PHARMACOLOGICAL MODULATION OF SPINAL SOMATOSENSORY PROCESSING FOLLOWING PERIPHERAL NERVE INJURY IN THE RAT

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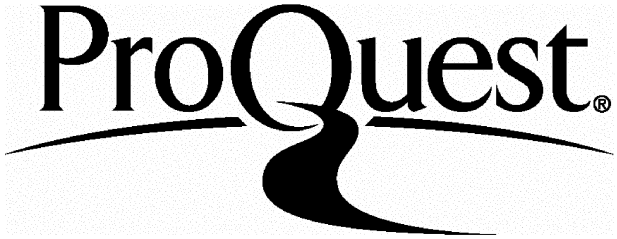

ABSTRACT

Chronic neuropathic pain state which arises from injury to the nervous system remains a significant problem. There have been few studies on the electrophysiological changes which take place in the spinal cord following peripheral nerve injury. I employed *in vivo* electrophysiological techniques to investigate the effect of nerve injury on the responses of spinal dorsal horn neurones in a rat model of neuropathy (Kim & Chung 1992).

Following surgery for spinal nerve ligation, behavioural tests were carried out postoperatively in spinal nerve ligated (SNL) and sham operated rats. SNL rats displayed signs of mechanical and cold allodynia on the ipsilateral hindpaw which was maintained for 2 weeks. Sham operated rats displayed no changes in mechanical / cooling sensitivity on either hindpaw. Electrophysiological studies were conducted at PO 1 or 2 weeks (Dickenson & Sullivan 1986) and extracellular recordings of dorsal horn neurones were made under halothane anesthesia to characterise the responses of spinal neurones to electrical and natural stimuli. Following nerve injury, the C-fibre and mechanical evoked responses (von Frey filaments, prod) were significantly reduced compared to sham controls. In contrast, the acetone-evoked response and the receptive field size of spinal neurones to low-intensity mechanical stimuli were significantly increased in SNL rats. Additionally, a greater proportion of neurones exhibited high levels of spontaneous activities in SNL rats.

Another objective was to study the effect of neuropathy on several pharmacological systems (opioid, N-methyl-D-aspartate and adenosine) through the use of selective agonists or antagonists. Overall, NMDA antagonists (ketamine, MK-801, memantine) produced greater inhibitions of the neuronal responses (wind-up, postdischarge, mechanical and thermal evoked responses) in SNL rats. Similarly, the adenosine agonist (CPA) and the adenosine kinase inhibitor (A200702.21) reduced the neuronal responses in both animal groups (input, wind-up, C-fibre and natural evoked responses). In sham operated rats, however, CPA produced a marked facilitation of the Aδ-fibre evoked response, however, this was absent after nerve injury. Interestingly, intrathecal morphine produced strong inhibitions of the neuronal responses and the reductions tended to be greater as compared to sham operated or naïve rats. Systemic morphine produced smaller reductions of the neuronal responses as compared to the intrathecal route. The altered somatosensory processing of the pain transmitter systems may reflect the spinal neuronal plasticity which takes place following nerve injury. Agents which target these systems may prove to be have possible therapeutic roles for the treatment of neuropathic pain states.

DEDICATION

Este trabajo se lo dedico a las personas que más tiempo me han dedicado en la vida, mis padres.

This is dedicated especially to my parents, Akira and Sakiko Suzuki, who made all things possible. Dedico este trozo de mi vida a vosotros.

To my sister, Mika, who has continuously given me support and encouragement during the writing of this thesis. No hubiera podido realizar este sueño sin tu ayuda.

Te quiero siempre.

I would also like to dedicate this to my grandparents, Masanori and Toshiko Ikegami, and in the loving memory of Kunisuke and Hisa Suzuki.

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For all my friends, near and far, who have been the centre of my support throughout my life, during good times and bad times – Marga & family (as the song goes 'Stand by me'), Iskender & Doireann (couldn't have done it w/o you), Ella, Jin, & Safo (thanks for being there always), Natee, Chi, Tony, Fernando (para el loco Alcalaino – thanx for being a great friend), Guillermo, Fran, Li, Brandy, JC, Kjersti, Sandrine, Ray, Auragun, Liza, Xuan, Karen and Jen. My sincere thanks to Mr. Sheriff (for never giving up) and Mrs. Wettler for your wonderful guidance. For the Attersalls,, Haruko and Midori for their friendship. Last, but not least, for Lior, por demonstrarme su amistad en los momentos dificiles.

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ABBREVIATIONS

ADP	adenosine 5'-diphosphate
AMP	adenosine 5'-monophosphate
AMPA	alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid
ATP	adenosine 5'-triphosphate
cAMP	cyclic adenosine monophosphate
cGMP	guanosine 3'5'-cyclic monophosphate
CB	cholera toxin B
CCI	chronic constriction injury
CCK	cholecystokinin
CGRP	calcitonin gene-related peptide
CNS	central nervous system
CPA	N^6-cyclopentyladenosine
CSF	cerebrospinal fluid
DAMGO	[D-Ala2, N-Me-Phe4, Gly5-ol] enkephalin
DPDPE	[D-Pen2, D-Pen5]-enkephalin
DRG	dorsal root ganglion
DSTBULET	Tyr-D-Ser(OtBu)-Gly-Phe-Leu-Thr
GABA	γ-aminobutyric acid
GAP-43	growth associated protein-43
GN	gracile nucleus
HRP	horseradish peroxidase
i.p.	intraperitoneal
i.t.	intrathecal
i.v.	intravenous
L-NAME	N^{ω}-nitro-L-arginine methyl ester
NECA	5'-N-ethylcarboxamide-adenosine
NGF	nerve growth factor
NMDA	N-methyl-D-aspartate
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NOS	nitric oxide synthase
NPY	neuropeptide Y

OFQ	orphanin FQ
ORL-1	opioid receptor-like
PAG	periaqueductal gray
PCP	phencyclidine
PO	postoperative
PSDC	postsynaptic dorsal column
PSTL	partial sciatic tight ligation
R-PIA	R-phenylisopropyl-adenosine
s.c.	subcutaneous
SCT	spinocervical tract
SG	substantia gelatinosa
SMP	sympathetically maintained pain
SNL	spinal nerve ligation
STT	spinothalamic tract
TTX	tetrodotoxin
VDCC	voltage dependent calcium channel
VIP	vasoactive intestinal polypeptide
WDR	wide dynamic range

Chapter 1

Introduction

1. Introduction

Pain: An unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage.

- International Association for the Study of Pain, Pain Terminology, 1994

Pain has been a major concern in the clinic for many decades. In recent years, considerable progress has been made with respect to our understanding of both acute and chronic pain mechanisms and this has largely been attributed to advancements in molecular biology and genomic techniques, as well as the use of animal models which has allowed us to explore potential targets for pain. This has fundamentally altered our understanding of the pathophysiology of pain mechanisms and has led to the hope of development of novel analgesics. Despite this progress, however, the management of pain still remains inadequate in many cases and is a significant problem even to this day. Not only does it bring undesirable sensations, it can often impair the quality of living for many if not effectively treated.

In broad terms, pain can be divided into 2 categories, acute and chronic, which differ in their aetiology, mechanisms and pathophysiology. Acute pain and its associated responses are provoked by noxious stimulation produced by injury and/ or disease, or by abnormal function of muscle or vicerae which does not involve actual tissue damage. Although acute pain conditions may last for a considerable length of time if not treated effectively, many cases of acute pain often resolve within days or weeks. In contrast, chronic pain can persist for a long periods of time and results from pathology in the somatic structure or viscerae, or from dysfunction or lesions to the nervous system.

1.1 Neuropathic pain

Neuropathic pain is a relatively common clinical disorder which results as a consequence of injury to the nervous system. According to the terminology guide of the International Association of Pain, neuropathic pain is defined as *'pain initiated or caused by a primary lesion, dysfunction in the nervous system'*. Neuropathy can be divided broadly into peripheral and central neuropathic pain, depending on whether the primary lesion or dysfunction is situated in the peripheral or central nervous system. In the periphery, neuropathic pain can result from disease or inflammatory states that affect peripheral nerves (e.g. diabetes mellitus, herpes zoster) or alternatively due to neuroma formation (amputation, nerve transection), nerve compression (e.g. tumours, entrapment) or other injuries (e.g. nerve crush, trauma) (Ralston 1998). Central pain syndromes, on the other hand, result from alterations in different regions of the brain or the spinal cord. Examples include tumour or trauma affecting particular CNS structures (e.g. brain stem and thalamus) or spinal cord injury. Both the symptoms and origins of neuropathic pain are extremely diverse. Partly due to this variability, neuropathic pain syndromes are often difficult to treat. Some of the clinical symptoms associated with this condition include spontaneous pain, tactile allodynia, hyperalgesia and sensory deficits.

Spontaneous pain can be either continuous or paroxysmal; continuous spontaneous pain is prominent in patients with central pain and peripheral neuropathies and is often characterised as a persistent ongoing pain localised within the innervation area of the lesioned nerve/ nerve root, or a part of this region. The pain sensation felt by the patient appears to differ depending on the origin of the ongoing pain and is often described as *'cramping, burning* or *stabbing'*. Paroxysmal spontaneous pain, on the other hand, is episodic and has a shorter duration. Patients often describe a *'shooting electric pulse-like'* pain. Episodic pain is common in patients with central pain, but can also occur in patients with peripheral nerve damage.

Allodynia is the perception of pain to a stimulus, which, under normal conditions, does not provoke pain. There is evidence to suggest that allodynia is largely mediated by large diameter, low-threshold Aβ-fibres (Ossipov *et al.,* 1999). Tactile allodynia can present a huge problem to neuropathic patients since a gentle brushing of the skin or even contact with clothes can evoke intense pain in these patients. Hyperalgesia, on the other hand, is a term used to describe an increased pain response to a given noxious stimulus. A noxious stimulus can create an exaggerated response, far beyond the normal response to the stimulus. Hyperalgesia therefore reflects an increased pain sensitivity to suprathreshold stimulation. It is important to distinguish that whilst hyperalgesia represents a mere quantitative change, allodynia rather represents a qualitative change in the perception of pain.

Rather paradoxically, another feature of neuropathic pain states is sensory deficits, which result from a partial or complete loss of afferent sensory function. Hence patients can often suffer sensory loss, whilst at the same time exhibit certain hyperphenomena such as allodynia or hyperalgesia. Neuropathic pain syndromes differ extensively between individuals and the symptoms outlined above can occur either singly, or in various combinations. Furthermore, the quality of the neuropathic pain sensation may also differ from that of a normal pain sensation and patients often report a strangeness to their pain, which has a distinct quality to that experienced normally. The existence of multiple kinds of abnormal pain sensations in neuropathic patients suggests that more than one mechanism underlies this clinical condition. Despite the continuing search for the development of novel analgesics, current treatment of neuropathic pain states remains inadequate and complete pain control is rarely achieved. Indeed, a long term follow-up survey of amputee patients suffering from chronic pain revealed that patients had been subjected to some 40 different pain therapies of which, only 1% reported lasting benefits (Sherman *et al.,* 1984).

1.2 Animal models of neuropathic pain

The continued progress for the search of possible treatments for neuropathic pain states requires the development of a valid animal model. Over the past decade, there has been a growing interest in developing animal models of neuropathy, in an attempt to further our understanding of the mechanisms underlying this clinical condition.

In order for such a model to be valid, there are a number of criteria which must be fulfilled. Firstly, the model must provide reproducible and quantifiable behavioural data that correlates with pain in man. In addition, the result of the nerve injury must produce behaviours in the animal that resemble some of the neuropathic pain syndromes observed in man (e.g. allodynia, hyperalgesia). Through the use of these animal models, we can broaden our understanding of the mechanism underlying this pathological condition and possibly identify or develop potential agents for its treatment.

1.2.1 Models of partial denervation of the hindpaw

In recent years, models of partial denervation involving injury to the sciatic nerve have been developed. The 3 main models which have been widely employed include the chronic constriction injury (CCI) model (Bennett & Xie 1988), partial sciatic tight ligation (PSTL) model (Seltzer *et al.*, 1990) and the selective spinal nerve ligation (SNL) model (Kim & Chung 1992). A schematic diagram of the sites of nerve injury produced by the three models is illustrated in Fig. 1.

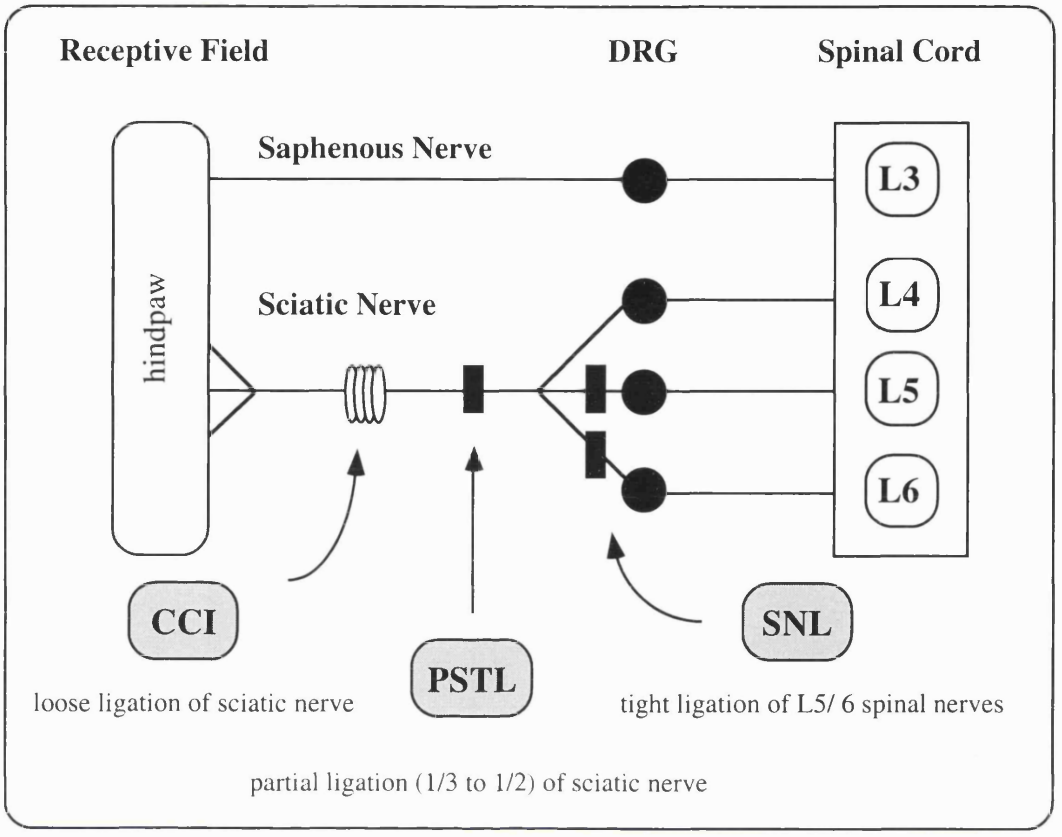


Figure 1. Animal models of neuropathy. All three models involve injury to the sciatic nerve, which innervates the hindpaw of the rat. Animals develop signs of neuropathic pain, which are maintained for 2-3 months. Abbreviations: CCI (chronic constriction injury); PSTL (partial sciatic tight ligation); SNL (spinal nerve ligation)

1.2.1.1 Chronic Constriction Injury (CCI) model of neuropathy

The first sciatic nerve injury model to be introduced was the Chronic Constriction Injury (CCI) model described by Bennett and Xie in 1988. Partial denervation of the hindpaw is produced by placing four loose 4-0 or 5-0 chromic gut sutures unilaterally around the common sciatic nerve, proximal to the trifurcation. The tightness of the ligatures is such that the nerve is barely constricted but not tight enough to obliterate the superficial epineurial vasculature supply. It has been proposed that the chromic gut suture, when placed around the nerve, may cause chemical irritation and produce an inflammatory reaction, which may contribute to the development of behavioural abnormalities (Kawakami *et al.,* 1994). Hence, CCI may represent a model of inflammatory neuropathy. Following surgery, rats show spontaneous pain-like behaviour, consistent with the presence of ongoing neuropathic pain and exhibit signs of allodynia and hyperalgesia (Bennett & Xie 1988). CCI rats display lowered thresholds and exaggerated responses to mechanical and thermal stimuli which are maintained up to 90 days following nerve injury (Attal *et al.,* 1990; Bennett & Xie 1988). Some rats also appear to display autotomy on the ipsilateral hindpaw where animals show signs of self-mutilation, possibly in an attempt to rid themselves of the unpleasant sensory experience coming from the partially denervated limb. The incidence of autotomy varies widely between studies. While Bennett and Xie (1988) reported 70% incidence of autotomy (n=148), Attal et al. Only reported 5 cases in a study of 133 rats (4%) (Attal *et al.,* 1990). Similarly, only 1 case in a study of 72 rats (14%) was observed in another study (Ro & Jacobs 1993). Autotomy is considered to be a response to pain or dysesthesia (Coderre *et al.,* 1986). However, the clinical relevance of this behaviour in human neuropathic pain states remains debatable. Neuropathic patients rarely exhibit such self-mutilation behaviour and it is therefore not possible to make direct relevant analogies with a human neuropathic pain state. One of the problems associated with this model is the considerable variation in the amount of nerve damage following the application of the loose ligatures. The variation arises mainly due to the difficulty in placing ligatures with a consistent degree of

compression and this could affect the number and type of injured afferent fibres (Basbaum *et al.*, 1991; Carlton *et al.*, 1991). This may reflect the considerable variability in the incidence of autotomy observed between studies.

1.2.1.2 Partial Sciatic Tight Ligation (PSTL) model of neuropathy

Partial denervation of the hindpaw is produced by inserting a 8-0 silk suture into the common sciatic nerve and tightly ligating one third to one half of the nerve thickness (Seltzer *et al.*, 1990). Similar to the CCI model, there is an immediate onset of allodynia and hyperalgesia, as well as behavioural signs of spontaneous pain which persist for several months. Rats exhibit a change in foot posture of the ipsilateral hindpaw and also display signs of guarding behaviour. Autotomy has not been observed in this model.

One of the problems associated with this model is that the number and type of sciatic nerve axons which are ligated differs between animals since it is not possible to damage exactly the same proportion of the nerve in each animal. Hence there may be a considerable degree of variability associated with this model.

1.2.1.3 Selective Spinal Nerve Ligation (SNL) model of neuropathy

The third model to be introduced is the selective spinal nerve ligation model which involves placing tight ligatures on two (L5 and L6) of the three spinal nerves which make up the sciatic nerve on one side of the rat using a 3-0 silk thread (Kim & Chung 1992). Selective spinal nerve ligation (L5 /L6) produces long-lasting allodynia and hyperalgesia on the ipsilateral hindpaw which persist for several weeks (5-10 weeks) (Kim & Chung 1992). Rats often exhibit signs of spontaneous pain and also display a guarding behaviour of the ipsilateral hindpaw. Autotomy is not observed in this model.

As compared to the other two models of sciatic nerve injury (Bennett & Xie 1988; Seltzer *et al.,* 1990), this model produces less variability between experiments since the same spinal nerves (L5 /L6) are ligated in each animal. The only potential variability may arise from differences among individual rats in the proportion of the sciatic nerve contributed by its 3 spinal segments.

One key feature of this model is that the location of the injured fibres is in completely separate spinal segments from the uninjured fibres (Kim & Chung 1992). Hence the dorsal root ganglia (DRG) which contains the injured nerves is separated from the neighbouring DRG which contains intact neurones. This allows us to selectively manipulate inputs from injured and intact fibres to spinal segments in an independent manner. This is unique to this model of nerve injury since in the CCI and PSTL models, the injured and intact primary afferent neurones are mixed within all the DRGs innervating the sciatic nerve territory.

1.2.1.4 Comparison of the sensory abnormalities between animal models of partial denervation

Unlike models of total denervation, involving complete spinal transection or dorsal root rhizotomy, models of partial nerve injury (Bennett & Xie 1988; Kim & Chung 1992; Seltzer *et al.,* 1990) preserve at least some of the sensory information passing into the spinal cord. The three models of partial sciatic nerve injury described above (Bennett & Xie 1988; Kim & Chung 1992; Seltzer *et al.,* 1990) have been widely employed as a tool for studying the mechanisms underlying neuropathic pain. These animal models produce sensory abnormalities in rats, some of which resemble those observed in human neuropathic pain states. The table below shows a comparison of the abnormal sensory behaviours (spontaneous and stimulus-evoked) observed in rats following peripheral nerve injury in various models of neuropathic pain.

Table 1. Spontaneous and stimulus-evoked behavioural abnormalities following peripheral nerve injury in animal models of partial denervation

Behavioural Abnormality	**CCI**	**PSTL**	**SNL**
Spontaneous pain behaviour			
Autotomy	+/-	-	-
Paw Biting / Claw Pulling		+	+
Guarding Behaviour	+	+	+
Stimulus evoked pain behaviour			
Mechanical Allodynia	+	+	+
Cold Allodynia	+	+	+
Thermal Hyperalgesia	+	+	+

Abbreviations: CCI= chronic constriction injury; PSTL= partial sciatic tight ligation; SNL= spinal nerve ligation.

Symbols: Empty cells denote models in which the given behaviour has not been studied. + denotes the presence of the behaviour in a model; +/- denotes that the behaviour was present in some but not all cases; - denotes that the behaviour was studied but not found in the model.

Following peripheral nerve injury, rats display signs of guarding behaviour which are exhibited in rats from all three models. Guarding behaviour may represent a protective behaviour of the rats to avoid aversive stimulation of the injured hindpaw. Hence following the application of a stimulus to the ipsilateral hindpaw, some rats escape from the site of stimulation in an attempt to prevent further stimulation. Furthermore, rats also display changes in the foot posture of the

ipsilateral hindpaw, whereby the toes are held clasped together. Under normal conditions, the toes of an intact animal are fully extended since this enables the rat to spread the weight borne by the hindpaw over a large area. Following partial hindpaw denervation, however, the ipsilateral hindpaw bears less weight than the contralateral paw, and rats often shift their weight to the uninjured paw. Furthermore, the denervated hindpaw may be held at an elevated position to avoid contact with the floor, suggesting that even such contact may become an aversive stimulus.

In addition to these behavioural changes, all three models produce sensory abnormalities to evoked stimuli, including mechanical allodynia and thermal hyperalgesia. Allodynia is manifested as a significant reduction in the withdrawal threshold to a mechanical stimulus (Attal *et al.,* 1990; Bennett & Xie 1988; Kim & Chung 1992; Seltzer *et al.,* 1990). Stimulation of the ipsilateral hindpaw is often accompanied by nocifensive behaviours such as licking of the hindpaw and repeated flicking of the paw (Zeltser & Seltzer 1994). Similarly, thermal hyperalgesia is manifest as a decreased withdrawal latency to noxious thermal stimuli, and animals display an increase in the intensity and/ or duration of certain nocifensive pain behaviours.

Although the origin of the nerve injury (sciatic nerve) is similar in all 3 models of partial denervation, the degree to which the neuropathic pain-like behaviours are manifested appears to differ between the animal models. Autotomy is one such example and so far, it has only been reported for the CCI model of nerve injury (Bennett & Xie 1988). Autotomy is claimed to be an expression of spontaneous neuropathic pain which appears to result from the activation of neuronal pools that represent the denervated or deafferented limb within the somatotopic map of the brain (Zeltser & Seltzer 1994). The self-mutilation behaviour is said to represent the animal's attempt to free itself of the dysesthetic sensations caused by these inputs.

There is also a considerable variability in the degree of spontaneous pain behaviours across various animal models of neuropathic pain. Spontaneous pain behaviour, which is observed in all three models, can be assessed by measuring the frequency of foot lifts of the affected hindpaw off the floor within a given period of

1.2.2 Other models of neuropathy

A model of central pain has been developed by Xu and colleagues (1992) which involves ischemic spinal cord injury. A photosensitive dye is injected systemically and ischemia is induced photochemically by Argon laser irradiation (Xu *et al.,* 1992). The model produces allodynia-like behaviours and the severity of the sensory abnormality can be titrated by changing the duration of the light exposure.

Various models have been recently developed to study the complications associated with the clinical condition of diabetes mellitus. The most widely employed is streptozocin-induced diabetes produced by a single injection of streptozocin, which results in an insulin-dependent diabetic neuropathy (Ahlgren & Levine 1993). Studies have shown that the pharmacological characteristics of this model corresponds well to the clinical experience from patients with painful diabetic neuropathy, therefore making it a useful model to study the mechanisms underlying this condition (Courteix *et al.,* 1993).

time (Kim *et al.*, 1997a). The behavioural sign of spontaneous pain is reported to be most pronounced in CCI rats, while it is less marked in SNL rats (Kim *et al.*, 1997a).

There may also be a difference in the incidence of cold allodynia between these animal models. Whilst cold allodynia has been described in the CCI and SNL models (Attal *et al.*, 1990; Bennett & Xie 1988; Kim & Chung 1992), it was not observed in the original report by Seltzer and Zeltser (1990). In a later study where comparisons were made on the various pain behaviours produced by the different models, however, PSTL rats were demonstrated to exhibit cold allodynia, with a similar time-course and magnitude to SNL rats (Kim *et al.*, 1997a). The variability in the manifestation of these pain–like behaviours between animal models may possibly reflect a difference in the mechanism underlying neuropathy, induced by the various types of nerve lesion.

1.2.3 Use of present models of neuropathic pain: benefits and problems

The development of animal models of neuropathic pain has largely contributed to the understanding of the mechanisms underlying this clinical condition. The difficulty in treating neuropathy is largely attributed to the wide range of pathology associated with this condition, which, in turn results in an even greater variability in the clinical presentation of the symptoms. Animal models enable the replication of some of the symptoms associated with human neuropathic pain states and they have been invaluable in providing information on the pathophysiological processes occurring after nerve injury. Furthermore, animal models have contributed to the clinical management of neuropathic pain states by providing information on the therapeutic value of existing drugs, as well as revealing potential novel targets.

Despite the apparent benefits of these animal studies, it is important to be aware of some of the limitations and problems associated with the use of these models. One must be very cautious when extrapolating data from animal studies to clinical application. Although animal models produce behavioural responses which resemble those of human neuropathic pain states, it is not clear how these

behavioural features correspond to the human perception of pain. We can only assume that the behavioural manifestation of the animal represents a similar clinical presentation of the pain in a neuropathic patient.

Another limitation of an animal model is that it is not possible to distinguish or discriminate the different types of pain sensations of the animal. Whilst clinicians can rely on descriptions given by patients to determine the clinical characteristic of the pain (e.g. 'burning' pain or 'shooting' pain), information from animal studies is confined to observations made on physical features and behavioural responses that are suggestive of neuropathic pain.

A question which one needs to bear in mind when employing animal models is, which model relates to which clinical condition? Whilst some animal models represent specific clinical conditions (trigeminal CCI-induced neuropathy and diabetic neuropathy model), others are not as specific. Furthermore, even when the model appears to have a close resemblance to a specific condition, it is difficult to determine whether it represents the same pathological entity as that seen in patients and whether it would respond to the same pharmacological interventions. Hence the direct clinical application of animal findings may not necessarily be straightforward.

1.3 Mechanisms of neuropathic pain

Following nerve injury, there is considerable plasticity in the peripheral and central nervous system, which may be related to the pathogenesis of neuropathic pain states. The mechanisms underlying this chronic pain state are heterogeneous, both in terms of its aetiology and anatomical site. The complex nature of the syndrome is largely responsible for the poor management of this pain state.

The sequence of events which follow peripheral nerve injury, and consequently contribute to the development of neuropathic pain, can be seen at various levels of the nervous system, both peripherally and more centrally. Some alterations associated with nerve injury, include anatomical, neurochemical, pharmacological and electrophysiological changes, and these will be discussed in more detail below.

Anatomical changes

There is substantial evidence to suggest that nerve injury induces changes in the anatomy of the peripheral nerve (Dougherty & Lenz 1994). Studies have reported a disruption of the myelination of the peripheral axon which may affect the propagation of electrical signals, leading to an aberrant conduction or excitability. Decreased conduction velocity has been demonstrated in sensory fibres reflecting changes in Schwann cell density and distribution (Stohr *et al.*, 1977). Demyelination is thought to occur through phagocytosis by macrophages which invade the site of injury following nerve lesion (Garry & Tanelian 1997).

In addition to the removal of myelin, there also appears to be a disruption in axonal transport which is essential for the maintenance and repair of axonal processes (Garry & Tanelian 1997). Axonal transport is essential for carrying cellular products to and away from the cell body, hence disruption of this process is likely to affect the normal functioning of a peripheral nerve.

Neurochemical changes

In addition to the anatomical changes, sensory neurones in the DRG also respond to nerve injury by altering the synthesis of various proteins which are required for the regeneration of injured axons (Garry & Tanelian 1997). Synthesis of neuroactive agents, such as peptides, takes place in the cell bodies of the DRG and they are subsequently transported to the peripheral or central terminals of primary afferents. Nerve injury induces a change in the synthesis and transport of these compounds and these changes show a complex pattern. Studies have reported a marked reduction in the levels of substance P and calcitonin gene-related peptide (CGRP) in the DRG (Bisby & Keen 1986; Cameron *et al.*, 1997; Nothias *et al.*, 1993; Villar *et al.*, 1989). Several mechanisms have been proposed for this decrease, including a degeneration of primary afferent terminals and depletion of neuropeptides due to constant release. Following nerve injury, there is a marked increase in the level of spontaneous activity in DRG cells (Kajander *et al.*, 1992), and this may cause a constant release of the peptides, therefore leading to its depletion from the nerve terminal. In addition, studies have reported a decreased level of substance P mRNA in the DRG following CCI (Marchand *et al.*, 1994; Nahin *et al.*, 1994), sciatic nerve section (Nielsch *et al.*, 1987) and nerve crush (Nielsch & Keen 1989). A similar decrease in mRNA and peptide level has been reported for CGRP (Nothias *et al.*, 1993), however, the changes which occur with this peptide are unclear (Kajander & Xu 1995; Munglani *et al.*, 1995; Villar *et al.*, 1989). A recent study employing the L5 spinal nerve ligation model of neuropathy demonstrated that CGRP mRNA expression is downregulated in L5 DRG, however in complete contrast, the uninjured L4 DRG exhibited a marked increase in its expression (Fukuoka *et al.*, 1998). In addition to its excitatory role in potentiating the effect of substance P or noxious stimuli on rat dorsal horn neurones (Biella *et al.*, 1991), CGRP has been shown to increase the Ca^{2+} conductance of DRG neurones (Ryu *et al.*, 1988) and enhance the release of aspartate and glutamate from spinal cord perfusate (Kangrga *et al.*, 1990). Hence the upregulation of CGRP mRNA expression in L4 DRG may contribute to the facilitation of sensory transmission through the L4 spinal nerve in this model (Fukuoka *et al.*, 1998).

In contrast to the decreased expression of SP and CGRP, some peptides exhibit an increase following nerve injury. There is evidence for an upregulation of vasoactive intestinal polypeptide (VIP) in the ipsilateral dorsal horn and DRG following transection of the sciatic nerve (McGregor *et al.*, 1984; Shehab & Atkinson 1986). Studies have also demonstrated an increase in the synthesis of its precursor (prepro-VIP) and the rate of its breakdown to VIP (Shehab & Atkinson 1986). Normally, the DRG of primary afferent neurones do not express this peptide, although its expression is induced following nerve injury (Nielsch & Keen 1989). VIP is involved in the stimulation of glycogenolysis and the increased expression of this peptide may reflect the regenerative processes which take place during nerve injury. Similarly, galanin, which under normal conditions is expressed in low levels in a limited population of cells, is markedly increased mainly in small diameter DRG neurones following nerve lesion (Hokfelt *et al.*, 1987; Villar *et al.*, 1989). The functional significance of increased galanin levels is still unclear. In a recent study employing the L5 spinal nerve ligation model of neuropathy, neuropeptide Y (NPY) gene expression was shown to be markedly induced in medium and large diameter DRG neurones (Marchand *et al.*, 1999). The novel induction of NPY gene expression on large diameter Aβ-fibres, together with the accumulation of Y_2 receptor binding sites proximal to the site of nerve ligation, suggest a potential pathophysiological role for NPY in mediating neuropathic pain symptoms following nerve injury.

Hence there is a reciprocal up- and down-regulation of VIP /galanin /NPY and tachykinin gene expression following peripheral nerve injury, which appears to follow a similar time course. The mechanisms underlying these alterations remain unclear, although nerve growth factor (NGF) has been proposed to play a role in regulating the synthesis of at least some of these peptides (Lindsay *et al.*, 1989). NGF is produced by peripheral targets and is subsequently internalised at axonal endings, following which it is carried to the cell bodies of DRG neurones through retrograde transport (Nothias *et al.*, 1993). Sensory neurones (small to medium sized) express the high affinity TrkA receptor and NGF is able to bind to this receptor and trigger a signal transduction cascade. NGF is essential for neuronal survival and may also be implicated in the regulation of neuronal phenotypes such as the expression of neuropeptides, including substance P. An early decrease in

NGF protein level (Lee *et al.*, 1998) and decreased expression of TrkA receptors has been demonstrated in L5 spinal nerve ligated rats (Shen *et al.*, 1999). These changes appear to return to normal levels over the postoperative period between 2 days (NGF) and 2 months (TrkA receptor). Nerve lesion brings about a disruption in the transport of these trophic factors and this may partly account for the altered neuropeptide expression in both the dorsal horn and DRG. In support of this view, the reduction in substance P levels in the rat DRG following sciatic nerve section was shown to be partially reversed by the infusion of NGF (Fitzgerald *et al.*, 1985). In studies where levels of substance P were compared following different sciatic nerve injuries (nerve crush or nerve section), nerve section was shown to produce a more profound depletion of substance P levels, compared to nerve crush (Nielsch *et al.*, 1987; Nielsch & Keen 1989). The level of substance P also remained low until at least 64 days post-injury after nerve section, whereas substance P levels exhibited a partial recovery following nerve crush. The variability in the recovery of substance P levels may reflect the capability of injured nerves to undergo regeneration following different nerve injuries. Nerve crush injury permits axonal regeneration which consequently allows nerves to establish contacts with their peripheral targets, therefore supplying it with NGF and subsequently restoring substance P synthesis (Nielsch *et al.*, 1987; Nielsch & Keen 1989).

Hence peripheral nerve damage induces a complex alteration in the expression of neuropeptides in the DRG, and these changes appear to be related to the degenerative and regenerative processes which take place in the central and peripheral branches of the sensory neurone. This has raised the question of whether these histochemical changes are related to the behavioural changes that accompany nerve injury. A previous study investigated whether a correlation exists between morphological alterations and the onset and magnitude of the behavioural changes (Cameron *et al.*, 1997). The results revealed that certain histochemical changes (e.g. substance P) parallel the manifestation of thermal hyperalgesia following nerve injury, although no significant correlations were found between the two (Cameron *et al.*, 1997). Hence this reflects the complexity of the mechanisms underlying neuropathic pain- the relationship between histochemical changes and behavioural changes which accompany this condition remains unclear.

Abnormal nociceptor sensitisation

There is evidence to suggest that following nerve injury, primary afferent fibres acquire abnormal sensitivity to mechanical, thermal and chemical stimuli (Burchiel 1984a; Garry & Tanelian 1997; Scadding 1981). Several studies have reported that gentle mechanical stimulation of the neuroma often evokes a repetitive discharge which lasts for a brief period which outlasts the duration of the stimuli (Burchiel 1984a; Scadding 1981). Following nerve injury, axons also appear to develop responsiveness to algesic compounds such as bradykinin, histamine, prostaglandins and leukotrienes (Garry & Tanelian 1997). The local release of these mediators may contribute to the ectopic hyperexcitability seen after nerve injury and may have a role in the development of hyperalgesia.

Ectopic discharge

It has been reported that damage to peripheral nerves brings about marked changes in the electrical properties of peripheral and central neurones, which consequently alters the processing of somatosensory information (Titmus & Faber 1990). Following nerve injury, there is an increase in the excitability of primary afferents and this is manifested by an increase in the level of ongoing spontaneous activity (Burchiel 1984a; Devor 1989; Devor 1991; Kajander & Bennett 1992; Leem *et al.,* 1997). Ectopic impulses appear to originate from the DRG, as well as from the injured peripheral nerve, including the neuroma and demyelinated regions of the axon (Devor & Bernstein 1982; Tal & Eliav 1996; Wall & Devor 1983). Abnormal ectopic activities have been reported across many studies, following peripheral nerve section (Burchiel 1984a; Devor & Bernstein 1982; Wall & Devor 1983) and partial denervation (Petersen *et al.,* 1996; Sotgiu *et al.,* 1994b; Study & Kral 1996; Tal & Eliav 1996; Xie *et al.,* 1995).

The incidence of spontaneous activity has been shown to vary between individual experimental preparations and this appears to depend on several factors, including animal species, time elapsed from injury onset, type of nerve injury and the nerve studied. So far, there has been a degree of variability in the onset and duration of the spontaneous activity between results from previous studies. Whilst ectopic activity has been demonstrated from the site of nerve injury, 2 days after

CCI (Tal & Eliav 1996), other studies have observed spontaneous discharges from dorsal horn neurones as early as 1 hour following surgery (Sotgiu *et al.*, 1994b). In contrast, Govrin-Lippmann and Devor (1978) demonstrated that following sciatic nerve section, virtually no spontaneous discharge was observed for the first 1-2 days after nerve section. The level of the spontaneous activity, however, gradually increased to higher levels over the postoperative period (PO 1-10 weeks). The time course of spontaneous discharge exhibited different patterns between myelinated A-fibres and unmyelinated C-fibres (Govrin-Lippmann & Devor 1978).

Evidence suggests that ectopic discharge arises from all fibre classes, including Aβ-, Aδ- and C-fibres (Kajander & Bennett 1992; Study & Kral 1996; Xie *et al.*, 1995), however, there is considerable variability in the contribution of different nerve fibre types to ectopic afferent barrage between studies. Electrophysiological recordings of primary afferent axons in CCI rats revealed that 35% Aβ-fibres, 15% Aδ-fibres and 3% C-fibres became spontaneously active at PO 1-3 days, which coincided with the onset of painful behaviours in these rats (Kajander & Bennett 1992). A slightly different pattern was reported in another study where recordings were made from DRG neurones of CCI rats, 3-14 days after injury (Study & Kral 1996). Here, it was shown that 18% Aβ-, 66% Aδ- and 42% C-fibre neurones exhibit ongoing activities (Study & Kral 1996). DRG neurones typically showed spontaneous membrane voltage fluctuations of the resting potential, and this has been suggested to be the trigger for the random action potential activity seen in many spontaneously active neurones (Study & Kral 1996). The pattern of spontaneous activity is random in most neurones and further does not appear to be specific for any particular cell. However, the spontaneous activity originating from injured fibres appears to have a primarily fast, regular continuous or bursting pattern whilst activity from the DRG shows a much slower and random pattern (Burchiel 1984a; Study & Kral 1996; Wall & Devor 1983).

It is not clear to what extent the aberrant activity contributes to the onset of painful symptoms associated with neuropathic pain states, however, correlations between spontaneous firing and injury-induced pain have been reported in humans (Gracely *et al.*, 1992; Nystrom & Hagbarth 1981) and in animal models (Seltzer *et al.*, 1991a). Peripheral nerve injury in humans is often associated with ongoing or episodic abnormal pain sensations and this may in part be caused by abnormal

ectopic impulses in peripheral sensory neurones.

Sodium channels

Substantial evidence exists for alterations in the expression of ion channels on peripheral afferents following nerve injury (Devor *et al.*, 1993; Devor *et al.*, 1994b). Immunohistochemical studies have shown that following nerve section, there is remodelling of axolemmal sodium channels, both in terms of their distribution and their membrane density (Devor *et al.*, 1993). Sodium channels have been demonstrated to accumulate in neuromas, especially in demyelinated regions of the injured axon and in end bulbs (Devor *et al.*, 1993). The change in expression of voltage-gated sodium channels may account for the electrogenesis of ectopic discharges and subsequently contribute to the hyperexcitability of neuroma afferents seen after nerve injury. In support of this idea, increased sodium channel density was shown to correlate with the occurrence of peak ectopic activity following neuroma formation (Devor *et al.*, 1993). One site of spontaneous discharge appears to be in regions of demyelination, which permits the invasion of sodium channels being transported along the axon (Burchiel 1980; Calvin *et al.*, 1982). The principle permissive factor for sodium channel insertion appears to be the removal of myelin, and *in vitro* studies have shown that contact with Schwann cell cytoplasm promotes its accumulation (Joe & Angelides 1993).

DRG neurones are known to express multiple distinct sodium channels encoded by different genes (Waxman *et al.*, 1999). Sodium channel expression in DRG neurones is highly dynamic and changes are seen not only during development, but also in various pain states (Okuse *et al.*, 1997). Two types of sodium currents have so far been identified in the DRG, the tetrodotoxin (TTX)-resistant (NaN, SNS) and TTX-sensitive (types I, IIA, III, PN1, NaCh6) currents, which differ in their kinetics (rate of recovery from inactivation) and sensitivity to TTX (Waxman *et al.*, 1999). The fast-inactivating TTX-sensitive current is found in all types of DRG cells, while the more slowly inactivating TTX-resistant current is preferentially expressed in a subpopulation of small diameter neurones. The preferential expression of SNS and NaN in small DRG neurones has led studies to suggest that these channels may represent unique targets for the pharmacological

treatment of pain (Waxman *et al.*, 1999). Whilst evidence is accumulating for alterations in the expression of various sodium channels following nerve injury (Black *et al.*, 1999; Dib-Hajj *et al.*, 1999; Okuse *et al.*, 1997), the relative contribution of individual sodium channel subtypes towards altered sensory processing remains unclear (Porreca *et al.*, 1999). Changes in the expression and function of these sodium channels may make an important contribution to the establishment of certain chronic pain states. In the CCI and SNL model of neuropathy, transcripts for the TTX-resistant sodium channels, NaN and SNS, were shown to be significantly reduced in small diameter DRG neurones (Dib-Hajj *et al.*, 1999; Novakovic *et al.*, 1998; Okuse *et al.*, 1997). The loss of SNS/ NaN immunolabelling from the DRG appears to be correlated with the redistribution and accumulation of the channel protein within the peripheral nerve, proximal to the site of injury (Novakovic *et al.*, 1998). Recent studies have implicated the SNS sodium channel in the pathophysiology of nerve-injured states, and any interference in its expression or function has been proposed to attenuate some of the symptoms of neuropathic pain (Porreca *et al.*, 1999). Furthermore, nerve injury induced a switch in the type of sodium channel produced by DRG cells, and this was demonstrated by the emergence of a previously silent type III sodium channel gene (Black *et al.*, 1999; Dib-Hajj *et al.*, 1999). The type III sodium channel mRNA is usually only expressed in embryonic neurones and is subsequently downregulated with development (Black *et al.*, 1996). This channel is thought to underlie the TTX-sensitive rapidly repriming Na^+ current in DRG neurones following peripheral nerve injury (Black *et al.*, 1999) and displays a 4-fold acceleration of recovery from inactivation, due to a reduced refractory period (Cummins & Waxman 1997). Activation of these previously quiescent channels may therefore induce abnormal repetitive firing in injured neurones and may potentially act as ectopic impulse generators. A previous study demonstrated that TTX-sensitive channels are necessary for the manifestation of ectopic neuronal activity in neuromas and DRG (Omana-Zapata *et al.*, 1997). These findings therefore support a role for TTX-S sodium channels in the development of neuropathic pain, and this is reflected by the inhibitory effect of TTX on neuronal excitability in neuromas (Matzner & Devor 1994). Thus, the upregulation of TTX-S channels, together with the loss of TTX-R current following nerve injury, implies that there is an increase in the relative

amount of TTX-S current available for activation. Since the TTX-S current after nerve injury reprimes relatively rapidly, this predisposes the injured neurone to abnormal firing, allowing them to sustain higher firing frequencies (Cummins & Waxman 1997). Hence, one factor which may contribute to neuronal hyperexcitability after nerve injury, is an alteration in the kinetics and voltage-dependent characteristics of sodium currents, which could arise as a result of plasticity in sodium channel expression (Black *et al.,* 1999; Cummins & Waxman 1997). Additionally, post-injury hyperexcitability can also be attributed to the pathological accumulation of voltage-gated sodium channels at the neuroma or tips of injured neurones which may promote inappropriate action potential initiation (Devor *et al.,* 1989; England *et al.,* 1994). Consistent with these findings, local anaesthetics have been demonstrated to relieve symptoms of neuropathic pain in animal models (Dougherty *et al.,* 1992; Mao *et al.,* 1992b; Sotgiu *et al.,* 1994a; Sotgiu *et al.,* 1994b). Remodelling of other membrane-associated channels and proteins (e.g. K^+ channels) by a similar process could also contribute to altered sensory processing in injured nerves. It has been shown that a block of K^+ conductance increases spontaneous activity in experimental neuromas (Devor 1983b; Kajander *et al.,* 1992). Further studies are needed to investigate their extent of contribution to ectopic impulse generation following nerve injury.

Ephatic transmission

Under physiological conditions, primary afferent neurones are normally functionally isolated and act as independent sensory communication channels. Unmyelinated nerve fibres are separated by Schwann cell processes and this anatomical configuration allows individual nerve fibres to be insulated from adjacent nerve fibres. Nerve injury, however, produces a disruption in the myelination of damaged axons and as a consequence, functional interactions between neighbouring axons can now develop, leading to an alteration in the discharge pattern and ectopic impulse generation (Devor & Bernstein 1982; Devor & Wall 1990). Studies have demonstrated that impulses carried in one nerve fibre can be transmitted to a neighbouring fibre through a phenomenon called *cross-talk* (Blumberg & Janig 1982; Seltzer & Devor 1979). Following nerve section, ephatic

fibre-to-fibre cross-talk between axon endings develop several weeks later (Blumberg & Janig 1982; Devor & Bernstein 1982; Lisney & Pover 1983; Seltzer & Devor 1979). Coupling between electrically interacting fibres appears to result from close membrane apposition and not through any specialised anatomical structures (Blumberg & Janig 1982; Devor & Bernstein 1982).

In addition, in *in vivo* DRG cell preparations, where primary sensory neurones are anatomically isolated, cross-depolarisation (or cross-excitation) has been shown to occur between neighbouring DRG neurones (Amir & Devor 1996). Hence despite the lack of synaptic interconnections, DRG neurones can be coupled in an activity-dependent manner (Devor & Wall 1990; Utzschneider *et al.*, 1992). Several mechanisms have been proposed for cross-depolarisation, which include a transient rise in $[K^+]_o$ as well as a non-synaptic release of neurotransmitters from stimulated neurones (Devor & Wall 1990). Cross-depolarisation occurs not only under pathological conditions but also under normal physiological conditions, although its role in physiological conditions remains unclear. It is clear, however, that during nerve injury, excitatory coupling among DRG neurones can accelerate spontaneous firing of cells as well as recruiting activity in silent neurones, thereby contributing to afferent hyperexcitability (Devor & Wall 1990). Interactions between adjacent neurones may have important clinical implications since the resulting abnormal afferent discharge could contribute to the sensory abnormality observed in many neuropathic pain states.

Axonal and collateral sprouting

One feature seen in injured nerves is axonal sprouting, where sensory axons of damaged nerves undergo regeneration and reinnervate target peripheral tissues including the deafferentated territory. Regeneration of sensory axons requires the presence of promoting substances in the outgrowth region, which include NGF and apolipoprotein E. In addition to these extrinsic factors, nerve axons themselves release various proteins which are essential for axonal outgrowth. Hence both intrinsic and extrinsic neural factors appear to influence nerve regeneration after nerve injury. All nerve fibres are able to undergo nerve regeneration following nerve injury, although the degree of functional recovery may

depend on the specific type of nerve fibre. Evidence suggests that small size fibres have a greater capacity for regeneration as compared to large myelinated fibres (Navarro *et al.,* 1994). In addition, the time course and latency of nerve regeneration depends on both the type and extent of the nerve lesion. The severity of the injury influences the extent of axonal regeneration and consequently, the recovery of target organ function. Studies have shown that reinnervation is more successful after nerve crush than after complete section of the nerve, thus suggesting that the degree of functional recovery of an injured nerve decreases with the severity of nerve lesion (Navarro *et al.,* 1994; Navarro *et al.,* 1997).

Interestingly, regeneration occurs not only with axons of injured nerves but also in uninjured primary afferents. Numerous studies have reported *collateral sprouting,* where sensory fibres of neighbouring nerves gain access to adjacent territories and expand into an area previously innervated by the lesioned nerve (Brenan 1986; Devor *et al.,* 1979; Doucette & Diamond 1987; Kingery & Vallin 1989; Kinnman & Aldskogius 1986; Markus *et al.,* 1984). Hence nerve injury can induce adjacent undamaged sensory axons to sprout collateral fibres into an area which has been denervated due to the nerve lesion over a limited distance. Collateral sprouting has been observed in both large and small diameter fibres although there appears to be differences in the pattern of reinnervation between different nerve fibres (Brenan 1986; Devor *et al.,* 1979). Evidence suggests that small axons sprout and reinnervate larger territories than large myelinated axons (Navarro *et al.,* 1994; Wiesenfeld-Hallin *et al.,* 1989).

Collateral sprouting has been reported in several studies following nerve crush or transection (Brenan 1986; Jackson & Diamond 1981; Markus *et al.,* 1984; Navarro *et al.,* 1994; Navarro *et al.,* 1997). A similar case may also occur in cases of partial denervation where collaterals may sprout from the remaining intact afferents of the damaged nerve, or alternatively, from afferents of undamaged nerves. It appears that such collateral sprouting contributes to the early return of sensation in the affected area following nerve injury. Collateral sprouting has been suggested to be responsible for the occurrence of pain-related behaviours in rats with nerve injury (Kingery & Vallin 1989; Markus *et al.,* 1984). Sectioning the saphenous nerve at the time of or within one week of nerve ligation abolishes the hyperalgesia of CCI rats (Ro & Jacobs 1993). This suggests that hyperalgesia seen

after CCI may result from collateral innervation of the saphenous nerve. In humans, a partial recovery of sensation has been reported in patients who have had traumatic or surgical nerve section, and this was attributed to reinnervation of the denervated skin following collateral sprouting of neighbouring uninjured nerves (Inbal *et al.*, 1987).

It is important to note that reinnervation following peripheral nerve injury, either through axonal regeneration or collateral sprouting, does not necessarily lead to a successful functional recovery. Thus the correlation between morphology and effective functional recovery is not straightforward. Many injured axons fail to reinnervate the appropriate target tissue and there is a high incidence of misdirected regrowth leading to an atypical peripheral reinnervation (Koerber *et al.*, 1989). In addition, there is evidence to suggest that abnormal changes also occur in the central connections. One study has reported that there are alterations in the mechanoreceptor input to certain areas of the brain following nerve injury (Paul *et al.*, 1972). Hence, misdirected regeneration of nerve fibres may lead to a disruption in central spatial representation, thereby resulting in misinterpretation of tactile localisation. This aberrant central connection could, in part, contribute to the development of abnormal pain sensation in neuropathic pain patients.

Aβ-fibre sprouting

It has been suggested that following axotomy, there is structural reorganisation of the spinal cord, such that the central terminals of axotomised Aβ-fibres sprout into lamina II, an area of the cord that normally only processes C-fibre input (Shortland & Woolf 1993; Woolf *et al.*, 1992). Using cholera toxin-conjugated horseradish peroxidase (CB-HRP), which labels the central terminals of myelinated afferents, Woolf and co-workers (1992) demonstrated that there was a dorsal extension of the CB-HRP label from lamina III to lamina II, 1-15 weeks after nerve injury. Intact nerves displayed no labelling in lamina II, however, staining was observed in deeper layers. Hence, as a result of these changes, it was proposed that low-threshold mechanoreceptive afferent terminals may establish functional contacts with cells that normally would have a C-nociceptor input, leading to inappropriate responses to innocuous peripheral stimuli. Aβ-fibre sprouting has

been observed after axotomy (Woolf *et al.,* 1992), L5/ L6 spinal nerve ligation (Lekan *et al.,* 1996) and CCI (Nakamura & Myers 1999). The mechanism by which Aβ-fibres sprout into lamina II are unclear, although several mechanisms have been proposed. Firstly, transganglionic degeneration of C-fibre terminals, possibly due to a lack of peripherally-derived growth factors, may allow Aβ-fibres to sprout into the vacant space in lamina II (Doubell *et al.,* 1997). This hypothesis, however, does not seem likely, since a recent study demonstrated that creation of synaptic space within lamina II, through dorsal rhizotomy, does not induce sprouting in either intact or injured A-fibres (Mannion *et al.,* 1998). An alternative explanation was therefore suggested, where it was proposed that the central terminals of injured C-fibres release a factor, which could act as a chemoattractant for A-fibres (Doubell *et al.,* 1997; Mannion *et al.,* 1998). Furthermore, there may be an upregulation of growth-related proteins, such as GAP-43, which may promote the growth of Aβ-fibres into the denervated area (Woolf *et al.,* 1990). Sprouting of large diameter Aβ-fibres has been shown to exhibit differential sensitivity to nerve growth factors. Whilst the exogenous administration of NGF prevented Aβ-fibre sprouting following axotomy, intrathecal treatment with BDNF was ineffective in preventing these changes (Bennett *et al.,* 1996). Hence one possible mechanism for A-fibre sprouting may be the deficit of peripherally derived NGF due to an interruption of retrograde transport. However, this explanation is unlikely since only a small proportion of the large DRG cells with myelinated axons express the high affinity trkA receptor. The large DRG cells, however, express the trkB receptor but BDNF had no effect on A-fibre sprouting (Bennett *et al.,* 1996). Such structural reorganisation of primary afferent central terminals, which may result in the formation of novel inappropriate synaptic contacts between low threshold afferents and dorsal horn neurones, has been proposed to underlie the development of some of the sensory abnormalities associated with peripheral nerve injuries (Woolf *et al.,* 1992).

Despite the substantial evidence which exists for the phenomenon of Aβ-fibre sprouting, these findings have been questioned in a recent study, where it was demonstrated that the increased CB-HRP labelling reported by Woolf *et al.* (1992) may represent an enhanced uptake and transganglionic transport of the tracer in small DRG neurones, rather than sprouting of large diameter neurones from deeper laminae (Tong *et al.,* 1999). Tong and co-workers (1999) provided evidence that

(continued overleaf)

there is a marked increase in the number of CB- or CB-HTP-labelled neuronal profiles in the ipsilateral DRG, 18 days after peripheral axotomy in the rat and monkey (Tong *et al.,* 1999). Whilst under normal conditions, only large neuronal profiles are labelled with CB-HTP, axotomy induced labelling in both small and large neuronal profiles (Tong *et al.,* 1999), and this could account for the reported increase in laminae II immunostaining after nerve injury (Woolf *et al.,* 1992). Bennett and coworkers (1996), however, have provided evidence against this argument by showing that there was no difference in the cell size distribution of CB-containing DRG neurones between normal and axotomised animals. CB binds to the cell membrane glycoconjugates, GM1, hence the increased uptake of CB after nerve injury reported by Tong and coworkers (1999) may possibly reflect a novel expression of the GM1 receptor on small neuronal profiles. The significance of elevated GM1 levels is unclear, however, this suggests that CTB-HRP may not be an appropriate marker to study the events underlying Aβ-fibre sprouting, since the tracer may not be strictly selective for large diameter fibres after nerve injury (Tong *et al.,* 1999).

Sympathetic system

Under normal physiological conditions, the DRG is innervated by some catecholaminergic fibres (Stevens *et al.*, 1983) although there is little evidence for functional coupling between sympathetic fibres and primary afferent fibres (Burchiel 1984b). Following nerve injury, however, there is a formation of abnormal terminal arborizations, or 'basket-like' structures which surround the large diameter cell bodies in the DRG (McLachlan *et al.*, 1993). Morphological studies have demonstrated that sprouts of sympathetic axons form physical contacts with the DRG somata of primary afferents (Chung *et al.*, 1993), and this results in a functional coupling between the two, allowing DRG neurones to be activated through sympathetic stimulation (Devor & Janig 1981; Devor *et al.*, 1994a; McLachlan *et al.*, 1993). Immunohistochemical studies have shown that there is an increase in the number of sympathetic fibres following selective spinal nerve (L5/ L6) ligation, 3-5 days after surgery (Chung *et al.*, 1993). Based on these observations, sympathetic sprouting was proposed to contribute, at least in part, to the development of pain-like behaviours following nerve injury. However, the extent of its contribution has been questioned in recent studies, and there is accumulating evidence suggesting it may be a non-specific event following nerve injury, without direct relevance to the development of neuropathic pain (Kim *et al.*, 1999). In support of this argument, it was demonstrated that the degree of sympathetic dependence does not correlate with the extent of sympathetic fibre sprouting (Kim *et al.*, 1998). Additionally, a recent study demonstrated that sympathetic sprouting was not evident at PO 10 days, although animals displayed marked allodynia (Marchand *et al.*, 1999). These observations suggest that remodelling of sympathetic fibres within the DRG may not play a pivotal role to the production of pain associated with nerve injury.

In addition, studies have reported an increase in adrenergic sensitivity of peripheral afferent fibres following nerve injury, which may also contribute to the occurrence of injury-induced pain (Korenman & Devor 1981; Petersen *et al.,* 1996). Previous studies have shown that spontaneous activity originating from the neuromas of an injured nerve is enhanced following the application of adrenergic agonists (Burchiel 1984b; Korenman & Devor 1981; Leem *et al.,* 1997; Scadding 1981; Welk *et al.,* 1990; Xie *et al.,* 1995). Whilst sympathetic stimulation of injured afferent fibres produces a significant excitation of primary afferents, intact afferent fibres from naive rats do not respond to sympathetic stimulation (Leem *et al.,* 1997; Xie *et al.,* 1995). The development of adrenergic sensitivity was also reported in *in vitro* skin nerve preparation, where uninjured C-fibre nociceptors were shown to acquire alpha-adrenergic sensitivity in L6 spinal nerve ligated monkeys (Ali *et al.,* 1999). Several lines of evidence suggest that α2-adrenoceptors are involved in the mechanisms underlying the increased adrenergic chemosensitivity, and complex changes in α2 adrenergic receptor expression have been reported after nerve injury (Birder & Perl 1999; Cho *et al.,* 1997). Whilst $\alpha 2_A$-adrenergic receptors are upregulated in medium-large DRG neurones following sciatic nerve section, $\alpha 2_C$-adrenergic receptors, on the other hand, appear to be downregulated (Birder & Perl 1999). Similar changes have been reported for the expression of genes encoding these receptors in the SNL model (Cho *et al.,* 1997). The significance of these changes is unclear although alterations in the expression of these receptor subtypes may contribute to the generation of neuropathic pain.

In recent years, studies have investigated the role of the sympathetic nervous system in neuropathic pain states using sympathectomy, either through the surgical removal of sympathetic chains or through the administration of sympathetic blocking agents, such as guanethidine and phentolamine. Pain that can be relieved through sympathetic manipulation has been termed sympathetically maintained pain (SMP). Guanethidine produces a sympathetic block through the depletion of noradrenergic terminals and it has been demonstrated to produce a long lasting effect in both humans (Wahren *et al.,* 1991) and in animal models (Kim *et al.,* 1993; Neil *et al.,* 1991a; Shir & Seltzer 1991). Phentolamine, on the other hand, is a competitive α-adrenergic blocker. In addition, surgical sympathectomy has

been shown to attenuate mechanical allodynia in SNL rats (Kim & Chung 1991; Kim *et al.*, 1993). When performed prior to spinal nerve ligation, sympathectomy completely prevented the development of allodynia and hyperalgesia (Kim *et al.*, 1993). The SNL model of neuropathy has therefore been proposed to represent a model of sympathetically maintained pain. Similar effects have been reported following guanethidine administration in PSTL (Shir & Seltzer 1991) and CCI rats (Neil *et al.*, 1991b). In direct contrast to these studies, however, other studies have demonstrated that neither sympathectomy nor sympatholytic agents reverse behavioural signs of neuropathic pain in L5 spinal nerve ligated rats, 1-3 weeks after nerve injury (Ringkamp *et al.*, 1999a; Ringkamp *et al.*, 1999b). Hence there appears to be a degree of discrepancy between studies. Sympathetic sprouting in the DRG may have a delayed onset (> PO 10 days) (Marchand *et al.*, 1999), hence the failure of chemical or surgical intervention to reverse allodynia in the above studies may be due to the fact that sympathetic remodelling had not yet become evident, and therefore, may not form a large component of the allodynia at this time-point.

Thus, *de novo* or increased adrenergic sensitivity of sensory neurones following peripheral nerve injury may play a pivotal role in the genesis of spontaneous impulse generation, and contribute to the development of pain in causalgia and related sympathetic dystrophies (Campbell *et al.*, 1994; Devor 1983a; Petersen *et al.*, 1996). This provides a rationale for the effectiveness of sympathetic block or sympathectomy in these clinical conditions (Campbell *et al.*, 1994). Although sympathetic block has been shown to be effective in some patients (Bonica 1990b), the role of the sympathetic nervous system in neuropathic pain states is still a complex and controversial issue. The effectiveness of sympathectomy appears to vary depending on the timing of treatment with respect to nerve injury (Bonica 1990b). Whilst sympathectomy performed long after nerve injury (10 months-15 years) has been reported to be successful in some patients (Wahren *et al.*, 1991), it is generally believed that early sympathectomy has a higher success rate compared to a later one. Furthermore, there is evidence to suggest that strain difference in rats may also influence the degree of adrenergic dependency of pain behaviours (Lee *et al.*, 1999).

Nitric oxide and Nitric oxide synthase

Nitric oxide (NO) is a neuronal messenger which, unlike other messengers, is not stored in synaptic vesicles, but is produced on demand by the enzyme nitric oxide synthase (NOS). It is synthesised from L-arginine by NOS, which is found throughout the CNS and is thought to act as an intercellular, and possibly an intracellular messenger (Garthwaite & Boulton 1995). There is now accumulating evidence for the role of NO in nociceptive processing during states of enhanced neuronal excitability, such as inflammation or neuropathy (Malmberg & Yaksh 1993a; Meller *et al.,* 1992). Activation of the NMDA receptor results in an increase in $[Ca^{2+}]_i$, stimulation of NOS and production of NO. One target for NO is the enzyme guanylate cyclase, which, when activated, produces cGMP and subsequently exerts numerous effects on neuronal function (Meller & Gebhart 1993). Hence this raises the possibility that NO may be involved in the cascade of events underlying NMDA receptor-mediated central sensitisation, and the generation of persistent abnormal pain states (McMahon *et al.,* 1993; Meller *et al.,* 1992).

In recent years, the role of NO in neuropathic pain states has become increasingly recognised. Following the unilateral ligation of L5/ L6 spinal nerves, a significant increase in neuronal nitric oxide synthase (nNOS) mRNA (Luo *et al.,* 1999) and NOS-immunoreactivity has been reported in the L5/ L6 ganglia, which develops progressively over the postoperative period (Steel *et al.,* 1994). This was further supported by observations of an increased functional NOS activity in the DRG of SNL rats (Choi *et al.,* 1996). These changes were observed as early as 3 days after surgery and maintained for at least 2 weeks (Steel *et al.,* 1994). Similar results have also been demonstrated across other models of nerve injury (Gonzalez-Hernandez & Rustioni 1999; Verge *et al.,* 1992). The significance of increased NOS expression is unclear although a recent study has proposed a dual role for NO after peripheral nerve injury - firstly, as a mediator of mechanisms related to the regenerative processes of injured axons at the lesion site, and secondly, as a neuromodulator of abnormal primary sensory input in the DRG to maintain a hyperexcitable sensitised state (Gonzalez-Hernandez & Rustioni 1999). Hence

these results suggest that NOS activity may be important in the genesis and/ or maintenance of altered pain behaviours following peripheral nerve injury (Choi *et al.*, 1996). In support of this view, inhibitors of NOS, such as N^{ω}-nitro-L-arginine methyl ester (L-NAME), have been shown to relieve some of the behavioural signs of neuropathic pain through peripheral and central actions (Meller *et al.*, 1992; Niedbala *et al.*, 1995; Yoon *et al.*, 1998). Furthermore, systemic administration of L-NAME was demonstrated to attenuate ectopic discharge resulting from peripheral nerve transection (Wiesenfeld-Hallin *et al.*, 1993). Rather paradoxically, however, in a study employing L5 spinal nerve injured rats, it was demonstrated that blockade of NO synthesis with L-NAME (non-specific NOS inhibitor) or 7-nitroindazole (nNOS-specific inhibitor) produces either no effect or excitation of the ongoing activity in afferent fibres (Habler *et al.*, 1998). Similarly, a recent study provided evidence for a clear separation between nNOS regulation and development of allodynia (Luo *et al.*, 1999). Selective (L5/ L6) spinal nerve ligation produced a marked upregulation of nNOS mRNA in the DRG, however, treatment with 7-nitroindazole failed to reverse allodynia in SNL rats (Luo *et al.*, 1999). In addition, SNL rats which fully recovered from allodynia continued to show elevated levels of nNOS expression (Luo *et al.*, 1999). Hence, although the alteration in nNOS expression represents neuroplasticity in the DRG following nerve injury, whether it is responsible for the development of allodynia remains unclear. Further studies are required to assess the complete role of this mediator in neuropathic pain states.

1.4 Current approaches to the treatment of neuropathic pain

The current mainstay treatments of neuropathic pain are antiepileptics (e.g. carbamazepine, lamotrigine, gabapentin) and tricyclic antidepressants (e.g. imipramine, desipramine) (Sindrup & Jensen 1999). Controlled studies using tricylic antidepressants have shown that these agents are effective against neuropathic pain states, such as diabetic neuropathy (Max *et al.*, 1991; Sindrup *et al.*, 1990) and postherpetic neuralgia (Watson *et al.*, 1992), and their analgesic effects appear to be independent of their antidepressant effect (Kingery 1997). One drawback in their use is the occurrence of side effects, which include cardiac arrhythmias, postural hypotension and sedation. This may limit their use in patients

with cardiovascular problems (Rowbotham *et al.*, 1998). The sodium channel blocker, mexiletine, has also been examined in various neuropathic pain states although results from these studies remain somewhat unclear since some have reported good effects (Dejgard *et al.*, 1988), whilst others observed little or no effect (Stracke *et al.*, 1992). Similarly, there is evidence for the effectiveness of anticonvulsants, of which carbamazepine has been the most frequently used, for conditions such as postherpetic neuralgia (Killian & Fromm 1968). Lamotrigine, which acts by stabilising the slow inactivated conformation of sodium channels, has also become increasingly recognised as a potential treatment for neuropathic pain states (Canavero & Bonicalzi 1996; Eisenberg *et al.*, 1998a; Zakrzewska *et al.*, 1997). Recently, attention has focused on the novel anticonvulsant, gabapentin (1-(aminomethyl)cyclohexane acetic acid; Neurotonin), which has established efficacy in patients with diabetic neuropathy (Backonja *et al.*, 1998) and postherpetic neuralgia (Rowbotham *et al.*, 1998). Unlike conventional antiepileptic agents, gabapentin does not affect voltage-dependent sodium channels, nor does it possess high affinities for the $GABA_A$ /$GABA_B$ receptor, or GABA uptake sites (Gee *et al.*, 1996). Despite the extensive research, the mechanism of action of this novel agent remains largely unclear. In recent biochemical studies, it was demonstrated that gabapentin may possibly interact with the $\alpha 2\delta$ subunit of voltage-dependent calcium channels (Gee *et al.*, 1996). The $\alpha 2\delta$ subunit appears to be common to all voltage-dependent calcium channels hence gabapentin may conceivably modulate the activity of more than one neuronal calcium channel (Stefani *et al.*, 1998). At present, however, the physiological role of this subunit is not well understood and the relevance of the interaction of gabapentin at the $\alpha 2\delta$ subunit to the clinical utility of the drug remains unclear. Other potential targets which may be used possibly in conjunction with the above agents include opioids (morphine, oxycodone), alpha-2 adrenergic agonists (clonidine), NMDA receptor antagonists (ketamine, memantine) and topical agents (capsaicin, EMLA). The use of adjuvant analgesics may enhance the therapeutic effects of current treatments (anticonvulsants and antidepressants), and may prove to be beneficial in the future. Meanwhile, the potential use of these agents as first-line treatments for neuropathic pain states has not yet been firmly established and further trials are needed to assess their long-term effects.

1.5 The anatomy and physiology of pain

Primary afferent fibres

The somatosensory primary afferent fibre, which conveys sensory information to the spinal cord can be classified into several classes, according to the transduction properties of the individual nerve fibre. The properties of each afferent fibre are summarised in the table below.

Table 2. Classification of somatosensory primary afferent fibres innervating the skin.

Primary Afferent Fibre type	Mean Diameter (μm)	Myelination	Mean Conduction Velocity (m/s)
Aβ	6 - 12	Myelinated	25 -70
Aδ	1 - 5	Thin Myelination	10 - 30
C	0.2 - 1.5	None	< 2.5

The afferent fibres differ in their conduction velocity and degree of myelination, and can be distinguished by the size of their diameter. The large diameter Aβ-fibres are myelinated by Schwann cells and hence has a fast conduction velocity. This group of nerve fibres innervates receptors in the dermis and is involved in the transmission of low-threshold, non-noxious information, such as touch. The Aδ-fibre is less densely myelinated and conveys both non-noxious and noxious sensory information. The unmyelinated C-fibre conveys high-threshold noxious inputs and has the slowest conduction velocity of all 3 fibre types.

Upon entry into the spinal cord, each primary afferent fibre (Aβ-, Aδ- or C-fibre) exhibits a specific termination pattern in the dorsal horn, and this has been studied extensively. The termination pattern of large and small diameter fibres can be studied through the use of use of specific markers, such as horseradish peroxidase (HRP), conjugated to choleragenoid and wheat germ agglutin, respectively (LaMotte *et al.,* 1991). Dorsal root afferents send most of their collaterals into the segment of entry. However, there is also a degree of rostrocaudal distribution and some collaterals may spread to several segments above or below the target segment.

Aβ-fibres

The large diameter Aβ-fibres enter the spinal dorsal horn through the medial division of the dorsal root. Following entry, they then descend through the medial region of lamina I or II, or alternatively, curve around the medial edge of the dorsal horn to enter the ventral horn. On reaching deeper laminae, laminae IV and V, the Aβ-fibres ascend back up into laminae III and IV where they repeatedly subdivide and form a characteristic termination pattern (Sorkin & Carlton 1997). The densest arborisation appears to occur in lamina III. Axons originating from specialised muscle stretch receptors have collaterals that pass ventrally to make monosynaptic connections with neurones of laminae V, VI and VII. Some also extend to laminae VIII and IX of the ventral horn where they synapse directly onto motor neurones and form the basis of monosynaptic reflexes (Bonica 1990a).

Aδ-fibres

The termination pattern exhibited by Aδ-fibres is entirely different from that of large Aβ-fibres. Aδ-fibres travel extensively in Lissauer's tract and their terminals form a plexus at the surface of the spinal cord (Gobel *et al.,* 1981). Aδ-fibres from high-threshold mechanoreceptors distribute to laminae I, IIo and V. Projections also appear to terminate on the contralateral side, in laminae V. Aδ-fibre innervations from deep tissues (muscles and joint) have been shown to terminate exclusively in lamina I, or in laminae IV and V (Mense & Prabhakar 1986).

C-fibres

Extensive studies have investigated the organization and termination patterns of C-fibres, employing various techniqes including Golgi staining (Rethelyi 1977), degeneration techniques (LaMotte 1977) and HRP transport (Gobel *et al.*, 1981). Unmyelinated C-fibres enter the spinal cord through the lateral part of the dorsal white matter, including the Lissauer's tract. Studies have shown that unmyelinated primary afferents terminate in the superficial dorsal horn, although there is conflicting evidence as to whether the terminations are restricted to lamina II or whether it includes both laminae I and II. Current evidence suggests that lamina II is the main termination area for cutaneous primary afferent C-fibres (Sugiura *et al.*, 1986), while that for Aδ-fibres is in lamina I (Sorkin & Carlton 1997).

1.6 Sensory transmission in the spinal cord

The spinal cord is arranged in such a way that primary afferents originating from different regions of the body display specific somatotopic organisations upon entry into the cord. Hence in any given segment, there is a definite laterality (ipsilateral/ contralateral) and a three dimensional organisation (rostrocaudal, mediolateral, dorsoventral) of the afferent terminations.

The spinal cord can be classically divided into white and grey matter. The white matter of the spinal cord consists of axons, while the grey matter contains cell bodies and their processes. The grey matter can be organised into 10 different laminae, which run continuously along the entire length of the spinal cord. Within a given section of a spinal cord, each lamina can be seen as a layer of functionally distinct cells. These laminar boundaries are not exact and rigid hence dendrites of a given neurone whose cell body is located in one lamina may extend to a neighbouring lamina. Laminae I to VI compose the dorsal horn, laminae VII to IX the ventral horn, and lamina X is the substantia grisea centralis which surrounds the central canal.

1.6.1 Morphology of the spinal cord dorsal horn

Lamina I

Lamina I forms the outer layer of the dorsal horn and contains the large marginal cells of Waldeyer. Lamina I plays an important role in nociception since it is the layer in which some nociceptive afferents terminate. It contains a number of cell types, including nociceptive-specific neurones, which receive Aδ- and C-fibre input, neurones which only respond to innocuous thermal stimuli and wide dynamic range (WDR) neurones. Many marginal cells appear to be projection neurones, which contribute to the lateral spinocervical (SCT), spinoreticular and spinothalamic tracts (STT) (Sorkin & Carlton 1997). The projections also extend to the periaqueductal gray (PAG), parabrachial nucleus and the nucleus submedius.

Lamina II

Lamina II is also known as the substantia gelatinosa (SG) and can be divided into 2 layers, the outer layer (IIo) and the inner layer (IIi). This layer is densely packed with small neurones and lacks myelinated axons. Neurones with cell bodies in IIi receive inputs from low-threshold mechanoreceptive primary afferents, while those in IIo respond to inputs from high-threshold and thermoreceptive afferents (Bonica 1990a). The intrinsic cells which compose the SG are predominantly stalk and islet cells. Stalk cells are found located in lamina IIo, particularly on the border of lamina I, and most of their axons have ramifications in lamina I although some also project to deeper layers. These cells are thought to predominantly relay excitatory transmission (Bennett *et al.*, 1980). Islet cells, on the other hand, are located in IIi and have been demonstrated to contain the inhibitory neurotransmitter, γ-aminobutyric acid (GABA) in their dendrites. Hence these cells have been proposed to be inhibitory interneurones. Other cell types in lamina II include arboreal cells, II-III border cells, and spiny cells.

Lamina III

The cell bodies in lamina III are generally larger and less densely packed than those in the substantia gelatinosa. The main cell type of lamina III include projection cells, which contribute to the SCT and postsynaptic dorsal column (PSDC) (Bennett *et al.,* 1984). The dendrites of SCT cells are confined to lamina III and do not reach laminae I and IIo, however, those of PSDC are not flattened in the mediolateral plane and extend to laminae I and II, thus forming monosynaptic connections with small primary afferent fibres (Sorkin & Carlton 1997).

Laminae IV to VI

Lamina IV is composed of heterogeneous sized cells and is less densely packed than lamina III due to the number of nerve axons passing in this layer. At least 3 types of neurones have been identified in lamina IV, based on different dendritic projection patterns and these include SCT and PSDC cells. Another cell type has been described which has a dendritic pattern similar to SCT and PSDC, but with local axon terminations. Somas of STT cells are also found in lamina IV (Sorkin & Carlton 1997).

The cells composing lamina V are more diverse than those of lamina IV and their dendrites extend vertically toward the superficial layers (Sorkin & Carlton 1997). Cell bodies in lamina V contribute to three projection pathways, the SCT, PSDC and STT, however, the STT cells appear to be the predominant in this lamina. Lamina V plays an important role in nociception since it receives both Aδ- and C-fibre inputs. Some cells in lamina V also respond to cutaneous low- and high-threshold mechanical stimuli and receive nociceptive inputs from the viscera.

Lamina VI forms the base of the dorsal horn and can be found only in certain levels of the spinal cord, the cervical and lumbar regions. Little data has been reported on the cell composition of lamina VI. Cells of lamina VI are small in size compared to those of lamina V and some axons appear to contribute to the STT and SCT pathways.

(continued overleaf)

Lamina X

Most cells in lamina X are small cells, which possess a large number of short axon collaterals that often extend bilaterally. Lamina X cells appear to take part in propriospinal systems and a small number of projection cells terminate in medullary and pontine reticular formations, as well as the periaqueductal gray and thalamus (Sorkin & Carlton, 1997). Additionally, it has been demonstrated that lamina X receives bulbar descending projections from brainstem structures (Du 1989). High levels of kappa opioid receptor mRNA (Schafer *et al.,* 1994), 5HT receptors (Marlier *et al.,* 1991) and CGRP binding sites are expressed in lamina X (Yashpal *et al.,* 1992), and these neurones may therefore be implicated in the control of nociceptive transmission at the spinal level.

1.7 Pharmacology of the spinal cord

Nociceptive sensory information arriving from primary afferent fibres enter via the dorsal horn and on entering the spinal cord, undergoes considerable convergence and modulation. The pharmacology of the spinal cord is extremely rich and contains a diverse range of neurotransmitters and receptors, which may be excitatory or inhibitory, depending on the consequence of their activation and their location on neuronal circuitry. The transmission of pain can therefore be seen as a complex process involving the interplay between excitatory and inhibitory systems acting at different levels of the central nervous system. Excitatory systems are responsible for the generation of pain and its transmission, and these include the NMDA receptor system. Activation of excitatory receptors will result in increased transmitter release and consequently increased neuronal excitability. Activation of inhibitory receptors, on the other hand, will decrease neuronal firing, inhibit transmitter release and consequently bring about a reduced excitability of neurones. These may include the GABAergic and opioid receptor systems. Hence, activation of inhibitory transmitter systems or alternatively, blockade of excitatory systems can produce analgesia and may be an important strategy for pain control. All these systems are subject to plasticity, and alterations in pharmacological systems may occur during pathological conditions.

The spinal cord is an important site at which various nociceptive signalling systems undergo convergence and modulation. The spinal cord is under ongoing control by peripheral inputs, interneurones and descending controls. One consequence of this modulation is that the relationship between stimulus and response to pain is not always straightforward. The response of output cells could be greatly altered via the interaction of various pharmacological systems in the spinal cord.

1.7.1 Excitatory transmission

1.7.1.1 N-methyl-D-aspartate (NMDA) receptor system

The excitatory amino acids, glutamate and aspartate, have been implicated in the transmission of nociceptive information in acute and chronic pain states (Dickenson 1994a). Several receptors for glutamate have been identified in the brain and spinal cord, including the N-methyl-D-aspartate (NMDA), alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA), kainate and metabotropic glutamate receptors. Immunohistochemical studies have shown that all three receptor types have a prominent localisation in the superficial dorsal horn (laminae I-III) (Bonnot *et al.*, 1996; Yung 1998). In addition, staining was also observed in deeper layers (laminae IV-VI), which may correspond to receptors located on WDR neurones. The parallel neuroanatomical distribution of these ionotropic receptors in laminae I-III of the spinal cord provides further support for functional interactions between NMDA and non-NMDA receptors in modulating nociceptive transmission.

The excitatory amino acids are found in most sensory fibres, including both large and small diameter fibres where, in the latter case, they are colocalised with peptides such as substance P (Battaglia & Rustioni 1988; De Biasi & Rustioni 1988). The coexistence of these two transmitters suggest that they are released together in response to a noxious stimulus hence contribute to the transmission of pain. Whilst AMPA receptors are activated in response to brief acute stimuli and are involved in the fast events of pain transmission, NMDA receptors are only activated following repetitive noxious inputs, under conditions where the stimulus is maintained (Dickenson 1994a). NMDA receptors have been implicated in the spinal events underlying 'wind-up' (Mendell 1966), whereby the responses of dorsal horn neurones are significantly increased after repetitive C-fibre stimulation despite the constant input (Dickenson 1995). Thus the activation of this class of receptors brings about a marked increase in neuronal excitability and is responsible for the amplification and prolongation of neuronal responses in the spinal cord (Dickenson *et al.*, 1997a; Dickenson *et al.*, 1997b).

Table 3. Classification of glutamate receptors. Abbreviations: N-methyl-D-aspartate (NMDA), alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA), L(+)-2 amino-3-phosphonopropionic acid (L-AP3), 6-cyano-7-nitroquinoxaline (CNQX), 2,3-dihydroxy-6-nitro-7-sulfamyl-benzo-f-quinoxaline (NBQX), 3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP), 7 Chlorokynureic acid (7-CK).

	Metabotropic	**Ionotrophic**		
		NMDA	**AMPA**	**Kainate**
Endogenous Agonist	Glutamate	Glutamate	Glutamate	Glutamate
Other Agonists	Quisqualate	NMDA	AMPA Kainate	Kainate
Antagonists	L-AP3	MK-801 Memantine Ketamine Dextrophan CPP 7-CK (glycine site)	CNQX NBQX	LY382884

The NMDA receptor has a heteromeric structure composed of two subunit types; the NR1 subunit and one of four subunits (NR2A-NR2D). It is an ionotropic receptor coupled to a cation channel, which is blocked by physiological levels of Mg^{2+} at the resting membrane potential. The channel is blocked in a voltage-dependent manner thus the receptor can only operate after sufficient repeated depolarisation. The removal of the Mg^{2+} block is mediated by tachykinins, which are coreleased with glutamate. After a brief acute stimulus, pain transmission from C-fibres is largely mediated by the action of glutamate on AMPA receptors. When the stimulus is sustained or its intensity is increased, however, the action of substance P on NK-1 receptors produces sufficient membrane depolarisation so that the Mg^{2+} block can now be removed and the NMDA receptor activated (Dickenson *et al.,* 1997b). These events underlie central hyperexcitability and result in a significant amplification of the response. Substance P therefore plays an important role in recruiting NMDA receptors and contribute to the cascade of events leading to the enhancement and prolongation of the neuronal response. Indeed, the administration of substance P antagonists have been shown to produce antinociception and decrease spinal excitability (De Koninck & Henry 1991; Toda & Hayashi 1993; Yashpal *et al.,* 1993).

Functional modulation of the receptor can be achieved through actions at various recognition sites including the primary transmitter site (competitive), the phencyclidine site (uncompetitive), polyamine modulatory site and the strychnine insensitive glycine site (Fig. 2). Potentially, there are several ways in which the effect of released glutamate can be antagonised through NMDA receptor blockade. Numerous studies have investigated the potential use of antagonists acting through the different recognition sites, however, due to the ubiquitous nature of the receptor, it has often been difficult to achieve therapeutic effects at the target site, in the absence of adverse side effects.

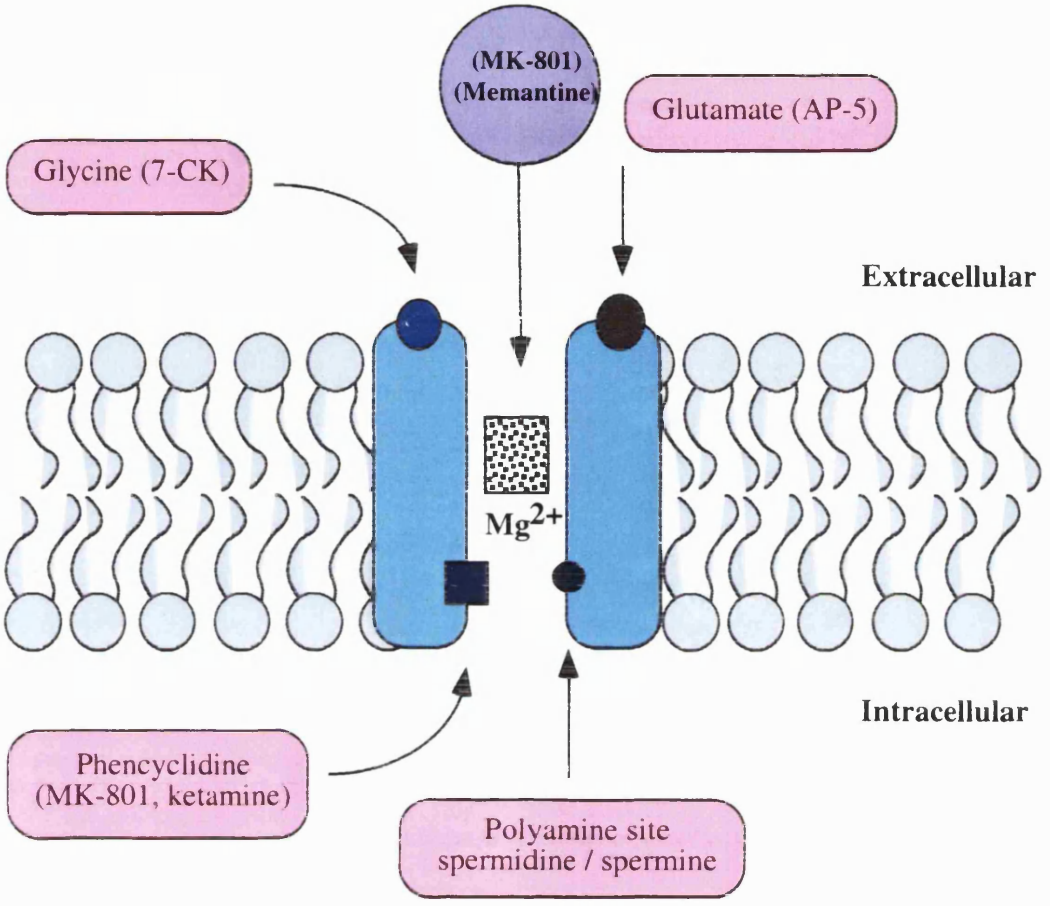


Figure 2. Structure of the NMDA receptor-channel complex. The receptor has a complex structure and this is highlighted through the presence of many pharmacologically distinct binding sites through which its receptor activity can be modulated. The channel associated with the receptor is blocked by Mg2+ at resting potential (-70mV). Receptor activation requires the removal of this Mg2+ block (voltage-gated) as well as the binding of glutamate and the coagonist, glycine (ligand-gated). The different binding sites (glutamate, phencyclidine, polyamine, glycine) are illustrated, and together with antagonists which act at the various sites (in parenthesis). The polyamine site is an intracellular site which modulates the affinity of other agonists and antagonists.

Substantial evidence exists for the involvement of NMDA receptors in various pathological pain states. Studies have demonstrated the effectiveness of NMDA receptor antagonists in animal models of inflammation (Coderre & Melzack 1992a; Eisenberg *et al.,* 1994; Ren *et al.,* 1992a), neuropathic pain (Mao *et al.,* 1993), allodynia (Yaksh 1989) and ischemia (Sher *et al.,* 1992). Both pre- and post-surgical administration of antagonists were shown to be effective, suggesting that the induction and maintenance of these ongoing pain states are dependent on NMDA receptor-mediated events.

It is now clear that neuropathic pain states are, at least in part, mediated by NMDA receptor-mediated events, based on earlier findings from animal studies (Davar *et al.,* 1991; Mao *et al.,* 1992b; Seltzer *et al.,* 1991b; Yamamoto & Yaksh 1992a). Following nerve injury, there appears to be a greater contribution of the NMDA receptor system to neuronal activity, and this may play a role in the spinal hyperexcitability which underlies this condition. Neuropathy may produce a prolonged activation of NMDA receptors, due to a sustained afferent input to the spinal cord, and this may result in a relatively small, but continuous increase in the extracellular level of glutamate. Increased glutamate levels have been reported in the ipsilateral dorsal horn of CCI rats 4-14 days after surgery (Kawamata & Omote 1996) and furthermore, there is evidence for an upregulation of glutamate receptors following nerve injury (Croul *et al.,* 1998; Harris *et al.,* 1996; Popratiloff *et al.,* 1998). Hence, a greater proportion of channels are likely to be in their open state during neuropathy, and this could enable NMDA channel blockers to exert greater effects, due to their use-dependency.

1.7.2 Inhibitory transmission

In addition to these excitatory events, several inhibitory systems also exist in the spinal cord which are in dynamic equilibrium with other intrinsic systems. When activated, inhibitory transmitter systems can suppress nociceptive transmission and effectively produce analgesia. The opioid receptor system represents one of the main inhibitory systems in the spinal cord and extensive studies have been conducted over the past decades. Other inhibitory systems include the GABAergic and monoaminergic systems, for which numerous receptor subtypes have been described (Millan 1999).

1.7.2.1 Opioid system

The discovery and use of opium dates back for many centuries. However, even today, there is still a wide general interest in this area of research and recent years have seen a remarkable advance in the understanding of opioid analgesia and ways in which it can be further improved for the treatment of difficult pain states (e.g. chronic neuropathic pain).

To date, four opioid receptor subtypes have been cloned and isolated, which include the μ, δ, κ receptors (Dickenson 1994b; Dickenson & Suzuki 1999), and the recently identified ORL-1 (Opioid Receptor-Like) receptor (Peluso *et al.*, 1998; Wick *et al.*, 1994) (Table 4). The endogenous opioid peptides for these receptors are endorphin, enkephalin, dynorphin and nociceptin, respectively (Dickenson & Suzuki 1999). The recently discovered ORL-1 receptor, which exhibits considerable sequence homology with the other three 'classical' opioid receptors, shows unique pharmacological properties since it exhibits only a low affinity for naloxone, a universal opioid receptor antagonist. The 17 amino acid endogenous peptide for the receptor has been termed nociceptin, or orphanin FQ (Meunier *et al.*, 1995; Reinscheid *et al.*, 1995), and although its functional role is still somewhat unclear, extensive studies are currently being conducted to elucidate its role in pain modulation (Carpenter & Dickenson 1998; Connor *et al.*, 1996;

Darland *et al.*, 1998; Taylor & Dickenson 1998; Vaughan & Christie 1996).

Table 4. Opioid receptors and their ligands. Abbreviations: [D-Ala2, N-Me-Phe4, Gly5-ol] enkephalin (DAMGO); [D-Pen2, D-Pen5]-enkephalin (DPDPE); Tyr-D-Ser(OtBu)-Gly-Phe-Leu-Thr (DSTBULET); orphanin FQ (OFQ)

	μ	δ	κ	**ORL-1**
Endogenous Agonist	β-endorphin Endomorphin	Met-enkephalin Leu-enkephalin	Dynorphin $A_{(1-8)}$ Dynorphin $A_{(1-13)}$ Dynorphin B	Nociceptin / OFQ
Other Agonists	Morphine DAMGO	DPDPE DSTBULET	U50488H	-
Antagonists	Naloxone	Naltrindole Naloxone	Naloxone Nor-BNI	$[Phe^1\Psi(CH_2NH)$ $Gly^2]NC_{(1-13)}NH_2$

The cloning and isolation of opioid receptors was a major breakthrough in understanding the molecular basis of opioid actions as well as their localisation on nerve fibres. Autoradiographic and immunohistochemical studies have shown opioid receptors to be localised primarily in the superficial dorsal horn (laminae I and II), and a smaller population has been demonstrated in deeper layers (Besse *et al.*, 1990a; Rahman *et al.*, 1998). The relative proportion of opioid receptor subtypes in the rat spinal cord has been reported to be approximately 70%, 20-30%, and 5-10 % for μ, δ and κ receptors, respectively (Besse *et al.*, 1990a; Dickenson & Suzuki 1999; Rahman *et al.*, 1998). The majority of these receptors appear to be located on presynaptic terminals of fine afferent fibres (Dickenson & Suzuki 1999). Hence, the predominant presynaptic localisation suggests for a main presynaptic action of opioids through the inhibition of transmitter release. Indeed, over 70% of the total μ receptor sites in the spinal cord are localised presynaptically on primary afferent terminals and only 25% of the receptor population are found on

postsynaptic sites, on interneurones or on dendrites of deep cells in the C-fibre terminal zone (Besse *et al.*, 1990a). There is, however, functional data from electrophysiological and behavioural studies supporting the postsynaptic actions of opioids (Dickenson 1994b; Hylden & Wilcox 1983; Lombard & Besson 1989).

The mechanism of action of opioids can be broadly divided into presynaptic and postsynaptic, based on the location of receptors on the neuronal circuitry within the spinal cord. Clearly, the predominant mode of action by which opioid analgesia is produced is through presynaptic mechanisms, whereby the release of transmitters from afferent terminals (e.g. substance P, CGRP) is reduced by receptor activation, consequently resulting in an inhibition of nociceptive transmission (Hirota *et al.*, 1985; Kangrga & Randic 1991). The selective action of opioids on nociceptive transmission is mainly due to the absence of opioid receptors on large diameter Aβ-fibre terminals. The synthesis of opioid receptors takes place in the cell bodies of small diameter fibres in the DRG and they are subsequently transported both centrally and peripherally. The activation of these receptors thus results in the selective inhibition of high threshold noxious inputs, while activity mediated by low threshold Aβ-fibres remain relatively unaffected.

In addition to these presynaptic effects, opioids can also act postsynaptically at sites located on interneurones or on projection neurones (cell body/ dendrites), and consequently inhibit nociceptive transmission. Whilst activation of receptors on interneurones or on dendrites of projection neurones can produce a selective inhibition of the nociceptive input, hyperpolarisation of the cell body of projection neurones is expected to result in an overall inhibition of the cell response, including both noxious and non-noxious inputs. This is supported by evidence from electrophysiological studies where morphine administration produced a small reduction of the Aβ-fibre evoked response (Dickenson & Sullivan 1986). Furthermore, opioids can also exert indirect postsynaptic actions through a disinhibitory effect on the inhibitory interneuronal system (enkephalin and GABA neurones) in the substantia gelatinosa. In a system where an inhibitory interneurone is held under inhibitory control by another interneurone, activation of opioid receptors on the first neurone would result in hyperpolarisation, thereby disinhibiting the second neurone, producing a net inhibition. Hence, through the combined effect of these mechanisms, opioids can produce powerful antinociceptive

effects and this has been demonstrated in a large number of studies in various pain states (Attal *et al.*, 1991; Kayser *et al.*, 1991; Ossipov *et al.*, 1995a).

Substantial evidence exists for the effectiveness of opioids in animal models of acute and persistent pain, and also in neuropathic pain states although to variable extents (Dickenson 1994b; Ossipov *et al.*, 1997a). It is known that the opioid system is subject to a considerable degree of plasticity following various pain states (Ossipov *et al.*, 1997a). Hence, whilst inflammation results in an overall increase in the analgesic effect of opioids, neuropathic pain states following nerve injury often display decreased opioid sensitivities, leading to difficulties in achieving good opioid analgesia. The degree of analgesia achieved from opioid administration therefore depends partly on the clinical characteristic of the pain state. The issue of opioid responsiveness in neuropathic pain states has been somewhat controversial and it has been a subject of much debate over the past decade. Reports on the efficacy of opioids have been conflicting and various effects have been reported, ranging from no analgesia (Arner & Meyerson 1988), to adequate pain relief following sufficient dose-escalation (Portenoy *et al.*, 1990). However, it is now generally acknowledged that neuropathic pain states are not completely refractory to opioid treatments, and clinical reports have demonstrated beneficial effects in some patients (Rowbotham *et al.*, 1991). Various factors are responsible for bringing about these changes in opioid actions following nerve injury and these will be discussed later in Chapter 7.

1.7.2.2 Adenosine receptor system

The role of the purinergic system in regulating nociceptive sensory processing has been recognised for a long time (Duggan & Griersmith 1979; Keele 1970). The purines, adenosine and ATP (adenosine 5'-triphosphate) have been implicated in the modulation of nociceptive trasmission, both in the periphery and in the CNS (Sawynok 1998).

The release of ATP from peripheral endings of primary afferent fibres was originally demonstrated by Holton and Holton (1954) and studies have reported the release of ATP from nerve terminals within the spinal cord (White *et al.*, 1985). The observation that capsaicin evokes the release of ATP in the dorsal horn (Sweeney *et al.*, 1989) suggests that release may occur from capsaicin-sensitive small diameter primary afferents (Sawynok & Sweeney 1989). Following its release, ATP can be converted to adenosine through a series of metabolic pathways involving various enzymes, including Ca^{2+} Mg^{2+} ATPase, ADPase and 5'-nucleotidase (Salter *et al.*, 1993b) (Fig. 3). Although there is evidence for adenosine-like immunoreactivity in the substantia gelatinosa (Braas *et al.*, 1986), it is not yet clear whether adenosine is stored in a classical manner, akin to other neurotransmitters.

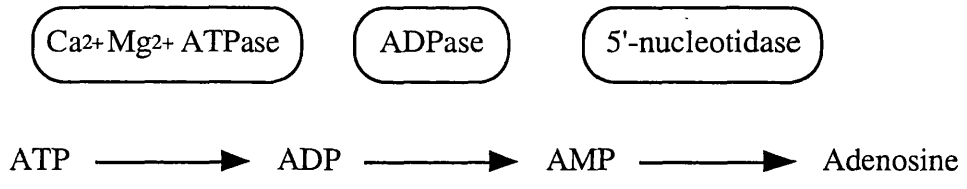


Figure 3. Extracellular metabolism of ATP. Abbreviations: ATP (adenosine 5'-triphosphate), ADP (adenosine 5'-diphosphate), AMP (adenosine 5'-monophosphate)

Receptor sites for adenosine in the spinal cord have been identified in the substantia gelatinosa where they are localised primarily on intrinsic neurones (Geiger *et al.*, 1984; Goodman & Synder 1982). Two main subclasses of adenosine receptors (A_1/ A_2) have so far been described and the A_2 receptor has been classified further into A_{2a} and A_{2b} subtypes (Bruns *et al.*, 1986; Choca *et al.*, 1988; Choca *et al.*, 1987; Londos *et al.*, 1980; Ralevic & Burnstock 1998). The two receptor subtypes differ in their effects on adenylate cyclase activity. A_1 receptors are linked to the G proteins, G_i and G_o, and exert their actions through the inhibition of adenylate cyclase. A_2 receptors, on the other hand, are coupled to G_s and stimulate adenylate cyclase activity (Salter *et al.*, 1993b; Sawynok 1999). Although evidence exists for the involvement of A_2 receptors in spinally-mediated antinociception (DeLander & Hopkins 1987), it appears to be predominantly the A_1 receptor subtype which plays a major role in inhibiting the nociceptive input in the dorsal spinal cord (Reeve & Dickenson 1995b; Sawynok *et al.*, 1986).

The predominant localisation of adenosine A_1 receptors in the spinal cord appears to be on postsynaptic sites, and only a minor population has been observed presynaptically on afferent terminals (Choca *et al.*, 1988; Geiger *et al.*, 1984). Adenosine exerts its actions through the inhibition of excitatory postsynaptic sensory pathways, by activating K^+ channels and consequently producing hyperpolarisation (Doi *et al.*, 1987; Li & Perl 1994; Ocana & Baeyens 1994; Salter *et al.*, 1993a). A presynaptic action of adenosine has also been proposed on the basis of an inhibition of primary afferent Ca^{2+} currents and the spinal release of neuropeptides in various experimental preparations (Santicioli *et al.*, 1992; Santicioli *et al.*, 1993; Sjolund *et al.*, 1997). In the brain, adenosine has been reported to decrease aspartate and glutamate release from rat hippocampal slices (Corradetti *et al.*, 1984). However, the majority of the effects of the purine appear to be mediated by postsynaptic actions. The mechanism by which adenosine produces antinociception involves an interaction with the excitatory amino acids and the neurokinins. Hyperpolarisation of transmission neurones through the activation of postsynaptic A_1 receptors reduces the excitatory actions of glutamate or substance P which also act on postsynaptic sites and consequently produces an inhibition of synaptic transmission (Doi *et al.*, 1987). In agreement with this, electrophysiological studies have demonstrated that A_1 receptor agonists inhibit

wind-up, an NMDA receptor-mediated event (Reeve & Dickenson 1995b).

Furthermore, it has been proposed that the spinal analgesic action of morphine is partially mediated by adenosine (Sawynok *et al.*, 1989). The observation that the adenosine receptor antagonist, methylxanthine produces inhibition of morphine mediated analgesia (Sweeney *et al.*, 1987b) led to the speculation that morphine may cause the release of adenosine, which in turn produces analgesia through activation of spinal adenosine receptors. The release of adenosine in response to morphine was subsequently demonstrated *in vivo* from intact rat spinal cord (Sweeney *et al.*, 1987a), as well as from synaptosomes prepared from the dorsal spinal cord (Sweeney *et al.*, 1987b). The neuronal origin of the released adenosine appeared to be from terminals of capsaicin-sensitive small diameter primary afferents in the dorsal horn (Sweeney *et al.*, 1989). Hence this suggests that adenosine forms a significant component in mediating the spinal antinociceptive action of morphine and further supports the functional role of this purine in nociception.

There is a large body of evidence from animal behavioural studies demonstrating the antinociceptive effects of adenosine analogues in acute nociceptive tests using the hot plate and tail flick assays (Ahlijanian & Takemori 1985; Aran & Proudfit 1990; Contreras *et al.*, 1990; DeLander & Hopkins 1987; DeLander & Wahl 1988; Doi *et al.*, 1987; Fastbom *et al.*, 1990; Holmgren *et al.*, 1983; Holmgren *et al.*, 1986; Karlsten *et al.*, 1990; Karlsten *et al.*, 1991; Ocana & Baeyens 1994; Post 1984; Sjolund *et al.*, 1997; Sosnowski *et al.*, 1989; Yang *et al.*, 1995; Yarbrough & McGuffin-Clineschmidt 1981). Similarly, in models of inflammation, adenosine analogues attenuated carageenan-induced thermal hyperalgesia (Poon & Sawynok 1998) and reduced pain-related behaviours following formalin injection (Karlsten *et al.*, 1992; Malmberg & Yaksh 1993b). The administration of adenosine analogues has been shown to reduce carageenan-induced c-Fos protein expression in the spinal dorsal horn (Honore *et al.*, 1998). Furthermore, adenosine-mediated antinociception can be enhanced through the use of adenosine kinase and adenosine deaminase inhibitors, which prevent the degradation of adenosine. The administration of these compounds have been shown to be effective in producing antinociceptive effects in nociceptive (Keil & DeLander 1994; Keil & DeLander 1992), inflammatory tests (Poon & Sawynok 1995; Poon &

Sawynok 1998). Evidence from electrophysiological studies has also demonstrated that adenosine analogues produce inhibitions of both acute (Reeve & Dickenson 1995a) and more persistent nociceptive responses of dorsal horn neurones (Reeve & Dickenson 1995b). Similarly, adenosine analogues inhibited noxious evoked activity in the absence and presence of sensitisation induced by mustard oil application (Sumida *et al.*, 1998). Although substantial evidence exists from behavioural studies, current electrophysiological data on the effects of adenosine analogues is still lacking (Reeve & Dickenson 1995a). Nevertheless, these results observed across several models of pain, strongly support the potential clinical use of these agents in various pain states.

There is now a growing interest in the development of therapeutic agents which interact with adenosine systems for the treatment of neuropathic pain. It has been demonstrated that patients with neuropathic pain have a deficiency in the plasma and cerebrospinal fluid (CSF) adenosine level (Guieu *et al.*, 1996). Neuropathic pain states are associated with neuronal hyperexcitability (Dray *et al.*, 1994), hence adenosine administration may offer a beneficial approach in attenuating excessive neuronal activity through interactions with the NMDA receptor system. The direct and indirect manipulation of the adenosine system may prove to be a useful approach in producing spinally mediated antinociception during neuropathy.

1.7.2.3 γ-aminobutyric acid (GABA) system

Together with the opioid receptor system, GABA (γ-aminobutyric acid) forms the major inhibitory transmitter system within the spinal cord. GABA is found extensively within the CNS and appears to exert tonic inhibitory controls on excitatory transmission. GABA has been localised in the superficial dorsal horn (laminae I-III) where it is found mainly within interneurones (islet cells) (Dickenson *et al.*, 1997b). GABAergic terminals have been shown to contact mainly Aδ- and nonglomerular C-fibre terminals (Bernardi *et al.*, 1995) and are implicated in the modulation of nociceptive transmission. Evidence suggests that GABA may coexist with other inhibitory transmitters including glycine (Todd 1996; Todd *et al.*, 1996), galanin (Simmons *et al.*, 1995), met-enkephalin (Todd *et al.*, 1992) and neuropeptide Y (Rowan *et al.*, 1993).

Various receptors for GABA have been identified and these have been classified into 2 subtypes, the $GABA_A$ and $GABA_B$ receptor. A third subtype, the $GABA_C$ receptor has been described in the retina (Enz & Cutting 1999; Fletcher *et al.*, 1998; Lukasiewicz 1996) and possibly in the CNS (Boue-Grabot *et al.*, 1998), however, its functional role outside the retina is still unknown. The $GABA_A$ receptor is a ligand-gated chloride channel, which possesses allosteric binding sites for various modulators, including benzodiazepines and barbiturates (Schofield 1989). $GABA_B$ receptor on the other hand, is a G protein (G_o) coupled receptor linked to membrane Ca^{2+} and K^+ channels (Campbell *et al.*, 1993). Both these receptors produce inhibitions of neuronal activity, either through an increased Cl^- conductance or by reducing Ca^{2+} influx into nerve terminals, and they have been shown to coexist pre- and postsynaptically on Aδ- and C-fibre primary afferents (Desarmenien *et al.*, 1984).

GABAergic interneurones appear to exert their inhibitory effects through presynaptic actions via axo-axonal synapse with primary afferent terminals (Alvarez *et al.*, 1992) or postsynaptically via axo-dendritic synapses in the dorsal horn (Magoul *et al.*, 1987). There is substantial evidence demonstrating the role of

GABA receptors in the inhibition of transmitter release (Bourgoin *et al.*, 1992; Henry 1982; Johnston *et al.*, 1980; Malcangio & Bowery 1994; Potashner 1978). GABA also exerts postsynaptic effects through hyperpolarisation of dorsal horn neurones and holds the neurone near resting membrane potentials, consequently preventing the generation of excitatory postsynaptic potentials (Alvarez *et al.*, 1992).

There is increasing evidence for the role of the GABA inhibitory system in the modulation of nociceptive transmission at the spinal level (Buritova *et al.*, 1996; Clavier *et al.*, 1992; Dickenson *et al.*, 1985; Dirig & Yaksh 1995). It has been proposed in a previous study that a low endogenous level of GABA inhibitory control exists in the spinal cord (Dirig & Yaksh 1995). However, an alternative hypothesis has been proposed in a more recent study where it was postulated that GABAergic modulation of spinal nociceptive processing is maximally driven under normal physiological conditions (Dickenson *et al.*, 1997b). Alterations in the disposition of GABA and glycine may therefore lead to the generation or maintenance of persistent pain states. This is supported by previous studies where the administration of $GABA_A$ antagonists produced allodynia (Sivilotti & Woolf 1994; Yaksh 1989). During inflammation, there appears to be an increase in the GABAergic inhibitory control, as demonstrated by an increase in GABA immunoreactivity following carageenan-induced inflammation (Castro-Lopes *et al.*, 1994). The increase in inhibitory control after inflammation may possibly reflect a mechanism by which the enhanced neuronal excitability is counteracted. In direct contrast, there appears to be a significant decrease in spinal GABA levels and the number of GABA immunoreactive neurones in the dorsal horn following transection of the sciatic nerve (Castro-Lopes *et al.*, 1993), or chronic constriction injury (Ibuki *et al.*, 1997). The reduction in GABA content and GABA-immunoreactive cells is thought to result from the diminished sensory input to the spinal cord after nerve injury (Castro-Lopes *et al.*, 1993). Additionally, a decrease in $GABA_B$ receptor binding has also been reported in lamina II of the dorsal horn 2 to 4 weeks following nerve transection, and this was attributed to atrophic changes on primary afferent terminals (Castro-Lopes *et al.*, 1995). Interestingly, $GABA_A$ receptor binding, on the other hand, was significantly upregulated in the same group of animals. This may reflect a compensatory mechanism in response to the reduced level of GABA

acting on $GABA_A$ receptors (Castro-Lopes *et al.*, 1995). A decrease in the expression of γ2 subunit mRNA of $GABA_A$ receptors has also been reported in medium to large sized L5 DRG neurones of L5 spinal nerve ligated rats (Fukuoka *et al.*, 1998). In the CCI model of neuropathy, the antinociceptive action of the $GABA_B$ receptor agonist, baclofen was markedly increased following nerve injury (Smith *et al.*, 1994a). However, no changes were observed in $GABA_B$ receptor expression (Smith *et al.*, 1994a). The response of the GABAergic system to inflammation is therefore, markedly different from that seen after nerve injury. Unlike after inflammation where a compensatory mechanism exists to counteract increased afferent input via the activation of endogenous inhibitory systems, such protective mechanisms do not operate in the spinal cord after nerve injury. This has important implications since the loss of endogenous inhibitory controls may disturb the physiological equilibrium between excitatory and inhibitory transmitter systems and cause presynaptic disinhibition of the primary afferent terminals. This may facilitate the generation of spontaneous discharge in the spinal cord, and contribute to the induction of central hyperexcitability following nerve injury (Laird & Bennett 1993).

1.8 Aims of this project

The aim of the experiments presented in my thesis was to investigate the behavioural and electrophysiological consequences of peripheral nerve injury produced by the selective ligation of L5 and L6 spinal nerves. The introduction of animal models of neuropathy has largely contributed to a better understanding of the mechanisms underlying this clinical condition. To date, numerous studies have reported the effect of pharmacological manipulations on the behavioural manifestations of nerve injury. However, there is still little data on the electrophysiological changes which take place in the responses of dorsal horn neurones following selective (L5/L6) spinal nerve ligation.

The aim of my study was to replicate the SNL model of neuropathy described by Kim and Chung (1992) and examine the behavioural changes which follow spinal nerve ligation. Following the 1- 2 week behavioural testing period, I

(continued overleaf)

conducted electrophysiological studies on the operated animals to characterise the changes that occur in the responses of dorsal horn neurones within the spinal cord. Since the spinal cord is the primary relay site of sensory information to the central nervous system, recordings from these neurones may reveal an important link between the behavioural alterations and neuronal plasticity following peripheral nerve injury. Furthermore, I examined whether there is plasticity in the various pharmacological systems that are involved in somatosensory transmission, including the NMDA, adenosine and opioid systems. These systems are important for the processing of nociceptive information hence the selective manipulation of these targets may have therapeutic relevance for the treatment of var ious pain states, including neuropathy.

Chapter 2.

Methods

2. Methods

2.1 Animals

All experiments were conducted on male Sprague-Dawley rats (Central Biological Services, University College London, UK). All procedures were approved by the Home Office and follow the guidelines of the International Association for the Study of Pain (Zimmermann 1983). Animals were divided into unoperated and operated experimental groups. The operated group of rats were subject to either spinal nerve ligation (SNL) or sham operation.

2.2 Surgical procedures for spinal nerve ligation

The procedure for spinal nerve ligation was performed according to those previously described by Kim and Chung (1992). Male Sprague-Dawley rats weighing 140-150g at the beginning of the study were employed. Animals were housed in groups of 4-5 in plastic cages under a 12/ 12h day/ night cycle for a week prior to surgery. Animals were divided into a control group (sham operation) and an experimental group (L5/ L6 spinal nerve ligation).

Anaesthesia was induced in rats with 1.2 - 2.5% halothane in 50% N_2O and 50% O_2. The rat was placed in a prone position and the left paraspinal muscles were separated from the spinous processes at the L4-S2 levels. A part of the L6 transverse process was carefully removed with rongeurs and the L4-L6 spinal nerves were exposed and identified. In rats that received spinal nerve ligation, the left L5 and L6 spinal nerves were isolated and tightly ligated with a 6-0 silk thread (Davis & Geck). The ligatures were placed at a site distal to the DRG and proximal to the trifurcation of the sciatic nerve. Rats that received a sham operation were operated in a similar way, however, the spinal nerves were left intact. The L4-L6 spinal nerves were identified and the presence of L5 and L6 spinal nerves confirmed. Care was taken not to damage the nerve in any way and minimal surgical intervention was given to the spinal nerves. A complete hemostasis was confirmed and the

wound was sutured. The total duration of anaesthesia in each animal did not exceed 20 minutes. Following surgery, rats were placed in temperature regulated recovery boxes where they were allowed to recover from anaesthesia. Careful examinations were made on the posture of the ipsilateral hindpaw, signs of limping as well as the general behaviour of the rat.

2.3 Behavioural studies

After surgery, rats were maintained under the same conditions as during the preoperative period. The weight gain and general behaviour of the operated rats were carefully monitored throughout the postoperative (PO) period. Careful observations were made on the position and posture of the foot. Behavioural tests were carried out in the morning, postoperatively at 2, 3, 5, 7, 9, 12 and 14 days. Rats were placed in transparent plastic cubicles on a mesh floor and a period of acclimatisation was allowed prior to testing. The sensitivity of the rats' hindpaw to mechanical and cooling stimuli were assessed through two forms of behavioural testing.

2.3.1 Foot withdrawal response to repeated mechanical stimuli

Mechanical sensitivity was assessed through the measurement of foot withdrawal frequencies to a sequential series of calibrated von Frey filaments of weights 1, 5 and 9g (9.9, 49.5, 89.1mN, respectively) applied to the plantar surface of the foot. A single trial of stimuli consisted of repeated applications of a von Frey filament 10 times, each application not lasting for more than 3 seconds. Von Frey filaments were applied in an ascending order and each test was separated by a period of 3 minutes. The occurrence of foot withdrawal for each trial was quantified and expressed as the difference score: *difference score = (number of foot withdrawals on contralateral paw) - (number of foot withdrawals on ipsilateral paw).*

2.3.2 *Foot withdrawal response to cooling stimuli*

The sensitivity of the hindpaw to cooling stimuli was assessed through the application of a drop of acetone onto the plantar region of the foot using a syringe connected to a small polyethylene tubing. Care was taken not to apply the acetone in an abrupt squirt thus evoking a mechanical response rather than a response to the cooling effect of acetone itself. Each trial consisted of 5 applications of acetone per hindpaw, and a period of 5 minutes was allowed between each application. The number of foot withdrawals was expressed as the difference score as described above.

The behavioural testing was carried out over a 2 week postoperative period, after which, the operated animals were subsequently used for electrophysiological studies.

2.4 Electrophysiological studies

Electrophysiological studies were conducted following the experimental procedures previously described (Dickenson & Sullivan 1986). Animals which had been operated for spinal nerve ligation or sham operation (PO 7-10 and 14-17 days), or unoperated naive rats (Central Biological services, University College London, UK, weight 200 - 250g) were employed for the electrophysiological study.

Anaesthesia was induced in rats with 2.0-2.5% halothane in a gaseous mixture of 66% N_2O and 33% O_2. The rat was placed in a nose cone and a complete areflexia was confirmed by gently pinching the toes in each hindpaw. The trachea was exposed through blunt dissection using a pair of tooth forceps and tracheal cannulation was subsequently performed. A small incision was made to the trachea and a thin polyethelene tubing (non-sterile, inside diameter 1.57mm, length 5cm) was inserted into the incision and secured tightly with a silk thread (Pearsalls sutures, braided untreated silk 3/0). The rat was subsequently placed in a stereotaxic frame secured by ear bars to ensure stability during electrophysiological recordings. Using rongeurs, a laminectomy was performed at the L1-3 level and segments L4-5

of the spinal cord were exposed, which corresponded to the area which received afferent input from the toe region of the rat. The cord was held rigid by clamps caudal and rostral to the exposed section and two rods were inserted into the lateral processes of the vertebrae to improve stability at the recording site. The level of anaesthesia was subsequently reduced to 1.2-1.8% halothane and was maintained at this level throughout the experiment, generally lasting around 8 hours. The core body temperature of the rat was monitored and maintained (36.5-37^{o}C) by means of a heating blanket connected to a rectal thermal probe via an automatic feedback control unit.

2.4.1 Extracellular recordings of convergent dorsal horn neurones

Extracellular recordings of convergent dorsal horn neurones were made with parylene coated tungsten electrodes (AM systems) descended through the cord by a SCAT microdrive (Digitimer) in 10μm steps. In SNL or sham operated animals, recordings were made from neurones ipsilateral to the nerve ligation or sham procedure and from both the ipsilateral and contralateral sides in naive animals. The depth of the neurone from the surface of the dorsal horn of the spinal cord was recorded for each neurone. The toe region of the hindpaw was tapped gently as the electrode was lowered, until a single convergent dorsal horn neurone was isolated. All neurones studied had discrete receptive fields confined to the toes and plantar region of the ipsilateral hindpaw. Once a neurone was isolated clearly from other background activity, 2 needles were inserted to the receptive field and a train of 16 electrical stimuli was applied transcutaneously. Electrical stimulation was delivered by a stimulus isolator module, which was driven by a Neurolog system consisting of a period generator, digital width and pulse buffer. On commencement of an electrical test, the period generator gives rise to a stimulus every 2 seconds. The width (2ms) and amplitude of the stimulus are controlled by the digital width and pulse buffer, respectively. Criteria for a spinal convergent neurone to be used for electrophysiological studies was a A-fibre evoked activity, followed by C-fibre evoked activity in response to electrical stimulation. A train of sixteen stimuli was applied at three times the threshold current for C-fibres (0.5 Hz),

and a post-stimulus histogram was constructed. The evoked response elicited by the different fibres was separated and quantified through latency measurements (0 - 20 ms Aβ-fibre; 20-90ms Aδ-fibre; 90 - 300 ms C-fibre), based on the known conduction velocities for the various fibre types. Any neuronal response occurring after the C-fibre latency band resulting from hyperexcitability of the neurone (300-800 ms) was taken to be the 'postdischarge' of the neurone. Wind-up was calculated as the difference between the number of action potentials evoked at three times the C-fibre threshold after 16 stimuli and the baseline. The baseline response (the non-potentiated response that would have occurred in the absence of wind-up) was calculated as the number of action potentials in the response produced by the first stimulation, multiplied by the total number of stimuli in the stimulus train (16).

2.4.2 Data capture and recording apparatus

The signal generated from the isolated cell was sent to the headstage where it was differentiated, amplified and filtered through a Neurolog system. The headstage received signal from the electrode (A signal), which included both the activity from the recording neurone, as well as any background activity in the spinal cord. It also received a second signal from the B lead which was attached to the animal itself, and carried activity from the animal's cardiovascular and respiratory systems, as well as electrical interference. The output of the headstage was amplified by the preamplifier and subsequently differentiated (A signal - B signal) to minimise background activity and interference. The differentiated signal was amplified further by an AC / DC Amp and filtered. The final output was sent to an auditory Amp, an oscilloscope and a window discriminator so as to obtain an auditory and visual representation of the signal. The window discriminator allowed cells to be differentiated according to their amplitude so as to exclude any background activity from the recording. The window height was adjusted so that only the neuronal activity of the isolated neurone is recorded. The output from the window discriminator was sent to a CED 1401 interface (CED, Cambridge) coupled to a Pentium computer, and data was captured using Spike 2 software (Rate and Post-stimulus histogram functions).

2.4.3 Characterisation of the responses of spinal neurones to electrical and natural stimuli

Following the isolation of a single convergent neurone, the responses of the neurone to a wide range of natural and electrical stimuli were characterised. The threshold current for A- and C-fibre firing was established and a train of 16 electrical stimuli was given at 3 times the threshold current for both A- and C-fibres. A post-stimulus histogram was constructed and the responses evoked by the different fibres were quantified (Aβ-, Aδ-, C-fibres), together with the postdischarge and wind-up of the neurone.

Following the characterisation of the electrical evoked responses, the responses of spinal neurones to natural stimuli were determined. The spontaneous activity was recorded over a period of at least 5 minutes or until response stabilisation. All natural stimuli were applied to the centre of the receptive field for a period of 10 seconds and sufficient interval was given between individual tests. A wide range of natural stimuli was employed, encompassing both the innocuous and noxious range, including brush, prod (5mm diameter, $4Ncm^{-2}$), acetone, von Frey hairs (1, 5, 9, 15, 20, 25, 50g) and heat (35, 40, 42, 45, 47, $50^{o}C$). Thermal stimulation involved the application of a constant jet of water stream onto the centre of the receptive field. The response to cooling was determined through the application of a drop of acetone using a syringe (1ml) connected to a plastic piece of tubing.

All evoked neuronal responses to natural stimuli were normalised by subtracting the spontaneous activity of the individual cell recorded at the beginning of the test.

2.5 Drugs

Morphine sulphate was obtained from Evans Medical. N^6-cyclopentyladenosine and MK-801 were obtained from Research Biochemical International (RBI), and A200702.21 from Abbott Laboratories. Memantine [1-amino-3,5-dimethyladamantane hydrochloride], ketamine hydrochloride, naloxone hydrochloride and theophylline were obtained from Sigma. All drugs were kept refrigerated at $4^{o}C$ and stock solutions were made in distilled water. Dilutions of the stock solutions were subsequently made with 0.9% saline.

2.6 Statistical Analysis

Data are presented as mean± standard error of mean (S.E.M) unless stated otherwise. All natural stimuli evoked responses are expressed in Hz. Statistical analysis of neuronal characteristics was performed using Mann-Whitney test. χ^2 test was employed to make comparisons on the population of cells responding to a given natural stimuli. Statistical analysis of drug effects was performed using Wilcoxon matched paired test. Mann-Whitney test was employed for the comparison of drug effects between different experimental groups. The level of significance was taken to be $p \leq 0.05$.

Chapter 3.

The behavioural consequences of peripheral nerve injury in the selective spinal nerve ligation model of neuropathy

3. The behavioural consequences of peripheral nerve injury in the selective spinal nerve ligation model of neuropathy

3.1 Introduction

Despite the continuing search for an effective treatment for neuropathic pain, the mechanisms underlying this clinical condition still remain unclear. In recent years, animal models of neuropathic pain have been developed, which involve injury to the peripheral or central nervous system, and these have become widely used as a tool to investigate the mechanisms underlying this pathological condition. Furthermore, these models have allowed us to explore the potential therapeutic value of different agents for the treatment of neuropathic pain, and this has led to a better understanding of this clinical condition. Following the introduction of earlier animal models involving the complete denervation of the hindpaw through spinal transection or dorsal rhizotomy (Zeltser & Seltzer 1994), three models were further described, based on the partial denervation of the hindpaw through sciatic nerve injury (Bennett & Xie 1988; Kim & Chung 1992; Seltzer *et al.*, 1990) (see Chapter 1.2.1).

The model which I employed for my study is the selective spinal nerve ligation model of neuropathy, which involves the tight ligation of two of the three spinal nerves (L5/ L6) which make up the sciatic nerve (L4-L6). Unlike the former two models (Bennett & Xie 1988; Seltzer *et al.*, 1990), this model offers less variability between studies and has been shown to produce reproducible behaviours in rats (Kim *et al.*, 1997a; Kim & Chung 1992). The SNL model of neuropathy offers the advantage that the same proportion of the sciatic nerve (2/3) is injured in every animal, and the degree of subjectivity which may exist in other models can therefore be avoided. Since there is a complete separation in the levels of the injured (L5 and L6) and intact (L4) spinal segments in this model, this allows different segments (injured / intact) to be studied independently of each other.

3.2 Methods

Behavioural testings were conducted on a total of 209 male Sprague-Dawley rats (SNL, n=98; sham operated, n=111) to assess the mechanical and cooling sensitivities of the ipsilateral and contralateral hindpaws of operated rats. The surgical procedures for spinal nerve ligation or sham operation were conducted following methods previously described by Kim and Chung (1992). The mechanical and cooling sensitivity of the rats' hindpaws was assessed over a 2 week postoperative period on PO 2, 3, 5, 7, 9, 12 and 14 days. Furthermore, the general behaviour of rats (e.g. signs of autotomy, abnormal growth of toe nails) and their weight gain at PO 7 and 14 days, were also monitored during this period.

3.3 Results

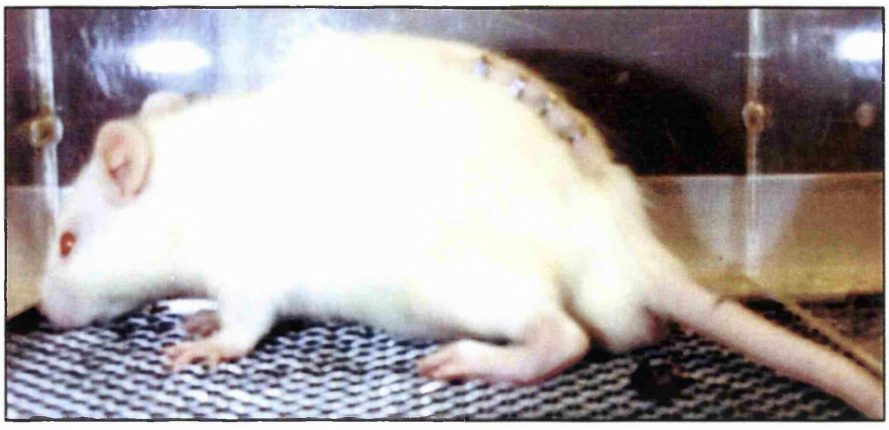
3.3.1 General behaviour

Following surgery, rats maintained good health and exhibited normal weight gain (Table 5). Rats displayed no signs of distress, abnormal aggressive behaviour or autotomy. SNL rats exhibited a 'guarding behaviour' of the ipsilateral hindpaw whereby the toes of the ipsilateral paw were held clasped together (Figs. 4 and 5). While resting, SNL rats frequently displayed licking or pulling of the ipsilateral hindpaw in the absence of any external stimuli. SNL rats tended to shift their body weight onto the contralateral hindpaw, rather than on the ipsilateral paw. Such behaviour was not observed in sham operated rats. Toe nail growth was normal in both groups of animals.

Table 5. The mean weight of operated rats following surgery for spinal nerve ligation or sham operation at PO 0, 7 and 14 days. Rats exhibited normal weight gain which was comparable between the animal groups.

Postoperative Day	Weight (g)	
	Sham operated	SNL
Day 0	148 ± 1	148 ± 1
Day 7	199 ± 2	192 ± 2
Day 14	260 ± 3	253 ± 2

A.



B.

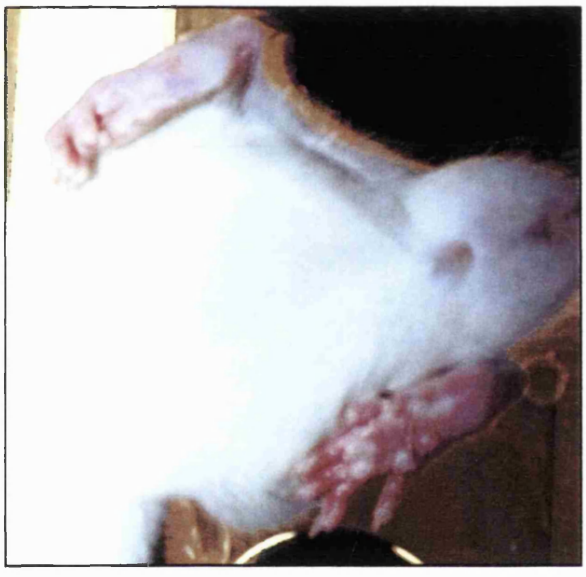
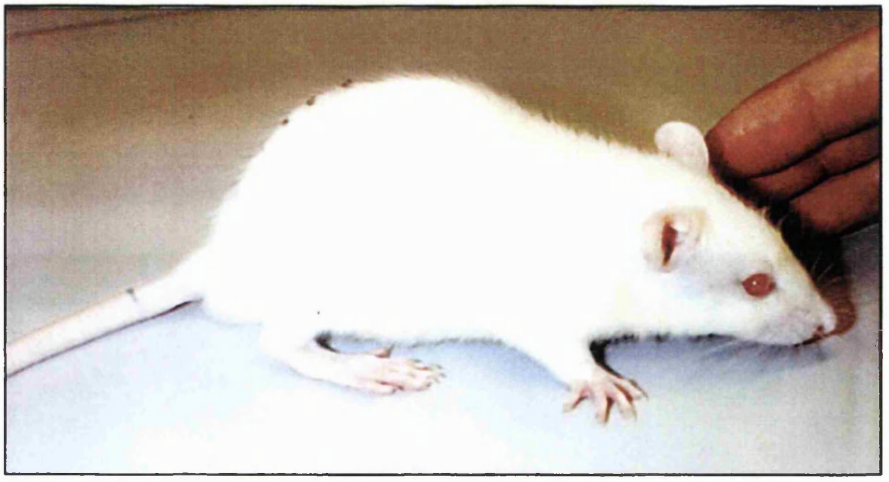


Figure 4. (A) Transparent plastic cubicle used for assessing the mechanical and cold sensitivity of rats' hindpaw. Behavioural testing was carried out postoperatively on 2, 3, 5, 7, 9, 12 and 14 days. A period of acclimitisation was allowed prior to testing. (B) A view of the ipsilateral and contralateral hindpaws from below. Following surgery, there was a change in the foot posture of the ipsilateral hindpaw in SNL rats. Rats exhibited 'guarding behaviour' whereby the toes of the ipsilateral hindpaw were held clasped together. The contralateral hindpaw did not show abnormality and were fully extended under resting conditions.

A.



B.

C.

Figure 5. Changes in foot posture following spinal nerve ligation. A comparison of the foot posture of the (A) contralateral and (B) ipsilateral hindpaws of a SNL rat at PO 14 days. (C) A closer view of the ipsilateral hindpaw. At rest, rats tended to shift their body weight onto the contralateral hindpaw. These behavioural alterations were observed as early as 1 day after surgery, and were maintained throughout the 2 week postoperative period.

3.3.2 Mechanical and cold allodynia

SNL rats displayed behavioural signs of mechanical and cold allodynia of the ipsilateral hind paw (Fig. 6). SNL rats exhibited abrupt and brisk foot withdrawals in response to a normally innocuous mechanical stimulus (von Freys 1, 5 and 9g) and to the application of acetone. Von Frey 9 grams, when applied to human skin, produces only a faint sense of pressure and is considered to be innocuous. Foot withdrawal in the SNL rats was often accompanied by aversive behaviours such as shaking and licking of the ipsilateral paw. No vocalisation was observed on application of the stimulus and the application of von Frey filaments (1, 5 or 9grams) rarely evoked a response on the contralateral hindpaw of SNL rats. These exaggerated behavioural responses were interpreted to be manifestations of mechanical and cold allodynia and were observed as early as day 2 after surgery. There was a gradual increase in the level of allodynia over the postoperative period and this was maintained throughout the whole of the testing period (2 weeks). The contralateral paw of SNL rats did not develop modified mechanical or cold sensitivity. Similarly, sham operated rats rarely responded to the application of innocuous mechanical stimuli (von Frey 1, 5 or 9 grams) or acetone on either hindpaw. On a rare occasion where a foot withdrawal was observed, the response was not accompanied by aversive behaviours (licking and shaking of the hindpaw) such as those exhibited by SNL rats.

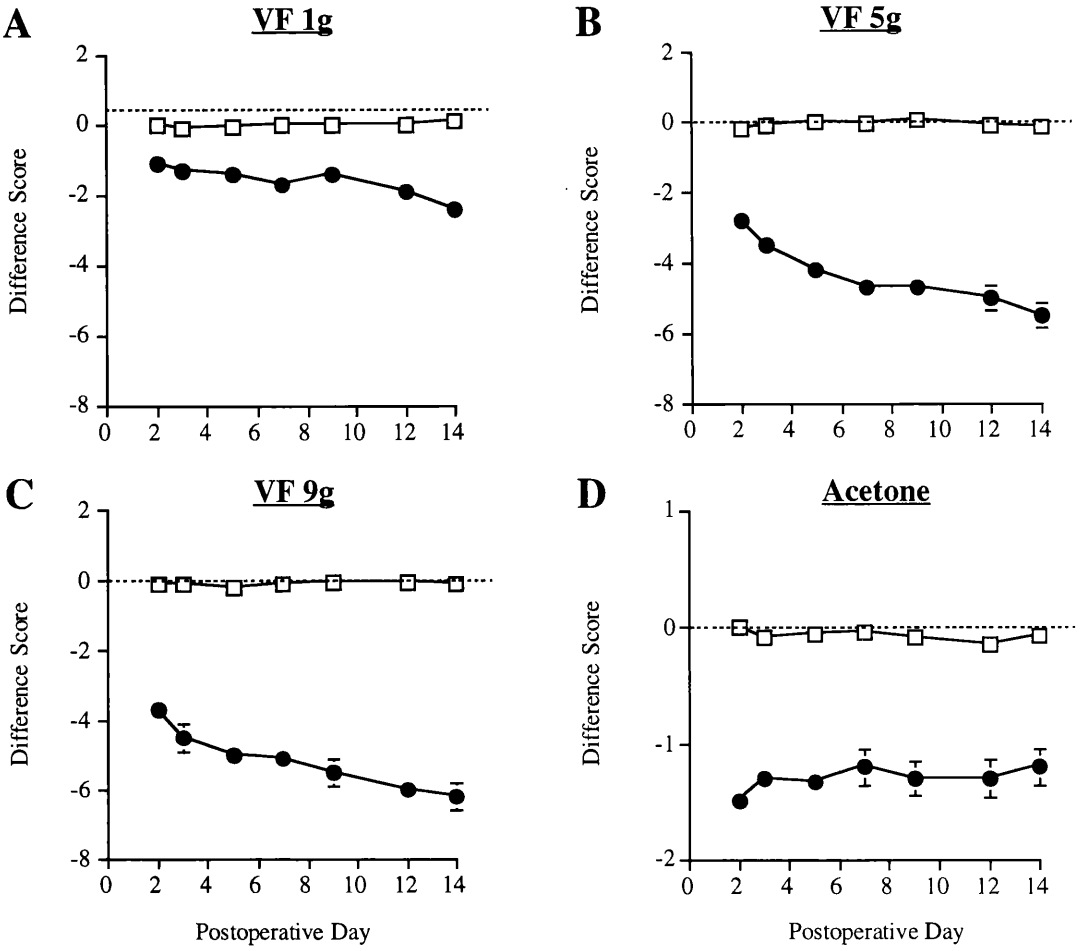


Figure 6. The difference score of foot withdrawal frequencies to the application of (A) von Frey 1g, (B) von Frey 5g, (C) von Frey 9g and (D) a drop of acetone in SNL (filled circles) and sham operated rats (open squares). Difference score was calculated as: difference score = (no. of foot withdrawals on contralateral paw) - (no. of foot withdrawals on ipsilateral paw). Negative values indicate a greater frequency of foot withdrawal responses on the ipsilateral hindpaw, and was interpreted to be a manifestation of mechanical allodynia. SNL rats showed a significantly greater foot withdrawal frequency as compared to sham operated rats at all postoperative time-points ($p<0.0001$). The onset of allodynia was early, and was observed as early as 2 days after nerve injury. Data is presented as the mean foot withdrawal frequency ± S. E. M. The smallest error bars are contained within the symbol.

3.4 Discussion

Here I have replicated the selective spinal nerve (L5/ L6) ligation model of neuropathy (Kim & Chung 1992) and investigated the behavioural consequences of peripheral nerve injury over a 2 week postoperative period. Following spinal nerve ligation, rats displayed an increased sensitivity to cooling and mechanical stimuli on the ipsilateral hindpaw. The application of a drop of acetone or a normally innocuous mechanical stimulus evoked an exaggerated behavioural response in these animals which was often accompanied by aversive behaviours, such as licking and shaking of the hindpaw. These abnormal pain behaviours were interpreted to be manifestations of cold and mechanical allodynia and displayed an early onset of 2 days after surgery. The level of allodynia gradually increased over the postoperative period and was maintained for at least 2 weeks. The contralateral hindpaw of SNL rats did not show such changes in either the mechanical or cooling sensitivity and a response was rarely evoked. Similarly, sham operated rats did not develop any abnormal pain behaviours or modifications in mechanical/ cooling sensitivity. These findings are consistent with those originally reported by Kim and Chung (1992) where mechanical allodynia, cold allodynia and thermal hyperalgesia were demonstrated on the ipsilateral hindpaw of SNL rats. Mechanical allodynia was reported as early as 1 day after surgery, and was maintained for up to 112 days (Kim & Chung 1992). The onset of allodynia was reported to be between 12- 20 hours after ligation. Interestingly, foot withdrawal responses were also reported on the contralateral hindpaw in SNL rats when tested with a von Frey filament of 186.7mN (bending force 18.8g). Contralateral responses became prominent at 2 weeks after surgery and were maintained for up to 98 days. Although smaller in magnitude, these contralateral signs followed a similar time course to those on the ipsilateral paw (Kim & Chung 1992). In my study, I did not any observe contralateral effects in SNL rats. However, these changes were reported after 2 weeks postsurgery in the original studies, therefore it is unlikely that they would be manifested during the length of my behavioural testing period (PO 14 days). In addition, the mechanical stimulus (186.7mN, von Frey 18.8g) with which these contralateral effects were observed, is of a higher intensity than that employed in my study (89.1mN, von Frey 9g). Thus these effects may not be seen with stimuli of low intensities.

Furthermore in the study by Kim and Chung (1992), a sham surgery was performed contralaterally in SNL rats. It is unlikely, however, that the sham surgery produced the contralateral changes since in a separate group of animals where a sham operation was performed, no changes in mechanical sensitivity were observed on the operated paw (Kim & Chung 1992).

In addition to the changes observed in the mechanical and cooling sensitivities, SNL rats frequently licked their toes and pulled the nails of the ipsilateral hindpaw. These behaviours were interpreted to be signs of spontaneous pain and were observed in the absence of any external stimuli.

Although not studied in the present study, it has been reported that ligation of L5/ L6 spinal nerves also induces thermal hyperalgesia, which is manifested as a decrease in the withdrawal latency to suprathreshold heat stimuli (Kim & Chung 1992). Increased sensitivity to heat developed soon after nerve injury (PO 3 days) and was maintained for at least PO 5 weeks. In recent studies, however, a lack of heat hyperalgesia following spinal nerve ligation has been reported, although mechanical allodynia was observed in these studies (Kontinen *et al.,* 1998; Roytta *et al.,* 1999). Dissociation of thermal and mechanical allodynia/ hyperalgesia has also been described in other models of neuropathy (Luukko *et al.,* 1994), suggesting differences in the mechanisms underlying the two measures.

When employing animal models for the study of mechanisms underlying neuropathic pain, it is important to consider the degree of distress inflicted to the animal as a result of the surgery, since changes in mood or activity may affect the assessment of sensory behaviours. The behavioural consequences of spinal nerve ligation were investigated in a recent study which measured depression and anxiety in SNL rats (Kontinen *et al.,* 1999). According to this study, it appears that there are no differences in the measures of anxiety or depression between the animal groups (SNL and sham operated rats) after surgery. When compared to the preoperative period, however, rats generally showed a decrease in motor activity during the postoperative period. Several factors could account for this observation, including habituation to the testing method, increased anxiety or age (Kontinen *et al.,* 1999).

Hence, the selective spinal nerve (L5/ L6) ligation model of neuropathy produces a rapid onset of abnormal pain-related behaviours, which include mechanical and cold allodynia, and possibly thermal hyperalgesia. These behaviours resemble symptoms of causalgia in human patients and so the model appears to be a useful approach for studying mechanisms underlying neuropathic pain. The injury does not appear to produce generalised distress in the animals, which could confound sensory testing in behavioural studies, and does not produce measurable changes in mood, anxiety or depression as demonstrated in a wide range of tests.

3.4.1 Behavioural testing method for mechanical allodynia

The hindpaw withdrawal reflex used to assess mechanical sensitivity, as demonstrated in this study, is a useful method in determining the changes in pain sensitivity to mechanical or thermal stimuli in animal models. The assessment of mechanical sensitivity through the stimulation of cutaneous mechanoreceptors using calibrated von Frey filaments has been commonly used to test for mechanical allodynia in animal models of neuropathy (Kim & Chung 1992; Na *et al.*, 1993; Sheen & Chung 1993). Following the ligation of L5/ L6 spinal nerves, the position and weight bearing of the affected limb is considerably altered. A recent study demonstrated that these changes do not affect hindlimb withdrawal thresholds and therefore allow a reliable measurement of mechanical allodynia in nerve injured rats (Kauppila *et al.*, 1998). The decreased mechanical threshold observed on the ipsilateral hindpaw of SNL rats is therefore independent of the weight bearing.

3.4.2 *Histological changes following spinal nerve ligation*

The morphological changes which occur in the peripheral nerves following tight ligation of L5/ L6 spinal nerves has been described in a recent study by Roytta and coworkers (1999). Spinal nerve ligation produced a dramatic decrease in the number of myelinated fibres and Wallerian degeneration was observed distal to the site of nerve ligation with macrophages and regeneration of axonal sprouts (Roytta *et al.*, 1999). There is evidence to suggest that Wallerian degeneration is associated with symptoms of neuropathic pain (Ramer *et al.*, 1997). Morphological changes have also been described in the CCI model of neuropathy, where a dramatic decrease in myelinated fibres has been reported distal to the nerve ligation (Basbaum *et al.*, 1991; Gautron *et al.*, 1990; Munger *et al.*, 1992). The concomitant presence of allodynia and loss of myelinated fibres appears rather paradoxical since allodynia is presumed to be mediated by myelinated large diameter fibres. This discrepancy may reflect a strong amplification of the afferent input within the spinal cord, which arrive via the remaining large diameter myelinated fibres. Alternatively, there may be a loss of central inhibitory controls which may facilitate nociceptive transmission (Pertovaara *et al.*, 1997).

3.4.3 *Comparison with other animal models of neuropathic pain*

To date, two other animal models of neuropathy involving partial sciatic nerve injury have been described (Bennett & Xie 1988; Seltzer *et al.*, 1990). The CCI model proposed by Bennett and Xie (1988) involves the application of 4 loose ligatures around the sciatic nerve (Bennett & Xie 1988), whilst the PSTL model, on the other hand, tightly ligates 1/3 to 1/2 of the sciatic nerve (Seltzer *et al.*, 1990). Following surgery, rats show signs of spontaneous pain, allodynia and hyperalgesia, lasting up to several months. A proportion of CCI rats also developed autotomy, where animals self-mutilate the denervated zones of the affected hindpaw in an attempt to get rid of the unpleasant sensation. This behaviour is not seen in the PSTL (Seltzer *et al.*, 1990) or SNL models of neuropathy (Kim & Chung 1992).

As previously discussed (see Chapter 1.2.1), both these models may produce variability in the extent of nerve damage between studies, which could potentially influence the behavioural consequences of the injury. In the CCI model, for example, there may be subjectivity in the tension of the ligatures applied to the sciatic nerve. Similarly, the proportion of the ligated nerve can vary from animal to animal (1/3 to 1/2) in the PSTL model of neuropathy. This individual variability may reflect the difficulty in producing the same extent of nerve damage in every animal, hence care must be taken when making comparisons between studies.

In contrast, the model developed by Kim and Chung (1992) overcomes some of these difficulties since the same proportion of the sciatic nerve is ligated in each animal therefore avoids the potential experimental variability between studies. One possible disadvantage of this model is that the surgery is more extensive compared to the other two models. However, the model is highly reproducible and is therefore a useful tool for the study of neuropathic pain. Indeed, this model has proved useful for testing the antiallodynic or antihyperalgesic effects of therapeutic agents for the treatment of neuropathic pain states (Bian *et al.*, 1995; Kim *et al.*, 1993; Ossipov *et al.*, 1997b).

Chapter 4.

Electrophysiological characterisation of dorsal horn convergent neurones: The response properties of spinal neurones to electrical and natural stimuli and changes produced following peripheral nerve injury

4. Electrophysiological characterisation of dorsal horn convergent neurones: The response properties of spinal neurones to electrical and natural stimuli and changes produced following peripheral nerve injury

4.1 Introduction

There is increasing evidence to suggest that peripheral nerve injury induces a number of alterations in the peripheral nervous system which could contribute to the occurrence of multiple symptoms associated with neuropathic pain states. Studies employing various animal models have reported aberrations in somatosensory processing which appear to result from a combination of factors including, reorganisation of myelinated large diameter fibres, generation of ectopic discharges, alterations in neuronal phenotypes as well as various neurochemical changes (see Chapter 1.3). These results suggest for a high degree of plasticity in the peripheral nervous system following nerve injury.

To date, there have been a number of studies reporting changes in the neuronal response characteristic following nerve injury. In the CCI model of neuropathy, loose ligation of the sciatic nerve induced abnormal mechanical sensitivity and increased spontaneous activity in dorsal horn neurones (Laird & Bennett 1993). A proportion of neurones was reported to have no definite peripheral receptive fields (Laird & Bennett 1993). Furthermore, the mechanical stimulus required to elicit a response in these cells (i.e. mechanical neuronal threshold) was considerably higher in CCI rats as compared to sham operated rats. A similar study employing the CCI model of neuropathy demonstrated that profound changes occur in the response characteristics of spinothalamic tract (STT) neurones 1-2 weeks following nerve injury (Palecek *et al.*, 1992b). STT neurones from neuropathic rats exhibited high background activities and increased afterdischarges to mechanical and thermal stimuli. Recordings from dorsal column nuclei neurones (gracile nucleus) also showed that the properties of neurones in naive and CCI rats were considerably altered at PO 10-14 days (Miki *et al.*, 1998). Gracile nucleus (GN) neurones from CCI rats exhibited high levels of spontaneous

activity, increased afterdischarges following mechanical and thermal stimulation, and a proportion of neurones did not have any detectable mechanical receptive fields. Similarly, loose ligation of the inferior alveolar nerve produced an increased number of spontaneously active neurones, which exhibit mechanical sensitivity (Bongenhielm & Robinson 1998).

In the partial sciatic nerve ligation model of neuropathy (Seltzer *et al.*, 1990), electrophysiological recordings of lumbar dorsal horn wide dynamic range (WDR) neurones have been reported at 5 or 16 weeks after nerve ligation (Takaishi *et al.*, 1996). The responses of WDR neurones to noxious thermal stimuli were comparable between nerve injured and sham operated rats, and little or no spontaneous activity was recorded from either group.

There have been several studies reporting changes in the response characteristic of dorsal horn neurones in the selective spinal nerve (L5/ L6) ligation model of neuropathy (Kim & Chung 1992). Electrophysiological recordings of WDR neurones demonstrated that both the spontaneous activity and neuronal responses to mechanical stimuli were enhanced in SNL rats, as compared to naive rats (Leem *et al.*, 1995; Pertovaara *et al.*, 1997). Similarly, in L7 ligated monkeys, ipsilateral STT neurones displayed a high level of background activity and an increased responsiveness to innocuous mechanical stimuli (Palecek *et al.*, 1992b).

Results from these electrophysiological studies suggest that there is a high degree of plasticity in the somatosensory processing system following nerve injury, which produces alterations in the response characteristics of sensory neurones. Overall, there appears to be a complex change in the pattern of neuronal responses, which includes both increases and decreases in the neuronal measures. Whilst there appears to be a general increase in the level of spontaneous activity after nerve injury, changes in the response to mechanical or thermal stimuli appear to be variable between studies, and may reflect differences in the recording method, model of neuropathy or time elapsed since nerve injury.

To date, there have only been a few studies where the responses of dorsal horn neurones to a broad range of peripheral stimuli (electrical/ natural) have been fully characterised in the SNL model of neuropathy. Dorsal horn sensory neurones can be classified in several ways, based on their responses to natural peripheral stimuli, the laminar location of the cell body or their projection site. According to the intensity-dependent categorization, dorsal horn enurones can be classified into low-threshold, nociceptive-specific, and WDR neurones. Low threshold cells respond to light touch, pressure and hair movement. This class of cells do not increase firing in response to noxious stimulation and the majority of their input is mediated by large diameter Aβ-fibres. On the contrary, nociceptive specific cells are high threshold cells which respond exclusively to noxious stimuli. These cells are found commonly in STT, SMT and spinohypothalamic tracts. Finally, the WDR neurones receive both low and high threshold inputs via Aβ-, Aδ- and C-fibres and differentially encode for the intensity of the stimulus. The highest concentration of WDR neurones is found in lamina V, and projection cells of this type are common in the STT, SMT, SRT, SCT, and spinohypothalamic tracts.

In this chapter, I investigate the electrophysiological changes which take place in the responses of WDR neurones to a wide range of controlled natural and electrical stimuli at 2 postoperative time-points following SNL (1 and 2 weeks post-surgery). I chose to employ WDR neurones for my study since these cells comprise more than 50% of the neurones in the STT tract (Besson & Chaouch 1987), which is considered to be one of the most important ascending tract relaying sensory information to higher brain centres. Furthermore, a previous study in primates has demonstrated that the activity of WDR neurones is a better predictor of thermal perception than that of nociceptive-specific neurones (Dubner *et al.*, 1989). In addition, the convergence of low and high threshold inputs onto these neurones make them a logical substrate for some of the changes that may underlie hyperalgesia and allodynia.

Although a large number of studies have investigated the behavioural consequences of peripheral nerve injury (allodynia, spontaneous pain, hyperalgesia) in animal models of neuropathy, the mechanism underlying these changes still remains unclear. Since the spinal cord is the primary relay site of sensory information to the central nervous system, recordings from dorsal horn neurones

(continued overleaf)

may reveal an important link between behavioural alterations and corresponding neuronal plasticity within the spinal cord.

4.2 Methods

Surgical procedures for the selective spinal nerve (L5/ L6) ligation were performed according to those described previously by Kim and Chung (1992). Following surgery, behavioural testing was conducted to assess the sensitivity of the rats' hindpaw to mechanical and cooling stimuli over a period of 1-2 weeks. Electrophysiological studies were subsequently carried out on the operated rats at two postoperative time-points (PO 7-10 days and PO 14-17 days).

4.2.1 Characterisation of the responses of dorsal horn neurones to electrical stimuli

Electrophysiological recordings of spinal neurones were made from operated rats, ipsilateral to the spinal nerve ligation or sham operation, and comparisons were made with those from naive rats. Once a single dorsal horn neurone was isolated, sufficient time was allowed for cell stabilisation, following which the electrically evoked response of the individual cell was characterised. A total of 558 neurones were characterised in the study, of which 267 were from SNL rats (PO 7-10 days n=65; PO 14-17 days n=202), 216 from sham operated rats (PO 7-10 days, n=43; PO 14-17 days, n=173) and 75 from naive rats. The threshold currents for Aβ- and C-fibre activation were carefully determined and the latency for Aβ- or C-fibre activation was recorded. An electrical test was carried out at 3 times the Aβ-fibre threshold and subsequently repeated for C-fibres. A post-stimulus histogram (PSTH) was constructed following a train of 16 electrical stimuli and the resulting neuronal responses were separated and quantified on the basis of latency measurements (Aβ-, Aδ- and C-fibre). The postdischarge of the neurone was similarly recorded and the wind-up of the neurone following suprathreshold Aβ- or C-fibre stimulation was calculated for each individual neurone.

4.2.2 Characterisation of the responses of dorsal horn neurones to natural stimuli

After electrical tests were conducted, the neurone was allowed to settle for several minutes, after which, the spontaneous activity was recorded over a period of at least 5 minutes or until the response reached stability. A total of 112 spinal neurones (PO 7-10 days, n=43; PO 14-17 days, n=69) were characterised in SNL rats for responses to natural stimuli; similarly, 89 were studied in sham operated rats (PO 7-10 days, n=31; PO 14-17 days, n=58) and 30 in naive rats.

The responses of the neurones to a wide range of natural stimuli were characterised including brush, prod, acetone, mechanical (von Frey weights 1, 5, 9, 15, 20, 25, 50g) and thermal stimuli (35, 40, 42, 45, 50^{o}C). All stimuli were applied for 10 seconds onto the most responsive part of the receptive field and sufficient interval was allowed between each stimulus application. The frequency of the evoked neuronal response was quantified and subsequently normalised by the subtraction of any spontaneous activity. In addition, the threshold of spinal neurones to mechanical and thermal stimuli was also estimated - this was taken to be the weakest stimulus, which was required to evoke a response in the cell.

4.2.3 Characterisation of the receptive field size of spinal neurones

The receptive field size of spinal neurones was quantified in a group of animals (SNL, n=37; sham operated, n=41; naive, n=24). A total of 150 neurones were characterised in this study, of which 56 were from SNL rats (PO 14-17 days), 63 from sham operated (PO 14-17 days) and 31 from naive rats.

The area of the neuronal receptive field was mapped by probing the skin of the hindpaw with three von Frey hairs of different weights (9, 15 and 75 grams). If a neuronal response greater than 1Hz was evoked through the application of the von Frey hair, the area was considered to be within the receptive field. The receptive field area for each von Frey hair was mapped on a standard diagram of the projected area of the plantar surface of the paw. The diagrams were subsequently copied to plain copier paper (80g/m^2, Kymi Oy, Kuusankoski, Finland) and marked areas were carefully cut and weighed. The receptive field areas were measured as the weight of the particular area and finally, expressed as a percentage of the mean weight of 20 control diagrams of the whole paw (79.8±0.2 mg).

The distribution of the receptive field in the plantar region of the paw was classified as medial (containing toe 1-2, or the medial side of the sole), central (toes 3-4, or only central area of the sole) or lateral (toe 5, or lateral side of the sole), depending on the localisation of the response to the lightest stimulation that evoked a response.

The data presented in this part of the study was obtained in conjunction with Dr. V. Kontinen and Miss E. Matthews.

4.3 Results

4.3.1 Characterisation of the responses of dorsal horn neurones to electrical and natural stimuli

Cell depth

The mean depth of neurones used in this study was 791±24μm (PO 7-10 days) and 754±14μm (PO 14-17 days) for SNL rats and 837±28μm (PO 7-10 days) and 786±14μm (PO 14-17 days) for sham operated rats. Similarly, the mean cell depth for naive rats was 797±17μm. All convergent neurones were located deep in the dorsal horn and corresponded to laminae V of the spinal cord.

4.3.1.1 The responses of dorsal horn neurones to electrical stimuli

All neurones employed for this study had a clear short latency Aβ-fibre evoked response followed by a longer latency C-fibre evoked response. A typical example of a post-stimulus histogram (PSTH) following a single electrical test is given in Fig 7.

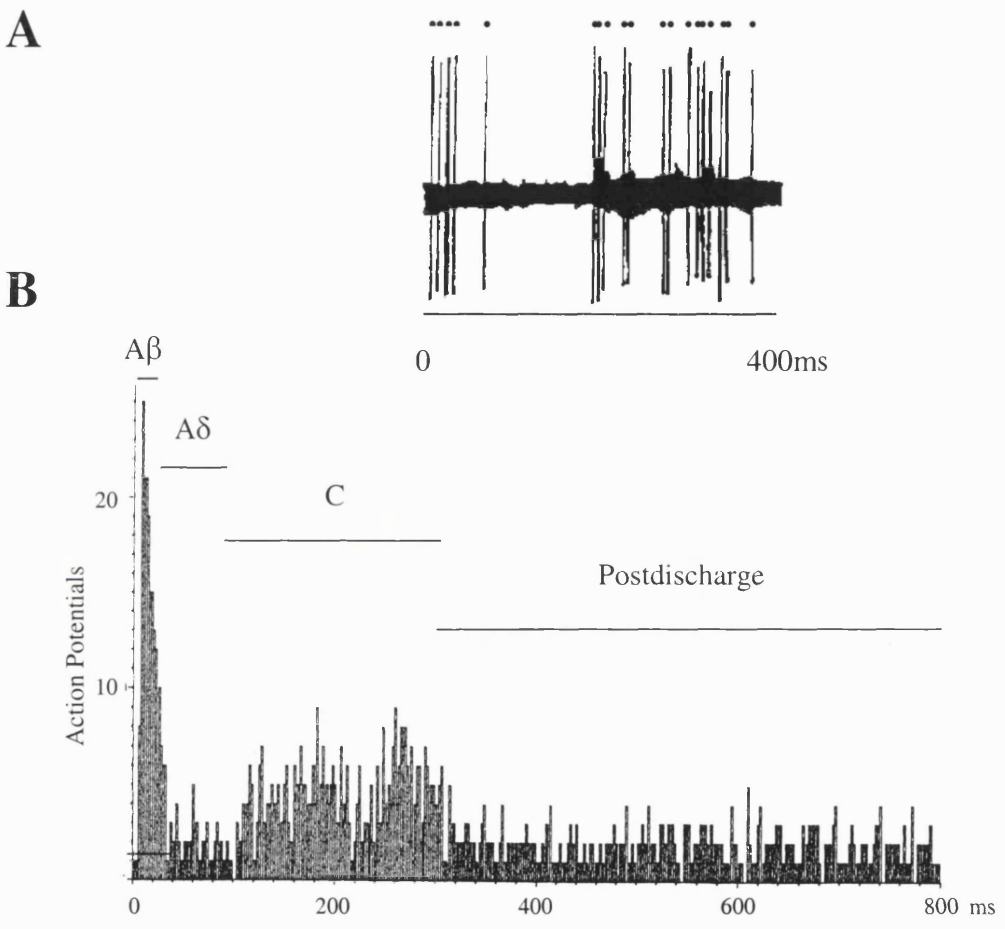


Figure 7. (A) A diagram of a single sweep oscilloscope trace of the response of a convergent dorsal horn neurone to a single electrical stimulus given at 3 x C-fibre threshold. (B) An example of a typical PSTH following a train of 16 electrical stimuli at 3 x C-fibre threshold (0.5Hz, 2ms pulse width). The responses evoked by different fibre types were separated and quantified on the basis of latency measurements (Aβ-fibre, Aδ-fibre, C-fibre). Any neuronal responses occurring after the C-fibre latency band (i.e. 300-800ms) were taken to be the postdischarge (PD) of the neurone.

The mean thresholds and latencies of neuronal responses following Aβ- and C-fibre stimulation are given in Table 6. The neuronal thresholds for Aβ- and C-fibres were comparable in all animal groups at both time-points. Similarly, the C-fibre latency of spinal neurones was similar between the three groups at PO 7-10 days. At PO 14-17 days, however, there was a significant difference in the C-fibre latency between SNL and sham operated rats (p=0.03). There was no difference in the C-fibre latency between SNL rats (PO 14-17 days) and naive rats.

Table 6. A comparison of the mean thresholds and latencies of Aβ-fibre and C-fibre evoked neuronal responses of naive, sham operated and SNL rats. The neuronal responses were characterised at two post-operative time-points (PO 7-10 and PO 14-17 days) for the sham and SNL group.

		PO 7-10 days		PO 14 -17 days	
	Naive	**Sham**	**SNL**	**Sham**	**SNL**
Aβ-fibre threshold (mA)	0.16± 0.02	0.13± 0.01	0.14± 0.02	0.15± 0.01	0.16± 0.01
Aβ-fibre latency (ms)	7.9± 0.4	8.0± 0.5	10.6±0 .4	8.2± 0.3	9.2± 0.2
C-fibre threshold (mA)	1.5± 0.1	1.1± 0.1	1.2± 0.1	1.6± 0.1	1.6± 0.1
C-fibre latency (ms)	187± 5	190± 5	201± 5	184± 3	193± 3

The mean neuronal responses following a train of 16 electrical stimuli (3 x Aβ-fibre or C-fibre threshold) are given in Table 7.

Table 7. A comparison of the mean evoked neuronal response following a train of 16 electrical stimuli at 3 x Aβ-fibre or C-fibre threshold in naive, sham operated and SNL rats. The neuronal responses evoked by the different fibres (Aβ-, Aδ- and C-fibre) were quantified together with the postdischarge and wind-up of the neurone. AP (Action Potentials).

		PO 7-10 days		PO 14 -17 days	
	Naive	**Sham**	**SNL**	**Sham**	**SNL**
3 x Aβ-fibre threshold					
Aβ-fibre evoked response (AP)	70±5	71±5	69±4	77±3	74±3
Wind-up (AP)	-5±2	-5±2	-8±2	-6±2	-6±2
3 x C-fibre threshold					
Aβ-fibre evoked response (AP)	100±4	93±6	93±5	99±3	95±3
Aδ-fibre evoked response (AP)	58±4	50±6	82±6	66±3	69±3
C-fibre evoked response (AP)	315±14	296±18	263±15	318±11	261±9
Post-discharge	183±19	174±19	164±17	210±13	192±11
Wind-up	281±30	227±24	186±20	269±16	215±14

Aβ-fibre evoked response

The mean Aβ-fibre evoked response following electrical stimulation at 3 times Aβ-fibre threshold was comparable in all animal groups (PO 7-10 and 14-17 days). Similarly, there was no significant difference in the Aβ-fibre evoked response between animal groups following electrical stimulation at 3 times C-fibre threshold.

Aδ-fibre evoked response

The Aδ-fibre evoked response of SNL rats was significantly greater than that of sham operated ($p=0.0003$) or naive rats ($p=0.002$) at PO 7-10 days. At the later time-point (PO 14-17 days), however, the magnitude of the response was comparable in all animal groups.

C-fibre evoked response

The C-fibre evoked response of SNL rats was comparable with that of sham operated rats at PO 7-10 days, however, at the later time-point, the magnitude of the response was significantly smaller in SNL rats ($p=0.0002$). When compared to naive rats, the C-fibre evoked response of SNL rats was smaller both at the earlier ($p=0.01$) and later time-points ($p=0.0007$).

Postdischarge

The postdischarge of spinal neurones was comparable between all animal groups and there was no significant difference at either time-point.

Wind-up

There was no wind-up of spinal neurones following electrical stimulation at 3 times the threshold for Aβ-fibres and this was true for all animal groups. The wind-up of spinal neurones following electrical stimulation at 3 times C-fibre threshold was comparable between SNL and sham operated rats at PO 7-10 days, however, at the later time-point, it was significantly smaller in SNL rats ($p=0.002$). When compared to naive rats, the wind-up of SNL rats was significantly smaller both at the earlier ($p=0.0009$) and later time-points ($p=0.001$).

4.3.1.2 The responses of dorsal horn neurones to natural stimuli

All neurones recorded in the study responded to a wide range of stimuli, both innocuous and noxious. Table 8 shows the proportion of neurones responding to each natural stimulus.

Table 8 A comparison of the proportions of spinal neurones responding to various natural stimuli in naive, sham operated and SNL rats.

		PO 7-10 days		PO 14 -17 days	
	Naive	**Sham**	**SNL**	**Sham**	**SNL**
Spontaneous Activity	17 %	30 %	56 %	19 %	67 %
Brush	80 %	71 %	67 %	79 %	77 %
Prod	100 %	100 %	100 %	98 %	98 %
Acetone	46 %	29 %	64 %	48 %	73 %

Fig. 8A shows an example of the typical response profile of a dorsal horn neurone to a range of mechanical and thermal stimuli.

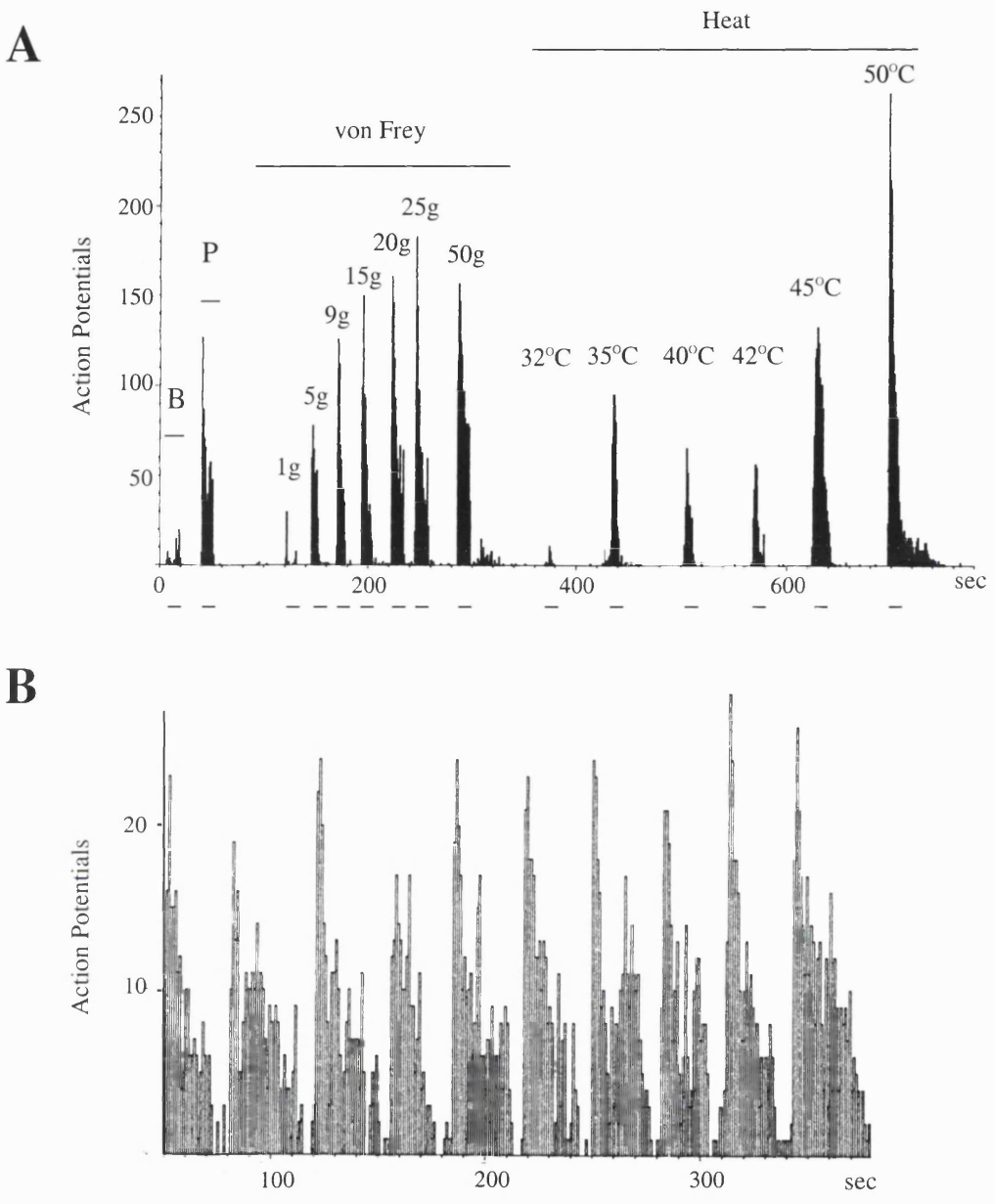


Figure 8. (A) A typical rate recording of the response of a single dorsal horn neurone to mechanical (brush (B), prod (P), von Frey) and thermal stimuli in a SNL rat at PO 14-17 days. All stimuli were applied for a period of 10 seconds as indicated by the horizontal bars, and the evoked neuronal response was quantified and subsequently normalised by the subtraction of any spontaneous activity recorded prior to the start of the recording. (B) A rate recording of the spontaneous activity of a single dorsal horn neurone from a SNL rat (PO 14-17 days). The cell shows a continuous 'tonic' pattern of ongoing activity. The interval between the successive spikes was uniform and a peak in the activity was seen approximately every 30 seconds.

A comparison of the spontaneous activity and the mean evoked neuronal responses to natural stimuli (prod, acetone brush) is given in Figure 9.

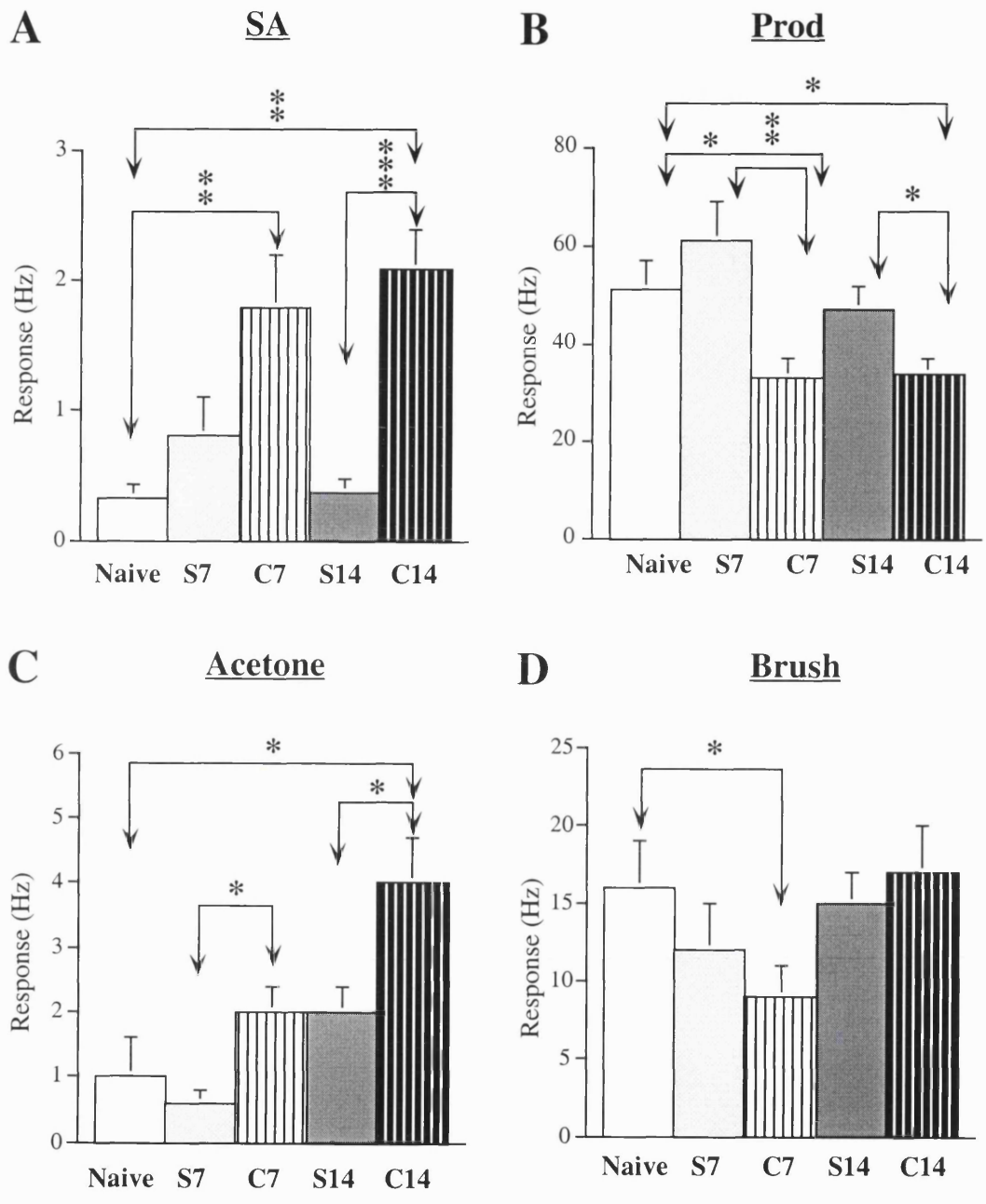


*Figure 9. A comparison of the (A) spontaneous activity and the mean evoked neuronal responses to (B) prod, (C) acetone and (D) brush in naive, sham operated and SNL rats at PO 7-10 and 14-17 days. * $p \leq 0.05$; ** $p < 0.001$. Abbreviations: Sham (S) Chung (C)*

Spontaneous Activity

Most spinal neurones recorded from SNL rats exhibited spontaneous activities with a wave-like periodical pattern of firing. A typical example of this firing pattern is illustrated in Figure 8B.

The mean level of spontaneous activity recorded from spinal neurones was comparable between SNL and sham operated rats at PO 7-10 days, although the proportion of neurones exhibiting ongoing activities was significantly greater in SNL rats, as compared to sham operated rats ($p=0.02$). At PO 14-17 days, both the level ($p<0.0001$) and proportion of neurones with spontaneous activity ($p<0.0001$) were significantly greater in SNL rats than in sham operated rats.

The level of spontaneous activity was significantly greater in SNL rats compared to naive rats, both at the earlier ($p<0.0001$) and later time-points ($p<0.0001$). A significantly higher proportion of spinal neurones exhibited spontaneous activity in SNL rats as compared to naive rats, both at PO 7-10 and 14-17 days ($p<0.0001$).

Brush

Overall, there was no significant difference in the mean brush evoked response between SNL and sham operated rats at both time-points. The magnitude of the brush evoked response of SNL rats was, however, significantly smaller compared to that of naive rats at PO 7-10 days ($p=0.04$). At PO 14-17 days, the responses were comparable between SNL and naive rats. The proportion of spinal neurones responding to the brush stimulus was similar in all animal groups.

Prod

The mean neuronal evoked response to prod was significantly smaller in SNL rats as compared to sham operated rats, both at PO 7-10 days (p=0.002) and at PO 14-17 days (p=0.004). Similarly, the magnitude of the prod evoked response was significantly smaller in SNL rats, compared to naive rats (PO 7-10 days, p=0.02; PO 14-17 days, p=0.009). Virtually all neurones characterised in the study responded to prod.

Acetone

The magnitude of the acetone evoked response was significantly greater in SNL rats as compared to sham operated rats at PO 7-10 (p=0.03) and 14-17 days (p=0.04). A larger proportion of neurones responded to the application of acetone in SNL rats, compared to sham operated rats (PO 7-10 days, p=0.008; PO 14-17 days, p=0.01).

In contrast, the magnitude of the acetone evoked response was comparable between SNL and naive rats at PO 7-10 days, as was the proportion of neurones responding to the stimulus. At PO 14-17 days, the acetone evoked response of SNL rats was significantly greater than that of naive rats (p=0.01) and a larger proportion of neurones responded to the stimulus (p=0.03).

Mechanical stimuli

Overall, the mean neuronal response to mechanical punctate stimulus (von Frey filaments 1-50 g) was smaller in SNL rats, as compared to either sham operated or naive rats (Fig. 10). This difference was more pronounced over the noxious range of stimuli. Furthermore, the mechanical stimulus required to evoke a response in spinal neurones (mechanical neuronal threshold) was lower in SNL rats as compared to sham operated or naive rats. At PO 7-10 days, the mechanical threshold of SNL rats (6.5± 0.8g) was comparable with that of sham operated (7.5± 1.1g) or naive rats (7.2± 1.2g). However, at the later time-point, the mean mechanical threshold of spinal neurones was significantly lower in SNL rats (5.0± 0.5g) as compared to either sham operated (7.0± 0.8g; p=0.05) or naive rats (7.2±

1.2g; p=0.05). Thus there appears to be a gradual drop in the mechanical neuronal threshold over the post-operative period and this may partly account for the development of mechanical allodynia observed behaviourally in SNL rats (Chapter 3.3.2).

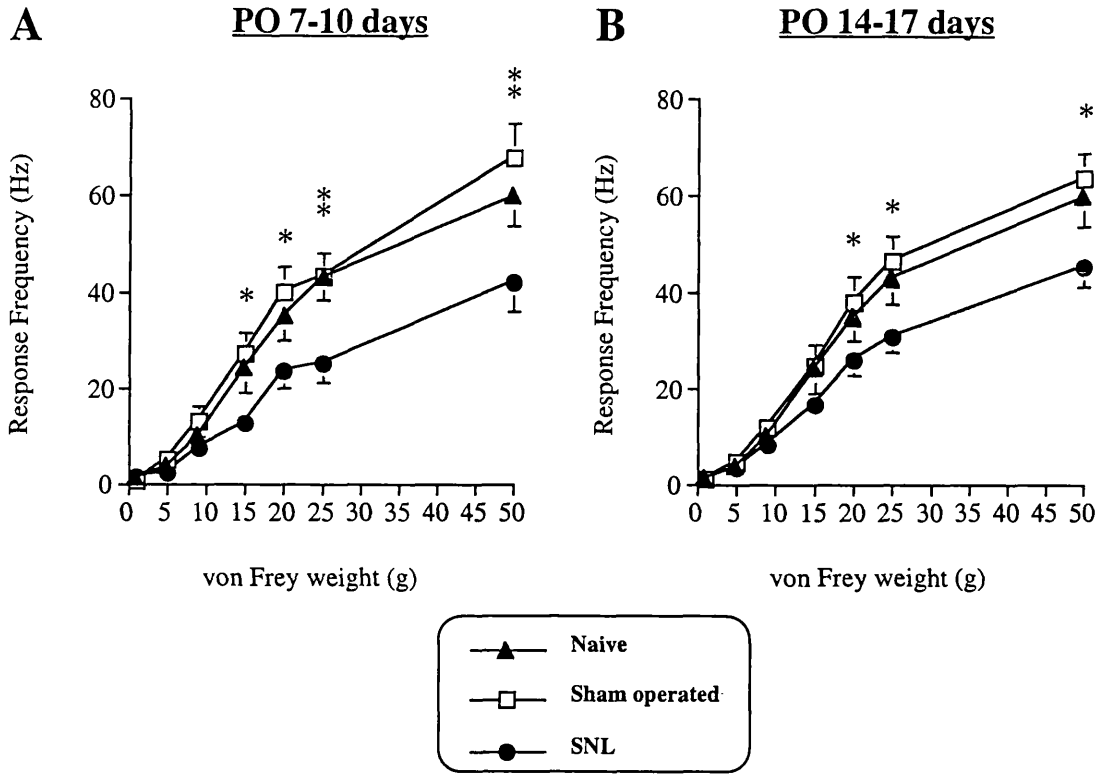


*Figure 10. The responses of spinal neurones to mechanical punctate stimuli applied peripherally to the hindpaw in SNL, sham operated and naive rats at (A) PO 7-10 and (B) PO 14-17 days. * $p \leq 0.05$; ** $p < 0.001$*

Thermal Stimuli

In contrast to the right shift observed with the mechanical evoked response, the responses of spinal neurones to thermal stimuli were similar in all animal groups and no dramatic shifts were observed after nerve injury. The stimulus response curves of SNL rats, however, tended to be slightly reduced compared to those of either sham operated or naive rats.

The mean thermal threshold of spinal neurones tended to be lower in SNL rats (PO 7-10 days, 40.9± 1.2°C; PO 14-17 days, 41.8± 0.8°C), however, there was no significant difference between the animal groups (sham operated, PO 7-10 days, 43.7± 0.6°C; PO14-17 days, 43.9± 0.9°C; naive, 44.4± 0.9°C). The drop in neuronal threshold to thermal stimulus may partly contribute to the thermal hyperalgesia which has previously been reported in the present animal model (Carlton *et al.*, 1994; Kim & Chung 1992).

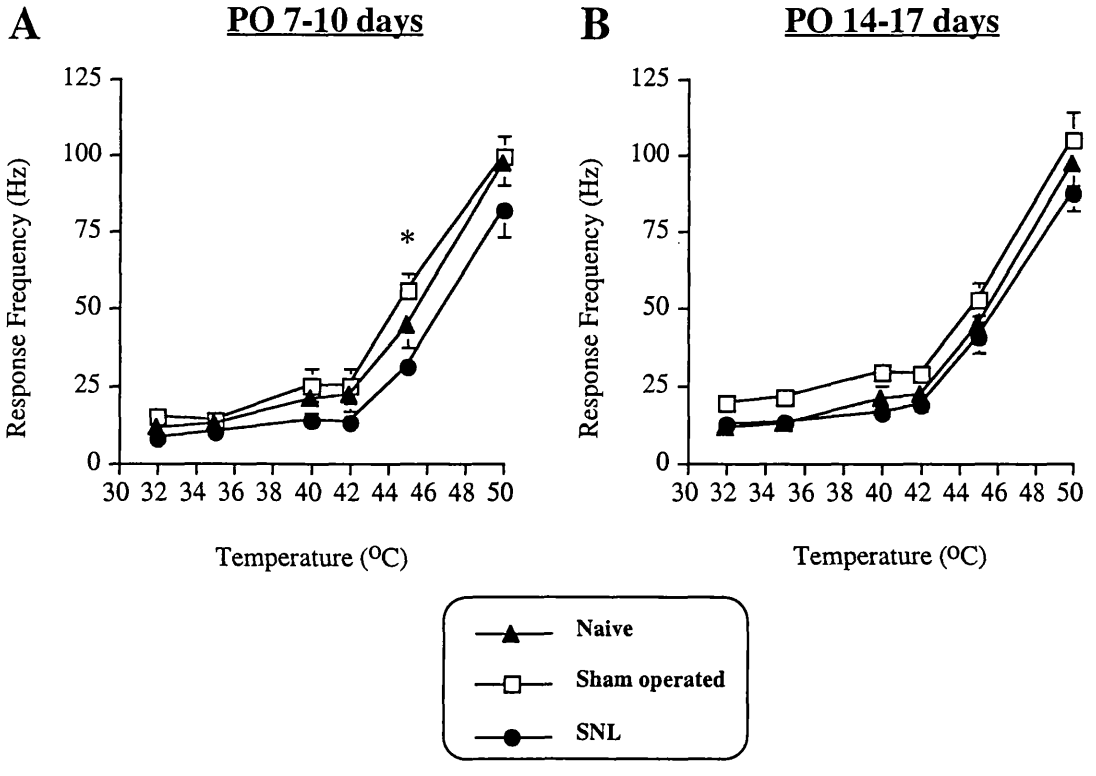


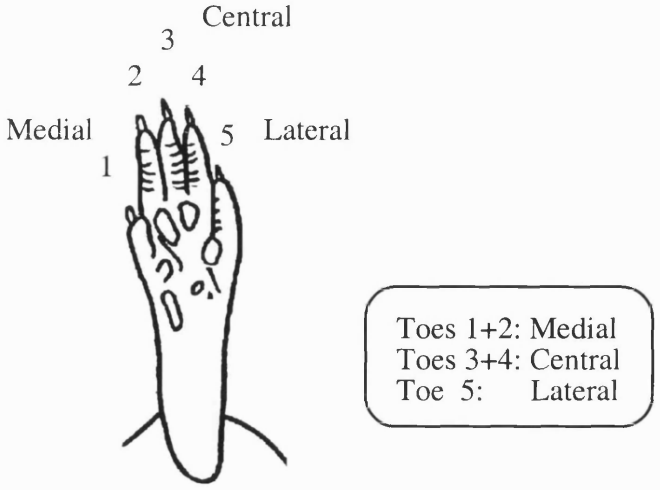
*Figure 11. The response of spinal neurones to thermal stimuli in SNL, sham operated and naive rats at (A) PO 7-10 and (B) PO 14-17 days. * p≤0.05.*

4.3.2 The receptive field size of dorsal horn neurones

All receptive fields studied in this part of the study were located on the plantar surface of the hindpaw, mostly in the toe regions. The majority of the receptive fields were continuous although, in 3 cases, discontinuous receptive fields were found. The distribution of the receptive field area was similar in the three animal groups.

Table 9. Above: The distribution of the receptive field areas of dorsal horn neurones in naive, sham operated (PO 14-17 days) and SNL rats (PO 14-17 days). Below: The distribution of the receptive field was classified as medial, central or lateral, depending on the localisation of the response to the lightest mechanical stimuli that evoked a neuronal response.

	Naive	**Sham operated**	**SNL**
Medial	19% (6/ 31)	24% (15/ 63)	29% (16/ 56)
Central	47% (15/ 31)	44% (28/ 63)	50% (28/ 56)
Lateral	28% (10/ 31)	32% (20/ 63)	21% (12/ 56)



The receptive field sizes of dorsal horn neurones, mapped using von Frey filaments of different bending force (89.1, 148.5, 742.5mN; 9, 15 and 75g) are shown in Fig. 12. Overall, the mean receptive field size of spinal neurones was greater in SNL rats, compared to either sham operated or naive rats. The receptive field area for 9g von Frey was significantly greater in SNL rats, as compared to either sham operated ($p=0.05$) or naive rats ($p=0.05$). The mean receptive field size for von Frey 9g was 37% larger than that in the sham operated or naive group. The receptive field sizes for mechanical stimuli of higher intensities (von Frey 15 and 75g) were also slightly greater in SNL rats, however, this did not reach significance level. Examples of the receptive fields of dorsal horn neurones are illustrated in Fig. 13.

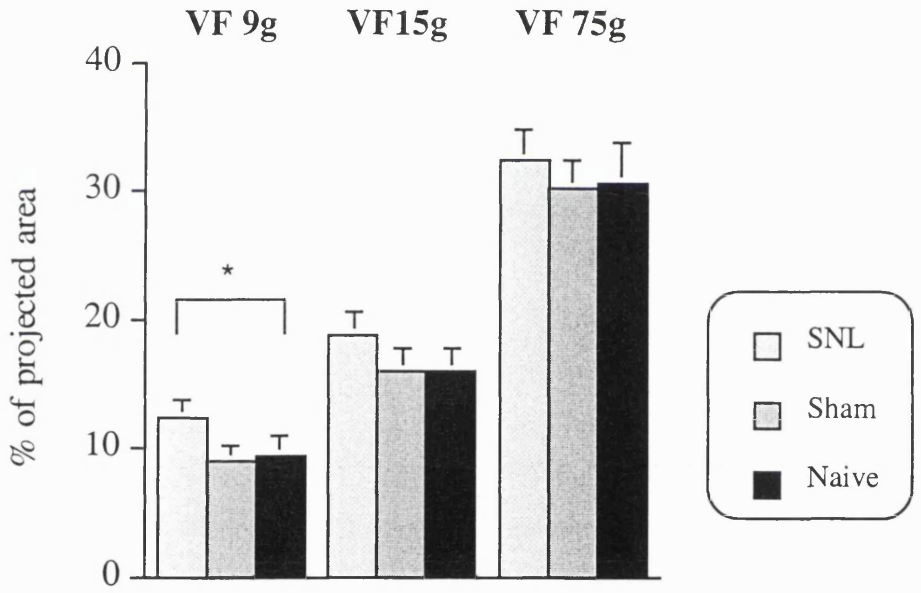


*Figure 12. A comparison of the mean receptive field size of spinal neurones in SNL (PO 14-17 days), sham operated (PO 14-17 days) and naive rats, mapped using von Frey 9, 15 and 75 grams. The receptive field area is expressed as a percentage of the projected area of the plantar surface of the paw. * $p \leq 0.05$.*

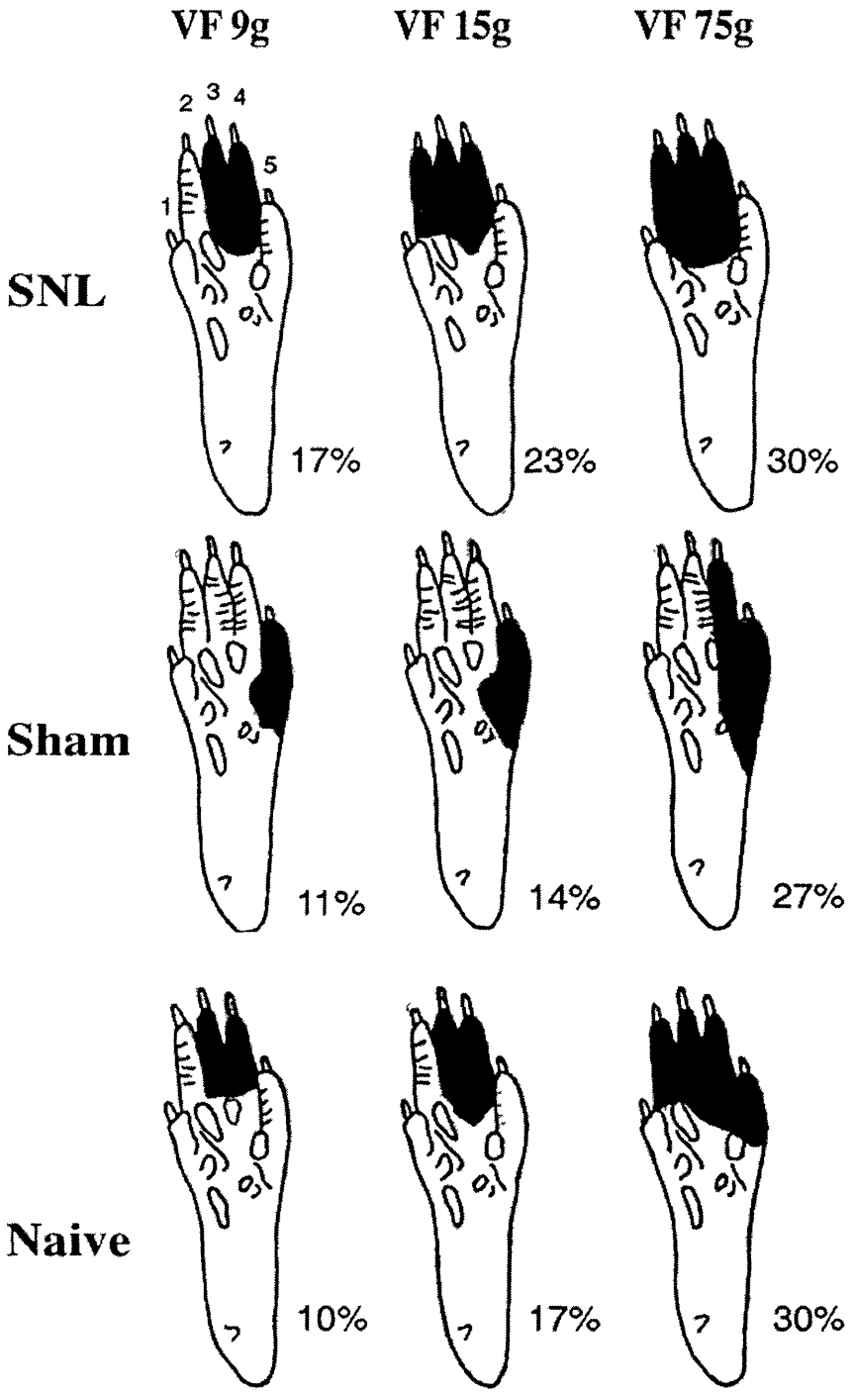


Figure 13. Typical examples of the receptive fields of dorsal horn neurones, mapped using von Frey filaments 9, 15 and 75 grams in SNL, sham and naïve rats. The area as percentage of the whole projected area of the plantar surface of the paw is given below each diagram.

4.4 Discussion

In this chapter, I report on my investigations of the effect of peripheral nerve injury on the responses of dorsal horn neurones to natural and electrical stimuli, using the selective spinal nerve (L5/ L6) ligation model of neuropathy (Kim & Chung 1992). Electrophysiological characterisation of neuronal responses was conducted at 2 postoperative time-points corresponding to the developmental (PO 7-10 days) and peak maintained phase (PO 14-17 days) of behavioural allodynia, to investigate the temporal changes which take place in the spinal somatosensory processing of noxious and innocuous information following nerve injury. In addition, I also investigated whether there are alterations in the receptive field sizes of dorsal horn neurones at 2 weeks after nerve injury. Comparisons of the mean receptive field sizes were made in a separate group of animals (PO 14-17 days) using a range of von Frey hairs encompassing the innocuous and noxious range.

4.4.1 Changes in the spontaneous and evoked neuronal responses following spinal nerve ligation

One of the marked changes seen after peripheral nerve injury was the development of a high level of spontaneous activity in spinal neurones. Whilst neurones of sham operated and naive rats exhibited only low levels of ongoing activity (< 0.8 Hz), neurones from SNL rats developed a high level of spontaneous activity within 1 week of nerve injury (1.8 Hz), and this was further increased after 2 weeks post-surgery (2.1 Hz). The proportion of neurones exhibiting spontaneous activity was also markedly increased following spinal nerve ligation (>50%), and nearly 70% of neurones developed abnormal activity by 2 weeks. Hence this suggests that there is a temporal development of spontaneous activity following nerve injury which is reflected both in the frequency of the firing as well as in its incidence. The increased activity observed in these dorsal horn neurones after spinal nerve ligation may contribute to the spinal excitability associated with neuropathic pain states.

The modifications seen in the evoked neuronal responses to peripheral natural stimuli following nerve injury were complex and appeared to be both modality- and time-dependent. Spinal nerve ligation induced an increase in the acetone-evoked response of SNL rats. At PO 14-17 days, a significantly higher proportion of neurones from SNL rats responded to acetone application as compared to sham operated or naive rats, and the magnitude of the response was similarly greater in these animals. This may contribute to cold allodynia, where exaggerated behaviours are observed in response to acetone application.

In contrast to the enhanced responsiveness of dorsal horn neurones after spinal nerve ligation, neuronal responses to prod and mechanical punctate stimuli were considerably reduced following nerve injury. The magnitude of the prod evoked response was significantly smaller in SNL rats compared to either sham operated or naive rats, although there was no decrease in the actual proportion of neurones responding to the stimulus. The mechanical stimulus response function to a range of von Frey hairs was similarly reduced in SNL rats and this was more pronounced in the noxious range. Furthermore, there was a drop in the threshold of spinal neurones to mechanical stimuli 1 week after spinal nerve ligation, and this was reduced further at 2 weeks post-injury. The lowered mechanical threshold of spinal neurones may account for the occurrence of mechanical allodynia seen behaviourally in SNL rats in the present model. In contrast, the thermal stimulus response function was not shifted after nerve injury and the magnitude of the response was comparable in all groups of animals. The thermal threshold of spinal neurones was, however, reduced in SNL rats, though this did not reach significance. This may underlie the thermal hyperalgesia reported in previous studies (Kim & Chung 1992), although the presence of this behaviour has been questioned (Kontinen *et al.,* 1998; Roytta *et al.,* 1999). The combination of a lower thermal threshold, yet overall unchanged heat evoked responses would make this behavioural change hard to gauge. It is worth noting that the method of thermal stimulation used in this study is likely to include a mechanical component due to the force of water from the water jet. However, as can be seen from Fig. 11, the contribution of the mechanical component is relatively small as compared to the heat response observed at higher temperatures. In addition, this mechanical component will be consistent at all temperatures.

4.4.2 *Changes in the mean receptive field size of dorsal horn neurones following nerve injury*

In addition to the changes in the response characteristics of dorsal horn neurones after nerve injury, changes were also observed in the receptive field sizes of spinal neurones at PO 14-17 days. Two weeks following spinal nerve ligation, there was an expansion of the receptive field size of dorsal horn neurones to mechanical stimuli, which was most prominent for the low intensity stimulus (von Frey 9g). The receptive field sizes to higher intensity mechanical stimuli (von Freys 15 and 75g) also tended to be larger in SNL rats compared to either sham or unoperated controls, however, this did not reach significance. The distribution of the receptive fields over the plantar surface was similar in all animal groups and did not show any changes after nerve injury. Receptive field organisation is an established characteristic of neuronal plasticity after nerve injury and may be an important factor contributing to the development of neuropathic pain (Devor & Wall 1981). This may have important clinical implications since a given low intensity stimulus will recruit more neurones, leading to a greater afferent input and possibly enhanced pain transmission.

Overall, these results suggest that there is a complex change in the pattern of the evoked neuronal responses, as well as in the receptive field size of dorsal horn neurones following nerve injury. The alterations in the response profile of spinal neurones observed here were modality dependent and were characterised by both increases and decreases to selected peripheral stimuli. Some modifications appeared to develop over the postoperative period and were more pronounced at the later time-point (spontaneous activity, mechanical threshold), whilst other changes were already marked at PO 7-10 days (mechanical, prod and C-fibre evoked responses). It is not clear what underlies this differential plasticity of spinal neurones observed in the present study, although it is likely that the changes result from both *de novo* acquired neuronal responses as well as from alterations in the existing response profiles of spinal neurones. The reduction in neuronal activity to peripheral stimuli

is not surprising since two-thirds of the spinal input from the sciatic nerve is lost in this model of neuropathy. (Kim & Chung 1992). Ligation of L5/ L6 spinal nerves produces a complete block of sensory transmission through the nerves and produces a significant reduction in the input to these segments of the spinal cord. Using pre-synaptic opioid receptors as markers of presynaptic terminals, a previous study quantified the contribution of afferent fibres in a single dorsal root to adjacent spinal segments in the cervical spinal cord (Besse *et al.*, 1991). This study showed that following entry to the spinal cord, primary afferent fibres terminate in two segments rostral (37%) and one segment caudal (16%) to the target segment of entry of the nerve (38%) (Besse *et al.*, 1991). If the same pattern holds for the lumbar spinal cord, it could be expected that the overall input to the L4 segment (which receives rostral innervations from L5/ L6 spinal nerves, as well as from the intact L4 spinal nerve) is considerably reduced by about 37% as a result of the partial deafferentation. However, this may only relate to unmyelinated small diameter fibres since opioid receptors are predominantly located on these fibres and not on large diameter myelinated fibres. Hence, although the exact contribution of large myelinated fibres from adjacent spinal segments is unknown, a large proportion of input from unmyelinated fibres is expected to be lost in L4-6 segments, as a result of spinal nerve ligation. It is interesting to note that despite this marked loss of afferent input in this present model, the overall changes in the responses of spinal neurones were comparatively small at PO 1-2 weeks. This may represent an increase in spinal cord excitability which could compensate for the loss of afferent drive. The high level and incidence of spontaneous activity seen in SNL rats may be one contributing factor to the global spinal hyperexcitability in these animals. This activity may well produce an ongoing level of transmitter release in the spinal cord, which may in turn, favour hyperexcitability of responses to subsequent evoked stimuli. Furthermore, there was a gradual drop in the mechanical threshold of spinal neurones after nerve injury, which could facilitate neuronal activation with a low intensity stimulus. This, together with an enlargement of neuronal receptive fields to innocuous mechanical stimuli (von Frey 9g), could form the electrophysiological basis for the mechanism underlying the behavioural manifestation of allodynia in SNL rats.

4.4.3 Comparisons of behavioural and electrophysiological studies

Previous studies from animal models of neuropathy have demonstrated that there is a temporal development of abnormal pain behaviour following peripheral nerve injury, including mechanical/ cold allodynia, hyperalgesia and spontaneous pain (Attal *et al.*, 1990; Bennett & Xie 1988; Kim & Chung 1992; Seltzer *et al.*, 1990). The onset of these behavioural manifestations is seen as early as 2 days after injury and is maintained up to several months. What are the mechanisms underlying these abnormal behavioural changes? Evidence from previous electrophysiological studies suggests that there is an increase in the responsiveness of neurones to mechanical stimuli following nerve injury (Laird & Bennett 1993; Palecek *et al.*, 1992a; Pertovaara *et al.*, 1997). This is reflected by a lowered threshold of neurones to mechanical stimuli, as well as by an increase in the mean mechanically evoked response over the innocuous and noxious range (Palecek *et al.*, 1992a; Pertovaara *et al.*, 1997). The results from my present study also demonstrated a similar drop in the mechanical threshold of dorsal horn neurones in SNL rats (neuronal thresholds: SNL, von Frey 5-6.5g; sham, 7-7.5g). The lowered neuronal threshold to mechanical stimuli could, in part, underlie the occurrence of mechanical allodynia in nerve injured animals where decreased withdrawal thresholds to innocuous mechanical stimuli are frequently observed. Furthermore, several studies have reported an increase in the mean mechanically evoked response following nerve injury (Palecek *et al.*, 1992a; Pertovaara *et al.*, 1997), as well as a prolonged afterdischarge following mechanical or thermal stimulation (Laird & Bennett 1993; Miki *et al.*, 1998; Palecek *et al.*, 1992b). This could also contribute to the overall increase in mechanical responsiveness of neurones and account for the exaggerated behavioural responses of nerve injured animals to innocuous mechanical stimulation. In contrast to previous findings, however, I did not observe such enhancements in the mechanical sensitivity of dorsal horn neurones, and on the contrary, found an overall decrease in the mechanical stimulus response function following spinal nerve ligation. Previous studies have also reported an increased brush evoked response following nerve injury (Palecek *et al.*, 1992a; Palecek *et al.*, 1992b). However, such changes were not seen in my study.

Another factor which could contribute to the occurrence of allodynia is the change in receptive field size of dorsal horn neurones. There is evidence to suggest that there is an enlargement of the mean receptive field size 1-5 weeks (Behbehani & Dollberg-Stolik 1994; Cumberbatch *et al.*, 1998) and 4-5 months after nerve injury (Tabo *et al.*, 1999; Takaishi *et al.*, 1996). The expansion of neuronal receptive field implies that with a given mechanical stimulus, a greater number of neurones can be recruited, resulting in a greater afferent input to the spinal cord and therefore, enhancing pain transmission. In support of these findings, I also found a similar increase in the receptive field size of dorsal horn neurones to low-intensity mechanical stimuls (von Frey 9g) 2 weeks after nerve injury. This same mechanical stimulus produced an exaggerated pain-like response when applied behaviourally prior to electrophysiological recordings.

In addition to the exaggerated behavioural responses to innocuous mechanical stimuli, studies have also reported the occurrence of cold allodynia following peripheral nerve injury (Bennett & Xie 1988; Kim & Chung 1992). Abnormal neuronal responses to cold stimuli have been reported in spinothalamic tract (STT) cells following L7 spinal nerve ligation, where more than 50% of the neurones developed cooling sensitivity 2 weeks after nerve injury (Palecek *et al.*, 1992a). Responses to innocuous cooling stimuli using acetone have also been reported in dorsal horn neurones of CCI rats (Laird & Bennett 1993). This is consistent with the results from my study where there was an increase in both the proportion and magnitude of the acetone evoked response following spinal nerve ligation. The exaggerated neuronal responses to cooling stimuli may account for the development of cold allodynia in nerve injured animals.

The occurrence of thermal hyperalgesia seen behaviourally in nerve injured animals, on the other hand, is more difficult to explain. Changes in neuronal responses to thermal stimuli have been demonstrated in animal models of neuropathy (Palecek *et al.*, 1992a; Tabo *et al.*, 1999), however, the majority of the electrophysiological evidence to date suggests there is little change in either the threshold (Laird & Bennett 1993; Palecek *et al.*, 1992b; Takaishi *et al.*, 1996), or the magnitude of the neuronal response to thermal stimuli (Pertovaara *et al.*, 1997; Takaishi *et al.*, 1996). Palacek et al. (1992) demonstrated that there was a drop in the thermal threshold, as well as an increased responsiveness to suprathreshold

thermal stimuli in STT neurones of L7 spinal nerve ligated monkeys 2 weeks after nerve injury (Palecek *et al.,* 1992a). A slight non-significant drop in thermal threshold has also been reported in dorsal horn neurones of CCI rats (neuropathic, 45.2± 0.9°C; sham operated, 47.6± 1.5°C), 9-14 days after nerve injury (Laird & Bennett 1993). These results are comparable to those from my study where I observed a small drop in the thermal threshold of SNL rats 2 weeks after nerve ligation (SNL, 41.8± 0.8°C; sham operated 43.9± 0.9°C; naive 44.4± 0.9°C). Tabo and co-workers (1999) have recently reported that there is a marked increase in the responses of dorsal horn neurones to thermal stimuli (38- 52°C), 5 and 22 weeks after constriction of L4-L6 dorsal roots. In this study, however, both ipsilateral and contralateral neurones displayed increased responsiveness to heat - the mechanisms underlying this bilateral change are unclear (Tabo *et al.,* 1999). In direct contrast, other studies have shown that the responses of dorsal horn neurones to thermal stimuli remain largely unchanged in the SNL and PSTL models of neuropathy (Pertovaara *et al.,* 1997; Takaishi *et al.,* 1996). In agreement with these findings, the thermal stimulus response functions of dorsal horn neurones were similar between SNL and sham operated rats at PO 1-2 weeks in my present study. The mechanisms underlying thermal hyperalgesia in nerve injured animals remain unclear.

Whilst mechanical allodynia and thermal hyperalgesia have been frequently reported in different models of neuropathic pain, there appears to be a considerable variability in the manifestation of various pain behaviours across these models (see Chapter 1.2.1.4). Spontaneous pain behaviour is most frequently reported in the CCI model (Attal *et al.,* 1990; Bennett & Xie 1988) and autotomy behaviour is not observed in either the SNL or the PSTL model of nerve injury. The electrophysiological mechanisms underlying the abnormal pain behaviours are unclear, yet the high level of spontaneous activity (Laird & Bennett 1993) seen after nerve lesion may possibly play a role in both the spontaneous and evoked pain behaviours of these animals.

Clearly, the correlation between neuronal plasticity and abnormal pain behaviour following nerve injury is complex, involving an interaction of multiple factors. The temporal profile of the electrophysiological changes appears to be similar to the profile of the allodynia development in SNL rats. However, a direct comparison between electrophysiological and behavioural studies cannot be made, and the relationship between spinal cord plasticity and clinical symptoms of neuropathic pain states remains unclear. One issue here is that it is impossible to know whether anaesthesia alters the sensory responses. In comparison to the exaggerated behavioural changes that accompany this animal model, the electrophysiological changes I observed after spinal nerve ligation were relatively small. In this study, there was no dramatic alteration in the response profile of spinal neurones, although a greater proportion of neurones exhibited spontaneous activities. However, we still do not know what level of ectopic activity is required to elicit the pain-like behaviours in nerve injured animals, and similarly, the abnormal pain sensations in neuropathic patients. It is possible that the electrophysiological changes observed here in the present study are sufficient to evoke pain in these animals. Hence, only a few hyperexcitable axons with ectopic activity may be all that is needed to evoke the conscious sensation of paresthesia and pain in humans.

4.4.4 *Previous electrophysiological studies in animal models of neuropathy*

Although there have been many animal studies investigating the behavioural consequences of nerve injury in models of neuropathic pain, little electrophysiological evidence exists on the changes which take place in the responses of spinal neurones following nerve lesion. The electrophysiological data which have so far been described, have employed various models of nerve injury, including CCI, PSTL and SNL.

Earlier recordings made in rats with spinal nerve section showed that an ongoing spontaneous activity was present in the majority of dorsal rootlets which became prominent at 3 days post-surgery (Wiesenfeld & Lindblom 1980). Spontaneous activity was also reported in the L5 dorsal root fibres (Aβ- and C-fibres) of CCI rats 11-52 days following loose ligation, where it was shown to originate proximal to the site of injury (Xie *et al.*, 1995). The level of the ongoing activity was sensitive to changes in temperature and could be altered by either cooling or heating. The dorsal root fibres also developed adrenergic sensitivity following peripheral nerve injury and sympathetic stimulation was shown to increase the abnormal activity of these fibres (Xie *et al.*, 1995). In another study where the responses of STT neurones were studied in CCI rats, a high level of background activity and an increased afterdischarge following mechanical and thermal stimulation was reported (Palecek *et al.*, 1992b). The magnitude of the response to brush stimulation was not altered by nerve injury, nor was the mean threshold of STT cells to thermal stimuli. However, there was a decrease in the response of neurones to noxious mechanical and thermal stimuli (49°C), which was most prominent at 7 and 14 days after nerve injury. Similar observations were reported in another study where the responses of dorsal horn neurones were recorded in CCI rats at PO 9-11 days (Laird & Bennett 1993). Neurones from CCI rats exhibited abnormal characteristics, including high levels of spontaneous activities, afterdischarge, increased mechanical sensitivity to gentle touch and absence of detectable receptive fields. The mean thresholds of spinal neurones to mechanical and thermal stimuli were not altered by nerve injury and there was a

smaller proportion of neurones responding to low-intensity stimuli in CCI rats (Laird & Bennett 1993). In another study, where recordings were made from gracile nucleus (GN) neurones, high levels of spontaneous activity and afterdischarge were found in cells both ipsilateral and contralateral to the nerve injury (Miki *et al.*, 1998). The receptive field size of GN neurones was not significantly altered by chronic constriction injury.

There is also evidence from the SNL model of neuropathy demonstrating changes in the neuronal properties of STT and dorsal horn WDR neurones (Palecek *et al.*, 1992a; Pertovaara *et al.*, 1997). Recordings of STT neurones in primates revealed that the background activity of ipsilateral neurones was markedly increased, as compared to the contralateral sham operated side, 2 weeks after nerve injury (Palecek *et al.*, 1992a). Furthermore, there was a significant increase in the responsiveness of ipsilateral neurones to innocuous mechanical stimuli, as well as an enhanced sensitivity to cooling and suprathreshold heat stimuli. This was accompanied by a decreased mechanical/ thermal neuronal threshold. Similar results have been reported in WDR neurones where high levels of spontaneous activity, together with a leftward shift in the mechanical stimulus response function, were found (Pertovaara *et al.*, 1997). The responses of spinal neurones to thermal stimuli were, however, unaltered.

In the PSTL model of neuropathy, recordings of dorsal horn WDR neurones revealed that there is a significant increase in the receptive field area of spinal neurones, both ipsilateral and contralateral to the ligation, 16 weeks after nerve injury (Takaishi *et al.*, 1996). Neither the threshold, or magnitude of the neuronal response to heat were altered by nerve injury.

Taken together, the data from my present study and previous electrophysiological studies indicate that there is a considerable degree of plasticity in the somatosensory system following nerve injury. This brings about complex changes in the response properties of neurones in the spinothalamic tract, dorsal column nuclei and spinal cord dorsal horn. Overall, the modification in the neuronal response profile after spinal nerve ligation appears to involve both an increase and a decrease, depending on the modality being studied. As indicated by the presence of abnormal spontaneous activity in dorsal horn neurones, there is a considerable increase in the level of neuronal activity in the spinal cord 1-2 weeks after injury, which may represent a global increase in the spinal excitability of SNL rats. These changes in the somatosensory system are likely to contribute to the mechanisms underlying the manifestation of abnormal pain behaviours in nerve injured animals, and may be relevant to the pathophysiology of neuropathic pain states in humans.

It is interesting to note, however, that concomitant to the global increase in spinal excitability induced by nerve injury, there is also evidence for a marked reduction in neuronal activity, as exemplified by the decreased responses to selected stimuli (prod, mechanical), encompassing both the noxious and the innocuous range. This may seem rather paradoxical, yet a similar situation is seen clinically where patients with neuropathic pain report a combination of sensations, including sensory deficits and hyperphenomenas, such as allodynia, hyperalgesia and spontaneous pain. Thus the concomitant presence of enhancements and reductions of neuronal activity are reminiscent of clinical situations where pain and sensory loss coexist. The difficulties associated with the treatment of neuropathic pain states are likely to arise from the multiplicity of symptoms and mechanisms underlying this condition.

Chapter 5.

Comparison of the effects of MK-801, ketamine and memantine on the responses of dorsal horn neurones following peripheral nerve injury in the rat

5. Comparison of the effects of MK-801, ketamine and memantine on the responses of dorsal horn neurones following peripheral nerve injury in the rat

5.1 Introduction

There is substantial evidence to suggest that N-methyl-D-aspartate (NMDA) receptors are implicated in the processing of afferent nociceptive information at the level of the spinal cord (Dickenson *et al.*, 1997b; Kristensen & Gordh 1997). Using immunohistochemical methods, glutamate binding sites and NMDA receptors have been identified in the outer lamina of the dorsal horn (laminae I-III), which receives terminations from nociceptive primary afferents (Bonnot *et al.*, 1996). Evidence for a role of excitatory amino acids in nociceptive transmission has been demonstrated from electrophysiological studies where the application of glutamate produced depolarisation of spinal neurones (Bernardi *et al.*, 1972; King *et al.*, 1988). Behavioural studies have shown that spinally applied excitatory amino acids induce hyperalgesia, which can be attenuated by NMDA receptor antagonists (Aanonsen & Wilcox 1987). NMDA receptors have been implicated in the spinal events underlying 'wind-up' and related changes which enhance and prolong sensory transmission (Dickenson 1995; Kristensen & Gordh 1997; Seltzer *et al.*, 1991b). Numerous studies have demonstrated the role of these receptors in the development and maintenance of pathological pain states, hence antagonism of this receptor system may be a useful target for the treatment of pain.

To date, there have been numerous behavioural studies demonstrating the effect of NMDA receptor antagonists in animal models of pain. NMDA receptor antagonists have been shown to attenuate hyperalgesia following formalin (Coderre & Melzack 1992a; Coderre & Melzack 1992b; Murray *et al.*, 1991) and carrageenan-induced inflammation (Ren *et al.*, 1992b), ischaemia (Sher *et al.*, 1992) and NMDA administration (Aanonsen & Wilcox 1987; Mjellem *et al.*, 1991). In support of these findings, electrophysiological studies have demonstrated that the administration of these antagonists produces inhibitions of the wind-up (Dickenson & Sullivan 1987; Dickenson & Sullivan 1990), and reduce the NMDA-induced hyperexcitability of dorsal horn neurones (Sher & Mitchell 1990).

Based on these findings, the therapeutic values of NMDA receptor antagonists have been assessed in numerous clinical studies. Ketamine is an intravenous anaesthetic which binds to the phencyclidine (PCP) site of the NMDA receptor-channel complex and produces analgesia at subanaesthetic doses (Klepstad *et al.,* 1990). The administration of ketamine has been shown to be effective in experimental (Andersen *et al.,* 1996; Ilkjaer *et al.,* 1996; Park *et al.,* 1995; Pedersen *et al.,* 1998; Warncke *et al.,* 1997), postoperative (Abdel-Ghaffar *et al.,* 1998; Choe *et al.,* 1997; Mathisen *et al.,* 1995; Stubhaug *et al.,* 1997) and ischemic pain states (Segerdahl *et al.,* 1994), however, it produces severe cognitive disturbances even at relatively low doses, therefore limiting its clinical use (Maurset *et al.,* 1989). The side effects often associated with ketamine include dizziness, illusions, visual disturbances and a sense of detachment (Kristensen & Gordh 1997). Dextromethorphan has also been tested clinically, however, its effectiveness in various pain states appears to differ between reported studies. Whilst it was shown to attenuate temporal summation of electrical and thermal evoked second pain (Price *et al.,* 1994), dextromethorphan produced no effect against experimental pain (Ilkjaer *et al.,* 1997; Kauppila *et al.,* 1995; Kinnman *et al.,* 1997). Memantine, is another clinically licensed drug, which acts through an uncompetitive antagonism of the NMDA receptor. This 1-amino-3, 5 dimethyl-adamantane derivative is currently used for the treatment of movement disorders such as Parkinson's and spasticity (Parsons *et al.,* 1999). Unlike ketamine or MK-801, it has a low side effect profile and is considered to be a relatively safe drug. To date, little clinical data is available for the use of memantine in the treatment of pain states (Eisenberg *et al.,* 1998b).

In recent years, attention has focused on NMDA receptor antagonists as potential therapies for neuropathic pain states, based on earlier findings from animal studies (Davar *et al.,* 1991; Mao *et al.,* 1992b; Seltzer *et al.,* 1991b; Yamamoto & Yaksh 1992a). MK-801 is a standard uncompetitive NMDA receptor antagonist used widely for experimental purposes. As with many NMDA receptor antagonists, it has a high side effect profile which makes it unsuitable for clinical use. There is substantial evidence demonstrating the effectiveness of MK-801 against hyperalgesia and spontaneous pain behaviour in CCI rats (Davar *et al.,* 1991; Kawamata & Omote 1996; Mao *et al.,* 1992a; Mao *et al.,* 1992b; Mao *et al.,* 1995b;

Munglani *et al.*, 1999; Smith *et al.*, 1994b; Yamamoto & Yaksh 1992a; Yamamoto & Yaksh 1992b; Yamamoto & Yaksh 1993). Similar effects have been reported in other models of nerve injury such as SNL (Lee & Yaksh 1995; Wegert *et al.*, 1997), PSTL (Seltzer *et al.*, 1991b), ischaemic spinal cord injury (Hao & Xu 1996; Hao *et al.*, 1991b) and unilateral transection of the superior caudal trunk (Kim *et al.*, 1997b). Furthermore, there have been extensive studies investigating the effects of the clinically available NMDA receptor antagonists, ketamine and memantine, following peripheral nerve injury. In the CCI (Eisenberg *et al.*, 1995; Mao *et al.*, 1993; Yamamoto & Yaksh 1992a) and SNL model of neuropathy (Burton *et al.*, 1999; Carlton & Hargett 1995; Qian *et al.*, 1996), both agents have been reported to attenuate nociceptive pain behaviours when administered preemptively, or postsurgery in nerve injured animals. Hence these results support the therapeutic role of NMDA antagonists in the treatment of neuropathic pain states.

In this chapter, I investigate the systemic effects of three uncompetitive NMDA receptor antagonists MK-801, ketamine and memantine, on the responses of dorsal horn neurones to natural and electrical stimuli in the SNL model of neuropathy (Kim & Chung 1992). These compounds exert their actions through their ability to bind to the open ion channel of the NMDA receptor-complex and exhibit use-dependency. Since these antagonists block channels and reduce synaptic transmission preferentially from active neurones, they may tend to preserve normal levels of synaptic transmission, yet reduce dorsal horn neuronal hyperexcitability associated with nerve injury. Furthermore, I investigated whether memantine produces similar inhibitions of the evoked neuronal responses, compared to MK-801 or ketamine in SNL rats, and assessed the potential therapeutic value of this drug in neuropathic pain states. The side effect profile of memantine is more favourable than that of other agents, therefore, if memantine could be shown to be as effective, it may offer a useful approach to the treatment of this clinical condition.

5.2 Methods

A total of 48 male Sprague-Dawley rats (PO 14-17 days, SNL, n=24; sham operated, n=24) were employed in this study. Recordings were made from dorsal horn neurones to determine the effect of NMDA receptor antagonists on the electrical and natural (mechanical/ thermal) evoked responses. Furthermore, in cells that exhibited ongoing activities, the effect of the antagonists on the level of this activity was also investigated.

Memantine (Sigma, UK) was administered to the rat subcutaneously (cumulative doses 1, 5 and 20 mg/kg) in a volume of 0.25 ml to the scruff of the neck (SNL, n=7; sham operated, n=8). The effect of the drug was followed over a period of 60 minutes per dose and tests were carried out at 10 minute intervals. Ketamine (Sigma, UK) was administered intravenously (cumulative doses 1, 5, 10 mg/kg) through a cannula inserted to the right jugular vein of the rat (SNL, n=8; sham operated, n=10). A test was made initially at 5 minutes after drug administration and subsequently at 10 minute intervals over a period of 40 minutes per dose. Similarly, MK-801 (RBI, UK) was administered intravenously (cumulative doses 0.1, 1, 5 mg/kg) and its effect was followed at 10 minute intervals over a period of 60 minutes per dose (SNL, n=9; sham operated, n=6).

Maximal effects of all doses of the antagonists were seen between 20-30 minutes (ketamine) or 20-50 minutes (MK-801 or memantine) after drug administration, justifying the 40 or 60 minute intervals between cumulative dosing. The plasma half-life of ketamine is reported to be relatively short (10-15 minutes). In this study, however, the doses of ketamine were taken to be cumulative (and not single doses of 1, 4 and 5mg/kg) since peak drug effects were still observed at later time points (~30 minutes after administration). Hence with this approach, I avoided overestimating the drug effect.

5.3 Results

The mean depths of the spinal neurones employed in this study were similar between SNL (755±42 μm) and sham operated rats (753±27μm).

5.3.1 Effect of MK-801, ketamine and memantine administration on the spontaneous activities of spinal neurones

Neurones exhibiting very high levels of spontaneous activities (>5Hz) were not employed in the study since controlled responses to natural stimuli could not be analysed when background activity was high. However, of the 15 spinal neurones tested with MK-801, 4 neurones (SNL, n=3; sham operated, n=1) exhibited spontaneous activities greater than 0.1 Hz (mean frequency of firing: SNL, 3.7±3 Hz; sham operated, 1.7 Hz). MK-801 produced inhibitions of the spontaneous activities in both SNL (maximal inhibition 78±13%) and sham operated rats (maximal inhibition 57%).

Of the 18 spinal neurones tested with ketamine, 5 neurones (SNL, n=3; sham operated, n=2) exhibited spontaneous activity (SNL, 7.2±6 Hz; sham operated, 1.1 and 2.1 Hz). The spontaneous activity of spinal neurones of both SNL (maximal inhibition 86±12%) and sham operated rats (maximal inhibitions: 43 and 25%) were reduced by the administration of ketamine.

Of the 15 spinal neurones tested with memantine, 4 neurones exhibited ongoing spontaneous activities in SNL rats (8±5 Hz). Memantine produced a 95±4% inhibition of the spontaneous activity of these neurones.

5.3.2 Effect of MK-801 on the evoked neuronal responses to electrical and natural stimuli

MK-801 inhibited the wind-up of spinal neurones in SNL (1 and 5mg/kg, p=0.04) and sham operated rats (1 and 5mg/kg, p=0.03). The magnitude of the inhibitions were comparable for both groups of animals (maximal inhibitions: SNL, 52±11%; sham operated, 48±11%) (Fig. 14).

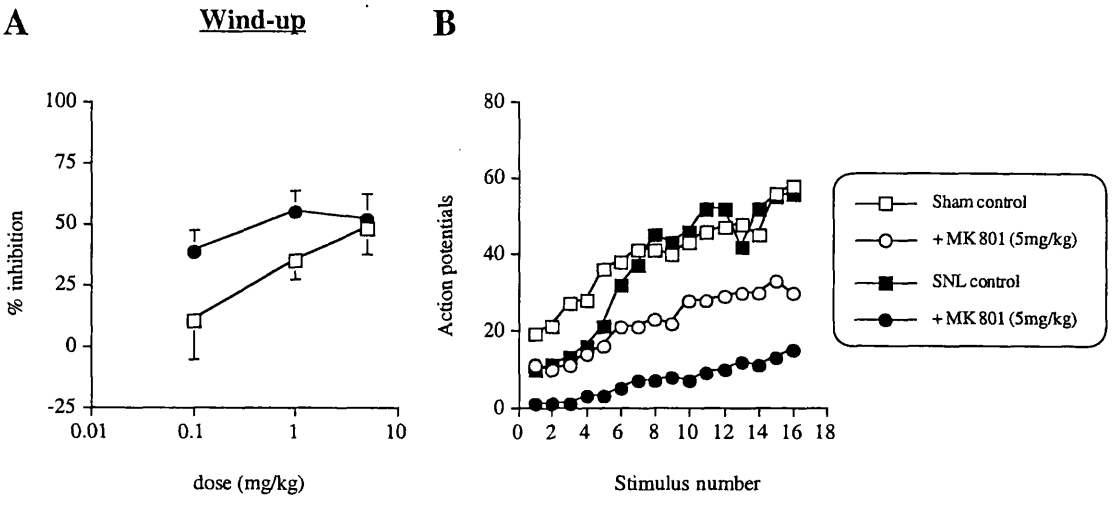


Figure 14. The effect of systemic MK-801 administration on the (A) wind-up of spinal neurones in SNL and sham operated rats. Data are presented as percentage inhibitions of pre-drug control values ± S.E.M. (B) A typical example of the effect of MK-801 (5mg/kg) on the wind-up of a single dorsal horn neurone. Trains of 16 stimuli (0.5 Hz) were given at three times the threshold current for C-fibres. The number of action potentials evoked per stimulus were plotted against the stimulus number, before (squares) and after (circles) drug administration. MK-801 produced dose-dependent inhibitions of the wind-up in SNL (filled symbols) and sham operated rats (open symbols).

Similarly, the postdischarge of spinal neurones was reduced dose-dependently in SNL (1mg/kg, p=0.03; 5mg/kg, p=0.04) and sham operated rats (1 and 5mg/kg, p=0.04). The inhibitions were similar between the animal groups (maximal inhibitions: SNL, 45±9%; sham operated, 58±9%; Fig. 15A). Compared to the inhibitions on the postdischarge, MK-801 produced only minor reductions of the Aβ, Aδ- and C-fibre evoked responses. The Aβ-fibre evoked response was reduced significantly in SNL (0.1, 1 and 5mg/kg, p=0.04) and sham operated rats (5mg/kg, p=0.02) following administration of MK-801. The effects were comparable between the two animal groups (maximal inhibitions: SNL, 37±6%; sham operated, 25±4%) (data not shown). MK-801 produced non-significant effects on the C-fibre evoked response of SNL and sham operated rats and its effect was comparable between the two groups (maximal inhibitions: SNL, 13±9%; sham operated, 18±8%; Fig. 15B). Similarly, MK-801 reduced the Aδ-fibre evoked response of SNL (0.1mg/kg, p=0.03) and sham operated rats (0.1mg/kg, p=0.04; 1 and 5mg/kg, p=0.03). Again, the inhibitions were comparable between the two groups (maximal inhibitions: SNL, 28±6%; sham operated, 48±10%) (data not shown).

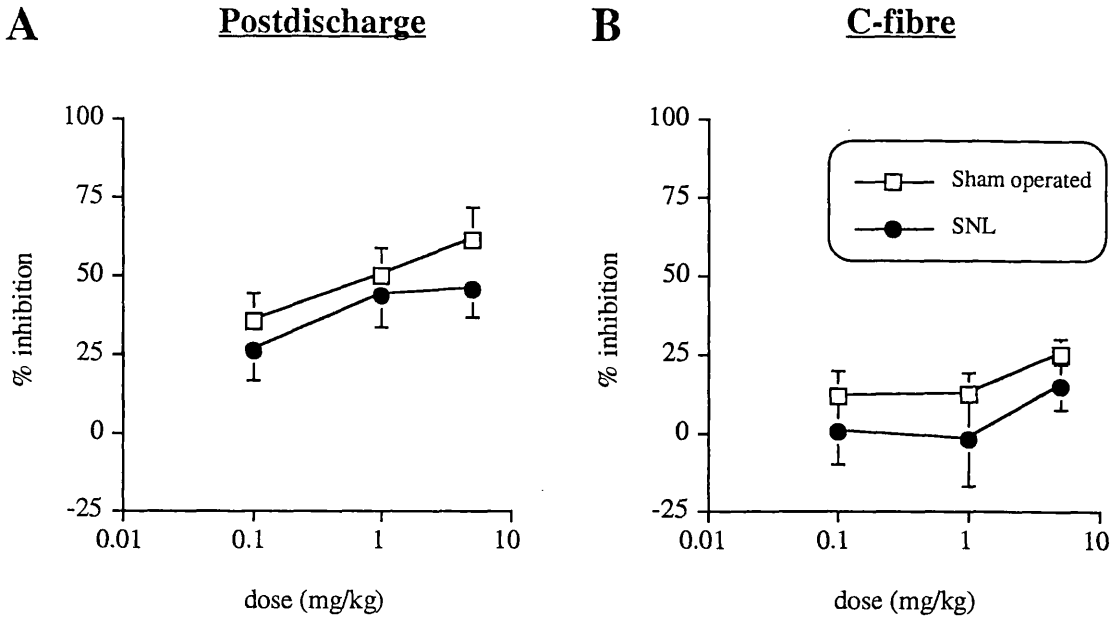


Figure 15. The effect of systemic MK-801 administration on the (A) postdischarge and (B) C-fibre evoked response of SNL and sham operated rats. Data are presented as percentage inhibitions of pre-drug control values ± S.E.M.

MK-801 produced inhibitions of the low and high intensity mechanical evoked responses of SNL and sham operated rats (Fig. 16). The 9g von Frey evoked neuronal response was reduced by MK-801 in SNL and sham operated rats (1 and 5mg/kg, p=0.03). The inhibition of the 9g von Frey response was greater in SNL rats (maximal inhibition 79±10%), than in sham operated rats (maximal inhibition 48±8%), although this was not a significant difference. Similarly, MK-801 reduced the noxious 50g von Frey evoked response of SNL (1 and 5mg/kg, p=0.04) and sham operated rats (5mg/kg, p=0.01). The inhibition of the 50g von Frey evoked response was comparable between the two animal groups (maximal inhibitions: SNL, 54±6%; sham operated, 43±9%).

MK-801 significantly reduced the thermal evoked response of SNL (0.1, 1 and 5mg/kg, p=0.04) and sham operated rats (0.1 and 5mg/kg, p=0.04). The magnitude of the reduction was comparable for both animal groups (maximal inhibition: SNL, 71±9%; sham operated 53±13%).

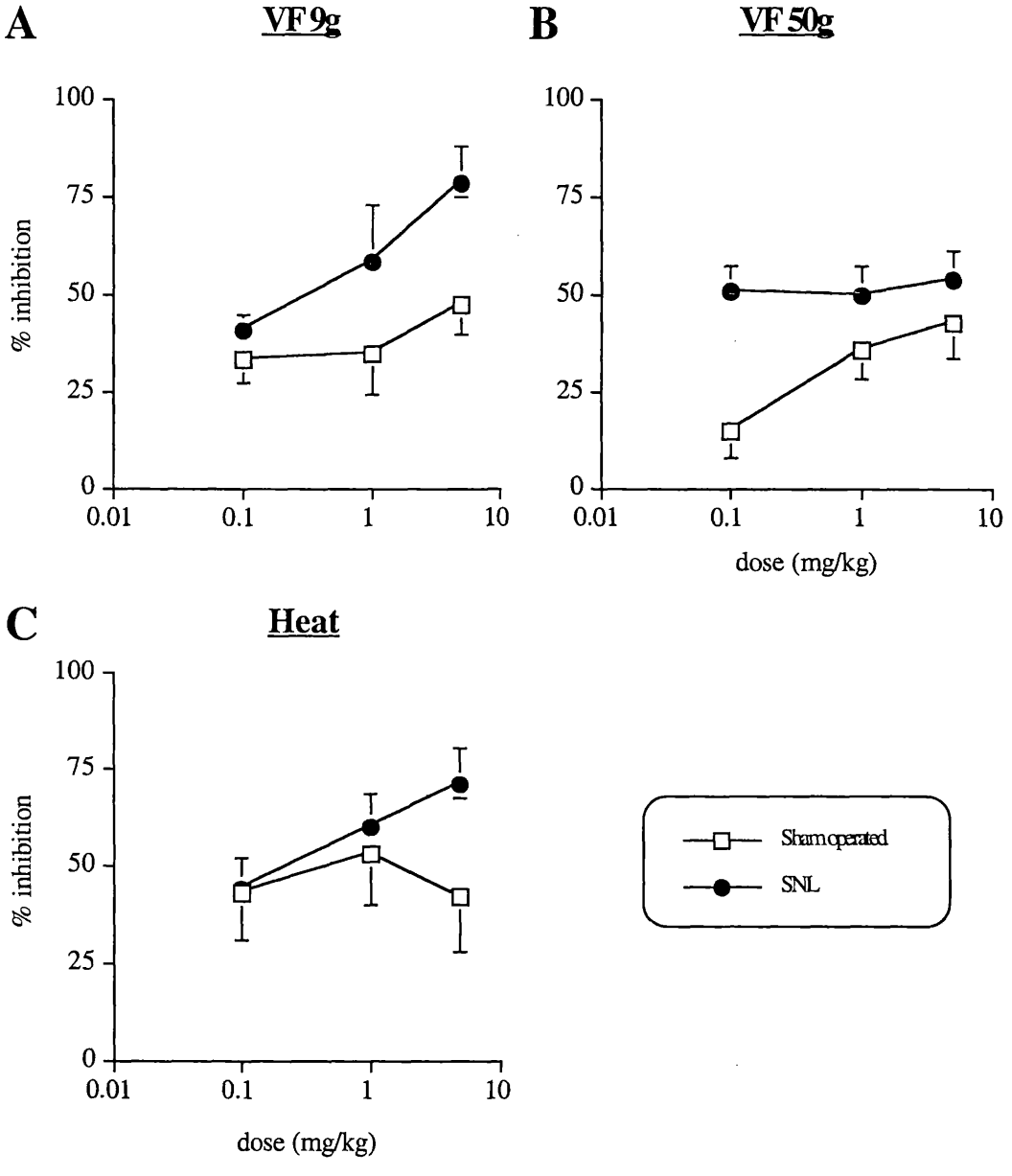


Figure 16. The effect of systemic MK-801 on the evoked responses of spinal neurones to (A) von Frey 9g, (B) von Frey 50g and (C) thermal stimuli in SNL and sham operated rats. Data are presented as percentage inhibitions of pre-drug control values ± S.E.M.

5.3.3 Effect of ketamine on the evoked neuronal responses to electrical and natural stimuli

Ketamine produced a significant inhibition of the wind-up of spinal neurones in SNL (1 and 5mg/kg, p=0.04) and sham operated rats (1 and 5mg/kg, p=0.02). The extent of the inhibitions were comparable between the animal groups (maximal inhibitions: SNL, 65±5%; sham operated, 48±10%) (Fig 17).

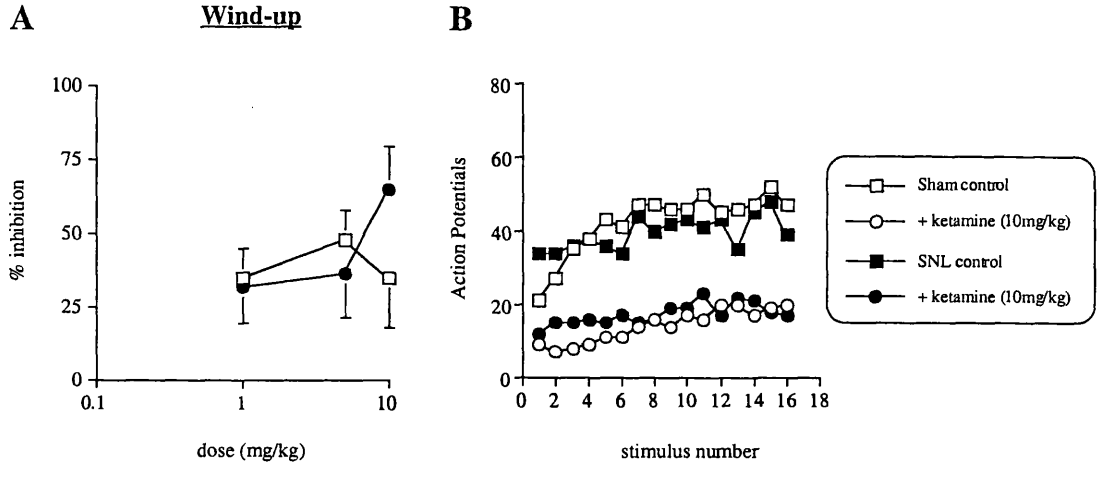


Figure 17. The effect of systemic ketamine administration on the (A) wind-up of spinal neurones in SNL and sham operated rats. Data are presented as percentage inhibitions of pre-drug control values ± S.E.M. (B) A typical example of the effect of ketamine (10mg/kg) on the wind-up of a single dorsal horn neurone. The number of action potentials evoked per stimulus were plotted against the stimulus number, before (squares) and after (circles) drug administration. Ketamine reduced the wind-up of spinal neurones in a dose-dependent manner in SNL (filled symbols) and sham operated rats (open symbols).

Similarly, the postdischarge was reduced dose-dependently following ketamine administration in SNL (1mg/kg, p=0.03; 5mg/kg, p=0.02) and sham operated rats (5mg/kg, p=0.005; 10mg/kg, p=0.02) (Fig. 18A). The effects were comparable between the animal groups at the lower doses of the drug (1 and 5mg/kg). At the highest dose of the drug (10mg/kg), however, the inhibitions were greater in SNL rats (maximal inhibition 71±13%), compared to sham operated rats (maximal inhibition 38±13%).

Ketamine produced only minor effects on the C-fibre evoked response of SNL (5mg/kg, p=0.02; 10mg/kg, p=0.03) and sham operated rats (Fig. 18B). The inhibitions were significantly greater in SNL rats (maximal inhibition 26±7%), compared to sham operated rats (maximal inhibition 7±10%) (5mg/kg, p=0.03; 10mg/kg, p=0.04). Similarly, only small reductions of the Aβ-fibre evoked response were observed following ketamine administration in SNL (1 and 5mg/kg, p=0.008) and sham operated rats (5mg/kg, p=0.02) (data not shown). The effects were comparable between both animal groups (maximal inhibitions: SNL, 21± 9%; sham operated, 13± 6%). Ketamine reduced the Aδ-fibre evoked response of SNL (1mg/kg, p=0.03; 5mg/kg, p=0.02; 10mg/kg, p=0.04) and sham operated rats (data not shown). At the lower doses of the drug, the inhibitions were comparable between the two groups, however, at the highest dose, the magnitude of the inhibition was greater in SNL rats (p=0.03; maximal inhibitions: SNL, 57±10%; sham operated, 24±13%).

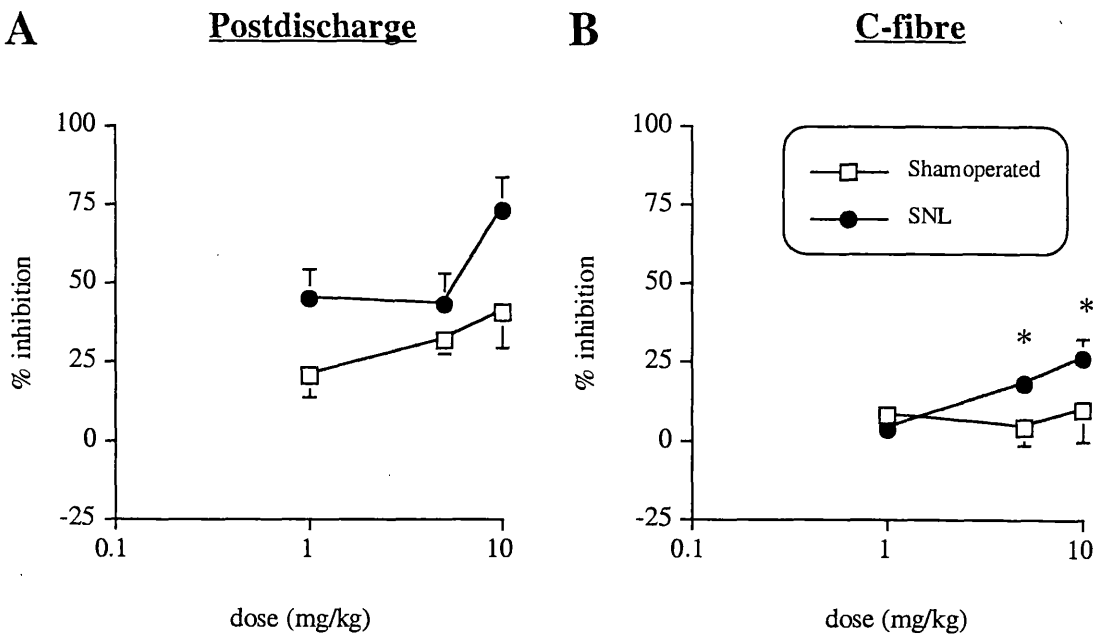


*Figure 18. The effect of systemic ketamine administration on the (A) postdischarge and (B) C-fibre evoked response of SNL and sham operated rats. Data are presented as percentage inhibitions of pre-drug control values ± S.E.M. * $p \leq 0.05$.*

Ketamine significantly reduced the 9g von Frey evoked neuronal response of SNL (1 and 5mg/kg, p=0.01; 10mg/kg, p=0.04) and sham operated rats (1mg/kg, p=0.005; 5mg/kg, p=0.003; 10mg/kg, p=0.008). The inhibitions were significantly greater in SNL rats (maximal inhibition 87±5%), compared to those in sham operated rats (maximal inhibition 62±9%) (1mg/kg, p=0.04; 5mg/kg, p=0.01; 10mg/kg, p=0.05). Similarly, ketamine produced significant inhibitions of the noxious 50g von Frey evoked response of SNL (1 and 5mg/kg, p=0.01; 10mg/kg, p=0.03) and sham operated rats (1mg/kg, p=0.04; 5mg/kg, p=0.005; 10mg/kg, p=0.007). The inhibition of the 50g von Frey evoked response was significantly greater in SNL rats (maximal inhibition 67±4%) than in sham operated rats (maximal inhibition 35±8%) (5mg/kg, p=0.02; 10mg/kg, p=0.03).

The thermal evoked responses were similarly inhibited by ketamine in SNL (1 and 5mg/kg, p=0.02) and sham operated rats (1mg/kg, p=0.02; 5 and 10mg/kg, p=0.005). The inhibitions were comparable at lower doses of the drug (1 and 5mg/kg), however, ketamine produced a greater effect in SNL rats (p=0.02) at the highest dose (maximal inhibitions: SNL, 75±6%; sham operated, 37±8%).

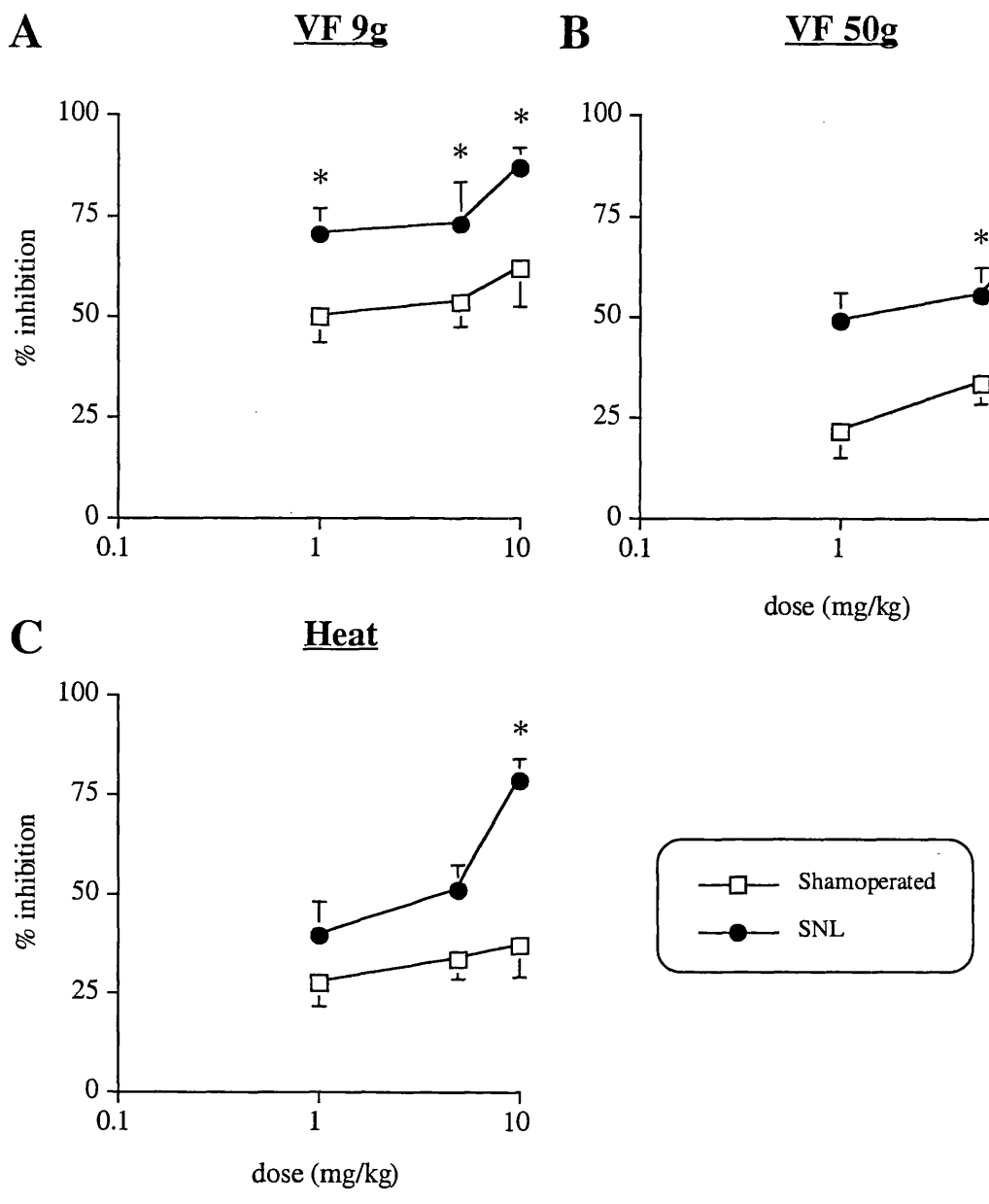


*Figure 19. The effect of systemic ketamine administration on the evoked responses of spinal neurones to (A) von Frey 9g, (B) von Frey 50g and (C) thermal stimuli in SNL and sham operated rats. Data are presented as percentage inhibitions of pre-drug control values ± S.E.M. * $p \leq 0.05$.*

5.3.4 Effect of memantine on the evoked neuronal responses to electrical and natural stimuli

Memantine reduced the wind-up of spinal neurones in both SNL (1, 5 and 20mg/kg, p=0.03) and sham operated rats (20mg/kg, p=0.03) (Fig. 20). Whilst memantine produced a large inhibition of the wind-up in SNL rats (maximal inhibition 74±6%), it produced a significantly smaller effect in sham operated rats (maximal inhibition 30±11%) (5mg/kg, p=0.02).

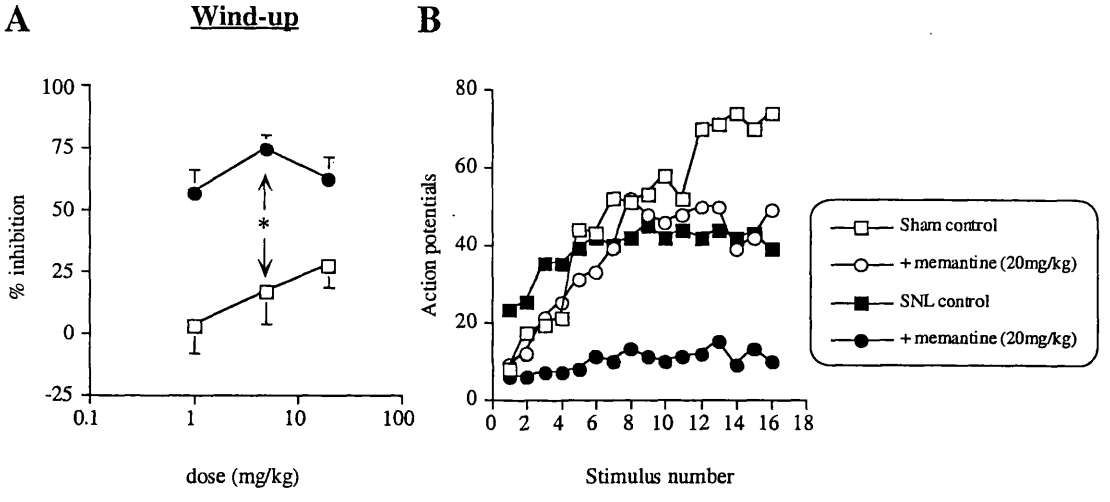


*Figure 20. The effect of systemic memantine administration on the (A) wind-up of spinal neurones in SNL and sham operated rats. Data are presented as percentage inhibitions of pre-drug control values ± S.E.M. * p≤0.05. (B) A typical example of the effect of memantine (20mg/kg) on the wind-up of a single dorsal horn neurone. The number of action potentials evoked per stimulus were plotted against the stimulus number, before (squares) and after (circles) drug administration. Memantine reduced the neuronal wind-up of SNL rats (filled symbols), but had little effect on the wind-up of sham operated rats (open symbols).*

The postdischarge of spinal neurones was similarly reduced following the administration of memantine in SNL (1mg/kg, p=0.04; 5mg/kg, p=0.008; 20mg/kg, p=0.02) and sham operated rats (20mg/kg, p=0.04). This inhibition was greater in SNL rats (maximal inhibition 60±9%) compared to sham operated rats (maximal inhibition 34±8%). The C-fibre evoked response of SNL rats (20mg/kg, p=0.03) was dose-dependently reduced with memantine, yet memantine produced only minor effects in sham operated rats. The effect of the drug was significantly greater in SNL rats (maximal inhibition 28±9%) as compared to that in sham operated rats (maximal inhibition 18±6%) (5mg/kg, p=0.03).

Memantine produced small reduction of the Aβ-fibre evoked response in SNL rats (1 and 20mg/kg, p=0.04) (data not shown). The effect of memantine was greater in SNL rats (maximal inhibition 32±4%) compared to sham operated rats (maximal inhibition 13±7%) (1mg/kg, p=0.04; 20mg/kg, p=0.03). Memantine reduced the Aδ-fibre evoked response of SNL rats (1mg/kg, p=0.01; 5 and 20mg/kg, p=0.02), yet produced little effect on the Aδ-fibre evoked responses in sham operated rats (maximal inhibitions: SNL, 50±14%; sham operated, 11±18%).

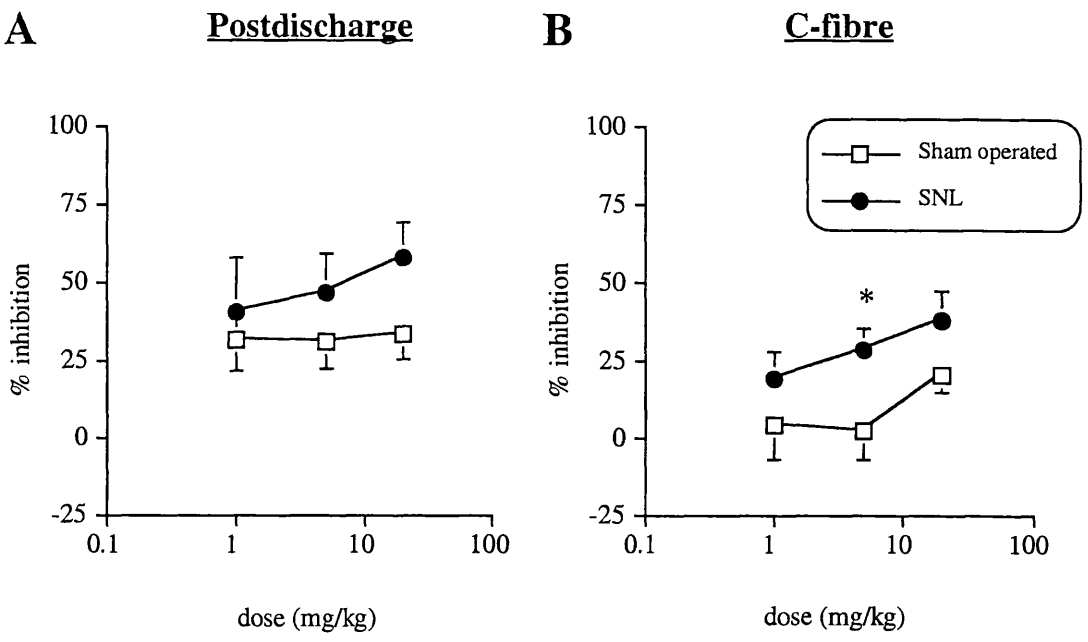


*Figure 21. The effect of systemic memantine administration on the (A) postdischarge and (B) C-fibre evoked response of SNL and sham operated rats. Data are presented as percentage inhibitions of pre-drug control values ± S.E.M. * $p \leq 0.05$*

Memantine reduced both the innocuous and noxious mechanical evoked neuronal responses of both animal groups (Fig. 22). The 9g von Frey evoked response was significantly reduced in SNL (5mg/kg, p=0.01; 20mg/kg, p=0.02) and sham operated rats (20mg/kg, p=0.03) and the inhibitions were similar in both experimental groups (maximal inhibitions: SNL, 74±8%; sham operated, 69±17%). Memantine produced a significant inhibition of the noxious 50g von Frey evoked response of SNL (1 and 20mg/kg, p=0.02; 5mg/kg, p=0.01) and sham operated rats (5mg/kg, p=0.03; 20mg/kg, p=0.04). The magnitude of the inhibition was greater in SNL rats, although this did not reach significance level (maximal inhibitions: SNL, 66±12%; sham operated, 40±10%).

The thermal evoked response of spinal neurones was reduced following memantine administration in SNL (1 and 5mg/kg, p=0.02; 20mg/kg, p=0.04) and sham operated rats (5mg/kg, p=0.03; 20mg/kg, p=0.04). Memantine produced comparable effects between the animal groups (maximal inhibitions: SNL, 65±16%; sham operated, 69±16%).

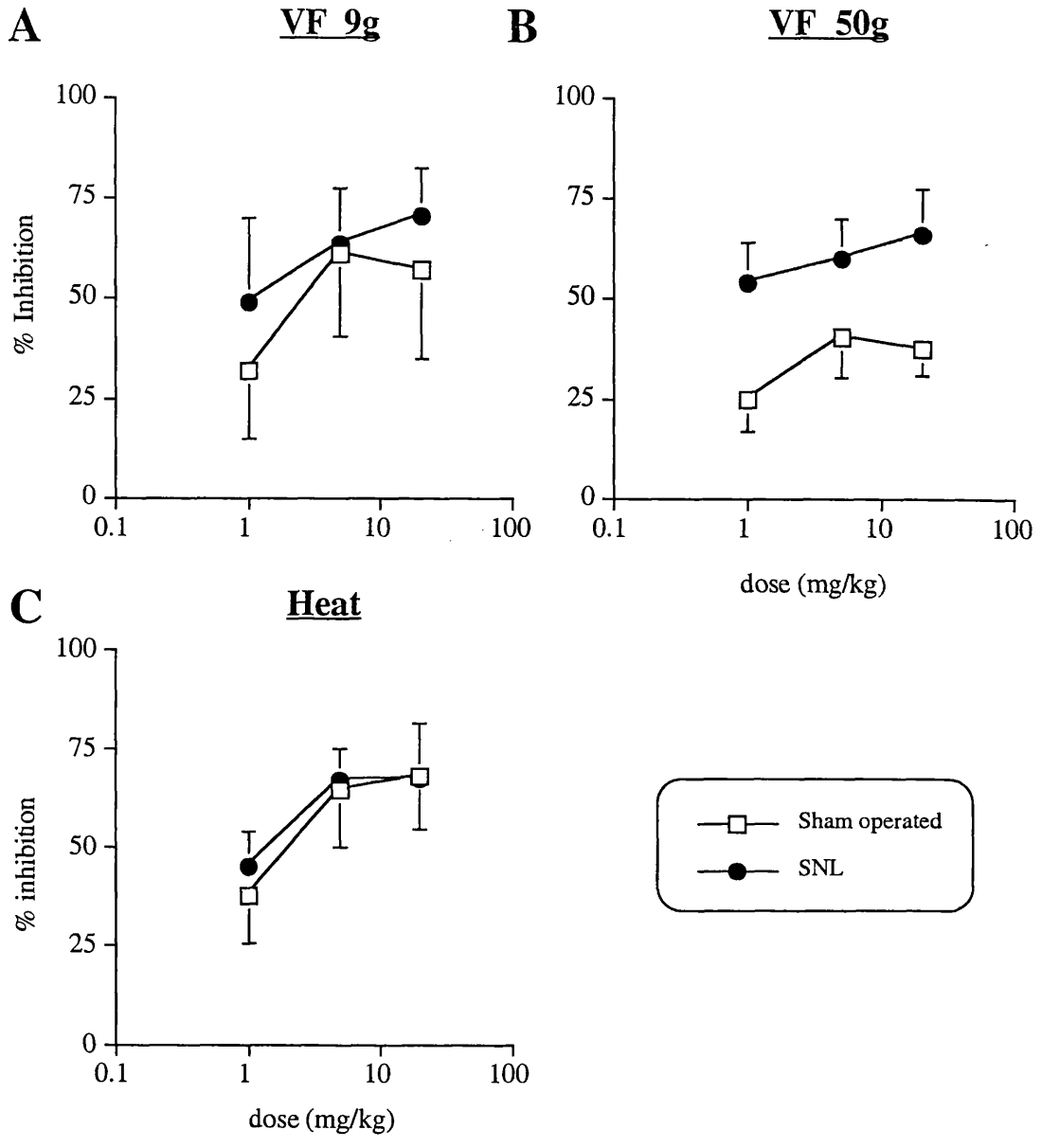


Figure 22. The effect of systemic memantine administration on the evoked responses of spinal neurones to (A) von Frey 9g, (B) von Frey 50g and (C) thermal stimuli in SNL and sham operated rats. Data are presented as percentage inhibitions of pre-drug control values ± S.E.M.

5.3.5 Comparisons of the effects of memantine with MK-801 and ketamine on the electrical and natural evoked responses of SNL rats

The effects of memantine on the responses of spinal neurones were compared with those produced by MK-801 or ketamine, to assess the therapeutic potential of the compound in neuropathic pain states. Figure 23 shows a comparison of the effect of the three antagonists on the electrical and natural evoked responses of spinal neurones in SNL rats.

All three NMDA receptor antagonists produced marked inhibitions of the wind-up and postdischarge of spinal neurones in SNL rats. Memantine (maximal inhibition 74%) produced the greatest inhibition of the wind-up of all three antagonists (ketamine, 65%; MK-801, 52%). Similarly, the effect of memantine (61%) on the postdischarge was slightly greater compared to MK-801 (55%), although its effect was smaller than that of ketamine (71%).

Memantine, MK-801 and ketamine produced relatively small reductions of the Aβ- (< 40%) and C-fibre evoked responses (< 40%) in SNL rats, and the magnitude of the effects were similar for all three antagonists. The inhibition of the Aδ-fibre evoked response was comparable for memantine (maximal inhibition 50%) and ketamine (57%). However, MK-801 (28%) produced a much smaller effect on this neuronal measure.

All three antagonists produced a comparable inhibition of the evoked neuronal response to low intensity mechanical stimuli (9g von Frey) in SNL rats (maximal inhibitions: memantine, 74%; MK-801, 79%; ketamine, 87%). Similarly, the inhibition of the noxious 50g von Frey evoked response was comparable for all antagonists (memantine, 66%; MK-801, 54%; ketamine, 67%). Memantine (65%) produced a slightly smaller reduction of the thermal evoked response compared to MK-801 (71%) or ketamine (75%).

These results therefore suggest that memantine is effective in inhibiting both the natural (mechanical / thermal) and electrical evoked responses of SNL rats, producing inhibitions which are comparable in magnitude to those produced by ketamine or MK-801.

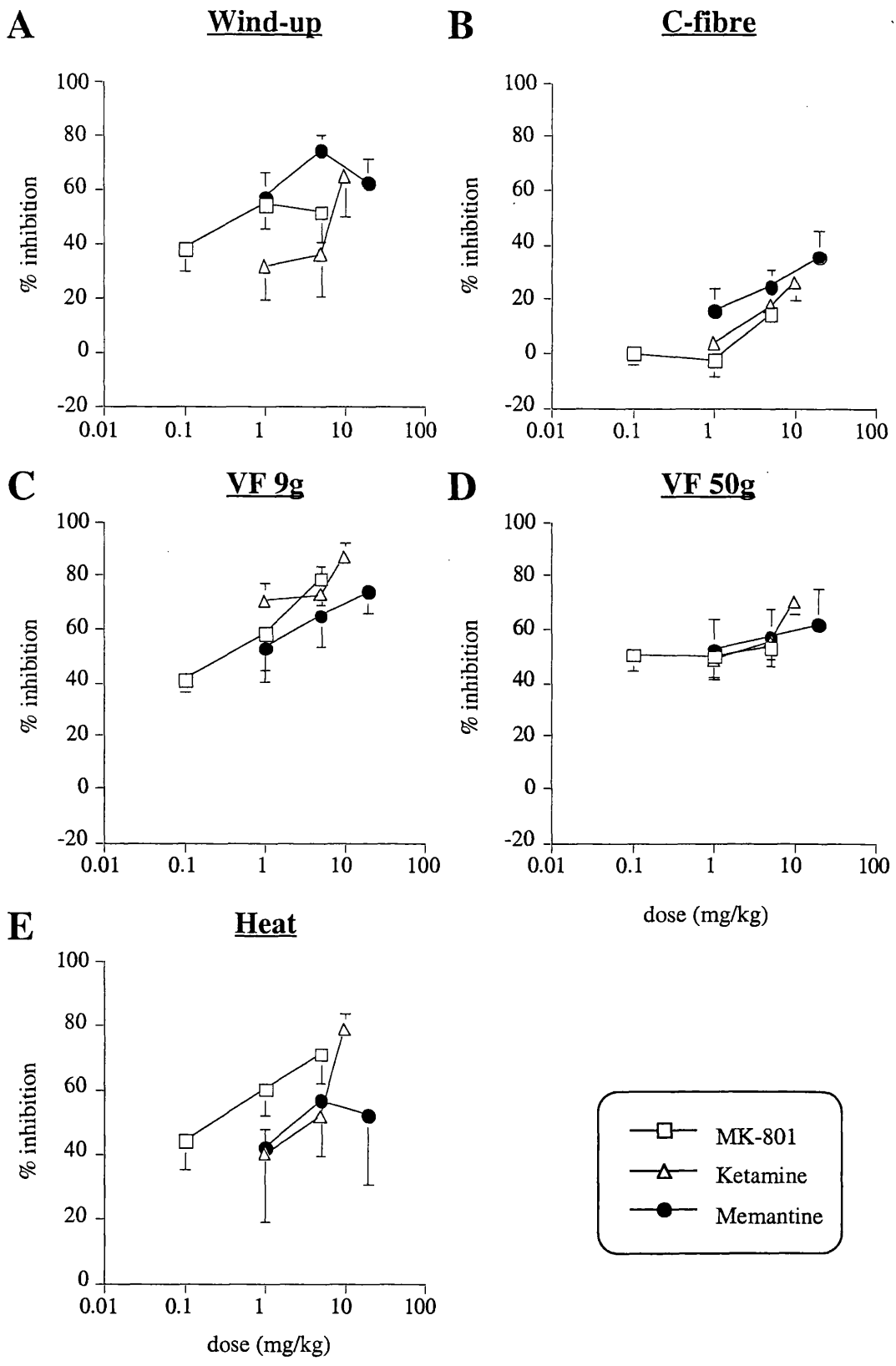


Figure 23. A comparison of the effects of NMDA antagonists on the electrical and natural evoked responses of SNL rats. Data are presented as percentage inhibitions of pre-drug control values ± S.E.M.

5.4 Discussion

Substantial evidence exists for the involvement of NMDA receptors in the induction and maintenance of certain pathological pain states, possibly via the sensitization of dorsal horn neurones in the spinal cord (Dickenson 1995; Kristensen & Gordh 1997). Results from animal behavioural models have demonstrated that NMDA receptor antagonists attenuate some of the symptoms of neuropathic pain, including allodynia and hyperalgesia (Carlton & Hargett 1995; Davar *et al.,* 1991; Yamamoto & Yaksh 1992a), further supporting the role of spinal NMDA receptors in the events underlying central sensitization. There is growing evidence, however, that descending influences from supraspinal sites contribute to the development and maintenance of central sensitization (Urban &Gebhart 1999). Recent studies have reported that descending facilitatory excitatory amino-acid influences can be produced from brainstem structures, in particular, the rostral ventromedial medulla (RVM), and activation of these pathways could facilitate spinal nociceptive transmission. Hence, central sensitization at the level of the spinal cord can be modulated by the RVM and so a supraspinal component to the effects of systemic NMDA receptor antagonists may be expected.

Here I provide electrophysiological evidence for the inhibition of the electrical and natural evoked responses of dorsal horn neurones through antagonism of the NMDA receptor. My results showed that the channel blockers, MK-801, ketamine and memantine, are effective in reducing both the electrical and natural (low and high intensity) evoked responses of SNL and sham operated rats. Overall, the inhibitory effects of these antagonists tended to be greater in SNL rats, as compared to sham operated rats, and a leftward shift in the dose response curves was seen with most neuronal measures. The effects of the three systemically administered antagonists are likely to be produced through effects at both spinal and supraspinal sites. The greater effectiveness of the drugs in SNL rats may be due to the greater contribution of the NMDA receptor system to neuronal activity following nerve injury. During pathological conditions, NMDA receptors can be activated with lower concentrations of glutamate (μM), and also for a longer period than under normal conditions, where mM concentrations of glutamate are required.

(continued overleaf)

Persistent injury states, such as neuropathy, may produce prolonged activation of the NMDA receptor due to a sustained afferent spinal input which results in a relatively small, but continuous increase in the extracellular level of glutamate. Increased glutamate levels have been reported in the ipsilateral dorsal horn of CCI rats at PO 4-14 days (Kawamata & Omote 1996) and furthermore, there is evidence for an upregulation of glutamate receptors following nerve injury (Croul *et al.*, 1998; Harris *et al.*, 1996; Popratiloff *et al.*, 1998). Therefore, a greater proportion of channels are likely to be in their open state during neuropathy, and this could enable NMDA channel blockers to exert greater effects in SNL rats through their use-dependency. Furthermore, ectopic impulse generation, alterations in the phenotype of damaged nerves, loss of GABA controls and reduced opioid sensitivities (Fields & Rowbotham 1994; Jensen 1996) may all contribute to a more efficient removal of the Mg^{2+} block of the NMDA channel complex. It is also worth noting that the pharmacological and electrophysiological properties of NMDA receptors can be altered through the subunit composition of the heteromeric complex (e.g. NR1A with various NR2 subtypes). If nerve injury induces modulation in the subunit composition of the NMDA receptor, this may potentially alter the channel open probability and play a role in mechanisms underlying spinal hyperexcitability.

My results are consistent with previous findings where the effectiveness of NMDA receptor antagonists has been demonstrated behaviourally. In the CCI model of neuropathy, both prophylactic and therapeutic administration of memantine (s.c.) was shown to attenuate thermal hyperalgesia (Eisenberg *et al.,* 1995). Similarly, memantine, administered chronically through osmotic pumps or by a bolus injection (i.p.) was demonstrated to be effective against mechanical hyperalgesia and allodynia in SNL rats (Carlton & Hargett 1995). Ketamine (i.t./ i.v.), another clinically available NMDA receptor antagonist, has been reported to attenuate nociceptive pain behaviours across various models of neuropathy, including CCI (Mao *et al.,* 1993; Yamamoto & Yaksh 1992a) and SNL (Burton *et al.,* 1999; Qian *et al.,* 1996). Although not in clinical use, there is substantial evidence for the antinociceptive effects of MK-801 (i.t./ i.p.) in CCI rats (Davar *et al.,* 1991; Kawamata & Omote 1996; Mao *et al.,* 1992a; Mao *et al.,* 1992b; Mao *et al.,* 1995b; Munglani *et al.,* 1999; Yamamoto & Yaksh 1992a; Yamamoto & Yaksh 1992b; Yamamoto & Yaksh 1993). Similar effects have been reported across other models of nerve injury, such as PSTL (Seltzer *et al.,* 1991b) and ischaemic spinal cord injury (Hao & Xu 1996; Hao *et al.,* 1991b). Interestingly, the time of drug administration relative to nerve injury appears to play a part in determining the effectiveness of MK-801. Studies have shown that the administration of MK-801 prior to nerve injury attenuates the development of thermal hyperalgesia (Mao *et al.,* 1992a) and mechanical allodynia in CCI rats (Munglani *et al.,* 1995; Smith *et al.,* 1994b), and delays the onset of allodynia in rats with unilateral transection of the superior caudal trunk (Kim *et al.,* 1997b). When administered post-surgically, however, only a temporary effect was achieved (Kim *et al.,* 1997b). In addition, there is conflicting evidence on the ability of MK-801 to suppress the different

behavioural measures between various studies. While postsurgical administration of MK-801 (i.t.) has been reported to relieve mechanical allodynia (Lee & Yaksh 1995) and thermal hyperalgesia in SNL rats (Wegert *et al.*, 1997), other studies have shown MK-801 to be ineffective against these measures (Nichols *et al.*, 1997; Wegert *et al.*, 1997). In support of the above behavioural findings where generally a single stimulus modality was studied, I found all three agents to be highly effective in inhibiting a wide range of evoked neuronal responses when administered postoperatively two weeks after nerve injury. The long term prophylactic and therapeutic effects of these agents support the idea that these agents block the development of central sensitisation mediated by NMDA receptors and therefore prevent the induction and maintenance of pain-related behaviours.

I have also demonstrated in this electrophysiological study that memantine produces inhibitory effects on the responses of dorsal horn neurones, which are similar in magnitude to the classical channel blockers, such as MK-801 and ketamine. This is consistent with a previous report where memantine was shown to be antinociceptive on spinal neurones (Neugebauer *et al.*, 1993). These results strongly support the potential use of this compound for the treatment of neuropathic pain states. Although all three antagonists (memantine, ketamine and MK-801) I employed in this study block the NMDA receptor via the same mechanism, (i.e. uncompetitive antagonism through the phencyclidine site), the tolerability of these agents have been shown to differ in animal models. Whilst memantine is well tolerated both in humans and in animals (Hesselink *et al.*, 1999), ketamine and MK-801 have less favourable side effect profiles which limit their clinical use. NMDA receptors are ubiquitous and are implicated in sensory perception, cognition and consciousness, hence the administration of NMDA receptor antagonists will inevitably result in unwanted psychotropic effects, often at doses within their putative therapeutic range. The difference in the balance between therapeutic and unwanted effects of these antagonists will be determined, in part, by the characteristics of their kinetics and voltage-dependent block.

MK-801 has a weak voltage-dependency and slow kinetics. The weak voltage-dependency of MK-801 implies that its actions are not highly dependent on synaptic activity, hence it will also tend to block physiological activation of the NMDA receptor, as well as that under pathological conditions. Additionally, the

unblocking kinetics of MK-801 are slower compared to Mg^{2+}, hence once bound to the channel-receptor complex, it is unable to leave the channel within the time-course of a normal NMDA receptor-mediated excitatory post-synaptic potential. These factors may account for the poor therapeutic profile of MK-801. By contrast, memantine, which in my hands was highly effective on the various neuronal measures, has a much favourable side-effect profile. Memantine has a weaker use-dependency compared to MK-801 although it exhibits high voltage-dependency and fast offset kinetics (Parsons *et al.*, 1993; Parsons *et al.*, 1995). Under normal resting conditions, memantine can produce a block of the NMDA receptor channel (Sobolevsky *et al.*, 1998). Following strong synaptic depolarisation, however, this block may be removed due to the strong voltage-dependency of the drug and its rapid unblocking kinetics. This allows memantine to rapidly leave the channel upon transient physiological activation by mM concentrations of glutamate, whilst blocking the sustained activation by μM concentrations of glutamate during pathological conditions. Memantine should therefore preferentially act to block channels in active neurones that are opening and closing rapidly, and effectively reduce pathological excessive synaptic activation of neuropathic pain states. In contrast, under normal conditions where there is a relatively lower level of synaptic transmission, memantine should exert less influence and produce fewer side effects. The profile of ketamine is similar to that of memantine, in terms of the biophysics of the channel block. However there is a marked difference in the profile of ketamine depending on the dose. The difference in the use-dependency of the channel block produced by memantine, and to some extent ketamine, compared to MK-801 (Parsons *et al.*, 1993) is a possible basis for the reduced side effects seen with memantine.

Hence there is a need for more selective NMDA receptor antagonists with regard to spinal nociceptive processing. One way in which this could be achieved is by the development of agents that act specifically on particular NMDA receptor subunits, thereby conferring regional specificity. Interestingly, it has been proposed in recent studies that the better therapeutic index of memantine may be partly related to the selective action of the drug on the NR1A/ NR2B receptor subtype (Parsons *et al.*, 1999). Memantine has been demonstrated to have a two to three fold greater potency at NR1A/ NR2B receptors over NR1A/ NR2A receptors

(Parsons *et al.*, 1999). The extent to which receptor subtype selectivity contributes to the therapeutic profile of NMDA receptor antagonists remains unclear.

To date, numerous studies have investigated the potential therapeutic value of NMDA receptor antagonists in neuropathic pain states, based on findings from animal studies. Ketamine, a clinically licensed drug, has been shown to be effective in various neuropathic pain states (Backonja *et al.*, 1994; Felsby *et al.*, 1996; Rabben *et al.*, 1999), including phantom limb pain (Nikolajsen *et al.*, 1996; Stannard & Porter 1993), post-amputation stump pain (Nikolajsen *et al.*, 1997) and neuropathic cancer pain (Mercadante *et al.*, 1995). Long term treatment with the drug produced beneficial effects in the absence of side effects or development of tolerance (Nikolajsen *et al.*, 1997). Ketamine was also reported to provide pain relief in patients with post-herpetic neuralgia (Eide *et al.*, 1995; Eide *et al.*, 1994; Hoffmann *et al.*, 1994; Klepstad & Borchgrevink 1997) and reflex sympathetic dystrophy (Lin *et al.*, 1998), including those who responded poorly to conventional therapies such as opioids (Mercadante *et al.*, 1995; Persson *et al.*, 1995; Takahashi *et al.*, 1998). In patients with chronic neuropathic pain of orofacial or trigeminal origin, however, mixed effects of ketamine have been reported - ranging from no analgesia to significant pain relief (Mathisen *et al.*, 1995; Rabben *et al.*, 1999). The beneficial effects of ketamine appeared to be more pronounced in patients suffering pain for less than 3-5 years, as compared to those with longer pain histories (Enarson *et al.*, 1999; Mathisen *et al.*, 1995).

The main drawback in the use of ketamine for the treatment of pain is the occurrence of intolerable side effects, which are frequently reported in many patients (Eide *et al.*, 1995; Eide *et al.*, 1994; Enarson *et al.*, 1999; Mathisen *et al.*, 1995; Nikolajsen *et al.*, 1996; Rabben *et al.*, 1999). At subanaesthetic doses, ketamine produces auditory, visual and proprioceptive disturbances producing a feeling of detachment (Backonja *et al.*, 1994; Oye *et al.*, 1992). This, together with reports of motor impairments in rats at high doses (Qian *et al.*, 1996), indicates a narrow therapeutic index of the drug.

Memantine, on the other hand, is a clinically well-tolerated NMDA receptor antagonist which is considered as a relatively safe drug. The possible therapeutic use of this compound for the treatment of various CNS disorders such as spasticity, dementia and Parkinsons had been recognised since the late 1970s, however, the primary mechanism by which it produces its therapeutic effect (i.e. via uncompetitive NMDA receptor antagonism) was only revealed 10 years later (Parsons *et al.*, 1999). To date, very little clinical data is available on the use of memantine for the treatment of neuropathic pain states. In a double-blind placebo-controlled study, however, memantine was reported to be ineffective in the treatment of postherpetic neuralgia (Eisenberg *et al.*, 1998b). This study used memantine doses of up to 20mg/kg and most patients reported no side effects. Hence, it is possible that the ineffectiveness of memantine may be due to insufficient dosing of the drug. In addition, it may be possible that postherpetic neuralgia is less likely to respond to NMDA receptor antagonists, as compared to other neuropathic pain syndromes (Eisenberg *et al.*, 1998b). Interestingly, amantadine, a related drug, has been shown to be effective in patients with cancer (Pud *et al.*, 1998) and chronic neuropathic pain (Eisenberg & Pud 1998). There is conflicting animal evidence on the potency of memantine. While some studies report memantine to be less potent than other NMDA receptor antagonists (ketamine) in depressing the neuronal responses to NMDA or peripheral noxious stimuli (Herrero *et al.*, 1994), other studies have shown it to produce comparable blocking effects to MK-801 using patch clamp techniques (Bormann 1989). Behaviourally, memantine has been shown to exert a greater antiallodynic effect in SNL rats, compared to either MK-801 or ketamine (Chaplan *et al.*, 1997). The results from my electrophysiological study demonstrates that memantine produces strong inhibitions of the evoked neuronal responses in SNL rats, which are similar in magnitude to ketamine. At doses which produce antinociception in rats, memantine causes little or no motor/ behavioural impairments (Carlton & Hargett 1995; Chaplan *et al.*, 1997; Eisenberg *et al.*, 1994) and it is also reported to lack abuse potential (Parsons *et al.*, 1999). At high doses of the drug (20- 40mg/kg), memantine may produce side effects in animals (e.g. ataxia), although these rarely occur at therapeutically relevant doses (Parsons *et al.*, 1999). Hence memantine

may offer an alternative approach to the treatment of neuropathic pain states, producing long-term analgesia in the absence of adverse side effects. Further clinical trials are needed to assess the therapeutic potential of this drug for treatment of chronic pain states.

Chapter 6.

The adenosine A_1 receptor system: the effects of an adenosine A_1 receptor agonist, N^6-cyclopentyladenosine and an adenosine kinase inhibitor, A200702.21 on the responses of dorsal horn neurones following peripheral nerve injury

6. The Adenosine A_1 receptor system: the effects of an adenosine A_1 receptor agonist, N^6-cyclopentyladenosine and an adenosine kinase inhibitor, A200702.21 on the responses of dorsal horn neurones following peripheral nerve injury

6.1 Introduction

Substantial evidence exists for a physiological role of adenosine in the modulation of primary afferent transmission in the spinal cord (Sawynok 1998; Sawynok & Sweeney 1989). Since the antinociceptive effects of adenosine were originally described in earlier behavioural studies (Crawley *et al.*, 1981), there has been a considerable interest in the development of adenosine analogues as potential analgesics for the treatment of various pain states. The analgesic action of these agents was attributed to actions at the spinal cord, based on the observation that adenosine analogues administered via the intrathecal route produce antinociception which is 10-20 times greater in potency than that via the systemic route (Holmgren *et al.*, 1986). Hence much attention has been focused on the direct intrathecal route of drug administration. Use of autoradiographic and binding techniques led to the identification of adenosine receptors in the substantia gelatinosa of the spinal cord where two subtypes of adenosine receptors (A_1/ A_2) were characterised (Choca *et al.*, 1988; Choca *et al.*, 1987; Ralevic & Burnstock 1998). These receptors were shown to be localised primarily on intrinsic neurones (Choca *et al.*, 1988; Choca *et al.*, 1987; Geiger *et al.*, 1984). Although evidence exists for the involvement of A_2 receptors in spinally-mediated antinociception (DeLander & Hopkins 1987), it appears to be predominantly the A_1 receptor subtype which plays a major role in inhibiting the nociceptive input in the dorsal spinal cord (Karlsten *et al.*, 1991; Lee & Yaksh 1996; Reeve & Dickenson 1995b; Sawynok *et al.*, 1986).

The antinociceptive properties of adenosine and receptor-selective analogues have been demonstrated across a wide range of animal models, including acute nociceptive tests (Ahlijanian & Takemori 1985; Aran & Proudfit 1990;

Contreras *et al.*, 1990; DeLander & Hopkins 1987; DeLander & Wahl 1988; Doi *et al.*, 1987; Fastbom *et al.*, 1990; Holmgren *et al.*, 1983; Holmgren *et al.*, 1986; Karlsten *et al.*, 1990; Karlsten *et al.*, 1991; Ocana & Baeyens 1994; Post 1984; Sjolund *et al.*, 1997; Sosnowski *et al.*, 1989; Yang *et al.*, 1995; Yarbrough & McGuffin-Clineschmidt 1981) and in models of inflammation following carageenan (Poon & Sawynok 1998) and formalin injection (Karlsten *et al.*, 1992; Malmberg & Yaksh 1993). Much of the existing evidence however, has been from behavioural studies and that from electrophysiogical studies is still sparse (Reeve & Dickenson 1995a; Reeve & Dickenson 1995b; Sumida *et al.*, 1998). These results, observed across several models of pain, strongly support the potential clinical use of these agents in various pain states. Furthermore, the manipulation of the endogenous adenosine system through the use of inhibitors of adenosine metabolism (e.g. adenosine kinase inhibitor) has been shown to induce antinociception in nociceptive assays (Keil & DeLander 1994; Keil & DeLander 1992) and after inflammation (Poon & Sawynok 1995). The administration of adenosine kinase inhibitors has been shown to decrease pain behaviour induced by putative pain neurotransmitters, such as substance P and kainic acid (Keil & DeLander 1996). Adenosine kinase catalyses the phosphorylation of adenosine to AMP, and inhibitors of this enzyme have been demonstrated to modulate adenosine release from the spinal cord (Golembiowska *et al.*, 1995; Golembiowska *et al.*, 1996). The localisation of adenosine kinase in the spinal cord is unknown. However, it appears to have a high affinity for adenosine and regulates its endogenous activity at the spinal level. Antinociception produced by adenosine kinase inhibitor is likely to be mediated via the activation of cell surface adenosine receptors since it is inhibited by methylxanthines (Keil & DeLander 1992).

In humans, systemic or intrathecal administration of adenosine produced a significant increase in the cutaneous heat pain threshold in healthy volunteers (Ekblom *et al.*, 1995) and has been shown to be effective against experimentally induced pain (Rane *et al.*, 1998; Segerdahl *et al.*, 1995; Segerdahl *et al.*, 1994). Furthermore, preoperative adenosine infusion has been demonstrated to reduce the anaesthetic requirements during surgery, whilst adenosine administration after surgery reduced the demand for postoperative analgesics (Segerdahl *et al.*, 1996).

In view of this wide spectrum of activity, there has been a growing interest in the development of therapeutic agents which interact with adenosine systems for the treatment of neuropathic pain. Patients with neuropathic pain have been reported to have a deficiency in plasma and CSF adenosine levels (Guieu *et al.*, 1996). The adenosine level in these patients was significantly lower than in those with nervous system lesions exhibiting no pain, or in patients with pain due to excessive nociception (Guieu *et al.*, 1996). The reduced adenosine level may be a result of an alteration in adenosine synthesis following neuropathy, or an increase in its metabolism through adenosine kinase or deaminase. Alternatively, it may reflect the spinal hyperexcitability that is associated with this pain state (Dray *et al.*, 1994), since increased neuronal activities would cause an enhanced transmitter release, eventually leading to the depletion of the purine. Previous studies using animal models of neuropathy have demonstrated the effectiveness of adenosine and its analogues against mechanical allodynia (Cui *et al.*, 1998; Cui *et al.*, 1997; Lavand'homme & Eisenach 1999; Lee & Yaksh 1996; Sjolund *et al.*, 1998; von Heijne *et al.*, 1998; Yamamoto & Yaksh 1991), although motor impairments have been reported (Lavand'homme & Eisenach 1999; Lee & Yaksh 1996). In addition, there is evidence to suggest that adenosine kinase inhibitors attenuate allodynia in nerve injured rats (Lavand'homme & Eisenach 1999). These results therefore support the therapeutic use of adenosine administration in human neuropathic pain states (Belfrage *et al.*, 1999; Belfrage *et al.*, 1995; Sollevi *et al.*, 1995). The direct and indirect manipulation of the adenosine system may offer a beneficial approach in producing spinally mediated antinociception in this clinical condition.

To date, there has only been one electrophysiological study demonstrating the effect of adenosine agonists on the responses of dorsal horn neurones following peripheral nerve injury (Behbehani & Dollberg-Stolik 1994). Further electrophysiological investigations are therefore needed to elucidate the mechanism underlying the antinociceptive action of these agents, and to understand how their analgesic effects could be separated from potential side effects. In this chapter, I examine the effect of N6-cyclopentyladenosine (CPA), an adenosine A_1 receptor agonist, and A200702.21, an adenosine kinase inhibitor, on the evoked neuronal responses to controlled natural (mechanical/ thermal) and electrical stimuli using the selective SNL model of neuropathy (Kim & Chung 1992). The effects of these

agents were compared between SNL and sham operated rats to investigate whether there is plasticity in the spinal purinergic system following peripheral nerve injury, and to provide a possible basis for the treatment of the multiple symptoms of neuropathic pain.

6.2 Methods

CPA (RBI) was applied topically, directly onto the exposed surface of the spinal cord in cumulative doses (0.5, 5 and 50 μg) in a volume of 50μl using a Hamilton syringe. This is akin to an intrathecal injection. The effect of the drug was followed over a period of 40 minutes per dose and tests were carried out at 10 minute intervals to determine its effects on the electrical and natural evoked responses. The effect of CPA was reversed using a non-specific antagonist, theophylline (i.t. 1000μg), applied 40 minutes after administration of 50μg CPA, and followed for a further 40 minutes. A200702.21 (Abbott Laboratories) was given subcutaneously to the scruff of the neck (0.1, 1 and 10mg/kg) and tests were made every 10 minutes for 60 minutes.

6.3 Results

6.3.1 The effect of CPA on the electrical and natural evoked responses of dorsal horn neurones

Recordings were made from a total of 12 dorsal horn neurones in SNL (n=6) and sham operated rats (n=6), 2 weeks after surgery for spinal nerve ligation or sham operation. The mean depth of the spinal neurones was comparable between SNL (638±12μm) and sham operated rats (710±38μm). The maximal effects of all doses of CPA were observed between 20 to 30 minutes after drug administration, justifying the 40 minute intervals between cumulative dosing (Fig. 24).

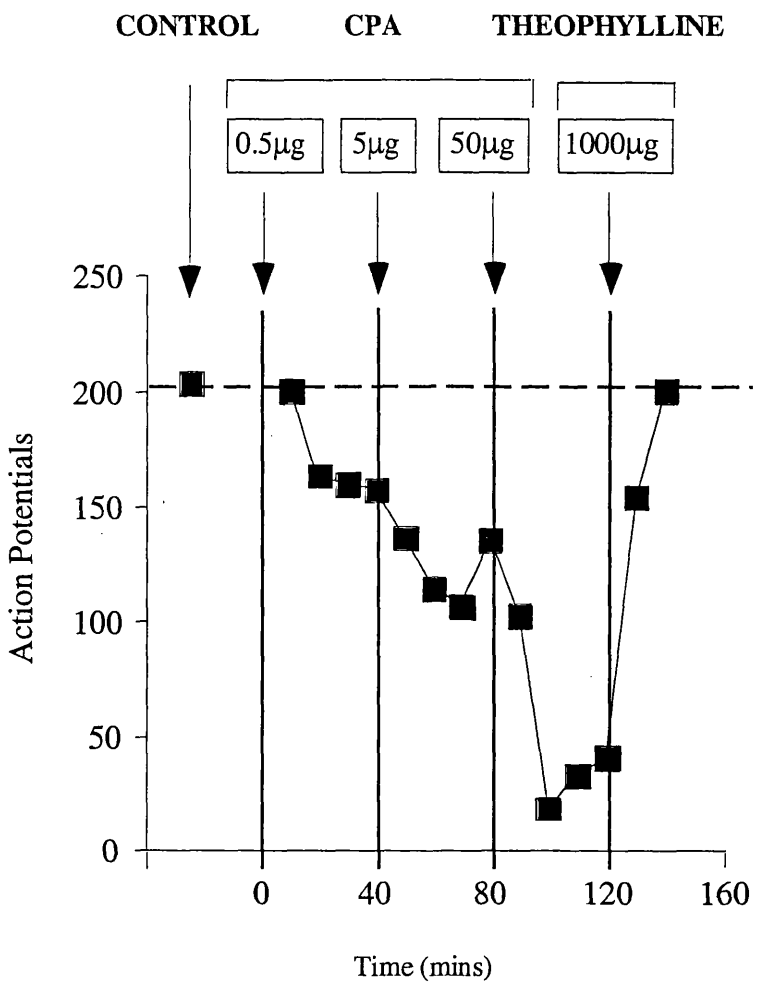


Figure 24. Typical time-course of the effect of CPA on the C-fibre evoked response of a single dorsal horn neurone. Cumulative doses of CPA were administered intrathecally and its effect was followed over a period of 40 minutes per dose. CPA produced inhibitions of the C-fibre evoked response and the inhibition was reversed with spinal theophylline after the final dose of CPA (50µg).

The administration of CPA produced only minor inhibitions of the Aβ-fibre evoked response in both SNL (maximal inhibition, 20± 4%) and sham operated rats (maximal inhibition, 23± 5%). The inhibition did not exceed 25% even with the highest dose of CPA employed in this study, and the effects were comparable between the two animal groups (Fig. 25A).

In contrast, CPA produced a significant inhibition of the C-fibre evoked response in SNL (0.5μg, p=0.03; 5 and 50μg, p=0.02) and sham operated rats (0.5, 5, 50μg, p=0.03). The magnitude of the inhibition was greater in SNL rats (maximal inhibition, 72± 7%) compared to sham operated rats (maximal inhibition, 55± 16%), however, this was not significant (Fig. 25B).

Similarly, the initial input of spinal neurones was dose-dependently inhibited by CPA in both SNL (0.5μg, p=0.03; 5 and 50μg, p=0.02) and sham operated rats (0.5, 5 and 50μg, p=0.03). The dose response curves for the C-fibre evoked response and input showed a similar pattern over the dose range of CPA used (Fig. 25C).

CPA produced a significant inhibition of the postdischarge of spinal neurones in SNL (0.5μg, p=0.03; 5 and 50μg, p=0.02) and sham operated rats (50μg, p=0.03). CPA produced a greater inhibition of the postdischarge in SNL rats (maximal inhibition, 94± 3%), as compared to sham operated rats (maximal inhibition, 70± 18%) although this difference did not reach significance (Fig. 25D). As with the C-fibre evoked response, the increased effect of CPA after nerve injury was manifested as a parallel leftward shift in the dose-response curve.

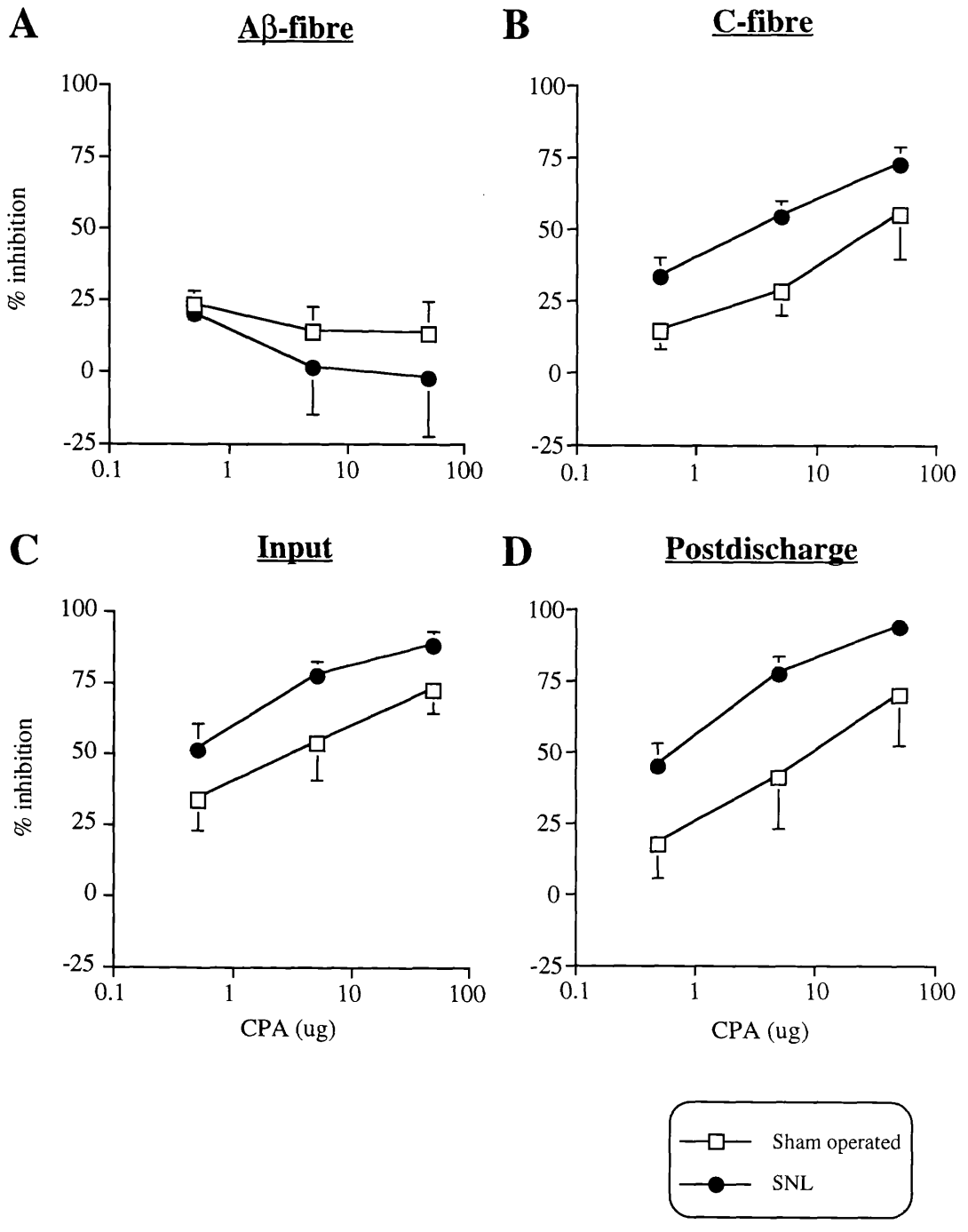


Figure 25. The effect of intrathecal CPA administration on the (A) Aβ-fibre evoked response, (B) C-fibre evoked response, (C) input and (D) postdischarge of spinal neurones in SNL and sham operated rats. Data are presented as percentage inhibitions of the pre-drug control values ± S.E.M.

The wind-up of spinal neurones was reduced dose-dependently by CPA in SNL rats (5 and 50μg, p=0.03). The inhibition of the wind-up was again greater in SNL rats (maximal inhibition, 85± 9%) than in sham operated rats at higher doses of the drug (maximal inhibition, 56± 19%) (5μg, p=0.04; Fig. 26A). Following the administration of 50μg CPA, the wind-up was dramatically reduced or abolished in most neurones of SNL rats. An example of the effect of CPA on the wind-up of a single dorsal horn neurone is illustrated in Fig 26B.

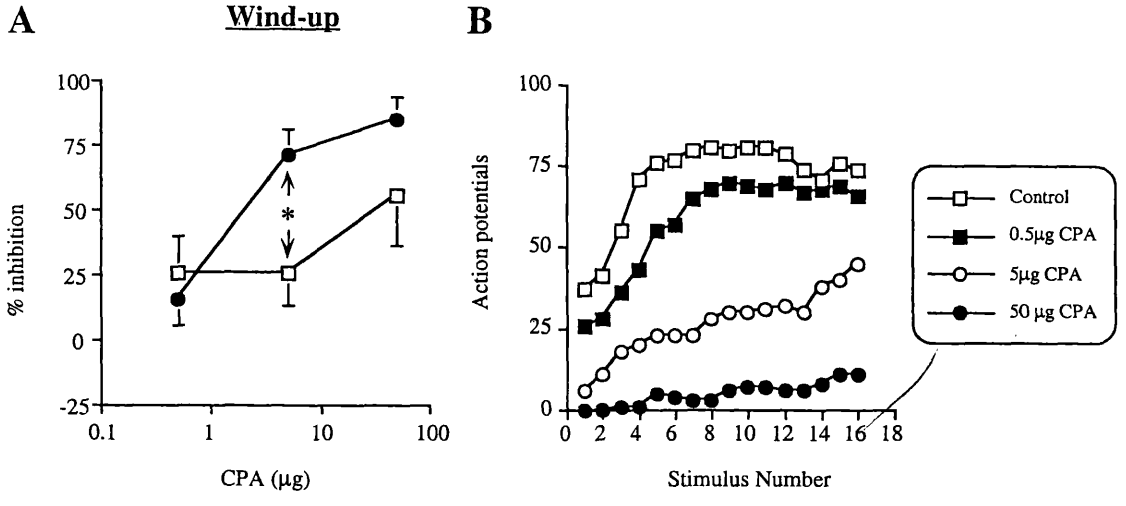


*Figure 26. (A) The effect of intrathecal CPA administration on the wind-up of spinal neurones in SNL and sham operated rats. Data are presented as percentage inhibitions of pre-drug control values ± S.E.M. Level of significance: * $P \leq 0.05$. (B) An example of the effect of CPA (0.5, 5 and 50μg) on the wind-up of a single dorsal horn neurone in a SNL rat. Trains of 16 electrical stimuli were given at 3 times the threshold current for C-fibres and the number of action potentials evoked per stimulus were plotted against the stimulus number, before and after drug administration.*

Interestingly, the effect of CPA on the Aδ-fibre evoked response was markedly different in SNL and sham operated rats (Fig. 27). In contrast to the dose-related inhibitory effects seen with CPA on the C-fibre evoked response and measures of hyperexcitability (wind-up and postdischarge), CPA produced a marked facilitation of the Aδ-fibre evoked response in sham operated rats. At the highest dose of the drug (50μg), the Aδ-fibre evoked response was facilitated to 194± 55% of the pre-drug control value. Remarkably, in complete contrast to this facilitatory effect, CPA produced little effect on the Aδ-fibre evoked response of SNL rats and the facilitation was entirely absent in this group of animals.

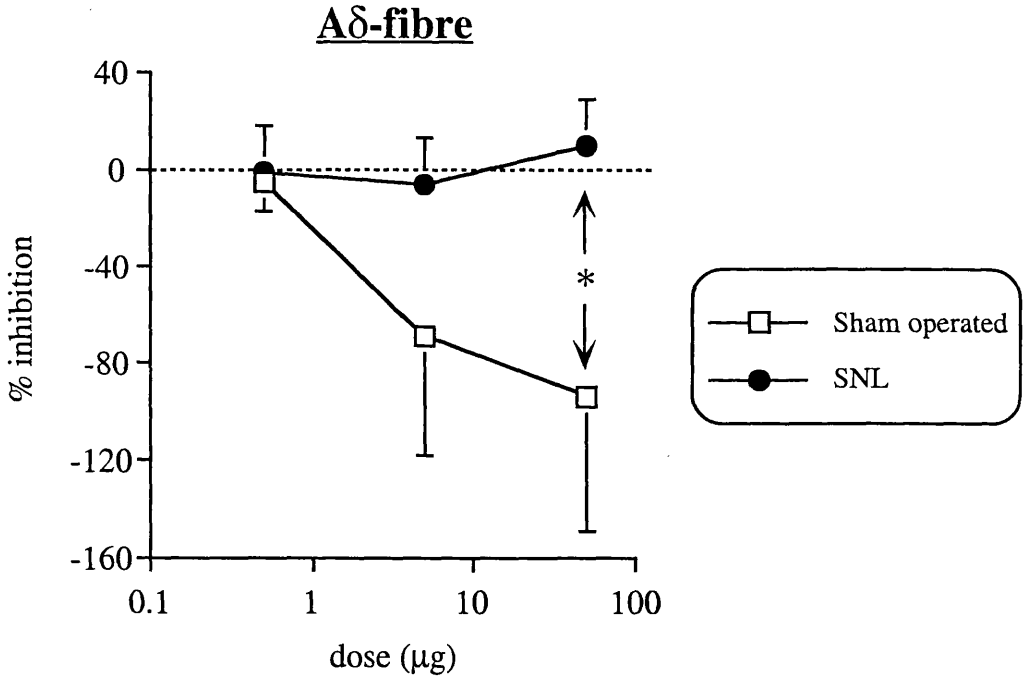


*Figure 27. The effect of intrathecal CPA on the Aδ-fibre evoked response of spinal neurones in SNL and sham operated rats. The Aδ--fibre evoked response of sham operated rats showed a marked facilitation following the administration of CPA. This facilitation was completely absent in SNL rats. Data are presented as percentage inhibitions of pre-drug control values ± S.E.M. * p≤0.05.*

The natural (mechanical / thermal) evoked response of SNL and sham operated rats was also inhibited dose-dependently by the administration of CPA. CPA produced a significant reduction of both the low (9g von Frey; 0.5, 5 and 50μg, p=0.03) and high intensity mechanical evoked response of SNL rats (50g von Frey; 0.5μg, p=0.03; 5 and 50μg, p=0.02). Similarly in sham operated rats, both the 9g von Frey (5 and 50μg, p=0.03) and 50g von Frey evoked responses were significantly reduced (5 and 50μg, p=0.03). The inhibition of the 9g von Frey evoked response was significantly greater in SNL rats (5μg, p=0.01; Fig. 28A). The inhibition of the noxious 50g von Frey evoked response was also greater in SNL rats (maximal inhibition, 88± 6%) than in sham operated rats (maximal inhibition, 77± 5%), however, this difference was non-significant (Fig. 28B).

Similarly, CPA produced a dose dependent inhibition of the thermal evoked response of SNL (0.5, 5 and 50μg, p=0.03) and sham operated rats (0.5, 5 and 50μg, p=0.04; Fig. 28C). At lower doses of the drug, CPA produced a greater inhibition of the thermal evoked response in SNL rats (maximal inhibition, 88± 7%) compared to sham operated rats (maximal inhibition, 74± 10%) (0.5μg, p=0.03; 5μg, p=0.04).

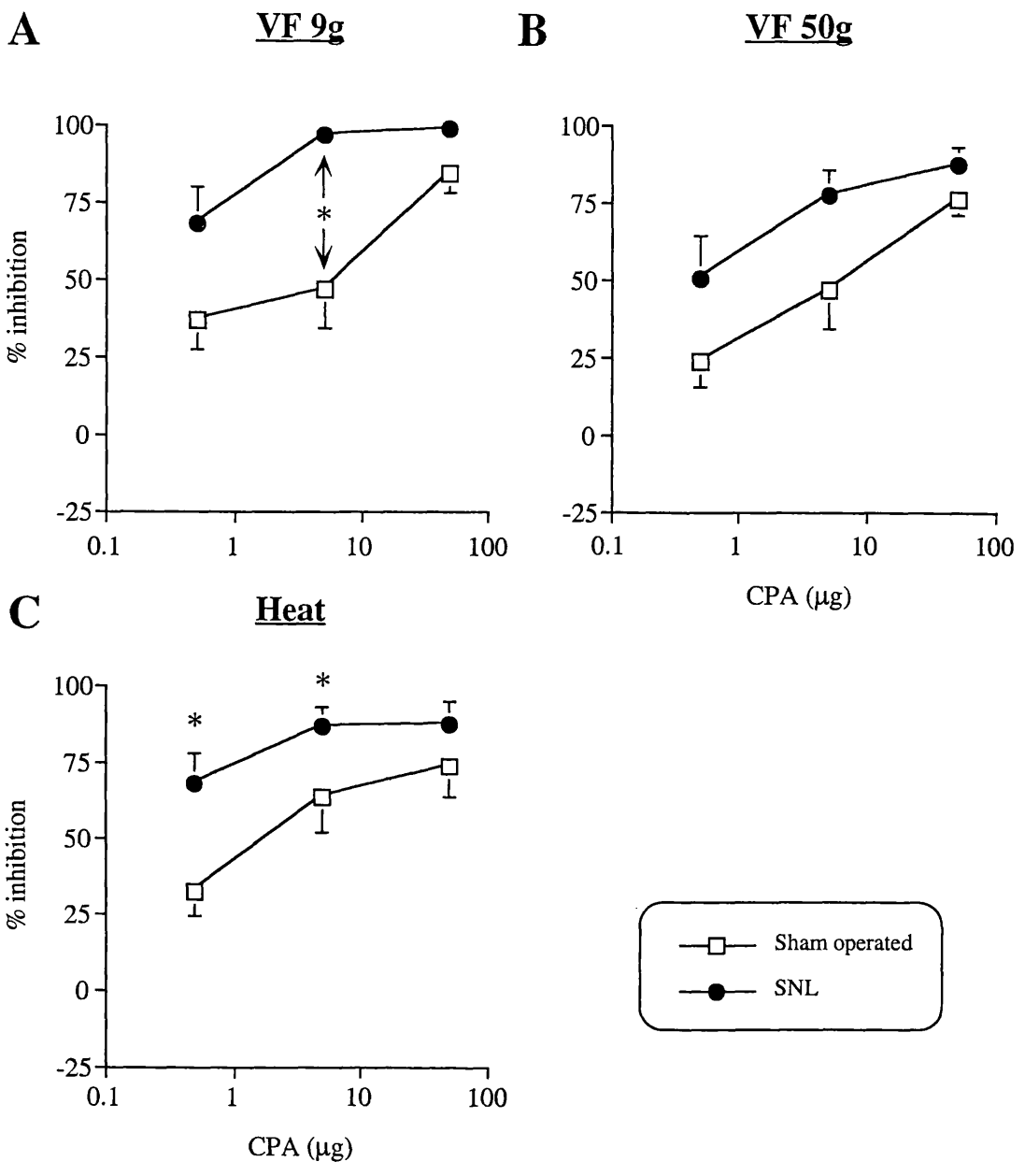


*Figure 28. The effect of intrathecal CPA on the evoked responses of spinal neurones to (A) von Frey 9 g, (B) von Frey 50g and (C) heat (45°C) in SNL and sham operated rats. Data are presented as percentage inhibitions of pre-drug control values ± S.E.M. Level of significance: * $P \leq 0.05$.*

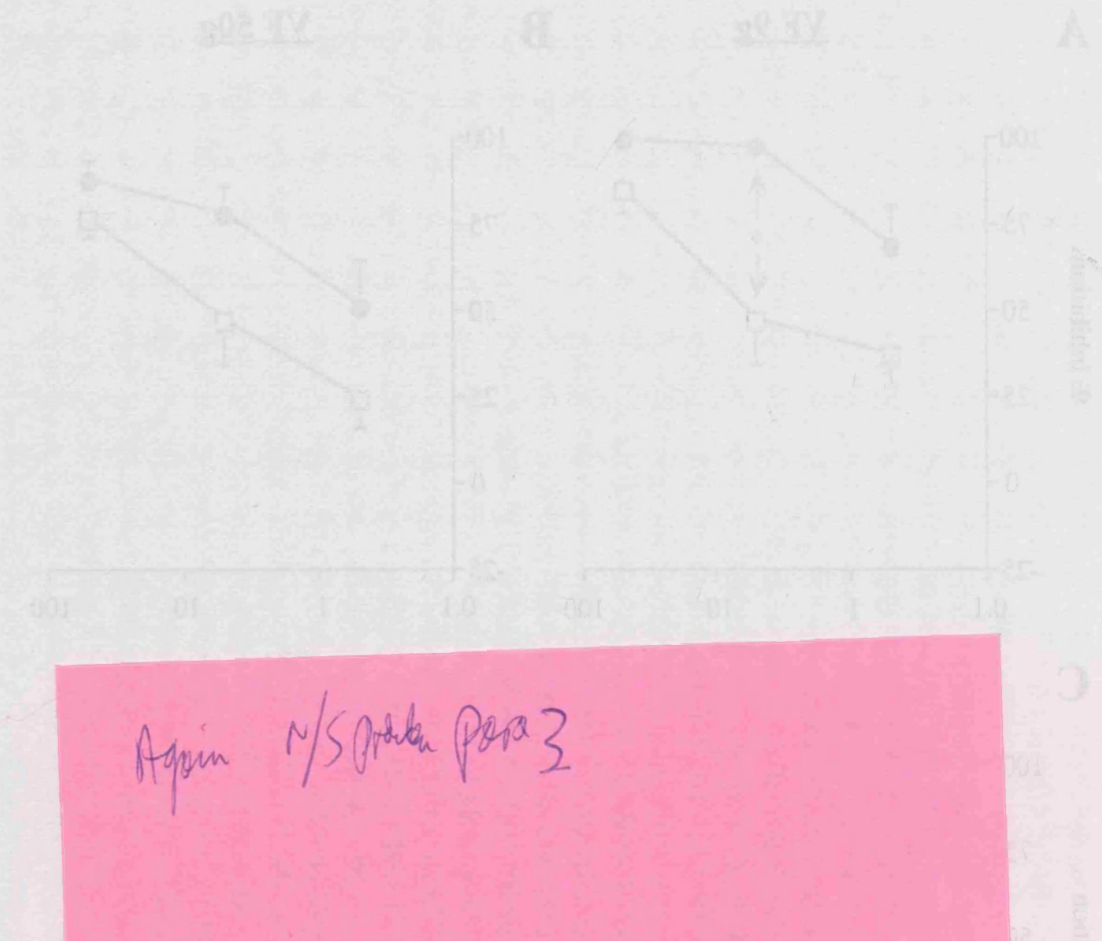


Figure 26. The effect of intrathecal CPA on the evoked responses of spinal neurones to (A) von Frey 9 g, (B) von Frey 50g and (C) heat (45°C) in SNL and sham operated rats. Data are presented as percentage inhibitions of predrug control values ± S.E.M. Level of significance: * $P \leq 0.05$.

The inhibitory effect of CPA was reversed to near pre-drug control values with spinal theophylline (1000μg) in both groups of animals. For example, the inhibition of the C-fibre evoked response and the 50g von Frey evoked response after 50μg CPA were reversed to 101±2 and 97±4% of pre-drug control values with the administration of theophylline, respectively (see Fig.24). The facilitation of the Aδ-fibre evoked response, however, was not reversed with theophylline and remained at 164± 38% of pre-drug control.

6.3.2 The effect of A200702.21 on the electrical and natural evoked responses of dorsal horn neurones

Recordings were made from a total of 12 dorsal horn neurones in SNL (n=6) and sham operated rats (n=6) at PO 14-17 days, with mean depths of 781 ± 51μm and 733 ± 33μm, respectively.

The administration of A200702.21 produced only a minor inhibition of the Aβ-fibre evoked response in both SNL and sham operated rats, and did not exceed 25% even with the highest dose of A200702.21 employed in this study (Fig. 29A). The effect of the drug on the Aβ-fibre evoked response was comparable in both groups of animals. Similarly, A200702.21 produced relatively small reductions of the C-fibre evoked response of spinal neurones (SNL: 10mg/kg, p=0.005; Fig. 29B). A200702.21 produced a slightly greater inhibition of the C-fibre evoked response in SNL rats (maximal inhibition, 36±3%) as compared to sham operated rats (11±11%). The difference was not significant.

A200702.21 produced a significant inhibition of the input in SNL (1mg/kg, p=0.03, 10mg/kg, p=0.005) and sham operated rats (10mg/kg, p=0.008; Fig. 29C). Similarly, the wind-up of spinal neurones was reduced in both groups of animals (SNL: 10mg/kg, p=0.04; sham operated: 10mg/kg, p=0.04). The effect of the drug was comparable between the animal groups for both neuronal measures (Fig. 29D).

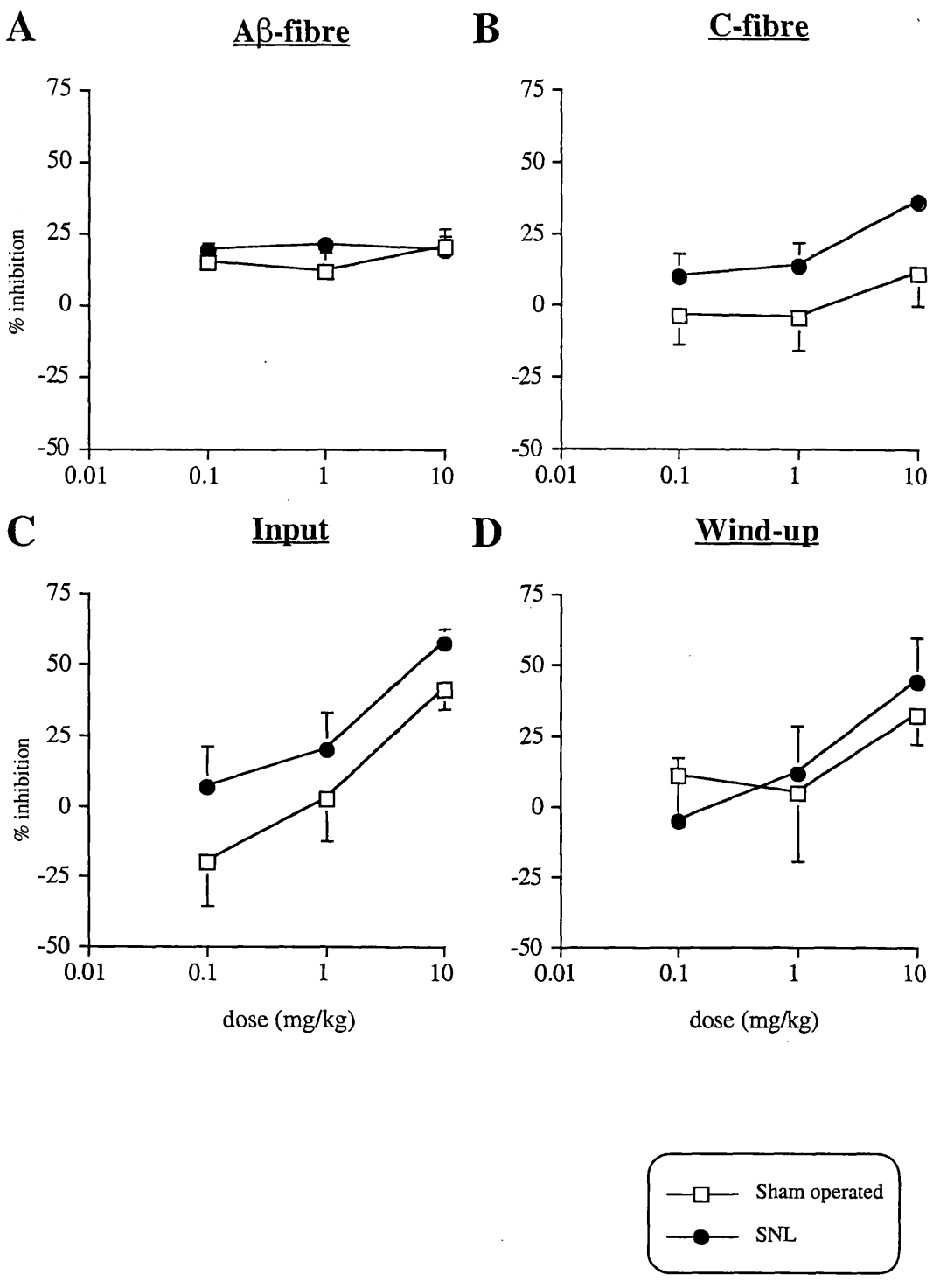


Figure 29. The effect of subcutaneous A200702.21 administration on the (A) Aβ -fibre evoked response, (B) C-fibre evoked response, (C) input and (D) wind-up of spinal neurones in SNL and sham operated rats. Data are presented as percentage inhibitions of pre-drug control values ± S.E.M.

A200702.21 produced inhibitions of the Aδ-fibre evoked response (10mg/kg, p=0.008) and postdischarge of spinal neurones in SNL rats (10mg/kg, p=0.005). In sham operated rats, however, both the Aδ-fibre evoked response and postdischarge were facilitated following the administration of A200702.21 (p>0.05). A200702.21 produced a greater inhibitory effect in SNL rats, as compared to sham operated rats although this difference was not significant.

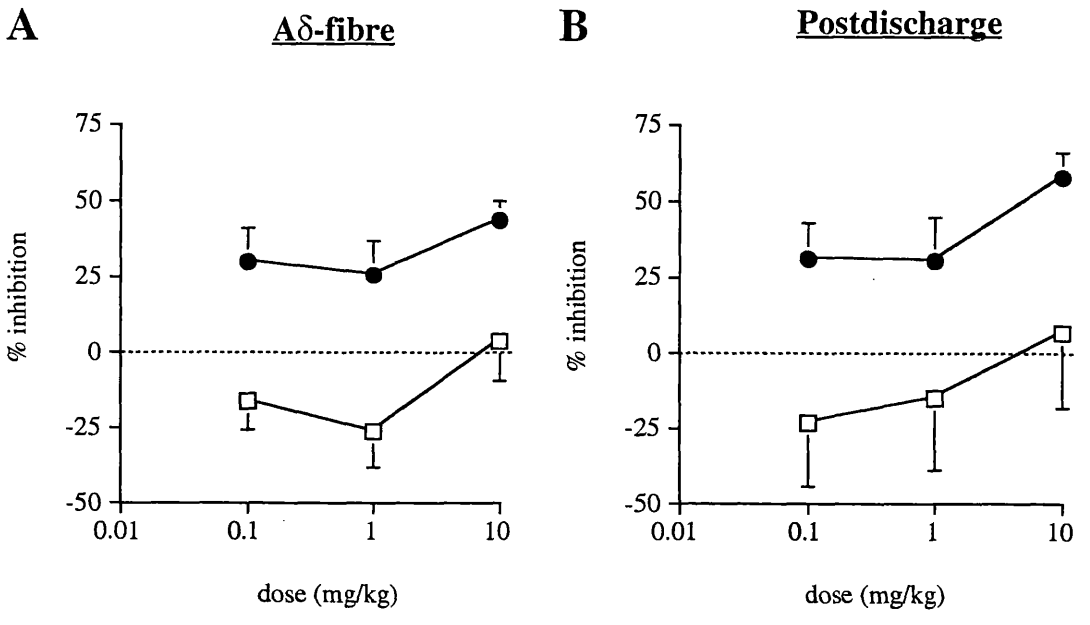


Figure 30. The effect of subcutaneous A200702.21 administration on the (A) Aδ -fibre evoked response and (B) postdischarge of spinal neurones in SNL and sham operated rats. Data are presented as percentage inhibitions of pre-drug control values ± S.E.M.

Both the innocuous (von Frey 9g) and noxious (von Frey 50g) mechanical evoked responses were reduced following the administration of A200702.21. A200702.21 produced a significant inhibition of the 9g von Frey evoked response in SNL (1 and 10mg/kg, p=0.02) and sham operated rats (1 and 10mg/kg, p=0.04). Similarly, the 50g von Frey evoked response was reduced by A200702.21 (SNL: 0.1mg/kg, p=0.03; 1 and10mg/kg, p=0.01; sham operated: 1 and 10mg/kg, p=0.03). Overall, A200702.21 produced a greater inhibitory effect in SNL rats, as compared to sham operated rats, however, this was not significant.

The thermal evoked response of spinal neurones was similarly reduced by A200702.21 in SNL (0.1, 1 and 10mg/kg, p=0.04) and sham operated rats (0.1mg/kg, p=0.04; 1mg/kg, p=0.03). The inhibition tended to be greater in SNL rats (p>0.05).

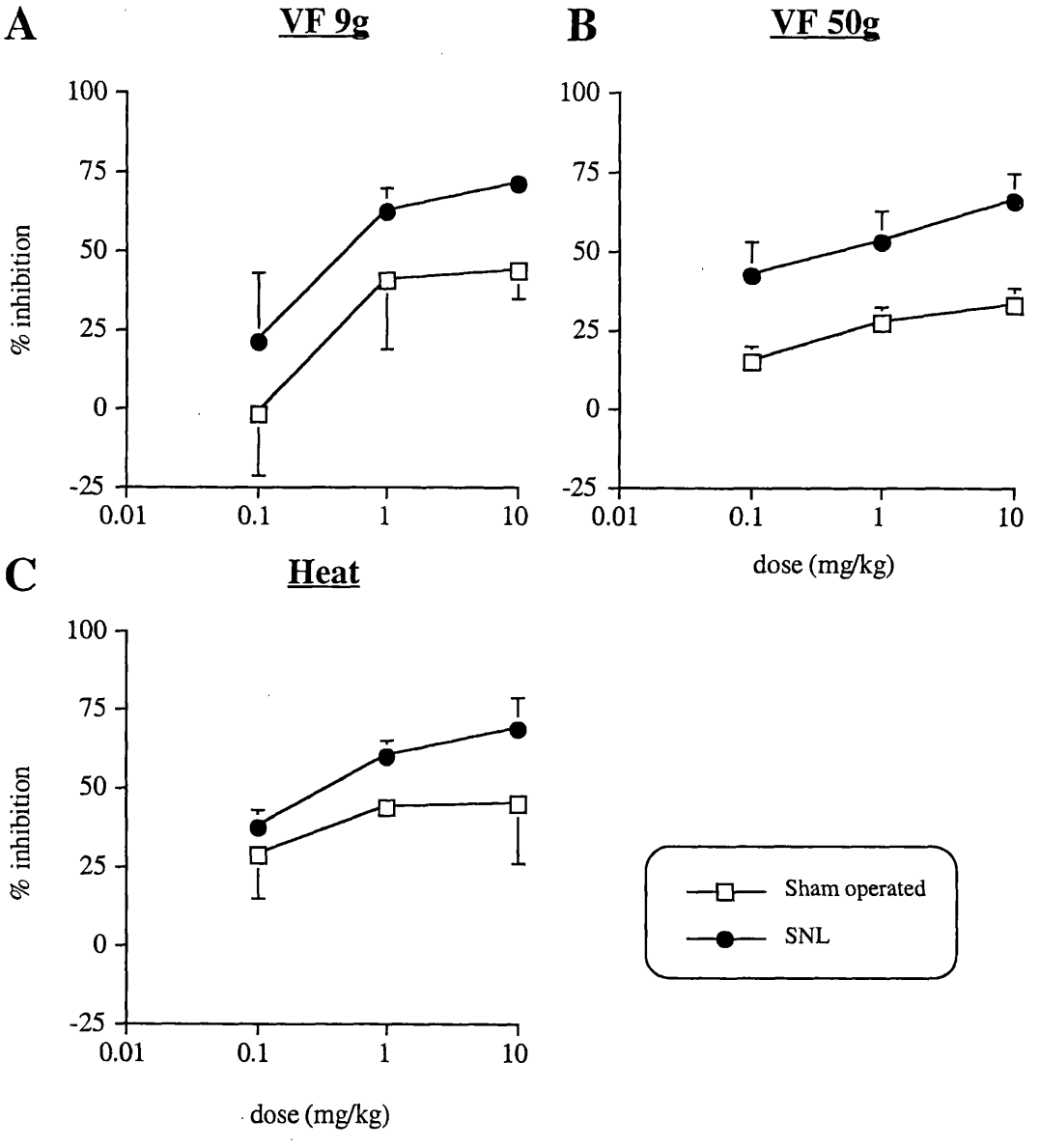


Figure 31. The effect of subcutaneous A200702.21 administration on the evoked responses of spinal neurones to (A) von Frey 9g, (B) von Frey 50g and (C) heat in SNL and sham operated rats. Data are presented as percentage inhibitions of pre-drug control values ± S.E.M.

6.4 Discussion

The results from this study provide further evidence for a role of the adenosine receptor system in nociceptive transmission. Intrathecal administration of the adenosine A_1 receptor agonist, CPA, produced powerful inhibitions of both the electrical and natural evoked responses of SNL and sham operated rats, 2 weeks after nerve injury. CPA produced significant inhibitions of the C-fibre evoked response, input, postdischarge and wind-up of spinal neurones, and reduced the evoked neuronal responses to low- and high-threshold mechanical and thermal stimuli. The Aβ-fibre evoked responses, however, remained relatively unaltered. The ability of CPA to produce marked inhibitions of the high-threshold C-fibre evoked responses yet unalter the low-threshold Aβ-fibre evoked responses suggests that its actions are selective for noxious inputs. However, rather paradoxically, despite the lack of effect on Aβ-fibre evoked response, CPA reduced the low intensity 9g von Frey evoked response in both groups of animals. It is likely that this natural mechanical stimulus, which may include a contribution from both Aβ- and Aδ-fibres, would produce a pattern of activity more amenable to inhibition than the suprathreshold synchronised responses elicited by the electrical activation of Aβ-fibres. These results therefore indicate a broad spectrum of action of CPA that may result from a combination of pre- and post-synaptic actions.

Adenosine exerts its actions primarily through postsynaptic events via the activation of K^+ channels which consequently produces membrane hyperpolarisation (Doi *et al.*, 1987; Li & Perl 1994; Ocana & Baeyens 1994; Salter *et al.*, 1993). A_1 receptors have been identified in the spinal cord where they are localised on intrinsic cells (Choca *et al.*, 1988). In support of these findings, I demonstrated that CPA reduces the wind-up of dorsal horn neurones, which reflects postsynaptic NMDA receptor-mediated activity. Furthermore, CPA also inhibited the evoked neuronal responses to natural stimuli and the dose-response curves were similar for all modalities. Hence, the ability of CPA to reduce these neuronal measures suggests an overall postsynaptic site of action of the drug. Although A_1 receptors are predominantly located in the superficial dorsal horn, their location on

interneuronal systems or dendritic trees of the deeper neurones I recorded from, could be responsible for the observed effects. CPA could therefore modulate sensory transmission through the activation of the A_1 receptor, thereby producing inhibitions of excitatory interneurones in the NMDA receptor polysynaptic pathway. Hence the indirect modulation of the excitatory nociceptive pathway may be one mechanism by which adenosine-mediated antinociception is mediated at the level of the spinal cord (Reeve & Dickenson 1995a; Reeve & Dickenson 1995b).

In addition to the inhibitory effects on postdischarge, wind-up and natural evoked responses, I observed marked reductions of the input and C-fibre evoked response following CPA administration. The inhibitions of these measures (input and C-fibre evoked response) are suggestive of a pre-synaptic A_1 receptor location, acting to inhibit transmitter release from small diameter afferent fibres. CPA also reduced neuronal responses to high intensity natural stimuli (von Frey 50g) which could also be explained through presynaptic effects of the drug. These results are consistent with previous findings where a presynaptic action of adenosine has been proposed on the basis of an inhibition of primary afferent Ca^{2+} currents and spinal release of neuropeptides (Santicioli *et al.*, 1992; Santicioli *et al.*, 1993; Sjolund *et al.*, 1997). Furthermore, there is evidence to suggest that adenosine modulates primary afferent transmission in the substantia gelatinosa of the spinal cord (Li & Perl 1994).

Overall, the inhibitory effects of CPA were greater in SNL rats compared to those in the sham operated group, and there was a leftward-shift in the dose-response curves after nerve injury. Previous studies have proposed possible interactions between adenosine and glutamate in the spinal cord (Dolphin & Prestwich 1985), and between NMDA receptor activation and adenosine release elsewhere in the brain (Craig & White 1992). Following neuropathy, there appears to be an enhanced role of the NMDA receptor system in both low- and high-threshold signalling in the spinal cord (Mao *et al.*, 1992; Qian *et al.*, 1996). Spinal hyperexcitability, which occurs partly as a result of a sustained afferent drive from the injury site, is likely to produce a greater degree of NMDA receptor activation following peripheral nerve injury. This, in turn, would lead to an increased release of adenosine, resulting in an eventual depletion of the purine. A deficiency in circulating and CSF adenosine levels has been reported in patients with neuropathic

pain (Guieu *et al.*, 1996). Previous studies have suggested the existence of an endogenous adenosine tone in the spinal cord which modulates sensory transmission (Keil & DeLander 1994; Keil & DeLander 1996). It could be envisaged that any disruption of the endogenous purinergic inhibitory tone as a result of nerve injury would augment spinal nociceptive transmission, thus contributing to facilitated sensory transmission (hyperalgesia) and miscoding of innocuous information (allodynia). Furthermore, any reduction in the level or release of adenosine would be expected to induce receptor upregulation. To date, changes in adenosine receptor expression following nerve injury have not been reported although the enhanced effectiveness of CPA seen here in SNL rats would support this idea.

In direct contrast to these powerful inhibitory effects, CPA produced a dose-dependent facilitation of the Aδ-fibre evoked response in sham operated rats, an observation also previously reported in naive rats (Reeve & Dickenson 1995a; Reeve & Dickenson 1995b). Since the A_1 receptor is inhibitory, the observed facilitation is likely to result from a postsynaptic disinhibitory action. The Aδ-fibre terminals in the spinal cord are known to be contacted by GABAergic neurones (Alvarez *et al.*, 1992; Bernardi *et al.*, 1995). Activation of the A_1 receptor on these inhibitory interneurones would cause hyperpolarisation therefore producing a reduction in the overall inhibitory tone. This would consequently result in a net facilitation of the Aδ-fibre evoked response (i.e. disinhibition), as observed after CPA administration in the present study (Fig. 32). During neuropathy, however, there appears to be a progressive loss of inhibitory interneurones such as the GABA system (Ibuki *et al.*, 1997; Ralston *et al.*, 1997). The loss of inhibitory controls may explain why the facilitation of Aδ-fibre evoked response was completely absent in SNL rats. Interestingly, in a recent study where adenosine was administered intrathecally to patients with chronic neuropathic pain, some reported transient pain on initial injection, which was later followed by a prolonged pain relief (Belfrage *et al.*, 1999). In addition to the mechanisms which have been proposed, it is possible that the pain experienced by these patients is due to the disinhibitory action of adenosine on Aδ-fibres. This would cause a net facilitation in areas of the spinal cord outside the neuropathic zone, consequently evoking a pain sensation (Belfrage *et al.*, 1999).

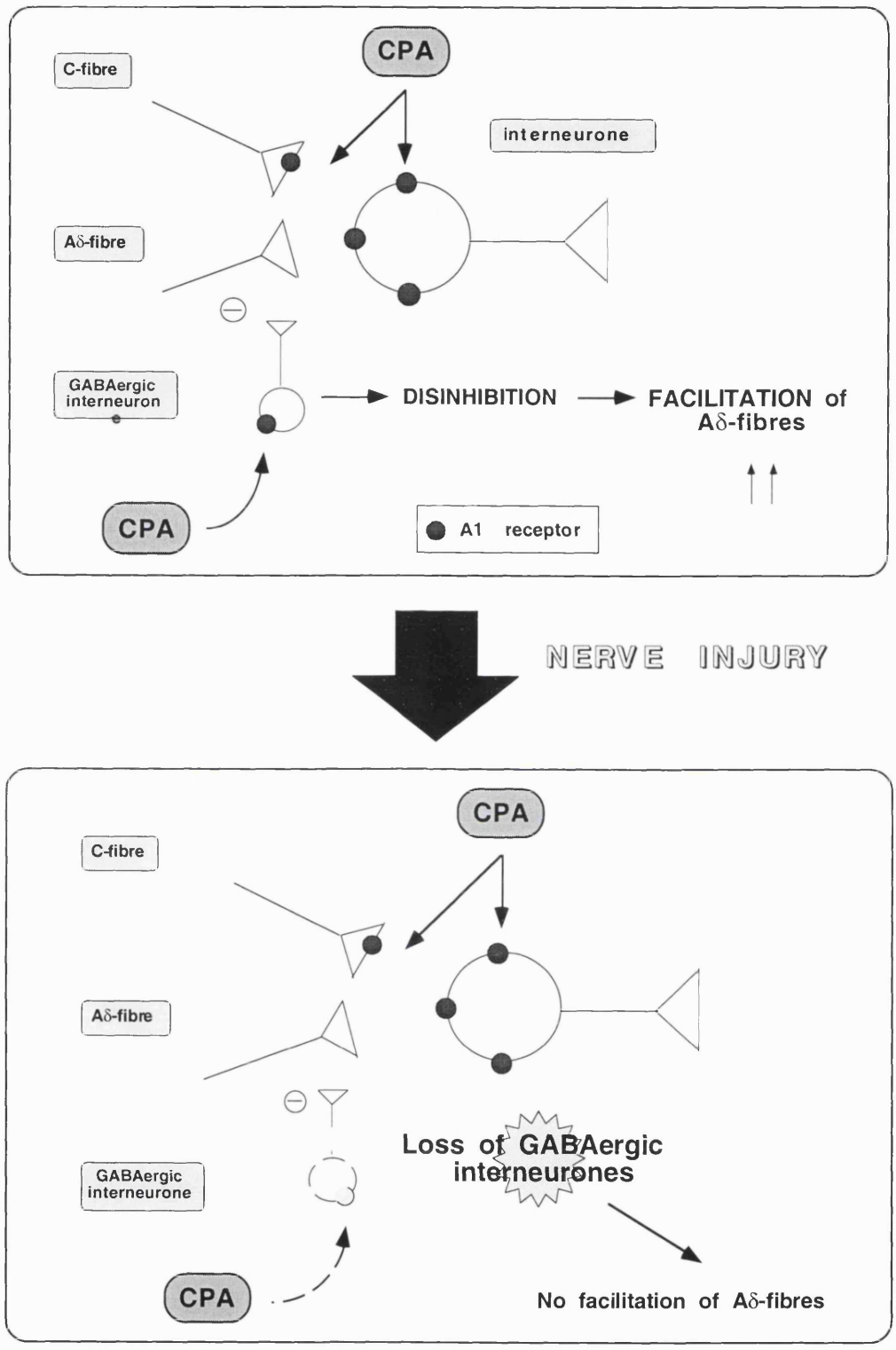


Figure 32. Possible mechanisms underlying the facilitation of Aδ--fibre evoked response following intrathecal CPA administration in sham operated rats, and changes after nerve injury.

In order to establish the role of the endogenous adenosine system after nerve injury, I investigated the effect of A200702.21, an adenosine kinase inhibitor, which acts to reduce the metabolism of adenosine. A200702.21 reduced both the electrical (Aδ- and C-fibre evoked response, input, postdischarge, wind-up) and natural (mechanical / thermal) evoked response of spinal neurones in SNL rats. Overall, the inhibitions tended to be slightly greater in SNL rats, as compared to sham operated rats, although this did not reach significance level. The enhanced effect of A200702.21 after nerve injury may possibly indicate an upregulation of A_1 receptors, which would also support the observation of a greater effectiveness of CPA in SNL rats. Alternatively, there may also be an increased release of adenosine in the spinal cord. The increased afferent input from the periphery after nerve injury gives rise to spinal hyperexcitability and may evoke a greater release of the purine, either from primary afferent fibres or intrinsic cells in the spinal cord. When it is protected from breakdown by A200702.21, this may result in an increased pool of adenosine available for interaction with spinal adenosine receptors, therefore allowing greater inhibitions to be attained. These results suggest that the manipulation of endogenous adenosine level induces antinociception and further supports the role of adenosine kinase in the modulation of nociceptive transmission. Interestingly, when compared to the effects of CPA, the A_1 receptor agonist, A200702.21 was not as effective in reducing the neuronal responses. Whilst CPA produced a near abolition of the natural evoked response (80-99%), A200702.21 in comparison, produced a smaller effect. Similarly, the inhibition of the electrical evoked response rarely exceeded 50-60% after A200702.21 administration. This difference may reflect access of adenosine released in the spinal cord to the target receptor and differences in both uptake and receptor activation between the transmitter and synthetic agonist (see Fig. 33).

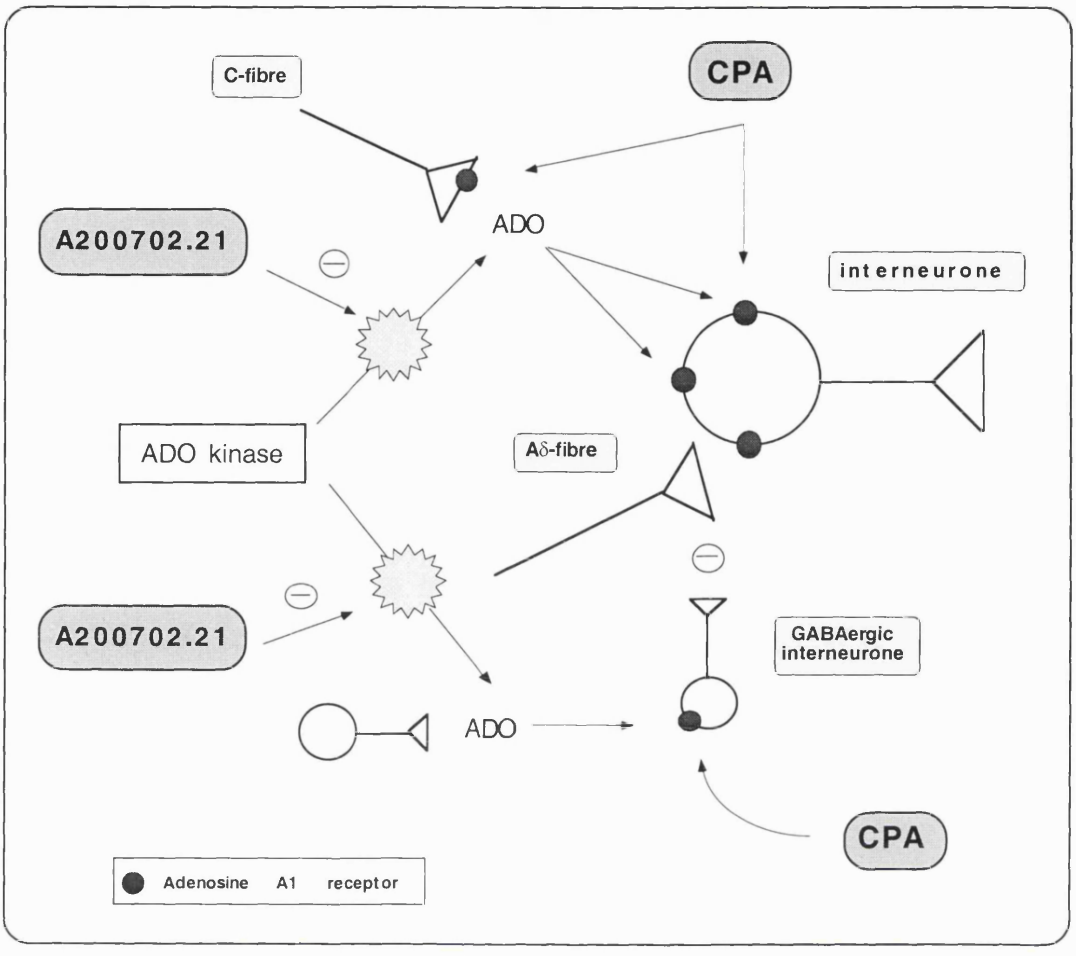


Figure 33. Possible sites of action of CPA and A200702.21 in the spinal cord. Abbreviations: ADO (adenosine)

These results support previous behavioural findings where adenosine analogues have been found to produce antinociception in animal models of neuropathy. Studies have demonstrated that the administration of R-PIA (R-phenylisopropyl-adenosine), an A_1 receptor agonist, attenuates the spontaneous scratching behaviour (Sjolund *et al.*, 1996) and allodynia in CCI rats (Cui *et al.*, 1997). Allodynia was also suppressed following R-PIA administration in SNL rats (Lavand'homme & Eisenach 1999; Lee & Yaksh 1996) and in rats with photochemically induced spinal cord injury (Sjolund *et al.*, 1998; von Heijne *et al.*, 1998). Development of tolerance to R-PIA was reported in a study after repeated intrathecal drug administration (von Heijne *et al.*, 1998). Similarly, spinal administration of NECA (5'-N-ethylcarboxamide-adenosine), a non-selective A_1/

A_2 receptor agonist, was also demonstrated to be effective against thermal hyperalgesia in CCI rats (Yamamoto & Yaksh 1991). In addition to these studies which have employed analogues of adenosine, the effects of adenosine itself have been investigated in a recent study, where it was shown to attenuate the allodynia of SNL rats without motor blockade (Lavand'homme & Eisenach 1999). In another study, however, intrathecal adenosine failed to reverse allodynia in spinally injured rats, although R-PIA proved to be effective (von Heijne *et al.*, 1999). Further studies are needed to clarify this discrepancy. In addition to these studies which employed the direct manipulation of the adenosine receptor system through exogenous administration of adenosine analogues, a recent study demonstrated that adenosine kinase inhibitors are also effective against allodynia (Lavand'homme & Eisenach 1999). None of these animal behavioural studies used a broad range of stimuli so that the complete role of the A_1 receptor has been difficult to assess, in terms of its effect on different sensory modalities. The findings of my electrophysiological study support these behavioural studies where adenosine analogues were found to be effective against some of the symptoms of nerve injury states, and possibly provide a neuronal basis for these findings. Consistent with previous studies, I demonstrated that CPA reduces the acute nociceptive responses of dorsal horn neurones and further extended these observations by demonstrating that controls exerted by the A_1 receptor include a large range of modalities (low and high intensity mechanical and thermal stimuli).

Clinically, there has been a considerable interest in the potential therapeutic use of adenosine and its agonists in neuropathic pain states. In patients with peripheral neuropathic pain, systemic infusion of adenosine was shown to provide significant relief of spontaneous pain and tactile allodynia, lasting from several hours to a few days (Belfrage *et al.*, 1995; Sollevi *et al.*, 1995). Similarly, intrathecal administration of adenosine (Belfrage *et al.*, 1999) and its analogue attenuated allodynia in patients with chronic neuropathic pain (Karlsten & Gordh 1995).

In conclusion, the present finding that CPA and A200702.21 produce inhibitions of the electrical and natural evoked responses of spinal neurones supports a role for these compounds as potential therapeutics for the treatment of neuropathic pain. One limitation in the use of adenosine analogues is the

occurrence of side effects (e.g. motor dysfunction and cardiac effects) which are associated with higher doses of the drug (DeLander & Hopkins 1987; Karlsten *et al.*, 1990; Lee & Yaksh 1996; von Heijne *et al.*, 1999). The motor dysfunction appears to be primarily an A_2 receptor-mediated effect in rats (Karlsten *et al.*, 1990), although, when given at very high doses, A_1 receptor agonists also produce similar impairments. The leftward shift in the dose-response curve for CPA after neuropathy favours the use of lower doses of the drug in nerve injury pain states. The spinal administration of adenosine kinase inhibitors, on the other hand, has been reported to be devoid of side effects (Lavand'homme & Eisenach 1999). The use of adenosine metabolism inhibitors allows the selective recruitment of the endogenous adenosine system and potentially minimise side effects. This may represent a more favourable approach than the administration of adenosine analogues. To date, the effect of adenosine modulators (e.g. adenosine kinase inhibitor) has not been investigated in humans and preclinical toxicity of these agents have not been reported. Further trials of the potential use of these compounds in neuropathic pain states are required. Another approach may be to employ adenosine in conjunction with other agents, through combination therapy. Previous studies have shown that coadministration of low doses of R-PIA and baclofen, a $GABA_B$ agonist, potentiates their antiallodynic effects (Cui *et al.*, 1998). Similarly, the combination of spinal adenosine and spinal morphine produced an additive enhancement of the antiallodynic effect in SNL rats, in the absence of side effects (Lavand'homme & Eisenach 1999). Hence the combined effect of adenosine, together with other analgesic agents (e.g. opioids) may provide a beneficial approach to the treatment of neuropathic pain states by reducing the dose requirements of each drug, and furthermore enhance opioid therapy in patients who respond poorly to opioid treatments.

Chapter 7.

The effectiveness of spinal and systemic morphine on the responses of dorsal horn neurones in the selective spinal nerve ligation model of neuropathy

7. The effectiveness of spinal and systemic morphine on the responses of dorsal horn neurones in the selective spinal nerve ligation model of neuropathy

7.1 Introduction

Despite the continuing search for novel and improved targets for the treatment of neuropathic pain, the clinical management of this chronic pain state still remains inadequate. Whilst the efficacy of opioids in acute pain conditions is well established, there is considerable debate over the issue of opioid sensitivity in neuropathic pain states. To date, clinical and animal studies have reported conflicting views on the effectiveness of morphine, ranging from no pain relief to potent analgesia. Early studies by Arnér and Meyerson (1988) demonstrated that neuropathic pain is unresponsive to treatment with opioids. In contrast, other studies reported that intravenous infusion of morphine produces adequate analgesia, provided that sufficient doses were given through dose-escalation (Portenoy *et al.*, 1990). In support of these findings, studies also show that intrathecal (Krames & Lanning 1993) and intravenous morphine (Rowbotham *et al.*, 1991) can be effective in patients with neuropathy, although the degree of analgesia attained was less, as compared to those with nociceptive pain (Jadad *et al.*, 1992).

The question 'Are opioids effective in neuropathic pain?' has been frequently raised in the clinic and numerous studies have been conducted over the past decade to resolve this controversy. Clearly, the major problem associated with the use of opioids is the occurrence of adverse effects, which include nausea, vomiting, dizziness, constipation and sedation. This can be partly overcome by selecting the appropriate route for administering the opioid, thus allowing the desired concentration of the drug to be achieved only at the target organ (Kalso 1999). The poor effectiveness of opioids seen in a proportion of neuropathic patients may therefore reflect the difficulty in achieving a sufficient dose-escalation, before side effects of the drug become intolerable.

Using animal models of neuropathic pain, the effect of opioids following nerve injury has been extensively studied. Systemic morphine has been shown to reverse allodynia, hyperalgesia and spontaneous pain behaviour in CCI rats (Backonja *et al.*, 1995; Hedley *et al.*, 1995; Jazat & Guilbaud 1991; Kayser *et al.*, 1995; Koch *et al.*, 1996). However, the intrathecal route of administration had reduced effectiveness against thermal hyperalgesia in the same model (Mao *et al.*, 1995; Yamamoto & Yaksh 1991). Similarly, in the selective spinal nerve (L5/ L6) ligation model of neuropathy (Kim & Chung 1992), morphine administration via systemic and supraspinal routes was effective in attenuating allodynia although intrathecal morphine had little effect (Lee *et al.*, 1995). Interestingly, intrathecal morphine was reported to have antinociceptive and anti-hyperalgesic effects, but no marked effects on allodynia in SNL rats (Wegert *et al.*, 1997). In a rat model of central pain, however, intrathecal morphine dose-dependently reversed mechanical allodynia, whereas systemic morphine had little effect on this measure (Hao *et al.*, 1991; Yu *et al.*, 1997). Thus, it appears that the efficacy of morphine in attenuating allodynia and/or hyperalgesia in these behavioural studies is largely dependent on the animal model, the measure and more importantly, the route of administration of the drug.

In this chapter, I investigate the effects of intrathecal versus systemic, administration of morphine on the evoked responses of spinal neurones in rats with peripheral nerve injury at two postoperative time-points. The effectiveness of morphine was assessed on a range of low- and high-intensity natural and electrical evoked neuronal responses in SNL rats, and this was compared to a population of naive and sham operated rats. Recordings were made at one and/or two weeks after surgery for spinal nerve ligation or sham operation. Thus, the overall aim was to compare the effects of morphine on a wide range of modalities of stimuli, to judge whether the effects of the opioid changed over time after the injury, and to compare spinal and systemic routes of administration. Any of these variables could contribute to the confusing clinical appraisal of morphine in neuropathic patients.

7.2 Methods

A total of 72 male adult Sprague-Dawley rats were employed in this study (SNL, n=33; sham operated, n=23; naive, n=16). The effects of morphine on the responses of spinal neurones were compared between SNL rats (PO 7-10 and 14-17 days), and either sham operated (PO 7-10 and 14-17 days) or naive rats.

For intrathecal administration, morphine was applied topically, directly onto the exposed surface of the spinal cord (0.1, 0.25, 1, 5μg/ 50μl). The effect of the drug was followed for a period of 40 minutes per dose and tests were carried out at 10 minute intervals to determine the effects of morphine on natural and electrical stimuli evoked neuronal responses. Tests for natural evoked responses consisted of the application of mechanical punctate stimuli (von Frey weights 9, 20, 50g) and heat (45°C). Following the application of the highest dose of morphine, the effects of morphine were challenged by intrathecal naloxone (5 μg and 50 μg).

Systemic morphine was administered through an indwelling intravenous cannula inserted in the right jugular vein of the rat. Cumulative doses of morphine (1, 3, 6 mg/kg) were administered to the rat and a test was made initially at 5 minutes after drug administration, and subsequently at 10 minute intervals over a period of 40 minutes per dose. Higher doses could not be used due to obvious respiratory depression in these anaesthetised animals. The effects of the highest dose of systemic morphine were challenged by intrathecal naloxone (5 and 50 μg).

7.3 Results

Recordings were made from 33 neurones in SNL rats (PO 7-10 days, n=17; PO 14-17 days, n=16), 23 neurones in sham operated (PO 7-10 days, n= 13; PO 14-17 days, n=10) and 16 neurones in naive rats. The mean depths of the spinal neurones were comparable for SNL (PO 7-10 days, 738±33μm; PO 14-17 days, 756±24μm), sham operated (PO 7-10 days, 790±37μm; PO 14-17 days, 795±24μm) and naive rats (831±20μm). Hence, all cells were found in a similar depth range of the dorsal horn.

The maximal effects of all doses of morphine were seen between 20-30 minutes after intrathecal administration, or 5-20 minutes after systemic morphine, justifying the 40 minute intervals between cumulative dosing. Figure 34 shows the time-course of the effect of intrathecal and systemic morphine on the C-fibre evoked response in a dorsal horn neurone.

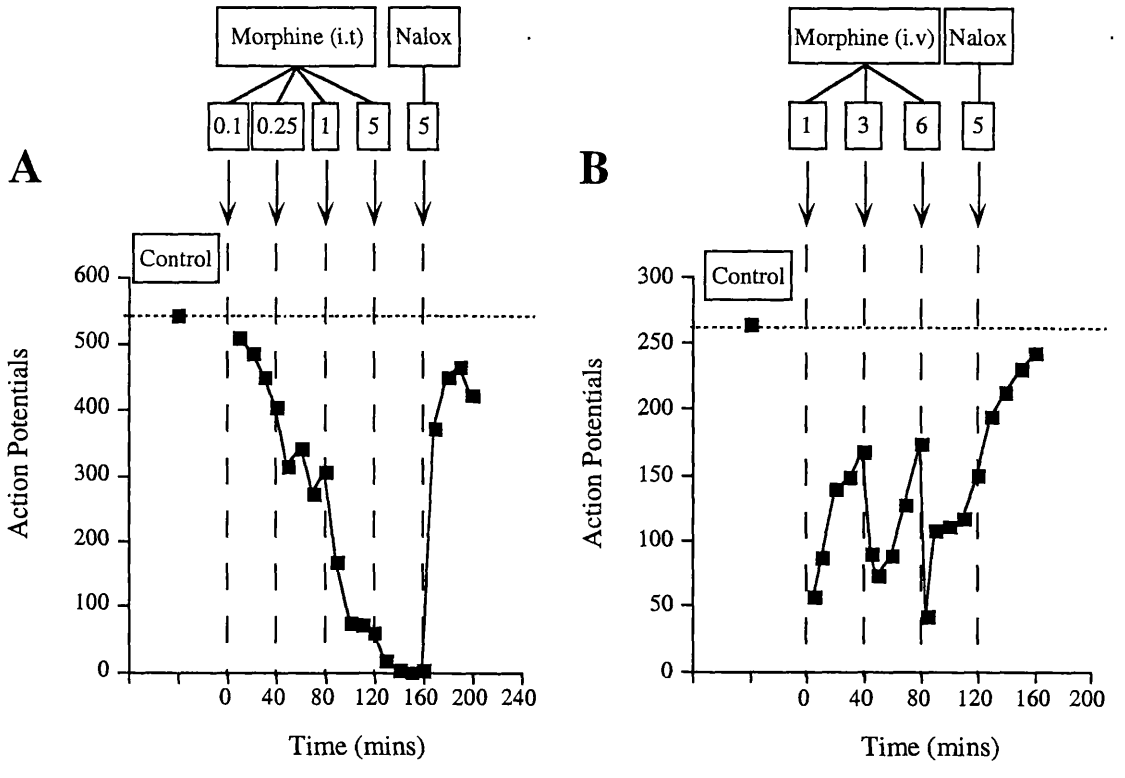


Figure 34. A typical example of the time-course of the effect of (A) intrathecal and (B) systemic morphine on the C-fibre evoked response of a dorsal horn neurone (SNL rat, PO 14-17 days). Maximal effects of the drug were seen between 20-30 minutes after drug administration for the intrathecal route, and between 5-20 minutes for the systemic route. The effects of morphine were reversed with spinal naloxone (5μg). Abbreviations: Nalox (Naloxone); i.t. (intrathecal); i.v. (intravenous).

7.3.1 Effect of intrathecal morphine administration on the spontaneous activity, and electrical and natural evoked responses of spinal neurones

The administration of intrathecal morphine produced a dose-dependent inhibition of the C-fibre evoked response in SNL rats, both at PO 7-10 (0.25μg p=0.008; 1μg, p=0.01; 5μg, p=0.02) and PO 14-17 days (0.25μg, p=0.02; 1 and 5μg, p=0.01). The dose-response curves for C-fibre inhibition were comparable at 1 and 2 weeks after nerve injury, and no dramatic shifts were observed between the two postoperative time-points. (Fig 35A) Similarly, the C-fibre evoked response of unoperated naive rats (5μg, p=0.03) and sham operated rats (PO 7-10 days: 5μg, p=0.04) were reduced after spinal morphine. (Fig 35A) Overall, intrathecal morphine produced greater inhibitions of the C-fibre evoked response in SNL rats, as compared to sham operated rats, and a leftward shift in the dose-response curve was observed following nerve injury. Similarly, the effect of morphine on the C-fibre evoked response tended to be greater in SNL rats as compared to naive rats, especially with lower doses of the drug. At the highest dose of the drug (5μg), however, comparable effects were seen. The magnitude of the C-fibre inhibition was significantly greater in SNL rats compared to that of naive (PO 7-10 days: 0.25μg, p=0.04; PO 14-17 days: 0.25μg, p=0.01) or sham operated rats, at both postoperative time-points (PO 7-10 days: 1μg, p=0.01; 5μg, p=0.04; PO 14-17 days: 0.25 and 1μg, p=0.008; 5μg, p=0.01).

Intrathecal morphine produced significant inhibitions of the input of spinal neurones in SNL rats (PO 7-10 days: 0.25μg, p=0.008; 1μg, p=0.01; 5μg, p=0.02; PO 14-17 days: 0.25μg, p=0.05; 1 and 5μg, p=0.008). The effect of morphine was comparable at both postoperative time-points. (Fig 35B) Similarly, the input of spinal neurones was reduced in naive (1 and 5μg, p=0.03) and sham operated rats (PO 7-10 days: 1μg, p=0.02; 5μg, p=0.03). (Fig 35B) The reductions were comparable for all groups of animals at the earlier time-point, however, morphine produced a greater effect in SNL rats, as compared to sham operated rats at PO 14-17 days (0.25μg, p=0.04; 1μg, p=0.01; 5μg, p=0.03).

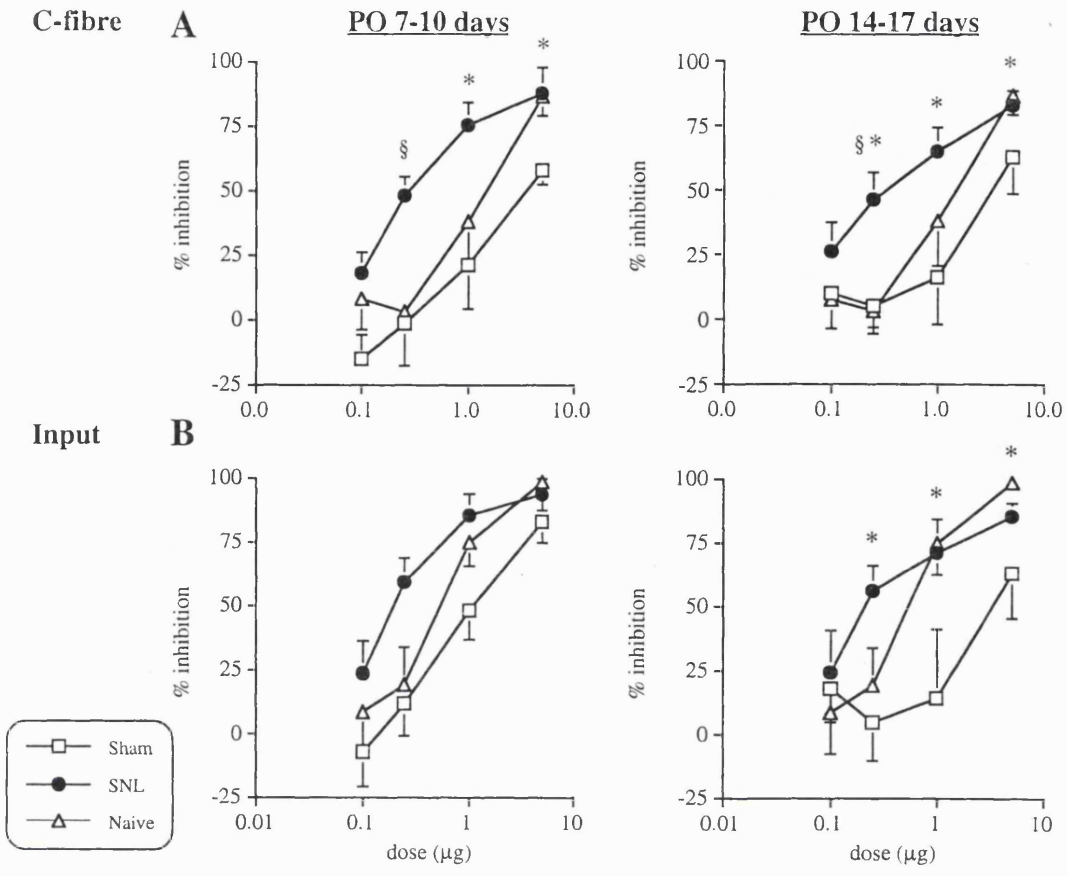


*Figure 35. The effect of intrathecal morphine administration on the (A) C-fibre evoked response and (B) input of spinal neurones at PO 7-10 and PO 14-17 days. Data are presented as percentage inhibitions of the pre-drug control values ± S.E.M. * indicates a significant difference between SNL and sham operated rats, while § indicates a difference between SNL and naïve rats.*

In direct contrast, spinal morphine produced only small inhibitions of the Aβ-fibre evoked response of SNL (PO 7-10 days: 0.25μg, p=0.04; 1μg, p=0.02; PO 14-17 days: 0.25 and 1μg, p=0.02), naive and sham operated rats (PO 7-10 days: 0.25μg, p=0.04). The effect of morphine on the Aβ-fibre evoked neuronal response was similar between SNL and naive rats. Morphine also produced comparable effects between SNL and sham operated rats PO 14-17 days, however, there was a significant difference at the earlier time-point (5μg, p=0.04). The magnitude of the inhibition of the Aβ-fibre evoked response did not exceed 40%, even with the highest dose of the drug in all animals groups.

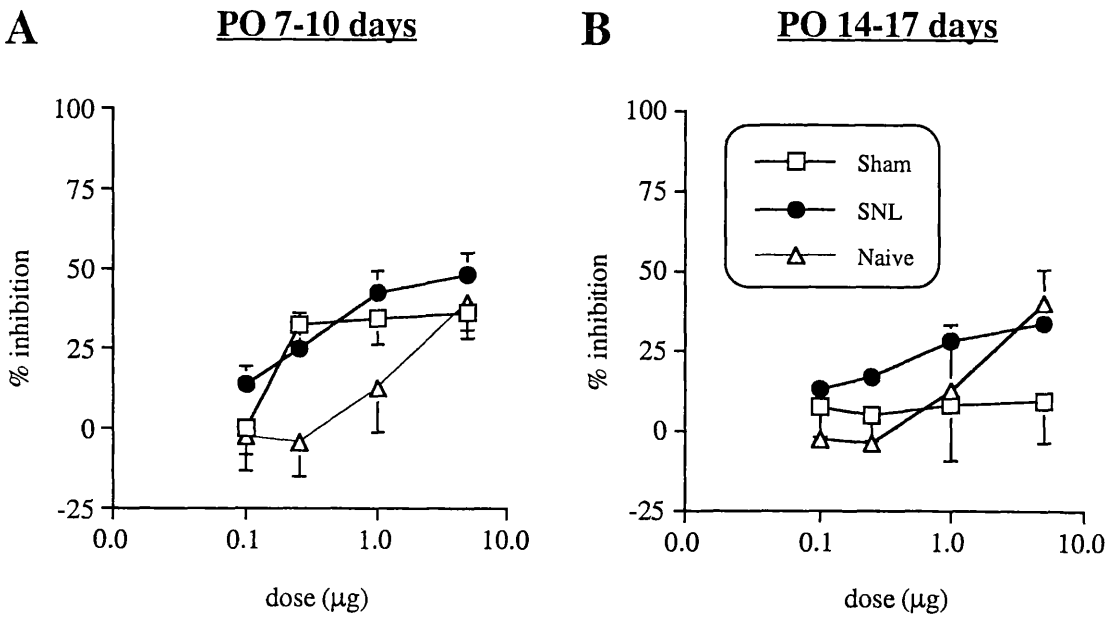


*Figure 36. The effect of intrathecal morphine administration on the Aβ-fibre evoked response of SNL, naive and sham operated rats at (A) PO 7-10 and (B) PO 14-17 days. Data are presented as percentage inhibitions of the pre-drug control values ± S.E.M. * indicates a significant difference between SNL and sham operated rats, while § indicates a difference between SNL and naïve rats.*

Intrathecal morphine produced dose-dependent inhibitions of the Aδ-fibre evoked response of SNL rats both at PO 7-10 days (0.25, 1 and 5μg, p=0.02) and at PO 14-17 days (0.25, 1 and 5μg, p=0.008). Similarly, the Aδ-fibre evoked responses of naive (1μg, p=0.03) and sham operated rats (PO 7-10 days: 1μg, p=0.03; 5μg, p=0.04; PO 14-17 days: 0.1μg, p=0.04) were reduced with morphine (data not shown).

The mechanical evoked response of spinal neurones was dose-dependently inhibited by intrathecal morphine in all groups of animals. Morphine produced significant inhibitions of the 9g von Frey evoked response of SNL rats at PO 7-10 (0.1, 0.25, 5μg, p=0.04) and PO 14-17 days (1μg, p=0.02). The effect of morphine (Fig 37A)
in SNL rats was comparable between both postoperative time-points. Similarly, spinal morphine reduced the 9g von Frey evoked response in both naive (0.25

and 1μg, p=0.03; 5μg, p=0.04) and sham operated rats (PO 7-10 days, 1 and 5μg, p=0.03). (Fig 37A) At PO 7-10 days, the inhibition of the innocuous 9g von Frey evoked neuronal response was significantly greater in SNL rats, as compared to naive (0.25μg, p=0.03) or sham operated rats (1μg, p=0.02). At the later time-point, however, the effect of morphine on the 9g von Frey evoked response was comparable in all groups.

Intrathecal morphine produced dose-dependent inhibitions of the 20g von Frey evoked response of SNL rats (PO 7-10 days: 0.1 and 1μg, p=0.04; 0.25μg, p=0.03; PO 14-17 days: 0.25 and 1μg, p=0.01). Similarly, the 20g von Frey evoked response of naive (0.25 and 1μg, p=0.03) and sham operated rats (PO 7-10 days: 0.25μg, p=0.04; 1 and 5μg, p=0.03) was similarly reduced following intrathecal morphine. (Fig 37B) Morphine produced a significantly greater inhibition of the 20g von Frey evoked response in SNL rats, as compared to sham operated rats at both PO 7-10 (1μg, p=0.03) and PO 14-17 days (0.1μg, p=0.05; 0.25 and 1μg, p=0.04). The effect of spinal morphine was comparable between SNL and naïve rats.

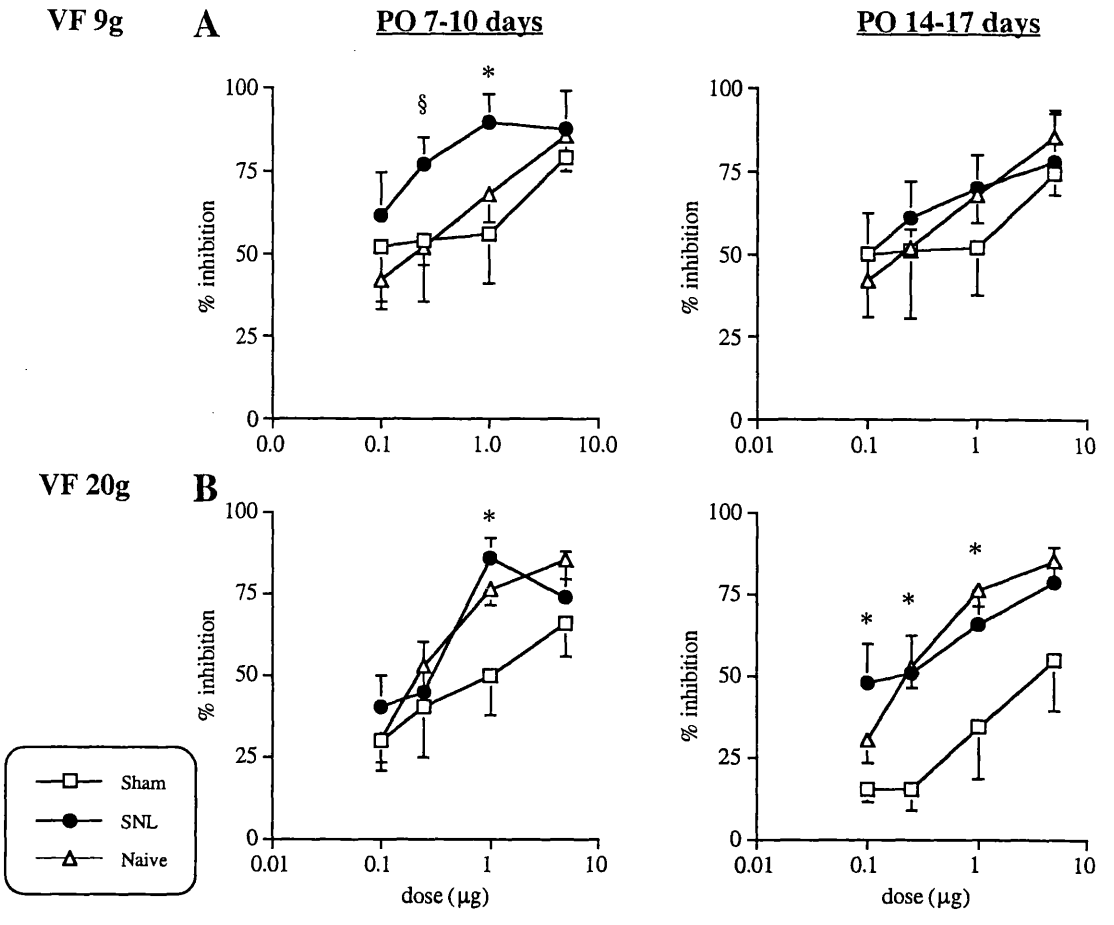


*Figure 37. The effect of intrathecal morphine administration on the responses of spinal neurones to (A) von Frey 9g and (B) von Frey 20g at PO 7-10 and PO 14-17 days. Data are presented as percentage inhibitions of the pre-drug control values ± S.E.M. * indicates a significant difference between SNL and sham operated rats, while § indicates a difference between SNL and naïve rats.*

Intrathecal morphine reduced the noxious 50g von Frey evoked response of SNL rats at PO 7-10 (0.25 and 1μg, p=0.01; 5μg, p=0.02) and PO 14-17 days (0.1μg, p=0.02; 0.25μg, p=0.01; 1 and 5μg, p=0.008). Similarly, in naive (1μg, p=0.02; 5μg, p=0.03) and sham operated rats (PO 7-10 days: 0.25μg, p=0.04; 1 and 5μg, p=0.03), morphine produced dose-dependent inhibitions of the 50g von Frey evoked response. The magnitude of the inhibition was significantly greater in SNL rats compared to naive rats at the earlier postoperative time-point (0.25μg, p=0.02). There was a tendency for morphine to be more effective in SNL rats, as compared to naïve rats at lower doses, however, this was not significant. Similarly, intrathecal

morphine produced greater effects on the 50g von Frey evoked response of SNL rats, compared to those of sham operated rats (PO 7-10 days: 0.25 μg, p=0.04; 1 and 5μg, p=0.02; PO 14-17 days: 0.1μ g, p=0.03; 0.25 μg, p=0.01; 1μ g, p=0.008; 5μ g, p=0.04).

The administration of intrathecal morphine produced dose-dependent inhibitions of the thermal evoked response in SNL (PO 7-10 days: 0.1 and 0.25μ g, p=0.03; PO 14-17 days: 0.1, 0.25, 1 and 5 μg, p=0.04), naive (0.25 and 5μg, p=0.03; 1μg, p=0.02) and sham operated rats (PO 7-10 days: 0.25, 1 and 5μ g, p=0.04). The effect of morphine on the thermal evoked response was comparable between SNL and naive rats. When compared to sham operated rats, morphine produced a greater inhibitory effect in SNL rats at PO 14-17 days (0.25μg, p=0.03; 1 and 5μg, p=0.05).

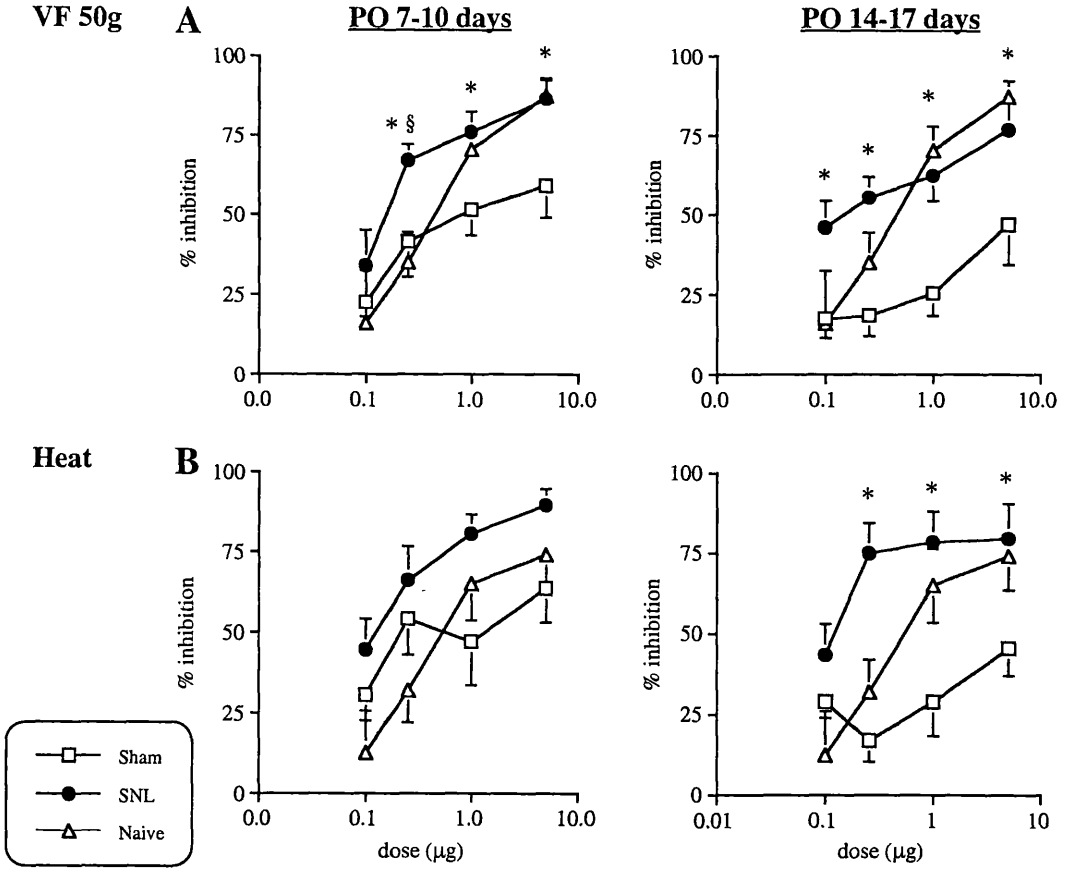


Figure 38. The effect of intrathecal morphine administration on the responses of spinal neurones to (A) von Frey 50g and (B) heat at PO 7-10 and PO 14-17 days. Data are presented as percentage inhibitions of the pre-drug control values ± S.E.M.

A high proportion of spinal neurones exhibited spontaneous activities in SNL rats. At PO 7-10 days, 4 of 9 neurones (44%) tested with spinal morphine exhibited ongoing activities with a mean level of 3.1±1.8 Hz. Similarly, 4 of 8 spinal neurones (50%) displayed spontaneous activities at the later time-point (mean level of firing, 3.1±2.4 Hz). In sham operated rats, however, only a small proportion of cells exhibited ongoing activities (PO 7-10 days, 33%; PO 14-17 days, 20%) and the level of the firing was generally lower (PO 7-10 days, 0.4±0.3 Hz; PO 14-17 days, 0.5 Hz). No spontaneous activity was observed in neurones of unoperated naive rats in this study.

The intrathecal administration of morphine produced inhibitions of the spontaneous activity in SNL rats (maximal inhibitions: PO 7-10 days, 76±14% and PO 14-17 days, 83±21%). The extremely low spontaneous activity observed in the sham operated rats precluded analysis of the effects of morphine on this measure.

All observed inhibitory effects were reversed by spinal naloxone (5μg and 50μg) to near control values; for example, the inhibitions of the C-fibre and 50g von Frey evoked response produced by 5μg morphine in SNL rats (PO 14-17 days) were reversed to 96% and 101% of pre-drug control values by naloxone (5μg), respectively.

7.3.2 Effect of systemic morphine administration on the spontaneous activity, and electrical and natural evoked responses of spinal neurones

The administration of systemic morphine produced dose-dependent inhibitions of the C-fibre evoked response in SNL rats (PO 7-10 days: 6mg/kg, p=0.03). (Fig 39A) The effect of morphine was comparable at PO 7-10 and PO 14-17 days. Similarly, the C-fibre evoked response of naive (1 and 6mg/kg, p=0.02; 3mg/kg, p=0.01) and sham operated rats was reduced following systemic morphine (PO 7-10 days: 6mg/kg, p=0.03; PO 14-17 days, 1 and 6mg/kg, p=0.04). (Fig 39A) The effect of the drug was comparable between all groups of animals.

The administration of morphine produced non-significant effects on the input and Aδ-fibre evoked response of spinal neurones. Systemic morphine produced comparable effects on the input of spinal neurones between SNL (maximal inhibitions: PO 7-10 days, 63%; PO 14-17 days, 46%) and sham operated rats (PO 7-10 days, 56%; PO 14-17 days, 54%) at both time-points. (Fig 39B) The inhibition of input tended to be greater in naive rats (89%) as compared to SNL rats, however, this difference was non-significant.

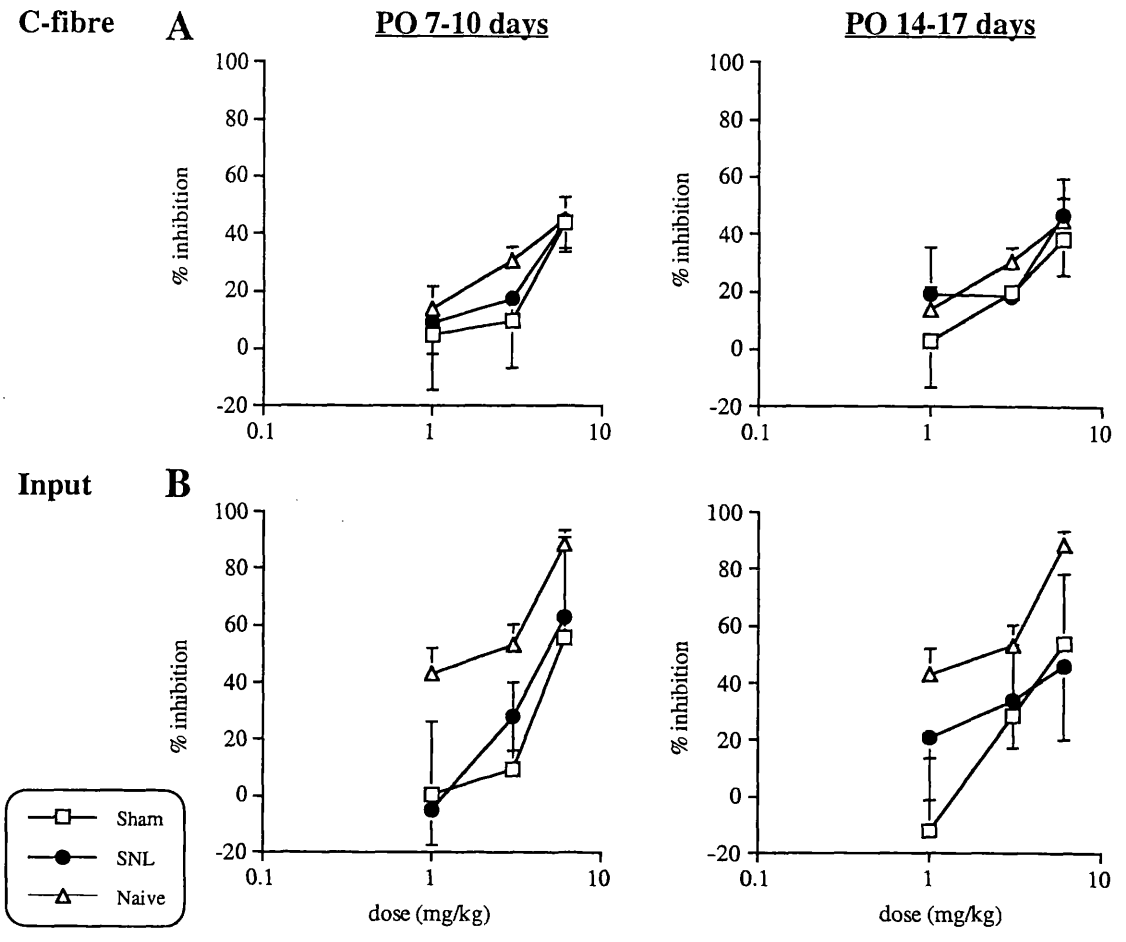


Figure 39. The effect of systemic morphine on the (A) C-fibre evoked response and (B) input of spinal neurones at PO 7-10 and PO 14-17 days. Data are presented as percentage inhibitions of the pre-drug control values ± S.E.M.

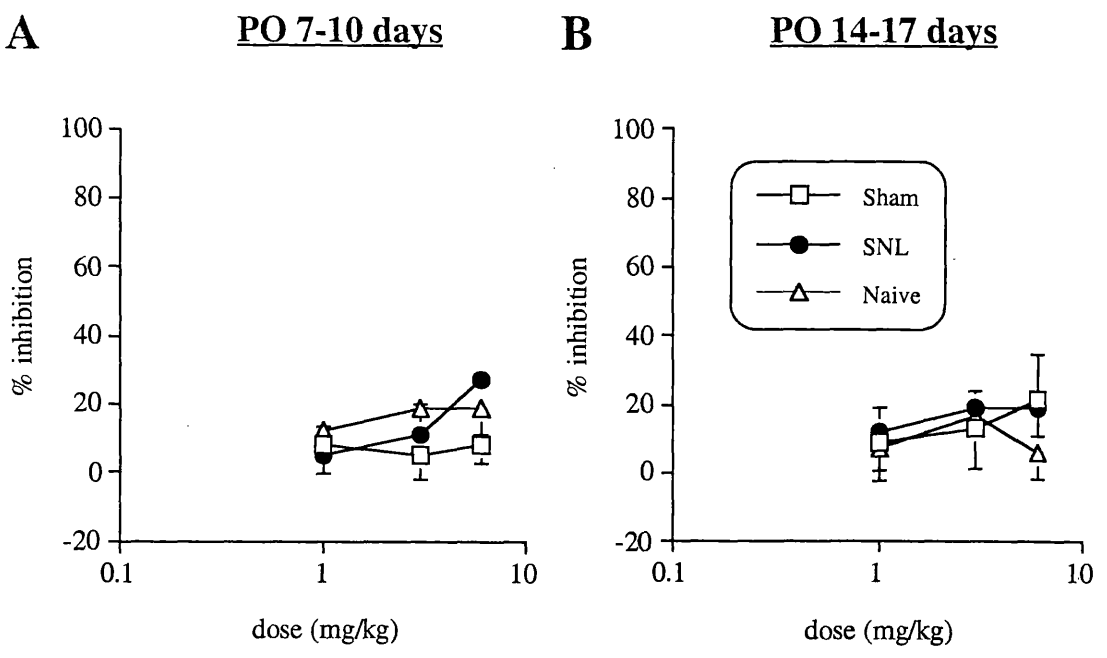


Figure 40. The effect of systemic morphine administration on the Aβ-fibre evoked response of SNL, naive and sham operated rats at (A) PO 7-10 and (B) PO 14-17 days. Data are presented as percentage inhibitions of the pre-drug control values ± S.E.M.

Systemic morphine produced only minor effects on the Aβ-fibre evoked response of SNL (PO 7-10 days: 6mg/kg, p=0.03; PO 14-17 days: 3mg/kg, p=0.02), sham operated and naive rats (1 and 3mg/kg, p=0.01; 6mg/kg, p=0.02). Even at the highest dose of morphine studied (6 mg/kg), the inhibition of Aβ-fibre evoked response did not exceed 25%. The inhibitions were comparable in all groups of animals.

Systemic morphine produced inhibitions of the mechanical evoked response in all groups of animals. The mechanical evoked response of SNL rats were non-significantly reduced at PO 7-10 days. At PO 14-17 days, systemic morphine produced significant inhibitions of the innocuous 9g von Frey evoked response in SNL rats (1 and 3mg/kg, p=0.04), and similar effects were observed in both naive (1 and 3mg/kg, p=0.03) and sham operated rats (3mg/kg, p=0.04). Overall, the inhibition of the 9g von Frey evoked response of SNL rats was greater at PO 14-17 days (maximal inhibition 72%) than at the earlier time-point (41%). The inhibitions were comparable for all animal groups at both time-points.

The 20g von Frey evoked response was reduced following systemic morphine in SNL (PO 14-17 days: 3mg/kg, p=0.02; 6mg/kg, p=0.04), naive (1 and 3mg/kg, p=0.02; 6mg/kg, p=0.04) and sham operated rats (PO 7-10 days: 6mg/kg, p=0.03; PO 14-17 days: 1, 3 and 6mg/kg, p=0.04). The effect of morphine was comparable in all groups of animals. (Fig 41B)

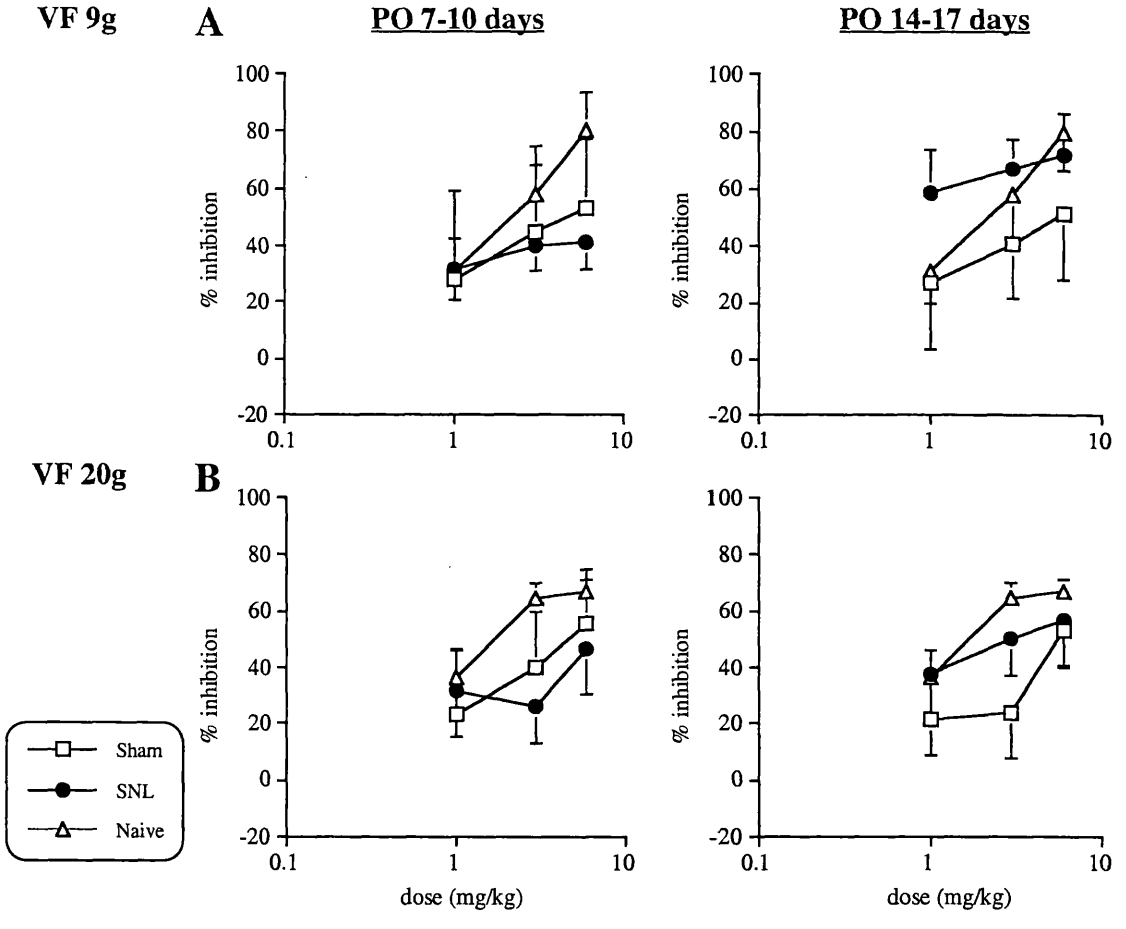


Figure 41. The effect of systemic morphine on the responses of spinal neurones to (A) von Frey 9g and (B) von Frey 20g at PO 7-10 and PO 14-17 days. Data are presented as percentage inhibitions of the pre-drug control values ± S.E.M.

Systemic morphine produced significant reductions of the noxious 50g von Frey evoked response in SNL (PO 14-17 days: 1 and 3mg/kg, p=0.03), naive (1 and 3mg/kg, p=0.03; 6mg/kg, p=0.04) and sham operated rats (PO 7-10 days: 3mg/kg, p=0.04; 6mg/kg, p=0.03; PO 14-17 days: 3 and 6mg/kg, p=0.04). In SNL rats, the inhibition of the 50g von Frey evoked response was greater at PO 14-17 days (59%) compared to that at the earlier time-point (29%). At PO 7-10 days, systemic morphine produced a significantly greater inhibition in naive rats, as compared to SNL rats (3mg/kg, p=0.02; 6mg/kg, p=0.01). At the later time-point, however, the effect of the drug was comparable between the two groups. Systemic morphine produced similar inhibitions of the noxious mechanical evoked response (von Frey 50g) in SNL and sham operated rats, at both postoperative time-points.

Systemic morphine produced inhibitions of the thermal evoked response of SNL rats at PO 7-10 (1 and 3mg/kg, p=0.03) and PO 14-17 days (1 and 3mg/kg, p=0.02; 6mg/kg, p=0.04). Again, the effect of morphine tended to be greater at PO 14-17 days (maximal inhibition 69%), as compared to the earlier time-point (49%). Similarly, the thermal evoked responses of naive (1mg/kg, p=0.02; 3mg/kg, p=0.03) and sham operated rats (PO 7-10 days: 6mg/kg, p=0.03; PO 14-17 days: 1, 3 and 6mg/kg, p=0.04) were reduced after systemic morphine administration. Morphine produced comparable effects in all animal groups.

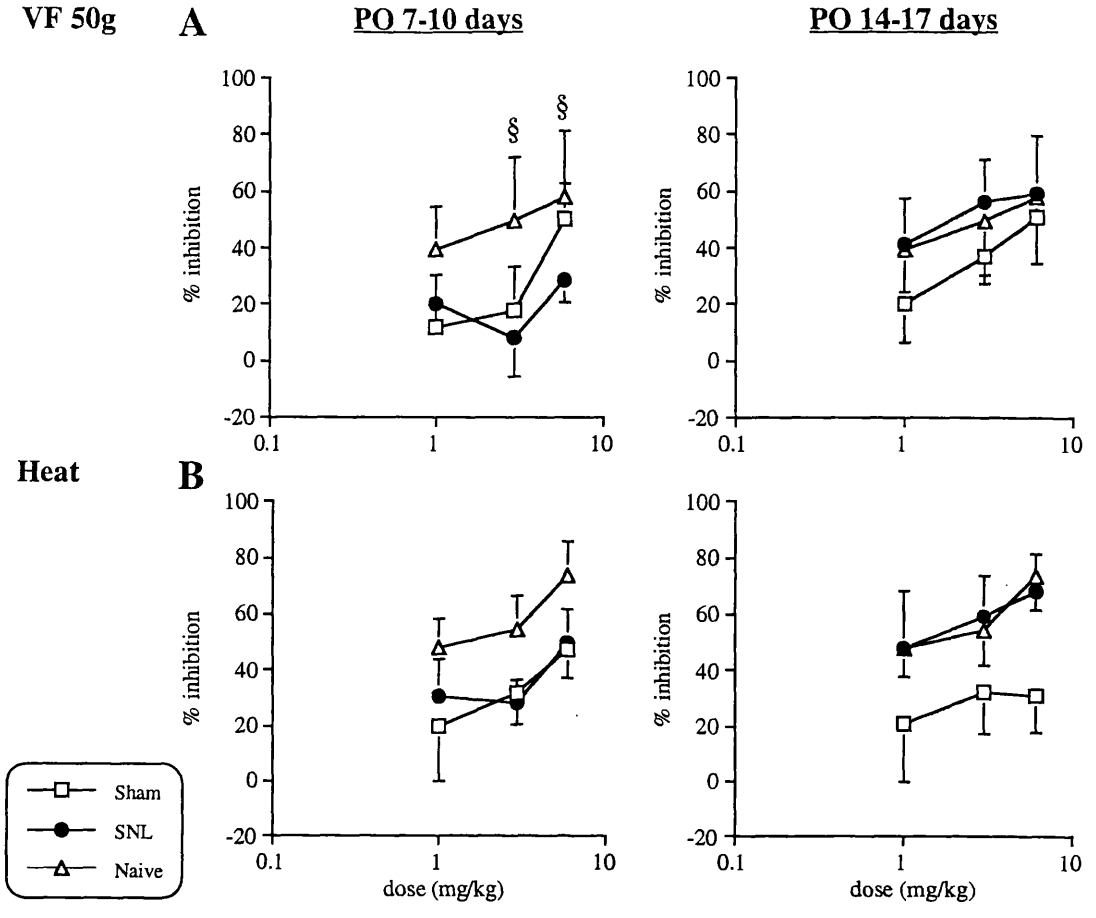


Figure 42. The effect of systemic morphine on the responses of spinal neurones to (A) von Frey 50g and (B) heat at PO 7-10 and PO 14-17 days. Data are presented as percentage inhibitions of the pre-drug control values ± S.E.M.

In 2 of the 8 neurones tested with systemic morphine in SNL rats (PO 7-10 days), a low level of spontaneous activity was observed, with a mean firing frequency of 1.8 Hz. In these cells, systemic morphine produced different effects between the two - in one cell, the ongoing activity was facilitated to 330% of pre-drug control values, whilst in another, the activity was almost completely abolished (7% of pre-drug control). Similarly, 3 of 8 neurones studied in SNL rats at the later time-point (PO 14-17 days) also exhibited ongoing activities (2.6±0.8 Hz). The highest dose of systemic morphine (6mg/kg) produced a 68±24% inhibition of the spontaneous activity in these cells.

The inhibitions produced by systemic morphine (6mg/kg) were partly reversed by spinal naloxone (5μg and 50μg); for example, the C-fibre and 50g von Frey evoked responses were reversed to 66% and 98% of pre-drug control values in SNL rats (PO 14-17 days) following administration of intrathecal naloxone.

7.3.3 Comparison of the effects of morphine on the evoked neuronal responses through different routes of drug administration (intrathecal vs systemic)

C-fibre evoked response

Overall, my results showed that the C-fibre evoked response of SNL rats was reduced to a much greater extent following intrathecal administration, than via the systemic route, both at PO 7-10 (maximal inhibitions: i.t., 88%; i.v., 45%) and at PO 14-17 days (i.t., 83%; i.v., 47%). The magnitude of the inhibitions produced by the systemic route was considerably smaller than that by the spinal route and the reductions did not exceed 50% even with the highest dose of the drug. Higher doses of systemic morphine (>6mg/kg) could not be administered to these animals, due to marked respiratory depression in these anaesthetised animals. In naive rats, morphine administered via the intrathecal route was also shown to reduce the C-fibre evoked response (87%) to a much greater extent, than via the systemic route (45%). A similar pattern was observed for sham operated rats, and this difference was more pronounced at the later time-point (maximal inhibitions: i.t., 63%; i.v., 40%) than at the earlier time-point (i.t., 58%; i.v., 44%).

Aβ-fibre evoked response

Morphine produced only minor inhibitions of the Aβ-fibre evoked neuronal response in all groups of animals. In SNL rats, intrathecal morphine (maximal inhibitions: PO 7-10 days, 48%; PO 14-17 days, 34%) produced a greater inhibition of the Aβ-fibre evoked response compared to systemic morphine at both postoperative time-points (PO 7-10 days, 27%; PO 14-17 days, 17%). Similarly, the inhibition of the Aβ-fibre evoked response was greater with intrathecal morphine (40%) than with systemic morphine in naive rats (19%), and a similar pattern was observed for sham operated rats at the earlier time-point (i.t., 37%, i.v., 8%). At PO 14-17 days, however, the systemic route of morphine administration produced greater effects (21%) as compared to the intrathecal route (9%).

Mechanical evoked response

Morphine produced differential spinal versus systemic inhibitory effects of mechanical evoked responses in SNL rats. At PO 7-10 days, intrathecal morphine produced a greater inhibition of the innocuous 9g von Frey evoked response in SNL rats (maximal inhibition: 90%), as compared to systemic morphine (41%). At the later time-point, however, morphine produced comparable inhibitions via the two routes (i.t., 78%; i.v., 72%). A similar pattern was also observed for the noxious 50g von Frey evoked response, which was reduced to a greater extent with intrathecal morphine at the earlier time-point (i.t.,87%; i.v., 29%). At PO 14-17 days, however, morphine produced comparable effects via the two routes (i.t., 78%; i.v., 60%).

In naive rats, morphine produced similar inhibitions of the 9g von Frey evoked response via the intrathecal (85%) and systemic routes (80%). For the noxious 50g von Frey evoked response, however, the intrathecal route of administration (88%) produced greater effects, as compared to the systemic route (58%). Similarly, in sham operated rats, morphine produced greater reductions of the 9g von Frey evoked response via the intrathecal route at both PO 7-10 (i.t., 79%; i.v., 53%) and PO 14-17 days (i.t., 75%; i.v., 52%). The 50g von Frey evoked

responses, on the other hand, were reduced to similar extents via the two routes (PO 7-10 days: i.t., 59%; i.v., 50%; PO 14-17 days: i.t., 47%; i.v., 51%).

Thermal evoked response

Morphine produced a greater inhibition of the thermal evoked response via the intrathecal route (maximal inhibitions: PO 7-10 days: 90%; PO 14-17 days: 79%), as compared to the systemic route in SNL rats at both time-points (PO 7-10 days: 50%; PO 14-17 days: 69%). In naive rats, however, morphine produced comparable effects between the two routes of administration (i.t., 74%; i.v., 74%). The inhibition of the thermal evoked response of sham operated rats was also comparable via the two routes, however, intrathecal morphine generally tended to produce a slightly greater effect (PO 7-10 days: i.t., 64%; i.v., 47%; PO 14-17 days: i.t., 46%; i.v., 31%).

7.4 Discussion

In this chapter, I investigated the effects of morphine on the electrical and natural evoked responses of spinal neurones, and compared these effects between SNL and sham operated rats at 2 postoperative time-points (PO 1 and 2 weeks). In addition, comparisons were made with a group of unoperated naive animals. Morphine was administered via two routes - intrathecal and systemic - and the antinociceptive effects of the drug via the various routes were studied. The results from my study confirmed previous findings where morphine was shown to inhibit the responses of dorsal horn neurones in naive rats (Dickenson & Sullivan 1986). Furthermore, I extended these observations by demonstrating that morphine also reduces, to varying extents, the electrical and natural evoked responses of SNL rats at 1 and 2 weeks after nerve injury. Morphine produced greater inhibitory effects on the C-fibre evoked response, as compared to the Aβ-fibre evoked response, and reduced the responses of spinal neurones to both low- and high-intensity natural stimuli in all animal groups. In general, the dose-response curves for the electrical evoked responses were comparable in SNL rats at both time-points, and the

responsiveness of spinal neurones to morphine (spinal/ systemic) did not alter dramatically between 1 to 2 weeks after nerve injury. There was, however, a tendency for systemic morphine to be more effective in reducing the natural evoked responses at PO 14-17 days, as compared to the earlier time-point. Intrathecal morphine, on the other hand, produced comparable effects at both time-points.

Overall, my results showed that intrathecal morphine produces a greater inhibitory effect in SNL rats, as compared to sham operated rats at both time-points. The responsiveness of spinal neurones to intrathecal morphine was markedly enhanced following nerve injury, as demonstrated by the greater effectiveness of the drug in SNL rats. In contrast, systemic morphine produced comparable effects between SNL and sham operated rats, although SNL rats exhibited a reduced sensitivity to systemic morphine when compared to naive animals. Generally, the inhibitory effect of systemic morphine on the neuronal responses was considerably smaller compared to the intrathecal route of administration in SNL rats. Although behavioural studies have demonstrated systemic morphine to be effective in producing antinociception in animal models of neuropathy (Bian *et al.*, 1995; Lee *et al.*, 1995), my electrophysiological results showed that it produces relatively small inhibitions of the evoked neuronal responses, when compared to intrathecal morphine. The ability of systemic morphine to produce potent behavioural antinociceptive effects, yet smaller effects on neuronal responses, could suggest that morphine may be acting at an additional site apart from the spinal cord, such as at supraspinal sites (Yaksh 1997). Dorsal horn neurones are known to be under strong regulatory influence of midbrain/ brainstem structures and activation of supraspinal pathways can therefore produce powerful modulatory effects on spinal nociceptive processing. One mechanism through which opioids are thought to mediate antinociception is by action on PAG, which is held under tonic inhibitory control by GABAergic neurones. The action of opioids on GABAergic neurones results in a lift of the tonic inhibitory control, and increases the outflow of PAG to the medulla, thereby activating bulbospinal projections, and enhancing descending inhibitory controls (Yaksh 1997). Other supraspinal mechanisms have also been proposed, which involve the direct modulation of the ascending projections to higher centres. The powerful antinociceptive effects of systemic morphine reported in previous behavioural studies could therefore be attributed to the combination of these

supraspinal mechanisms. Although it is possible that the inhibitory effect of systemic morphine is, in part, mediated via the activation of descending inhibitory controls, it is unlikely that the latter mechanism (direct inhibition of ascending projections) contributes to the spinal inhibitory effect of systemic morphine observed in the present study. However, large contributions from supraspinal sites, whatever the mechanism, under these experimental conditions are unlikely since *spinal* naloxone produced large reversals of the inhibitory effects of *systemic* morphine. This indicates primary spinal actions of intravenous morphine under these conditions.

Another possibility is that the behavioural effects are more marked since they reflect actions on threshold whereas the neuronal responses are suprathreshold – the greater magnitude of neuronal activity may need higher doses for modulation.

The route-dependent differences in opioid effectiveness seen after spinal or systemic morphine administration, may also reflect the local tissue concentration of the drug attained via the two routes. It may be envisaged that following intrathecal administration, there is a higher spinal concentration of morphine as compared to that achieved with the systemic route of administration, thus producing a greater spinal opioid receptor occupancy (Matos *et al.,* 1995). Autoradiographic studies have shown a significant early (PO 2-5 days) increase in the ipsilateral μ opioid receptor binding in the CCI model of nerve injury, which then declines to control levels at later time-points (Besse *et al.,* 1992; Goff *et al.,* 1998; Stevens *et al.,* 1991). If such early increases in receptor number occur in the present model, this may account for the enhancement of intrathecal morphine sensitivity, as observed in my study. However, a recent study provided evidence for a discrete localised decrease in opioid receptor immunoreactivity in the SNL model of neuropathy, corresponding to the L6 segment of the ipsilateral dorsal horn (Porreca *et al.,* 1998). Based on the findings of this study, there appears to be a loss in opioid receptor expression 7 days after spinal nerve ligation, and this is confined to a discrete segment of the spinal dorsal horn, rather than occurring at a generalised multi-segmental level (Porreca *et al.,* 1998). A decrease in opioid receptor expression has also been reported following dorsal rhizotomy (Gouarderes *et al.,* 1991; Ninkovic *et al.,* 1981; Zajac *et al.,* 1989), peripheral nerve section (deGroot *et al.,* 1997; Zhang *et al.,* 1998) and tight ligation of the sciatic nerve (Goff *et al.,* 1998), and this has

been proposed to contribute to the loss of opioid efficacy in neuropathic pain states. The lack of antiallodynic effect of spinal morphine has been demonstrated behaviourally where the opioid displays a reduced efficacy compared to systemic morphine (Bian *et al.*, 1995; Lee *et al.*, 1995). Even a dose of 100μg morphine failed to reverse allodynia in SNL rats (Bian *et al.*, 1995), whilst systemic morphine produced a dose-dependent reversal of this measure. Side effects were, however, reported at higher doses (Lee *et al.*, 1995; Martin *et al.*, 1998). It is unlikely, however, that the highly localised loss of opioid receptors reported in the SNL model (Porreca *et al.*, 1998) could solely be responsible for the loss of efficacy of intrathecal morphine associated with nerve injury states. Interestingly, the changes which occur following nerve injury are temporary and the level of the receptors returns to its control level within 2 weeks (Besse *et al.*, 1992; Stevens *et al.*, 1991) to 1 month after nerve lesion (deGroot *et al.*, 1997). In spite of this, however, behavioural studies show that opioid sensitivity does not show a significant recovery at this postoperative time-point. The extent to which the reorganisation of μ opioid receptors influences opioid sensitivity is unclear, since these changes appear to be specific for the type of nerve injury (CCI, SNL, peripheral nerve section, dorsal rhizotomy). The time-course of these changes is also dependent on the model of nerve injury. Furthermore, there is evidence to suggest that the regulation of opioid receptor expression following nerve injury also varies between species, and a greater reduction has been reported in monkeys, as compared to rats (Zhang *et al.*, 1998).

Despite the lack of allodynic efficacy, intrathecal morphine has been shown to produce antinociceptive effects against acute nociceptive stimuli in SNL rats, although with a reduced potency compared to that in sham operated rats (Ossipov *et al.*, 1995a; Ossipov *et al.*, 1995b; Wegert *et al.*, 1997). A similar pattern has also been demonstrated in the CCI model of neuropathy (Mao *et al.*, 1995a; Yamamoto & Yaksh 1991). The ability of morphine to produce a differential antiallodynic and antinociceptive effect following nerve injury may reflect the difference in the mechanisms underlying these behavioural measures. Studies have suggested a mechanistic difference between tactile allodynia and hyperalgesia, which can be distinguished by the type of afferent fibres involved (Ossipov *et al.*, 1999). Whilst hyperalgesia is largely mediated through capsaicin-

sensitive primary afferents, allodynia on the other hand, appears to be due to inputs from large diameter fibres (Ossipov *et al.,* 1999). Ample evidence exists for the localisation of μ opioid receptor mRNA and binding sites in DRG cell bodies and C-fibre afferents (Besse *et al.,* 1990a; Mansour *et al.,* 1995; Mansour *et al.,* 1994), however, the presence of these receptors on large diameter Aβ-fibres is lacking. The specific action of morphine on noxious inputs is therefore largely attributed to its selectivity on small diameter primary afferents, and on cell bodies of second order neurones (Dickenson 1994b). This paradoxically, is beneficial in non-neuropathic pain states since it allows the preservation of tactile sensitivity, yet it may account for the poor therapeutic effect of morphine against allodynia during neuropathy. Hence the population of opioid receptors which may be relevant in attenuating the transmission of allodynia is that which occurs postsynaptic to the primary afferents, consisting of 30-40% of the total spinal μ and δ opioid receptor population (Besse *et al.,* 1990b). Consistent with this finding, my results showed that morphine produced only minor effects on the Aβ-fibre evoked responses in all groups of animals. Interestingly, despite the lack of inhibition of the Aβ-fibre evoked response, the innocuous 9g von Frey evoked response was clearly reduced with morphine administration. The small proportion of postsynaptic opioid receptors on spinal neurones (Besse *et al.,* 1990a) may not be sufficient to modulate Aβ-fibre inputs when they are evoked by synchronised electrical stimulation, but may inhibit the weaker responses evoked by 9g von Frey stimulation. Indeed, the activation of postsynaptic receptors appears to require higher doses of morphine (Lombard & Besson 1989), hence this may explain the need for dose-escalation in some clinical neuropathic pain states where presynaptic receptors have been lost.

In contrast to previous behavioural findings where intrathecal morphine has generally been found to exhibit a reduced efficacy following nerve injury, there is also evidence demonstrating the effectiveness of intrathecal morphine in an animal model of spinal cord injury (Yu *et al.,* 1997). Interestingly, systemic morphine did not significantly reverse allodynia in this model at non-sedative doses (Hao *et al.,* 1991a; Yu *et al.,* 1997). Similarly, intrathecal administration of morphine (50μg) was demonstrated to alleviate allodynia in rats with nerve crush, although at lower doses (25μg), it was found to be ineffective (Przewlocka *et al.,* 1999).

Overall, there appears to be a degree of discrepancy between the results of some previous behavioural studies and my current electrophysiological findings. Behaviourally, intrathecal morphine (30-100μg) has been generally shown to display a reduced efficacy following nerve injury, although my present electrophysiological results demonstrate that spinal morphine (5μg) is effective in reducing neuronal responses at 1-2 weeks post-surgery. Furthermore, the effectiveness of the drug appeared to be enhanced after spinal nerve ligation. Many of the above behavioural studies used changes in withdrawal thresholds as measures of allodynia and morphine efficacy (Lee *et al.*, 1995; Wegert *et al.*, 1997). This clearly contrasts my present study which assessed the ability of morphine to inhibit neuronal responses evoked by various natural stimuli (mechanical/ thermal). It is feasible that morphine may not exert effects on the withdrawal thresholds to mechanical/ thermal stimuli, whilst still having inhibitory effects on the suprathreshold firing of spinal neurones. Additionally, although the reductions in neuronal responses seen after morphine are likely to represent a decreased sensory response to the stimulus, the remaining activity within the spinal circuitry may still exceed levels required to elicit a withdrawal reflex. The correlation between neuronal activity and behavioural response remains unclear and it is difficult to assess to what extent spinal neuronal activity must be reduced, in order to suppress behavioural withdrawal reflexes. In addition, it is interesting to note that in my study, the effects of morphine on the electrical and natural evoked responses of sham operated rats were consistently less than those of naïve rats. A sham surgery may produce a small degree of damage to minor nerve branches and local tissue. However, the exact clinical relevance of the sham operation is still unclear, and although it represents an essential control for the SNL surgery, its physiological basis is obscure and therefore makes discussion of results difficult in these animals.

The mechanisms underlying the potential reduced efficacy of morphine are complex and involve various components. There is compelling evidence to suggest that following peripheral nerve injury, neuronal plasticity occurs which interferes with the action of opiate drugs (Dickenson 1994b). In addition to anatomical changes, neuropathy results in an increased spinal level of cholecystokinin (CCK) (Nichols *et al.*, 1995; Stanfa *et al.*, 1994). Previous studies have shown that the diminished morphine responsiveness of rats with sciatic nerve section and spinal

nerve ligation results from increased levels of CCK, which physiologically antagonises morphine-induced antinociception (Nichols *et al.*, 1995; Xu *et al.*, 1993). A sufficiently high enough local concentration of morphine delivered to the spinal cord by intrathecal administration may overcome the CCK-mediated physiological antagonism of morphine analgesia. Systemic administration may not achieve the required local spinal concentration of morphine (Matos *et al.*, 1995) in the absence of adverse effects, which can prevent full dose-escalation under neuropathic conditions (Portenoy *et al.*, 1990). Hence, the nature of poor opioid sensitivity in neuropathic pain states (Arner & Meyerson 1988) may in part be that of insufficient dosing, which may prevent analgesic doses of morphine to be titrated, before side effects become intolerable.

While neuropathic pain has been reported to be resistant to the analgesic effects of morphine (Arner & Meyerson 1988), it is now generally acknowledged that neuropathy is not opioid resistant, but exhibits a reduced sensitivity to systemic opioids (Jadad *et al.*, 1992; Portenoy *et al.*, 1990). In recent years, the terminologies 'opioid-responsive pain' and 'opioid-non-responsive pain' have been developed to describe how well a pain state responds to opioid analgesics, and neuropathic pain has often been described as 'opioid-non-responsive' or 'opioid-resistant'. These terms are not entirely satisfactory since clinically, the situation is rarely clear cut and patients do not always fall into these broad categories. Perhaps the more appropriate term would be 'opioid-poorly responsive pain'. It is now generally agreed that the lack of response of neuropathic pain states to opioid therapy is not an *absolute* phenomenon, but rather, it is *relative*, thus if a sufficient dose of the opioid is administered, a partial response can at least be obtained in most patients (McQuay *et al.*, 1992). In a study using patient controlled analgesia, at least 84% of neuropathic patients reported moderate (46%) to good response (38%) following systemic morphine administration (McQuay *et al.*, 1992). Although placebo effects must be taken into account, these results support the idea that opioid treatment may have significant therapeutic value in at least some patients, and opioids should not be excluded from pain management in this pain state. The results from my present study clearly demonstrate that opioids are not ineffective in reducing neuronal responses that may be related to symptoms of neuropathic pain. The extent to which morphine can alter these responses appears to be dependent on

the route of administration. Hence, in my study, the intrathecal route of morphine administration produced greater inhibitions of the neuronal responses in SNL rats, as compared to the systemic route of administration. Furthermore, the systemic route was limited by adverse effects, which restricted dose-escalation. The clinically relevant routes of opioid administration include the oral, sublingual, buccal, subcutaneous and spinal (epidural / intrathecal) routes. Oral administration is generally the preferred route, since it is both economical and easy to administer, however, it may be associated with harmful metabolites from first-pass metabolism, such as the glucuronides. Both oral (Moulin *et al.*, 1996) and intrathecal administrations of morphine have been shown to be effective in providing long term analgesia in patients with chronic noncancer pain (Winkelmuller & Winkelmuller 1996). In addition, a recent study reported that intraspinal opioids administered via an implantable medication pump provided good pain relief in 66% of the patients with chronic non-malignant pain (Likar *et al.*, 1999).

These observations therefore suggest that intrathecal morphine may have therapeutic value in neuropathic pain conditions where systemic morphine fails to provide adequate analgesia. Clinically, the potential use of spinally administered opioids has been increasingly recognised, and it has been suggested to have a much smaller dose requirement, longer duration and possibly, a better side-effect profile than the systemic route of administration. The problem of opioid responsiveness in neuropathic pain may not simply be that of a reduced opioid sensitivity but, rather, the failure to deliver a sufficiently high concentration of the systemic opioid to the spinal cord in the absence of adverse side effects. Further trials of the effectiveness of spinal opioids in neuropathic patients may resolve this issue. The lack of consensus between the animal studies on the effect of morphine in a number of different models of neuropathic pain may be due to varying opioid sensitivities of the different types and symptoms of neuropathic pain. Even in humans, it is known that a wide inter-individual variation in opioid responsiveness exists, which may determine how much drug can be tolerated, before side effects limit its dose-escalation (Hanks & Forbes 1997). The time-course of the neuropathy is also an important consideration. Further studies are needed to assess whether the organisation of these receptors changes over time following the lesion, since this may influence opioid efficacy under various neuropathic pain states. In the present

study, the effect of morphine was studied over a two week period and opioid sensitivity did not differ significantly between the two time-points. Whether or not the clinical effectiveness of morphine is lost at later stages, my results suggest that early opioid intervention can be effective and an aggressive approach to the treatment of neuropathic pain with opioids may be beneficial.

Chapter 8

General Discussion

8. General Discussion

Neuropathic pain is a major clinical problem which is known to afflict millions of people every year. In a recent World Health Organisation (WHO) survey, the prevalence rate of persistent pain states (> 6months) was reported to be 22%, based on a cross-national data obtained from 14 countries (Gureje *et al.*, 1998). Thus, a significant proportion of these populations suffer from chronic pain states of various origins, some of which will be neuropathic, and despite recent advances in pain research, the treatment of neuropathic pain still remains far from being satisfactory. One of the main factors which complicates pain management is the complexity of the mechanisms underlying this clinical condition. Neuropathic pain is heterogeneous, both in terms of its aetiology and anatomical location in the nervous system. The mechanisms underlying this condition are multiple and most patients will often report several pains, with the result that each pain syndrome may respond differently to various treatments. It is not surprising, therefore, that pharmacological intervention with one drug does not always abolish the pain, and although some component of their pain may be significantly attenuated, patients may still report inadequate analgesia to the treatment.

The spinal nerve ligation (L5/ L6) model of neuropathy (Kim & Chung 1992) employed in this study produced reproducible behaviour in rats, which could easily be quantified to study the time course of the development of behaviours indicative of neuropathic pain (mechanical/ cold allodynia). The introduction of animal models has led to recent advances in the understanding of neuropathic pain and allowed us to explore potential therapeutic targets for the treatment of this condition. Although care must be taken in order to extrapolate data from animal studies to clinical applications, the models have proved to be invaluable research tools. To date, substantial evidence exists for the behavioural consequences of neuropathic pain, although there is only limited electrophysiological data on the modifications of central neuronal responses resulting from peripheral nerve injury. Hence my studies were aimed to investigate the potential electrophysiological changes which take place in the spinal dorsal horn neurones following spinal nerve ligation.

The overall results from my study suggest that there is considerable plasticity in the physiology and pharmacology of pain transmission at the level of the spinal cord following selective ligation of L5/ L6 spinal nerves. SNL induced a complex pattern of changes in the responses of dorsal horn neurones which became prominent at 1 to 2 weeks after nerve injury. One of the most marked features of the nerve injury was an increase in the level of spontaneous activity, which suggested an enhancement of spinal cord excitability. In addition, there was also a reduction in the mechanical and thermal evoked responses, which was observed both in the noxious and innocuous range of stimuli. Hence, these results indicate that there is plasticity in the spinal cord following spinal nerve ligation, which consequently produces changes in the response profile of spinal neurones, involving a combination of both increased and decreased neuronal responses. Although the combined presence of hyperexcitability and reduced neuronal responses appears to be somewhat paradoxical, a similar situation is also seen in the clinic where patients can display both positive (spontaneous pain, hyperalgesia, allodynia) and negative symptoms (sensory deficit).

In addition to the alterations in the neuronal response profile, my results demonstrated that there is plasticity in both spinal excitatory (NMDA receptor system) and inhibitory transmitter systems (adenosine, opioid) following nerve injury. This is reflected by the greater effectiveness of morphine (μ opioid receptor agonist), N^6-cyclopentyladenosine (adenosine A_1 receptor agonist) and NMDA receptor antagonists (memantine, ketamine, MK-801) in SNL rats as compared to sham operated rats. These findings are consistent with previous behavioural studies where both NMDA receptor antagonists (Davar *et al.*, 1991; Mao *et al.*, 1992b; Seltzer *et al.*, 1991b; Yamamoto & Yaksh 1992a) and adenosine A_1 receptor agonists (Cui *et al.*, 1997; Lee & Yaksh 1996; Sjolund *et al.*, 1998; von Heijne *et al.*, 1998) were demonstrated to be antinociceptive/ antiallodynic in animal models of neuropathy. I extended these observations by providing electrophysiological evidence that these agents produce inhibitions of the electrical and natural evoked responses of spinal neurones in SNL rats. Although these neuronal responses are not direct measures of allodynia or hyperalgesia, they may be related to some of the symptoms of neuropathic pain.

My finding that intrathecal morphine exerts greater effectiveness after nerve injury was somewhat surprising, since behavioural data to date employing the SNL model of neuropathy have suggested that intrathecal opioids are generally less effective in attenuating symptoms of neuropathic pain as compared to sham operated rats, in particular, against mechanical allodynia. Intrathecal morphine produced marked inhibitions of the C-fibre and natural (mechanical/ thermal) evoked responses of SNL rats, and the degree of inhibition was largely dependent on the route of drug administration. Overall, intrathecal morphine produced greater effects as compared to the systemic route of administration. These results support the idea that opioid responsiveness is a complex phenomenon, which is dependent not only on the characteristic of the pain syndrome (acute/ chronic), but also on other factors such as the route of administration, bioavailability of drug, and the timing of administration relative to nerve injury. These factors may, in part, account for the controversy of opioid sensitivity in neuropathic pain states (Dellemijn 1999), where some patients obtain pain relief following morphine administration, whilst others do not.

The figure below (Fig. 43) attempts to summarise the findings of my present electrophysiological study, together with results reported from previous behavioural, immunohistochemical, or autoradiographic studies in the selective spinal nerve ligation model of neuropathy.

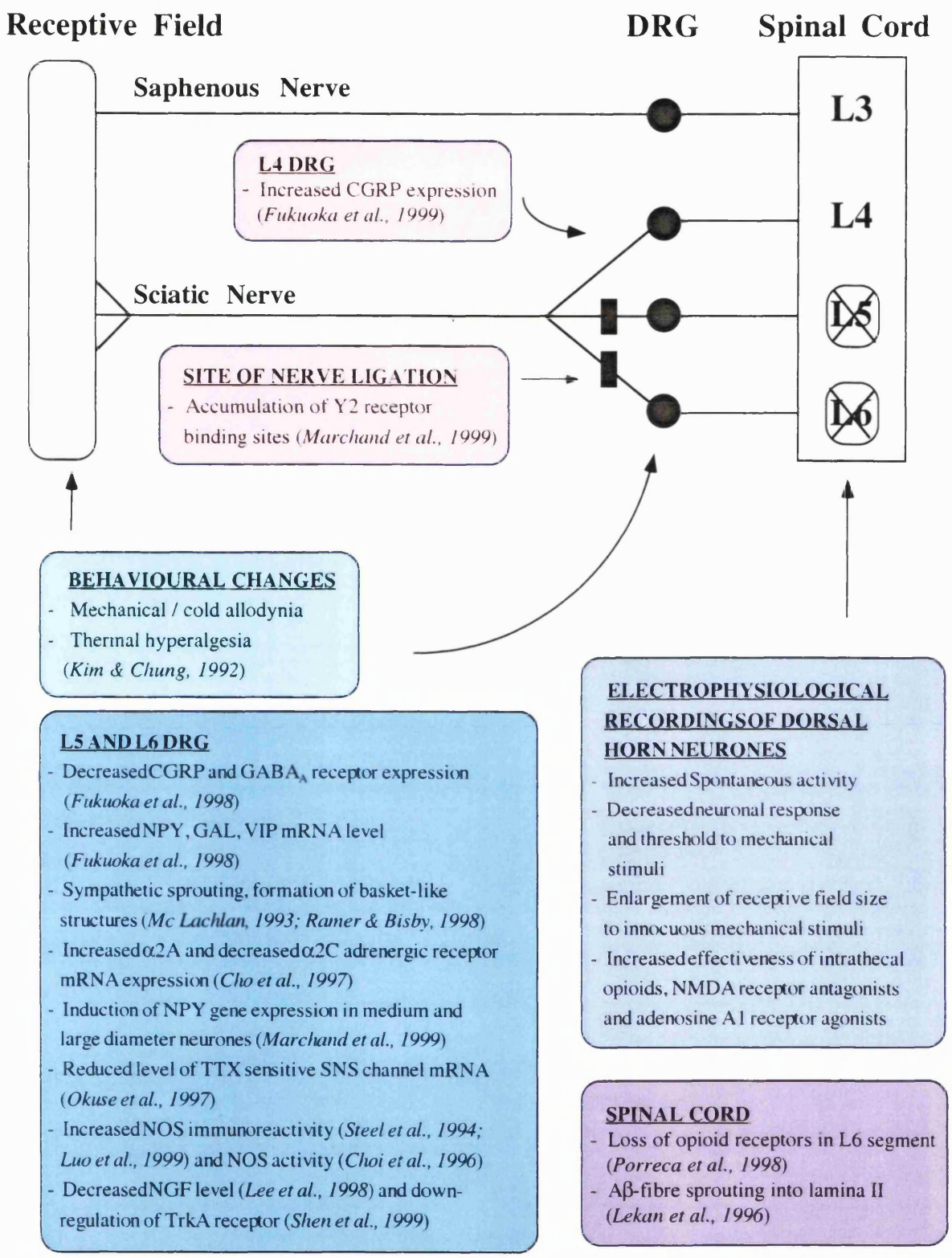


Figure 43. Plasticity of the peripheral and central nervous system following spinal nerve ligation: A summary of our current knowledge.

8.1 Neuropathic pain: current approaches and future directions

Hence, in spite of the considerable progress in basic pain research, the understanding of neuropathic pain still remains limited, as are the treatments currently available for this multiple pain syndrome. Tricyclic antidepressants and anticonvulsants are still the mainstay approach to the management of neuropathic pain. The effectiveness of these agents have been demonstrated clinically for conditions such as postherpetic neuralgia and diabetic neuropathy (Sindrup & Jensen 1999). However, dose-limiting side effects remain a problem for neuropathic pain patients. The use of anticonvulsants has been associated with adverse effects such as drowsiness and nausea. Similarly, tricyclic antidepressants can produce cardiac arrhythmias, postural hypotension and sedation, therefore it cannot be prescribed to patients with cardiovascular problems (Rowbotham *et al.*, 1998). Despite the clinical trial data demonstrating successful pain relief with several drug regimens, the management of this pain state remains difficult and there appears to be little consensus in the effectiveness of various agents for the treatment of this condition (Davies *et al.*, 1991). Lack of analgesic effect observed in some patients may be a result of inadequate dosing, failure to attain a favourable balance between analgesia and adverse side effects or development of drug tolerance (Kingery 1997). In addition, the drug may produce moderate analgesic effects, but may not attenuate the major component of the patient's pain, therefore resulting in an inadequate drug response.

It has now become clear that neuropathic pain does not represent a single mechanism but rather, a combination of multiple factors, and this is supported by clinical findings where a wide range of neuropathic phenomena coexist in an individual patient (Fields & Rowbotham 1994). Furthermore, neuropathic pain can arise from conditions as diverse as diabetes and HIV. This multi-factorial aspect is also suggested by findings from animal studies where various mechanisms have been proposed to underlie the development of different neuropathic pain behaviours (Ossipov *et al.*, 1999). It is not surprising, therefore, that a given pharmacological intervention can give rise to a heterogeneous response in patients since individuals

rarely exhibit an identical combination of pain symptoms. This has prompted studies to use a mechanism-based classification to provide specifically orientated therapy for the various sensory phenomena. By taking these pathological mechanisms into account, there is a potential for the development of new and improved therapies for the treatment of this clinical condition.

A better therapy for the long term treatment of neuropathic pain states may in part be achieved through combination therapy, which could potentially target different pain phenomena and consequently produce adequate analgesia. In recent years, increasing number of studies have recognised combination therapy as an useful approach for the management of this complex pain syndrome, and co-administration of adjuvant drugs have been shown not only to enhance analgesia, but also to reduce side effects. Hence in patients who respond poorly to opioids, early intervention and concurrent use of adjuvant drugs, such as anticonvulsants, may prove to be effective in providing pain relief. Recent evidence suggests that gabapentin, a novel anticonvulsant, is effective in relieving postherpetic neuralgia (Rowbotham *et al.*, 1998) and diabetic neuropathy (Backonja *et al.*, 1998). This novel compound has received much attention as a potential first line treatment of neuropathic pain states, due to its relatively well tolerated side effect profile, lack of organ toxicity or drug interaction with other medications. Its mechanism of action has not been fully elucidated, although recent studies have suggested possible interactions with the spinal cord neuronal calcium channels (Rowbotham *et al.*, 1998). However, in these studies with gabapentin, the degree of pain relief, although significant, was not that marked (Backonja *et al.*, 1998; Rowbotham *et al.*, 1998). The novel agents tramadol (a weak opioid) and lamotrigine (sodium channel blocker) may also have potential therapeutic value in controlling neuropathic pain (Zakrzewska *et al.*, 1997). Other targets which have also received considerable attention include sodium channel blockers, calcium channels blockers and cannabinoids.

Recent evidence has demonstrated that there is significant plasticity in the expression and function of sodium channels following peripheral nerve injury, and this has been suggested to form the molecular basis underlying abnormal sensory processing (Black *et al.*, 1999; Dib-Hajj *et al.*, 1999; Okuse *et al.*, 1997). One principle feature associated with neuropathic pain is the hyperexcitability and/or

increased baseline sensitivity of injured afferent fibres, which contributes to the generation of sustained ectopic activity. Abnormal repetitive firing in these fibres has been largely attributed to the accumulation and increased membrane density of sodium channels at the site of nerve injury (Devor *et al.*, 1993). Whilst evidence is accumulating for alterations in the expression of various sodium channels following nerve injury (Black *et al.*, 1999; Dib-Hajj *et al.*, 1999; Okuse *et al.*, 1997), the relative contribution of individual sodium channel subtypes toward altered sensory processing remains unclear. The TTX-resistant SNS sodium channel has recently been implicated in the pathophysiology of nerve injured states and interference of the expression or function of this channel has been proposed to attenuate primary symptoms of neuropathic pain (Porreca *et al.*, 1999). This was supported by evidence demonstrating that a selective inhibition of SNS protein expression with specific antisense oligodeoxynucleotides prevents mechanical allodynia in SNL rats (Porreca *et al.*, 1999). Since this channel exhibits a restricted distribution in sensory neurons, a selective inhibitor of the SNS sodium channel may potentially offer an alternative strategy for the treatment of neuropathic pain, with an improved side-effect profile (Porreca *et al.*, 1999). The change in expression of voltage-gated sodium channels may account for the electrogenesis of ectopic discharges following nerve injury and contribute to the hyperexcitability of injured neurones (Devor *et al.*, 1993). The administration of sodium channel blockers may therefore be effective in reducing at least some of the symptoms of neuropathic pain through the attenuation of excess neuronal activity.

Another potential target is the voltage-dependent calcium channel (VDCC), which controls many neuronal functions, including neurotransmitter release and neuronal excitation. To date, several classes of VDCCs (L-, N-, P-, Q-, R- and T-type) have been identified, which can be distinguished by their electrophysiological and pharmacological profiles (Olivera *et al.*, 1994). Substantial evidence exists for the effectiveness of N/ P-type calcium channel antagonists in animal models of neuropathy (Bowersox *et al.*, 1996; Chaplan *et al.*, 1994; Xiao & Bennett 1995). These compounds are thought to act by reducing the pathological hyperexcitability of neuronal circuitry in the spinal cord. Many of the naturally occurring peptides which produce a selective block of these calcium channels are toxins derived from marine snails (e.g. ω-conotoxin, ω-agatoxin), therefore making it unsuitable for

clinical applications. This has encouraged the development of synthetic analogues, which are able to selectively block neuronal N or P-type calcium channels in the absence of toxic effects. SNX-111 (N-type calcium channel blocker) is one such compound, and phase I/ II clinical studies have shown that it provides significant pain relief in chronic pain patients (Brose *et al.*, 1997). Encouraging results of preliminary clinical studies therefore support the use of these compounds for the treatment of persistent pain states, such as neuropathy. The discovery of a potential N-type VDCC isoform specific to the sensory system may provide a more suitable target for calcium channel blockers with minimal side-effects.

More recently, there has been a growing interest in the cannabinoid receptor system as a potential target for the treatment of pain (Martin *et al.*, 1999). To date, two cannabinoid receptor types have been identified (CB1 and CB2) and endogenous agonists for the receptors have been isolated from mammalian tissues (Felder & Glass 1998). Cannabinoid receptors are found within anatomical regions implicated in pain modulation including the spinal dorsal horn (Hohmann & Herkenham 1999; Tsou *et al.*, 1998) and exhibits both a pre- and post-synaptic localisation in the spinal cord (Hohmann *et al.*, 1999a). The observation that spinal injection of CB1 antagonists (Richardson *et al.*, 1997) or CB1 antisense treatment (Richardson *et al.*, 1998b) produces hyperalgesic effects led to the speculation that an endogenous cannabinoid tone exists, which dampens acute and tonic pain sensitivity (Strangman *et al.*, 1998). Several behavioural studies have demonstrated the role of cannabinoids in pain modulation using various stimulus modalities (Martin *et al.*, 1999; Richardson *et al.*, 1998a; Vivian *et al.*, 1998). This was further supported by electrophysiological evidence which demonstrated that cannabinoids selectively modulate the activity of nociceptive neurons at the spinal level by action at the CB1 receptor (Chapman 1999; Hohmann *et al.*, 1999b; Strangman & Walker 1999). These findings provide strong evidence that cannabinoids inhibit the transmission of brief and prolonged nociceptive stimuli, that lead to acute and persistent pain. One mechanism underlying the antinociceptive action of cannabinoids appears to be an inhibition of N- and P/Q-type voltage-gated calcium channels (Shen & Thayer 1998). The ability of cannabinoids to modulate pain sensitivity and inhibit the maintenance of central sensitization in response to repeated or prolonged noxious stimulation has raised the possibility that these

compounds may be useful for the treatment of neuropathic pain. In support of this view, several studies have provided evidence for the effectiveness of cannabinoids in animal models of neuropathy (Herzberg *et al.*, 1997; Zeltser *et al.*, 1991).

Cannabinoids may therefore represent a possible novel target for the treatment of neuropathic pain. Although the potent antinociceptive actions of cannabinoids had been recognised for a long time, their use in the clinic has largely been restricted due to their psychotropic effects. Provided their psychotropic effects can be separated from their analgesic effects, the potential exists for the clinical application of these agents as analgesics for the treatment of neuropathic pain.

In conclusion, the use of these agents as part of a multidrug regimen may be an effective approach to control the multiple symptoms of neuropathic pain. Further large scale randomised controlled studies are clearly needed to assess the efficacy of novel agents for long term use in neuropathic pain states. An improved therapy of this complex clinical condition may rely on the development of drugs specifically orientated towards certain pain phenomena, through mechanism-based therapy. In patients with neuropathic pain where therapeutic options are limited, such drugs could offer significant advantages over currently available treatments and may produce promising results.

Chapter 9

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