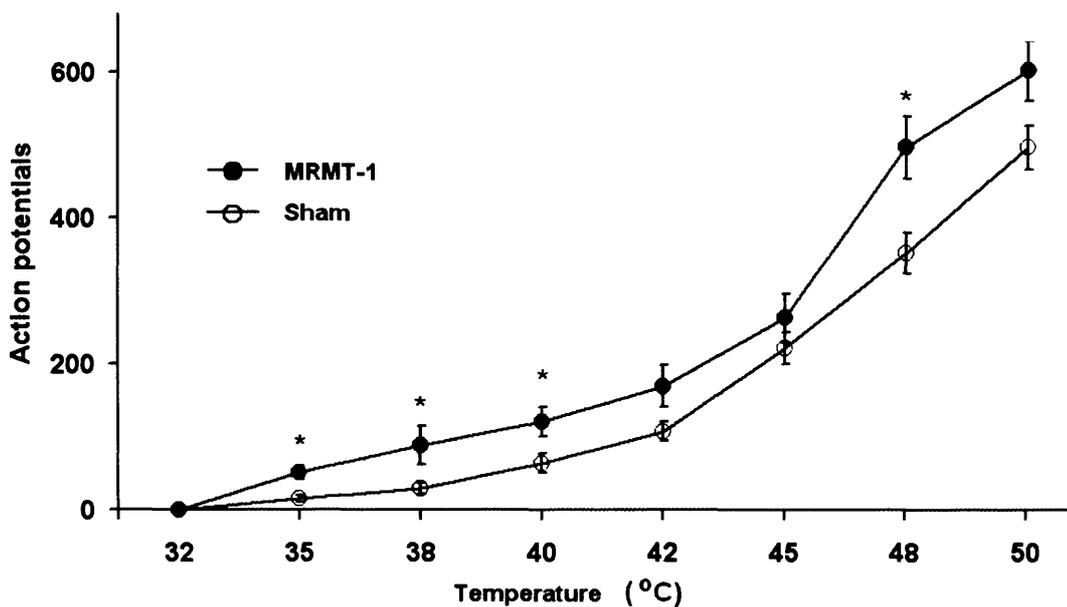
Figure 3.5 Response of DDH neurones in MRMT-1-injected and sham-operated rats to thermal stimulation of the peripheral receptive field. The mean response of DDH cells in MRMT-1-injected rats was significantly higher (P<0.05) than in sham-operated rats between 35 and 42 °C, and at 48 °C. Data are presented as mean number of action potentials ± SEM evoked during the 10 s duration of stimulus.
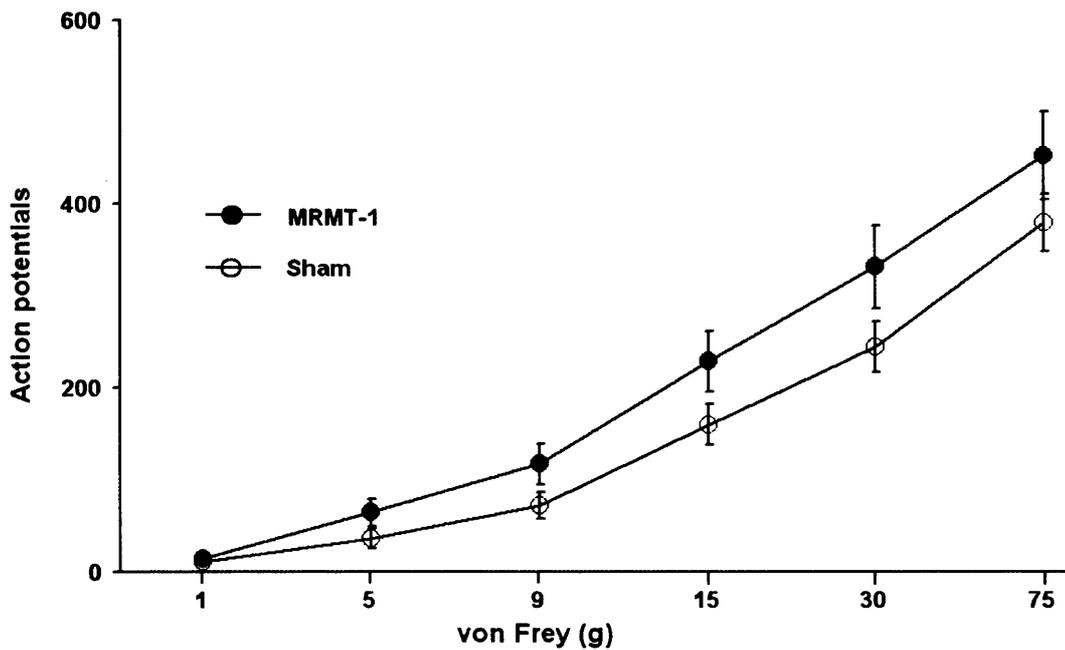
Figure 3.6 Response of DDH neurones in MRMT-1-injected and sham-operated rats to punctate mechanical stimulation of the peripheral receptive field. The mean responses of DDH neurones in MRMT-1-injected rats to von Frey stimulation tended to be higher than in sham-operated rats, but the difference was not significant at any force. Data are presented as mean number of action potentials ± SEM evoked during the 10 s duration of stimulus.

(ii) Superficial dorsal horn neurones   A total of 34 neurones in sham-operated rats, and 30 neurones in MRMT-1-injected rats were characterised. The mean depths of the neurones in the two groups were similar: 292 ± 10μm in sham animals and 295 ± 9μm in MRMT-1 animals. These depths fit within the range of depths of lamina I projection cells verified by antidromic stimulation and histology (Bester et al. 2000b). The responses of the neurones characterised here were also similar to those in the aforementioned study and it is therefore likely that the populations of cells described here were in lamina I of the dorsal horn.

All of the neurones had peripheral receptive fields over the plantar surface of the hind paw, which were mapped by response elicited by noxious pinch.

Unlike the DDH cells, there was a significant difference ($P<0.05$) between sham-operated and MRMT-1-injected animals with mean receptive field sizes of 20 ± 4% and 38 ± 7% respectively. Twenty-one percent of cells in the sham group and 17% of cells in the MRMT-1 group showed low levels of spontaneous activity (<3.0Hz).

SDH cells were classified as NS or WDR based on their responses to von Frey and heat as described previously. In the sham-operated rats 74% of the cells characterised were NS with the remaining 26% being WDR. In MRMT-1-injected rats 53% were NS and 47% WDR, indicating a substantial shift from NS to WDR response in CIBP.

The mean C-fibre thresholds of SDH cells were greater than those of DDH neurones, but similar between the sham (3.2 ± 0.1mA) and MRMT-1 (3.1 ± 0.1 mA) groups. There was no difference in electrical-evoked responses of NS neurones between the groups, whilst for WDR neurones Aβ- and C-fibre responses were significantly elevated ($P<0.05$) in the MRMT-1 group compared to shams (Table 3.4). The increased Aβ-fibre-evoked response is of particular note as this was not altered in the DDH neurones.

Similar to DDH neurones, the calculated wind-up mean was notably higher in the MRMT-1-injected group but not significantly different. Analysis of individual wind-up curves (Figure 3.7) revealed that in these populations this was also a result of an elevated primary-evoked response mirrored in the increased mean input for MRMT-1 rats.

Table 3.4 Comparison of mean electrical-evoked responses of SDH neurones in MRMT-1-injected and sham-operated rats.

	Sham NS	MRMT-1 NS	Sham WDR	MRMT-1 WDR
Input	103 ± 16	124 ± 26	139 ± 36	239 ± 26 *
A-beta	86 ± 6	93 ± 12	75 ± 8	120 ± 13 *
A-delta	23 ± 4	33 ± 6	46 ± 13	42 ± 6
C-fibre	124 ± 15	162 ± 29	147 ± 17	243 ± 24 *
Post-discharge	47 ± 10	62 ± 15	41 ± 10	121 ± 28 *
Wind-up	85 ± 16	119 ± 32	73 ± 14	176 ± 47

Values presented as mean action potentials ± SEM.
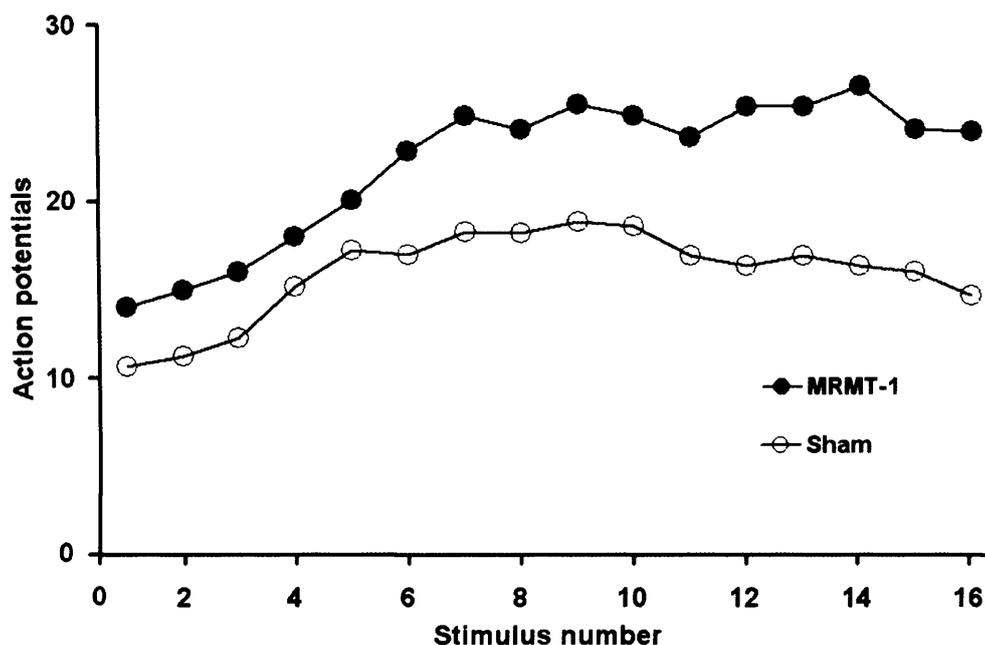
Figure 3.7 Wind-up curves for individual SDH neurones in MRMT-1-injected and sham-operated rats. An increase in the primary-evoked response results in a shift of the wind-up curve in the positive direction of the Y-axis but with the general shape and slope of the plot remaining the same. This may explain why notable changes in calculated wind-up were not significantly different.

The majority of SDH neurones responded to brush and noxious cold, and the proportions were similar in the sham-operated (76% and 79% respectively) and MRMT-1-injected (77% and 97% respectively) rats. However, the mean brush-evoked response in SDH WDR neurones of MRMT-1-injected rats at 403 ± 39 action potentials was significantly greater (P<0.05) than in the sham group (239 ± 26). The response of SDH NS cells to mechanical and thermal stimulation did not differ between the two groups (Figure 3.8) whilst the WDR cells showed significantly greater (P<0.05) mechanical and thermal evoked responses in MRMT-1-injected rats compared to shams (Figure 3.9).
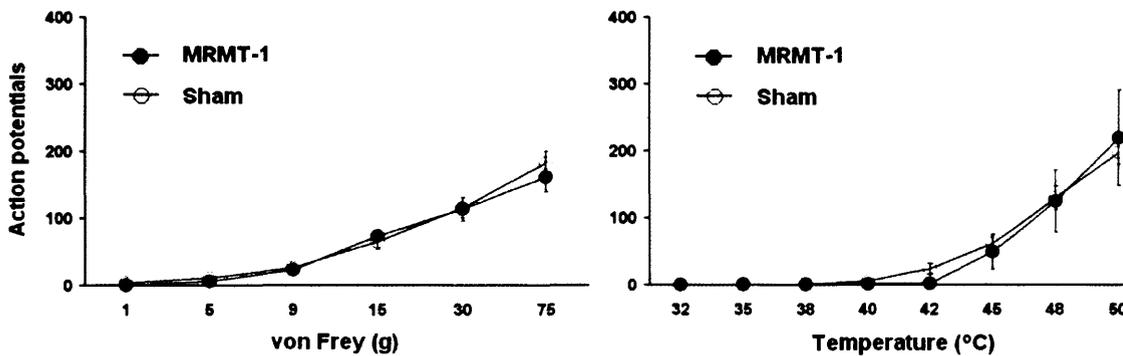
Figure 3.8 Responses of NS SDH neurones to mechanical and thermal stimuli in MRMT-1-injected and sham-operated rats. The mean responses of superficial NS cells to von Frey or thermal stimuli did not differ between MRMT-1-injected and sham-operated rats. Data are presented as mean number of action potentials ± SEM evoked during the 10 s duration of stimulus.
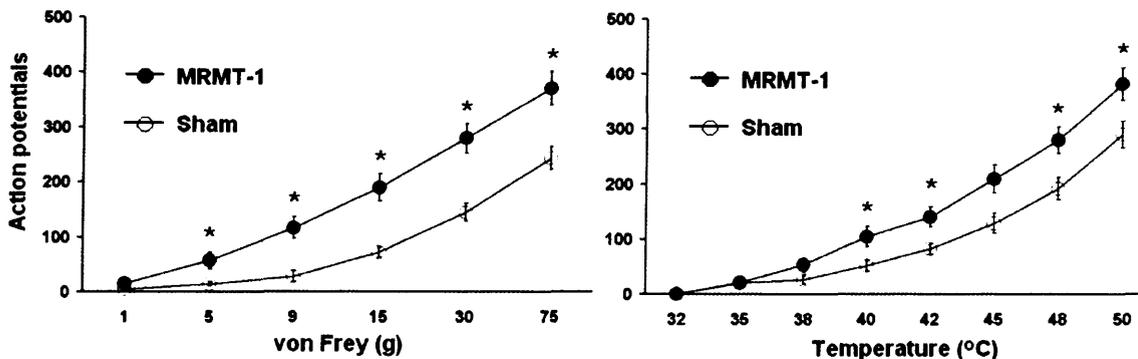
Figure 3.9 Responses of WDR SDH neurones to mechanical and thermal stimuli in MRMT-1-injected and sham-operated rats. The mean responses of superficial WDR cells to von Frey and thermal stimuli were significantly greater (P<0.05) in MRMT-1-injected compared to sham-operated rats. Data are presented as mean number of action potentials ± SEM evoked during the 10 s duration of stimulus.

3.4 Discussion

The results demonstrate clearly that localised injection of MRMT-1 mammary carcinoma cells into the tibia of Sprague-Dawley rats results in the progressive development of mechanical hyperalgesia and allodynia and cold allodynia as shown by the increasing withdrawal responses to von Frey filaments and acetone. The behavioural changes emerge by day 9 and are significant for all stimulus modalities by day 11. The mechanical and cold allodynia are paralleled by the emergence of ambulatory-evoked pain assessed on the rotorod, maximal 18 days after inoculation with MRMT-1. The pain behaviours and their temporal development, and the absence of any pain behaviours in sham-operated rats, are comparable to other models of CIBP in mice and rats (Medhurst et al. 2002; Sabino et al. 2003; Schwei et al. 1999; Wacnik et al. 2003).

The studies presented here are the first electrophysiological characterisations of dorsal horn neuronal responses in a model of CIBP. Around the time of maximum pain behaviour in MRMT-1-injected rats, there are also altered evoked responses of WDR neurones in the deep and superficial dorsal horn. Neuronal responses in sham-operated rats were the same as in normal animals, extensively characterised within this laboratory (Flatters et al. 2002; Stanfa et al. 2000b; Suzuki et al. 2001).

The results show that dorsal horn neurones are hyperexcitable in the CIBP state, in agreement with the idea of an ongoing central sensitization (Clohisy and Mantyh 2003). As the periosteum, mineralised bone, and bone marrow all receive sensory innervation, it is clear that there would be primary afferent nerve damage within a tumour-bearing bone. Inflammatory mechanisms must also contribute to CIBP, as described previously cancer cells release a cocktail of growth factors, cytokines, interleukins, chemokines, prostanoids, and endothelins, resulting a reduction of pH to below 5 and thus deformation of primary afferents (Griffiths 1991; Safieh-Garabedian et al. 1995; Sorkin et al. 1997; Suzuki and Yamada 1994; Watkins et al. 1994; Woolf et al. 1997). In spite of this, the dorsal horn neuronal response was not the same as in

neuropathy or inflammation. Repetitive stimulation of primary afferent fibres generates long-lasting post-discharge, and this excitability is increased in various models of chronic pain including the chronic constriction injury (CCI) model of neuropathic pain (Palecek et al. 1992), but only in some neurones in the carrageenan model of inflammatory pain (Stanfa et al. 1997) and was robust in this model of CIBP. Other features however are different from neuropathy, such as the significant increase in peripheral receptive field only in SDH neurones and not in the DDH, in contrast to findings in the sciatic nerve ligation (SNL) model of neuropathic pain (Suzuki et al. 2000). Responses to natural stimuli and the electrical C-fibre-evoked response in CIBP also differ from the SNL model where there is a significant reduction in responses of DDH neurones (Chapman et al. 1998b). This contrast may be explained by the differing degrees of denervation in the two models, being far more substantial in the SNL model. The increased neuronal responses to thermal stimuli reported here are in contrast to findings of initial thermal hypoalgesia in a murine model of CIBP (Menendez et al. 2003a) but are consistent with the enhanced neuronal responses to natural and electrical stimuli. Although less is known about the dorsal horn neuronal response in models of inflammation, pharmacological studies do suggest fundamental differences in the mechanisms driving CIBP and inflammatory pain (Luger et al. 2002). The data presented here are therefore in agreement with CIBP being a unique pain state.

Perhaps the most interesting finding in these investigations is the alteration of the NS:WDR ratio in the SDH, with the increased WDR population in CIBP driving the dorsal horn hyperexcitability. The sham group reflected the normal SDH ratio with 74% of cells being NS, as reported by other groups (Bester et al. 2000b; Han et al. 1998). In animals with CIBP on the other hand the WDR population almost doubled to 47% and, in addition, the responses of these cells were hyperexcitable. This is a change that had not previously been reported in any chronic pain model. Recently however, studies in a murine model of CIBP have also shown hyperexcitability in WDR neurones and not NS cells confirming my results (Khasabov et al. 2004). Of particular note among the hyperexcitable responses is the increased Aβ-fibre

response to electrical stimulation in WDR SDH neurones, consistent with the increased brush-evoked response in this group.

The increased input responses and spinal hyperexcitability may result in a reduced threshold for activation which could explain the shift from NS to WDR in SDH neurones, as well as the increased peripheral receptive field area in this population. Changes in the GABAergic system may also contribute to neuronal hyperexcitability (Reeve et al. 1998). Furthermore, the increased excitability may be generated in both spinal and supraspinal pathways as lamina I NK_1-expressing neurones have recently been shown to be the origin of a spinal-supraspinal loop important in evoking descending facilitation (and inhibition) back down to superficial and deep dorsal horn laminae (Suzuki et al. 2002). Thus the changes in the SDH in CIBP may contribute to the increased excitability of the DDH. Also, lamina I project preferentially to the PAG, parabrachial area and CVM which are important circuits contributing to the affective and nocifensive responses to noxious stimuli (Bernard et al. 1995; Craig 1995; Hylden et al. 1985; Mouton and Holstege 1998). This suggests that in CIBP there is increased activation of these areas as well as access of lower threshold inputs.

In summary, these results show that intra-tibial injection of MRMT-1 results in a reliably reproducible model of CIBP in which mechanical and cold allodynia, and ambulatory-evoked pain, can be measured behaviourally. Electrophysiological characterisations reveal a significantly hyperexcitable dorsal horn in MRMT-1-injected rats compared to shams, probably driven by a population of WDR SDH neurones. The dorsal horn pathophysiology is different to that in both inflammatory and neuropathic models confirming that CIBP is a unique pain state.

PART TWO: TEMPORAL CORRELATES OF BEHAVIOURAL AND NEURONAL ALTERATIONS.

3.5 Introduction

The relationship between behavioural and neuronal alterations has not yet been widely studied in chronic pain states, a void that is also present in other areas of neuroscience. However, in murine and rat models of CIBP unique neurochemical changes in segments of spinal cord receiving afferent input from the tumour-bearing bone have been found to occur concurrently with osteoclastic destruction of the bone and pain behaviour (Medhurst et al. 2002; Schwei et al. 1999). The dorsal horn neurone characterisations in the previous study revealed interesting changes that seem to be driven by shifting populations of SDH neurones, a phenomenon not reported before in other pain models.

As discussed in previous chapters, NK_1-expressing SDH neurons form the origin of a spinal-supraspinal loop modulating excitatory and inhibitory pathways to the dorsal horn (Suzuki et al. 2002). These neurons have also been shown to be involved in the generation of pain behaviour following spinal cord injury, as their ablation resulted in significant decreases in the onset and severity of pain-related behaviour after intraspinal injection of quisqualic acid (Yezierski et al. 2004).

The aim of the next investigation was therefore to monitor the time course of behavioural hyperalgesia and allodynia in parallel with evoked SDH neuronal responses in the rat model of CIBP, in order to assess possible temporal correlates of neuronal and behavioural alterations.

3.6 Methods

Behavioural testing and electrophysiological characterisations of SDH neurones were carried out as described previously. In MRMT-1-injected rats electrophysiological studies were performed on post-operative day 7 before significant behavioural changes, day 9 at which point there is the emergence of hyperalgesia, and day 11 by which time significant hyperalgesia and

allodynia are evoked by all stimulus modalities. These data were compared also to the results from characterisations on days 15-17 at the time of maximal pain behaviour. In order to reduce the number of animals used, electrophysiological characterisations in shams were only done between days 15 to 17 as there were no behavioural alterations at any time point.

3.7 Results

At least 30 SDH neurones were characterised at each time point. The mean depths in the MRMT-1 and sham-operated groups were 290 ± 10μm and 300 ± 12μm respectively. As with the previous characterisations these depths fit within the range for lamina I neurones, verified by antidromic stimulation and histology (Bester et al. 2000b).

On day 7 in the MRMT-1 group, 23% of SDH neurones were characterised as WDR and the remaining 77% NS (Figure 3.10).
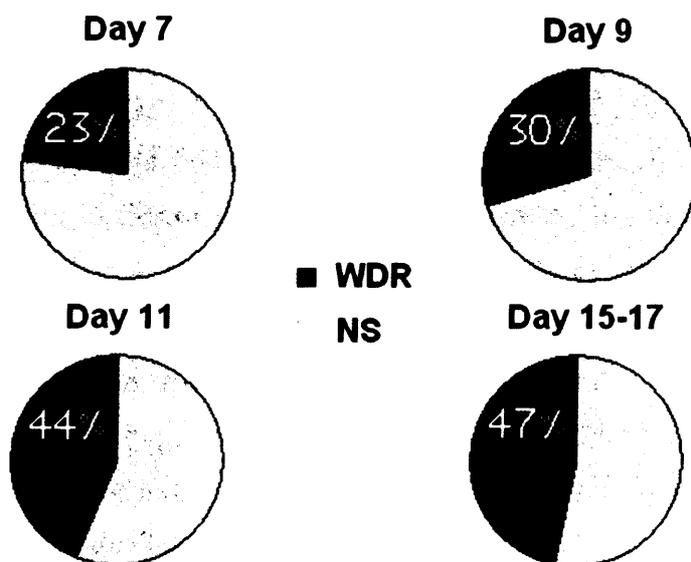
Figure 3.10 Ration of WDR : NS neurones in the SDH following intratibial injection of MRMT-1 in rats. There is a marked shift from NS to WDR in the superficial dorsal horn in CIBP occurring in parallel with behavioural signs of allodynia.

By day 11 the WDR population in the MRMT-1 group had increased to 44% (Figure 3.10) and the cells showed significantly elevated responses to electrical, mechanical and thermal stimuli (Figures 3.11 - 3.13), akin to the characterisations made on days 15-17 (Table 3.4 and Figures 3.9 and 3.11-3.13). At this time point also, the evoked responses of NS cells in the MRMT-1 group were not different to shams.
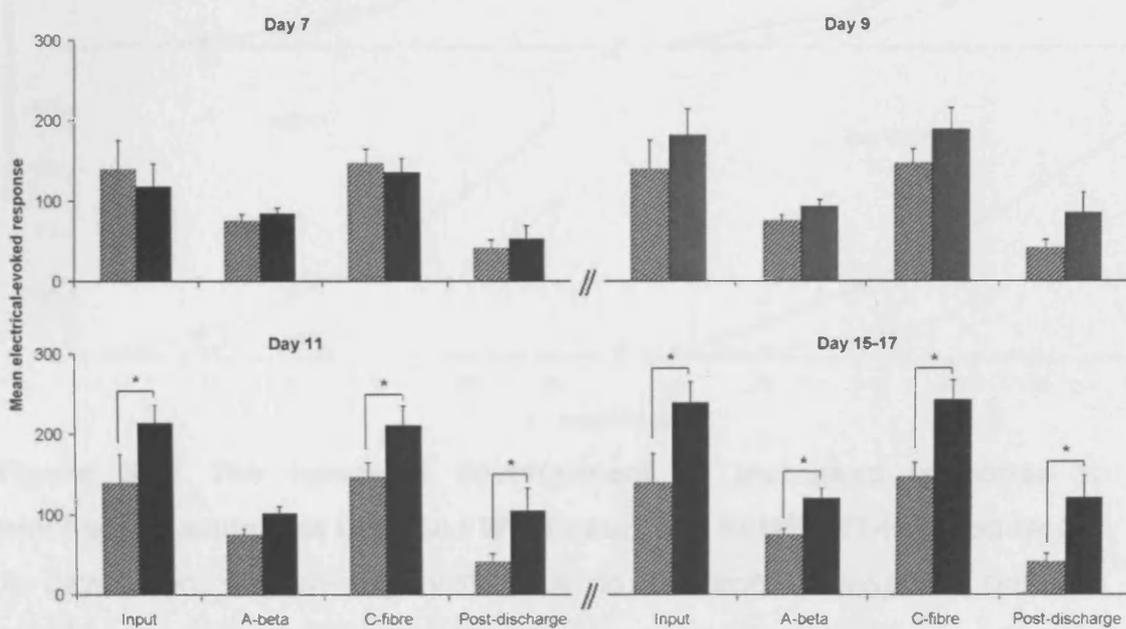
Figure 3.11 The temporal development of increased electrical-evoked responses in Lamina I WDR neurones MRMT1-injected rats.

At days 7 and 9 post-surgery, MRMT1-injected rats (solid black bars) show no difference to sham-operated animals (diagonally striped) in the electrical-evoked neuronal response. By day 11 the input, C-fibre and post-discharge responses are significantly increased in MRMT1-injected compared to sham rats and between days 15 to 17 the A-beta fibre electrical-evoked response is also significantly increased. ($^*P<0.05$).
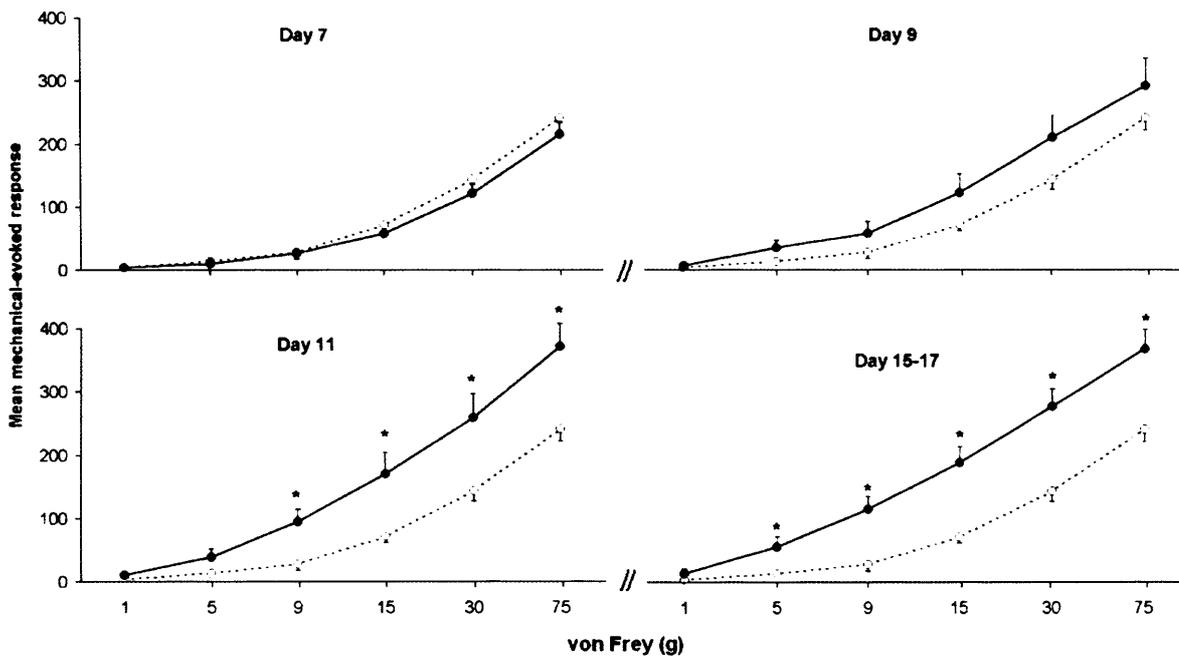
Figure 3.12 The temporal development of increased response to mechanical stimuli of Lamina I WDR neurones in MRMT1-injected rats.

At days 7 and 9 post-surgery there is no difference in response between MRMT1-injected (solid black) and sham-operated rats. By day 11, the MRMT1-injected rats show significantly increased neuronal responses to von Frey 9g and above, and between days 15 to 17 the response to von Frey 5g was also significantly increased. (*P<0.05)
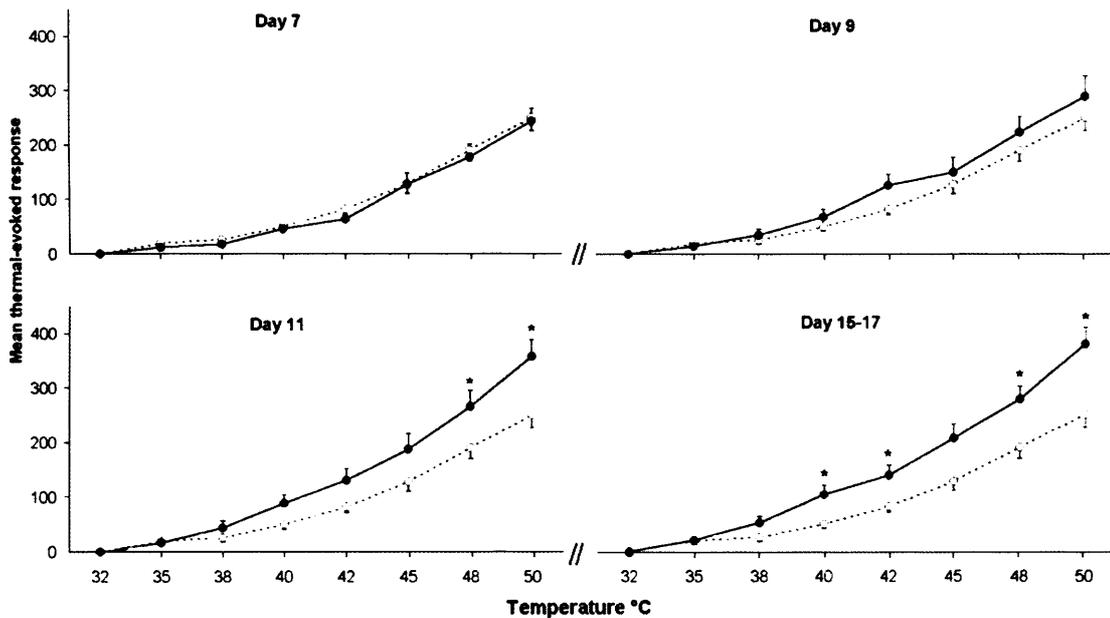
Figure 3.13 The temporal development of increased response to thermal stimuli of Lamina I WDR neurones in MRMT1-injected rats.

At days 7 and 9 post-surgery there is no difference in response between MRMT1-injected (solid black) and sham-operated rats. By day 11 the noxious intensity thermal-evoked neuronal response is significantly increased in MRMT1-injected animals and by days 15 to 17 the response is also significantly increased at lower intensities. ($^*P<0.05$)

3.8 Discussion

The results from this set of investigations confirm our previous findings of progressive development of hyperalgesia and allodynia as assessed by the behavioural tests, and an altered superficial dorsal horn neuronal response and NS:WDR ratio revealed by electrophysiological characterisation.

The temporal development of pain behaviour is very similar to another rat model of CIBP (Medhurst et al. 2002) where animals injected with MRMT-1 show a significant reduction of weight bearing on the affected limb by day 11 onwards, and general loss of activity by day 15 – the same time points at which we have observed significant behavioural allodynia and increased neuronal responses to all stimulus modalities.

These are the first studies to show development of behavioural allodynia concurrently with changes in dorsal horn electrophysiology. The hyperexcitability of the dorsal horn seems to be driven by a shift in cellular populations with previously NS neurones becoming WDR. The relationship between behavioural alterations and neuronal responses indicates the latter as the most suitable substrate for pharmacological study, being suprathreshold responses more clinically relevant than the threshold level behavioural responses. Furthermore, the close correlation between the temporal development of pain behaviour and increased dorsal horn excitability suggests that this would be a suitable model in which to study changes in gene expression in hyperalgesia and allodynia.

Finally, these investigations contribute to evidence for a major role of SDH neurones in the regulation and behavioural expression of pain sensitivity (Mantyh et al. 2004; Yezierski et al. 2004).

DESCENDING EXCITATORY CONTROL OF CIBP: A PHARMACOLOGICAL STUDY WITH ONDANSETRON.

4.1 Introduction

As discussed in section 1.5, descending pathways from the brain to the dorsal horn of the spinal cord are important in the modulation of nociceptive information, and have been demonstrated to have key roles in chronic pain states. Study of these facilitatory and inhibitory pathways has not only improved our understanding of the mechanisms underlying these states, but also afforded new approaches to pharmacotherapy in these clinically important areas.

Most of the work in this area has concentrated on the often tonically active inhibitory pathways, but more recently there has been growing interest in a facilitatory pathway mediated by 5-HT$_3$ receptors.

4.1.1 5-HT in pain 5-HT, when initially discovered in the first half of the 20th century, was called serotonin (a name still used widely) as a reference to its origin and biological action; a powerful *serum* vasoconstrictor inducing muscle *tone*. It is now known to be located also in the GI tract and CNS, having important roles as a neurotransmitter and a local hormone, involved in an array of physiological functions including sleep, wakefulness, mood, and control of sensory transmission, thus 5-HT is probably a more appropriate name.

5-HT pathways comprise one of the major components of descending controls and, as mentioned in section 1.6, electrophysiological, anatomical, and pharmacological studies of these pathways have highlighted areas of the medulla, particularly the RVM (containing the nucleus raphe magnus (RMg) and reticular formation (including the nucleus reticularis magnocellularis - NRMC), as the major output for descending influences (Gebhart 2004).

Direct evidence for the involvement of 5-HT systems in pain came from observing the effects of electrical stimulation or lesion of serotonergic brainstem nuclei; electrical stimulation of the PAG produced sufficient analgesia to carry out abdominal surgery in the rat in the absence of general

anesthesia (Reynolds 1969) and stimulation in the region of the dorsal raphe blocked excitatory locus coeruleus responses to noxious stimuli (Segal 1979), whilst electrolytic lesions of the RMg have been shown to cause a significant reduction in autoanalgesia (behaviourally activated analgesia) (Chance 1980). However, recent studies would suggest that although 5HT can mediate inhibitory effects, its predominant action is more likely to be facilitatory.

Cells in the RVM have been classified, according to responses to rat tail withdrawal to noxious heat, into 'on', 'off' and 'neutral' groups (Fields et al. 1983). After a long period of thermal stimulation, there is increased 'on' cell discharge suggesting that these cells facilitate nociception via a descending pathway (Morgan and Fields 1994). Furthermore, selective activation of these cells using low doses of neurotensin has been shown to produce thermal hyperalgesia confirming a facilitatory role for 'on' cells (Neubert et al. 2004). Some of these cells probably contain 5-HT as following formalin injection and noxious heat stimulation of the rat hind paw, 5-HT-positive RMg neurones are also labeled with FOS (Suzuki et al. 2002). However there is contradictory evidence suggesting that serotonergic raphe magnus cells responding to noxious heat are neither 'on' nor 'off' (Gao and Mason 2000). This illustrates the fact that descending 5-HT pathways and their pharmacology represent an area which needs further investigation to be clarified and better understood.

As mentioned in the introduction, more than 15 subtypes of 5-HT receptors have been identified which not only suggests a diverse role for 5-HT systems in pain processing but also goes some way to account for the complicated pharmacology. For example, $5-HT_{1A}$ receptors are coupled negatively to AC and hyperpolarise neurones by opening K^+ channels, whilst $5-HT_{2A}$ and $5-HT_3$ receptors activate PLC and depolarise cells by closing K^+ channels and/or opening VGCCs (Millan 1999). Therefore, when co-localised on neurones, these receptors will have opposing modulatory effects on nociceptive transmission in the DH.

4.1.2 5-HT₃ receptor-mediated excitation   Pharmacological studies have demonstrated the 5-HT$_3$ receptor to be pronociceptive in the spinal cord, as selective blockade of these receptors results in a reduction of nociceptive neuronal and behavioural responses (Ali *et al.*, 1996).

This excitatory serotonergic system has been shown to be enhanced following formalin-induced inflammation (Green *et al.*, 2000) and peripheral nerve injury (Suzuki *et al.*, 2004), but not following carrageenan-induced inflammation (Green *et al.*, 2000; Rahman *et al.*, 2004) suggesting that, in some pain states, selective inactivation of descending facilitatory pathways might offer analgesia that is complementary to the more traditional method of activating the descending inhibitory pathways (Millan 1999).

In addition, it has been demonstrated that lamina I dorsal horn (DH) neurones expressing the NK$_1$ receptor form the origin of a spino-bulbo-spinal loop which, relaying in the rostroventral medulla (RVM) and having a facilitatory action at spinal 5-HT$_3$ receptors, modulates mechanical and thermal nociceptive transmission and is necessary for full coding of inputs by deep DH neurons (Suzuki *et al.*, 2002). Furthermore, this circuit will receive input from centres such as the parabrachial area and amygdala involved in the affective component of pain (i.e. the fear, anxiety, emotional aspect) to amplify and prolong the sensation to a painful stimulus so that emotional state may alter the perception of pain (Suzuki et al. 2004b).

This is particularly significant with respect to CIBP. In the DH characterisation studies in the previous chapter, I have shown that the general hyperexcitability of the spinal cord is driven by changes in SDH populations with previously NS cells becoming WDR. As some of these superficial cells will be NK$_1$-expressing projection neurones it translates to increased transmission of signals to the brain areas involved in the affective component of pain, as well as access of lower threshold inputs. This does in fact fit with the clinical condition of CIBP correlating with increased anxiety and depression (Mercadante 1997) and the low threshold access may be

important in terms of the mechanical allodynia seen behaviourally in the animal models.

The aim of this next study was to measure the effects of spinally administered ondansetron, a selective 5-HT$_3$ receptor antagonist, on electrical- and natural-evoked DH neuronal responses in CIBP, in order to assess the role of the descending serotonergic facilitatory pathway.

4.2 Methods

Electrophysiological experiments were carried out between days 15-17 in MRMT-1-injected and sham-operated rats as described in Chapter 2, at which time all animals in the cancer group show significant allodynia. Tests on superficial and DDH neurones, carried out every 20 minutes, consisted of electrical stimuli followed by natural stimuli (brush, von Frey, thermal, and cold). After three consecutive stable control trials (< 10% variation) neuronal responses were averaged to give pre-drug control values. Ondansetron (Zofran; Glaxo-Wellcome: 10, 50 and 100μg) was administered directly onto the exposed spinal cord in a volume of 50μl using a Hamilton syringe. The response of the neurone was followed for 50 minutes (testing at t = 10, 30, and 50 minutes) before the next dose was given.

4.3 Results

As described in chapter 3, all MRMT-1-injected rats showed progressive development of hyperalgesia and allodynia to mechanical and cooling stimuli, as well as ambulatory-evoked pain assessed on the rotorod, whilst sham-operated rats showed no change from pre-operative baseline behaviour (data not shown). A total of 39 DH neurones were used for pharmacological study and were separated according to depth and animal group, thus 6 neurones

were used in each of the sham-operated rat groups (DDH, NS SDH, WDR SDH) and 7 in the MRMT-1-injected rat groups as above.

4.3.1 Electrical-evoked responses   Ondansetron had no significant effect on the control electrical-evoked responses for either SDH or DDH neurones (Table 4.1) at any of the doses tested.

Table 4.1 Mean control electrical-evoked responses of superficial and deep dorsal horn neurones in sham-operated and MRMT-1-injected rats.

	Cell group	Input	A-beta	A-delta	C-fibre	Post-discharge	Wind-up
SHAM	DDH WDR (n=6)	301 ± 45	102 ± 5	95 ± 10	374 ± 21	198 ± 16	256 ± 49
	SDH NS (n=6)	90 ± 26	96 ± 11	29 ± 9	129 ± 36	49 ± 12	80 ± 26
	SDH WDR (n=6)	153 ± 34	85 ± 9	48 ± 12	133 ± 25	54 ± 22	94 ± 14
MRMT	DDH WDR (n=7)	421 ± 27	112 ± 14	112 ± 15	428 ± 20	279 ± 32	331 ± 42
	SDH NS (n=7)	151 ± 42	94 ± 21	34 ± 11	158 ± 39	63 ± 17	123 ± 23
	SDH WDR (n=7)	247 ± 36	133 ± 8	45 ± 9	248 ± 26	136 ± 49	161 ± 32

4.3.2 Natural-evoked responses

The highest dose of ondansetron (100μg) significantly reduced ($P<0.05$) noxious mechanical-evoked neuronal responses to von Frey 75g of DDH and SDH in sham-operated rats (Figure 4.1).

In MRMT-1-injected rats, ondansetron also significantly reduced noxious mechanical-evoked neuronal responses to von Frey 30g and 75g in DDH neurones ($P<0.001$). In WDR SDH neurones there were marked effects with significant reduction by the middle dose of ondansetron (50μg) of neuronal responses to von Frey 9g ($P<0.05$), 15g ($P<0.001$), 30g ($P<0.05$) and 75g ($P<0.001$). The mechanical-evoked responses of NS SDH neurones were also sensitive to ondansetron at 50μg (von Frey 30g, $P<0.05$) and 100μg (von Frey 30g and 75g, $P<0.05$). Figure 4.2 shows the effects of ondansetron on mechanical-evoked responses of DH neurones in MRMT-1-injected rats.
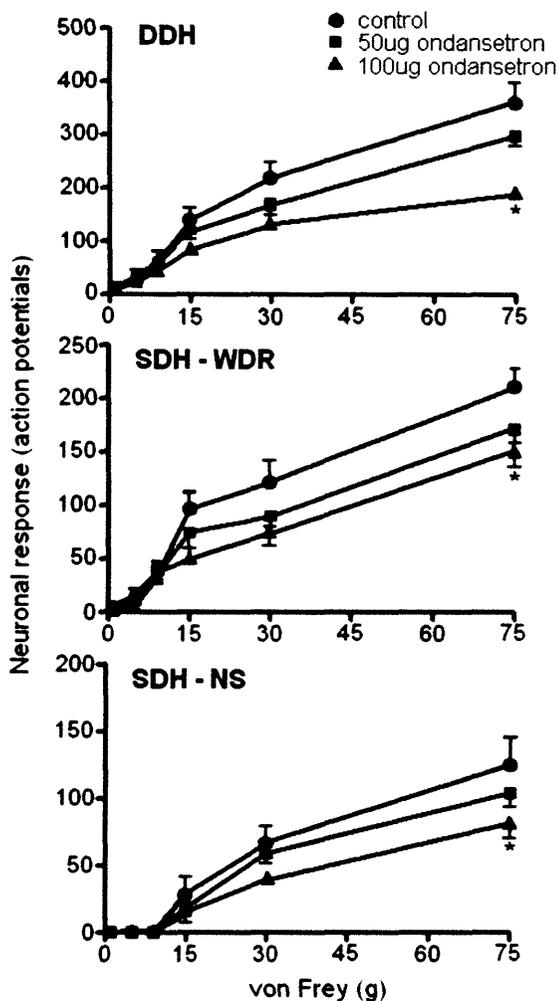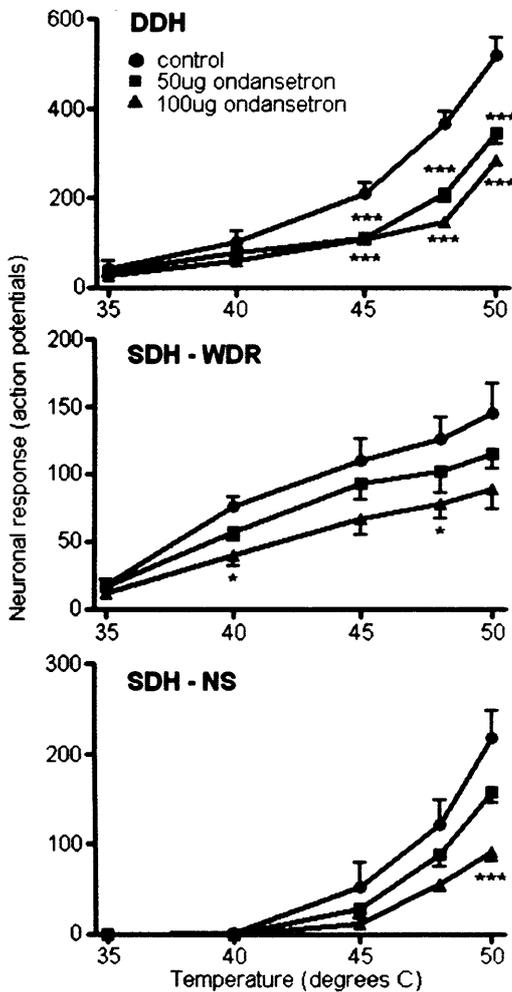

Figure 4.1 Mechanical-evoked DH neuronal responses are mediated by 5HT₃ receptors in sham-operated rats. Spinal ondansetron reduced mechanical-evoked responses in sham-operated rats. n = 6, *($P<0.05$) significant reduction compared to pre-drug control.
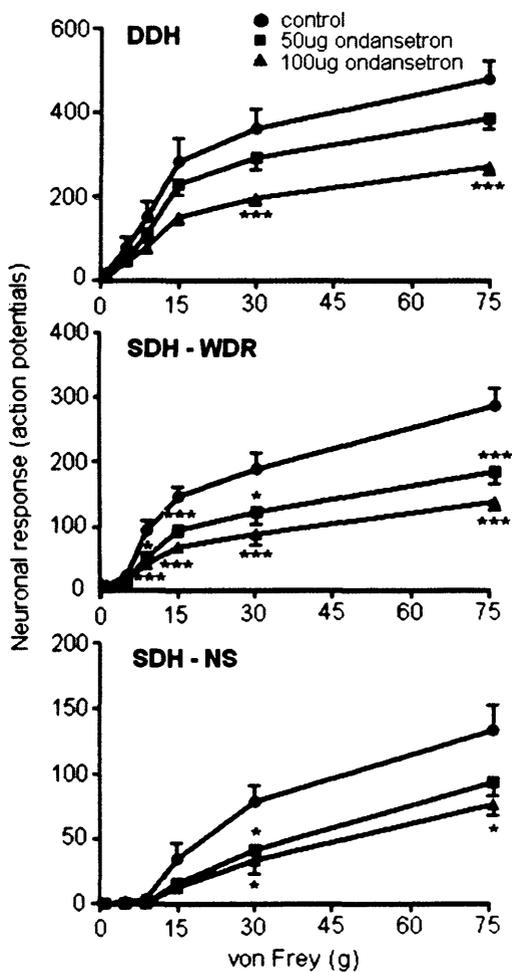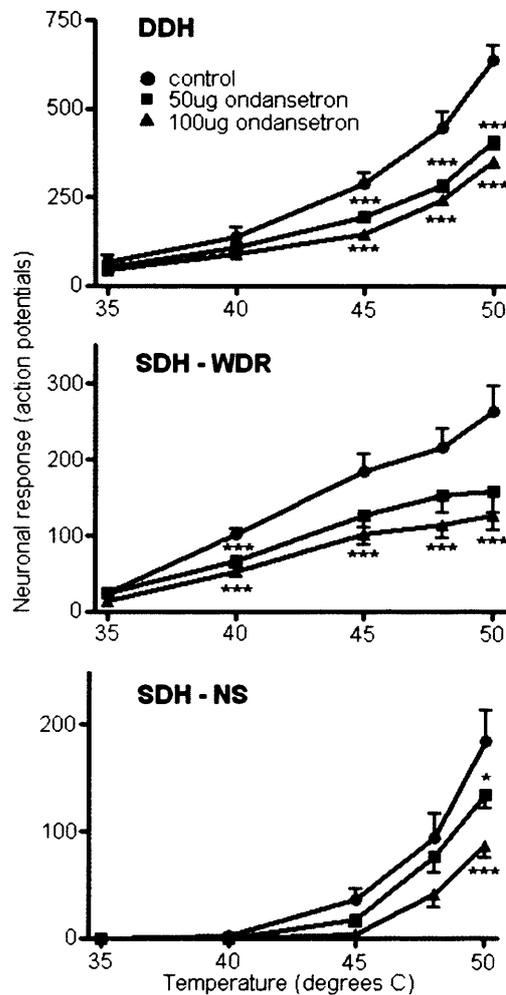
Figure 4.2 5HT₃ receptor-mediated facilitation of mechanical-evoked neuronal responses is enhanced in CIBP. Spinal ondansetron reduced mechanical-evoked responses of DDH and SDH neurones in rats with CIBP at a lower dose (50μg) and over a wider range of stimuli than in sham-operated rats, suggesting an enhanced drug effect in this pain state. n = 7. *(P<0.05), ***(P<0.01) significant reduction compared to pre-drug control.

Similar effects were seen with ondansetron in the thermal-evoked DH neuronal responses. In sham-operated rats, the middle and top doses of ondansetron significantly reduced (P<0.001) DDH responses to thermal stimuli in the noxious range of 45-50°C. In WDR SDH neurones the top dose of ondansetron significantly reduced (P<0.05) thermal-evoked responses at innocuous (40°C) and noxious (48°C) temperatures. In NS SDH neurones the top dose of ondansetron significantly reduced (P<0.05) the thermal-evoked response to 50°C. The effects of ondansetron on thermal-evoked responses in sham animals are shown in Figure 4.3.

In MRMT-1-injected rats ondansetron had similar effects on DDH neurones as in the sham-operated rats with significant reductions (P<0.01) to thermal stimuli in the noxious range between 45°C and 50°C. In WDR SDH neurones the effects were more marked than in shams with the middle dose significantly reducing (P<0.001) the response to 40°C, and the top dose significantly reducing (P<0.001) responses to thermal stimuli from 40-50°C.

In NS SDH cells the response to 50°C was significantly reduced by the middle (P<0.05) and top dose (P<0.001) of ondansetron.

Figure 4.3 Thermal-evoked DH neuronal responses are mediated by 5HT₃ receptors in sham-operated rats. Spinal ondansetron reduced thermal-evoked dorsal horn neuronal responses in sham-operated rats. n = 6, ***(P<0.01) significant reduction compared to pre-drug control.

Figure 4.4 5HT₃ receptor-mediated facilitation of thermal-evoked neuronal responses is enhanced in CIBP. Spinal ondansetron reduced thermal-evoked responses of SDH neurons in rats with CIBP at a lower dose (50μg) and over a wider range of stimuli than in sham-operated rats, suggesting an enhanced drug effect in this pain state. n = 7. *(P<0.05), ***(P<0.01) significant reduction compared to pre-drug control.

4.4 Discussion

All animals injected with MRMT-1 developed behavioural signs of hyperalgesia, allodynia and ambulatory-evoked pain, and the electrical- and natural-evoked responses in these animals were elevated compared to sham-operated rats (Table 4.1, Figures 4.1 - 4.4). These results therefore confirm the findings from the characterisation study that this is a reliably reproducible model of CIBP, in which mechanical allodynia, cold allodynia, and ambulatory-evoked pain can be measured behaviourally, and with a characteristic electrophysiological feature of dorsal horn hyperexcitability.

Research into descending inhibitory and facilitatory influences on spinal nociceptive processing has improved our understanding of chronic pain states and provided new approaches to their pharmacological treatment. This study provides electrophysiological evidence of a role for descending facilitation in a rat model of CIBP.

Antagonism of 5-HT$_3$ receptors, using ondansetron, has previously been shown to inhibit neuronal activity and pain behaviour (Ali et al. 1996; Green et al. 2000; Rahman et al. 2004; Suzuki et al. 2004a). These results clearly demonstrate that ondansetron selectively inhibits natural-evoked DH neuronal responses in sham-operated rats suggesting that this pathway is normally active. The effects however were much greater in animals injected with MRMT-1 with significant reductions at lower doses of ondansetron and encompassing both the innocuous and the noxious range, thus clearly showing that the effect of ondansetron is enhanced in CIBP. These findings are comparable with findings in other chronic pain models as denoted below.

A model of spinal cord injury, in which chronic tactile hyperalgesia and allodynia are observed in the absence of changes in density or distribution of primary afferent fibres in the region surrounding the injury, has been shown to alter descending antinociceptive serotonergic tracts that modulate pain transmission, with decreased 5-HT immunoreactivity caudal to the injury site and a threefold increase rostral to it (Bruce et al. 2002). Subsequent studies

in the same model showed that intrathecally administered ondansetron produced a robust long-term reduction of mechanical allodynia whereas the 5-HT$_3$ agonist m-chlorophenylbiguanide (m-CPBG) exacerbated allodynia (Oatway et al. 2004). The reduction of allodynia with exogenous 5-HT was only transient compared to the long-term reduction with ondansetron and was opposed by methysergide, the non-selective 5-HT$_1$/5-HT$_2$ receptor antagonist. This suggests that increased 5-HT fibre density immediately rostral to the injury site in this model could have transient effects to reduce mechanical allodynia via actions at 5-HT$_1$ and/or 5-HT$_2$ receptors, whereas the more long-lasting effects of this enhanced serotonergic input may facilitate chronic allodynia via the 5-HT$_3$ receptor.

In a model of carrageenan-induced inflammation it has been shown that intrathecal ondansetron has no effect on electrical-evoked responses but reduces punctuate mechanical and thermal-evoked responses of DDH WDR neurones in both sham and carrageenan-inflamed rats (Rahman et al. 2004). However, unlike in CIBP, there was no difference in drug effect between the animal groups. In a spinal nerve ligation model (SNL) of neuropathic pain, however, it has been shown that intrathecal ondansetron selectively inhibits mechanical and thermal-evoked responses to a greater degree in SNL compared to sham-operated rats (Suzuki et al. 2004a). As with the carrageenan and CIBP models, the electrical-evoked responses were not affected in either group of animals in the SNL model. The inability of ondansetron to reduce the electrical-evoked responses may be due to the intensity of the stimulus. In all of these studies transcutaneous electrical stimuli were given at three times the threshold for C-fibre activation. Although ondansetron clearly reduced noxious level mechanical and thermal stimuli these were delivered over a 10 second period, whilst the electrical pulses were 2 milliseconds wide, therefore representing a much more intense time-locked stimulus that would be harder to inhibit.

It is interesting to note that in the inflammatory model electrophysiological experiments were carried out 3 hours after intraplantar injection of carrageenan when pre-drug characterisation revealed no difference in

evoked responses of dorsal horn neurones between inflamed and sham-operated rats. In the neuropathy model however, experiments were carried out two weeks after surgery when it has been demonstrated that DH neurons are electrophysiologically distinct to those in sham-operated rats (Chapman et al. 1998b). In this model electrophysiology experiments were carried out a similar length of time after surgery when we have also shown DH neurons to be distinct from those in sham-operated animals, though the changes are different to those observed in the model of neuropathy.

This would therefore suggest that plasticity of the descending facilitatory pathway, contributing to abnormal natural-evoked responses in neuropathy and CIBP, does not occur immediately after tissue injury and is more likely to be involved in the maintenance rather than the generation of these chronic pain states.

Results in the previous chapter demonstrated that neuronal alterations in the SDH in CIBP correspond temporally with behavioural signs of allodynia and it has been suggested that inappropriate activation of descending excitatory pathways may be a contributory mechanism to abnormal behaviour associated with chronic pain states (Porreca et al. 2002). The results presented here are therefore in agreement with a role of descending facilitation in pain behaviour; the elevated neuronal responses characteristic of CIBP, occurring only when behavioural alterations are present, were more sensitive to ondansetron than the normal neuronal responses of the sham-operated animals which do not at any point show behavioural deficits. Given the temporal correlation between neuronal and behavioural deficits in this model, it may therefore be of interest to study the effects of ondansetron at earlier time points in CIBP to establish when serotonergic facilitatory control begins to exert its effect on spinal processing and pain behaviour.

In summary, these findings suggest a role for descending facilitation in the elevated mechanical and thermal-evoked responses observed in CIBP and, by virtue of the correlation of this hyperexcitability with behavioural allodynia,

provide further evidence for the involvement of 5-HT$_3$ receptor systems in pain behaviour.

GABAPENTIN: EFFECTS ON DORSAL HORN NEURONAL RESPONSES AND PAIN BEHAVIOUR.

5.1 Introduction

Gabapentin, 1-(aminomethyl)cyclohexane acetic acid, (Neurontin™) was designed as a structural analogue of GABA for the treatment of epilepsy, and introduced in the UK in 1993 as an anticonvulsant to reduce seizure frequency when co-administered with existing antiepileptic drugs. However, the drug was also found to be an effective antihyperalgesic and in May 2000 it became the first agent in the UK to be approved for the treatment of all types of neuropathic pain. By 2002 gabapentin had been approved for treatment of neuropathic pain in five more European countries as well as Australia, New Zealand, and several parts of Latin America, and in September 2001 Morgan Stanley predicted sales of US $1871 million in 2002 falling to US $413 million in 2006 (Wheeler 2002).

As well as being used widely in clinical neuropathies, gabapentin has been shown to be effective in a variety of animal models of chronic pain. The work in this chapter focuses on the effects of systemic gabapentin on evoked dorsal horn neuronal responses and pain behaviour in CIBP.

5.1.1 Structure and function of GBP Gabapentin (GBP) is derived by the addition of a cyclohexyl group to the backbone of GABA (Taylor 1997) resulting in a lipophilic GABA analogue that is able to cross the blood brain barrier. At physiological pH, GBP is a zwitterion, i.e. an amino acid containing both acidic and basic groups in the same molecule that undergoes an internal acid-base reaction, existing primarily as a dipolar ion (Taylor et al. 1998).

GBP, although a structural analogue of GABA, does not interact with either $GABA_A$ or $GABA_B$ receptors (Taylor 1997). It does not have an effect on [^{3}H]-GABA uptake into neuronal or glial cultures (Su et al. 1995) nor does it inhibit GABA transaminase, the GABA-metabolising enzyme (Taylor et al. 1998). Furthermore, the antiallodynic actions of GBP are not sensitive to either $GABA_A$ or $GABA_B$ receptor antagonists (Hwang and Yaksh 1997) and, unlike

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several other antiepileptics, GBP does not affect voltage-gated Na^+ channels. However, there is some evidence that it acts as an agonist at the $GABA_B$ gb1a-gb2 heterodimer coupled to inwardly rectifying K^+ channels (kir3.1/3.2) in *Xenopus* oocytes (Ng et al. 2001).

Autoradiography studies have shown that [^3H]-GBP binds with high affinity to the outer layers of the cortex (frontal, parietal, occipital and entorhinal) with lower levels in the white matter, and some binding in the dentate gyrus, cerebellum, and CA1 region of the hippocampus (Hill et al. 1993). The high affinity binding site was identified as the $\alpha_2\delta$ subunit of VGCCs (Gee et al. 1996). Some other drugs acting at VGCCs, such as flunarizine, have also been shown to prevent seizures in animal models, but GBP is different to the other ligands in that it acts at the $\alpha_2\delta$ rather than the α_1 subunit (Gee et al. 1996).

5.1.2 The $\alpha_2\delta$ subunit of VGCCs   As mentioned in Chapter 1, VGCCs are made up of a number of subunits; the α_1-subunit forms the basic structure of the channel containing amino acids that form the pore and voltage sensor determining the major functional properties of the channel and its subtype, and the auxiliary subunits (β, $\alpha_2\delta$, and γ) modulate the function of the α_1-subunit (Catterall 2000b). As shown in the figure below, the δ subunit is anchored into the lipid bilayer, but most of the α_2 amino acids are exposed extracellularly.

The $\alpha_2\delta$ family consists of three genes; the first subunit, $\alpha_2\delta$-1, was identified in rabbit skeletal muscle in the late 1980s (Ellis et al. 1988) and two more family members, $\alpha_2\delta$-2 and $\alpha_2\delta$-3, were identified in the late 1990s (Klugbauer et al. 1999). GBP has been found to bind specifically to the $\alpha_2\delta$-1 subunit (Gee et al. 1996), with a much lower affinity for $\alpha_2\delta$-2 and no binding to $\alpha_2\delta$-3 (Marais et al. 2001). R217A mutant mice, which have a single amino acid substitution at position 217 in the α_2 protein, have been shown to be insensitive to the actions of GBP after neuropathy (Bramwell et al. 2004). Autoradiography studies with [3H]-GBP in R217A mice have shown a global

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reduction in signal (compared to wild type litter mates) with 80% reduction in the cortex and hippocampus, 70% reduction in the spinal cord, and 30% reduction in the cerebellum (Melrose et al. 2004). This study highlighted the superficial dorsal horn as an area of intense binding which, as discussed in previous chapters, is a particularly important junction in the nociceptive pathway.
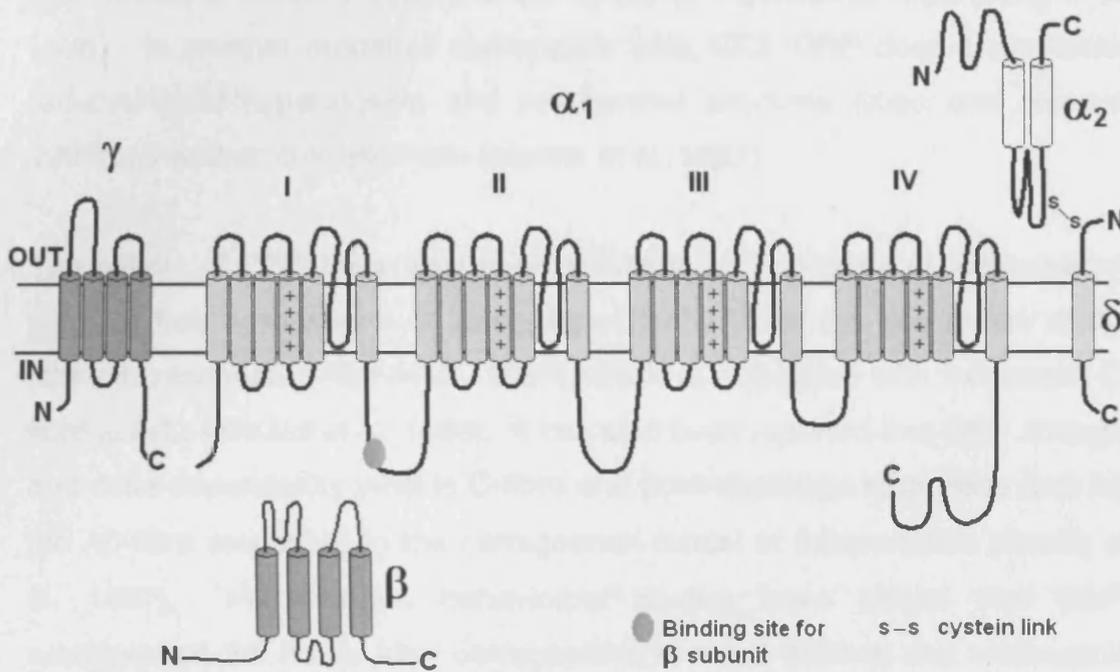
Figure 5.1 Schematic representation of the transmembrane topology of calcium channel subunits. The pink oval area on the intracellular loop between domains I and II of α_1 represents the binding site for the β subunit. The transmembrane regions of the α_1 subunit with + symbols are the S4 segments, which contain positively charged amino acids and are thought to make up the voltage sensors. Adapted from (Canti et al. 2003).

5.1.3 GBP in animal models of pain　　　GBP has been shown to reduce neuronal responses and nocifensive behaviour in a number of pain models. In the SNL model of neuropathy for example, GBP reduced withdrawal responses to von Frey (Hunter et al. 1997), and in other studies it inhibited electrical- and mechanical-evoked DDH neuronal responses

(Chapman et al. 1998a; Matthews and Dickenson 2002). The latter investigation underlined the antiallodynic effects of GBP which had significant effects in neuropathic rats, mild effects in sham-operated rats (perhaps indicating the presence of some minor post-operative inflammation), but was totally ineffective in normal rats. In the same model, GBP had no effect on spontaneous ectopic discharges in the injured peripheral nerve suggesting a mechanism of action primarily at the spinal or supraspinal level (Abdi et al. 1998). In another model of neuropathic pain, CCI, GBP dose-dependently reduced heat hyperalgesia and mechanical allodynia (Xiao and Bennett 1995), as well as cold allodynia (Hunter et al. 1997).

The effects of GBP have also been investigated in models of inflammatory pain. It has been shown to dose-dependently block the late phase of the formalin response (Field et al. 1997) which is correlated with increased C-fibre activity (McCall et al. 1996). It has also been reported that GBP strongly and dose-dependently inhibits C-fibre and post-discharge responses (but not the Aβ-fibre response) in the carrageenan model of inflammation (Stanfa et al. 1997). Furthermore, behavioural studies have shown that GBP, administered 2.5 hours after carrageenan, reduces thermal and mechanical hyperalgesia (Field et al., 1997).

As CIBP shares some features of neuropathic and inflammatory pain, the aim of this study was to determine the efficacy of systemic GBP on the hyperexcitable dorsal horn neuronal responses and behavioural alterations in the rat intra-tibial MRMT-1 model, and to gauge the relationship between spinal plasticity and behaviour.

5.2 Methods

Preparation of the MRMT-1 cell line and surgery to induce CIBP (or sham surgery) were carried out as described in Chapter 2.

5.2.1 Drug administration   GBP (a gift from Pfizer, Sandwich,UK) was dissolved in saline to give 30mg kg^{-1} (in a volume of 1ml kg^{-1}) for the chronic behavioural study, and 10mg kg^{-1}, 30mg kg^{-1} and 100mg kg^{-1} (in the same volume of 250 µl) for the acute pharmacologic study. All injections were given subcutaneously in the scruff of the back of the neck.

Rats were divided into three treatment groups. The first group (n = 15), all having received intratibial injection of MRMT-1 cells, were given bi-daily injections (every 12 hours 8am and 8pm) of GBP 30mgkg^{-1} between days 11 and 14 post-surgery, with a single injection on the morning of day 15. Twelve of the animals in this group were subsequently used for electrophysiological characterisation of superficial dorsal horn neurones. A second group (n = 6) acting as a control for the GBP-treated animals were inoculated with MRMT-1 cells and received bi-daily injection of saline according to the same schedule. The third group of animals made up of MRMT-1-injected (n = 7) and sham-operated rats (n = 6) received no post-operative treatment and were used for electrophysiological pharmacologic study of the acute systemic effects of GBP.

5.2.2 Behavioural testing   The methods for behavioural testing were exactly as described in Chapters 2 and 3. Tests were performed pre-operatively and on post-operative days 1, 5, 7, 11, 12, 13, 14, 15, and 18. Between days 11 to 15 behavioural testing took place 1 hour after drug administration (morning).

5.2.3 Spinal cord electrophysiology   Characterisation of SDH neurones was carried out on the final day of GBP treatment (i.e. post-operative day 15) and on the third day after cessation of treatment (i.e. post-operative day 18) exactly as described in Chapter 2. Acute pharmacology

studies with GBP were carried out in the same way as described in Chapter 4 for ondansetron, with the exception that GBP was given systemically (see above).

5.2.4 GBP autoradiography studies

Two sham-operated and two MRMT-1-injected rats were used for the autoradiography studies. On post-operative day 17 the rats were deeply anaesthetized with an intra-peritoneal injection of 200 mg ml^{-1} pentobarbitone (Rhone Merieux). Upon cessation of respiration a thoracotomy was performed to expose the heart. The right atrium was cut and the left ventricle cannulated for transcardial perfusion which, initially, was with 0.9% saline and then 4% paraformaldehyde in 0.2M phosphate buffer (pH 7.4). The lumbar spinal cord was exposed and spinal insertion points L4-L6 were identified and removed, a pin was inserted to make a hole in the dorsal area of the spinal cord contralateral to the tumour so that ipsi- and contralateral sides could thereafter be easily identified. Tissue was post-fixed for at least 1 hour in 4% paraformaldehyde and cryoprotected for at least 24 hours in 20% sucrose.

For cutting, tissue was covered in OTC embedding medium (Tissue-Tek) and frozen on a freezing sledge microtome at -16°C (Leica, Switzerland). Transverse sections were cut at 30μm thickness and collected freefloating in 0.1M phosphate buffered saline with 5% sucrose and 0.02% azide in 96 well plates, 1 section per well. Sections were mounted serially onto glass slides such that each slide contained eight sections 360μm apart. Slides were then left to dry at room temperature and stored at -70°C until autoradiography experiments.

Before the binding assay slides were thawed at room temperature for approximately 30 minutes and pre-incubated in 10mM Hepes pH 7.4 at 4°C for 15 minutes. Slides were then incubated in the same buffer containing 40nM [^{3}H]-GBP (50Ci/mmol, Amersham Biosciences, custom synthesis) at room temperature for 30 minutes. Adjacent sections were incubated in the presence of 10μM [3H]-pregabalin (78Ci/mmol, Amersham Biosciences,

custom synthesis) to define non-specific binding. The slides were washed four times for 1 minute in 0.1M NaCl followed by a final rinse in cold dH_2O to remove excess salt. The slides were dried at room temperature and exposed along with a [³H] microscale ranging from 0.09 to 95.44 nCi/mg (Amersham) to [³H] hyperfilm (Amersham) for 4 weeks. Films were developed in a dark room with Kodak D19 developer for 3 minutes, washed in tap water for 30 seconds and then fixed for 5 minutes with Unifix (Kodak). The film was then rinsed with running water and hung to dry.

Optical densitometry was performed using a microcomputer imaging device (MCID-M5+ 5.1 Rev 1.2) image analysis system and software (Imaging Systems Incorporated, St. Catherine's, Ontario, Canada). Illumination of film images was supplied by the Northern Light Precision Illuminator model B95 (Imaging Systems Inc). A Sony CCD monochrome camera model XC-77 (512x480 pixels, 30Hz frame) with a micro-Nikkor 55mm lens (Nikon, Japan) was used to capture the images.

Quantification of film images allows the rapid gathering of anatomical binding data at the regional level. The optical density of the film images produced by the labelled sections is related to the regional radioactivity concentration and therefore represents the relative amount of bound ligand present, although this is not necessarily a linear relationship. For accurate quantification of film images it is necessary to correct for illumination and shading errors. Illumination and shading differences were corrected by digitizing a blank field (flat field correction) with the lighting set so that optical density of the blank field was around the mid range of the values encountered in the analysis. To obtain relative values, the values produced by the images must be compared with those produced by samples of known radioactivity content. A calibration curve was constructed by plotting densitometry measurements (relative optical density) obtained from [³H] standards (which were co-exposed in the cassette with the slides) against the given concentration of radiation (nCi/mg) for each standard.   Once the calibration is established all sample measurements are collected in nCi/ng.   The MCID software flags measurements that are at the level of film saturation.

At the end of the autoradiography study sections were stained with toluidine blue for standard photography of the slides. Sections were dehydrated in absolute alcohol for 3 minutes and then industrial methylated spirit (IMS) for a further 3 minutes. The sections were then rehydrated in running tap water for 5 minutes before incubation in Toluidine blue for 1 minute. The slides were then rinsed in tap water, dehydrated in (IMS) for 30 seconds, absolute alcohol for another 30 seconds, and cleared in xylene for 5 minutes. Slides were mounted using DPX permanent mountant.

5.3 Results

5.3.1 Chronic systemic GBP – behaviour
A total of 21 MRMT-1-injected rats were used in the chronic behavioural study, 15 of which received bi-daily injection of GBP 30mgkg^{-1} between post-operative days 11 to 15, and 6 of which received bi-daily injection of saline. The GBP-treated MRMT-1 animals showed an attenuation of the enhanced withdrawal responses to mechanical stimuli caused by CIBP compared to saline-injected animals, which was significant ($P<0.05$) by post-operative day 12 for von Frey 1g (Figure 5.2A) and by day 13 for higher von Frey filaments (Figure 5.2B). We would interpret this as indicative of inhibition of allodynia/hyperalgesia. It is notable that GBP did not abolish the withdrawal response to mechanical stimuli but returned it to the pre-operative/sham baseline level (Figure 5.2B). The results for the ambulatory-evoked pain (as assessed by the rotorod test) mirrored the von Frey results with a significant reduction of the deficits ($P<0.05$) in the GBP-injected group compared to saline-injected rats by post-operative day 13 (Figure 5.2C). The withdrawal response to acetone was also significantly lower ($P<0.05$) in the GBP-treated animals by day 14 (Figure 5.2D). Unlike with the mechanical and ambulatory-evoked pain responses, GBP did not normalise the response to acetone but prevented it from further increasing beyond the level it had reached at the time treatment was initiated.

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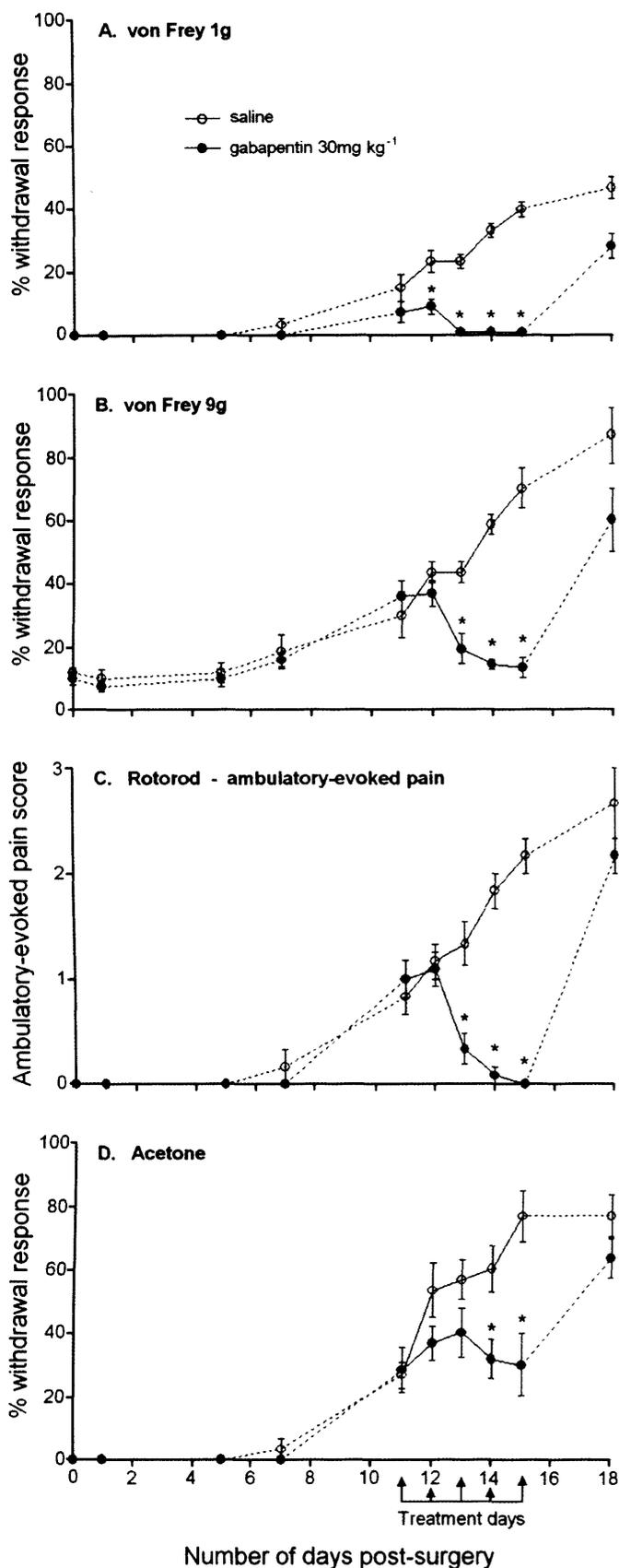
Figure 5.2 The effect of chronic GBP treatment on mechanical allodynia (1A, 1B), ambulatory-evoked pain (1C) and cold allodynia (1D) in MRMT-1-injected rats. Each data point in the saline-treated group (open circle) represents the mean ± s.e.m. of 6 rats and in the GBP-treated group (closed circle) the mean ± s.e.m. of 15 rats. Animals received bi-daily subcutaneous (s.c.) injection of saline/GBP 30mgkg^{-1} between post-operative days 11 to 14 inclusive at 12-hour intervals, with a final injection on the morning of day 15. GBP-treated rats show a reduction in withdrawal responses to von Frey 1g (1A) compared to saline-treated rats significant (P<0.05) from day 12, and to von Frey 9g (1B) significant from day 13. The ambulatory-evoked pain score (1C) in GBP-treated rats was also significantly reduced compared to saline-treated rats from day 13, and the withdrawal response to acetone (1D) from day 14. On day 18, two days after cessation of treatment, there was no longer a significant difference between the groups with GBP-treated rats returning to the same level of mechanical and cold allodynia, and ambulatory-evoked pain, as saline-treated rats.

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The results from day 18 (from a total of 9 rats, two days after cessation of GBP treatment) revealed the re-emergence of hyperalgesia and allodynia in this group that was equivalent to the saline-treated rats (i.e. rats never having received GBP) for all of the behavioural tests (Figures 5.2A-D).

In four normal animals treated with GBP according to the same dosing regime, there was no alteration in motor function (i.e. normal performance on the rotorod) nor responses to Von-Frey filaments or acetone when compared to non-treated, or GBP solute (normal saline) injected normal animals (data not shown).

5.3.2 Chronic systemic GBP – SDH neurone characterisations

A total of 30 SDH neurones were characterised (from 6 rats) on post-operative day 15 after the final dose of GBP at the time point when pain behaviour was reduced to the level of sham-operated rats. A total of 32 cells (from another 6 rats) were characterised on post-operative day 18 (and 2 days after cessation of GBP) by which time pain behaviour had returned to the same level of allodynia / hyperalgesia as the saline-treated MRMT-1 rats. Thirty cells were characterised from 6 animals receiving MRMT-1 cells and normal saline vehicle, and a total of 31 cells from 6 sham operated animals.

In the initial characterisation study (Chapter 3), in sham operated animals the ratio of WDR:NS was 26%:74%, which altered in MRMT-1 treated animals to 47%:53%. Here, almost identical results were seen in that the ratio of WDR:NS in GBP-treated CIBP rats on day 15 was 33%:66% and on day 18 had returned to 50%:50% (Figure 5.3). This suggests that chronic treatment with GBP attenuates spinal cord plasticity and so shifts the abnormally high WDR neuronal population (characteristic of this rat model of CIBP) back towards the predominant high-threshold situation seen in sham animals. Importantly, cessation of treatment and the re-emergence of pain-related behaviour, results in a return to the CIBP state and increased WDR ratio.
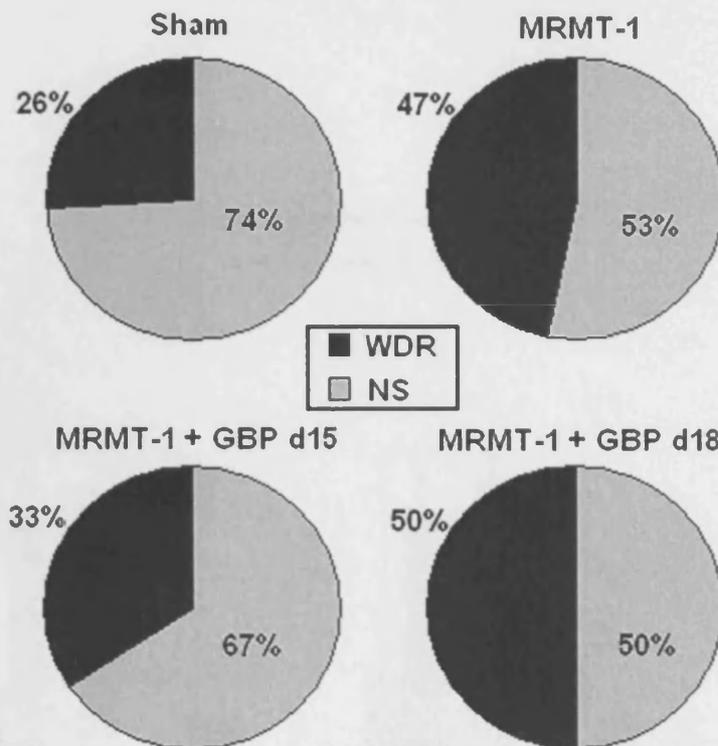
Figure 5.3 The effect of chronic GBP treatment on proportions of WDR and NS neurones in the SDH of MRMT-1-injected rats. In MRMT-1-injected rats receiving GBP treatment (bi-daily s.c. injection GBP 30mgkg^{-1} post-operative days 11-14 and a single injection on day 15), characterisation of superficial DH neurones on day 15 (n=30 cells), when pain-related behaviour was returned to a normal (i.e. sham-operated rat) level, revealed a WDR:NS ratio of 33%:67% approaching the normal ratio seen in sham-operated animals. Characterisation on post-operative day 18 (n=32 cells), two days after cessation of GBP treatment when animals had returned to the same level of pain behaviour as saline-treated rats revealed a return to the characteristic CIBP state with a WDR:NS ratio of 50%:50%.

The WDR cells characterised in GBP-treated rats on day 15 (number of animals=6, total of 30 neurones, of which 10 were WDR) showed significantly reduced ($P<0.05$) input and C-fibre evoked responses to electrical stimuli compared to cells characterised in MRMT-1-injected animals receiving no post- operative drug treatment (number of animals=6, total number of neurones 30, of which 14 were WDR) (Figure 5.4).
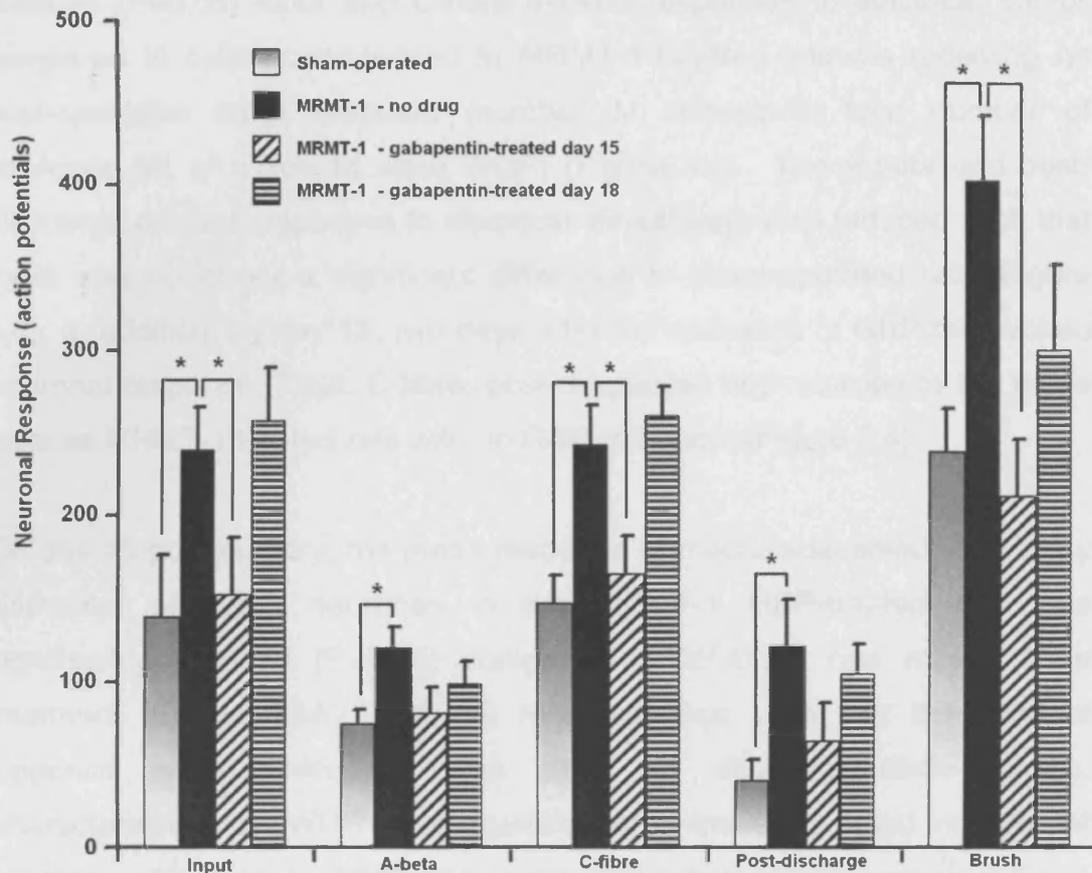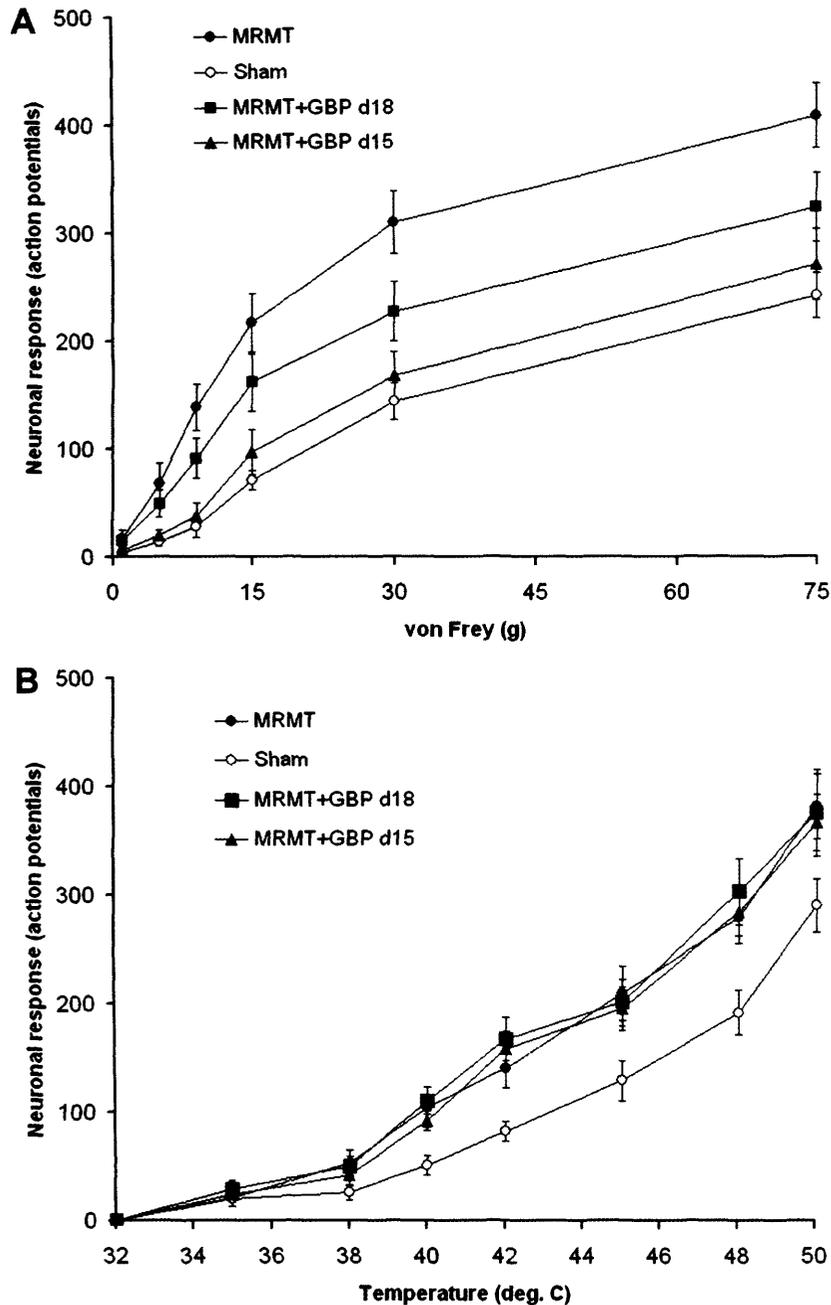
Figure 5.4 The effect of chronic GBP treatment on electrical- and natural-evoked responses of WDR SDH neurones in MRMT-1-injected rats. Electrophysiological characterisation of WDR SDH neurones in MRMT-1-injected rats having had chronic GBP treatment (bi-daily s.c. injection GBP 30mgkg^{-1} post-operative days 11-14 with a single injection on day 15) on the final day of treatment (n=10 cells) showed a significant reduction (P<0.05) in electrical- (input, C-fibre) and brush-evoked responses compared to MRMT-1-injected rats receiving no drug treatment. The A-beta and post-discharge evoked responses to electrical stimuli were reduced such that there was no longer a significant difference to the level of response of neurones in sham-operated rats. Characterisation of WDR SDH neurones in MRMT-1-injected rats two days after cessation of GBP treatment (post-operative day 18, n=16 cells) revealed that the electrical- and natural-evoked responses had returned to the same level as in MRMT-1-injected rats receiving no drug treatment. The input and C fibre evoked responses were significantly increased (P<0.05) compared to the responses at day 15 (final day of GBP treatment).

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The WDR cells characterised in GBP-treated rats on day 15 (number of animals=6, total of 30 neurones, of which 10 were WDR) showed significantly reduced ($P<0.05$) input and C-fibre evoked responses to electrical stimuli compared to cells characterised in MRMT-1-injected animals receiving no post-operative drug treatment (number of animals=6, total number of neurones 30, of which 14 were WDR) (Figure 5.4). The A-beta and post-discharge evoked responses to electrical stimuli were also reduced such that there was no longer a significant difference to sham-operated rats (Figure 5.4). In addition, by day 18, two days after the cessation of GBP the evoked neuronal response (input, C-fibre, post-discharge) had returned to the same level as MRMT-1 treated rats with no GBP treatment (Figure 5.4).

On day 15 post-surgery, the mean response to mechanical stimuli (von Frey filaments) of WDR neurones in the MRMT-1 GBP-treated rats was significantly reduced ($P<0.05$) compared to MRMT-1 rats receiving no treatment (Figure 5.5A), and this reduction was such that the level of response was similar to that seen in sham-operated animals. Characterisation of WDR cells (number of animals =6, total number of neurones =32 of which WDR=16) on day 18 (2 days after cessation of GBP) revealed that the mechanical-evoked response was returning to the level of response in untreated MRMT-1 rats (Figure 5.5A).

Similar normalisation of the CIBP-induced increases in brush responses was also seen suggesting that the drug can influence both the abnormal static and dynamic mechanical responses. It is of note that GBP normalises both the A-beta response to noxious electrical stimulation and the brush-evoked response in CIBP. However in order to investigate the effect of GBP on A-beta fibre evoked responses it would be necessary to carry out the test at supra-A-beta thresholds not at the supra-C-fibre thresholds used here. Chronic GBP treatment did not have a significant effect on the thermal-evoked response of WDR neurones at either day 15 or day 18 (Figure 5.5B).

Figure 5.5 The effect of chronic GBP treatment on mechanical- (A) and thermal- (B) evoked responses of WDR SDH neurons in MRMT-1-injected rats. WDR SDH neurones in MRMT-1-injected rats (n=13 cells) show significantly increased (P<0.05) responses to mechanical- (A) and thermal- (B) evoked stimuli compared to sham-operated rats (n=9 cells). The mean response to mechanical stimuli (A) of WDR neurones in the MRMT-1 GBP-treated rats (bi-daily s.c. injection GBP 30mgkg^{-1} post-operative days 11 to 14 with a single injection on day 15) on the day of the final injection was significantly reduced (P<0.05) compared to MRMT-1 rats receiving no treatment, such that the level of response was similar to that seen in sham-operated animals. Characterisation of WDR cells (n=16) on day 18 (2 days after cessation of GBP) revealed that the mechanical-evoked response was returning to the level of response in untreated MRMT-1 rats. Chronic GBP treatment had no significant effect on thermal-evoked responses of WDR SDH neurones at any time point (B).

5.3.3 Acute systemic GBP – electrophysiology   In each of the two groups, MRMT-1 operated animals (n=7) and sham operated animals (n=6), a single WDR neurone was isolated per rat per experiment. These cells showed increased responses in the MRMT-1 group compared to shams, consistent with the previous characterisations and drug study. This was particularly notable for input and C-fibre-evoked responses to electrical stimuli where cells in the sham group gave average pre-drug control action potential (AP) values of 131 ± 19 and 133 ± 9 respectively, and those in the cancer group were 201 ± 23 and 206 ± 41 respectively. Post-discharge and wind-up responses also showed a tendency to be increased in MRMT-1-injected animals. Again, corresponding with earlier findings, the non-noxious brush-evoked and the noxious mechanical-evoked responses were greater in the cancer group compared to sham (Figure 5.6).

GBP had no significant effect on A-fibre-evoked responses to electrical stimuli, or thermal-evoked responses; mean control $A\beta$- and $A\delta$-fibre responses to electrical stimuli and the mean control response to $50°C$ were 112 ± 28, 46 ± 9, and 340 ± 42 respectively, and after 100mg kg^{-1} GBP these were 91 ± 27, 51 ± 10, and 311 ± 51 respectively. The input, C-fibre, post-discharge and wind-up responses to electrical stimuli in the MRMT-1 group were significantly reduced ($P<0.05$) compared to shams with the highest dose of GBP (100mg kg^{-1}). Furthermore the C-fibre-evoked response was significantly reduced ($P<0.05$) (113 ± 11 AP in sham-operated rats and 82 ± 11 AP in MRMT-1-injected rats) with GBP 30mgkg^{-1} (Figure 5.6).

The increased responses to noxious mechanical stimuli seen in MRMT-1-injected animals were reduced to sham level with 30mg kg^{-1} GBP (Fig 5.6). The brush-evoked response was also reduced in MRMT-1-injected rats from 468 ± 36 AP to 247 ± 34 AP (sham pre-drug control was 243 ± 74 AP) with 100mg kg^{-1} GBP (Fig 5.6).

As found with the chronic treatment study, GBP did not have a significant effect on thermal-evoked responses at any dose.
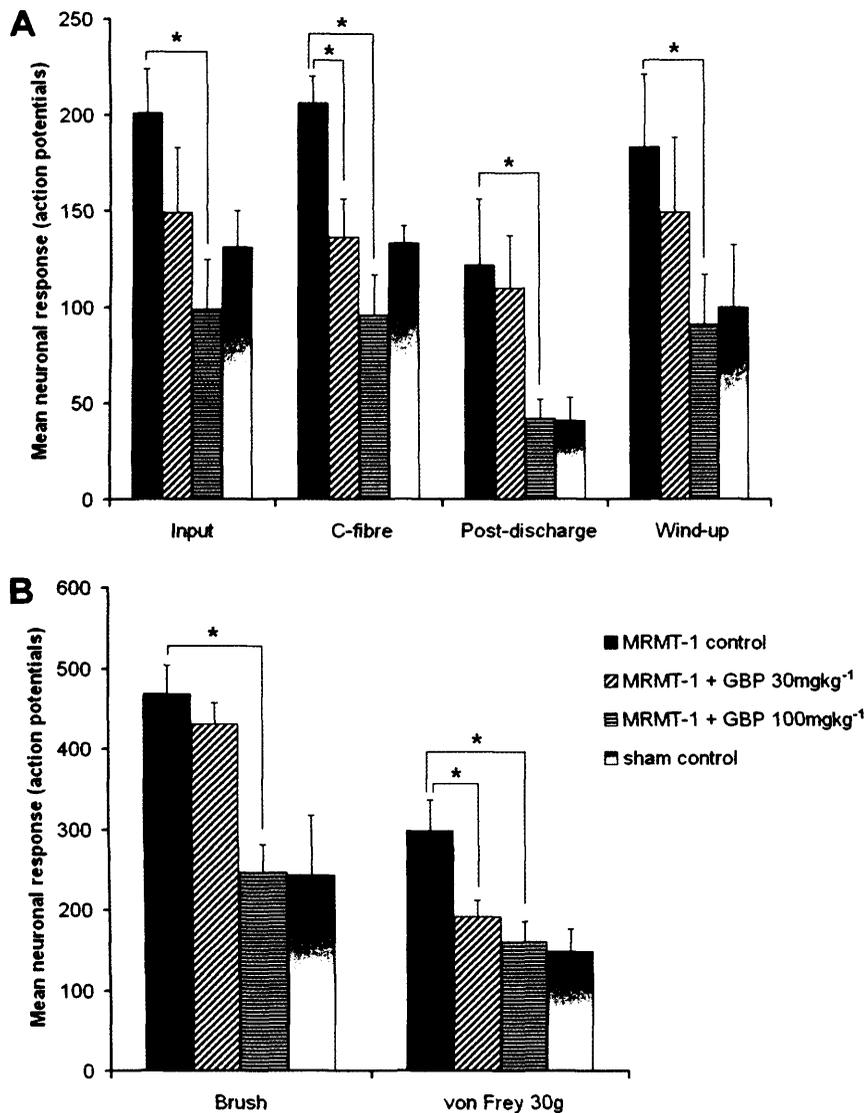
Figure 5.6 The effect of acute systemic GBP on electrical- (A) and mechanical- (B) evoked responses of WDR SDH neurones in MRMT-1-injected rats. Electrical-evoked responses (A) of WDR SDH neurones (n=7) in MRMT-1-injected rats were reduced compared to pre-drug control values, significant (P<0.05) at 30mgkg^{-1} GBP for C-fibre-evoked responses and 100mgkg^{-1} for input, post-discharge and wind-up. The reduction was such that the level of response was similar to sham-operated rat controls. Mechanical-evoked responses (B) were also significantly reduced (P<0.05) with GBP (at 30mgkg^{-1} for von Frey 30g and 100mgkg^{-1} for brush).

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5.3.4 GBP Autoradiography These preliminary studies revealed binding of GBP in the superficial dorsal horn layers of both sham-operated and MRMT-1-injected rats. Sections co-incubated with pregabalin showed no radioactivity on the films. There was no difference in intensity or area of binding between the ipsilateral and contralateral side (with respect to injected limb) of the cord in sections from sham-operated rats (Figure 5.7).
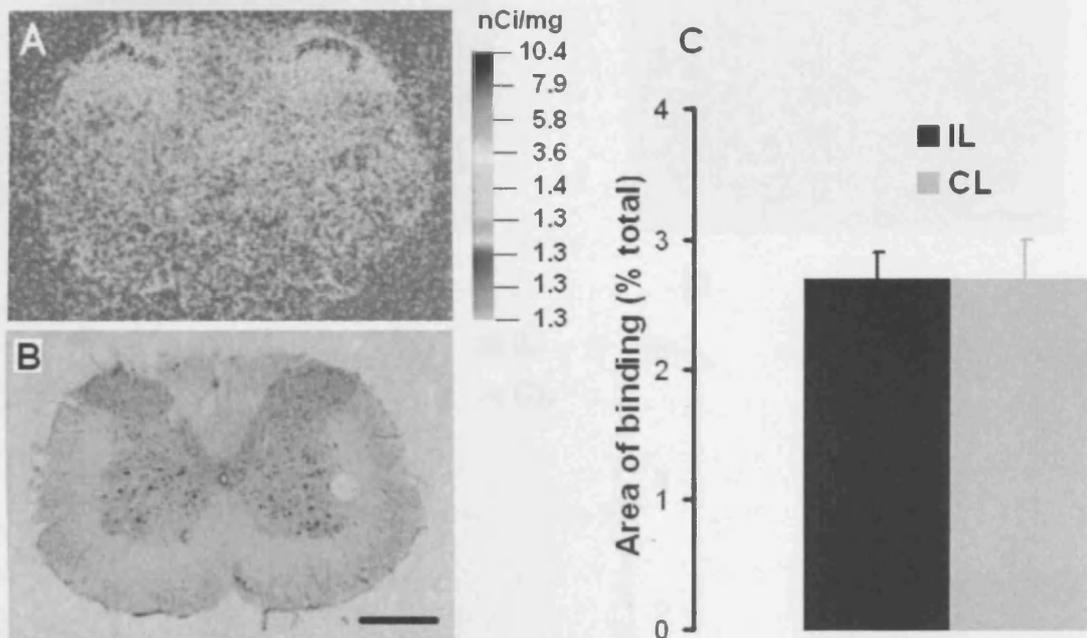
Figure 5.7 [^{3}H]-GBP binding in the SDH of sham-operated rats. Panel A shows a pseudo-colour photomicrograph of whilst panel B shows a black and white photograph of the same section after staining with toluidine blue. The pale circle on the right-hand dorsal horn is the pin hole in the contralateral side. Graph C shows that there is no difference in the area of binding between ipsilateral and contralateral sides. Scale bar = 1mm.

However, in spinal cord sections from MRMT-1-injected rats, there was a marked increase in the area of binding on the ipsilateral compared to contralateral side, whilst the intensity of binding in these areas was the same (Figure 5.8).
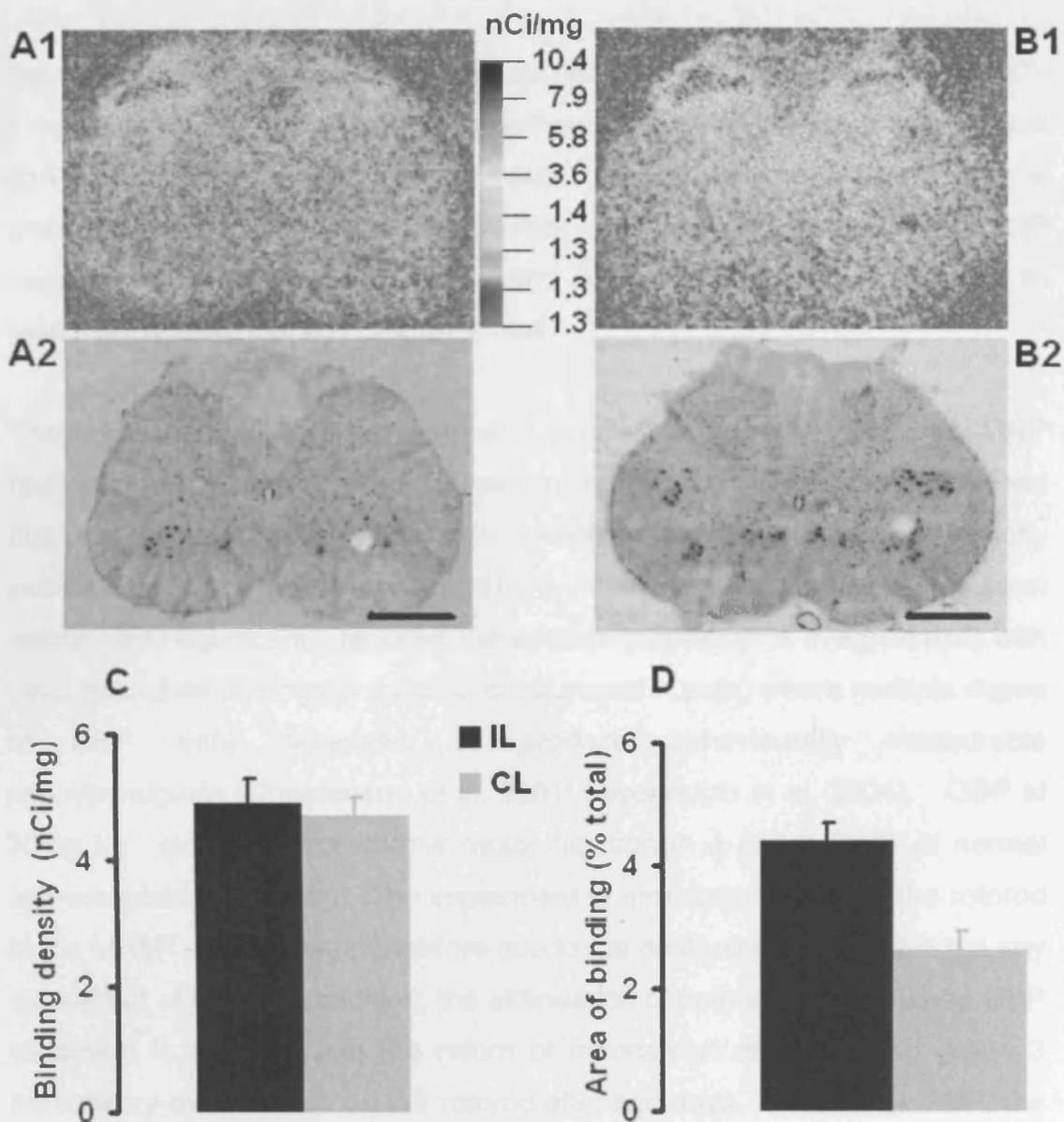
Figure 5.8 [³H]-GBP binding in the SDH of MRMT-1-injected rats. Panels A1 and B1 show pseudo-colour photomicrographs of [H³]-GBP binding in spinal cord sections from MRMT-1-injected rats whilst panels A2 and B2 show black and white photographs of the same sections after staining with toluidine blue. The contralateral sides are identified by the pale circular areas in the ventral part of the cord. As shown in graph C, there is no difference in binding intensity between ipsilateral and contralateral dorsal horn. However, there seems to be a marked increase in the area of binding on the ipsilateral side (graph D).

5.4 Discussion

As with the previous chapter, the results here confirm that injection of MRMT-1 cells into the rat tibia results in the progressive development of mechanical and cold allodynia as demonstrated by behavioural testing, and a characteristic dorsal horn pathophysiology of increased proportion of WDR neurones in the superficial lamina and hyperexcitability of these cells in response to electrical and natural stimuli.

These behavioural studies show that chronic systemic administration of GBP reduces the aberrant behavioural responses to mechanical-stimuli (von Frey filaments) to normal pre-operative baseline level and also significantly reduces cold allodynia as assessed by the acetone test. The 2 day lag seen before GBP significantly reduced behavioural responses is in agreement with other published findings in a model of neuropathic pain, where multiple doses of GBP were necessary to produce behaviourally measurable antihyperalgesia (Christensen et al. 2001; Levendoglu et al. 2004). GBP at 30mg kg^{-1} did not affect normal motor function in a group (n=4) of normal animals (data not shown). The impairment of ambulation seen on the rotorod in the MRMT-1 group was therefore due to the destruction of the tibia not any side-effect of GBP. In addition, the attenuation of pain is reversed when GBP treatment is stopped with the return of mechanical allodynia and grade 3 ambulatory-evoked pain on the rotorod after two days. Thus, after GBP, the improvement in the rotorod performance is most likely to be due to an attenuation of the pain-evoked motor impairment caused by the bone cancer.

Characterisation of SDH neurones at the time of maximum anti-hyperalgesia (day 15 post-surgery and the fifth day of GBP treatment) confirmed that GBP reversed the dorsal horn pathophysiology, significantly reducing electrical- and mechanical-evoked responses of WDR neurones as well as normalising the ratio of WDR to NS neurones. Two days after cessation of GBP treatment the dorsal horn returned to the hyperexcitable state with an increased proportion of superficial WDR neurones. This shows that the changes occurring after GBP treatment are not due to permanent changes

such as anatomical reorganisation. Indeed, the rapid return to the uninhibited pain state would suggest that neuronal physiology may be a major determinant of the behavioural allodynia/hyperalgesia. These findings provide further evidence for a correlation between the dorsal horn neuronal pathophysiology and pain behaviours in CIBP. In addition, they suggest a pivotal role for plasticity in superficial SDH neurones in CIBP pain behaviour development.

The shift in the ratio of NS:WDR would appear to be due to a *de novo* gaining in the ability of low threshold mechanical inputs to activate NS neurones (either due to an increase in excitatory inputs and/or a reduction in inhibition). This is paralleled by increased responsiveness of the overall WDR neurone population at the same time as pain-related behaviour. GBP abolished the aberrant behaviour and re-set the neuronal responses. This is consistent with the idea that the allodynia/hyperalgesia seen after CIBP is in part due to spinal hyperexcitability and that central sensitisation is responsible for the pain behaviour. GBP removed the behaviour but rather than abolishing the neuronal responses it normalised them. Finally, the data further support the concept that GBP is antihyperalgesic as when the treatment ceased and the neuronal excitability was re-established, there was a re-emergence of pain behaviour.

The neuronal plasticity probably results from peripheral mechanisms that then drive central spinal changes. For example, peripheral mechanisms may reflect a large area of primary hyperalgesia, extending from the tibia into the adjacent paw. In addition the increased afferent drive into the spinal cord and the subsequent excitation and central sensitisation could evoke a secondary hyperalgesia over the hind paw. Although with the methods used here it is impossible to prove or disprove these ideas, one important factor must be the increased primary afferent input into the superficial (and thus indirectly to the deep) lamina. The shifting ratio of WDR:NS neurones in the superficial lamina that appears to drive the CIBP behavioural changes may be due to a combination of increased excitation (arising from primary afferents and/or descending facilitation) and/or decreases in intrinsic

inhibition. Chronic treatment with GBP causes a decrease in the number of WDR neurones along with a reduction of the hyperexcitable state of the remaining WDR neurones.

As detailed in the introduction, GBP binds to the $\alpha_2\delta$ subunit of VGCCs (Gee et al. 1996) and thus the expected action would be a reduction of transmitter release and neuronal activity. The significantly increased input response (representing the excitability produced by afferent activity) seen in MRMT-1-injected rats could be indicative of increased neurotransmitter release as a result of the cancer-induced osteoclast activation and subsequent bone destruction (Mantyh et al. 2002) which this study has shown to be normalised by GBP. N- and P-type calcium channels have been reported to be involved in neuropathic and inflammatory pain states (Chaplan et al. 1994b; Cizkova et al. 2002; Diaz and Dickenson 1997; Malmberg and Yaksh 1994; Matthews and Dickenson 2001). In addition, nerve injury-induced upregulation of the $\alpha_2\delta$-1 subunit is reported to correlate with the anti-allodynic effects of GBP (Luo et al. 2001). The ability of GBP to normalise and reduce these enhanced responses could result from potential actions on altered VGCC activities so reducing transmitter release and the monosynaptic activation of the spinal superficial neurones that drive the plasticity.

The acute effects of systemic GBP in CIBP can be compared directly with other animal models of neuropathy and inflammation. GBP has been shown to reduce the evoked responses of dorsal horn neurones and have anti-allodynic effects in the spinal nerve ligation (SNL) and chronic constriction injury (CCI) models of neuropathic pain (Abdi et al. 1998; Chapman et al. 1998a; Hunter et al. 1997; Matthews and Dickenson 2002). It has also been shown to block the late phase of the formalin response (Field et al. 1997), inhibit C-fibre and post-discharges (Stanfa et al. 1997), and reduce both thermal and mechanical hyperalgesia in the carrageenan model of inflammation (Field et al. 1997).

As discussed in previous chapters, the dorsal horn alterations in CIBP have been shown to have unique characteristics that differentiate it from neuropathy or inflammation (Medhurst et al. 2002; Schwei et al. 1999). The data here show the acute effect of GBP in CIBP is similar to that reported in both neuropathy and inflammation, although in CIBP there was no effect on the neuronal responses to thermal stimuli, which agrees with some studies in animal models of inflammatory and neuropathic pain (Nagakura et al. 2003; Villetti et al. 2003), as well as clinical investigations (Attal et al. 1998; Werner et al. 2001), but not others (Cho et al. 2002; Field et al. 1997; Partridge et al. 1998). In addition, the acute doses of GBP used in this study are the same as those used in an inflammatory pain model (Stanfa et al. 1997) and the chronic behavioural dose used was the minimum effective dose in the acute study. The data suggest that GBP inhibits hyperexcitable neuronal response in animal models of chronic pain 'regardless' of underlying pathology. Thus, at normal adjuvant doses GBP may be helpful in controlling CIBP in humans. It is of interest that a single systemic dose of GBP 30mg/kg had no effect on behavioural responses (shown by the lag period), although had some effect on reducing DH excitation (deep lamina C-fibre responses). This could be an effect of dose as for a single dose of GBP to have an effect on all DH responses it was required to be given at 100mg/kg. The acute reduction in DDH C-fibre responses may not be sufficient to translate into behavioural responses, however with multiple doses GBP (30mg/kg) normalises the DH response and this neuronal change may be pre-requisite for behavioural responses to alter.

The autoradiography studies were preliminary and so limited conclusions can be drawn from them. However, the effects seen were consistent for tissue from both of the animals in each group. In agreement with findings from other investigations (Melrose et al. 2004), GBP binding was highest in the superficial laminae of the DH. Although there was no increase in binding intensity on the ipsilateral compared to contralateral spinal cord in MRMT-1-injected rats, there seemed to be a marked increase in the area of binding in the SDH which implies an increased number of binding sites and thus is consistent with the upregulation of the $\alpha_2\delta$ subunit shown to correlate with

antiallodynic effects of GBP in neuropathic pain (Luo et al. 2001). It is interesting that the area of binding seems to be expanding in the part of the dorsal horn that undergoes significant plasticity in CIBP.

As detailed previously, one of the characteristic features of the rodent models of CIBP is massive astrocyte hypertrophy, (Medhurst et al. 2002; Schwei et al. 1999). Astrocytes express VGCCs, calcium currents in cultured astrocytes have been shown to be enhanced by cAMP which also induces the morphological transformation from flat to process-bearing (MacVicar 1987). It is clear from the results that it is necessary to further investigate GBP autoradiography in CIBP, and it would also be of interest to be able to compare these binding assays with glial fibrillary acidic protein staining in the same sections in order to assess whether this increased area of GBP-binding is a result of interaction with activated astrocytes.

In summary, these data show that GBP normalises DH neuronal responses and pain behaviour in the rat MRMT-1 model of CIBP. That increased excitation, arising from primary afferents and/or descending facilitatory pathways, may be responsible for the plasticity in superficial DH neuronal populations with an increase in the WDR population. The results provide strong evidence to support a correlation between SDH hyperexcitability and behavioural allodynia / hyperalgesia. Preliminary GBP binding studies demonstrate that there seem to be changes in the total area of binding in CIBP consistent with upregulation of $\alpha_2\delta$ in this pain state. Finally, the ability of GBP to normalise SDH neuronal responses and pain behaviour in this model at clinically relevant doses suggests that it could be effective in treating humans with CIBP.

MORPHINE: EFFECTS ON DORSAL HORN NEURONAL RESPONSES AND BEHAVIOUR.

6.1 Introduction

Opium, extract of the poppy plant *Papaver somniferum*, has been used medicinally and socially for thousands of years. The second-century Greek physician Galen administered it to relieve pain from headaches, gallbladder maladies, colic and kidney stones (Snyder 1996). Morphine, a derivative of opium, was first obtained from the poppy plant in 1805 by 20-year-old German chemist Friedrich Sertürner who named it after Morpheus, the Greek god of dreams (Snyder 1996). It has become the most widely used analgesic for severe pain, the 'gold standard' to which other drugs are compared.

Opioids are one of the mainstay therapies (along with radiotherapy and bisphosphonates) in the clinical treatment of CIBP (Mercadante 1997; Portenoy and Lesage 1999). Although effective in the treatment of background pain, control of incident pain remains a problem. The presence of breakthrough pain is in fact predictive of reduced opioid efficacy (Portenoy et al. 1999) and at doses sufficient dose to control the movement induced pain the patient will suffer severe side-effects (Mercadante 1997). Some of the lack of efficacy is due to the pharmacokinetics of morphine, with a relatively slow onset and prolonged action, meaning that clinically the pain will have peaked and may be fading before the opioid has any effect. Even oral-transmucosal fentanyl citrate, which has a faster onset and shorter duration of action than morphine, is often ineffective at controlling severe movement-induced pain (Rhiner et al. 2004). One important issue here is that another reason for the reduced efficacy of morphine on movement-induced or breakthrough pain may be the underlying neuronal substrates compared to those for pain at rest. Thus, a given dose of opioid may be sufficient to inhibit sensory neurones active at rest. When movement commences, inputs and so neuronal activity will increase, and compounding the problem will be the neuronal hyperexcitability to both low and high threshold inputs induced by CIBP that I have described in chapter 3. Higher doses may predictably be required to control breakthrough pain but the actions of opioids on neuronal activity in this model have never been examined.

As discussed in section 1.4.6, opioids act at both peripheral and central sites. It has been demonstrated that the peripheral actions of opioids are an important active pathway in CIBP, as local administration of morphine and loperamide have been shown to abolish thermal hyperalgesia in the 2472 murine model (Menendez et al. 2003b). Systemic morphine has also been shown to reduce the behavioural signs of hyperalgesia in the same model (Luger et al. 2002; Vermeirsch et al. 2004).

The actions of morphine vary widely between different pain states with enhanced efficacy reported after inflammation and poor efficacy in neuropathic pain (Matthews and Dickenson 2002; Stanfa and Dickenson 1995; Suzuki et al. 1999). In CIBP the local destruction of the bone, activation and sensitization of peripheral nerves, and dorsal horn pathophysiology indicate that this pain state is unique but has elements of both neuropathy and inflammation.

Although there is evidence from the 2472 murine model of CIBP that higher doses of morphine are required to control pain from cancer in the bone compared with inflammation (Luger et al. 2002), there as been nothing published on the effect of chronic morphine on pain behaviour or dorsal horn pathophysiology. This study investigates the effect of chronic systemic morphine administration on behaviour and dorsal horn neuronal responses. As with the GBP study, systemic morphine was commenced after hyperalgesia had been established, akin to the clinical situation. As peripheral and central actions of morphine would all contribute to the altered behaviour, acute spinal morphine was also investigated to establish the effect locally on the dorsal horn.

6.2 Methods

Preparation of the MRMT-1 cell line and surgery to induce CIBP (or sham surgery) were carried out as described in Chapter 2.

6.2.1 Drug administration Morphine sulphate (Evans Medical) was prepared for use in chronic (3 mg kg^{-1}) and acute (0.1µg, 1µg and 10µg in a volume of 50µl) studies. Chronic morphine injections were administered subcutaneously in the scruff at the back of the neck, whereas in the acute studies morphine was applied directly to the spinal cord, i.e. the intrathecal route.

A total of 27 animals were used in the chronic morphine behavioural study, 6 of which were sham-operated and 21 receiving intratibial injection of MRMT1. MRMT1-injected rats were divided into three groups. The first group (n=9) received bi-daily (7.30am / 7.30pm) subcutaneous injection of morphine (3 mg kg^{-1}) commencing on day 11 continuing through to day 14, with a single injection on the morning of day 15. The second group (n=6) received bi-daily injection of saline according to the same schedule. The final group of cancer animals (n=6) received a single injection of morphine (3 mg kg^{-1}) on the morning of post-operative day 15.

The sham-operated rats received bi-daily injection of morphine according to the same chronic schedule as described for the cancer animals.

Behavioural testing was carried out exactly as described for gabapentin, with the exception that this experiment was terminated on the fifth day of drug treatment, i.e. post-operative day 15, on which day electrophysiological characterisations were made of SDH neuronal responses. Four out of the 9 MRMT-1-injected rats chronically treated with morphine were used for the characterisations and an average of 5 neurones was characterised per rat.

For the acute study 7 rats were assigned to each experimental group (DDH cancer and sham, SDH WDR cancer and sham, SDH NS cancer and sham).

Pharmacological study of SDH and DDH neurones with morphine was as described for ondansetron and gabapentin, recording electrical- and natural-evoked responses and testing at t=10, 30 and 50 minutes after each dose. After the maximum effect of morphine was observed with the top dose, naloxone (10μg in a volume of 50μl) was applied to the spinal cord, the neuronal response followed as for morphine and the maximum reversal was recorded.

6.3 Results

6.3.1 Chronic systemic morphine – behaviour

The sham-operated animals, receiving bi-daily injection of morphine 3mg/kg between post-operative days 11 to 14 and a single dose on day 15, showed no changes to the stable baseline responses indicating that this dose of morphine does not cause somnolence (rotorod) or changes in normal baseline sensitivity to mechanical or cold stimuli (data not shown).

Of the 21 animals in the cancer group, 6 received bi-daily injection of saline between post-operative days 11 to 14 and a single dose on day 15 and 9 received morphine 3mg/kg according to the same treatment schedule. The final 6 rats received a single injection of morphine on day 15. The cancer animals not receiving morphine injections (i.e. those injected with saline or those only injected with morphine on day 15) developed mechanical allodynia and cold sensitivity over the ipsilateral hind paw and ambulatory-evoked pain as described previously for non-treated animals in this model (Figure 6.1). Conversely, the cancer animals receiving bi-daily morphine injection did not show development of pain behaviour as responses to all behavioural tests were maintained at the pre-operative baseline (Figure 6.1).

A. von Frey 1g

··⊖·· saline
—●— morphine 3mg/kg

100
80
60
40
20
0

0 3 6 9 12 15

% Max. withdrawal response

B. von Frey 15g

100
80
60
40
20
0

0 3 6 9 12 15

C. Acetone

100
80
60
40
20
0

0 3 6 9 12 15

D. Rotorod

AEB score

3
2
1
0

0 3 6 9 12 15

Post-operative day
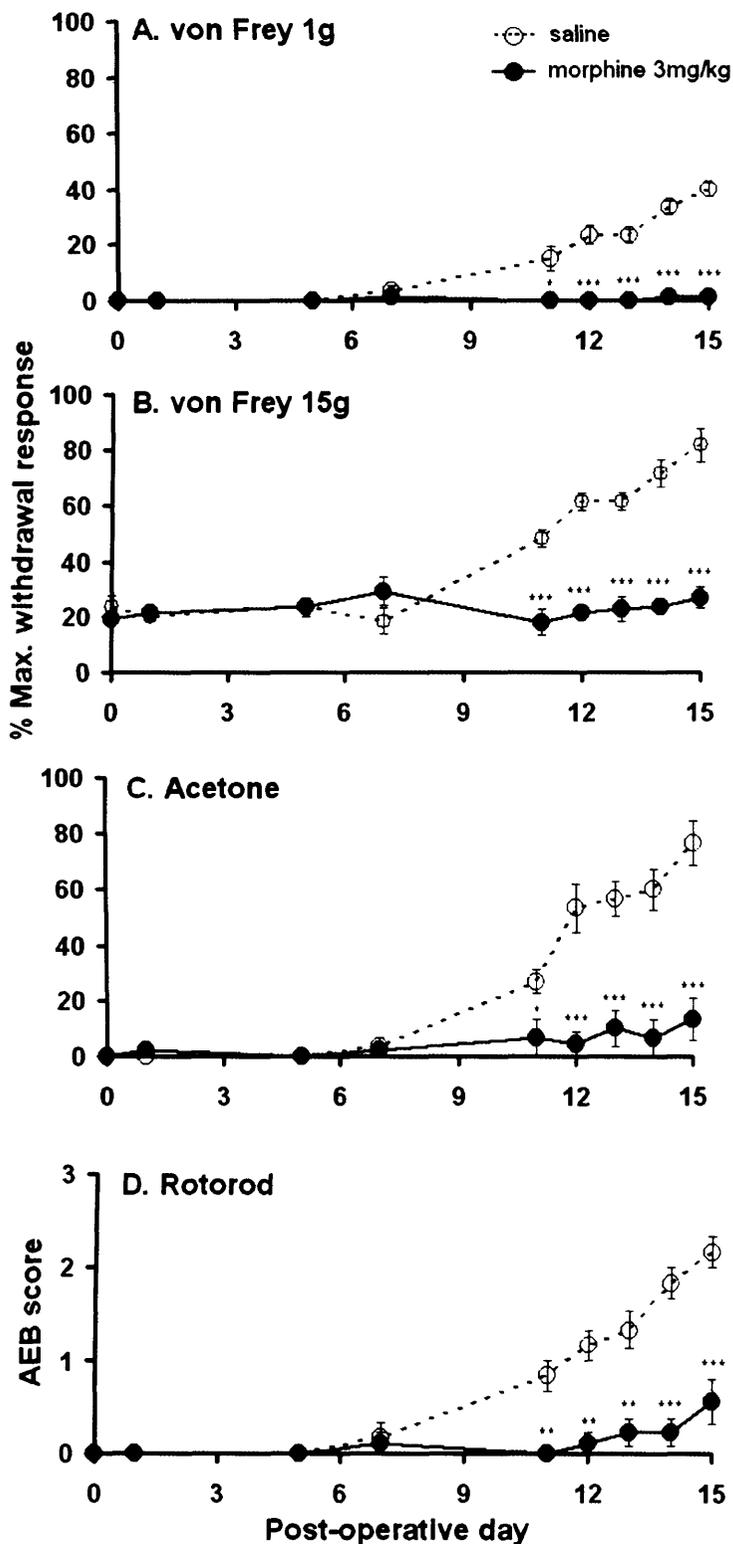
Figure 6.1 The effect of chronic systemic morphine (3mg/kg) on mechanical allodynia (1A, 1B), cold allodynia (1C) and ambulatory-evoked pain (AEB) (1D) in MRMT-1-injected rats. Each data point in the saline-treated group (open circle) represents the mean ± s.e.m. of 6 rats and in the morphine-treated group (closed circle) the mean ± s.e.m. of 9 rats. Animals received bi-daily subcutaneous (s.c.) injection of saline or morphine 3mgkg⁻¹ between post-operative days 11 to 14 inclusive at 12-hour intervals, with a final injection on the morning of day 15. Morphine-treated rats show a reduction in withdrawal responses to von Frey 1g (A) and to von very 15g (B) from the first day of treatment. The response to acetone (C) in morphine-treated rats was also significantly reduced compared to saline-treated rats from the first injection onwards, as was the ambulatory-evoked pain score (D). *$P<0.05$, *$P<0.01$, *$P<0.001$.

Pre-injection compared with post-injection behavioural testing on day 15 showed a significant difference in the chronically treated morphine group suggesting that the effects of the drug are wearing off between injections. Although these data show that the level of analgesia is not stable, it is clear that some analgesic action remains with the chronic morphine injection schedule as there was still a significant difference in von Frey withdrawal response between vehicle treated animals and morphine treated animals tested prior to injection of the drug on day 15 (Figure 6.2).
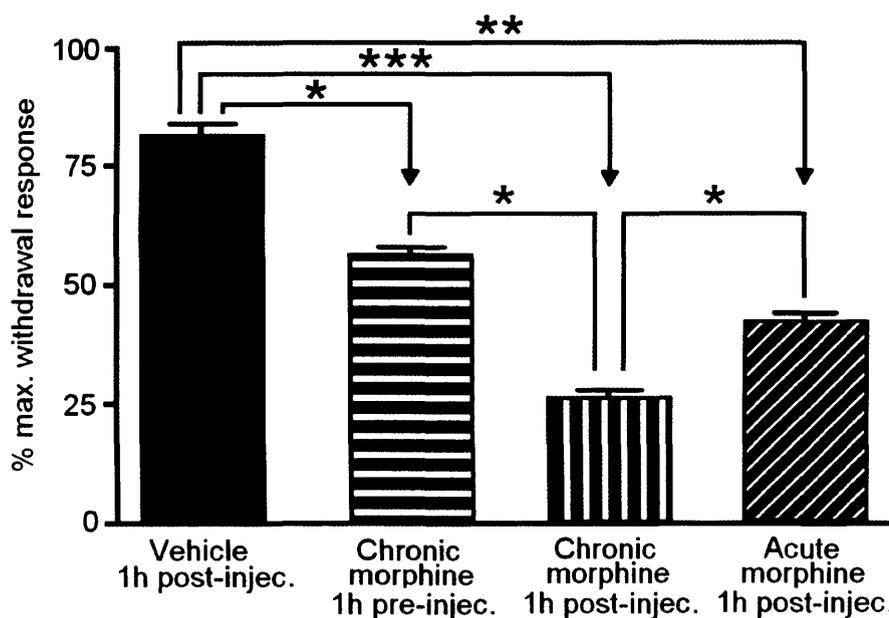
Figure 6.2 Withdrawal responses to von Frey 15g on post-operative day 15 in MRMT-1-injected rats. Withdrawal responses to von Frey 15g pre- and post-morphine in the chronically treated group (n=9) and 1hour post-morphine in the acutely treated group (n=6) were all significantly lower than in rats chronically treated with saline (vehicle, n=6). There was also a significant difference between the pre- and post-morphine withdrawal responses in the chronically treated group suggesting that the analgesic effect is waning between doses. However, there is a significant difference between the post-morphine responses in the acute and chronic groups suggesting that there is some advantage to chronic dosing. *P<0.05, **P<0.01, ***P<0.001

Acute injection of morphine on day 15 also resulted in a significant reduction of behavioural responses, although to a lesser extent than animals receiving chronic morphine treatment (Figure 6.2).

6.3.2 Chronic systemic morphine – SDH neurone characterisations   A

total of 20 SDH neurones were characterised from 4 of the MRMT1-injected rats chronically treated with morphine on post-operative day 15 after the final injection of morphine. For statistical analysis these data were compared with the previous characterisations of SDH cells in MRMT-1 and sham-operated rats detailed in Chapter 3.

The characterisations on the final day of morphine treatment in MRMT1-injected rats revealed that the ratio of WDR SDH neurones is still abnormally high compared to shams but that some of the hyperexcitable responses have been normalized (Table 6.1).

Table 6.1 Electrical-evoked responses of WDR SDH neurones in MRMT-1-injected rats treated chronically with systemic morphine (3mg/kg).

	% of population	Input	A-beta	C-fibre	Post-discharge
Sham	26	139±36	75±8	162±29	62±15
MRMT-1	53	*239±26	*120±13	*243±24	*121±28
MRMT-1 + morphine	45	176±23	86±9	189±12	66±20

Results are the mean ± S.E.M action potentials evoked by electrical stimuli at three times C-fibre threshold. *Significantly elevated responses compared to sham-operated rats ($P<0.05$).

6.3.3 Acute intrathecal morphine   A single dorsal horn neuron

was isolated for experimentation per animal, giving a total of 42 neurones. Consistent with previous characterisations, WDR neurones located in both the SDH and DDH showed hyperexcitable responses compared to sham-operated rats.

Acute spinal morphine had similar actions in all groups of neurones with no significant effect on electrical-evoked A-beta fibre responses or brush-evoked responses (Tables 6.2-4) but dose-dependent reductions of all other electrical and natural-evoked responses (Tables 6.2-4; Figures 6.3-6.5).

Table 6.2 Comparison of the effects of morphine on the electrical-evoked responses of DDH neurones in MRMT1-injected and sham-operated rats.

	Morphine			
SHAM	**0.1μg**	**1μg**	**10μg**	**Naloxone**
A-beta	93 ± 15	98 ± 16	107 ± 11	102 ± 17
A-delta	94 ± 15	53 ± 11	*28 ± 19	103 ± 10
Input	95 ± 15	*37 ± 11	**19 ± 14	137 ± 23
C-fibre	89 ± 16	*49 ± 16	**16 ± 7	107 ± 15
Post-discharge	102 ± 18	**37 ± 16	**27 ± 14	124 ± 16
Wind-up	84 ± 23	*35 ± 19	**15 ± 8	117 ± 19
MRMT	**0.1μg**	**1μg**	**10μg**	**Naloxone**
A-beta	116 ± 11	127 ± 10	130 ± 13	120 ± 12
A-delta	97 ± 16	70 ± 9	**32 ± 12	81 ± 10
Input	102 ± 22	58 ± 17	**10 ± 18	82 ± 12
C-fibre	99 ± 12	77 ± 10	**14 ± 12	74 ± 17
Post-discharge	124 ± 12	68 ± 11	**33 ± 13	113 ± 18
Wind-up	105 ±10	75 ± 11	**20 ± 12	116 ± 11

Data shown are mean action potentials ± SEM from a total of 7 rats in each group. Responses are expressed as % of pre-drug control value.

The magnitude of effect of morphine on SDH NS cells was the same in sham-operated and cancer animals (Table 6.3, Figures 6.3-6.5). However, for WDR cells only the top dose of morphine (10μg) significantly reduced the hyperexcitable electrical-evoked responses in MRMT1-injected rats, whereas the electrical-evoked responses in sham animals were sensitive to a lower dose of 1μg (Tables 6.2 and 6.4).

Table 6.3 Comparison of the effects of morphine on the electrical-evoked responses of SDH NS neurones in MRMT1-injected and sham-operated rats.

	Morphine			
SHAM	**0.1μg**	**1μg**	**10μg**	**Naloxone**
A-beta	89 ± 23	88 ± 21	91 ± 19	103 ± 24
A-delta	82 ± 16	64 ± 13	*52 ± 12	126 ± 23
Input	94 ± 24	58 ± 10	*40 ± 12	128 ± 22
C-fibre	80 ± 10	**54 ± 9	**48 ± 8	94 ± 10
Post-discharge	66 ± 17	**26 ± 16	**11 ± 10	80 ± 21
Wind-up	55 ± 27	*34 ± 15	**9 ± 14	99 ± 18
MRMT	**0.1μg**	**1μg**	**10μg**	**Naloxone**
A-beta	104 ± 11	129 ± 16	95 ± 24	105 ± 13
A-delta	103 ± 26	64 ± 10	*49 ± 6	101 ± 10
Input	127 ± 21	55 ± 18	*28 ± 21	141 ± 26
C-fibre	88 ± 15	*51 ± 17	*41 ± 12	97 ± 21
Post-discharge	71 ± 18	*33 ± 21	*24 ± 18	93 ± 16
Wind-up	66 ± 26	*43 ± 22	*21 ± 18	111 ± 11

Data shown are mean action potentials ± SEM from a total of 7 rats in each group. Responses are expressed as % of pre-drug control value.

Table 6.4 Comparison of the effects of morphine on the electrical-evoked responses of SDH WDR neurones in MRMT1-injected and sham-operated rats.

	Morphine			
SHAM	0.1μg	1μg	10μg	Naloxone
A-beta	90 ± 7	89 ± 10	69 ± 16	106 ± 12
A-delta	81 ± 14	*47 ± 14	**32 ± 10	90 ± 11
Input	82 ± 12	**44 ± 13	**15 ± 9	102 ± 19
C-fibre	111 ± 11	*58 ± 13	**28 ± 13	83 ± 11
Post-discharge	113 ± 19	51 ± 11	**18 ± 12	71 ± 28
Wind-up	123 ± 16	59 ± 11	**20 ± 12	88 ± 20
MRMT	0.1μg	1μg	10μg	Naloxone
A-beta	109 ± 19	102 ± 17	88 ± 17	103 ± 9
A-delta	116 ± 16	95 ± 15	*49 ± 9	95 ± 10
Input	139 ± 28	72 ± 14	*37 ± 10	92 ± 14
C-fibre	115 ± 18	87 ± 17	*40 ± 8	114 ± 32
Post-discharge	136 ± 35	79 ± 23	*38 ± 11	83 ± 27
Wind-up	99 ± 23	67 ± 23	**34 ± 11	92 ± 23

Data shown are mean action potentials ± SEM from a total of 7 rats in each group. Responses are expressed as % of pre-drug control value.
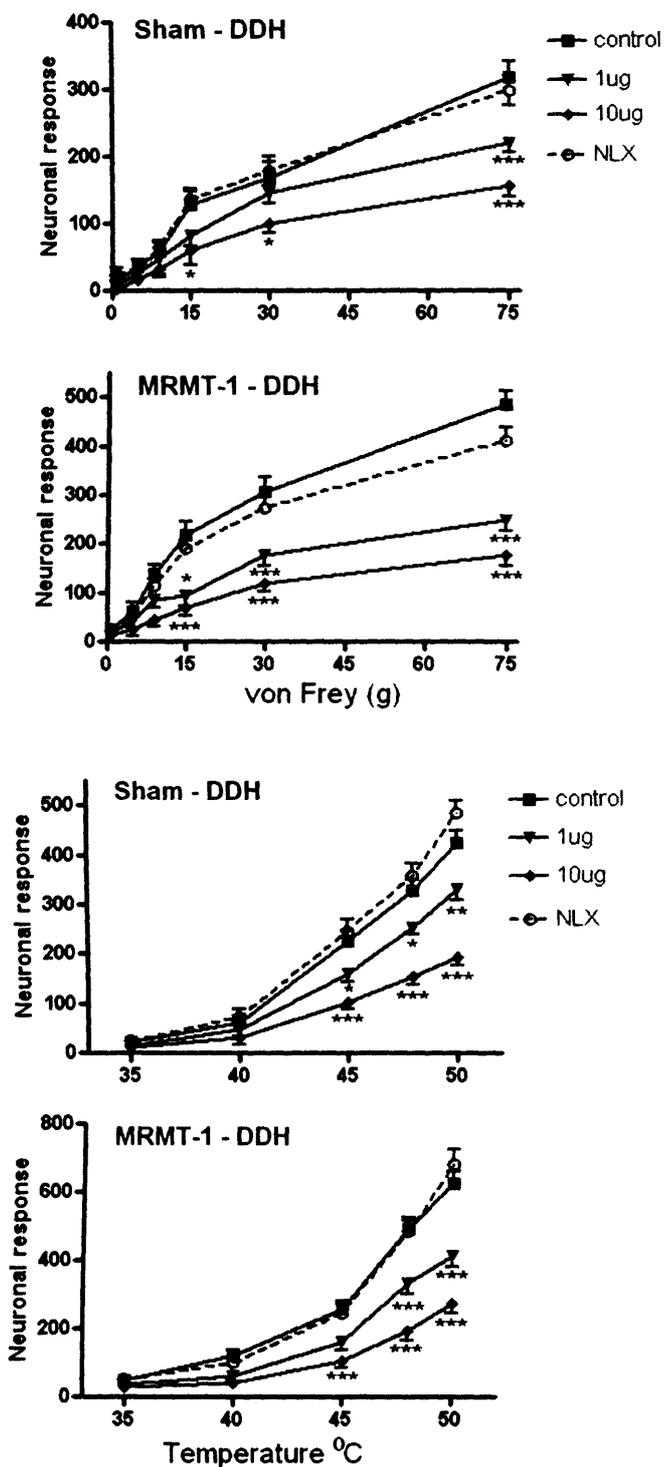
Figure 6.3 The effect of morphine on natural-evoked responses of DDH neurones in sham-operated and MRMT-1-injected rats. In both sham-operated and MRMT-1-injected rats morphine (1µg and 10µg) significantly reduced both mechanical- and thermal-evoked responses of DDH neurones. Each data point represents the mean ± S.E.M of 7 animals. *P<0.05, *P<0.01, *P<0.001.

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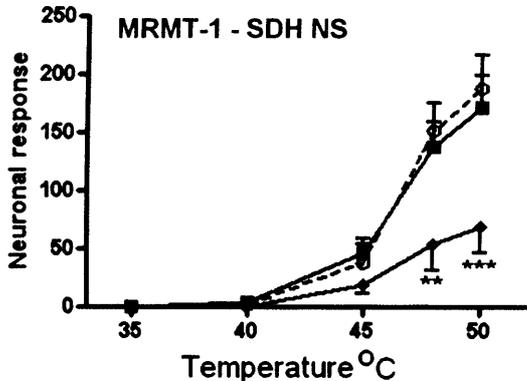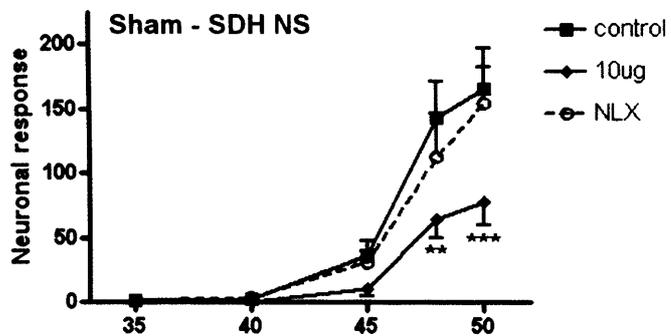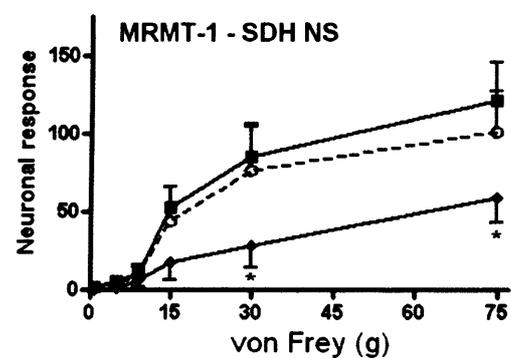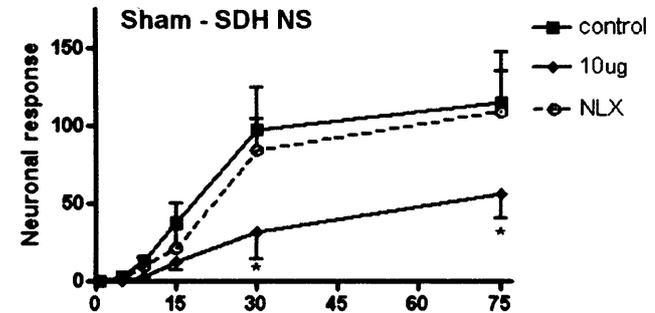
Figure 6.4 The effect of morphine on natural-evoked responses of SDH NS neurones in sham-operated and MRMT-1-injected rats. In both sham-operated and MRMT-1-injected rats 10µg morphine significantly reduced both mechanical- and thermal-evoked responses of SDH NS neurones. Each data point represents the mean ± S.E.M of 7 animals. *P<0.05, *P<0.01, *P<0.001.
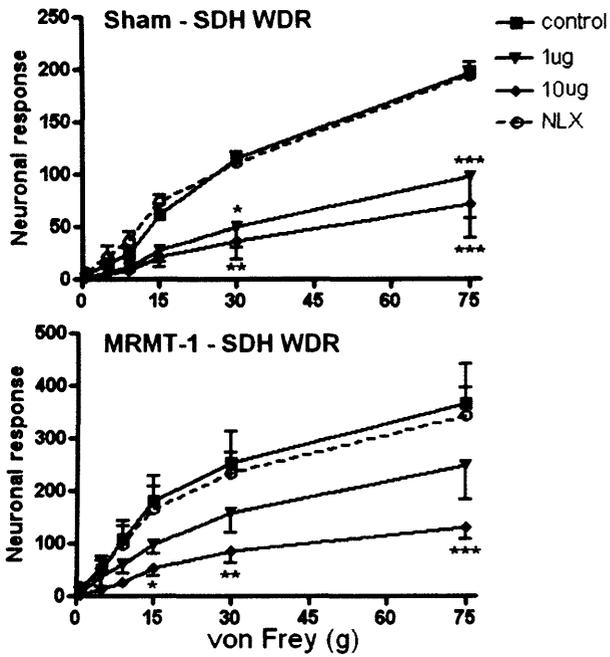
Figure 6.5 The effect of morphine on natural-evoked responses of SDH WDR neurones in sham-operated and MRMT-1-injected rats. In both sham-operated and MRMT-1-injected rats morphine (1μg and 10μg) significantly reduced both mechanical- and thermal-evoked responses of SDH WDR neurones. Each data point represents the mean ± S.E.M of 7 animals. *$P<0.05$, *$P<0.01$, *$P<0.001$.

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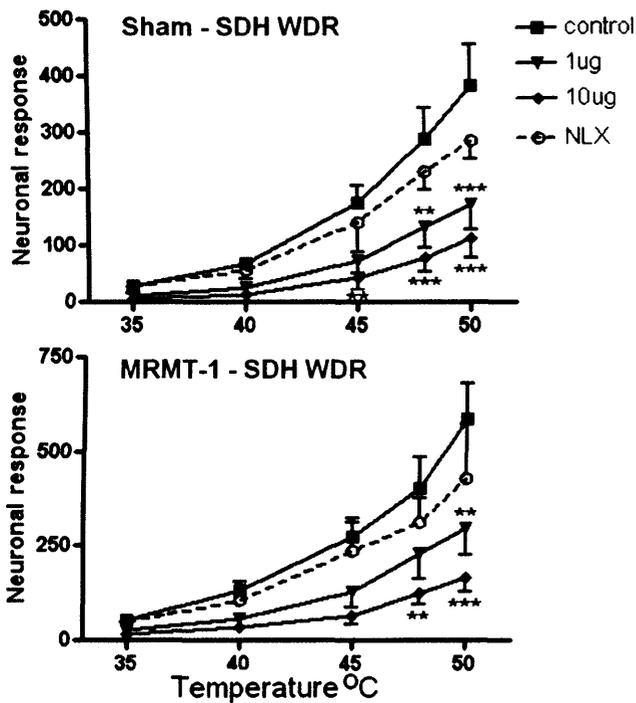
6.4 Discussion

This study demonstrates that acute spinal administration of morphine in the rat tibia model of CIBP significantly reduces electrical- and natural-evoked DH neuronal responses. Reductions of similar magnitude were also seen in sham-operated animals (which have neuronal and behavioural responses similar to normal rats) and at lower doses than in cancer animals, thus suggesting that the efficacy of morphine is lower in CIBP than in normal states. This is in contrast with results from acute spinal administration of morphine (at similar doses to those administered in this study) in the spinal nerve ligation (SNL) model of neuropathy in rat where it has been reported that there is enhanced potency of intrathecal morphine after nerve injury (Suzuki et al. 1999). In the same study it was shown that systemic morphine was less effective at inhibiting the evoked neuronal responses of spinal nerve ligated rats compared to shams. This finding was mirrored by another investigation in the same model which showed systemic morphine to be less effective at reducing evoked neuronal responses in neuropathic rats (Matthews and Dickenson 2002). Electrophysiological studies have shown the antinociceptive effects of spinal morphine to be more potent following carrageenan inflammation in rats compared to normal animals (Stanfa and Dickenson 1994). All of these results therefore suggest that the efficacy of morphine at attenuating dorsal horn neuronal responses is related, at least in part, to the route of administration with spinal administration possibly being a more appropriate approach to pain control in neuropathic and inflammatory states since high local concentrations will be achieved in areas critical for opioid analgesia. The effects of spinal morphine in this CIBP model are therefore more like those seen in inflammatory pain than neuropathic pain, but this finding still underlines the fact that although CIBP shares some features of neuropathy and inflammation it is in fact a unique pain state (Luger et al. 2002; Schwei et al. 1999) reflected by the differing efficacies of spinal morphine in SNL, carrageenan inflammation, and malignant bone pain models.

These data also show that in a rat model of CIBP, systemic morphine (at a dose that does not cause somnolence or alter normal withdrawal responses) is effective at attenuating behavioural signs of pain. For all of the MRMT1-injected rats receiving bi-daily subcutaneous injection of morphine (3mg/kg) between post-operative days 11 to 15 the behavioural responses were maintained at the pre-operative baseline level, significantly different to the responses of MRMT1-injected rats receiving saline injection showing progressive development of mechanical and cold allodynia as well as ambulatory-evoked pain. Systemic morphine has been used, with varying efficacy, in other rodent models of CIBP. In a murine model employing injection of sarcoma cells into the femur, doses of systemic morphine required to block pain behaviours were ten times higher (30mg/kg) than needed to block peak inflammatory behaviour in the complete Freund's adjuvant (CFA) model (Luger et al. 2002). In a similar model using intratibial injection of osteolytic fibrosarcoma cells in mice, systemic administration of both morphine and the peripherally acting opioid loperamide have been shown to attenuate behavioural thermal hyperalgesia (Menendez et al. 2003b). More recently, another group using the mouse femur model has also shown that systemic morphine (10mg/kg) significantly reduced spontaneous flinching behaviour in cancer animals and the rather high dose of 40mg/kg significantly improved limb use on the rotorod (Vermeirsch et al. 2004). The overall picture, therefore, is that systemic morphine does attenuate pain behaviours in the different models of CIBP, although the doses required vary widely between the models. This is probably due, at least in part, to the different animal species being used and the different cell lines.

The advantage with the chronic dosing schedule used here, over acute administration at just a couple of time points at advanced stages of pain behaviour as employed by other studies, is that it has enabled the monitoring of the efficacy of morphine over time. Interestingly, the pre- versus post-injection behavioural testing carried out on the final day of morphine treatment showed that although there was a significant difference, indicating that the analgesic effects are wearing off between doses, some drug action remained as the withdrawal responses prior to the final morphine injection

were still significantly lower than in cancer animals receiving saline injections according to the same schedule. The loss of analgesic effect with the 12-hour dosing schedule is unsurprising given that systemic morphine in Sprague-Dawley rats has a half life of 15 minutes and morphine-6-glucoronide (its active metabolite) has a half life of 11.2 minutes (Hasselström et al. 1996). Despite the slight wearing off of drug effect, over this 5 day schedule it is clear that no dose escalation is necessary to achieve the same level of analgesia suggesting that there is no development of tolerance to the analgesic effect of morphine. It would be interesting however to carry out the same study over a longer period in order to assess whether, and if so when, dose escalation would become necessary to maintain stable analgesia.

Acute systemic injection of morphine on post-operative day 15 significantly reduced pain behaviour although not to the same extent as the chronically treated cancer animals at this time point. Furthermore there was no significant difference in behavioural responses between the pre-injection tests in cancer rats chronically treated with morphine or those of cancer rats receiving acute systemic morphine on day 15. These data not only support a chronic opioid treatment schedule in CIBP, but may also add to the explanation of why higher doses of systemic morphine were necessary in the other studies to attenuate pain as they were carried out on later post-operative days when the pain has reached a more severe level, and which although was sensitive to acute morphine in this investigation, was less so than with chronic administration.

The SDH cell characterisations in MRMT1-injected rats chronically treated with morphine demonstrate that evoked neuronal responses that were sensitive to acute spinal morphine were also significantly reduced with chronic systemic administration. It should be noted however that in the acute electrophysiological study it is the immediate effect of morphine that is being measured with evoked neuronal responses being followed for 1 hour after each dose tested. With the chronic morphine electrophysiological characterisations on the other hand, the neuronal responses are being

recorded at the earliest 3 hours after systemic injection of the final dose of morphine and up to 7 or 8 hours after injection for the last cell characterised in an animal. These characterisations are therefore representative of the effects of chronic morphine administration rather than the acute drug effect on the dorsal horn. Although chronic systemic morphine reduced the hyperexcitablility of SDH cells, the abnormally high ratio of WDR neurones characteristic of CIBP was not reversed unlike the case with chronic systemic gabapentin treatment. So although my study shows that morphine can attenuate neuronal activity in this model, its inability to reverse the induced neuronal hyperexcitability may relate to the problems of control of breakthrough pain. The analgesic effect of chronic morphine differed from that of chronic gabapentin treatment since with gabapentin although there was a lag before it significantly reduced pain behaviours, once the antihyperalgesic effect was established it was maintained throughout the treatment schedule. The effects of morphine, as discussed tended to 'reset' between the daily tests. These points further substantiate the proposal that pain behaviours in CIBP are driven by changes in populations of SDH cells with abnormally high numbers of hyperexcitable WDR cells in this pain state, which may provide a pharmacological target. However, it implies that for more adequate pain control in CIBP combination therapy of an opioid with gabapentin for example may be useful, particularly if as has been shown to be the case in neuropathic pain there is a synergistic action between morphine and gabapentin (Matthews and Dickenson 2002).

In summary, the data presented here agree with the findings that CIBP, although sharing some features of neuropathy and inflammation, is a unique pain state reflected by the differences in efficacy of spinal and systemic morphine between these models. The data show that a chronic morphine treatment regime is necessary to treat CIBP but suggest that in order to re-set the dorsal horn to a totally normal state combination therapy of opioids with another drug class would be necessary.

Chapter 7.

GENERAL DISCUSSION.

The dorsal horn of the spinal cord is an important hub in the intricate network of nociceptive pathways that run from the peripheral nociceptors at the site of the pain, through the spinal cord circuits and then on to higher centres where perception is established. Investigation of neuropathological alterations occurring in this area in chronic pain states is helping to shed light on mechanisms driving these conditions, and hand in hand with behavioural studies is identifying novel drug candidates for use in the clinic. Although pain itself is a product of conciousness, the requisite activity in the higher centres relies on inputs to the forebrain and cortex that can be dramatically altered by spinal mechanisms, underlining the importance of understanding dorsal horn mechanisms in pain states. Clearly, for such investigations it is essential to have a reliable animal model that is representative of the clinical condition. The recent development of suitable rodent models of CIBP has brought this major clinical problem to the forefront, and the aim of this thesis was to contribute to a better understanding of a pain state that severely decreases the quality of life of so many patients.

The results from the characterisation studies in Chapter 3 have agreed with previous findings in the rat MRMT-1 model of CIBP that there is progressive development of behavioural hyperalgesia and allodynia that emulates the clinical progression of disease (Medhurst et al. 2002). I have demonstrated here that the electrophysiological characteristics of DH neurones in CIBP are different to those reported in any other pain state, in line with the unique neurochemical signature reported in this state (Medhurst et al. 2002; Schwei et al. 1999). Around the time of maximum pain behaviour in MRMT-1-injected rats, there are also altered evoked responses of WDR neurones in the deep and superficial dorsal horn. My results show that dorsal horn neurones are hyperexcitable in the CIBP state, in agreement with the idea of an ongoing central sensitization (Clohisy and Mantyh 2003).

Perhaps the most interesting finding from the characterisations was the shift in SDH neuronal populations in favour of an abnormally high proportion of WDR cells in the pain state compared to sham animals (which reflect the normal non-malignant pain-free state). This has been interpreted as

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previously NS cells becoming WDR. However, it is important to recognise that other cellular populations do exist in the DH and the view presented here is a simplified one based on the types of neurones that can be identified with this methodology. It is possible that the changes in populations involve previously silent cells becoming active and it would be very interesting to use a multi-electrode setup to test this hypothesis. Regardless of the exact dynamics between populations, it seems to be clear that the shift to the abnormal WDR state in the SDH is driving the general spinal hyperexcitability.

This raised the possibility that increased excitability may be generated in both spinal and supraspinal pathways; lamina I NK_1-expressing neurones have been shown to be the origin of a spinal-supraspinal loop important in evoking descending facilitation, via spinal 5-HT_3 receptors, back down to superficial and DDH laminae, which is necessary for full coding of inputs by deep DH neurons (Suzuki et al. 2002). The changes in the SDH in CIBP may therefore contribute to the increased excitability of the DDH. Also, as lamina I neurones project preferentially to the PAG, parabrachial area and CVM which are important circuits contributing to the affective and nocifensive responses to noxious stimuli (Bernard et al. 1995; Craig 1995; Hylden et al. 1985; Mouton and Holstege 1998), it suggested that in CIBP there is increased activation of these areas as well as access of lower threshold inputs.

The monitoring of SDH changes in parallel with behavioural signs of hyperalgesia showed that the two are occurring in parallel in this pain state. These results agree with a key role of the SDH in the regulation and behavioural expression of pain sensitivity (Mantyh and Hunt 2004; Yezierski et al. 2004) and also validated neuronal responses as the most suitable substrate for pharmacological study, being suprathreshold responses more clinically relevant than the threshold level behavioural responses.

In order to assess the role of the descending serotonergic facilitatory pathway in CIBP, I measured the effects of spinally administered

ondansetron, a selective 5-HT$_3$ receptor antagonist, on electrical- and natural-evoked DH neuronal responses in MRMT-1-injected and sham-operated rats. Ondansetron inhibited the natural-evoked responses in sham-operated rats indicating that this pathway is active in the normal pain-free state. The effects however were much greater in animals injected with MRMT-1, significant reductions being seen at lower doses of ondansetron and encompassing both the innocuous and the noxious range, thus showing that the effect of ondansetron is enhanced and suggesting that there is increased activation of this excitatory serotonergic pathway in CIBP. Comparing these results to other pain models it seems that the effects of ondansetron are only enhanced at longer time points after induction of the model in question when alterations have been reported in DH neuronal responses, implying that plasticity in the 5-HT$_3$ descending pathway does not occur immediately after tissue injury and is more likely to be involved in or produced by the maintenance phase rather than the generation phase of chronic pain.

A common thread to the investigations throughout this thesis has been comparison of the results to observations in neuropathic and inflammatory pain states, due to the supposed contribution of these processes to CIBP. The neurochemical and dorsal horn signatures of CIBP do confirm that some features are common to these pain states, but show that the individual profile of each is unique. GBP having been demonstrated to be effective in models of neuropathy and inflammation was therefore an interesting subject for pharmacological study.

Acute systemic GBP at higher doses was able to attenuate some of the hyperexcitable responses of WDR neurones in both the SDH and DDH and had no effect on responses in the remaining NS cells in MRMT-1-injected rats (that are not hyperexcitable), or any cells in sham-operated rats, thus fitting the anti-hyperalgesic profile of this drug. Chronic systemic administration of GBP (at a clinically relevant dose), starting when significant pain behaviour had developed, brought behavioural responses back to the pre-operative normal baseline after a 2 day lag. As GBP normalised rather

than abolished withdrawal responses, these studies provided further confirmation of the antihyperalgesic action of the drug. Cessation of treatment, after another 2 days, resulted in a return to the uninhibited pain state showing that permanent changes, such as anatomical reorganisation, are unlikely to account for CIBP and that neuronal physiology probably has a more prominent role.

As well as removing pain behaviour, chronic systemic GBP re-set the DH neuronal responses causing a decrease in the number of WDR neurones along with a reduction of the hyperexcitable state of the WDR cells still remaining. A single systemic dose of GBP 30mg/kg had no effect on behavioural responses (shown by the lag period), although had some effect on reducing DH excitation. The acute reduction in DDH C-fibre responses may not be sufficient to translate into behavioural responses, however with multiple doses GBP (30mg/kg) normalises the DH response and this neuronal change may be pre-requisite for behavioural responses to alter, supporting the idea that abnormal SDH neuronal responses correlate with pain behaviour.

Preliminary autoradiography studies with [^{3}H]-GBP demonstrated that there seem to be changes in the total area of binding in CIBP which would be consistent with an upregulation of the $\alpha_2\delta$-1 subunit. In a model of neuropathy, upregulation of DRG $\alpha_2\delta$ has been shown to correlate with allodynia, and expression of distinct $\alpha_2\delta$ subunits in DRG and spinal cord with presence of neuronal $\alpha_2\delta$ mRNA in both places suggests that the subunit is synthesised in a tissue-specific manner, possibly having a different functional role in each (Luo et al. 2001). It would therefore be of great interest to further study gabapentin autoradiography in the CIBP model, first of all to confirm the changes in area of binding and secondly to see if there are changes in the DRG.

Comparison of the effects of GBP in CIBP with other chronic pain models raises an important question: is a mechanism-based approach essential in

treating pain? All of the work presented in this thesis, along with many published studies, show that CIBP, inflammation, and neuropathy although sharing some features, are unique pain states with different underlying processes. GBP, however, has been shown to be effective at reducing pain and neuronal responses in animal models of all of these states, with the common substrate seeming to be neuronal hyperexcitability. Unfortunately, GBP does not provide the 'magic bullet' for pain in the clinic as, whilst it seems to be effective in 100% of animals in these different pain models, it is only effective in about 30% of patients with neuropathic pain. This is probably a reflection of the genetic similarity of animals used as well as the identical nature of the neuropathy, inflammation, or general tissue injury in the models. For example, within Sprague-Dawley rats, one substrain shows a marked fast onset persistent allodynia after nerve injury whereas the other displays a short weak allodynia (Valder et al. 2003). It is not certain whether genes protect or predispose, but it does seem that in mice genes contribute to symptoms rather than neuropathy or inflammation per se (Mogil et al. 2000). Clearly systematic investigation requires repeatability in the laboratory, but it does not reflect the patient population with a non uniform genetic background and widely varying underlying injuries, diseases and symptoms, as well as very different locations, areas, and durations of their pains.

The final pharmacological study presented here is with morphine. Acute spinal administration of morphine significantly reduced electrical- and natural-evoked DH neuronal responses. Reductions of similar magnitude were seen in sham-operated animals but at lower doses than in cancer animals, thus suggesting that the efficacy of morphine is lower in CIBP than in normal states, consistent with breakthrough pain correlating with reduced opioid efficacy (Mercadante 1997). SDH cell characterisations in MRMT1-injected rats chronically treated with morphine demonstrated that evoked neuronal responses that were sensitive to acute spinal morphine were also significantly reduced with chronic systemic administration. Chronic systemic morphine significantly reduced pain behaviour and pre- versus post-injection behavioural testing carried out on the final day of morphine treatment showed

that although there was a significant difference, indicating that the analgesic effects are wearing off between doses, some drug action remained as the withdrawal responses prior to the final morphine injection were still significantly lower than in cancer animals receiving saline injections. However, these data imply that for more adequate pain control in CIBP combination therapy of an opioid with gabapentin for example may be useful, particularly if as has been shown to be the case in neuropathic pain there is a synergistic action between morphine and gabapentin (Matthews and Dickenson 2002).

In conclusion the neuronal plasticity seen in CIBP probably results from peripheral mechanisms, that then trigger central spinal changes. Peripheral mechanisms may produce a large area of primary hyperalgesia, extending from the tibia into the adjacent paw. The increased afferent drive into the spinal cord and the subsequent excitation and central sensitisation could then evoke a secondary hyperalgesia over the hind paw. Although with the methods used here it is impossible to prove or disprove these ideas, one important factor must be the increased primary afferent input into the superficial (and thus indirectly to the deep) lamina. The shifting population of WDR neurones in the superficial lamina that appears to drive the CIBP behavioural changes may be due to a combination of increased excitation (arising from primary afferents and/or descending facilitation) and/or decreases in intrinsic inhibition.

These changes in SDH neurones have important implications in terms of CIBP in its clinical context. As mentioned in the introductory chapter, an important facet of pain is the affective component. Although this is separable from sensory aspects of pain sensation, the pathways that are involved are interlinked. SDH neurones project preferentially to the parabrachial area which contacts the amygdala and hypothalamus. These regions are involved in the emotional and autonomic responses to pain. Lower threshold and hyperexcitable inputs to these areas in this model of CIBP correspond with increased anxiety and depression in patients with breakthrough pain. The

spinal-supraspinal loop that feeds back to the dorsal horn via a descending excitatory serotonergic pathway is activated in CIBP thus increasing the hyperexcitability and amplifying the pain. The emotional state of patients is therefore probably important in determining the severity of their pain by the level of activity in this descending excitatory pathway. This suggests that in order to treat chronic pain more effectively the emotional and sensory aspects should be considered and treated with equal importance. Figure 7.1 provides a schematic diagram summarising the processes driving CIBP discussed above.

The aims of this thesis have been met as the work set out here has provided some novel insights into mechanisms involved in CIBP, correlating behavioural signs of pain with alterations in SDH neuronal responses, and highlighting GBP as a possible novel treatment for CIBP in the clinic. The studies presented here have also raised many questions and there is obviously still much to be learned about CIBP. What is clear is that pharmacological modulation of neuronal responses in animal models is a valuable tool in the quest to understand, and thus alleviate, chronic pain.
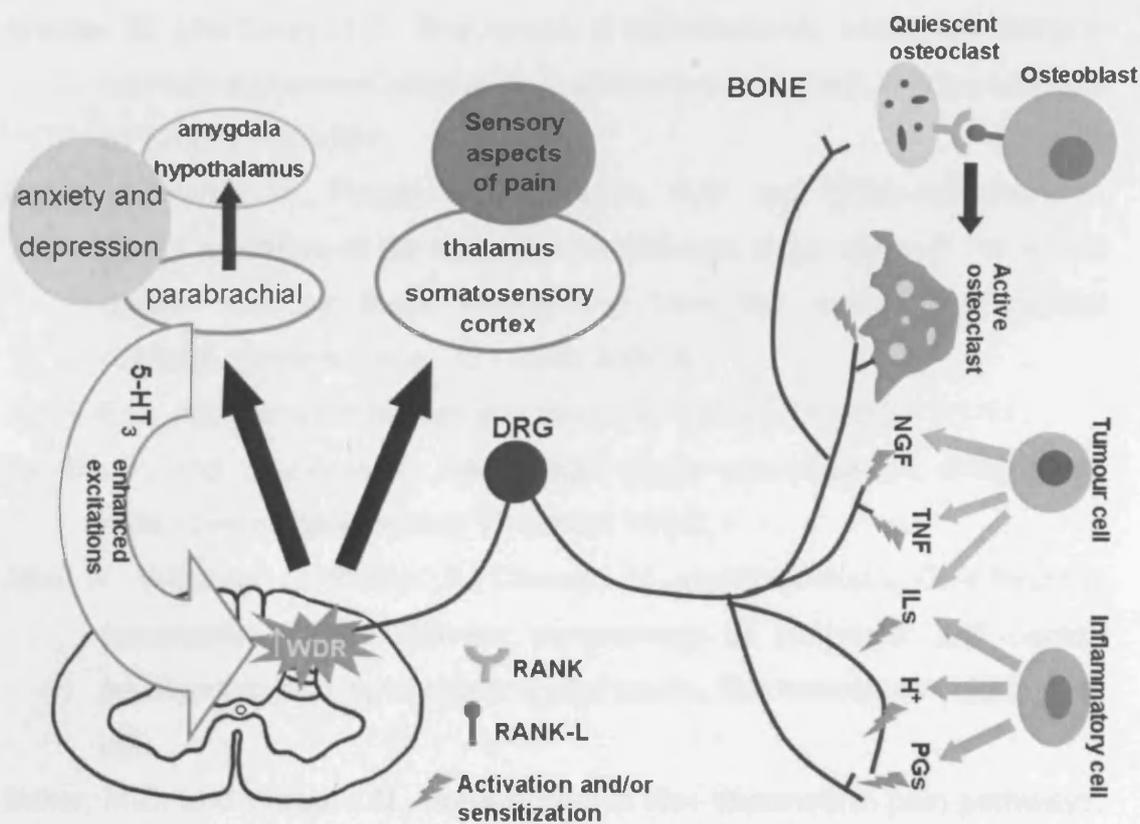
Figure 7.1 Schematic summary of the processes involved in driving CIBP.

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Appendix 1.

PUBLICATIONS

FENS Forum 2004

Poster 250 - Tue 13/07, 16:00 - Hall I
Session 192 - Pain IV
Abstract A192.33, published in *FENS Forum Abstracts, vol. 2, 2004*.
Ref.: *FENS Abstr., vol.2, A192.33, 2004*

Author(s)   **Urch C., Donovan-Rodriguez T. & Dickenson A.**

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Title   Correlation between behavioural responses and dorsal horn pathophysiology in a rat model of cancer-induced bone pain.

Text   The development of animal models of cancer-induced bone pain (CIBP) has shed light on the underlying pathophysiology. We have used in vivo electrophysiology in a rat model of CIBP to investigate plasticity of spinal dorsal horn nociceptive neurones correlation with bone destruction and behavioural alterations.
Intra-tibial injection of MRMT-1 carcinoma cells resulted in progressive bone destruction and pain behaviour. Withdrawal responses to low mechanical stimuli and cold increased significantly over the post-operative period (significant by day 11).
Dorsal horn (DH) neurones were characterized at days 7, 9, 11, 15. At the time of maximal pain behaviour (15-17days) deep DH had significantly increased C-fibre and thermal evoked responses in MRMT-1-injected rats compared to sham injected rats. Superficial DH cells were classed as wide dynamic range (WDR) or nociceptive specific (NS). The proportion of WDR cells doubled in the MRMT-1 group to 47%. Responses of NS cells did not differ between the groups. The WDR cells in MRMT-1 rats had significantly increased A-beta, C-fibre, thermal and mechanical response. Receptive field size was also significantly increased.
The plasticity of superficial WDR cells in MRMT-1 rats developed during the post-operative period in parallel with the behavioural hyperalgesia and allodynia and bone destruction. In CIBP the spinal cord is significantly more excitable compared to sham animals, possibly driven by changes in the characteristics and response profiles of superficial DH neurones. It is of note that many of these neurones project to areas of the brain involved in emotional responses to pain and drive some descending pathways.
Urch et al, Pain 103, 2003
Donovan-Rodriguez T et al, Neurosci. Lett. 2004
Syngeneic MRMT-1 rat mammary gland carcinoma cells were donated by Novartis, London. This work was supported by The Wellcome Trust and a Pfizer PhD studentship.

Theme   Sensory systems
Pain / Spinal cord processing

Correlation between behavioural responses and dorsal horn pathophysiology in a rat model of cancer-induced bone pain.

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INTRODUCTION

- Cancer-induced bone pain (CIBP) is the most common cause of cancer-related pain and poses a major clinical problem [1].
- The development of reliable models of CIBP has revealed a unique neuropathophysiology [2,3].
- We have reported electrophysiological changes in dorsal horn (DH) spinal cord in a rodent model of CIBP, with an overall increased excitability and a generalised shift in superficial dorsal horn (SDH) neuronal responses such that a greater number are wide dynamic range (WDR), relaying innocuous as well as noxious sensory information directly to central pain centres [4].
- Here we have studied the time course of the behavioural allodynia and hyperalgesia in parallel with the monitoring of WDR SDH cellular responses. We wished to assess the temporal correlates of the neuronal and behavioural alterations.

METHODS

SURGERY:
Intratibial injection of MRMT-1 tumour cells in halothane-anaesthetised male Sprague-Dawley rats. Sham-operated animals received cell medium alone.

BEHAVIOURAL TESTING:
Behavioural stimuli applied to the plantar surface of the hind paws.
Testing for mechanical allodynia/hyperalgesia via von Frey filaments (1-15g).
Testing for cold allodynia via application of acetone.
All testing carried out pre-operatively (baseline) and on post-operative days 2, 4, 7, 9, 11, 14, 16 & 18.

ELECTROPHYSIOLOGICAL CHARACTERIZATION:
Single unit extracellular recordings made from WDR SDH neurones on post-operative days 7, 9, 11 and 15-18 (Fig. 1). Responses to electrical, mechanical and thermal stimuli recorded.
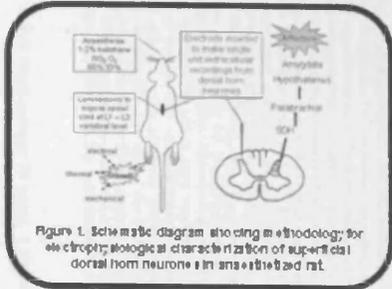
Figure 1. Schematic diagram showing methodology for electrophysiological characterization of superficial dorsal horn neurones in anaesthetized rat.

RESULTS
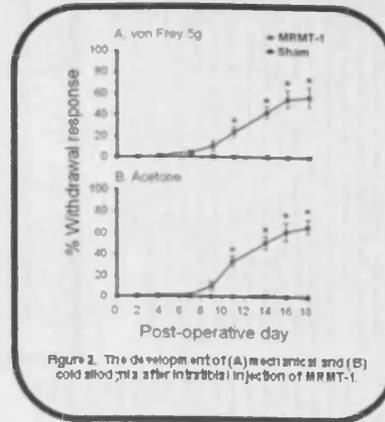
Figure 2. The development of (A) mechanical and (B) cold allodynia after intratibial injection of MRMT-1.

Figure 3. The development of increased electrical-evoked responses in SDH neurones of MRMT-1-injected rats.

On post-operative day 11 and between days 15 and 17 when there were significant behavioural changes to all von Frey stimuli and acetone, SDH cells in MRMT-1 animals showed significantly increased (P<0.05) input, A-beta, C-fibre and post-discharge responses to electrical stimuli (Fig. 3) and significantly increased responses to mechanical and thermal stimuli (data not shown) compared to sham.

BEHAVIOUR

MRMT-1-injected rats showed significantly increasing withdrawal responses to mechanical stimuli throughout the post-operative period compared to the sham-operated animals which showed no change in baseline withdrawal response.

Responses to von Frey 9g and 15g became significantly different (P<0.05) from day 9 onwards, and to von Frey 1g and 5g (Fig. 2A) and acetone (Fig. 2B) from day 11 onwards.

ELECTROPHYSIOLOGY

At time points before significant behavioural changes (post-operative days 7 and 9) there was no difference in cellular response between MRMT-1-injected and sham-operated animals for any of the stimuli (Fig. 3).

CONCLUSIONS

Here we have confirmed the temporal development of behavioural hyperalgesia/allodynia and the altered SDH neuronal response (increased excitability) in CIBP, as we previously reported [4]. This study is the first to show that in this model the behavioural alterations occur in parallel with the changes in DH electrophysiology. The close temporal correlation between behavioural and electrophysiological changes in CIBP suggests that this may be a suitable model in which to study changes to gene expression in hyperalgesia and allodynia.

The correlation between behavioural and neuronal responses confirms that the latter provides an excellent substrate for pharmacological studies on suprathreshold responses in this model, more able to elicit pain states than the established behavioural responses.

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Gabapentin normalises the dorsal horn neuronal response in a rat model of cancer-induced bone pain

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INTRODUCTION

- Cancer-induced bone pain (CIBP) is a major clinical problem in which current treatments lack full efficacy.
- Recently developed rodent models which mimic the clinical progression of disease and pain have shown that CIBP has a unique neuropathophysiology, but share some features of neuropathic and inflammatory pain states [1-3]. Gabapentin (GBP) is licensed for use in neuropathic pain and is more effective against inflammatory stimuli in animals. This drug may therefore provide a novel treatment for CIBP.
- We have reported previously that superficial dorsal horn (SDH) neuronal plasticity develops in parallel with behavioural hyperalgesia and allodynia in CIBP [4].
- Here we investigate the effects of acute systemic GBP on SDH and deep dorsal horn (DDH) neuronal responses in a rat model of CIBP.

METHODS

SURGERY:

- Experiments were carried out on male SD rats weighing approximately 170g at the time of surgery.
- 3x10⁵ mammary carcinoma (MRMT-1) cells in a volume of 10μl injected into the distal tibia. Controls were anaesthetised and the hole filled with bone restorative material.
- Sham-operated animals received cell medium alone.

ELECTROPHYSIOLOGY:

- Single unit extracellular recordings made from DH neurons on post-operative days 15-18 (Fig. 1) when all MRMT-1 injected animals showed significant mechanical and cold allodynia. Responses to electrical, mechanical and thermal stimuli were recorded.
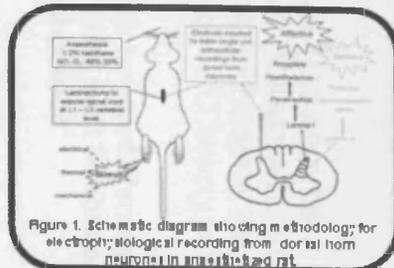

Figure 1. Schematic diagram showing methodology for electrophysiological recording from dorsal horn neurones in anaesthetized rat

- After 3 consecutive stable trials (<10% variation), neuronal responses were averaged to give pre-drug control values. Cells were classified as nociceptive specific (NS) or wide dynamic range (WDR) according to responses to mechanical and thermal stimuli.
- GBP (10, 30 and 100 mg/kg-1) was given subcutaneously in the scruff of the back of the neck in a volume of 250μl, and the neuronal response monitored for 50 mins (t=10, 30, 50 mins)

RESULTS

- In agreement with previous findings [3,4], electrical-evoked responses of WDR dorsal horn neurones (superficial and deep) were significantly greater in MRMT-1 injected rats compared to sham-operated animals (Fig. 2) whereas there was no difference in responses of NS SDH cells between the groups.
- GBP did not have a significant effect on evoked responses of any cells in sham-operated animals, or NS WDR cells in MRMT-1 injected animals, for any stimulus at any dose (data not shown).
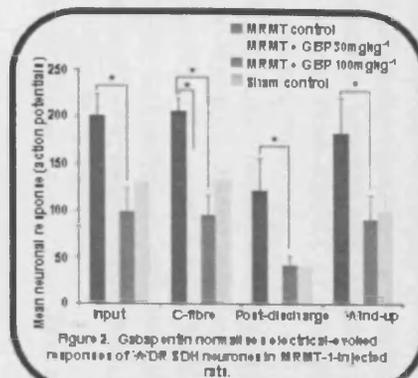

Figure 2. Gabapentin normalises electrical-evoked responses of WDR SDH neurones in MRMT-1-injected rats.

- The input, post-discharge and wind-up responses of WDR SDH neurones to electrical stimuli in the MRMT-1 group were significantly reduced (P<0.05) compared to shams with GBP 100 mg kg-1 (Fig. 2).
- The C-fibre response to electrical stimuli was significantly reduced with GBP 30 mg/kg-1.
- Input, C-fibre and post-discharge responses of WDR DDH neurones to electrical stimuli, and responses to mechanical stimuli, were significantly reduced with GBP 100 mg kg-1 (data not shown).
- GBP reduced the abnormally high electrical-evoked neuronal responses in the MRMT-1 group to the same level of response as seen in sham animals.
- As reported previously [3,4] the mechanical (Fig. 3) and thermal-evoked responses of WDR neurones were significantly higher in MRMT-1-injected rats compared to shams.
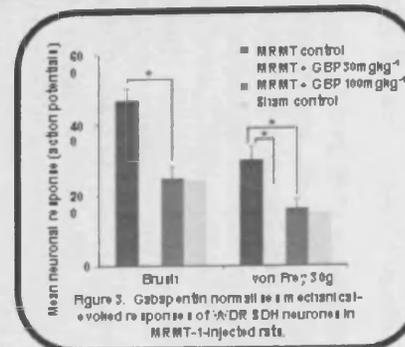

Figure 3. Gabapentin normalises mechanical-evoked responses of WDR SDH neurones in MRMT-1-injected rats.

- GBP significantly reduced (P<0.05) the mechanical (Fig. 3) but not the thermal-evoked responses of WDR SDH neurones in MRMT-1-injected rats.
- Mechanical-evoked responses of WDR DDH neurones were also significantly reduced with GBP (data not shown).
- As with the electrical-evoked responses, the abnormally high mechanical-evoked responses in MRMT-1-injected rats were reduced to sham level.

CONCLUSIONS

- Here we have confirmed our previous findings that WDR DH neurones are hyperexcitable in CIBP [3,4].
- GBP had significant effects only in cellular populations that are hyperexcitable in CIBP, reducing the electrical- and mechanical-evoked responses to sham level, thus fitting the anti-hyperalgesic profile of this drug.
- The significant reduction of the input response would agree with a predominantly postsynaptic site of action.
- As we have shown that the SDH neuronal pathophysiology occurs in parallel with behavioural allodynia [4] and demonstrate here that GBP reduces the neuronal hyperreactivity in CIBP, it suggests that this drug may provide a novel treatment in the clinic.

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