Peripheral nerve injury-induced plasticity of spinal voltage-dependent calcium channels: an electrophysiological study of dorsal horn neurones in the rat.

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ABSTRACT

Neuropathic pain can involve exaggerated pain responses often accompanied by sensory deficits with some patients responding poorly to traditional analgesics. Dysfunctional mechanisms, and thus potential drug targets, are conceivably located on peripheral nerves and central neurones. Of interest here are alterations in spinal neuronal excitability contributing to the plasticity of transmission and modulating systems, manifest as altered nociception. Counterparts of clinical symptoms can be studied in animal models. Spinal nerve ligation, employed here, involves unilateral tight ligation of L5/6 spinal nerves of the sciatic nerve and reproducibly induces mechanical and cold allodynia. In vivo electrophysiology was subsequently used to record evoked dorsal horn neuronal responses to electrical and natural stimuli. Activation of voltage-dependent calcium channels (VDCCs) is critical for neurotransmitter release and neuronal excitability, and blockers are antinociceptive in behavioural and clinical studies. Here, effects of N-, P/Q-, T- and L-type VDCC blockers (ω-conotoxin GVIA, ω-agatoxin IVA, ethosuximide and nifedipine, respectively) on evoked neuronal responses were investigated in an attempt to unravel their physiological and pathophysiological roles in sensory transmission. Spinal ω-conotoxin GVIA produced prolonged inhibitions of the evoked neuronal responses in neuropathic and control rats, in a manner increased after neuropathy. Spinal ω-agatoxin IVA exerted inhibitory actions but to a lesser extent. Minimal inhibitions were achieved with spinal ethosuximide and nifedipine. Mechanisms of neuropathic pain are diverse; indicating that combination therapy could be beneficial. The anticonvulsant gabapentin, effective in the clinical treatment of neuropathic pain, has been demonstrated to bind the VDCC α2δ subunit. Here, gabapentin inhibited neuronal responses in neuropathic, but not normal, rats, in line with neuropathy-induced VDCC plasticity. In the presence of gabapentin, morphine exhibited greater inhibitions, again more pronounced after neuropathy. These studies indicate a prominent role for N-type VDCCs in sensory transmission, which is increased after neuropathy, suggestive of a key role in the increased central excitability.
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CHAPTER 1

INTRODUCTION
Pain is an unpleasant sensory and emotional experience associated with actual or potential tissue damage. It is subject to various influencing factors, including the physical, psychological and social experiences of the patient, and is more complicated than a simple fixed stimulus-response relationship. On encountering a potentially harmful or 'noxious' stimulus the final perception of pain is only established once the higher cortico-limbic brain centres receive the information. This pathway involves a substantial degree of encoding and modulation that can occur in the peripheral nervous system, the dorsal horn of the spinal cord and supraspinal relay and modulating centres, including the thalamus, midbrain and brain stem.

Under normal physiological conditions pain is useful and serves as a warning to protect the organism. In such circumstances pain is termed 'acute', in that the undesirable sensations created by injury persist for only days or, in some cases, weeks. The pain transmission pathways are, however, subject to dysfunction such that pain can become exaggerated or 'chronic', persisting after resolution of the injuring modality, and outweighing its biological purpose. Chronic pain is often associated with expanded receptive fields; thus the amplitude of response to a given stimulus can increase (hyperalgesia) and pain can be elicited by normally innocuous stimuli (allodynia). Pain can also spread and become non-segmental. Such maladaptive pain states can arise from damage to the nervous system and when resulting from nerve injury are termed 'neuropathic'.

Acute pain can be successfully controlled by a variety of pharmacological agents, yet chronic pain is a major clinical concern and often responds poorly to traditional therapeutic approaches such as non-steroidal anti-inflammatory drugs (NSAIDs) and opioids. The development of effective analgesics is made possible by extensive research in the clinical and pre-clinical setting. Exploitation of continually developing pharmacological, immunohistochemical, molecular and genomic techniques, furthers knowledge of the molecular and cellular mechanisms that underlie the pain systems, along with its pathophysiological changes. Much of the understanding surrounding pain transmission comes from the extensive study into cutaneous sensory input, and although non-cutaneous pain is also very important it is
1.1 Neuropathic Pain in the Clinic

1.1.1 Incidence and Causes of Neuropathic Pain

Neuropathic pain is a generic term which refers to a group of painful disorders characterized by pain due to dysfunction or disease of the peripheral and/or central nervous system (CNS). It has been estimated that in the UK 1% of the population experience some form of neuropathic pain, which rises to probably 50% in the over 65 age bracket (Bowsheer, 1991). Persistent pain syndromes offer no biological advantage and cause suffering and distress and since relatively few neuropathic pain patients adequately benefit from pharmacological interventions, they are therefore worthy of investigation. Disorders that frequently result in neuropathic pain are from diverse backgrounds (Ralston, 1998). Peripherally they range from metabolic disorders, such as diabetes mellitus (diabetic neuropathy), infections, such as herpes zoster (post-herpetic neuralgia) and human immunodeficiency virus, to nerve compression due to entrapment or invading tumours, as well as neuroma formation arising from amputation or nerve transection. Centrally mediated neuropathic pain can result from disorders of the spinal cord such as multiple sclerosis and trauma or tumours of the spinal cord, brainstem and hemispheric injuries.

1.1.2 Symptoms of Neuropathic Pain

Irrespective of the heterogeneous aetiologies of neuropathic pain the clinical picture exhibits common characteristic symptoms. Paradoxically the coexistence of sensory deficits and increased sensations is often manifest. As might be expected from peripheral nerve damage, impaired axonal conduction properties can arise resulting in areas showing partial or complete loss of sensation. This so-called ‘negative’ symptom, may or may not correspond to the somatic area of pain showing ‘positive’ phenomena, commonly localized to the territory innervated by the damaged nerve.
One of the most commonly used terms to describe the positive symptoms experienced is allodynia. This applies to the perception of pain evoked by a normally non-noxious stimulus. Even very gentle somatosensory stimuli, such as bending of hairs due to the wearing of clothes or a breeze, can be painful. The cutaneous region of allodynia usually shows no visible signs of injury. Allodynia can be further defined in terms of the evoking stimulus, such that ‘dynamic’ (or tactile) allodynia arises from a moving stimulus in contrast to the ‘static’, ‘pressure’ and ‘punctate’, allodynia, produced by large or fine-tipped probes, respectively. The term hyperalgesia is often confused or misinterpreted as allodynia, but this describes an enhanced pain perception, such that there is an increased pain response to, or lowered threshold to, a normally noxious stimulus. Allodynia describes a change in quality of sensation, whereas hyperalgesia is indicative of a quantitative change, and both of these represent stimulus-evoked pain. In contrast, pain perception emanating from the innervation area of the injured nerve can occur in the absence of an external stimulus and is termed ‘spontaneous’. Spontaneous pain can be continuous (ongoing, non-relenting), or paroxysmal (episodic) and is often described as having ‘tingling, crawling, cramping, burning or stabbing’ components.

Abnormal sensations are described as paraesthesias, such that the quality of sensation is different to that experienced in the absence of a neuropathic syndrome, and these can be spontaneous or stimulus-evoked. In particular, dysesthesia relates to unpleasant yet non-painful sensations that are qualitatively unrelated to the stimulus, whereas hyperesthesia describes non-painful heightened sensations. Other features of neuropathic pain include after-sensation and summation, whereby repetitive stimulation with a mildly noxious stimulus, such as pin prick, evokes a delayed pain (hyperpathia) and a progressive worsening of pain, respectively.

In an individual neuropathic pain patient not all of the various types of pain exist, rather a combination of these phenomena will be present in many conditions. For example, in a study of 63 patients with post-herpetic neuralgia, 49 showed some form of mechanical allodynia or hyperalgesia, but only 16 exhibited dynamic, pressure and punctate allodynia, the other 33 patients showed only one or two of the forms in various combinations. Furthermore, 18 of the 63 patients reported thermal allodynia, 13 reported cold allodynia and only 6 displayed both (Pappagallo et al.,
The variety of aberrant sensations observed in neuropathic pain indicates that there are either multiple pathophysiological processes or common processes affecting various components of the peripheral and central nervous system that nonetheless cause common symptoms of allodynia and hyperalgesia, possibly explaining its complicated clinical management.

1.1.4 Pharmacological Based Therapeutic Approaches to Neuropathic Pain

Traditionally, the treatment of neuropathic pain is guided by a disease/trauma based criteria dependent of the type of nerve damage. It is now becoming apparent that a symptom based approach may be more appropriate, as underlying dysfunctional mechanisms often differ in their molecular, cellular and anatomical basis (discussed in section 1.3). Since hyperexcitability, peripherally or centrally, is an essential part of many types of neuropathic pain, treatments have focused on drugs that might reduce it. However, again, neuronal hyperexcitability is not simply generated by one mechanism alone, but rather it is a culmination of several different alterations to various parts of the sensory pathway.

Interpretation of clinical evidence in order to determine effective analgesic drugs for use in defined neuropathic pain states has proven difficult. However, over the last decade numerous controlled trials have highlighted possibilities for new therapeutic strategies as well as helping to uncover possible links between different mechanisms and specific pain symptoms and pathologies (Woolf et al., 1998). One of the most useful methods employed to assess analgesic efficacy is 'NNT' (numbers needed to treat), which refers to the number of patients that have to be treated before one patient experiences more than 50% pain relief. One limitation to this method concerns drugs with side-effect profiles that prohibit optimal dosing with respect to efficacy. Side-effects are a major concern and thus although NNT may not truly reflect efficacy they do bear relevance on the usefulness of a drug.

Traditional analgesics, such as opiates and NSAIDs, are rarely used for the treatment of neuropathic pain, since they have been shown to exert limited
effectiveness, although there is considerable debate over the former. The current mainstay treatments are therapeutic agents utilized for other neurological disorders, namely depression and epilepsy, which have been observed to exhibit analgesic properties in chronic pain states. Tricyclic antidepressants have been demonstrated in controlled clinical studies effective in various neuropathic pains, with clinical profiles different to those observed for depression (Kingery, 1997). These include post-herpetic neuralgia and diabetic neuropathy with overall NNTs of 2.3 and 3, respectively (McQuay et al., 1996). Tricyclic antidepressants, some of which act by enhancing α2-adrenergic transmission by inhibition of noradrenaline and serotonin re-uptake, and newer antidepressants, which are serotonin-selective, show no advantage over other treatments (McQuay et al., 1996), and some are purported to possess NMDA (N-methyl-D-aspartate) receptor blocking activity (Eisenach et al., 1995). Side-effects, such as cardiac arrhythmias, postural hypotension and sedation limit their use (Rowbotham et al., 1998).

Neuropathic pain and epilepsy both share neuronal hyperexcitability as a common underlying mechanism. There are established anticonvulsants that target the generation of neuronal hyperexcitability via non-specific block of Na+ channels and some of these have been proven effective in the treatment of various forms of neuropathic pain. For example, carbamazepine has been shown to be effective in diabetic neuropathy, yet its NNTs range from 2 - 9.4; thus is not always beneficial (McQuay et al., 1995). Lamotrigine is a newer anticonvulsant displaying subtype/conformational Na+ channel selectivity and is proving to have potential in the treatment of neuropathic pain (Eisenberg et al., 1998). Of great interest, is the novel anticonvulsant gabapentin (GBP), which although originally designed as a γ-aminobutyric acid (GABA) analogue, does not interact with the GABA system or at the traditional Na+ channel site of other antiepileptics (Taylor et al., 1998). Its mode of action remains somewhat elusive, with VDCCs being possible targets, nonetheless GBP has been proven an effective analgesic in randomized, double-blind studies of diabetic neuropathy (Backonja et al., 1998) and post-herpetic neuralgia (Rowbotham et al., 1998) with NNTs of 3.7 and 3.2, respectively. Advantages of GBP include its well-tolerated side-effect profile and its lack of adverse drug interactions, and thus it represents a useful add-on therapy.
There are other clinically licensed drugs that show potential for the treatment of neuropathic pain. These include NMDA antagonists, such as ketamine and memantine, and α2-adrenergic agonists, such as clonidine although their use is hindered by adverse side-effects (Sang, 2000). The widely accepted notion of 'opioid-resistant neuropathic pain' has been re-addressed and neuropathic pain may only show reduced opioid sensitivity, overcome with increasing dose, but again this approach is not without side-effects (Portenoy et al., 1990; Jadad et al., 1992). In these cases utilization of such drugs with other therapies may be of benefit. Additionally, there are emerging novel, but as yet unlicensed, drugs for the treatment of neuropathic pain, such as the N-type VDCC blocker SNX-111, currently undergoing phase III clinical trials, which shows promise (Mathur et al., 1998).

1.2 Animal Models of Neuropathic Pain

The development of several animal models of neuropathic pain has been critical in elucidating its complex causal mechanisms. Pre-clinical models can be used to provide an unanaesthetized, intact system, with preserved neuronal pathways and spinal/supraspinal interactions, which realistically relates to the integrative process of nociception. In addition to aiding the understanding of dysfunctional mechanisms, behaviourally, animal models serve to define of the pharmacology of neuropathic pain states and can be used to assess the therapeutic benefit of existing drugs as well as the analgesic potential of novel drugs. Likewise, at a neuronal level, in vivo electrophysiological techniques can be used to investigate pathological changes involved in neuropathic pain, which is also useful for pharmacological assessment and identification of possible new drug targets.

These approaches do provide useful predictive information regarding the treatment of neuropathic pain, especially in the case of novel therapies before transfer into clinical setting. However, animal studies cannot provide descriptive information about the quality of pain, which may bear some relevance to underlying mechanisms, and thus the therapeutic approach applied clinically. Furthermore, differences in receptor types and distributions between animals used and humans may also complicate the extrapolation of pre-clinically obtained data to the human
situation. The various animal models also show differences between each other in the extent of behavioural symptoms manifest and underlying nervous system changes, as is the case in human patients. It is important to appreciate this when making comparisons to human neuropathic pain states, as in both scenarios the type of injury and observed symptoms or behaviours often reflect differences in causal mechanisms which can result in differential responsiveness to pharmacological interventions. Thus, attention should be paid not to the model per se, but perhaps to these individual characteristics.

Animal models of neuropathic pain can broadly be divided into peripheral mononeuropathy, peripheral polyneuropathy and central neuropathic pain and these will now be summarized.

1.2.1 MODELS OF PERIPHERAL MONONEUROPATHY

There are three main established rat models of peripheral mononeuropathy that produce partial denervation of one hindpaw. These are the chronic constriction injury model (CCI) (Bennett & Xie, 1988), the partial sciatic nerve ligation model (PSL) (Seltzer et al., 1990), and the selective spinal nerve ligation model (SNL) (Kim & Chung, 1992), which are represented diagrammatically in Figure 1. In contrast to models of complete denervation, such as spinal section and dorsal root rhizotomy (Zeltser & Seltzer, 1994), some sensory input into the spinal cord is maintained allowing successful behavioural testing. Each produces behavioural signs reminiscent of the clinical picture, ipsilateral to nerve injury. These include tactile allodynia, indicated by hindpaw withdrawal to cold or innocuous mechanical stimuli such as von Frey filaments, and hyperalgesia, indicated by a shortened withdrawal latency to mechanical and thermal stimuli. Often these evoked pain behaviours are associated with other nocifensive manners, such as licking and biting of the injured hindpaw in the presence or absence of an external stimulus, which in the latter case is indicative of ongoing pain.
Figure 1. Animal models of neuropathic pain. SNL (spinal nerve ligation) involves tight ligation of L5/6 spinal nerves; CCI (chronic constriction injury) involves placing 4 loose ligatures around the common sciatic nerve; PSL (partial sciatic nerve ligation) involves tight ligation of 1/3 to 1/2 of the thickness of the common sciatic nerve. For SNL, the L4, L5 and L6 spinal nerves are exposed by removal of part of the L6 transverse process. (Modified from Tabo et al. (1999).)
1.2.1.1 Chronic Constriction Injury

CCI involves loosely tying four ligatures of 4-0 or 5-0 chromic gut suture unilaterally around the sciatic nerve at the level of the popliteal fossa (back of the knee joint), which is proximal to its branching into the L4, L5 and L6 spinal nerves (Bennett & Xie, 1988). Loose ligation is performed such that the superficial epineurial vasculature is maintained, however this ‘looseness’ imparts a degree of experimental variability into the model. Depending on the extent of constriction exerted by the ligatures, varying proportions of fibre types within the sciatic nerve innervating the hindpaw could be injured (Basbaum et al., 1991; Carlton et al., 1991). Nonetheless, behavioural studies following surgery reveal that CCI rats show signs of allodynia and hyperalgesia to mechanical and thermal (both hot and cold) stimuli. These evoked responses are apparent within 1-2 days and maintained for a post-operative period of up to 3 months, and are accompanied by spontaneous nociceptive behaviours, such as contact avoidance foot lifts exerted ipsilateral to nerve injury in the absence of external stimulus, indicative of on-going pain. The ipsilateral hindlimb also displays a characteristic ‘guarding’ posture. Instead of fully extending toes mediating an even spread of weight over the entire hindpaw, the toes are held clasped together, often raised probably to avoid contact with the floor, and weight bearing shifts predominantly to the uninjured contralateral side. Autotomy has also been reported, but to varying extents, following CCI (Bennett & Xie, 1988; Attal et al., 1990) and is always seen in the complete denervation models. This self-mutilation may reflect the animal’s response to pain or unpleasant sensation (Coderre et al., 1986) by endeavouring to rid itself of the painful hindpaw, but this is not a common clinical observation and not considered relevant to the human condition. The CCI-induced behavioural abnormalities appear to have some sympathetic component (discussed in section 1.3) since sympathectomy has been shown to reduce thermal allodynia and to a lesser extent mechanical hyperalgesia (Perrot et al., 1993). However this is not consistently reported and CCI is considered to produce relatively sympathetic-independent pain. It has been suggested the clinical correlates of CCI are most likely peripheral nerve injuries and complex regional pain syndrome (see Wallace, 2001).
1.2.1.2 Partial Sciatic Nerve Ligation

PSL involves ipsilaterally passing a 8-0 silk suture through the sciatic nerve and tying it such that one third to a half of the nerve thickness is tightly ligated (Seltzer et al., 1990). In accordance with the experimental variability encountered using loose ligatures in the CCI model, PSL also inconsistently injures varying proportions and types of sciatic nerve afferent fibres between animals. However, reliable behavioural characteristics are exhibited, similar to those produced by CCI, such as long-lasting spontaneous and evoked nociceptive behaviours, including tactile allodynia and thermal hyperalgesia which are relieved to a certain extent by sympathectomy (Shir & Seltzer, 1990). Although not documented in the original paper, cold allodynia has since been described (Kim et al., 1997). 'Guarding' posture is displayed, but autotomy is not reported. Again, like CCI, the clinical correlates are suggested to be peripheral nerve injuries and complex regional pain syndrome (see Wallace, 2001).

1.2.1.3 Selective Spinal Nerve Ligation

SNL involves unilaterally tying a tight ligature, using 3-0 silk suture, around two (L5 and L6) of the three spinal nerves (L4 - L6) that comprise the sciatic nerve, inbetween their conjunction to form the sciatic nerve but proximal to their DRG (Kim & Chung, 1992). This results in a highly reliable tactile allodynia, thermal hyperalgesia and cold allodynia, and to a lesser more variable extent, spontaneous pain behaviours. Rats also show 'guarding' positioning of the injured hindpaw, but no autotomy. Stable allodynia is established within 2 days of surgery and maintained for 2 - 3 months. In comparison to CCI and PSL, the magnitude of mechanical allodynia, assessed by withdrawal frequency to innocuous mechanical stimuli (von Frey filaments), is greatest and most reliable in the SNL model. Conversely CCI, which displays the least reliable mechanical allodynia, shows more substantial spontaneous nociceptive behaviours than observed after SNL and PSL (Kim et al., 1997). Sympathectomy is reported to relieve neuropathic pain behaviours induced by SNL (Kim & Chung, 1992; Choi et al., 1994; Kim et al., 1997), and this has been demonstrated to be notably greater than that achieved for CCI and PSL (Kim et al., 1997).
In comparison to CCI and PSL, the selective tight ligation of SNL minimizes experimental variability to some extent, however individual rats anatomy may differ in the contribution that each spinal segment makes to the sciatic nerve. Furthermore the SNL model preserves segregation of injured and uninjured primary afferent input proximal to the formation of the sciatic nerve such that the corresponding spinal segments and DRG for each spinal segment contain either injured (L5 and L6) or intact (L4) fibres. SNL represents a model of partial denervation but in contrast to CCI and PSL, where partial denervation damage to the common sciatic nerve results in all three spinal segments and their DRG containing a combination of damage and undamaged fibres, spinal input from damaged afferents can be selectively handled. The clinical correlates of SNL are suggested to be nerve root injury and plexus avulsion injury but may well also relate to other syndromes where nerve compression occurs.

1.2.2 Models of Peripheral Polyneuropathy and Central Neuropathic Pain

1.2.2.1 Diabetic Neuropathy

This model was developed to study the neuropathic pain state of peripheral diabetic neuropathy observed in some patients with diabetes mellitus. Subcutaneous injection of streptozotocin in rats induces a hyperglycaemia and glucosuria that results in a peripheral polyneuropathy, whereby animals show allodynia, not reversed by sympathectomy (Ahlgren & Levine, 1993), and decreased nociceptive threshold to paw pressure (Malmberg et al., 1993) within one week.

1.2.2.2 Central Neuropathic Pain

There are two main animal models of central neuropathic pain that involve the generation of spinal ischaemia. This can be induced transiently by occlusion of the descending aorta (Marsala & Yaksh, 1994), or permanently by localized Argon laser irradiation of the spinal cord following injection of a photosensitive dye (Hao et al., 1992a). Animals exhibit dynamic tactile, and cold allodynia. This model is proposed to be representative of spinal cord injury, such as that induced by
ischaemia, trauma or radiation. Additionally there is another central neuropathic pain model not involving ischaemia but rather inhibition of inhibitory glycine or GABA_A receptors (discussed in section 1.3) by intrathecal injection of strychnine or bicuculline, respectively (Yaksh, 1989; Sherman & Loomis, 1994). This only results in prominent tactile allodynia localized to the spinal dermatomes affected by the spinal injection (hindlimbs and lower back). This is also suggested to be a model of human spinal cord injury.

The next section reviews many of the mechanisms, suggested or otherwise proven, to underlie many of the symptoms experienced, or exhibited in the case of animal models, in neuropathic pain. Much of the information has been obtained or consolidated with observations made in the above mentioned pre-clinical models.

1.3 MECHANISMS OF NEUROPATHIC PAIN

In the normal situation, the perception of pain requires that information concerning a noxious stimulus at a peripheral cutaneous site must be conveyed to the higher centres of the brain. The sensory pathway begins with primary afferent neurones. The primary afferent neurone comprises a cell body (within the dorsal root ganglia (DRG)) and a stem process that extends both to the target tissue, via a peripheral nerve, and to the dorsal horn of the spinal cord. It is at the level of the dorsal horn that afferent spatial and temporal characteristics of the stimulus are encoded and subject to excitatory and inhibitory modulation before projection to higher brain centres via ascending tracts. The qualitative changes in sensation allude to long term reorganization of sensory pathways and evidence from clinical and pre-clinical studies reveals that changes in both peripheral nerves and central neurones may underlie the causal mechanisms and altered drug sensitivities displayed by neuropathic pain.

I will now go on to discuss the peripheral and central processes involved in the transmission of 'physiological' pain alongside probable and established dysfunctional mechanisms encountered within this pathway that result in 'pathophysiological' neuropathic pain.
1.3.1 Peripheral Processes

1.3.1.1 The Primary Afferent Fibres

Primary somatosensory afferents can be classified into essentially Aβ-, Aδ- and C-fibre groups utilizing anatomical, histochemical, functional and physiological criteria. In skin they are present in proportions of about 20, 10 and 70%, respectively, and are differentially sensitive to noxious and non-noxious stimuli. Physiologically, the degree of axonal Schwann cell myelination results in differing conduction velocities and also permits discrimination by diameter size reflected by their cell bodies in the DRG (Table 1). Heavily myelinated and thus large diameter, rapidly conducting Aβ-fibres normally only transmit modes of non-noxious, low intensity mechanical stimuli such as touch, vibration, and pressure. Thinly myelinated, intermediately sized and conducting Aδ-fibres convey both innocuous and noxious information. Type I Aδ-fibres are specifically activated by noxious high-intensity, mechanical stimuli, whereas the less common type II respond preferably to noxious heat. Aδ-fibres more responsive to cooling are also reported. C-fibres lack myelination and are therefore the smallest and slowest conducting afferents specialized for transmission of noxious stimuli. Polymodal C-fibres respond to mechanical, heat and chemical activation. Additionally C-fibres preferentially responsive to either chemical, warming, cooling or low-threshold mechanical pressure stimuli have also been described.

Table 1. Properties of the sensory cutaneous primary afferent fibre types Estimated conduction velocities (Schouenborg, 1984; Fox, 1999) and DRG cell body diameters (Scroggs & Fox, 1992a) refer to those of adult rats.

<table>
<thead>
<tr>
<th></th>
<th>Aβ-FIBRE</th>
<th>Aδ-FIBRE</th>
<th>C-FIBRE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conduction velocity (m/sec)</td>
<td>7 - 75</td>
<td>2 - 7</td>
<td>0.5 - 1.5</td>
</tr>
<tr>
<td>Myelination</td>
<td>Heavy</td>
<td>Light</td>
<td>None</td>
</tr>
<tr>
<td>DRG cell body diameter (μm)</td>
<td>45 - 51</td>
<td>33 - 38</td>
<td>20 - 27</td>
</tr>
<tr>
<td>Stimulus conveyed</td>
<td>Non-noxious</td>
<td>Non-noxious/noxious</td>
<td>Noxious</td>
</tr>
</tbody>
</table>
Histochemically, C-fibre afferent neurones can be further divided into IB4 (plant-derived isolectin) -positive and TrkA (NGF (nerve growth factor) receptor) -positive populations. Functional differences may exist between these two nociceptor populations relating to particular facets of pain transmission (Stucky & Lewin, 1999) and differential sensitivities to trophic factors, critical not only during development but also for maintenance of afferent phenotypes in the adult nervous system. These differences may underlie fibre alterations that occur in persistent pain states (Silos-Santiago et al., 1995; Fundin et al., 1997; Molliver et al., 1997).

1.3.1.2 Neurochemical Changes of Primary Afferent Fibres

Within the sensory pathway, neurotransmission between nociceptive primary afferent neurones and secondary neurones in the dorsal horn of the spinal cord is dependent on neurotransmitters. Neurotransmitters are produced in the DRG-located cell bodies of primary afferent neurones. From here they are axonally transported to central terminals and stored in presynaptic vesicles awaiting depolarization-coupled release. Translocation to peripheral terminals can also occur and this is important in neurogenic inflammation (Holzer, 1988). Neurotransmitters comprise excitatory amino acids (EAAs), such as glutamate and aspartate, associated with fast excitatory neurotransmission and neuropeptides, such as calcitonin gene-related peptide (CGRP) and substance P, that can exert longer-lasting actions. Physical separation of EAAs and peptide transmitters exists such that EAAs are stored in visually small, clear vesicles, whereas neuropeptides are stored in larger, dense vesicles, and this allows differential release in response to varying axonal activity patterns. In order to discuss alterations in the different primary afferent neurochemical phenotypes it is first necessary to understand the normal situation. The majority of studies concerning neurotransmitter distribution patterns have been conducted in the rat, and this appears to be similar in most mammals studied (Merighi et al., 1990; Bonfanti et al., 1991). There is little information regarding neurotransmitters involved in human nociception, however data derived from primates may approximate.
**A/β-fibres**

Neurotransmitter types located in the primary afferents of A/β-fibres are unclear. Although at least 50% of DRG neurones stain positive for glutamate in primates (rather more in rats), most glutamate-positive cells are of small diameter. Co-localization of glutamate and aspartate has been reported in the DRG, but these amino acids appear to distribute to different populations of afferent fibre terminals in the dorsal horn (Merighi et al., 1991), therefore aspartate may be found within Aβ-fibres (Sorkin & Carlton, 1997). Peptides tend not to be localized in large diameter DRG cell bodies, although correlations between peptide immunoreactivity and neuronal conduction velocity has revealed a small proportion of Aβ-fibres are positive for CGRP (McCarthy & Lawson, 1990). Aβ-fibres do not express substance P.

**Aδ-fibres**

Aδ-fibre DRG cell bodies are positive for glutamate, aspartate and a wide range of peptides. Notably a third contain CGRP and a fifth contain substance P and co-localization is probable (McCarthy & Lawson, 1989). The C-fibre phenotype is much more clearly defined and may equally well describe the Aδ-fibre phenotype originating from high threshold cutaneous mechanoreceptors.

**C-fibres**

C-fibre DRG cell bodies give rise to central terminals that contain both small clear and large dense vesicles, which highlights the co-transmission of EAAs and neuropeptides. As concerns the EAAs, terminals containing glutamate are more abundant than those for aspartate, and these amino acids are not found together (Merighi et al., 1991). 50% of small diameter C-fibre DRG neurones do not contain peptides and these correlate to the IB4-staining population. Those expressing TrkA on cell bodies and central terminals (Averill et al., 1995) however do stain positive for CGRP and substance P (Molliver et al., 1995). Co-localization of CGRP with somatostatin and/or tachykinins has been demonstrated (Merighi et al., 1988; Merighi et al., 1991) and see (Sorkin & Carlton, 1997; Wilcox & Seybold, 1997).
Alongside transmitters already mentioned are a number of possible others. The presence of various peptide neurotransmitters is subject to positive and negative modulation following peripheral nerve injury, as will now be discussed.

**Neurochemical Down-Regulation**

In rat and monkey DRG approximately 45% and 75%, respectively, of neurones are CGRP-immunoreactive (McCarthy & Lawson, 1990; Wilcox & Alhaider, 1990) and these are primarily nociceptive primary afferents, as already mentioned. Likewise 40% of monkey DRG neurones synthesize substance P mRNA (see Wilcox & Seybold, 1997) and this is nociceptive fibre specific. Following nerve injury there is a marked reduction in the expression of both these peptides in the DRG (Villar et al., 1989; Nothias et al., 1993; Cameron et al., 1997). This is confirmed by a reduction of substance P mRNA in the DRG in models of CCI, sciatic nerve section and nerve crush (Nielsch et al., 1987; Nothias et al., 1993; Marchand et al., 1994). A reduction in these peptides and their mRNA can be explained by the transient loss of TrkA receptors on the peripheral terminals of TrkA-positive, peptidergic primary afferents and disrupted axonal transport of NGF to the DRG, a necessary requirement for CGRP and substance P expression. Furthermore, the development of spontaneous activity within the DRG after nerve injury (Kajander et al., 1992) may result in a depletion of presynaptic terminal stores of peptide due to increased activity-dependent vesicular release.

**Neurochemical Up-Regulation**

Other peptides appear to upregulated in sensory neurones following peripheral nerve damage and the majority of these changes probably serve to enhance pain transmission. Interestingly, in contrast to the substance P decrease in nociceptive fibres, substance P's previously quiescent expression in Aβ-fibres is induced after nerve injury (Noguchi et al., 1994; Miki et al., 1998), although overall the levels of substance P mRNA are downregulated. This fibre switch to a nociceptive mode from a previously tactile-specific function, may have relevance to touch-evoked pain. Vasoactive intestinal polypeptide (VIP) is not normally expressed in lumbar ganglia, in contrast to the sacral and cranial spinal nerves.
(Honda et al., 1983; Yaksh et al., 1988; Helke et al., 1990), however it does appear to be upregulated in ipsilateral DRG after sciatic nerve transection (Shehab & Atkinson, 1986). NGF may act to prevent expression of VIP (Mulderry, 1994), the inhibitory effect of which would be removed on the decrease of NGF after nerve injury. It is important in glycogenolysis and thus its upregulation may reflect active processes of nerve regeneration, however it appears to mediate a central excitatory role in pain transmission (Wiesenfeld-Hallin et al., 1992), see also (Dickinson & Fleetwood-Walker, 1999) for review. In intact animals, galanin is expressed in a small number of DRG neurones (Lawson et al., 1993), preferentially small diameter cells, although its nociceptive/non-nociceptive role is somewhat unclear, it generally enhances nociception. Following peripheral nerve damage, its expression is upregulated in small diameter neurones and induced in larger cells (Noguchi et al., 1993; Ma & Bisby, 1997) and now exerts inhibitory actions in the dorsal horn (Wiesenfeld-Hallin et al., 1992; Luo & Wiesenfeld-Hallin, 1995). Neuropeptide Y, not normally expressed in sensory neurones, is also induced after nerve injury (Wakisaka et al., 1992; Verge et al., 1993) in large diameter neurones. This is paralleled by an increase of corresponding \( Y_2 \) receptors on peripheral afferents and central A\( \beta \)-fibre terminals. Centrally it may produce antinociceptive effects, yet peripherally it may be pro-nociceptive (Munglani et al., 1996).

Nerve injury induces a complex pattern of neurochemical and receptor alterations in primary afferent fibres, often in a fibre type-specific manner. Some of the changes observed are purely targeted to the development of neuropathic pain, however some of those apparently associated with regeneration of damaged nerves may also contribute to enhanced nociception. Although a general consensus prevails, the extent and specifics of such changes maybe subject to slight variation in different pre-clinical models. For example, levels of substance P depletion were less profound after nerve crush compared to section (Nielsch et al., 1987; Nielsch & Keen, 1989). Not all changes may be permanent, indeed the reduction of NGF and decreased TrkA receptor expression after L5 ligation returned between 2 days and 2 months, respectively. Furthermore, endogenous return of the trophic mediators has been correlated with the return of substance P levels (also demonstrated with exogenous NGF application), again relating to extent of nerve damage.
Aside from neuropeptides, certain alterations to other neuromodulators also exist in primary afferent fibres, such as nitric oxide (NO). NO is a neuronal messenger found throughout the CNS and is produced on demand from L-arginine by nitric oxide synthase (NOS). Evidence suggests that it may be linked to events downstream of NMDA receptor activation, underlying central sensitization (discussed in section 1.3.2), (Meller et al., 1992; McMahon et al., 1993) as well as exerting peripheral actions and thus maybe important in chronic pain states. Following some types of peripheral nerve damage, small DRG neurones show an increase in NOS mRNA (Luo et al., 1999), which may be attributable to NGF loss, and NOS inhibitors have shown experimental antinociceptive effects (Yoon et al., 1998). However, changes in NOS do not parallel the behavioural allodynia (Luo et al., 1999).

However convincing these nerve injury-induced histochemical changes are, the relationship of these to the onset and nature of neuropathic pain behaviours and the clinical relevance they might serve still remains to be conclusively proven.

1.3.1.3 Anatomical Changes of Peripheral Nerves

Peripheral nerve damage usually results in the formation of a ‘neuroma’, whereby the distal end of the remaining primary afferent seals and swells, due to continued axonal transport. Disruption to Schwann cell distribution and density also occurs along the damaged axon and at the neuroma producing areas of demyelination. Nerve injury, much in the same way as damage to non-neuronal tissue mediates an inflammatory response, can induce infiltration of macrophages and immunocompetent cells to damaged nerves and DRG, and it is thought that phagocytotic action of macrophages is responsible for the reduction in myelin. Loss of this electrical insulation results in altered axonal conduction properties and abnormal excitability may ensue (see Garry & Tanelian, 1997).

As might be expected after nerve lesion there is substantial loss of sensory axons distal to the site of damage accompanied by sensory neuronal cell death in the DRG. Anatomical and electrical studies report this to be largely a myelinated A-fibre
loss of up to 85%, with little reduction in C-fibre numbers. Since axonal transport is critical for the transport of cellular products neuronal death is likely a result of target-derived trophic factor deprivation (see Garry & Tanelian, 1997).

1.3.1.4 Primary Afferent Sprouting

A characteristic of the sensory nervous system is its ability to regenerate after injury. Indeed, substantial deafferentation induced by peripheral nerve damage can initiate the reinnervation of target tissue, involving growth cone formation and subsequent elongation of the injured nerve. Axonal and Schwann cell-derived growth-enhancing substances are required for this process. These include NGF, known to be necessary for the survival of many nerve cells, and apolipoprotein E, involved in remyelination. Regeneration is not always lucrative and its success can depend on fibre type and extent of injury. It appears that large, myelinated Aβ-fibres are least likely to regenerate, in comparison to C-fibres, and this is increasingly hindered with increasing severity of nerve injury (Navarro et al., 1994; Navarro et al., 1997). In some cases regeneration fails to be initiated and sensory loss in the target area results (Koerber et al., 1989). In other situations inappropriate targeting of regenerating fibres can lead to innervation of low-threshold cutaneous receptors by nociceptive afferent neurones.

Another complication is the phenomena of 'collateral sprouting', whereby uninjured sensory afferents of adjacent uninjured nerves, or the damaged nerve itself, expand into the deafferented area once occupied by the lesioned nerve. This is mediated by all fibre types, yet smaller primary afferents, in comparison to heavily myelinated axons, appear to have the potential to expand over larger areas (Navarro et al., 1994). This may impact upon the development of hyperalgesia as opposed to the return of normal sensation after nerve injury. Hyperalgesia observed after CCI, a model showing marked myelinated fibre degeneration, was prevented by saphenous nerve section to prevent collateral sprouting, one week after nerve injury (Ro & Jacobs, 1993). Models of nerve crush or transection also demonstrate collateral sprouting which may be related to pain behaviours in the rat (Markus et al., 1984; Brenan, 1986; Kingery & Vallin, 1989).
In both cases of axonal regeneration and collateral sprouting erroneous reinnervation of deafferented target tissue could contribute to aberrant pain sensations and abnormal central spatial representation of tactile information in neuropathic pain sufferers.

1.3.1.5 Ectopic Activity

Damage to peripheral nerves results in alterations in their electrical transduction properties and 'ectopic' activity arises whereby sites different to the normal transduction elements of peripheral terminals of the primary afferent receptor generate impulses. This can be both spontaneous and stimulus-dependent in nature and can arise from afferent axonal sprouts innervating the neuroma, axonal areas of demyelination and DRG of A- and C-fibres (Kajander & Bennett, 1992; Xie et al., 1995; Study & Kral, 1996). Ectopic discharge emanating from the DRG is slow and erratic (Kajander et al., 1992), which contrasts to the high frequency rhythmic firing from injured afferents. The contribution of DRG-derived ectopic activity to the total amount appears to depend on the type of nerve injury model, and ranges from very little (Wall & Devor, 1983) to accounting for nearly all ectopic activity in the CCI model (Kajander et al., 1992). The injured peripheral nervous system provides several locations at which numerous pathophysiological changes can occur contributing to this aberrant activity.

Stimulus-dependent ectopic activity can arise in the injured or regenerating nerve at the neuroma or at axonal sites, whereby novel or increased sensitivity to thermal and chemical mechanical stimuli develops such that even blood vessel pulsation can evoke pain. Gentle mechanical stimulation of neuromas can produce bursts of firing that can extend beyond the stimulation period (Burchiel, 1984). Algesic chemicals such as bradykinin, histamine, prostaglandins and leukotrienes are inflammatory mediators normally only active at peripheral nerve terminals, not along axons, where they act as sensitizing agents. Subsequent to nerve injury axons become responsive to exogenous application of these substances, and this may be important in the development of hyperalgesia (Devor et al., 1992). Endogenously, peripheral nerve damage has been shown to bring about a local inflammatory
response (Tracey & Walker, 1995; Michaelis et al., 1997) involving the release of inflammatory mediators from immunocompetent cells and proliferating Schwann cells.

Spontaneous activity of primary afferent neurones occurs independently to a stimulus, and in an intact peripheral nervous system this is rarely observed. After injury, primary afferent axons develop spontaneous activity (Devor, 1991). Differences in the characteristics of spontaneous activity occur for its time of onset and proportions of fibres involved, and this is under the influence of primary afferent fibre type, type of nerve injury and species. Onset has been observed 2 days after nerve injury (Steel et al., 1994), and the proportion of nerves displaying such spontaneous activity appears to increase over the postoperative period (Govrin-Lippmann & Devor, 1978). Generally A-fibre mediated spontaneous activity peaks at an earlier time point, which is then followed by C-fibres (Devor, 1991; Kajander & Bennett, 1992), and after nerve section these were demonstrated to be 2 and 4 weeks, respectively (Govrin-Lippmann & Devor, 1978). Furthermore, up to about 30% of myelinated fibres tend to develop spontaneous activity, whereas only as little as 3% of unmyelinated fibres do and 3 days after CCI this was shown to coincide with the onset of painful behaviours (Kajander & Bennett, 1992). In the clinic, positive correlations have been made between the occurrence of spontaneous firing of nociceptors innervating the painful region and neuropathic pain (Nystrom & Hagbarth, 1981; Gracely et al., 1992). In both human neuropathic pain states and animal models, stimulus-independent pain is observed and spontaneous ectopic activity seems a likely contributing candidate.

1.3.1.6 Ion channel Plasticity

The flow of Na⁺, K⁺ and Ca²⁺ ions through their respective channels in the axonal membrane is critical for the normal functioning of peripheral nerves controlling excitability and the propagation of action potentials. It is logical that altered electrical properties and excitability of primary afferent fibres subsequent to peripheral nerve damage is thus resultant from modifications to expression and distribution of such ion channel proteins. Na⁺ channels have attracted substantial
investigation and a multiple alterations have been highlighted following nerve injury.

In the adult, non-pathological situation, DRG have been shown to express at least seven types of Na\(^+\) channels encoded by different genes. Based on their susceptibility to block by tetrodotoxin (TTX) they have been classified into TTX-sensitive (low threshold of activation; rapidly activating/inactivating) and TTX-resistant (high threshold of activation; slower activation/inactivation) types. Of these, the TTX-resistant SNS and NaN channels are specifically expressed upon nociceptive small diameter neurones. Interestingly, after peripheral nerve damage their gene expression is reduced (Oku et al., 1988; Novakovic et al., 1998; Dib-Hajj et al., 1999), yet translocation of SNS to the site of injury is enhanced (Novakovic et al., 1998). The lack of myelin at surrounding the neuroma allows insertion of translocated Na\(^+\) channels into the axonal membrane, and indeed increased axonal Na\(^+\) channel density correlates with the materialization of ectopic spontaneous discharge, also demonstrated to occur at demyelinated areas (Burchiel, 1980).

"Knock-down" of SNS protein function, utilizing antisense oligodeoxynucleotides, has been shown to prevent CCI-induced neuropathic pain behaviours in rats, yet SNS "knockout" mice retain neuropathic pain symptoms, which may be due to developmental compensatory increases (Porreca et al., 1999). In contrast, the expression of a silent TTX-sensitive α-III embryonic Na\(^+\) channel emerges in C-fibres. Re-expression of α-III is thought to mediate the rapidly repriming Na\(^+\) current displayed by DRG neurones following nerve injury (Black et al., 1999) and its kinetics permit repetitive firing in injured neurones that likely contribute to ectopic activity. A similar pattern of TTX-sensitive channel increase and TTX-resistant decrease is also mirrored in damaged Aβ-fibres (Rizzo et al., 1994).

Ectopic neuronal discharge can be reduced by systemic and topical administration of Na\(^+\) channel blockers. Anticonvulsants, such as carbamazepine, antiarrhythmics, such as mexilitine and local anaesthetics such as lidocaine non-selectively block Na\(^+\) channels in use-dependent manner. Studies employing these agents show that differential Na\(^+\) channels types expressed at the neuroma and within the DRG are responsible for ectopic activity and pre-clinical and clinical data reports a reduction in neuropathic pain behaviours or symptoms following Na\(^+\) channel
blockade (see Garry & Tanelian, 1997).

1.3.1.7 Pathologic Interneuronal Communication

Under normal physiological conditions single primary afferent fibres function independently of one another in the peripheral nervous system until convergence occurs in the dorsal horn. This is made possible by the myelination of A-fibres, and the presence of Schwann cell processes that separate unmyelinated C-fibres. However, as already discussed, nerve injury can result in a disruption of insulating Schwann cell density and distribution. At areas of axonal demyelination and neuromas apposition of denuded axons can allow the ‘cross-excitation’ of an impulse in one fibre to an adjacent fibre via ‘ephatic communication’ (see Garry & Tanelian, 1997).

Another non-synaptic mode of communication between neurones is ‘crossed-afterdischarge’ whereby the activity of a group of neurones alters the endogenous firing activity of their neighbours (Amir & Devor, 1992). This occurs in non-pathological settings in primarily large diameter neurones with limited effects due to minor depolarizations. Suggested mechanisms include diffusible factors, such as ATP (adenosine triphosphate), and an increase in extracellular K⁺ concentrations. Again, due to nerve injury-induced demyelination, neurones and neuromas become more susceptible to such substances and crossed-afterdischarge may also enhance any ectopic activity ongoing in damaged nerves as well a recruit silent neurones (Amir & Devor, 1997).

Both these non-synaptic modes of interneuronal communication have the potential to mediate abnormal interactions between different fibre types. Low-threshold Aβ-fibres may directly activate nociceptive C-fibres, such that an innocuous stimulus could evoke a painful sensation and could thus contribute to mechanical allodynia.
1.3.1.8 Sympathetically-Activated Sensory System

In the intact peripheral nervous system, the afferents of sensory primary afferents are functionally discrete from the efferent sympathetic system and its innervation of the DRG usually ceases within the encompassing vasculature, where it controls blood supply. After damage to peripheral nerve both anatomical and physiological studies have demonstrated atypical sensory-sympathetic coupling such that the sympathetic nervous system may be involved in ensuing sensory changes that result from neuropathy.

Anatomically, nerve injury can induce sympathetic sprouting resulting formation of 'basket' structures that surround DRG soma, such that close contact between sympathetic terminals are formed (Chung et al., 1997). In particular, those corresponding to Aβ-fibres are innervated, however transient basket formation around smaller neurones may also occur (Chung et al., 1996; Ramer & Bisby, 1997; Ramer et al., 1997). This process appears to rely upon NGF and cytokines that are produced by proliferating Schwann and immunocompetent cells following peripheral nerve injury. The resultant effect of this novel sympathetic innervation is noradrenergic modulation of DRG activity such that spontaneous or evoked firing is dramatically enhanced, and to a lesser extent non-active Aβ-fibres may become active. Via Aβ-fibres this may directly evoke alldynia, and via facilitation of a constant C-fibre input into the dorsal horn it may contribute to central sensitization.

Physiologically, primary afferents show increased responsiveness to exogenous and endogenous stimulation. Application of noradrenaline to the neuroma of humans has been shown to produce burning sensations and increased electrical discharges. Likewise in pre-clinical studies systemic administration of noradrenaline activated and sensitized intact C-fibres of the partially injured nerve and electrical stimulation of the appropriate sympathetic nerve trunk caused increased activity of the neuroma. Subtype-specific alterations in density and/or responsiveness of α2-adrenergic receptors appear to contribute to the increased sympathetic sensitivity (Cho et al., 1997; Birder & Perl, 1999). Following sciatic nerve section, type A receptors are upregulated and type C downregulated in DRG corresponding to A-fibres, paralleled by alterations in their gene expression observed after SNL.
Furthermore peripheral nerve injury altered the activity of a population of $\alpha_2$ adrenoceptors such that their activation reduced $K^+$ currents and increased excitability of small DRG neurones (see Abdulla & Smith, 1997).

Numerous clinical and pre-clinical studies, some utilizing surgical or chemical intervention, have confirmed a sympathetically-maintained component of some, but not all, types of neuropathic pain. In human neuropathic pain patients the occurrence of sympathetic-attributable symptoms, such as skin vasomotor and sweating anomalies and dystrophic changes in skin, hair, nails and bone can be observed and their neuropathic pain is worsened by sympathetic stimulation (see Taylor, 2001). More convincing is the ability of guanethidine, which depletes noradrenergic terminals and thus all sympathetic neurotransmitters, and adrenergic antagonists to produce long-lasting relief from sympathetically-maintained pain in the majority of patients (Torebjork et al., 1995). In the animal model of SNL, surgical sympathectomy performed after or before nerve ligation alleviated allodynia or prevented allodynia and hyperalgesia, respectively (Kim et al., 1993). In SNL and other nerve injury models similar effects have been observed using guanethidine (Shir & Seltzer, 1991; Kim et al., 1993), however reversal of neuropathic pain behaviours has not been successful in L5 ligated rats. Such controversy in the literature may well be explained by differences between pre-clinical models since types of peripheral nerve damage and animal strains may provide sources of variability in the onset and time course of sympathetic sprouting, thus requiring appropriate timing of sympathectomy for successful results. Alternatively, there may not be a sympathetic component to some types of injury. Allodynia is observed in the absence of sympathetic sprouting (Marchand et al., 1999) and a positive correlation between the extent of sprouting and sympathetic dependence was shown not to exist (Kim et al., 1998). Furthermore, not all neuropathic pain patients have benefited from sympatholytic procedure (Kingery, 1997) and for those cases which do, painful symptoms can return. The use of sympathectomy as a treatment of neuropathic pain is therefore debatable and not always suitable for every situation.
1.3.2 Central Processes

As we progress along the sensory transmission pathway from the periphery to the brain, primary afferent fibres must now terminate and make their first synapse with the CNS. This occurs in the dorsal horn of the spinal cord, a site where the peripheral input undergoes anatomical convergence and neurotransmitter system modulation, before projection to higher brain centres via ascending tracts. The spinal cord exhibits both excitatory and inhibitory systems mediated by a diverse array of neurotransmitters and receptors and the balance of the two is critical in determining the stimulus-response relationship. Nerve injury-induced changes to this part of the pain pathway will now be discussed.

1.3.2.1 Dorsal Horn Neuronal Response Characteristics

In addition to the altered peripheral processes mediated by primary afferent fibres, peripheral nerve damage can also lead to long-term changes in the CNS which contribute to the dysesthesias associated with neuropathic pain. After SNL the receptive fields of dorsal horn neurones are enlarged (Suzuki et al., 2000b), and implies that a greater number of spinal neurones would be recruited for a given stimulus. Nociceptive dorsal horn neurones also display de novo spontaneous activity and reduced mechanical stimuli thresholds in parallel with lowered magnitudes of responses (Chapman et al., 1998b). Furthermore, experimental nerve injury creates a loss of afferent input into the spinal cord (Castro-Lopes et al., 1990). These observations fit well with the clinical neuropathic pain profile of both allodynia and hyperalgesia together with sensory deficits, yet how altered peripheral and central neuronal responses contribute to these symptoms still remains to be thoroughly defined. What is clear is that in the presence of dramatically reduced afferent input, dorsal horn neuronal responses manage to sustain their response magnitudes, indicative of central neuronal compensatory mechanisms which may explain the positive symptoms of neuropathic pain despite loss of normal sensory input (see Dickenson et al., 2001).

The increased barrage of impulses arising from primary afferent fibres mediated by the discussed peripheral mechanisms is important in eliciting central
sensitization and hyperexcitability of dorsal horn neurones (Woolf & Mannion, 1999). Ongoing peripheral activity results in sustained neurotransmitter release and activation of neurotransmitter systems within the spinal cord. Plasticity in the modulatory systems and functional connectivity together contribute to a persistent pain state. Appreciation of these alterations first requires some description of the spinal cord organization and termination patterns of the primary afferent fibres.

1.3.2.2 Significance of Spinal Cord Laminar Organization

The spinal cord accommodates axons within its white matter and neurones and their processes within its grey matter. The grey matter is cytoarchitecturally subdivided into 10 horizontal laminae, and this serves to roughly group functionally related cells (Figure 2). Laminae I-VI receive sensory input and constitute the dorsal horn. Lamina I is the outer marginal layer. Ventral to this is lamina II - the substantia gelatinosa, further divided into outer (IIo) and inner (Ii) layers. Laminae III and IV are the nucleus proprius, V and VI (only defined in lumbo-sacral and cervical enlargements) are the deep dorsal horn layers. Laminae VII-IX contain motor neurones and compose the ventral horn, and lamina X surrounds the central canal (see Sorkin & Carlton, 1997).

Primary afferent fibres collectively enter the dorsal horn via dorsal roots and either terminate in the dorsal horn of the segment of entry or send rostro-caudal projections out of this segment, projections that terminate several segments away or as far as the dorsal column nuclei. Such Aβ-fibre afferent collaterals are in the medially-running dorsal columns, whereas Aδ- and C-fibre afferent collaterals run laterally in Lissauer's tract. Each primary afferent dorsal horn terminal has clearly-defined laminae targets creating a dorso-ventral organization which reflects both receptive field and stimulus modality (Molander & Grant, 1986). This identification was made possible by the use of the axonal tracers horse radish peroxidase (HRP) and wheat germ agglutinin, which selectively label large myelinated and small unmyelinated fibres, respectively.
Figure 2. Organization of the spinal cord laminae in lumbar segments and the termination patterns of cutaneous primary afferent fibre input. Noxious stimuli evoked afferent input mediates the release of peptide neurotransmitters SP (substance P) and CGRP (calcitonin gene-related peptide) from C-fibre terminals in the superficial laminae of the dorsal horn. Non-noxious and noxious input converges upon WDR (wide dynamic range) neurones, which then project to the brain via ascending tracts.
**Aβ-fibre Termination Pattern**

Aβ-fibre afferents, mediating non-noxious information, enter the spinal cord via the medial dorsal root. They descend to the deep laminae either medially through or curve round the superficial medial edge of the dorsal horn to enter at a more ventral location where the majority terminate in laminae III-V. Little or no projections are made directly into laminae I and II. In laminae III and IV Aβ-fibres terminate onto non-nociceptive, low-threshold second order neurones, which project into the spinocervical (SCT) and spinothalamic (STT) tracts and the postsynaptic dorsal column (PSDC). In lamina V Aβ-fibres also terminate upon wide dynamic range (WDR) neurones, which also receive C-fibre input (see Sorkin & Carlton, 1997).

**Aδ-fibre Termination Pattern**

Aδ primary afferent fibres differ in their termination pattern in a manner dependent on their peripheral terminal receptor type. Those having high-threshold mechanoreceptors distribute ipsilaterally to laminae I, IIo and V, with some projections to contralateral lamina V. Here they terminate upon predominantly WDR and nociceptive, high-threshold second order dorsal horn neurones which project to the STT, spinomesencephalic (SMT) and spinohypothalamic (SHT) tracts and to the parabrachial nucleus (PBN). Aδ-fibres that innervate hair cells terminate in lamina III (see Sorkin & Carlton, 1997).

**C-fibre Termination Pattern**

C-fibre primary afferent fibres terminate in the superficial dorsal horn. Furthermore, peptidergic TrkA-positive nociceptors distribute to laminae I and IIo, whereas the non-peptidergic IB4-positive population distribute to IIIi. Lamina I contains nociceptive-specific (mechanical, heat and cold) and WDR dorsal horn neurones, the majority of which project to the SCT, STT and spinoreticular tract (SRT) as well as the periaqueductal grey (PAG), PBN and nucleus submedius. In contrast, the cells of laminae II only project locally to surrounding segments and not to ascending tracts, thus they serve as interneurones and relay inputs to deeper...
laminae, such as the WDR neurones in V. Morphologically they can be distinguished into excitatory 'stalk' cells found in IIO and the inhibitory GABA neurotransmitter containing 'islet' cells found in III. Furthermore neurones of IIO receive input from high-threshold and thermoreceptive afferents and those of III receive low-threshold mechanical information (see Sorkin & Carlton, 1997).

Anatomically, and thus functionally, there exists a considerable amount of convergence within the dorsal horn of the spinal cord, and this is largely mediated by the WDR neurones located predominantly in lamina V (also IV, VI and some superficially). Non-noxious and noxious primary afferent inputs, directly from Aβ-fibres and via a multi-synaptic pathway from Aδ- and C-fibres, all terminate upon these multireceptorial cells. WDR neurones display a dynamic response over a wide stimulus range such that they can encode stimulus intensity. WDR cells of lamina V then project to STT, SMT, SRT, SCT, SHT and PSDC. The supraspinal targets of these ascending tracts are all important in the transmission of nociceptive information. In particular the STT terminates in the thalamus, which is a very important in encoding type, temporal pattern, intensity and topographical information regarding pain before relay to the cortex. Via interactions with cortical and limbic regions it is also responsible for sensory-discriminative and emotional facets of pain perception (see Craig & Dostrovsky, 1997, for further discussion).

1.3.2.3 Abnormal Dorsal Horn Reorganization

Damage to peripheral nerve can impact upon the anatomical organization of primary afferent termination patterns within the dorsal horn of the spinal cord such that abnormal changes arise that may underlie some of the experienced neuropathic pain symptoms and characteristics. As might be expected it is likely that peripheral nerve injury results in loss of primary afferent input into the dorsal horn, indeed after peripheral nerve transection a substantial loss of C-fibre terminals has been observed in lamina II (Castro-Lopes et al., 1990). Further to this deafferentation, nociceptive input to dorsal horn neurones is reduced, which could underlie the occurrence of sensory deficits experienced by neuropathic pain patients (Fields et al., 1998).
Contrary to this, deafferentation has the ability to induce primary afferent regeneration and repair activity, as observed in the periphery. Whilst this can be beneficial in restoring original circuitry, more often than not in the development of neuropathic pain, aberrant re-wiring may underlie the positive neuropathic pain symptoms such as allodynia. It has been demonstrated by the use of cholera toxin B-conjugated (CB)-HRP tracing that the central projections of intact Aβ-fibres within the deep dorsal horn, that convey non-noxious information, may make abnormal dorsal-oriented sprouts into lamina II after axotomy (Woolf et al., 1992). This is also evident after SNL (Lekan et al., 1996) and CCI (Nakamura & Myers, 1999). Alongside the previously mentioned induction of substance P expression in Aβ-fibres, sprouting may permit innocuous input to reach the superficial dorsal horn, a region synonymous with pain transmission, whereby their terminations may activate nociceptive-specific spinal cord neurones (see Woolf et al., 1995). This appears a rational mechanism for touch-evoked pain however it is difficult to prove clinically.

New evidence has come to light, which may support a role for anatomical sprouting in the dorsal horn. After nerve crush-induced neuropathic pain innocuous stimulation of the target tissue evoked c-fos, an immediate early gene product expressed after noxious neuronal activation, in superficial dorsal horn neurones and activated PBN neurones, a major supraspinal termination site in the rat for nociceptive-specific superficial dorsal horn neurones (Bester et al., 2000). Innocuously activated Aβ-fibres, could activate nociceptive specific neurones in the superficial dorsal horn if their termination pattern extended dorsally. Additionally or alternatively, activation of superficially terminating, low-threshold mechanoreceptive C-fibres could be involved (Vallbo et al., 1999). Also, low-threshold stimulation has been demonstrated to evoke synaptic potentials in lamina II neurones 3 weeks after nerve injury (Kohama et al., 2000), an area which normally only responds to high threshold stimuli.

Aβ-fibre sprouting could possibly be triggered by the physical absence of normal C-fibre afferent terminals in lamina II after their degeneration. (Doubell et al., 1997), yet dorsal rhizotomy produced lamina II vacancy was shown not to induce Aβ-fibre sprouting (Mannion et al., 1998). More likely is the involvement of trophic
factors and chemoattractants, yet this is still yet to be proven. Suggestions include a possible upregulation of growth related proteins, such as GAP-43 (Woolf et al., 1990), and injured C-fibre terminal secretion of an Aβ-fibre attractant (Doubell et al., 1997; Mannion et al., 1998). Aβ-fibre sprouting after axotomy has been demonstrated to be inhibited by application of NGF, but since large diameter DRG cells do not express the appropriate TrkA receptor, the familiar explanation of 'disrupted axonal transport of peripherally-derived NGF' seems unfeasible as a causal mechanism.

Certain doubts surround the phenomena of Aβ-fibre sprouting and its functional contribution to neuropathic pains. Some discrepancies have come to light concerning the specificity of CB-HRP utilized to identify Aβ-fibres and their sprouting. It has been proposed that after axotomy, small nociceptive neurones express novel cell membrane glycoconjugates that mediate the uptake of CB, evident as an altered neuronal labelling profile after nerve injury (Tong et al., 1999). This might imply that CB-HRP labelling in superficial laminae following peripheral nerve damage may not represent Aβ-fibre sprouting. However others do not describe injury-induced alterations in DRG neuronal cell size distribution of CB-HRP (Bennett et al., 1996). As to the functional relevance of sprouting, it cannot be the sole mediator of allostynia for several reasons. Electrophysiological studies have shown that established behaviourally antiallodynic drugs have little impact upon the Aβ-fibre-evoked response (Chapman et al., 1998a; Suzuki et al., 1999). Furthermore, the time scale of functional reorganization of Aβ-fibre terminals does not correlate with the rapidity with which allostynia can be manifest. Allostynia is observed 2 days after nerve injury (Bennett & Xie, 1988; Shir & Seltzer, 1990; Kim & Chung, 1992), yet CB-HRP labelling (Woolf et al., 1995) and low threshold evoked neuronal activity (Kohama et al., 2000) is not observed in lamina II until one and three weeks, respectively. Structural reorganization as a basis for allostynia cannot be responsible for the induction of allostynia yet it may have relevance to its maintenance since sprouting demonstrated by CB-HRP labelling is maximal two weeks after nerve injury and still apparent at six months (Woolf et al., 1995).
1.3.2.4 Excitatory Neuropeptide and Amino Acid Receptor Systems

**Substance P**

Substance P is the predominant excitatory neurotransmitter peptide and acts at the NK1 receptor to mediate slow depolarizations and sustained excitatory postsynaptic potentials (EPSPs), which are important in the recruitment of NMDA receptor for activation, as will be discussed later. NK receptor activation, via a G-protein mechanism, results in the release of Ca\(^{2+}\) from intracellular stores and protein kinase C (PKC)-mediated NMDA receptor phosphorylation (a further recruiting action). Downstream effects of elevated intracellular Ca\(^{2+}\) involve enhancement of neuronal excitability, including NO synthesis that is implicated in the pronociceptive actions of substance P on dorsal horn neurones (Radhakrishnan et al., 1995). Substance P containing neurones are localized to the superficial dorsal horn, in particular lamina II, where its mRNA is also found (Gibson et al., 1981; Hunt et al., 1981; Westlund et al., 1990). Rhizotomy studies have demonstrated that, as already discussed, nociceptive primary afferent fibres give rise to about half these substance P containing terminals, whereas intrinsic dorsal horn neurones give rise to the remaining half. NK1 receptors are found on spinal cord neurones in the superficial laminae and on deeper cells with dendrites located superficially (Todd et al., 2000). Substance P is only released in response to noxious and persistent stimulation (Duggan et al., 1988), mainly owing to the fact that C-fibres, not Aβ-fibres, express substance P. The actions of substance P may be enhanced after nerve injury. Upregulation of NK-1 receptors occurs in the superficial dorsal horn (Abbadie et al., 1996), neurokinin antagonists attenuate spinal sensitization after sciatic nerve section (Luo & Wiesenfeld-Hallin, 1995), and Aβ-fibres phenotypically transform allowing them to synthesize substance P, as already discussed (Noguchi et al., 1995). In contrast, a decrease in spinal substance P itself is observed after nerve injury (Na et al., 2001). In addition to this, mechanical hyperalgesia has been shown to be attenuated in NK-1 receptor knockout mice (Mansikka et al., 2000).

**Calcitonin Gene-Related Peptide**

CGRP is another important excitatory peptide although CGRP-containing terminals are less abundant than those for substance P. CGRP is found in lamina I, II,
V and X, but in contrast to substance P, unmyelinated and small diameter myelinated primary afferent fibres are its only source. Little CGRP is generated in intrinsic spinal cord neurones. Activation of CGRP dorsal horn receptors probably serves to enhance the actions of substance P, but its role is comparatively less well defined due to a lack of useful antagonists, and may involve positive coupling to adenylate cyclase (AC). CGRP, like substance P, produces slow membrane depolarizations and increased intracellular Ca^{2+} levels by both Ca^{2+} influx and release from internal stores (Miletic & Tan, 1988; Oku et al., 1988; Wimalawansa, 1996).

Excitatory Amino Acids

The EAAs comprise glutamate and aspartate, and as already mentioned are found in both small and large primary afferent fibres (Battaglia & Rustioni, 1988; Maxwell et al., 1990). Within the spinal cord, glutamate-containing fibres and terminals are found in laminae I-IV, the majority arising from primary afferent neurones, however some are intrinsic. Aspartate-containing terminals are found in laminae I-III and coexistence studies indicate that these are separate to the glutamate population (Merighi et al., 1991). The EAAs are considered to be fast neurotransmitters and their substantial localization to the superficial dorsal horn implies an important role in pain transmission. Glutamate is predominantly found co-localized with substance P (Battaglia & Rustioni, 1988; Merighi et al., 1991) ensuring that both are probably released from afferent nociceptive fibres upon noxious stimulation.

The glutamate receptors are subdivided into the slow, G-protein-coupled metabotropic receptors and the fast, ligand-gated, ionotropic receptors. Ionotropic receptors are further characterized by synthetic agonists into AMPA (which contribute to fast transmission of acute innocuous and noxious stimuli), kainate and NMDA receptors (see Ozawa et al., 1998). All three are prominently localized to the superficial dorsal horn, with some found in deeper laminae which may correspond to receptors on WDR neurones (Bonnot et al., 1996; Yung, 1998). The NMDA receptor has been the most extensively studied glutamate receptor owing to its ubiquitous CNS expression. It is an ionotropic receptor coupled to a cation channel, which under resting conditions is quiescent due to intra-channel Mg^{2+} block. Only upon the
binding of glutamate, in addition to the co-agonist glycine, and sufficient membrane depolarization to relieve the Mg\(^{2+}\) block, is the complex activated and Ca\(^{2+}\) influx occurs.

Prolonged peripheral input into the spinal cord via nociceptive C-fibre is known to enhance dorsal horn neuronal responses to subsequent afferent input – an experimental phenomenon known as ‘wind-up’ used to study central sensitization and spinal cord hyperexcitability (Dickenson, 1995). The NMDA receptor is critical for wind-up and thus is important in events that enhance and prolong sensory transmission (Seltzer et al., 1991; Dickenson, 1995). Central sensitization is characterized by reduced thresholds to stimuli and enlarged receptive fields (McMahon et al., 1993) and at the level of the spinal cord NMDA receptor activity is heavily implicated. The inherent properties of the NMDA receptor/channel complex described permit its role in the development of wind-up and central sensitization. Under conditions of a repetitive nociceptive afferent input, sustained release of glutamate, substance P and CGRP activate AMPA and neuropeptide receptors, the actions of which summate and relieve Mg\(^{2+}\) block, thus recruiting NMDA receptors into glutamatergic transmission. Ca\(^{2+}\) influx through the NMDA receptor itself mediates further membrane depolarizations recruiting more NMDA receptors ... and so on. These convergent events result in amplified activation of the NMDA receptor. Increased and sustained Ca\(^{2+}\) influx results, which sets in motion signal transduction cascades culminating in secondary modifications of ionic currents via receptor/ion channel phosphorylation, as well as long term alterations in receptor/neurotransmitter expression via gene transcription processes. Central sensitization is normally kept under control by inhibitory controls, yet in neuropathic pain central sensitization can persist and become pathological.

After nerve injury there is evidence to show an ipsilateral enhancement of glutamate release (Kawamata & Omote, 1996) and an upregulation of glutamate receptors (Harris et al., 1996; Croul et al., 1998). Administration of NMDA receptor antagonists in models of neuropathic pain have proved effective in reducing hyperalgesic (Mao et al., 1993) and allodynic (Yaksh, 1989) behaviours, as well as dorsal horn neuronal responses (Suzuki et al., 2001). Likewise, the hypothesized
roles for NMDA receptors in central sensitization and its link to the development of neuropathic pain have been proven in clinical neuropathic pain states with the use of antagonists, but with limiting side-effects (Sang, 2000).

1.3.2.5 Inhibitory Receptor Systems

Opioids

The endogenous inhibitory opioid system has been manipulated to provide pain relief since the discovery of opium, of which morphine is the main active component. Cloning and isolation techniques have established three classical opioid receptor types, namely $\mu$, $\delta$, $\kappa$, each showing considerable sequence homology to one another (Dickenson & Suzuki, 1999). The $\mu$ opioid receptors (and $\delta$, $\kappa$ and ORL1 receptors) have a seven transmembrane domain receptor structure and are coupled to G-proteins. Upon extracellular binding of the appropriate ligand a conformational change in the receptor is elicited that inhibits cAMP formation, via pertussis (PTX) sensitive G-proteins, that subsequently opens $K^+$ channels ($\mu$, $\delta$ and ORL1 receptors). The resultant neuronal hyperpolarization leads to a decrease in the opening of VDCCs. In the case of $\kappa$ opioid receptor activation this reduction of $Ca^{2+}$ influx is direct. The $\mu$, $\delta$ and $\kappa$ receptors are endogenously activated by the endorphin, enkephalin and dynorphin peptides, respectively, and thus mediate membrane hyperpolarization, reducing neuronal excitability. Manipulation of these by exogenous agonists is thus extremely valuable as a means of exerting analgesia (Table 2). A recent addition to the opioid receptor family is the opioid-like receptor (ORL-1) (Wick et al., 1994; Peluso et al., 1998). Endogenously activated by the peptide nociceptin (or orphanin FQ), this receptor shows substantial sequence homology to the others, yet it only displays a low affinity for the universal opioid receptor antagonist, naloxone. Its physiological function and pain modulation role and is not yet well defined, but under investigation (Carpenter & Dickenson, 1998; Darland et al., 1998).
Table 2. Agonists and antagonists of the opioid receptors.

<table>
<thead>
<tr>
<th></th>
<th>μ</th>
<th>δ</th>
<th>κ</th>
<th>ORL-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endogenous agonist</td>
<td>Endorphins</td>
<td>Enkephalins</td>
<td>Dynorphins</td>
<td>Nociceptin/OFQ</td>
</tr>
<tr>
<td>Synthetic agonist</td>
<td>Morphine</td>
<td>DAMGO</td>
<td>DPDPE</td>
<td>Enadoline</td>
</tr>
<tr>
<td>Antagonist</td>
<td>Naloxone</td>
<td>Naloxone</td>
<td>Naltrindole</td>
<td>Naloxone</td>
</tr>
</tbody>
</table>

Abbreviations: DAMGO ([D-Ala²,N-Me-Phe⁵,Gly-o¹]enkephalin); DPDPE ([D-Pen²,D-Pen⁵]-enkephalin); CTAP (D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH₂); OFQ (orphanin FQ); Pheψ ([Phe¹ψ(CH₂NH)Gly²]NCδ₁₃)NH₂).

 Autoradiographic and immunohistochemical techniques have demonstrated that within the spinal cord, opioid receptors are mostly located in the superficial dorsal horn (laminae I and II), with a smaller population in deeper layers (Besse et al., 1990b; Rahman et al., 1998). The contribution of μ, δ, and κ receptors to the total opiate binding throughout the spinal cord is estimated at 70%, 24% and 6%, respectively (Besse et al., 1990a,b; Backonja et al., 1994), at a predominantly (>70%) presynaptic location relating to the central terminals of only small diameter nociceptive primary afferents. Opioid receptors are synthesized in small diameter DRG cells bodies and transported centrally and peripherally. This implies that the main mechanism of spinal opioid analgesia, whether it be endogenous or exogenously mediated, is via activation of presynaptic opioid receptors, which act to selectively decrease transmitter release from nociceptive afferents and thus nociceptive transmission leaving innocuous-evoked activity intact. Indeed, spinally applied morphine can reduce substance P and CGRP release after noxious stimulation (Go & Yaksh, 1987) and excitatory but not inhibitory lamina II synaptic transmission in is inhibited by a presynaptic opioid mechanism (Kohno et al., 1999).

The remaining 30% of opioid receptors are located postsynaptically on interneurones, and the dendrites of projections cells (Besse et al., 1990a), visualized functionally as agonist-stimulated receptor internalization (Trafton et al., 2000). Here, any opioid-mediated cell hyperpolarization will not exert nociceptive-specific
effects, and from electrophysiological studies a small inhibition of Aβ-fibre evoked responses can be observed (Dickenson & Sullivan, 1986). Since this inhibitory effect is much less pronounced than that observed on the C-fibre evoked response, this confirms that the predominant site of action of spinal opioids is via presynaptic opioid receptors on central terminals of nociceptive afferents (Ossipov et al., 1997).

Activation of the opioid system as a whole can mediate considerable antinociception as demonstrated by various pre-clinical studies (Mao et al., 1995; Ossipov et al., 1995a; Ossipov et al., 1995b) and its widespread clinical manipulation (McQuay et al., 1992). Endogenous release of the endorphins and enkephalins from intrinsic spinal neurones is stimulated only by high-intensity stimuli (Cesselin et al., 1984; Lucas & Yaksh, 1990) and under normal conditions the opioid antagonist naloxone does not produce hyperalgesia (Yaksh, 1989), suggestive of no opioid-mediated tonic control. However, the opioid system displays a substantial amount of plasticity in persistent pain states. Whilst such changes are beneficial in the presence of inflammation (Ossipov et al., 1997), neuropathic pain due to peripheral nerve damage more often than not displays reduced sensitivity to opioids. This is evident both pre-clinically (Mao et al., 1995; Ossipov et al., 1995a) and clinically (Portenoy et al., 1990; Jadad et al., 1992), and is surrounded by much controversy. Mechanisms by which opioid sensitivity may be reduced after nerve injury include a reduction of spinal opioid receptors (Porreca et al., 1998), non-opioid receptor-expressing Aβ-fibre-mediated allodynia, increased cholecystokinin antagonism (Nichols et al., 1995) and NMDA-mediated dorsal horn neuronal hyperexcitability, likely requiring a greater opioid inhibitory counteraction (see Dickenson, 1997). These possibilities will be discussed further in chapter 7.

It should be briefly mentioned that opioid receptors are distributed throughout the CNS especially in areas other than the spinal cord also concerned with nociceptive processing, such as the thalamus and supraspinal midbrain and brainstem structures - the PAG and the RVM (rostroventromedial medulla). Exogenous application of morphine into these sites elicits antinociceptive effects by increasing the activity of inhibitory descending controls that terminate in the dorsal horn (Heinricher, 1997). The pathway that descends from the RVM contains mostly
enkephalin, serotonin, GABA and glycine containing fibres. The pharmacology of these modulatory systems in the dorsal horn will now briefly be mentioned.

**Inhibitory Amino Acids**

GABA is the major inhibitory neurotransmitter in the CNS and its extensive distribution permits it to tonically control spinal cord excitability. The majority of GABA terminals arise from interneurones, though some are from descending tracts (Millhorn *et al.*, 1987), and are most concentrated in the superficial dorsal horn (laminae I-III) (Hunt *et al.*, 1981; Magoul *et al.*, 1987). GABA-ergic terminals can also contain other substances, such as glycine (Todd *et al.*, 1996), galanin (Simmons *et al.*, 1995), enkephalin (Todd *et al.*, 1992) and neuropeptide Y (Rowan *et al.*, 1993) in different populations, and they contact mainly nociceptive Aδ- and C-fibre terminals (Bernardi *et al.*, 1995). There are two receptors for GABA in the spinal cord, namely GABA<sub>A</sub> and GABA<sub>B</sub>, both of which are found pre- and postsynaptically on nociceptive afferents (Desarmenien *et al.*, 1984). GABA<sub>A</sub> is a ligand-gated chloride channel that can also be modulated by barbiturates and benzodiazepines (Schofield, 1989). GABA<sub>B</sub> is a G-protein-coupled receptor linked to the inhibition of Ca<sup>2+</sup> influx via K<sup>+</sup> and Ca<sup>2+</sup> channel mediated actions. Activation of presynaptic GABA receptors, most likely GABA<sub>B</sub>, has been demonstrated to inhibit neurotransmitter release (Bourgoin *et al.*, 1992; Malcangio & Bowery, 1994) and postsynaptically, probably GABA<sub>A</sub>, they prevent EPSPs via neuronal hyperpolarization (Alvarez *et al.*, 1992).

Under normal conditions the GABA-ergic control of nociceptive transmission is near maximally activated, indeed block of GABA receptors can mediate nociceptive responses to previously innocuous stimuli (Dickenson *et al.*, 1997a). Loss of GABA-ergic control upsets the balance of excitations and inhibitions within in the spinal cord favouring the generation of hyperexcitability and therefore plasticity within this system is implicated in pathophysiological pain states. CCI and sciatic nerve transection have been shown to result in a decrease of spinal GABA content and immunoreactivity (Castro-Lopes *et al.*, 1993; Ibuki *et al.*, 1997) and subtype-selective alterations in GABA receptor expression appear to exist (Castro-Lopes *et al.*, 1995). GABA<sub>B</sub> agonists have been shown more effective than GABA<sub>A</sub>
agonists in ischaemia-induced rat spinal cord injury (Hao et al., 1992b; Xu et al., 1992), yet electrophysiologically midazolam, a positive allosteric modulator of GABA_A receptor function, was shown to be more effective in inhibiting nociceptive activity after SNL (Kontinen & Dickenson, 2000).

Glycine is another inhibitory amino acid and is found in neurones within the spinal cord localized to the superficial dorsal horn mostly in coexistence with GABA (Todd & Sullivan, 1990). It acts via a strychnine-sensitive receptor that is a ligand-gated chloride ion channel to inhibit dorsal horn neurones and spinal strychnine mediates allodynia and nociceptive behaviour in rats (Yaksh, 1989; Sivilotti & Woolf, 1994), thus its role in nociceptive transmission appears similar to that of GABA. Little is known regarding changes to the glycinergic system after nerve injury, however increased glycine levels have been reported (Satoh & Omote, 1996). Furthermore, in the dorsal horn of neuropathic pain patients the ratio of aspartate to GABA and glycine is increased, suggestive of an impaired excitatory/inhibitory balance (Mertens et al., 2000). In contrast glycine is a co-agonist at the NMDA receptor, but spinal levels are such that this affects its activation.

**Monoamines**

The majority of nociceptive pathways (including SRT and STT) are under the control of bulbospinal projections originating in brainstem nuclei. The nucleus raphe magnus (NRM) gives rise to serotonin (or 5-HT (5-hydroxytryptamine)) containing projections, the locus coeruleus and lateral tegmentum cell systems contain noradrenaline, and these are released in the spinal cord selectively in response to high-intensity stimulated afferent input (Dickenson et al., 1997b).

The role of descending serotonergic pathways in the modulation of pain transmission is somewhat complicated by the multiplicity of its target 5-HT receptors, activation of which exert both pro- and antinociceptive actions (see Wilcox & Seybold, 1997) dependent on the differing effector mechanisms and neuronal locations. Dense 5-HT labelling is found in laminae I and II, the majority arising from supraspinal sites, in addition to a small interneuronal population (see
Wilcox & Seybold, 1997). Stimulation of the nucleus NRM displays a biphasic, excitatory followed by inhibitory, influence on dorsal horn neurones (Zhuo & Gebhart, 1997). Importantly, these descending pathways display plasticity after nerve damage. Chronic pain patients show changes in spinal levels of 5-HT and deep laminae terminating serotonergic terminals sprout into lamina II (Wang et al., 1991; Marlier et al., 1992) such that nociceptive transmission is enhanced. Blockade of serotonin re-uptake by tricyclic antidepressants may account for their noted analgesic effects, as previously mentioned. In brief, 5-HT1B and 5-HT1D mediate selective inhibition of nociceptive neurones and are implicated in headache (Goadsby, 2000). 5-HT2C and 2A are positively coupled to phospholipase C and suppress K+ current. 5-HT3 increase intracellular Ca2+ either directly by activation of a cation channel that triggers the opening of VDCCs or via induction of phospholipase C. The localization of 5-HT2A, 2C and 3 on excitatory interneurones or primary afferent terminals mediates the pro-nociceptive actions of 5-HT or descending facilitation. 5-HT1A are found densely localized in the superficial dorsal horn, like 5-HT3, and increase K+ currents and decrease Ca2+ currents via negative coupling to AC thus inhibition of these receptors on inhibitory interneurones contribute to central sensitization and allodynia by disinhibition of WDRs (see Millan, 1997).

Noradrenaline containing spinal cord terminals are localized to laminae I, II and V, and arise purely from supraspinal sites (see Sorkin & Carlton, 1997). Within the spinal cord there are two α2-adrenergic receptors. α2C have a presynaptic location in primary afferents and α2A are found in secondary dorsal horn neurones (Nicholas et al., 1993). They inhibit dorsal horn neurones by presynaptic inhibition of neurotransmitter release (Pang & Vasko, 1986; Go & Yaksh, 1987), probably via a PTX-sensitive Gq/11-protein coupling and postsynaptically by hyperpolarization, mediated by coupling to inwardly rectifying K+ channels. Administration of the α-adrenoceptor antagonist phentolamine has been shown not to produce hyperalgesic effects (Yaksh, 1989), and thus like the endogenous noradrenergic system is unlikely to exert ongoing modulation of nociception. There is little evidence so far but changes after nerve injury enhance the analgesic effect of α2-agonists (Suzuki, personal communication).
Adenosine

The purines, adenosine, produced by ATP metabolism, alongside ATP itself, are implicated in the modulation of nociception, although centrally the role of the adenosine receptor system is more defined (Sawynok, 1998; Dickenson et al., 2000). Adenosine-like immunoreactivity is detected in lamina II of the dorsal horn. Three differentially G-protein-coupled receptors for adenosine have been characterised. The A1 receptor inhibits AC whereas A2 and A3 stimulate AC. Via mainly a postsynaptic location, the A1 receptor predominates the antinociceptive effects of spinally applied adenosine and adenosine analogues, exerted through K⁺ channel mediated hyperpolarization (Sawynok, 1998; Dickenson et al., 2000). Additionally those receptors found on the terminals of primary afferent fibres inhibit Ca²⁺ current and neuropeptide release. Manipulation of the adenosine system with analogues has been shown to be effective against neuropathic pain in both clinical and pre-clinical studies, in a manner that may be enhanced after nerve injury (Suzuki et al., 2000a). Furthermore, adenosine is able to inhibit NMDA receptor-mediated hyperexcitability (Dickenson et al., 2000). Interactions between adenosine and serotonergic modulation are apparent (Sawynok, 1998), yet the mechanisms behind such effects remain to be uncovered.

Galanin

Galanin-containing dorsal horn cells are found superficially, localized to interneurones, projection neurones and primary afferent terminals, sometimes in coexistence with substance P, CGRP, cholecystokinin (CCK) and NOS (see Sorkin & Carlton, 1997). There are three cloned galanin receptors, namely GalR1, 2 and 3. GalR1 and 3 couple to the G, G-protein and inhibit levels of cAMP, whereas GalR3 couples to Gq and activates phospholipase C. Both GalR1 and 2 mRNA have been demonstrated to be present in the spinal cord and DRG of rats (for review see Branchek et al., 2000). In normal animals galanin release seldom occurs in response to noxious and innocuous stimulation (Morton & Hutchison, 1989) and low levels are pro-nociceptive (Wiesenfeld-Hallin et al., 1992). As already mentioned, nerve injury upregulates galanin synthesis and high doses appear to be antinociceptive (Wiesenfeld-Hallin et al., 1992). Thus, the spinal administration of galanin has complex but predominantly inhibitory/antinociceptive effects, which are enhanced in
models of neuropathy (see Xu et al., 2000, for review).

**Neuropeptide Y**

The Y$_2$ receptors for neuropeptide Y normally occur in small diameter DRG neurones and their activation inhibits Ca$^{2+}$ currents probably via a G$_o$ coupling. Receptor activation can block the release of substance P from cultured neurones (Walker et al., 1988) and spinal neuropeptide Y has been reported to exert potent analgesic effects (Hua et al., 1991). Spinal neuropeptide Y largely arises in descending neurones, but as previously mentioned, nerve injury induces the normally quiescent expression of neuropeptide Y in sensory primary afferents, which is also paralleled by distributional shift of Y$_2$ receptor expression to include Aβ-fibre terminals. Thus the neuropeptide Y receptor system appears to display nerve injury-induced changes that are beneficial.

**Cannabinoids**

There are two identified cannabinoid receptors, CB$_1$ and CB$_2$. CB$_1$ is found in the spinal cord where its mRNA has been located to medium and large DRG cell bodies (Hohmann & Herkenham, 1999) and at both presynaptic and postsynaptic spinal sites (Hohmann et al., 1999). Its activation here has been demonstrated to release dynorphins (Houser et al., 2000) and may be involved in endogenous inhibitory tone (Chapman, 1999; Drew et al., 2000). Cannabinoid agonists are also antihyperalgesic in the presence of inflammation (Martin et al., 1999b; Drew et al., 2000) and nerve injury (Fox et al., 2001), possibly mediated via actions in both the CNS and in the periphery, and activity within this system may increased in the presence of persistent pain (Martin et al., 1999b).
1.4 Voltage-Dependent Calcium Channels

It appears that nerve injury resulting in a neuropathic pain state initiates many changes to the peripheral and CNS that are accountable for the various symptoms that manifest. To summarize these include changes in spinal cord connectivity, loss of intrinsic modulatory systems and upregulation of excitatory process including the emergence of spontaneous activity and central sensitization. Pivotal to many of these alterations is the concentration of intracellular Ca\(^{2+}\) ions. In addition to release from internal stores heavily implicated in 2\(^{nd}\) messenger systems and gene induction, the major influx route is across the plasma membrane via the NMDA-receptor/channel complex and VDCCs, in response to neurotransmitter receptor activation and membrane depolarization. VDCCs are critical to the sensory pathway in various aspects, but more specifically they permit the synaptic transmission of sensory information from the periphery to the brain via the control of depolarization-coupled neurotransmitter release.

Primary Structure of VDCCs

VDCCs can be found in the membrane of many cell types throughout the body where they mediate Ca\(^{2+}\) entry into the cell in response to membrane depolarization. Originally solubilized and purified from skeletal muscle (Curtis & Catterall, 1984), VDCCs are hetero-oligomeric complexes consisting of various combinations of an \(\alpha_1\) subunit with auxiliary \(\alpha_2\delta\), \(\beta\) and \(\gamma\) subunits (Figure 3). Hydrophobicity plots, glycosylation and biochemical analysis of the subunits has revealed their primary structure. The \(\alpha_1\) subunit forms the pore of the channel and is predicted to have four domain repeats (I – IV), each having six transmembrane segments (S1 – S6) with a membrane-associated loop between S5 and S6 lining the pore, and the S4 segment forming the voltage-sensor required for activation. The \(\beta\) subunit is predicted to have no transmembrane regions and associates with \(\alpha_1\) intracellularly, in contrast to the \(\gamma\) subunit which has four transmembrane segments. The \(\alpha_2\) subunit is entirely extracellular with many glycosylation sites and is anchored to the membrane through a disulphide bond with the \(\delta\) subunit. Using in vitro expression systems, generally the \(\alpha_1\) subunit is enough to form functional channels and the auxiliary subunits serve to modulate its gating and current characteristics via
enhancing expression levels, shifting voltage-dependence of activation and inactivation to more negative potentials and increasing the rate of inactivation. In vivo, non-neuronal VDCCs comprise all five subunits, whereas neuronal channels lack the γ subunit (Ahlijanian et al., 1990; Witcher et al., 1993; Liu et al., 1996).

Several different classes of VDCCs exist and these were originally identified by their electrophysiological and pharmacological profiles into the L-, N-, P-, Q-, R- and T-types, each subserving different functional roles relative to their cellular location. Subsequent advances in molecular biology have identified ten genes encoding the main pore-forming α1 subunit, termed α1A to α1I, and a recently devised nomenclature groups these into three families, Ca1, 2 and 3, based upon structural and functional characteristics (Ertel et al., 2000). Ca1 and Ca2 comprise the high voltage-activated (HVA) VDCCs, which allow Ca2+ influx upon substantial membrane depolarization, such as that mediated by an action potential, whereas Ca3 channels are the low voltage-activated (LVA) VDCCs, such that they permit Ca2+ flux at resting membrane potentials (Table 3).

**L-type VDCCs (Ca1)**

There are currently four Ca1 channels (Ca1.1, 1.2, 1.3, 1.4 or α1A, IC, ID, IF, respectively) which show 75% sequence homology amongst themselves. These comprise the L-type VDCCs and are selectively blocked by 1,4-dihydropyridines (DHPs), phenylalkylamines and benzothiazepines. They can be located throughout the body and functions include excitation-contraction coupling in skeletal and cardiac muscle, hormone secretion in endocrine cells (Milani et al., 1990) and tonic neurotransmitter release in the retina. α1C and α1D can be found in neurones, through which Ca2+ influx contributes to gene regulation and is important in the integration of synaptic inputs (Bean, 1989a). Immunohistochemical studies have demonstrated the neuronal presence of α1C and α1D subunits upon the cell bodies and dendrites in a variety of cell populations, including the spinal cord, where they are distributed throughout the deep dorsal and ventral horns (Ahlijanian et al., 1990; Hell et al., 1993; Westenbroek et al., 1998).
Figure 3. Subunit structure and composition of a voltage-dependent calcium channel. Predicted α helices are depicted as cylinders. As shown for domain III, 6 transmembrane segments form a domain and the 4 domains comprise the pore-forming α₁ subunit.
Table 3. Classification, cellular localization and functional significance of voltage-dependent calcium channels.

<table>
<thead>
<tr>
<th>VDCC type</th>
<th>Current type</th>
<th>α1 subunit</th>
<th>Tissue localization</th>
<th>Subcellular localization</th>
<th>Spinal cord distribution</th>
<th>Specific blocker</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca,1.1</td>
<td>L (HVA)</td>
<td>α1S</td>
<td>Skeletal muscle</td>
<td>T-tubules</td>
<td>x</td>
<td>DHPs</td>
<td>Excitation-contraction coupling</td>
</tr>
<tr>
<td>Ca,1.2</td>
<td>L (HVA)</td>
<td>α1C</td>
<td>Cardiac muscle, Smooth muscle, Neurones, Pancreas</td>
<td>Membrane surface, t-tubule sarcolemma, Cell soma, Cell soma and larger dendrites of neurones</td>
<td>✓ deep dorsal and ventral horns</td>
<td>DHPs</td>
<td>Excitation-contraction coupling, hormone secretion, gene regulation</td>
</tr>
<tr>
<td>Ca,1.3</td>
<td>L (HVA)</td>
<td>α1D</td>
<td>Neurones Endocrine tissue Neurones Retina</td>
<td>Membrane surface, t-tubule sarcolemma, Cell soma, Cell soma and larger dendrites of neurones</td>
<td>✓ deep dorsal and ventral horns</td>
<td>DHPs</td>
<td>Hormone secretion, gene regulation</td>
</tr>
<tr>
<td>Ca,1.4</td>
<td>L (HVA)</td>
<td>α1F</td>
<td>Neurones</td>
<td>Membrane surface, t-tubule sarcolemma, Cell soma, Cell soma and larger dendrites of neurones</td>
<td>x</td>
<td>Tonic release of neurotransmitters</td>
<td></td>
</tr>
<tr>
<td>Ca,2.1</td>
<td>P/Q (HVA)</td>
<td>α1A</td>
<td>Neurones</td>
<td>Nerve terminals and dendrites</td>
<td>✓ throughout</td>
<td>õ-agatoxin IVA</td>
<td>Neurotransmitter release</td>
</tr>
<tr>
<td>Ca,2.2</td>
<td>N (HVA)</td>
<td>α1B</td>
<td>Neurones</td>
<td>Nerve terminals and dendrites</td>
<td>✓ mostly concentrated in superficial laminae</td>
<td>õ-conotoxin GVIA</td>
<td>Neurotransmitter release</td>
</tr>
<tr>
<td>Ca,2.3</td>
<td>R (HVA)</td>
<td>α1E</td>
<td>Neurones</td>
<td>Soma, dendrites and terminals</td>
<td>✓</td>
<td>None</td>
<td>Neurotransmitter release</td>
</tr>
<tr>
<td>Ca,3.1</td>
<td>T (LVA)</td>
<td>α1G</td>
<td>Neurones</td>
<td>Soma and dendrites</td>
<td>✓ low levels throughout</td>
<td>None</td>
<td>Pacemaking, gradual depolarization for multiple APs and oscillatory behaviour, depolarizes cells to threshold for other channels</td>
</tr>
<tr>
<td>Ca,3.2</td>
<td>T (LVA)</td>
<td>α1H</td>
<td>Neurones</td>
<td>Soma and dendrites</td>
<td>✓ mostly restricted to superficial laminae</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>Ca,3.3</td>
<td>T (LVA)</td>
<td>α1I</td>
<td>Neurones</td>
<td>Soma and dendrites</td>
<td>✓ throughout, mainly in laminae III-IV</td>
<td>None</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: VDCC (voltage-dependent calcium channel); HVA (high voltage-activated); LVA (low voltage-activated); DHPs (dihydropiridines); APs (action potentials).
P/Q-type VDCCs (Ca\textsubscript{2.1})

There are three members of the Ca\textsubscript{2} family and these are purely localized to neuronal populations and show 70% sequence homology amongst themselves, but less than 40% with the Ca\textsubscript{1} channels. Ca\textsubscript{2.1} encompasses both the P- and Q-type currents (often referred to as P/Q-type). These are generated by alternative splicing of the \( \alpha_{1A} \) gene, differentially blocked, in a reversible manner, by \( \omega \)-agatoxin IVA, a 48 amino acid peptide isolated from the funnel-web spider of the \textit{Agelenopsis} genus (see Olivera \textit{et al.}, 1994, for review). P-type current, first recorded in Purkinje neurones (Linas \textit{et al.}, 1989), is specifically blocked over a 100 nM range, whereas Q-type current, first recorded in cerebellar granule neurones (Randell & Tsien, 1995), is blocked at concentrations over 1000 nM. P/Q-type VDCCs are localized to nerve terminals and dendrites in a number of neuronal cell types throughout the brain, as shown by antibody labelling and \textit{in situ} mRNA hybridization (Bertolino & Llinas, 1992; Mintz \textit{et al.}, 1992; Turner \textit{et al.}, 1992; Turner \textit{et al.}, 1993; Stea \textit{et al.}, 1994), and P/Q-type current has been identified in spinal and DRG cells (Mintz \textit{et al.}, 1992). The terminal localization of P/Q-type VDCCs within neurones is indicative of their well-documented importance in synaptic transmission. Ca\textsuperscript{2+} influx through P/Q-type VDCCs in response to neuronal depolarization initiates the release of fast neurotransmitters at synapses, as shown in synaptosome and \textit{in vitro} neuronal preparations (Turner \textit{et al.}, 1992). \( \omega \)-agatoxin IVA has been demonstrated to block excitatory (Luebke \textit{et al.}, 1993; Castillo \textit{et al.}, 1994; Yamamoto \textit{et al.}, 1994) and inhibitory (Takahashi & Momiyama, 1993) synaptic transmission. Thus, P/Q-type VDCCs may be involved in the release of glutamate, aspartate, dopamine, serotonin, noradrenaline, GABA and glycine (Turner \textit{et al.}, 1992; Takahashi & Momiyama, 1993; Turner \textit{et al.}, 1993; Kimura \textit{et al.}, 1995; Miljanich & Ramachandran, 1995). Furthermore, P/Q-type VDCCs can be modulated by various G-protein-coupled pathways that are important for regulation of synaptic transmission. For example, activation of GABA\textsubscript{B} receptors mediates a reduction in P-type current and a decrease in the rate of activation (Mintz & Bean, 1993).
N-type VDCCs (Ca,2.2)

Ca,2.2 is the N-type (α1B) current and is sensitive to block by the ω-conotoxins, which are 24 - 29 amino acid peptides isolated from the venom of fish-hunting marine snails of the Conus genus (see Olivera et al., 1994, for review). In particular, ω-conotoxin GVIA exhibits high affinity, irreversible block of N-type VDCCs via a competitive mechanism. Autoradiographical and electrophysiological studies have shown N-type VDCCs to be widely expressed throughout the brain in numerous cell types (Regan et al., 1991; Ishibashi et al., 1995) and in the spinal cord, most concentrated in laminae I and II of the superficial dorsal horn where nociceptive primary afferents synapse upon spinal pain transmission neurons (Kerr et al., 1988; Gohil et al., 1994), and in DRG (Regan et al., 1991). In vitro studies have demonstrated the requirement of Ca,2+ influx through N-type VDCCs for depolarization-coupled neurotransmitter release. Release of CGRP from rat spinal afferents (Santicioli et al., 1992); sensory neuropeptides from nociceptive dorsal spinal cord afferents (Maggi et al., 1990) and substance P from primary sensory neurones in culture (Holz et al., 1988) have been shown to be ω-conopeptide sensitive. Glutamatergic synaptic transmission between DRG cells and spinal cord neurones has also been inhibited by block of pre-synaptic N-type VDCCs (Gruner & Silva, 1994). N-type VDCCs are also modulated through G-protein-coupled pathways, more so than P-type, such that Ca,2+ current is reduced by a positive shift in voltage-dependence of activation and a slowed rate of activation. Inhibition of N-type VDCC current is observed following activation of a number of receptors, including opioid, GABA, and neuropeptide Y (see Lynch III, 1997), and appears to be mediated by the interaction of Gβγ at a number of sites on the α1B subunit (notably the intracellular loop between domains I and II). Additionally, in certain cells N-type current may be tonically inhibited (Kasai, 1991; Kasai, 1992). Conversely, activation of PKC by various neurotransmitter systems can overcome the G-protein-mediated inhibition of N-type VDCCs (Swartz, 1993; Swartz et al., 1993), via a mechanism that also appears to involve phosphorylation of the I-II linker (Zamponi et al., 1997).

Further substantiating evidence of a role for N- and P/Q-type channels in neurotransmission comes from their direct interaction with the SNARE protein.
complex at the synprint site in the intracellular II-III loop. This mediates the fusion of secretory vesicle and plasma membranes upon Ca\(^{2+}\) influx through VDCCs permitting neurotransmitter release, which in N-type VDCCs has also been shown to modulated by PKC and Ca\(^{2+}\)/calmodulin protein kinase II (Yokoyama et al., 1997), providing feedback inhibition. Whilst L-type VDCCs have been shown not to be involved in fast synaptic transmission (Daniell et al., 1983; Suszkiw et al., 1986; Pfrieger et al., 1992), recent studies show that these channels may also exhibit SNARE protein complex interactions that also show feedback modulation. This has importance in the endocrine secretion of hormones for which they are responsible (Wiser et al., 1999; Yang et al., 1999).

**R-type VDCCs (Ca\(_{v2.3}\))**

The third member of the Ca\(_{v}\)2 family is Ca\(_{v2.3}\), the R-type (\(\alpha_{1E}\)) current, resistant to all previously mentioned agents (Zhang et al., 1993a), thus its distribution, function and modulation are somewhat less defined. However residual HVA Ca\(^{2+}\) current after inhibition of L-, N-, P/Q-type current with their specific blockers, is presumably mediated by R-type VDCCs and can be further distinguished by its electrophysiological properties. R-type current is found on a number of neuronal populations on the cell bodies, dendrites and terminals, and important for Ca\(^{2+}\)-dependent action potentials and neurotransmitter release (Ishibashi et al., 1995).

**T-type VDCCs (Ca\(_{v3}\))**

There are three Ca\(_{v3}\) channels, Ca\(_{v3.1}\), 3.2 and 3.3 (\(\alpha_{1G}, \alpha_{1H}\) and \(\alpha_{1I}\), respectively), that mediate the T-type current, and these show 30% homology to HVA channel forming \(\alpha_i\) subunits (Cribbs et al., 1998; Perez-Reyes et al., 1998; Lee et al., 1999). T-type channels are kinetically distinct LVA VDCCs, such that they permit Ca\(^{2+}\) flux at resting membrane potentials in skeletal (\(\alpha_{1G}\)) and cardiac muscle (\(\alpha_{1G}\) and \(\alpha_{1H}\)) and neuronal cells (\(\alpha_{1G}, \alpha_{1H}\) and \(\alpha_{1I}\)). They activate at voltages near the resting membrane potential, inactivate rapidly, deactivate slowly and have a small single channel conductance (Huguenard, 1996). These unique gating properties of T-type channels permits their involvement in pacemaking, low amplitude oscillations, neuronal bursting, synaptic signal boosting, Ca\(^{2+}\) entry promotion and
lowering threshold for high-threshold spike generation. This Ca\(^{2+}\) current appears to play an important physiological role in near-threshold phenomena and regulation of neuronal excitability. *In situ* hybridization studies on the rat brain have shown that \(\alpha_{1G}, \alpha_{1H}\) and \(\alpha_{1I}\) have unique neuronal distributions, including the dorsal horn of the spinal cord and sensory ganglia (Talley *et al.*, 1999). This is complemented by reported T-type currents in primary sensory neurones (Carbone & Lux, 1984; Kostyuk *et al.*, 1992; Scroggs & Fox, 1992a; Todorovic & Lingle, 1998) and some superficial rat dorsal horn neurones (Ryu & Randic, 1990). Unlike the HVA Ca\(^{2+}\) channels, the functional roles of T-type channels have been hindered by a scarcity of specific pharmacological agents, as they only show preferential block by nickel ions.

*Diversity of VDCCs*

The existence and expression of numerous genes encoding the VDCC pore-forming \(\alpha_1\) subunit is not the only means of generating VDCC diversity, the auxiliary subunits also contribute by the expression of different encoding genes and the generation of splice variants. Currently there are four genes and various splice variants of the \(\beta\) subunit (see Hofmann *et al.*, 1999, for review), three \(\gamma\) subunit genes (Eberst *et al.*, 1997; Letts *et al.*, 1998; Black & Lennon, 1999), and three genes encoding for \(\alpha_2\delta\), again with numerous splice variants (Klugbauer *et al.*, 1999; Hobom *et al.*, 2000). These different subunit types also show differential cellular distributions and exert differential modulation upon different \(\alpha_1\) pore-forming VDCC subunits. Thus, the subunit composition of a VDCC has influence on its electrophysiological and pharmacological properties as well as its subcellular localization and interaction with regulatory proteins. Various combinations of \(\alpha_1\) subunits with the different auxiliary subunits contributes to the diversity of VDCCs observed amongst cell types.

Given their role in controlling neuronal excitability and neurotransmitter release, the role of VDCCs in the transmission of pain has been of great interest. VDCC antagonists have been demonstrated to be antinociceptive in models of inflammation, based on behaviour and *in vivo* electrophysiology, confirming a role for Ca\(^{2+}\) influx into neurones in the spinal processing of nociceptive information (see
Vanegas & Schaible, 2000, for review). To varying extents N-, P/Q- and L-type VDCC blockers have been shown to mediate antinociception in inflammatory behavioural studies (Malmberg & Yaksh, 1994; Malmberg & Yaksh, 1995; Bowersox et al., 1996; Sluka, 1997; Sluka, 1998). Enhanced neuronal responses induced by inflammation are also inhibited by N- and P/Q-type blockers, but not L-type, (Neugebauer et al., 1996; Diaz & Dickenson, 1997; Nebe et al., 1997; Nebe et al., 1998). In contrast, investigations into the role of VDCCs in sensory transmission utilizing models of neuropathy have been limited, however a few behavioural studies demonstrate that N-type VDCC block can reduce pain-related responses (Chaplan et al., 1994; Xiao & Bennett, 1995; Bowersox et al., 1996; White & Cousins, 1998). In contrast block of L- and P/Q-type Ca$^{2+}$ current in the presence of neuropathy has been shown to have little effect (Chaplan et al., 1994; White & Cousins, 1998).
1.5 AIMS OF THIS STUDY

The experiments undertaken for this thesis were performed in order to investigate the role of VDCCs in nociceptive transmission at the level of the spinal cord, and to assess any alterations in dorsal horn neuronal excitability and possible plasticity of the different VDCCs induced by peripheral nerve damage that may contribute to altered nociception. VDCC activation is critical for neurotransmitter release and neuronal excitability, and blockers are antinociceptive in behavioural and clinical studies, but as yet there remains little electrophysiological data regarding the role of spinal VDCCs in the processing of neuropathic pain, an important relay site of sensory information.

In order to achieve these goals I employed the SNL model of neuropathy (Kim & Chung, 1992), confirmed by behavioural testing over a postoperative period of two weeks, to induce a neuropathic state. After the establishment of neuropathy, in vivo electrophysiological studies of dorsal horn spinal neurones were made to investigate the effects of various spinally delivered VDCC blockers on a wide range of electrical and natural-evoked neuronal activity, in comparison to sham-operated and naïve rats. ω-conotoxin GVIA, ω-agatoxin IVA, ethosuximide and nifedipine were used to inhibit Ca^{2+} flux through N-, P/Q-, T- and L-type VDCCs, respectively.

Since neuropathic pain has multiple causes and symptoms, dysfunctional mechanisms are likely to be diverse. For these reasons I also aimed to investigate the benefits of combination therapy using clinically licensed drugs. The anticonvulsant gabapentin has proved effective in the clinical treatment of neuropathic pain. Although its mechanism of action is undetermined it does bind to the VDCC α2δ subunit and acts to inhibit excitation. Conversely morphine, not often beneficial in the treatment of neuropathic pain, acts to facilitate inhibitory systems, thus the effects of low doses of subcutaneous gabapentin and morphine in the same experimental setting were also investigated. Due to the difficulties in conducting combination studies in patients with neuropathic pain it was hoped that this approach may provide a guide to potential improvements in its treatment.
CHAPTER 2

METHODS
2.1 Spinal Nerve Ligation Model of Neuropathy

2.1.1 Surgery

Male Sprague-Dawley rats (University College London Biological Services), initially weighing 130 - 150g, were used for SNL surgery. All experimental procedures were approved by the Home Office and follow the guidelines under the International Association for the study of Pain (Zimmermann, 1983). Selective tight ligation of spinal nerves L5 and L6, and a sham procedure were performed as first described by Kim and Chung (1992). Under gaseous anaesthesia with a mixture of halothane (3.5% for induction, 1.5% for maintenance) and a 1:1 flow ratio of N₂O:O₂, the rat was placed in a prone position. A midline incision was made from L4 - S2 and the left paraspinal muscles were separated from the spinous processes. The L6 transverse process and the sacrum were made visible by scraping off attached ligaments. Part of the L6 transverse process was then removed to expose the L4 and L5 spinal nerves and L6 was identified lying just under the sacrum. Using 6-0 silk thread, the left spinal nerves L5 and L6 were tightly ligated distal to their dorsal root ganglion and proximal to their conjunction to form the sciatic nerve. Hemostasis was confirmed, the wound sutured and the animal recovered from anaesthesia. A sham operation was performed to produce a control group, whereby the surgical procedure was identical to that of the experimental group, but spinal nerve ligation was omitted.

2.1.2 Behavioural Testing

For two weeks following surgery the rats were housed in groups of 4, in plastic cages under a 12/12h day/night cycle and their general health monitored. Successful reproduction of the neuropathic model was confirmed by behavioural testing (postoperative (PO) days 2, 3, 5, 7, 9, 12 and 14). Rats were placed in transparent plastic cubicles on a mesh floor and allowed to acclimatize before initiating any tests.
2.1.2.1 Sensitivity to Punctate Mechanical Stimuli

Foot withdrawals to trials of ascending von Frey filaments (bending forces 1, 5 and 9 grams: 9.9, 49.5 and 89.1mN respectively), considered non-noxious under normal circumstances, were quantified. In a trial a single filament was applied 10 times to the plantar surface of the foot, through the mesh floor, for 2-3 seconds each time. A period of 3 - 4 minutes was left before commencing with the next filament. The number of foot withdrawals (scored out of 10) to each von Frey filament was measured on both the ipsilateral and contralateral hindpaws.

2.1.2.2 Sensitivity to cooling stimuli

Foot withdrawals to the application of a drop of acetone to the plantar region of the foot were quantified. In a trial a drop of acetone was squirted through the mesh floor via a syringe, 5 times, at 5 minute intervals. The number of foot withdrawals (scored out of 5) was measured on both the ipsilateral and contralateral hindpaws. The acetone was applied with a gentle squirt so as not to evoke a mechanical response.

2.2 Spinal Cord Electrophysiology

2.2.1 Surgery

Subsequent to behavioural testing (PO days 14 - 17), the operated rats were used for electrophysiological studies, as described previously (Dickenson & Sullivan, 1986). Anaesthesia was induced by first placing the rats in a sealed box and filling it with 3.5% halothane in a mixture of 66% N₂O and 33% O₂, until loss of the righting reflex. The rat was then placed on a heating blanket on its back and anaesthesia sustained at 3% via a nose cone. On confirmation of areflexia, the trachea was exposed and a cannula inserted though which anaesthesia was maintained for the duration of the experiment. The rat was placed in a stereotaxic frame and secured via ear bars and a tooth bar rest, and a rectal probe was inserted to regulate body temperature (36.5 - 37°C) via an automatic feedback unit connected to the heating blanket. Lumbar vertebrae L1 - L3 were located by rib positioning and a clamp, attached to the stereotaxic frame, was secured to the vertebrae rostral of L1.
This allowed a laminectomy to be performed removing most of L1 - L3 to expose segments L4 - L5 of the spinal cord, which receive afferent input from the toe region. Another clamp was positioned caudal to the exposed site and to the frame in order to maintain stability during electrophysiological recordings, and the level of halothane was finally reduced to 1.7% such that the rats breathed spontaneously and areflexia was maintained throughout the course of the experiment.

2.2.2 Electrophysiological Recordings

Extracellular recordings of single convergent neurones, located deep within the dorsal horn (> 500 μm) were made using a parylene coated tungsten electrode, recorded and analyzed with a NeuroLog (Digitimer) system (Figure 4). The electrode was held in a manoeuvrable headstage that was grounded by an earth lead to the stereotaxic frame and steel table. To minimize interference and non-neuronal electrical activity, the head stage received electrical signal from both the spinal cord (A) via the electrode and the general surroundings (B) via a crocodile clip attached to the rats skin, such that the latter was subtracted from the former. The resultant (A-B) signal was then filtered and amplified by a series of NeuroLog modules and finally fed through an audio amplifier to a speaker and oscilloscope to allow the neuronal activity to be monitored. Adjustment of the window discriminator permitted selective data capture over background activity, differentiating cells by their amplitude, such that only action potentials above a certain height triggered a counting pulse. This was relayed to the oscilloscope (apparent as a dot over the spike), the latch counters (showing cumulative count) and to the CED 1401 interface for analysis. The CED 1401 was further coupled to a Pentium computer and the data captured using Spike 2 software (Rate and Post-stimulus histogram functions).

2.2.2.1 Neurone Isolation

A single convergent dorsal horn neurone was isolated that either received input from the toe region ipsilateral to the spinal nerve ligation or sham procedure, or from either side of the spinal cord in non-operated, naïve rats. The electrode, held in its headstage, could be moved laterally and rostro-caudally outside of the spinal cord, and dorso-ventrally through the cord using gross and micromanipulator manual
Figure 4. Schematic representation of data capture, courtesy of Dr. K. J. Carpenter.
controls. In parallel to slow descent of the electrode through the cord, the peripheral hindpaw receptive field was tapped with the finger allowing location of the spinal cord area receiving input specifically from the toe region. A neurone was selected for an experiment if it responded to both noxious (pinch) and non-noxious (touch) stimuli and if its action potential amplitude could be counted over background. Two fine needles, attached to a stimulus isolator module, were then inserted into the centre of the cells receptive field, to allow transcutaneous electrical stimulation. After an interval of about ten minutes, the C-fibre threshold current was determined by giving single, electrical pulses of amplitude 0.1 – 3.3 mA incrementally until a C-fibre response was evoked (distinguished by latency after stimulus). Subsequently, electrical stimuli were given at 3-times this C-fibre threshold, which even at a possible maximum intensity of 9.9 mA was not observed to damage the hindpaw.

2.2.2.2 Neurone Characterization

An electrical test consisted of a train of 16 stimuli, of a 2 ms wide pulse at 0.5 Hz at 3-times the threshold required to evoke a C-fibre response, which were set using the period generator, digital width and pulse buffer modules respectively, and a post-stimulus histogram was constructed. Electrically-evoked action potentials, mediated by different fibre types, were separated on a latency basis, determined by their known conduction velocities, into Aβ-fibres (0 - 20 ms), Aδ-fibres (20 - 90 ms), C-fibres (90 - 300 ms). Action potentials arriving 300 - 800 ms after an electrical pulse were classed as 'post-discharge', a result of repeated stimulation leading to wind-up neuronal hyperexcitability. The latch counter was set, via the delay width module, to count cumulatively the C-fibre and post discharge activity evoked by each of the 16 stimuli (90 - 800 ms). These counts allowed calculation of the 'input' (non-potentiated response) and the 'excess spikes' (potentiated response, evident by increased neuronal excitability to repeated stimulation).

**Input** = (action potentials (90 - 800 ms) evoked by first pulse) x (total number of pulses (16)).

**Excess spikes** = (total action potentials (90 - 800 ms) after train of 16 stimuli) – Input.
After establishment of a cell’s response to electrical stimulation its response profile to a range of natural stimuli was determined. Any spontaneous activity exhibited by a neurone in the absence of external stimulus to its receptive field was recorded over a period of 10 minutes. The number of action potentials evoked over a 10 second period to constant application of various natural stimuli was quantified in response to both punctate mechanical (von Frey filaments 9 and 75 grams) and thermal (constant water jet at 45 °C) stimuli applied to the centre of the neurone’s receptive field. The thermal response to 45 °C was determined by subtracting the response to 32 °C (a non-noxious temperature so as to ascertain any mechanically-evoked response from the water jet) from the response to 45 °C. All responses to natural stimuli were normalized by the subtraction of any spontaneous activity measured over a period of 10 seconds before the application of each stimulus.

2.3 Pharmacological Studies

2.3.1 Drug Administration

The testing protocol, initiated every 10 minutes, consisted of an electrical test followed by the natural stimuli, as described above. Stabilization of the neuronal responses was confirmed on consecutively obtaining at least three consistent pre-drug responses (<10% variation), for all measures. These values were then averaged to generate pre-drug control values with which to compare the effect of drug administration on subsequent evoked responses. Drugs were applied either spinally, in a volume of 50 µl directly onto the exposed spinal cord, or sub-cutaneously in a volume of 250 µl. After drug application the testing protocol was subsequently continued for between 40 – 90 minutes per dose, to follow the effects until the evoked neuronal responses peaked or plateaued, dependent on the time course of action of the drug in use.
2.3.2 Drugs Employed

ω-conotoxin GVIA was obtained from Sigma-Aldrich Company Ltd., Poole, Dorset, U.K. The toxin was dissolved in 0.9% saline and stores in separate aliquots stored at -20 °C.

ω-agatoxin IVA was obtained from the Peptide Institute, Inc., Scientific Marketing Associates, Barnet, Hertfordshire, U.K. The toxin was dissolved in 0.9% saline and stores in separate aliquots stored at -20 °C.

Ethosuximide (2-Ethyl-2-methylsuccinimide) was obtained from Sigma-Aldrich Company Ltd., Poole, Dorset, U.K. A stock solution was prepared in 0.9% saline and stored at 4 °C.

Morphine sulphate was obtained from Evans Medical. A stock solution was prepared in 0.9% saline and stored at 4 °C.

Gabapentin, a gift from Parke Davis, was dissolved in 0.9% saline and stored at 4 °C.

Naloxone hydrochloride was obtained from Sigma-Aldrich Company Ltd., Poole, Dorset, U.K. A stock solution was prepared in 0.9% saline and stored at 4 °C.

Nifedipine was obtained from Sigma-Aldrich Company Ltd., Poole, Dorset, U.K. Due to solubility difficulties in saline, this was dissolved in a mixture of ethanol (0.07%), cremophor (33.33%) and water (60%). The stock solution was then stored protected from light at 4 °C.
2.4 **Statistical Analysis**

The effects of drugs on the electrically- and naturally-evoked responses were calculated as maximum percentage changes from the averaged pre-drug value for each neurone (% control) and the overall results for each dose were calculated as means ± standard error of mean (S.E.M.) of the normalized data. In most cases graphs display the results expressed as mean% inhibition ± S.E.M. (% inhibition = 100% -% control, such that 100% inhibition represents no response, and 0% inhibition represents control value). Statistical analysis of maximal drug effects at each dose compared to the averaged pre-drug value was determined by paired Student's *t*-test on raw data (number of evoked action potentials). Comparison of drug effects between different experimental groups (naïve, sham and SNL) were made using an unpaired Student's *t*-test on the normalized data. This comparison was deemed valid if the evoked number of action potentials determined for each neuronal measurement by pre-drug characterization was found not to be statistically different between groups using an unpaired Student’s *t*-test on raw data. The level of significance was taken as *P* < 0.05. All statistical analysis of data was performed using the computer programme Statview 4.5 (Abacus Concepts). Additional analyses used for particular experiments are described when necessary.
CHAPTER 3

BEHAVIOURAL CONSEQUENCES

AND DORSAL HORN NEURONE RESPONSE

CHARACTERISTICS FOLLOWING

SPINAL NERVE LIGATION
3.1 INTRODUCTION

The underlying pathophysiological mechanisms of neuropathic pain, and thus guidance towards improved therapeutic approaches, have been enlightened by the introduction of animal models of nerve injury. Early models of spinal transection or dorsal rhizotomy resulted in complete denervation of the hindpaw (Zeltser & Seltzer, 1994). However, more recently models that produce partial denervation have been developed more reminiscent of the clinical situation (see chapter 1.2). These involve constriction of the common sciatic nerve with 4 loose ligatures in rats in the CCI model (Bennett & Xie, 1988; Attal et al., 1990), tight ligation of 1/3 – 1/2 of the sciatic nerve in rats in the PSL model (Seltzer et al., 1990), and tight ligation of one or two of the spinal nerves in rats in the SNL model (Kim & Chung, 1992) or monkeys (Palecek et al., 1992a). Common to each of these neuropathies is the behaviourally observed manifestation of thermal hyperalgesia and mechanical allodynia ipsilateral to nerve injury, which can be used to assess the therapeutic benefit of existing drugs as well as the analgesic potential of novel drugs. Likewise, at a neuronal level, in vivo electrophysiological techniques can be used to investigate pathological changes involved in neuropathic pain, also useful for pharmacological assessment and identification of possible new drug targets.

It is widely acknowledged that nerve injury leads to alterations to the peripheral nervous system that culminate in aberrant sensory processing and neuropathic pain symptoms. Observations of many pre-clinical studies employing the various models of nerve injury have demonstrated that primary afferent fibres properties are pivotal and at the origins of characteristic changes in response to nerve injury, including the generation of ectopic discharges and anatomical reorganization (see chapter 1.3.1). Evidence is now also accumulating regarding nerve injury-induced alterations at the level of the spinal cord. In vivo electrophysiological studies in rats with SNL (Pertovaara et al., 1997; Chapman et al., 1998b; Suzuki et al., 2000b), PSL (Behbehani & Dollberg-Stolik, 1994; Takaishi et al., 1996), CCI (Palecek et al., 1992b; Sotgiu et al., 1992; Laird & Bennett, 1993; Sotgiu et al., 1994a; Sotgiu et al., 1994b), L4 – 6 dorsal root constriction (Tabo et al., 1999) or monkeys with L7 SNL (Palecek et al., 1992a) have revealed a complex pattern of changes in the properties of dorsal horn neurones ipsilateral to nerve injury. In
In this chapter I describe the behavioural and dorsal horn neuronal consequences induced by peripheral nerve injury, using the selective spinal nerve ligation (SNL) rodent model. This involves tight ligation of two (L5/6) of the three (L4/5/6) spinal nerves that comprise the sciatic nerve (Kim & Chung, 1992). In comparison to CCI and PSL, the magnitude of mechanical allodynia, assessed by withdrawal frequency to innocuous mechanical stimuli (von Frey filaments), is greatest and most reliable in the SNL model and experimental variability is minimized to some extent due to the tight, versus partial, ligation. The SNL model also preserves segregation of injured and uninjured primary afferent input proximal to the formation of the sciatic nerve such that spinal input from damaged afferents can be selectively handled (see chapter 1.2). Subsequent to the establishment of neuropathy in vivo electrophysiological recordings of WDR dorsal horn neurones were made and the evoked responses to a wide range of natural and electrical stimuli assessed such that possible alterations in the spinal processing of cutaneous inputs following SNL might be highlighted.

3.2 METHODS

3.2.1 BEHAVIOURAL ASSESSMENT

Male Sprague-Dawley rats, initially weighing 130 - 150 g were subject to either unilateral selective spinal nerve ligation (L5/6) or a sham operation (control group) as first described by Kim and Chung (1992). For a postoperative (PO) period of two weeks behavioural testing of both experimental groups was carried out on days 2, 3, 5, 7, 9, 12 and 14, in order to determine the sensitivity of both hindpaws to punctate mechanical (von Frey filaments) and cooling (acetone) stimuli.
3.2.2 Determination of Dorsal Horn Neurone Response to Electrical Stimuli

Subsequent to SNL or sham operation and behavioural assessment, electrophysiological recordings of ipsilateral convergent dorsal horn neurones were made at PO days 14 - 17. Additionally, neuronal responses were recorded from unoperated naïve rats. On isolation of a single cell and insertion of stimulating needles into the receptive field, a rest period of 10 minutes was allowed before characterization of the neurones response to electrical stimulation. The threshold current required to elicit a C-fibre mediated response was determined. An electrical test of 16 stimuli at 3 x C-fibre threshold was then performed and a post stimulus histogram (PSTH) constructed. Quantification of the Aβ- Aδ- and C-fibre mediated action potentials was performed on a latency basis, as was the post-discharge. Input and excess spikes measures were also calculated.

3.2.3 Determination of Dorsal Horn Neurone Responses to Natural Stimuli

Subsequent to the electrical stimulus a rest period of several minutes was allowed. Any spontaneous activity was then recorded over a period of 10 minutes. Following this, the neuronal response to innocuous and noxious punctate mechanical stimuli (von Frey 9 and 75 g, respectively) and noxious heat (45 °C) was determined upon application to the most responsive part of the receptive field for 10 seconds. A recovery interval was allowed between each test, and responses were normalized by subtraction of any spontaneous activity apparent before application of each stimulus.

3.2.4 Determination of Dorsal Horn Neurone Receptive Field Size

The area of the neuronal receptive field over the ipsilateral hindpaw was determined by probing the plantar surface with three von Frey filaments (9, 15 and 75 g). An evoked response greater than 1 Hz was the criteria for inclusion in the neurone’s receptive field, which was marked on a standard diagram of the hindpaw. For quantification of the mean receptive field size of neurones recorded from each experimental group, the diagrams were photocopied onto plain paper (80 g/m²) and the shaded areas cut out and weighed and expressed as a percentage of the weight of
a control diagram (determined as an average of 20 as 77.8 ± 0.2 mg). Localization of
the receptive field was also classified into lateral (comprising toes 1 and 2 and the
lateral side of the plantar surface of the hindpaw), central (comprising toes 3 and 4
and the central plantar surface of the hindpaw) and medial (comprising toe 5 and the
medial side of the plantar surface of the hindpaw), as determined by von Frey 9 g.

3.3 RESULTS

3.2.1 BEHAVIOURAL ASSESSMENT

During the postoperative period the animals showed normal weight gain and
maintained good general health. Rats subjected to SNL exhibited abnormal foot
posture ipsilateral to nerve injury whereby toes were held together in a 'guarding'
behaviour. This did not occur in either the contralateral hindpaw, or in the sham-
operated rats. Furthermore, in the absence of externally applied stimuli, SNL rats
often licked or pulled the ipsilateral hindpaw and body weight was shifted more to
the contralateral hindpaw. Autotomy, signs of distress and aggression were not
observed.

Successful replication of the nerve injury model was confirmed by
behavioural testing of 138 SNL rats and 81 sham-operated rats, which demonstrated
the development of mechanical and cooling allodynia of the injured hindpaw of SNL
rats (Figure 5). Evoked allodynia, in response to innocuous mechanical (von Frey
filaments bending force 1 - 9 g) and cooling (acetone) stimuli, was displayed as a
brisk withdrawal, accompanied in some cases by shaking and licking of the foot
ipsilateral to SNL. This was evident at PO day 2, reached maximum PO days 7 - 12
and still maintained at PO day 14. Consistent withdrawal responses were never
exhibited by the control group or by the contralateral hindpaw of the experimental
group, and when present were never accompanied by the pain-like behaviours
displayed to stimuli applied to the lesioned hindpaw of SNL rats.
Figure 5. Development of mechanical and cooling allodynia in the ipsilateral hindpaw over the 2 week postoperative period following SNL (●, n = 138) in comparison to sham control (∑, n = 81). Data is expressed as the mean difference score ± S.E.M. for the hindpaw withdrawal response to punctate mechanical stimuli (von Frey filaments bending forces (a) 1 g, (b) 5 g and (c) 9 g and (d) cooling stimulus (drop of acetone) applied to the plantar surface of the hindpaws (trials of 10 for the mechanical and 5 for the cooling). Difference score = (ipsilateral hindpaw response frequency) - (contralateral hindpaw response frequency).
3.2.2 **Response Characteristics of Dorsal Horn Neurones to Electrical Stimuli**

A total of 233 convergent dorsal horn neurones were characterized, of which 75 were from naïve animals, 54 were from sham operated rats and 104 were from rats subject to SNL (Table 4). All neurones recorded from operated groups had a receptive field over the left ipsilateral hindpaw. No difference between experimental groups was observed in the mean cell depth, which was for individual neurones between 500 and 1000 μm corresponding to laminae V of the spinal cord.

**Table 4.** Comparison of the mean electrical threshold required to evoke a C-fibre mediated response and the mean evoked dorsal horn neuronal action potentials (APs) in response to a train of 16 electrical stimuli at 3 x C-fibre threshold in naïve, sham-operated and SNL rats at post-operative days 14-17. Electrically-evoked APs are divided into groups based upon their mediating fibre type. Data is expressed as means ± S.E.M. and * denotes statistically significant in comparison to naïve.

<table>
<thead>
<tr>
<th></th>
<th>NAIVE</th>
<th>SHAM</th>
<th>SNL</th>
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<tr>
<td>Neurone depth (μm)</td>
<td>815 ± 25</td>
<td>879 ± 27</td>
<td>801 ± 19</td>
</tr>
<tr>
<td>C-fibre threshold (mA)</td>
<td>1.48 ± 0.27</td>
<td>1.18 ± 0.1</td>
<td>1.19 ± 0.07</td>
</tr>
<tr>
<td>C-fibre mediated response (AP)</td>
<td>355 ± 17</td>
<td>351 ± 21</td>
<td>345 ± 15</td>
</tr>
<tr>
<td>Aβ-fibre mediated response (AP)</td>
<td>107 ± 4</td>
<td>109 ± 5</td>
<td>112 ± 3</td>
</tr>
<tr>
<td>Aδ-fibre mediated response (AP)</td>
<td>62 ± 4</td>
<td>69 ± 5</td>
<td>*83 ± 5</td>
</tr>
<tr>
<td>Post-discharge (AP)</td>
<td>194 ± 15</td>
<td>204 ± 16</td>
<td>214 ± 15</td>
</tr>
<tr>
<td>Input (AP)</td>
<td>307 ± 19</td>
<td>282 ± 19</td>
<td>290 ± 16</td>
</tr>
<tr>
<td>Excess spikes (AP)</td>
<td>297 ± 27</td>
<td>309 ± 27</td>
<td>268 ± 41</td>
</tr>
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</table>

The current required to evoke a C-fibre mediated response was similar across all three groups. Examples of a representative response of a neurone to a single electrical pulse at 3 x C-fibre threshold (as seen on the oscilloscope) and a PSTH following a train of 16 electrical stimuli is shown in Figure 6.
Figure 6. (a) Representation of the evoked dorsal horn neuronal response to a single electrical stimulus and 3 times C-fibre threshold, as seen on the oscilloscope. (b) An example of a typical PSTH produced after a train of 16 electrical stimuli at a frequency of 0.5 Hz at 3 times C-fibre threshold. The action potentials evoked in a single dorsal horn neurone by the different primary afferent fibre types are displayed, and the postdischarge.
After a train of 16 electrical stimuli, generally the total evoked neuronal response mediated by Aβ-, Aδ- and C-fibres were comparable in SNL, sham and naïve rats (Table 4), however the Aδ-fibre response in SNL rats was found to be significantly larger than that of naïve rats \( (P < 0.05) \), but not in comparison to sham. The post-discharge, input and excess spikes measures were not significantly different between groups.

### 3.2.3 Response Characteristics of Dorsal Horn Neurones to Natural Stimuli

The level of ongoing spontaneous activity was found to be significantly higher \( (P < 0.0001, \text{Mann-Whitney}) \) in neurones in SNL rats compared to sham and naïve (Table 5). Furthermore 64% of neurones characterized in SNL rats exhibited spontaneous activity at a rate greater than 0.1 Hz in comparison to only 31% of characterized neurones in sham-operated rats and 27% of neurones in naïve animals \( (P < 0.001, \text{Fishers exact test}) \).

**Table 5.** Comparison of the mean dorsal horn neurone depth recorded from, the mean spontaneous activity (i.e., neuronal activity in the absence of external stimulus) and the mean evoked dorsal horn neuronal action potentials (APs) in response to innocuous (9 g) and noxious (75 g) von Frey filaments and noxious heat in naïve, sham-operated and SNL rats at post-operative days 14 - 17. Data is expressed as means ± S.E.M. and * indicates statistical significance \( (P < 0.0001) \) in comparison to naïve and sham groups.

<table>
<thead>
<tr>
<th></th>
<th>NAÏVE</th>
<th>SHAM</th>
<th>SNL</th>
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<tbody>
<tr>
<td>Spontaneous activity (Hz)</td>
<td>0.36 ± 0.13</td>
<td>0.33 ± 0.11</td>
<td>*3.51 ± 0.72</td>
</tr>
<tr>
<td>von Frey 9 g evoked response (Hz)</td>
<td>14.18 ± 1.89</td>
<td>13.2 ± 1.86</td>
<td>14.58 ± 1.45</td>
</tr>
<tr>
<td>von Frey 75 g evoked response (Hz)</td>
<td>67.4 ± 4.11</td>
<td>66.23 ± 4.92</td>
<td>65.73 ± 3.41</td>
</tr>
<tr>
<td>45 °C heat evoked response (Hz)</td>
<td>38.52 ± 3.88</td>
<td>37.55 ± 4.04</td>
<td>31.14 ± 2.57</td>
</tr>
</tbody>
</table>
A representative response of a neurone to punctate mechanical stimuli (von Frey 9 and 75 g) and noxious heat (constant water jet at 45 °C) is shown in Figure 7. Neurones displayed an increased response to noxious von Frey 75 g in comparison to non-noxious von Frey 9 g. The heat response was normalized by subtraction of evoked activity due to mechanical stimulation of the water jet at 32 °C, when present. Naturally-evoked neuronal responses were comparable across SNL, sham and naïve groups (Table 5).

**Figure 7.** A typical rate recording showing the evoked dorsal horn neuronal action potentials to natural stimuli applied for a period of 10 seconds each. The mechanically evoked response to innocuous von Frey 9 g and noxious von Frey 75 g are displayed. The response to noxious heat over 10 seconds (water jet at 45 °C) was normalized by subtraction of the response to water jet at 32 °C over 10 seconds so as to remove any mechanically evoked activity due to the water jet itself.
3.2.4 **Receptive Field Characteristics of Dorsal Horn Neurones**

A total of 72 neurones where used for determination of receptive field distributions, of which 16 were from naïve rats, 26 were from sham-operated rats and 30 were from SNL rats. Of the neurones recorded from, the relative proportions that had medially, centrally or laterally distributed receptive fields tended to be mainly centrally located within each of the three groups (Table 6). Between experimental groups there was a tendency for the receptive fields of neurones recorded from SNL rats to be centrally/medially distributed, in comparison to naïve and sham-operated rats which tended to be centrally/laterally distributed.

**Table 6.** The distribution of dorsal horn neurone receptive fields over the hindpaw ipsilateral to SNL or sham-operation at PO day 14 - 17 and also in un-operated naïve rats. Medial, central and lateral assignment was determined by probing with von Frey 9 g. Data is expressed as percentage of neurones characterized within each group.

<table>
<thead>
<tr>
<th></th>
<th>NAÏVE</th>
<th>SHAM</th>
<th>SNL</th>
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<tbody>
<tr>
<td>Medial</td>
<td>19%</td>
<td>19%</td>
<td>27%</td>
</tr>
<tr>
<td>Central</td>
<td>56%</td>
<td>50%</td>
<td>60%</td>
</tr>
<tr>
<td>Lateral</td>
<td>25%</td>
<td>31%</td>
<td>13%</td>
</tr>
</tbody>
</table>

The receptive field size of dorsal horn neurones, mapped with von Frey 9, 15 and 75 g, were generally comparable between SNL, sham-operated and naïve animals, two weeks after surgery where performed, and increased in size with increasing intensity (Figure 8). However, the receptive field size mapped to innocuous von Frey 9 g was significantly greater (by 40%) for neurones recorded from SNL rats in comparison to either sham or naïve rats ($P < 0.05$). Receptive field sizes mapped to greater von Frey intensities of 15 g and 75 g were comparable across the three groups. An example of the receptive fields of a neurone from each experimental group is shown in Figure 9.
Figure 8. The mean cutaneous receptive field sizes of WDR dorsal horn neurones recorded from naïve (n = 16), sham-operated (n = 26) and SNL (n = 30) rats, ipsilateral to surgery where performed. Data are expressed as mean % of total area of the plantar surface of the hindpaw ± S.E.M. * denotes statistically significant difference in comparison to sham and naïve (P < 0.05).
Figure 9. Examples of typical receptive fields to of dorsal horn neurones recorded from naive, sham-operated and SNL rats, ipsilateral to surgery where performed. Receptive fields were mapped to von Frey filaments 9, 15 and 75 g and the area as a percentage of the whole hindpaw area is given. The numbers assigned to the toes are shown in the top left-hand diagram and the receptive fields displayed are classed as lateral in naive, central in sham and medial in SNL.
3.4 DISCUSSION

3.4.1 BEHAVIOURAL CONSEQUENCES OF SNL

Here, I have demonstrated successful replication of the nerve injury model of SNL as first described by Kim and Chung (1992). Over the postoperative period of two weeks the rats subject to L5 and L6 spinal nerve tight ligation displayed behaviours indicative of neuropathic pain ipsilateral to nerve injury. These nociceptive behaviours were not manifest contralateral to surgery nor in the sham-operated control animals. Brisk withdrawal of the injured hindpaw to stimulation with normally innocuous punctate mechanical stimuli (von Frey filaments) and cooling stimuli (acetone) indicated the development of mechanical and cooling allodynia, consistent with the original findings (Kim & Chung, 1992). Nociceptive behaviours were often apparent on the first testing day at PO day 2, and the level of allodynias increased and were maintained over the two week behavioural assessment period. Mechanical allodynia has been reported as early as 12 hours after nerve injury and maintained for up to 15 weeks (Kim & Chung, 1992). Delayed development of mechanical sensitivity was additionally been reported in the hindpaw contralateral to nerve injury, prominent at 2 weeks and maintained for 14 weeks. In the original paper a sham operation was performed on the contralateral side, however the lack of sham-induced behavioural changes seen in a separate sham control group (Kim & Chung, 1992) and here most likely eliminates this as the cause. I observed no consistent withdrawal responses contralateral to nerve injury, and on the few occasions this did occur it was never associated with nociceptive behaviours such as licking or shaking of the foot, often observed ipsilateral to nerve injury. However, I only performed behavioural assessment for 2 weeks after surgery, whereas these contralateral changes observed previously were delayed in onset. Additionally, these contralateral changes were observed in response to von Frey 19 g, which is higher than the greatest von Frey employed in my study. Other models of peripheral nerve injury have also reported delayed onset contralateral changes (Takaishi et al., 1996; Tabo et al., 1999), and this may be representative of ‘mirror image’ pain observed contralateral to nerve injury clinically (Bonica, 1979).
Thermal hyperalgesia was also recorded in the original study of SNL, being apparent 3 days after surgery and maintained for at least 5 weeks (Kim & Chung, 1992). This was not assessed in my study, however others have failed to observe the development of increased sensitivity to suprathreshold thermal stimulus after SNL (Kontinen et al., 1998; Roytta et al., 1999) and other neuropathic pain models (Luukko et al., 1994). It appears that there may be some differences in the development of nociceptive behaviours dependent upon the evoking stimulus modality, which is likely mediated by differing transduction mechanisms displaying differential alterations in response to nerve injury.

In addition to the evoked allodynia observed in response to external stimuli, rats subject to SNL also displayed certain ongoing nociceptive behaviours. Most notable was the adopted ‘guarding’ posture of the injured hindpaw and associated shift of weight-bearing to the contralateral side observed in SNL and other nerve injury models. Guarding posture has been shown to be decreased by morphine and tricyclic antidepressants as expected for a pain-related behaviour (Jazat & Guilbaud, 1991; Ardid & Guilbaud, 1992). This postural characteristic has been suggested to be an attempt to avoid potentially painful mechanical stimulation of the paw (Bennett & Xie, 1988) and mechanical stimulation of the injury site itself (Laird & Bennett, 1993). Following CCI it has been shown that the rapid development of scar tissue around the ligatures tethers the injury site to adjacent muscle (Bennett & Xie, 1988) resulting in its visible movement upon of leg extension (Laird & Bennett, 1993). This then excited dorsal horn neurones responding to direct mechanical stimulation of the nerve injury site and was not due to joint movement. Therefore leg extension encountered during walking or standing was postulated a likely adequate stimulus for the mechanosensitive afferents in the neuroma (Laird & Bennett, 1993). They additionally demonstrated that the majority of these responsive dorsal horn neurones had a C-fibre input, concluding that some of the afferent input evoked by neuroma stimulation is mediated by nociceptive afferents.

Despite evidence for ongoing pain after SNL, it has been reported that these animals show no differences in levels of anxiety or depression in comparison to sham-operated animals (Kontinen et al., 1999), therefore this would have no impact
upon evoked behaviours. As regards the withdrawal reflex as a valid indicator of mechanical allodynia, it has been suggested that the shift of weight to the contralateral side might influence this assessment. However it has been demonstrated that decreased mechanical thresholds ipsilateral to nerve injury are independent of this factor, and thus validating quantification of mechanical allodynia by evoked hindpaw withdrawal (Kauppila et al., 1998a).

In comparison to other models of partial denervation of the hindpaw, the selective tight ligation of SNL results in the most reproducible and extensive mechanical allodynia, and minimizes, to some extent, the inter-experimental variability that is encountered in CCI and PSL models. CCI involves constriction of the common sciatic nerve with 4 loose ligatures (Bennett & Xie, 1988), whereas PSL involves tight ligation of 1/3 – 1/2 of the thickness of the sciatic nerve (Seltzer et al., 1990). Depending on the extent of constriction exerted by the ‘loose’ ligatures or proportion of the sciatic nerve ligated, respectively, varying proportions of fibre types within the sciatic nerve innervating the hindpaw could be injured (Basbaum et al., 1991; Carlton et al., 1991). In this respect, for SNL rats the only source of variability in the contribution that each spinal segment makes to the sciatic nerve can arise from anatomical differences between individual rats. Furthermore the SNL model also preserves segregation of injured and uninjured primary afferent input proximal to the formation of the sciatic nerve such that spinal input from damaged afferents can be selectively handled (see chapter 1.2). In CCI and PSL models partial denervation damage to the common sciatic nerve results in all three spinal segments and their DRG containing a combination of damage and undamaged fibres. Self-mutilating behaviour of the denervated regions of the hindpaw, thought to be an attempt to rid itself of the painful experience (Coderre et al., 1986), has been observed in CCI rats (Bennett & Xie, 1988; Attal et al., 1990) but not after SNL (Kim & Chung, 1992) or PSL (Seltzer et al., 1990). I did not observe such autotomy in my study.

Peripherally reported consequences of SNL and other models of nerve injury include the development of ongoing ectopic activity, generated at the site of injury or within the DRG, may be responsible for alterations to behaviour and
somatosensory processing associated with neuropathic pain (Wall & Devor, 1983; Burchiel, 1984; Kajander et al., 1992; Steel et al., 1994; Xie et al., 1995; Study & Kral, 1996). In the clinic, positive correlations have been made between the occurrence of spontaneous firing of nociceptors innervating the painful region and neuropathic pain (Nystrom & Hagbarth, 1981; Gracely et al., 1992). Primary afferent fibres have also been reported to undergo structural reorganization and exhibit neurochemical/receptor plasticity, such that large myelinated Aβ-fibres might acquire the ability to mediate nociceptive transmission, in particular allodynia (see chapter 1.3 for discussion). Paradoxically, SNL has been shown to decrease the number of myelinated primary afferent fibres (Roytta et al., 1999), which may imply facilitated spinal nociceptive processing of afferent input. This is substantiated by the fact that spinal application of different drugs can reduce mechanical allodynia that develops following SNL (Backonja et al., 1994; Bowersox et al., 1996; Chaplan et al., 1997; Hwang & Yaksh, 1997) and thermal hyperalgesia (Wegert et al., 1997).

3.4.2 DORSAL HORN NEURONAL CONSEQUENCES OF SNL

Subsequent to establishment of neuropathy the operated rats were then used for electrophysiological studies, alongside naïve, un-operated animals. Making in vivo recordings of ipsilateral WDR neurones located in lamina V of the dorsal horn I was able to characterize the responses of these neurones driven by electrical and natural stimulation of their cutaneous receptive fields in order to assess any changes occurring parallel to the observed behavioural consequences. Before explaining my observations I will briefly explain the conclusions that can be drawn from the different electrical measurements made throughout this thesis.

The majority of evoked WDR neurone responses recorded are ultimately under the peripheral drive of primary afferent fibre activity. Action potentials arriving in the spinal cord carried by the different fibre types evokes WDR neurone activity either by synapsing directly (Aβ-fibres) or via a relay of interneurones (C-fibres), such that non-nociceptive and nociceptive information converges (see chapter 1.3.2.2). The Aβ-, Aδ- and C-fibre measurements, reveal the evoked activity of the WDR neurone mediated by activity in each of the different fibre types. Aβ-
fibres carry touch evoked stimuli, C-fibres convey nociceptive information and Aδ-fibres carry possibly both. The 'input' is reflective of presynaptic activity and neurotransmitter release, and of the baseline resting postsynaptic C-fibre-evoked dorsal horn neurone response, quantifying activity in response to the first stimulus of the electrical train. It represents the excitatory effect of a single afferent barrage on spinal neuronal circuitry, prior to which the system is at rest and interneuronal amplification/hyperexcitability mechanisms, involving NMDA receptor recruitment, are not in play. 'Postdischarge' quantifies action potentials that cannot have been evoked by even the slowest-conducting afferent fibre inputs, and is therefore reflective of spinal cord hyperexcitability generally only apparent after repeated stimulation. This activity-dependent hyperexcitability can also be quantified as 'excess spikes', a measure of the additional action potentials recorded above the predicted constant baseline response. Excess spikes and postdischarge are therefore more reflective of postsynaptic activity and wind-up, where interneuronal amplification/hyperexcitability mechanisms, involving NMDA receptor recruitment, are now in play.

I found the overall magnitude of responses to innocuous mechanical, noxious mechanical and thermal, and supra-C-fibre threshold electrical stimuli were comparable between SNL, sham-operated and naïve animals, which is surprising considering the behavioural difference observed between SNL and sham-operated groups. It could be argued that the lack of obvious correlation between the behavioural changes and response properties of dorsal horn neurones is that neuronal types (nociceptive specific, non-nociceptive, STT neurones), other than WDR, might have exhibited changes more consistent with behaviour. However, I believe that WDR neurones are a reasonable population to study as they are a rational target for some of the changes underlying hyperalgesia and allodynia for a number of reasons. Activity in WDR neurones has been shown to be sufficient for pain perception (Mayer et al., 1975; Price & Mayer, 1975) and they comprise more than half of the neurones of the STT (Besson & Chaouch, 1987), a major ascending pain signalling pathway. Due to the convergence of low- and high-threshold inputs onto WDR neurones, they can signal sensory-discriminative aspects of pain (Maixner et al., 1986) and have been shown to be more accurate predictors of thermal perception (Dubner et al., 1989) in comparison to nociceptive-specific neurones.
The most obvious difference in neurones recorded from SNL rats here, was the level of ongoing, spontaneous activity exhibited by neurones in the absence of application of an external stimulus to their receptive fields. This was significantly greater in WDR neurones ipsilateral to nerve injury in comparison to neurones recorded from sham-operated and naïve animals, in both terms of frequency and the proportion of neurones displaying such activity. This is in agreement with observations made in other electrophysiological dorsal horn neuronal studies after SNL (Pertovaara et al., 1997; Chapman et al., 1998b). The incidence of spontaneous activity of ipsilateral dorsal horn neurones has also been reported in other rat models of nerve injury such as PSL (Behbehani & Dollberg-Stolik, 1994), CCI (Palecek et al., 1992b; Sotgiu et al., 1992; Laird & Bennett, 1993; Sotgiu et al., 1994b) and L4 – 6 dorsal root constriction (Tabo et al., 1999) and in monkeys with L7 SNL (Palecek et al., 1992a). Furthermore, its development has been shown to increase temporally (Chapman et al., 1998b; Tabo et al., 1999), in parallel with the time course of evoked behavioural allodynia (Chapman et al., 1998b). The spontaneous activity described here at the level of the spinal cord may be secondary to the previously described nerve injury-induced generation of ectopic activity in the periphery (Wall & Devor, 1983; Burchiel, 1984; Kajander et al., 1992; Steel et al., 1994; Xie et al., 1995; Study & Kral, 1996). In SNL animals it has been demonstrated that signals from injured primary afferent fibres into the spinal cord are critical for the development and maintenance of neuropathic behaviour (Sheen & Chung, 1993; Yoon et al., 1996). Although I did not assess the level of ongoing spontaneous pain behaviour after SNL, this has been previously described (Kim et al., 1997) and may relate to my observation of increased dorsal horn neuronal spontaneous activity following SNL.

The other significant difference I observed was an increase in the cutaneous receptive field to innocuous mechanical stimulation (von Frey 9 g) of WDR dorsal horn neurones ipsilateral to SNL, in comparison to those of sham-operated and naïve animals. Other electrophysiology studies have also described enlarged receptive fields after nerve injury. Following PSL the receptive field areas of neurones ipsi- and contralateral to nerve injury 3 - 5 weeks later were significantly larger compared to those of neurones recorded in un-operated rats (Behbehani & Dollberg-Stolik, 1994), yet in another study this was not observed until 16 weeks. After L4 – 6 dorsal
root constriction WDR dorsal horn neurones were shown to exhibit enlarged mechanical receptive fields at a later time point of 22 weeks (Tabo et al., 1999). Despite the presence of enlarged receptive fields for a number of dorsal horn neurones several of these electrophysiological studies after nerve injury also report that a fraction of neurones on the nerve injured side display no mechanical receptive fields (Palecek et al., 1992b; Laird & Bennett, 1993) which is indicative of partial deafferentation.

Reorganization of receptive fields is indicative of neuronal plasticity after peripheral nerve damage and is described both clinically and pre-clinically. These observations described electrophysiologically after experimental neuropathy fit well with the clinical observations. In patients a cardinal feature of neuropathic pain is a partial or complete loss of afferent sensory function and the paradoxical presence of certain hyperphenomena in the painful area (Lindblom, 1994). Neuropathic pain patients also have large areas of abnormal sensations and pain, not corresponding to any known nerve or root innervation territory or dermatomes (Jensen & Rasmussen, 1994). Studies in humans and animals (see Dubner & Basbaum, 1994, for review) have shown radiation aftersensations and expansion of receptive fields may be related to pathological changes in dorsal horn neurones. Psychophysical and neuronal recordings have shown that pain intensity is related to impulse discharges and numbers of neurones activated (Price et al., 1994). WDR neurones are in part characterized by small receptive zones that can be excited by non-noxious stimuli (touch, gentle pressure) surrounded by a much larger zone from which noxious stimuli (pinch, firm pressure, temperature > 45 °C) can evoke neuronal discharges. These large receptive field zones are overlapping and extend over several dermatomes and their receptive fields are a reflection of synaptic propriospinal interconnections in the spinal dorsal horn that extend over several segments. Therefore a noxious stimulus can activate several WDR neurones and increasing the stimulus intensity will result in activation of further WDR neurones rostrocaudally dispersed. A global increase in spinal cord excitability resulting from either an increase in synaptic efficacy of excitatory inputs or a decrease of inhibitory controls (or both) would have the potential to alter neuronal cutaneous receptive fields. Indeed, blockade of inhibitory neurotransmission by picrotoxin or strychnine can mediate expansion of receptive fields of spinal or medullary dorsal horn neurones.
(Yokota & Nishikawa, 1979; Markus & Pomeranz, 1987). Furthermore PAG stimulation was shown not to attenuate heat-evoked responses of spinal dorsal horn WDR neurones in the ligated side in comparison to normal which indicated that SNL induced a change in the descending inhibitory regulation originating in the PAG (Pertovaara et al., 1997). If receptive field size is increased then for any given stimulus a greater number of spinal neurones will be recruited, actually leading to greater afferent input and possibly enhancing pain transmission. Thus, alongside nerve injury-induced dorsal horn neuronal spontaneous activity these observations are indicative of such spinal cord hyperexcitability.

Despite the behaviourally observed mechanical and cooling allodynia consequences of SNL just prior to the electrophysiological recordings, I saw no overall increases in the electrically- and naturally-evoked dorsal horn neuronal response magnitudes in comparison to sham and naïve animals, as might be expected in the presence of increased pain responsiveness. Such lack of increases of dorsal horn neuronal responses has also been described in other studies. Recordings of WDR neurones made ipsilateral to SNL have demonstrated subtle intensity-dependent changes in the response of dorsal horn neurones to mechanical stimulus, compared to sham (Chapman et al., 1998b). The absolute magnitude of response to brush, prod and acetone were reduced two weeks after neuropathy, the evoked responses to innocuous punctate mechanical stimuli were comparable between experimental groups, yet those to a noxious were reduced. Electrically- and noxious heat-evoked dorsal horn neuronal responses were also reduced in SNL compared to sham-operated rats. However, the proportion of neurones responding to different innocuous modalities of stimuli were increased after SNL and not in sham. No injury-induced modifications to stimulus thresholds were noted even though behavioural studies have shown that the threshold of the ipsilateral paw is reduced in response to mechanical stimuli and as is the withdrawal latency to thermal stimuli following SNL (Kim & Chung, 1992; Wegert et al., 1997). After L4 – 6 dorsal root constriction no difference in response properties of dorsal horn neurones were observed after 5 weeks, even though behavioural mechanical hypersensitivity was maximal (Tabo et al., 1999). Although the mean threshold for eliciting a mechanically-evoked dorsal horn WDR neurone response was not altered by CCI (Laird & Bennett, 1993), the number of neurones sensitive to low intensity
mechanical stimulation (Laird & Bennett, 1993) and the magnitude of mechanically-evoked responses of the neurones were also reduced (Palecek et al., 1992b). Dorsal horn neurones ipsilateral to nerve injury have been shown to respond normally to heat exhibiting no significant changes in response threshold or magnitude of responses after SNL (Pertovaara et al., 1997), CCI (Palecek et al., 1992b; Laird & Bennett, 1993) and PSL (Takaishi et al., 1996), even in the presence of behaviourally observed thermal hyperalgesia. Furthermore, the heat-evoked responses to suprathreshold stimuli were actually lower in neuropathic animals (Palecek et al., 1992b).

In contrast, nerve injury-induced increases in evoked neuronal responses have been reported. Following L5/6 SNL in rats, WDR responses to mechanical stimuli were enhanced independent of stimulus intensity (Pertovaara et al., 1997). In monkeys previously receiving L7 SNL, ipsilateral spinothalamic tract neurones rostral to the ligated segment exhibited reduced mechanical and thermal thresholds and increased magnitude of response in comparison to the contralateral sham-operated side. However, neurones within the ligated segment had low responsiveness (Palecek et al., 1992a).

In the present study, the magnitude of evoked dorsal horn neuronal responses show remarkable comparability between the groups, in agreement with the majority of other electrophysiological studies that report no increase in responses after nerve injury. It has been demonstrated that damage to the peripheral nervous system actually results in substantial primary afferent fibre loss (Castro-Lopes et al., 1990), and therefore stimulus-evoked input into the spinal cord is conveyed along fewer fibres. Consistent with this, a large proportion of neurones ipsilateral to CCI possess no cutaneous receptive fields (Laird & Bennett, 1993). In the SNL model, ligation of L5 and L6 spinal nerves almost completely abolishes input into the corresponding spinal segments. However, both of these areas send input to the undamaged L4 segment, and based upon the estimated contribution of adjacent unmyelinated nerves to a segment (Besse et al., 1991), in L4 there could be a deficit of about 35% in terms of afferent input. If there are fewer dorsal horn neurones on the injured side with intact sensory input, and those still receiving input respond
normally to noxious stimuli, then one would predict a reduction, rather than the observed increase in sensitivity of the hindlimb observed behaviourally and the relatively minor alterations in the evoked responses of dorsal horn neurones. Clinically it is clear that allodynia is mediated by low-threshold myelinated Aβ-fibres and in order for these normally innocuous inputs to be perceived as painful, abnormal central processing of these inputs would have to occur (see Torebjork et al., 1995), yet no difference between experimental groups was observed in the magnitude of Aβ-fibres nor C-fibre and hyperexcitability measures of excess spikes and postdischarge. In a similar fashion no nerve injury-induced change in the magnitude of neuronal response to innocuous and noxious natural stimuli was observed.

In consolidation with the other studies already discussed, overall, nerve injury induces behaviourally observed enhanced pain responses alongside complex changes in dorsal horn neuronal response pattern. Whilst there appears to be a general increase in the level of neuronal spontaneous activity after nerve injury, changes in response to externally applied stimuli vary slightly between studies, however the predominant tendency appears to be towards little change, or perhaps even a reduction. The concomitant presence of enhancements and reductions of neuronal activity and behaviours are reminiscent of the clinical descriptions of neuropathic pain where sensory deficits and enhanced sensations can coexist. On further consideration, the lack of increased neuronal excitability may not be as unexpected as first thought. These experimental observations, together with the consideration of symptoms of neuropathic pain, suggest that evoked pains may arise from central compensations for the loss of normal sensory inputs, whereby spinal processing of activity from the remaining intact primary afferents must actually be increased in order to maintain the same magnitude of response evoked in control animals. Ongoing peripheral activity may well produce an ongoing level of neurotransmitter release in the spinal cord, as indicated by high levels of spontaneous activity, which may in turn favour hyperexcitability of responses to subsequent evoked stimuli. Thus, in neuropathic pain it is likely that aberrant peripheral activity is amplified and enhanced by spinal mechanisms. This possibly results in increased VDCC activity leading to more transmitter release and thus increased neuronal activity mediated by NMDA- and other receptors. The altered stimulus-response
relationship may be a basis for the allodynia and thermal hyperalgesia reported in this model.
CHAPTER 4

VOLTAGE-DEPENDENT CALCIUM CHANNELS:

THE CAV2 FAMILY
4.1 INTRODUCTION

Neuropathic pain can arise from injury- or disease-evoked damage to the peripheral or central nervous system and patients often experience sensory deficits, ongoing pain and stimulus-evoked pain (allodynia and hyperalgesia). Animal models of neuropathy have been critical in elucidating the complex causal mechanisms, involving plasticity in nociceptive transmission and modulating systems. The SNL model (Kim & Chung, 1992) involves tight ligation of two (L5 and L6) of the three spinal nerves that form the sciatic nerve. Behavioural consequences include thermal hyperalgesia, and mechanical and cooling allodynia (Kim & Chung, 1992; Chaplan et al., 1997).

Sensory neurones express a number of classes of VDCCs (L, N, P/Q, R and T), distinguished by their electrophysiological and pharmacological profiles. Ten genes encoding the main pore-forming $\alpha_1$ subunit have been identified, termed $\alpha_{1A}$ to $\alpha_{1I}$, and a recently devised nomenclature groups these into three families, Ca$_{v}1$, 2 and 3, based upon structural and functional characteristics (Ertel et al., 2000). Ca$_{v}1$ and Ca$_{v}2$ comprise the high voltage-activated (HVA) VDCCs, which allow Ca$^{2+}$ influx upon substantial membrane depolarization, whereas Ca$_{v}3$ channels are the low voltage-activated (LVA) VDCCs, such that they permit Ca$^{2+}$ flux at resting membrane potentials.

N-type VDCCs ($\alpha_{1B}$ or Ca$_{v}2.2$ current) are sensitive to block by $\omega$-conotoxin GVIA. $\omega$-conotoxin GVIA is specific for the N-subtype and binds in an irreversible manner (see Olivera et al., 1994; Lynch III, 1997, for reviews). P/Q-type VDCCs ($\alpha_{1A}$ or Ca$_{v}2.1$ current) are sensitive to block by $\omega$-agatoxin IVA. $\omega$-agatoxin IVA is specific for both P- and Q-subtypes, yet block of P-type Ca$^{2+}$ current is 10-fold more potent than that of Q-type. In contrast to the actions of $\omega$-conotoxin GVIA, $\omega$-agatoxin IVA mediated Ca$^{2+}$ current block is reversible (see Olivera et al., 1994; Lynch III, 1997, for reviews). Both N- and P/Q-type VDCCs are widely expressed throughout the brain and spinal cord (Kerr et al., 1988; Mintz et al., 1992; Gohil et al., 1994). More specifically, the N-type channel is concentrated in laminae I and II of the superficial dorsal horn, where nociceptive primary afferents synapse.
Kerr et al., 1988; Gohil et al., 1994). Staining of the dorsal laminae of the rat spinal cord has revealed a complementary distribution of α₁A and α₁B subunits in nerve terminals in the superficial laminae (Westenbroek et al., 1998). Within these neurones the α₁A and α₁B subunits are located predominantly along apical dendrites at presynaptic locations, with substantially lower levels of somal expression (Westenbroek et al., 1998). Further to this, in vitro studies have demonstrated the requirement of Ca²⁺ influx through N- and P/Q-type VDCCs for depolarization-coupled neurotransmitter release (Miljanich & Ramachandran, 1995). Spinal release of primary afferent peptides, CGRP and substance P, is ω-conotoxin sensitive (Holz et al., 1988; Maggi et al., 1990; Santicioli et al., 1992), and glutamate release is both ω-conotoxin and ω-agatoxin sensitive (Dickie & Davies, 1992; Turner et al., 1992; Turner et al., 1993). Additionally, Ca²⁺ influx into neurones can enhance excitability and produce intracellular changes including gene induction.

VDCC antagonists are antinociceptive in models of inflammation, based on behaviour and in vivo electrophysiology, confirming a role for Ca²⁺ influx into neurones in the spinal processing of nociceptive information (see Vanegas & Schaible, 2000, for review). In the formalin test, behavioural antinociception is observed to varying extents with N-, P/Q- and L-type VDCC blockers (Malmberg & Yaksh, 1994; Malmberg & Yaksh, 1995; Bowersox et al., 1996), and Diaz and Dickenson (1996) reported phase-dependent effects of N- and P/Q-type blockers on neuronal responses. Blockade of N- and P/Q-type VDCCs has been shown to reduce enhanced neuronal responses (Neugebauer et al., 1996; Nebe et al., 1997; Nebe et al., 1998) and nociceptive behaviours (Sluka, 1997) after knee joint inflammation and intradermal capsaicin (Sluka, 1998). Studies in models of neuropathy have been limited, however Ca²⁺ chelation (White & Cousins, 1998) and N-type VDCC block have been shown to be effective (Chaplan et al., 1994; Xiao & Bennett, 1995; Bowersox et al., 1996; White & Cousins, 1998). In contrast L- and P/Q-type antagonists show little effect (Chaplan et al., 1994; White & Cousins, 1998).

Currently there are no in vivo electrophysiological studies investigating the role of spinal VDCCs in the processing of neuropathic pain. This chapter investigates the role of N- and P/Q-type VDCCs in the transmission of sensory information at the
level of the spinal cord and plasticity of these channels that may occur following nerve injury. After behavioural establishment of a neuropathic state, using the SNL model (Kim & Chung, 1992), electrophysiological studies of dorsal horn neurones were made to investigate the effects of spinally delivered ω-conotoxin GVIA (N-type VDCC blocker) and ω-agatoxin IVA (P/Q-type VDCC blocker) on a wide range of electrically- and naturally-evoked neuronal activity.

4.2 METHODS

A total of 72 male adult Sprague-Dawley rats were used in this study, of which 27 were naïve, 19 were sham-operated and 26 were SNL. The animals were prepared in the normal way and in separate experiments the effects of ω-conotoxin GVIA and ω-agatoxin IVA were investigated on the evoked dorsal horn neuronal responses. The toxins were applied directly onto the exposed surface of the spinal cord (0.1, 0.4, 0.8, 1.2, 2.2 and 3.2 µg). The effects of each dose were followed until the responses plateaued (a minimum of 60 minutes), when the next dose would be applied cumulatively.

4.3 RESULTS

4.3.1 BEHAVIOURAL ASSESSMENT

Rats subjected to SNL exhibited 'guarding' foot posture ipsilateral to nerve injury and successful replication of the nerve injury model was confirmed by behavioural testing which demonstrated the development of mechanical and cooling allodynia of the injured hindpaw (see chapter 3 for combined behavioural data obtained from all studies used in this thesis). This was not displayed by either the contralateral hindpaw, or in the sham-operated rats. Upon establishment of a neuropathic state, animals were then used for in vivo electrophysiology and the pharmacological study at PO days 14 - 17.
4.3.2 **Spinal Cord Electrophysiology: Neurone Characterization**

The number of dorsal horn neurones recorded from in each group were 23 in SNL rats, 21 in sham-operated rats and 37 in naïve rats. All neurones had a receptive field over the hindpaw ipsilateral to surgery (when performed). The level of ongoing spontaneous activity was found to be significantly higher \((P < 0.05)\) in neurones in SNL rats \((6.36 \pm 2.24 \text{ Hz})\) compared to sham \((0.34 \pm 0.22 \text{ Hz})\) and naïve \((0.83 \pm 0.69 \text{ Hz})\). No significant differences were found between experimental groups in the mean cell depth of recorded neurones and the mean neuronal responses evoked by electrical and natural stimulation.

4.3.3 **Spinal Cord Electrophysiology: Effects of ω-conotoxin GVIA**

The effect of ω-conotoxin GVIA \((0.1 - 3.2 \mu g)\), applied directly onto the spinal cord, on the electrically- and naturally-evoked dorsal horn neuronal responses was tested in SNL, sham-operated and naïve animals. Since the sham-operation is the appropriate control for SNL, and for clarity, the results obtained from the naïve, non-operated, group shall not be displayed on the graphs.

At the higher doses, ω-conotoxin GVIA produced inhibitions of all the electrically- and naturally-evoked responses in neurones in all experimental groups (Figure 10). Interestingly, in the sham-operated group, low doses tended to facilitate the neuronal responses, especially those induced by electrical stimulation. For each dose \((0.1, 0.4, 0.8, 1.2, 2.2, \text{ and } 3.2 \mu g)\) the effects of the toxin were slow in onset with clear effects seen around 40 minutes, maximal inhibitions established at 70 – 90 minutes (Figure 11), and these were irreversible. In SNL rats, all doses of ω-conotoxin GVIA \((0.1 – 3.2 \mu g)\) elicited significant effects, compared to pre-drug control, of the C-fibre, excess spikes, input, post-discharge and Aδ-fibre, responses (Figures 10a and 12a - e, respectively; \(P < 0.05; n = 8\)), with similar mean maximal inhibitions at top dose in the range 86 ± 6% to 96 ± 3%. The Aβ-fibre response was significantly inhibited with 0.4 – 3.2 μg ω-conotoxin GVIA, but only reached a maximum inhibition of 41 ± 10% (Figures 10a and 12f).
Figure 10. The effect of spinally applied \( \omega \)-conotoxin GVIA on the electrically-evoked dorsal horn neuronal responses recorded from (a) SNL (\( n = 8 \)), (b) sham-operated (\( n = 6 \)) and (c) naive rats (\( n = 11 \)) at PO days 14 - 17. Data are expressed as the mean maximal % inhibition of the pre-drug control values \( \pm \) S.E.M.
Figure 11. Time course of the effect of spinally applied α-conotoxin GVIA on the evoked response of a typical dorsal horn neurone recorded from a sham-operated rat. Examples of the effect on the Aβ-fibre, C-fibre and von Frey 75 g measurements are shown with the cumulative dose indicated. Data are expressed as % of the averaged pre-drug control values.
Figure 12. Comparison of the effect of spinally applied ω-conotoxin GVIA on the electrically-evoked dorsal horn neuronal responses recorded from sham-operated (n = 6) and SNL (n = 8) rats at PO days 14 - 17. Data are expressed as the mean maximal % inhibition of the pre-drug control values ± S.E.M. * denotes a significantly greater inhibitory effect (P < 0.05) of the toxin at a specific dose in SNL rats compared to sham.
In contrast to SNL animals, sham-operated rats required higher concentrations of the toxin to achieve significant inhibition of the electrically-evoked responses, but still reached similar maximal levels of inhibition at the top dose of 3.2 μg. ω-conotoxin GVIA (0.8 - 3.2 μg) significantly inhibited the post-discharge and Aδ-fibre responses (Figures 10b and 12d - e; \( P < 0.05; n = 6 \)), whereas the C-fibre, excess spikes and input responses (Figures 10b and 12a – c) were significantly inhibited over the dose range 1.2 - 3.2 μg (\( P < 0.05; n = 6 \)). In a similar fashion to the sham-operated group, naïve animals also required higher concentrations of the toxin to achieve significant inhibition of the electrically-evoked responses (Figure 10c). ω-conotoxin GVIA (0.8 - 3.2 μg) significantly inhibited the C-fibre, input and Aδ-fibre responses (\( P < 0.05; n = 11 \)) and 2.2 - 3.2 μg produced a significant inhibition (\( P < 0.05; n = 11 \)) of the post-discharge and excess spikes responses. Maximum inhibitions ranged from 69 ± 12% to 89 ± 6%. As in SNL rats, ω-conotoxin GVIA had limited effects on the Aβ-fibre response in sham (Figure 12f) and naïve rats (Figure 10c) where the mean maximum inhibitions were 25 ± 7% and 44 ± 6%, respectively.

After neuropathy the inhibitory effects of ω-conotoxin GVIA on the electrically-evoked responses appeared to be more marked at the lower end of the dose range in comparison to sham-operated rats. This was statistically significant (\( P < 0.05; n = 8 \)) at 0.1 and 0.4 μg for the C-fibre, excess spikes, input, and post-discharge responses (Figures 12a - d) and at 0.4 μg for the Aδ-fibre response (Figure 12c). 0.1 and 0.4 μg ω-conotoxin GVIA had a significantly greater effect (\( P < 0.05 \)) on the: C-fibre response (Figure 12a) in SNL rats (29 ± 5% and 47 ± 9% inhibitions, respectively, \( n = 8 \)) compared to sham rats (7 ± 10% and 5 ± 15% facilitations, \( n = 7 \)); excess spikes response (Figure 12b) in SNL rats (63 ± 12% and 77 ± 8% inhibitions, respectively, \( n = 8 \)) compared to sham (14 ± 10% and 15 ± 17% inhibitions, \( n = 7 \)); input response (Figure 12c) in SNL rats (30 ± 5% and 54 ± 9% inhibitions, respectively, \( n = 8 \)) compared to sham (19 ± 15% and 14 ± 21% facilitations, \( n = 7 \)); post-discharge (Figure 12d) in SNL (50 ± 13% and 67 ± 10% inhibitions, respectively, \( n = 8 \)) compared to sham (6 ±11% and 20 ± 16% inhibitions, \( n = 6 \)). 0.4 μg ω-conotoxin GVIA had a significantly greater effect (\( P < 0.05 \)) on the Aδ-fibre (Figure 12e) in SNL rats (50 ± 10% inhibition, \( n = 8 \)).
compared to sham (21 ± 19% inhibition, n = 5).

ω-conotoxin GVIA also produced an inhibitory effect on the naturally-evoked neuronal responses (Figure 13). At 3.2 μg ω-conotoxin GVIA, the response to non-noxious mechanical stimulation (von Frey 9 g) was inhibited by 95 ± 3% in both SNL (n = 5) and sham rats (n = 5), and 75 ± 6% in naïve rats (n = 10) (Figure 13a, naïve not shown). The response to noxious mechanical stimulation (von Frey 75 g) was inhibited by 83 ± 5% in SNL rats (n = 7), 78 ± 12% in the sham control group (n = 6), and 69 ± 5% in naïve (n = 10) (Figure 13b, naïve not shown). The response to noxious thermal stimulation (water jet at 45 °C) was inhibited by 89 ± 5% in SNL rats (n = 6), 93 ± 5% in sham rats (n = 5) and 79 ± 9% in naïve (n = 8) (Figure 13c, naïve not shown). After neuropathy, ω-conotoxin GVIA had a greater inhibitory effect on the naturally-evoked neuronal responses at the lower end of the dose range compared to sham-operated animals. This was found to be statistically significant at 0.1 and 0.4 μg ω-conotoxin GVIA, for the von Frey 9 g evoked responses (Figure 13a) (55 ± 6% and 69 ± 7% inhibitions, respectively, in SNL rats, compared to 6 ± 12% facilitation and 17 ± 11% inhibition, respectively, in sham rats; P < 0.001; n = 8), and at 0.4 μg for the von Frey 75 g evoked responses (Figure 13b) (51 ± 6% inhibition in SNL rats compared to 21 ± 12% inhibition in sham; P < 0.05; n = 8).
Figure 13. Comparison of the effect of spinally applied \( \omega \)-conotoxin GVIA on the naturally-evoked dorsal horn neuronal responses recorded from sham \((n = 6)\) and SNL \((n = 8)\) rats. Data are expressed as mean maximal % inhibition of pre-drug control values \(\pm\) S.E.M. \(\ast\) denotes a statistically significant greater inhibitory effect \((P < 0.05)\) of the toxin at a specific dose in SNL rats compared to sham.
4.3.3 Spinal Cord Electrophysiology: Effects of ω-agatoxin IVA

ω-Agatoxin IVA inhibited the electrically-evoked neuronal responses measured in all experimental groups, but to a lesser extent than that achieved with ω-conotoxin GVIA (Figure 14). For each dose (0.1, 0.4, 0.8, 1.2, 2.2, and 3.2 μg) clear effects of the toxin were seen around 40 minutes, maximal inhibitions established at 70 – 90 minutes (Figure 15), and these were partially reversible. In SNL animals, at the highest dose, mean maximal inhibitions from pre-drug controls were in the range 37 ± 13% to 60 ± 10% for excess spikes, input, post-discharge and Aδ-fibre (Figures 14a and 16b - e), and only 18% for the C- and Aβ-fibre (Figures 14a and 16a & f, respectively), at which significance was achieved (P < 0.05; n = 7). Similar maximal inhibitions were observed in sham-operated rats, and there was no difference in drug effect at the lower doses in comparison to SNL animals (Figures 14b and 16). ω-agatoxin IVA had no significant inhibitory effects on the responses in naïve animals, however with the low doses (0.1 - 0.8 μg) there was a tendency for the electrically-evoked neuronal responses to be facilitated and this was found to be statistically significant (P < 0.05; n = 5) for the C-fibre response (Figure 14c).

The naturally-evoked neuronal responses were also inhibited by ω-agatoxin IVA (Figure 17). In SNL rats, at the highest dose, mean maximal inhibitions from the pre-drug control were in the range 57 ± 11% to 71 ± 12%, and this was statistically significant for von Frey 9 and 75 g (P < 0.05; n = 7). Again, no difference in the extent of inhibition was observed between experimental groups.

The spontaneous rate of activity, when present, was clearly inhibited by both toxins. Due to the low pre-drug baseline values and variability between neurones in this ongoing activity was not quantified.
Figure 14. The effect of spinally applied ω-agatoxin IVA on the electrically-evoked dorsal horn neuronal responses recorded from (a) SNL (n = 7), (b) sham-operated (n = 7) and (c) naive rats (n = 5) at PO days 14 - 17. Data are expressed as the mean maximal % inhibition of the pre-drug control values + S.E.M.
Figure 15. Time course of the effect of spinally applied \( \omega \)-agatoxin IVA on the evoked response of a typical dorsal horn neurone recorded from a sham-operated rat. Examples of the effect on the A\( \beta \)-fibre, post-discharge and von Frey 75 g measurements are shown with the cumulative dose indicated. Data are expressed as % of the averaged pre-drug control values.
Figure 16. Comparison of the effect of spinally applied ω-agatoxin IVA on the electrically-evoked dorsal horn neuronal responses recorded from sham-operated (n = 7) and SNL (n = 7) rats at PO days 14 - 17. Data are expressed as the mean maximal % inhibition of the pre-drug control values ± S.E.M.
Figure 17. Comparison of the effect of spinally applied α-agatoxin IVA on the naturally-evoked dorsal horn neuronal responses recorded from sham (n = 7) and SNL (n = 7) rats. Data are expressed as mean maximal % inhibition of pre-drug control values + S.E.M.
4.4 DISCUSSION

In this study I have addressed the role of members of the Ca\(_{\alpha}2\) family of VDCCs in the spinal processing of sensory information after nerve injury. Spinal \(\omega\)-conotoxin GVI\(_A\), an N-type (Ca\(_{\alpha}2.2, \alpha_{1B}\)) VDCC blocker, inhibited the electrically- and naturally- (innocuous and noxious) evoked dorsal horn neuronal responses. The effects of low doses were significantly enhanced after the establishment of neuropathy. In comparison, the P/Q-type (Ca\(_{\alpha}2.1, \alpha_{1A}\)) blocker \(\omega\)-agatoxin IVA inhibited the evoked neuronal responses to a lesser extent and its profile was unaltered after nerve injury.

As regards the concentration and specificity of the toxins used it has been demonstrated in vitro that N-type VDCCs are specifically and completely blocked by 0.1 – 0.5 \(\mu\)M \(\omega\)-conotoxin GVI\(_A\). Block of P-type Ca\(^{2+}\) current is blocked by 2 nM \(\omega\)-agatoxin IVA, which is 100-fold less than that required to block Q-type Ca\(^{2+}\) current (Lynch III, 1997). Although the concentrations of the toxins at their sites of action in vivo obviously cannot be accurately determined, the concentration applied to the surface of the spinal cord at a mid range dose (0.8 \(\mu\)g) was 5 \(\mu\)M for \(\omega\)-conotoxin GVI\(_A\) and 3 \(\mu\)M for \(\omega\)-agatoxin IVA. These concentrations are higher than the selective dose-ranges for these toxins in vitro, however in vitro the toxins are applied directly to the membrane. Within the spinal cord these concentrations will be several orders of magnitude less due to the diluting effect of diffusion into the surrounding tissue and vasculature. Additionally, since the toxins are large peptides access into the tissue is likely to be limited as demonstrated by the slow establishment of maximal inhibitions (Figures 11 & 15). In addition, the different profile of action of \(\omega\)-conotoxin GVI\(_A\) and \(\omega\)-agatoxin IVA further suggests that they are acting at the appropriate VDCC subtype.

Blockade of Ca\(^{2+}\) current through both N- and P/Q-type VDCCs inhibited, to differing extents, the electrically- and naturally-evoked neuronal responses in both naïve un-operated and sham operated animals as well as after the establishment of neuropathy in SNL rats. This is indicative of a role of Ca\(_{\alpha}2\) VDCCs in sensory transmission at the level of the spinal cord. This is in agreement with their known
importance in neurotransmitter release (Miljanich & Ramachandran, 1995) and their reported distribution within the spinal cord (Kerr et al., 1988; Mintz et al., 1992; Gohil et al., 1994). ω-conotoxin GVIA and ω-agatoxin IVA are likely to be acting at VDCCs located at a number of possible sites within the spinal cord that may be both pre- and postsynaptic, as will be discussed. Furthermore SNL appears to selectively impart plasticity on only N-type VDCCs, enhancing their role in sensory transmission.

4.4.1 Role of Spinal N-type VDCCs (CAv2.2, α1B)

I have demonstrated an increased inhibitory effect of ω-conotoxin GVIA at the low doses in SNL animals, compared to sham and naïve, on evoked dorsal horn neuronal responses. A possible mechanistic explanation for this is that if there are increases in the probability and/or frequency of opening of pre-existing VDCCs, the likelihood of a given concentration of ω-conotoxin GVIA blocking a functionally important N-type channel would be increased. Indeed, it is likely that Ca\(^{2+}\) influx via VDCCs on central terminals of peripheral nerves and neurones, and consequently neurotransmitter release and membrane depolarization (Smith & Augustine, 1988), are increased due to enhanced neuronal activity observed peripherally after neuropathy (Wall & Devor, 1983; Sheen & Chung, 1993). Furthermore, despite reduced afferent input via L5 and L6 spinal nerves, the magnitude of neuronal responses recorded was not diminished after SNL. Conversely, increased frequency and occurrence of spontaneous activity was observed alongside enlarged receptive fields of these neurones (as discussed in chapter 3). This suggests that there are compensatory increases in peripheral and/or spinal neuronal activity after neuropathy leading to a greater functional role of VDCCs in the observed neuronal responses.

N-type VDCCs are subject to modulation by G-proteins and protein kinases and this serves to fine tune neurotransmitter release. VDCCs can be tonically inhibited by several G-protein-activating neurotransmitter systems. Binding of the activated G-protein βγ subunit to the domain I-II linker region of N-type VDCCs, and to a lesser extent P/Q- and possibly R-types, results in a depression of Ca\(^{2+}\) current (see Zamponi & Snutch, 1998, for review). This tonic inhibition can be
overridden specifically in N-type VDCCs by activation of protein kinase C (PKC) (Swartz, 1993; Swartz et al., 1993; Zamponi et al., 1997; Barrett & Rittenhouse, 2000) via so-called PKC/G-protein cross-talk that is mediated by PKC phosphorylation of a single threonine residue (Thr-422) in the α1β domain I-II linker (Hamid et al., 1999). This has been shown to dramatically reduce somatostatin (and to a lesser extent opioid) receptor-mediated G-protein inhibition of N-type VDCC activity (Hamid et al., 1999; Cooper et al., 2000). Additionally, PKC activation can also enhance N-type Ca^2+ current in the absence of tonic inhibition (see Yang & Tsien, 1993).

Of interest here, is the fact that PKC has been implicated in persistent pain states. For example, inhibitors of spinal PKC exert anti-allodynic effects in neuropathic models of SNL (Hua et al., 1999) and diabetic neuropathy (Ahlgren & Levine, 1994) and prevent the development of thermal hyperalgesia induced by PSL (Ohsawa et al., 2000). PKC inhibitors simultaneously decrease enhanced levels of membrane bound PKC and neuropathic behaviours induced by nerve injury (Mao et al., 1992). Furthermore, mice lacking the PKCy isoform gene show normal acute pain responses in the absence of tissue or nerve injury, but exhibit a significant decrease in behavioural alldynia that occurs days after injury (Malmberg et al., 1997). Interestingly, PKCy is restricted to a population of local circuit interneurones in lamina II of the dorsal horn that are not inhibitory (Martin et al., 1999a) and its presence here is critical for sustained hyperexcitability of lamina V WDR neurones after mustard oil induced injury (Martin et al., 2001). PKC has also been directly linked to neurotransmitter release in a study that demonstrated PKC activation increased the capsaicin-evoked release of substance P and CGRP from spinal cord slices (Frayer et al., 1999). This indicates that PKC's role in pain behaviours likely results from its ability to increase nociceptive sensory transmission at the level of the spinal cord. Given: (1) the current-enhancing effect of PKC-mediated phosphorylation on N-type VDCCs; (2) the role of both N-type VDCCs and PKC in neuropathy; (3) the localization of both the N-type α1β subunit and PKCy to the superficial dorsal horn, (4) that the tonic inhibition of N-type VDCCs may be relieved due to nerve injury induced alterations in neurotransmitter systems responsible; it seems possible that the increased effectiveness of ω-conotoxin GVIA after nerve injury on the evoked neuronal responses I demonstrate here could be due
to an increased activity of existing N-type VDCCs mediated by PKC phosphorylation of the channel.

An alternative, or additional possibility is that rather than an increase in activity of existing VDCCs, via whatever mechanism, there may be an increase in VDCC expression. In support of this idea, the expression of the N-type VDCC $\alpha_{1B}$ subunit is increased in small DRG cells and in lamina II of the spinal cord after neuropathy (Cizkova et al., 1999). Furthermore, the mRNA and protein for the auxiliary $\alpha_2\delta$ subunit of VDCCs is also upregulated in the ipsilateral dorsal root ganglia and spinal cord of SNL rats (Luo et al., 2001). In addition to its ability to modulate VDCC kinetics (Hobom et al., 2000), $\alpha_2\delta$ can modulate the binding affinity of $\omega$-conotoxins to the N-type VDCC (Brust et al., 1993). Although it is generally believed that the $\omega$-toxins primarily affect the function of the $\alpha_1$ subunit (Olivera et al., 1994), evidence indicates that they can also bind the $\alpha_2$ component of the $\alpha_2\delta$ subunit (Barhanin et al., 1988). It remains to be determined whether upregulation of $\alpha_2\delta$ is responsible for the selective enhancement of $\omega$-conotoxin GVIA's inhibitory actions I report here.

As previously discussed in chapter 3, peripheral nerve damage leads to the generation of ectopic activity at the injury site (Wall & Devor, 1983) or within the DRG (Wall & Devor, 1983; Kajander et al., 1992). This provides an ongoing, continual barrage of neuronal activity via primary afferents into the spinal cord that could lead to central sensitization and hyperexcitability (Gracely et al., 1992; Sheen & Chung, 1993; Yoon et al., 1996). Evidence indicates that enhanced nociceptive transmission in neuropathic pain states is mediated, at least in part, by the action of excitatory amino acids, particularly at NMDA receptor sites (Dubner & Bennett, 1983; Dickenson & Sullivan, 1987; Coderre et al., 1993), and NMDA antagonists have been shown to produce antinociception in patients (Eide et al., 1994; Price et al., 1994). Thus, any increased neuronal depolarization, such as that produced by NMDA receptor events, would increase the likelihood of VDCC activity in spinal neurones since they are voltage-operated ion channels. Indeed, events such as these regulate the expression of new channels and receptor subunits.
It is evident here that blockade of N-type VDCCs results in a greater inhibition of electrically- and naturally-evoked dorsal horn neuronal responses after SNL, indicative of a role for N-type VDCCs in mechanisms of central sensitization of nociceptive dorsal horn neurones after neuropathy. Increased primary afferent activity after peripheral nerve damage could result in enhanced exocytosis of excitatory transmitters, which would in turn increase activation of receptor systems such as those for glutamate (NMDA), substance P and CGRP, which produce excitation of spinal cord neurones. Nerve terminals throughout the dorsal horn have been demonstrated immunoreactive for N-type VDCCs, often correlating with the presence of substance P (Westenbroek et al., 1998). Since N-type VDCCs are abundant on nerve terminals and crucial for neurotransmitter release (Smith & Augustine, 1988; Miljanich & Ramachandran, 1995) the simplest explanation for the effects of ω-conotoxin GVIA is that it blocks presynaptic N-type VDCCs and therefore Ca^{2+} influx and evoked neurotransmitter release (see Figure 18). This is supported by the observation that the non-potentiated 'input' response recorded in this study, that likely relates to the level of synaptic transmission between the central terminals of primary afferents and the neurones of the spinal cord dorsal horn, and non-NMDA receptor mediated postsynaptic events (discussed in chapter 3), is significantly inhibited by the spinal application of the toxin. Small DRG cells express N-type Ca^{2+} currents (Scroggs & Fox, 1992a; Scroggs & Fox, 1992b) and the release of CGRP (Santicioli et al., 1992); sensory neuropeptides (Maggi et al., 1990) and substance P in culture (Holz et al., 1988) are all ω-conopeptide sensitive. As for the control of glutamate release and subsequent NMDA receptor/channel activation, implicated in central hyperexcitability, the role of N-type VDCCs is debatable. Given the evidence for a role of N-type VDCCs in peptide release it would be expected that a similar situation would exist for glutamate, although some studies have reported that N-type VDCCs have a minimal role in mediating glutamate release (Pocock & Nicholls, 1992; Turner et al., 1992). On the other hand, glutamatergic synaptic transmission between DRG cells and spinal cord neurones has been demonstrated to be inhibited by blockade of pre-synaptic N-type VDCCs (Gruner & Silva, 1994), and ω-conopeptides can cause significant reduction in depolarization-evoked glutamate release (Terrian et al., 1990; Dickie & Davies, 1992).
Figure 18. Hypothetical sensory pathway through the dorsal horn of the spinal cord. Possible locations of VDCCs, μ opioid receptors (R) and effects of peripheral nerve injury are indicated. Neurotransmitter release is mediated by $Ca^{2+}$ influx via presynaptic N- and P/Q-type VDCCs, which can be inhibited by activation of presynaptic opioid receptors. Nerve damage causes ongoing afferent input and thus increased neurotransmitter release. In particular the role of N-type VDCCs is enhanced and implicated in altered sensory perception.
It is also likely that ω-conotoxin GVIA inhibits Ca\(^{2+}\) influx through N-type VDCCs at postsynaptic sites (Takahashi & Momiyama, 1993) and presynaptic locations on spinal cord interneurones such that enhanced N-type VDCC-mediated exocytosis of transmitters and modulators is inhibited, thereby reducing excitation of spinal cord neurones. Nociceptive signals arriving at the spinal cord from damaged peripheral nerves may be subject to amplification via relays of interneurones that project onto an output neurone (such as the WDR neurones recorded from here) resulting in the phenomena of wind-up and central sensitization. In support of a possible interneuronal site of action, is the fact that NMDA receptors are not considered to be directly postsynaptic to primary afferents (Davies & Watkins, 1983). Also, the most substantial inhibitions observed in this study, produced by the low doses of ω-conotoxin GVIA after SNL, were on the ‘excess spikes’ and ‘post-discharge’ neuronal responses, indicators of neuronal hyperexcitability and mediated by postsynaptic events (discussed in chapter 3). However, in the latter case, blockade of presynaptic transmitter release by the VDCC blockers could indirectly also prevent postsynaptic hyperexcitability due to decreased activation of postsynaptic NMDA receptors (see Figure 18).

In both control and SNL animals the A\(\beta\)-fibre mediated response was inhibited the least by ω-conotoxin GVIA compared to all the evoked responses. This is in agreement with the facts that substance P containing terminals within the superficial dorsal horn do not normally arise from A\(\beta\)-fibre primary afferents, yet they often do co-localize with N-type VDCC containing terminals (Westenbroek et al., 1998). Furthermore, nerve injury induces upregulation of the N-type VDCC \(\alpha_{\text{1B}}\) subunit in only small DRG cells and in lamina II of the spinal cord after neuropathy (Cizkova et al., 1999) that are not associated with large diameter A\(\beta\)-fibre cell bodies or normal termination patterns. An additional explanation is that the electrical stimulus employed in this study is applied at three times the threshold required to mediate a C-fibre evoked response. C-fibres are high threshold activated primary afferents that convey nociceptive information, in contrast to low-threshold A\(\beta\)-fibres. Thus the experimental methodology used here is suprathreshold for A\(\beta\)-fibres, rendering inhibition of their evoked neuronal response more difficult.
Other *in vivo* electrophysiology studies have only investigated the role of VDCCs in models of inflammation. Inflammation can result from non-neuronal tissue damage, and can induce a set of excitatory changes in the periphery and CNS, which can establish a more persistent, but often reversible hypersensitivity in the inflamed and surrounding tissue. In some cases, for example arthritis, inflammation becomes chronic and leads to a pathological pain state. The dysfunctional mechanisms at play are often different, commonly showing distinct and often contrary pharmacological sensitivities to those displayed in neuropathic pain. However, inflammation is often associated with hyperalgesia and expansion of receptive fields, indicative of central hyperexcitability (see chapter 3), similar to that seen in neuropathy. There are several animal models of inflammatory pain. Injection of formalin into the hindpaw results in a biphasic response characterized by acute (phase 1) and persistent (phase 2) nociceptive behaviours (see Porro & Cavazzuti, 1993). This is also observed neuronally, and NMDA receptor activity has been shown to be critical in the 2nd phase (Haley *et al.*, 1990). Of interest here, ω-conotoxin GVIA has been demonstrated to reduce the responses of dorsal horn neurones in both phases of the formalin test (Diaz & Dickenson, 1997). ω-conotoxin GVIA has also been shown to inhibit the evoked responses of dorsal horn neurones receiving both noxious and innocuous mechanical input from the knee joint in the presence and absence of inflammation induced by carrageenan or kaolin (Neugebauer *et al.*, 1996) and mustard oil (Nebe *et al.*, 1998). This evidence is in accordance within the present investigation in that blockade of N-type VDCCs has the ability to reduce evoked dorsal horn neuronal responses to both innocuous and noxious stimuli, indicating that the toxin is not selective with regard to stimulus under such circumstances. However in contrast to SNL, establishment of inflammation does not appear to alter the sensory processing profile of the N-type VDCC in comparison to control responses.

**4.4.2 Role of Spinal P-type VDCCs (Ca\(_{\text{v}2.1}, \alpha_{1A}\))**

In contrast to the actions of ω-conotoxin GVIA, the P/Q-type VDCC blocker ω-agatoxin IVA had limited inhibitory effects on the evoked neuronal responses and no difference was seen between experimental groups. Interestingly, a significant inhibition of 'excess spikes' and 'post-discharge' was seen with higher
doses in sham and SNL rats, although the maximum effect was lower than after ω-conotoxin GVIA. In naïve and sham-operated animals, the electrically-evoked responses tended to be facilitated at low doses and similarly in an in vivo electrophysiological study ω-agatoxin IVA increased neuronal responses in the absence of inflammation but mediated inhibition in its presence (Nebe et al., 1997). In vitro studies have demonstrated that ω-agatoxin IVA blocks excitatory (Luebke et al., 1993; Castillo et al., 1994; Yamamoto et al., 1994) as well as inhibitory (Takahashi & Momiyama, 1993) synaptic transmission, which may explain the facilitatory and inhibitory effects observed here. P/Q-type VDCCs may be involved in the release of glutamate, aspartate, dopamine, serotonin, noradrenaline, GABA and glycine (Turner et al., 1992; Takahashi & Momiyama, 1993; Turner et al., 1993; Kimura et al., 1995; Miljanich & Ramachandran, 1995).

I have demonstrated a limited role for P/Q-type VDCCs in the maintenance of neuropathy. The P/Q-type channel appears to be linked selectively to NMDA receptor-mediated events as here post-SNL administered ω-agatoxin IVA had most marked effects on NMDA receptor-mediated 'excess spikes' and 'postdischarge' measurements. This is in accordance with results from inflammatory studies. Spinally applied ω-agatoxin IVA has been shown to inhibit dorsal horn WDR neuronal responses only in the 2nd phase, not the 1st phase (Diaz & Dickenson, 1997). In other inflammation models, P/Q-type channels may be most important in the initiation of the facilitated pain state, since intrathecal administration of ω-agatoxin IVA to rats prior to capsaicin injection into the hindpaw (Sluka, 1997) and carrageenan injection into the knee joint (Sluka, 1998) has been behaviourally shown to prevent the development of hyperalgesia.

Evidence to support a postsynaptic role for P/Q-type VDCCs is based on the fact that postsynaptic currents in numerous CNS neurones are markedly suppressed by ω-agatoxin IVA and reduced to a lesser extent by ω-conotoxin GVIA (Takahashi & Momiyama, 1993). Presynaptic P/Q-type channels are also present, but interestingly within the superficial dorsal horn the distribution of P/Q-type channels complements rather than co-localizes with N-type VDCC containing neurones (Westenbroek et al., 1998). Immunolocalization has demonstrated the presence of
P/Q-type VDCCs primarily on the nerve terminals of dorsal horn neurones in laminae II-VI, but unlike N-type channels, rarely in substance P containing neurones (Westenbroek et al., 1998). Intriguingly, this may indicate that N-type channels regulate the release of neurotransmitters, including glutamate, from peptide-containing C-fibres, whereas P/Q-type channels could possibly be localized to non-peptide, IB4-positive neurones. Thus based on their physical location P/Q-type VDCCs are not implicated in the nociceptive transmission that occurs at the first synapse between primary afferent neurones and neurones of the superficial dorsal horn. This is supported by the observation here that the non-potentiated ‘input’ measurement, reflective of the level of activity encountered at the primary afferent terminal/superficial dorsal horn synapse was not as inhibited by ω-agatoxin IVA in comparison to the potentiated ‘post-discharge’ and ‘excess spikes’ measurements. Furthermore, both N- and P/Q-type VDCCs are found in the deeper laminae of the dorsal horn, suggesting localization on terminals of large fibres and spinal neurones (Westenbroek et al., 1998).

4.4.3 Supporting Evidence and Implications

There are numerous behavioural studies that support a role for Ca^{2+} influx into neurones in the spinal processing of nociceptive information. Non-specific VDCC blockers, lanthanum (LaCl₃) or neodymium (NdCl₃), administered intrathecally have been shown to be antinociceptive in acute tail flick and hot plate tests (Reddy & Yaksh, 1980), and have produced dose-dependent inhibition of both the acute phase 1 and tonic phase 2 of the formalin test (Malmberg & Yaksh, 1994; Malmberg & Yaksh, 1995). Further to this, specific block of N-type VDCCs has been shown to be antinociceptive against acute, high-threshold, thermal stimulus, using ω-conotoxin GVIA in the rat tail-flick test (Wei et al., 1996) and SNX-111 (also known as ziconotide), a synthetic ω-conopeptide that selectively and reversibly blocks N-type VDCCs, in the hot plate test (Malmberg & Yaksh, 1994; Malmberg & Yaksh, 1995; Wang et al., 2000b). Conversely, ω-agatoxin IVA was without effect (Malmberg & Yaksh, 1994).
In the formalin model of inflammation, intrathecal administration of SNX-111 has been shown to mediate potent inhibition of flinching behaviour in both phases in the majority of studies and appears to be dependent on chronic infusion (Malmberg & Yaksh, 1994; Malmberg & Yaksh, 1995; Bowersox et al., 1996). Acute intrathecal administration of SNX-111 appears to only inhibit the 2nd tonic phase (Bowersox et al., 1996; Wang et al., 2000b). In contrast P/Q-type VDCC block produced only modest inhibition of the acute phase, but potent inhibition of the prolonged phase (Malmberg & Yaksh, 1994; Malmberg & Yaksh, 1995). Likewise, in other models of inflammation, intrathecally delivered ω-conotoxin GVIA and ω-agatoxin IVA given prior to injection of capsaicin into the hindpaw plantar (Sluka, 1997) and carrageenan into the knee joint (Sluka, 1998) prevented the development of secondary mechanical hyperalgesia and allodynia and heat hyperalgesia, respectively. In all these models of inflammation blockade of P/Q-type VDCCs is only effective when the agent is administered before inflammation induction, whereas it appears that N-type block is effective both before and after its establishment. Intrathecal administration of SNX-111 has also been demonstrated to alleviate established heat hyperalgesia and mechanical allodynia in a rat model of post-operative pain, selective to the injured paw (Wang et al., 2000b).

This electrophysiological study, investigating the role of N- and P/Q-type VDCCs in sensory transmission at the level of the spinal cord after nerve injury, extends results from behavioural studies confirming that spinal N-type VDCCs are the predominant isoform involved in pre- and postsynaptic processing of nociceptive information. Tactile allodynia induced by the SNL model has been shown to be alleviated by intrathecal N-type blockers, SNX-111, -239 and -159, without effect when administered intravenously or regionally to the nerve. In contrast, ω-agatoxin IVA and SNX-230 (synthetic P/Q-type selective conopeptide homologue) were inactive (Chaplan et al., 1994; Bowersox et al., 1996). In the CCI model of nerve injury, SNX-111 and -124 reduced heat hyperalgesia and mechano-allodynia when delivered directly to the site of injury and these were without effect in the uninjured nerve (Xiao & Bennett, 1995). Mechanical hyperalgesia in rats with PSL was reduced by subcutaneous injection of SNX-111 into the receptive field whereas SNX-230 had no effect. Neither compound had any effect in control animals (White & Cousins, 1998). I found a broad spectrum of activity of the N-type channel
blocker, ω-conotoxin GVIA, on both electrically- and naturally-evoked neuronal responses encompassing the single modalities used in these behavioural studies. It has been suggested that in the brain N-type channels are located predominantly on presynaptic terminals with a smaller proportion found postsynaptically, whereas P/Q-type channels appear to have functional roles both pre- and postsynaptically (see Lynch III, 1997). If this holds for sensory pathways in the dorsal horn, then the present results would suggest that the shift in the dose-response curve for the N-type VDCCs but not the P/Q-type is indicative of considerable plasticity in the N-type channels located presynaptically with less pronounced changes at postsynaptic sites. It may then be that the peripheral neuropathy selectively impacts upon the terminals of the injured and remaining non-injured peripheral neurones. The increased activity of spinal N-type VDCCs would be expected to lead to increased neurotransmitter release promoting spinal mechanisms of hyperexcitability that contribute to the ensuing neuropathic syndromes (see Figure 18).

Recent studies have ventured into the role of N-type VDCCs in nociception in mice. The distribution of α1B subunits in murine DRG has been observed to be similar to that observed in rat (Murakami et al., 2001). Intrathecal administration of ω-conotoxin SVIB, a novel analogue of ω-conotoxin GVIA which blocks N-type VDCCs via an alternative mechanism, has been shown to be antinociceptive in the 2nd phase of the formalin test, whereas ω-conotoxin GVIA was antinociceptive in both phases (Murakami et al., 2001). Exploitation of mice permits the use of knock-out animals and recently the nociceptive responses of mice lacking the N-type channel α1B subunit have been characterized. CaV2.2/- mutant mice, not showing any motor deficits, have been shown to exhibit normal responses to acute nociceptive stimuli in one study (Saegusa et al., 2001), yet reduced responses to mechanical and thermal stimuli in another (Kim et al., 2001). Both studies demonstrated reduced nociceptive responses in the 2nd phase of the formalin test, but not in the acute 1st phase (Kim et al., 2001; Saegusa et al., 2001). Of most interest here, is the greatly reduced development of neuropathic pain symptoms, including thermal hyperalgesia and mechanical allodynia induced by SNL, in comparison to that observed after SNL in normal mice (Kim et al., 2001; Saegusa et al., 2001). This further substantiates the predominant role of N-type VDCCs in pathophysiological pain states.
Although not investigated in this study, due to the lack of any known specific blocking pharmacological tools, the third member of the Ca\(_{\text{v}}\)2 family, Ca\(_{\text{v}}\)2.3 or R-type, \(\alpha_{1E}\), has been investigated by use of mutant mice. Mice lacking the \(\alpha_{1E}\) subunit were shown to exhibit normal pain behaviours to acute mechanical, thermal and chemical stimuli, yet in comparison to mice expressing the \(\alpha_{1E}\) subunit, mutant mice displayed reduced response to somatic inflammatory pain (Saegusa et al., 2000). Neuropathic pain behaviours were not investigated, however this provides further evidence of a role of the neuronal Ca\(_{\text{v}}\)2 VDCC family in facilitated pain states.

Since in this study the rats were under halothane anaesthesia any disturbance to the general behaviour caused by the toxins, in particular motor effects, would go undetected. The doses utilized here are comparable to those used in behavioural studies where no obvious side-effects were noted. Limiting effects of the VDCC blockers observed in behavioural studies include intermittent spontaneous shaking behaviour and serpentine-like movement of the tail (Malmberg & Yaksh, 1994; Malmberg & Yaksh, 1995). In a phase I/II open-label clinical study SNX-111 delivered intrathecally to 31 patients with chronic pain conditions with diverse backgrounds including cancer, AIDS, spinal cord injury, thalamic pain and postherpetic neuralgia reported partial to complete pain relief in instances were opioids were found to be ineffective (Brose et al., 1996). The most common side-effects were nystagmus, mental confusion, difficulty in word finding, nausea, dizziness, headache and disturbance of gait and balance. Although intrathecal administration of SNX-111 may selectively target spinal cord N-type VDCCs and limit hypotensive effects that would be caused by a reduced sympathetic efferent action, from this clinical study it appears that there is still some spread of the drug from the spinal cord to the brain. However, it should be noted these adverse effects reversed upon discontinuation or decrease in dose of SNX-111 since this analogue of the natural toxin has reversible effects whereas \(\omega\)-conotoxin GVIA effects tend to be irreversible in nature. It appears that pharmacological targeting of these channels with toxin blockers as a potential therapy for the treatment of chronic pain is hindered by the adverse systemic side-effects (Penn & Paice, 2000) and the inconvenient spinal route of administration.

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The present results, together with a number of previous behavioural reports, emphasize the key roles of VDCCs in sensory events within the spinal cord. Here, I show that N-type, in comparison to P/Q-type, VDCCs are the predominant functional type in terms of a wide range of sensory modalities and exhibit plasticity following nerve injury. The increased ability of ω-conotoxin GVIA to inhibit evoked neuronal responses to a wide range of stimuli suggests that N-type VDCCs are a key component of increased central excitability that follows nerve injury.
CHAPTER 5

VOLTAGE-DEPENDENT CALCIUM CHANNELS:

THE CA_v3 FAMILY
5.1 Introduction

Neuropathic pain, arising from injury- or disease-evoked damage to the peripheral or central nervous system, often responds poorly to traditional analgesics. Patients can experience sensory deficits, persistent and stimulus-evoked pain (allodynia and hyperalgesia). Peripheral nerve damage provides abnormal input into the central nervous system that then leads to dorsal horn hyperexcitability. Under the influence of both excitatory and inhibitory neurotransmitter systems, the dorsal horn is a site of peripheral input modulation before projection to higher brain centres, thus it controls the stimulus-response relationship. Reduction of this excitability is a possible key to neuropathic pain management. In this context, as discussed in the introduction, calcium influx into neurones is one event that increases excitability.

High voltage-activated (HVA) Ca\(^{2+}\) channels, of the Ca\(_{\text{i}}\)l and 2 families (L-, N-, P/Q- and R-types), consisting of a pore-forming \(\alpha_1\) subunit and modulatory accessory subunits, \(\beta\), \(\alpha_2-\delta\) and \(\gamma\) (Walker & De Waard, 1998), are widely expressed throughout the brain and spinal cord (Kerr et al., 1988; Mintz et al., 1992; Gohil et al., 1994; Westenbroek et al., 1998). They are activated by relatively strong membrane depolarization and permit Ca\(^{2+}\) influx in response to action potentials. Consequential secondary actions include neurotransmitter release; thus these channels establish a major link between neuronal excitability and synaptic transmission. For these reasons HVA Ca\(^{2+}\) channels have been the focus of both acute and persistent pain transmission studies. Animal models have demonstrated the antinociceptive abilities of antagonists specific for L-, N- and P/Q-type Ca\(^{2+}\) channels (see also chapters 4 and 6) highlighting the differential role each subtype plays in nociception, often dependent on the nature of the pain state (see Vanegas & Schaible, 2000, for review).

In addition to HVA Ca\(^{2+}\) channels, kinetically distinct low voltage-activated (LVA) Ca\(^{2+}\) channels of the Ca\(_{\text{i}}\)3 VDCC family, or T-type channels, also exist both in neuronal and non-neuronal cells. T-type VDCCs are present on cardiac muscle, smooth muscle, secretory cells, fibroblasts and neurones (see Tsien et al., 1988; Bean, 1989a, for reviews), including mammalian sensory neurones (Bossu et al., 1985; Fedulova et al., 1985; Bossu & Feltz, 1986; Carbone & Lux, 1987; Kostyuk &
Shirokov, 1989). They are designated LVA since they activate at voltages near the resting membrane potential, and T-type for their transient kinetics whereby they inactivate rapidly, deactivate slowly and have a small single channel conductance (Huguenard, 1996). In contrast to the HVA VDCCs, these unique gating properties help determine firing frequency of cardiac pacemaking cells (Hagiwara et al., 1988). In neurones their unusual kinetics prohibit T-type channels alone in supporting neurotransmission, yet permit their involvement in low amplitude oscillations, neuronal bursting, synaptic signal boosting, Ca$^{2+}$ entry promotion and lowering threshold for high-threshold spike generation (Jahnsen & Llinas, 1984; White et al., 1989). Ca$^{2+}$ current through these channels appears to play an important physiological role in near-threshold phenomena and regulation of neuronal excitability.

Until recently the LVA current was considered a single entity. However the genes encoding subunits responsible for Ca$_{3.1}$ (α$_{1G}$), Ca$_{3.2}$ (α$_{1H}$) and Ca$_{3.3}$ (α$_{1I}$) Ca$^{2+}$ currents have now been cloned, showing 30% homology to HVA channel-forming α subunits (Cribbs et al., 1998; Perez-Reyes et al., 1998; Lee et al., 1999) and displaying hallmark native T-type Ca$^{2+}$ channel properties when expressed heterologously. In situ hybridization studies on the rat brain have shown that T-type Ca$^{2+}$ channels have unique distributions, including the dorsal horn of the spinal cord and sensory ganglia (Talley et al., 1999). This is complemented by the presence of T-type currents in primary sensory neurones (Carbone & Lux, 1984; Kostyuk et al., 1992; Scroggs & Fox, 1992a; Todorovic & Lingle, 1998) and some superficial rat dorsal horn neurones (Ryu & Randic, 1990), a site important for the processing and integration of sensory information including pain. T-type Ca$^{2+}$ channels were first described in peripheral sensory neurones of the DRG (Carbone & Lux, 1984), yet their function in sensory processing is still unclear. Although their unique biophysical properties make LVA currents relatively easy to study in vitro, the involvement of T-type channels in pain-related central sensitization has been hindered by a scarcity of specific pharmacological agents. Unlike the HVA Ca$^{2+}$ channels, no natural toxins or venom components have been identified that target T-type VDCCs selectively.
Neuropathic pain and epilepsy both share neuronal hyperexcitability as a common underlying mechanism. There are established antiepileptic drugs that target the generation of neuronal hyperexcitability in the brain and some of these have been proven effective in the treatment of various forms of neuropathic pain (Swerdlow & Cundill, 1981; McQuay et al., 1995). The succinimide derivative ethosuximide (2-ethyl-2-methylsuccinimide) is an anticonvulsant (Macdonald & McLean, 1986) effective in the treatment of absence epilepsy (Coulter et al., 1989c); a condition characterized by spike-wave rhythm likely generated by T-type Ca$^{2+}$ current. Ethosuximide has been demonstrated to be a relatively specific T-type VDCC antagonist in thalamic (Coulter et al., 1989b) and DRG neurones (Kostyuk et al., 1992). The present study uses the SNL model, confirmed by behavioural testing, to induce a neuropathic state, and subsequently electrophysiological studies of dorsal horn spinal neurones were made to investigate the effects of spinally delivered ethosuximide on responses to a wide range of electrically- and naturally-evoked neuronal activity.

5.2 Methods

A total of 24 male adult Sprague-Dawley rats were used in this study, of which 7 were naïve, 6 were sham-operated and 11 were SNL. The animals were prepared in the normal way and the effect of ethosuximide (5, 55, 555 and 1055 µg), applied directly onto the exposed surface of the spinal cord, was investigated on the evoked dorsal horn neuronal responses. The effects of each dose were followed until the responses plateaued (a minimum of 50 minutes), when the next dose was applied cumulatively.

5.3 Results

5.3.1 Behavioural Assessment

Rats subjected to SNL exhibited 'guarding' foot posture ipsilateral to nerve injury and successful replication of the nerve injury model was confirmed by behavioural testing which demonstrated the development of mechanical and cooling allodynia of the injured hindpaw (see chapter 3 for combined behavioural data
obtained from all studies used in this thesis). This was not displayed by either the contralateral hindpaw, or in the sham-operated rats. Upon establishment of a neuropathic state, animals were then used for in vivo electrophysiology and the pharmacological study at PO days 14 - 17.

5.3.2 Spinal Cord Electrophysiology: Neurone Characterization

The number of dorsal horn neurones recorded from in each group were 11 in SNL rats, 6 in sham-operated rats and 7 in naïve rats. All neurones had a receptive field over the hindpaw ipsilateral to surgery (when performed). No significant differences were found between experimental groups in the mean cell depth of recorded neurones and the mean neuronal responses evoked by electrical and natural stimulation. However, 45% of neurones characterised in SNL rats exhibited spontaneous activity at a rate greater than 0.1 Hz in comparison to only 17% of characterised neurones in sham-operated rats and 25% of naïve.

5.3.3 Spinal Cord Electrophysiology: Effects of ethosuximide

The effect of ethosuximide (5 – 1055 µg), applied directly onto the spinal cord, on the electrically- and naturally-evoked dorsal horn neuronal responses was tested in SNL, sham-operated and naïve animals. Since the sham-operation is the appropriate control for SNL, and for clarity, the results obtained from the naïve, non-operated, group shall not be displayed on the graphs. However, no difference in the effects of ethosuximide was observed between sham and naïve groups. Ethosuximide produced a dose-related inhibition of the electrically- and naturally-evoked responses in neurones in all experimental groups (Figures 19 - 21) and there was no difference in the extent of its effects between groups. Effects were seen around 40 minutes, with maximal inhibitions established at around 60 minutes. For sham and SNL groups, all doses of ethosuximide (5 – 1055 µg) elicited statistically significant inhibitions of the electrically-evoked responses, compared to pre-drug control values (Figure 19; P < 0.05; n = 6 - 11). For naïve animals statistically significant inhibitions were elicited by ethosuximide: at all doses (5 – 1055 µg) for the Aβ-fibre, Aδ-fibre, postdischarge and excess spikes measurements; at 55 – 1055 µg ethosuximide for the C-fibre response; and at 555 and 1055 µg for the input response (P ≤ 0.05; n = 7). In all 3
Figure 19. Comparison of the effect of spinally applied ethosuximide on the electrically-evoked dorsal horn neuronal responses recorded from sham-operated (n = 6) and SNL (n = 6 - 11) rats at PO days 14 - 17. Data are expressed as the mean maximal % inhibition of the pre-drug control values ± S.E.M.
Figure 20. Examples of the inhibitory effect of spinally applied ethosuximide on individual dorsal horn neurones exhibiting wind-up recorded from (a) a sham-operated and (b) a SNL rat. Data are expressed as the number of evoked action potentials, in the 90 - 800ms latency band, to each electrical stimulus.
Figure 21. Comparison of the effect of spinally applied ethosuximide on the naturally-evoked dorsal horn neuronal responses recorded from sham (n = 6) and SNL (n = 6 - 11) rats. Data are expressed as mean maximal % inhibition of pre-drug control values ± S.E.M.
groups the $\alpha\beta$-fibre response was least affected with mean maximal inhibitions at top dose in the range $17 \pm 6\%$ to $26 \pm 8\%$ (Figure 19f). In all 3 groups the C-fibre and $\alpha\delta$-fibre responses reached similar mean maximal inhibitions at top dose in the range $33 \pm 13\%$ to $47 \pm 17\%$ (Figures 19d & 19e, respectively). Greater inhibitory effects over the entire ethosuximide concentration range were observed on the input, postdischarge and excess spikes measurements (Figures 19a - c, respectively). At 1055 $\mu$g, ethosuximide maximally inhibited the input and excess spikes to within the range $48 \pm 8\%$ to $61 \pm 14\%$. The greatest effect was observed on postdischarge, which was maximally inhibited in SNL, sham and naive rats over the range $58 \pm 10\%$ to $75 \pm 13\%$.

Figures 20a and 20b are examples of the effects of ethosuximide on the wind-up of an individual neurone recorded from a sham and SNL rat, respectively. These particular neurones were selected as those displaying a clear wind-up response i.e. an increasing number of evoked action potentials (300 ms – 800 ms latency band) observed with increasing electrical stimulus number, and the drug effects are consistent with the rest of the neurones tested. It is apparent that ethosuximide reduces the wind-up, in a dose-related manner (evident by a flattening of the curve), with a much less pronounced effect on the initial C-fibre input (response to stimulus number 1) and no difference was observed between experimental animal groups.

Ethosuximide also produced an inhibitory effect on the naturally-evoked neuronal responses (Figure 21). In SNL animals this was significant ($P < 0.05$; $n = 6 - 11$) for all concentrations of ethosuximide employed in this study on the response to non-noxious mechanical stimulation (von Frey 9 g, Figure 21a), noxious mechanical stimulation (von Frey 75 g, Figure 21b) and noxious thermal stimulation (water jet at 45 °C, Figure 21c). In sham rats the von Frey 9 g and heat responses were significantly inhibited at all concentrations of ethosuximide, and the von Frey 75 g response was inhibited by 555 and 1055 $\mu$g ($P < 0.05$; $n = 5 - 6$). In naïve animals responses to both von Frey filaments were significantly inhibited by all concentrations of ethosuximide, and inhibition of the heat response reached significance at 555 and 1055 $\mu$g ($P < 0.05$; $n = 5 - 7$). No differences in the effects of the drug were apparent between experimental groups and the mean maximal
inhibitions established at top dose were in the range 61 ± 11% to 77 ± 10%.

5.4 DISCUSSION

In this study I have addressed the role of the Ca\textsubscript{\textit{LVA}} family of VDCCs (LVA T-type Ca\textsuperscript{2+} channels) in the spinal processing of sensory information after nerve injury. Spinal ethosuximide, a relatively specific T-type channel antagonist, mediated significant inhibition of the electrically- and naturally- (innocuous and noxious) evoked rat dorsal horn neuronal responses, suggesting some role for T-type Ca\textsuperscript{2+} channels in sensory transmission.

Extensive behavioural and electrophysiological studies into nociception have demonstrated an important role for HVA Ca\textsuperscript{2+} channels in the processing of pain (see Vanegas & Schaible, 2000). The contribution of N-, P/Q- or L-type channels appears to alter depending on the nature of the pain (acute or chronic, inflammatory or neuropathic in origin), and this has been established by the use of specific channel antagonists (see also chapters 4 and 6). A predominant nociceptive role for N-type channels has been shown, an action that is enhanced after neuropathy (Chaplan \textit{et al.}, 1994; Xiao & Bennett, 1995; Bowersox \textit{et al.}, 1996; Brose \textit{et al.}, 1997; White & Cousins, 1998). Upon substantial membrane depolarisation N- and P/Q-type VDCCs mediate the release of excitatory neurotransmitters, such as glutamate, substance P and CGRP, critical for wind-up and central sensitization, in the presence of constant afferent input (Dickenson, 1994).

This study highlights the role of Ca\textsuperscript{2+} influx via T-type channels in these nociceptive pathways, on which ethosuximide may be exerting its effects at a number of possible sites. The biophysical characteristics of the Ca\textsubscript{3} VDCCs have led to their main implication being in the regulation of cell excitability. The broad action of ethosuximide on all the dorsal horn neuronal responses observed here, with no marked selectivity for a particular modality or evoked response, is in keeping with actions on postsynaptically located Ca\textsuperscript{2+} channels. A postsynaptic effect would equally effect all responses due to convergence of differential afferent inputs that occurs at this level. Since LVA Ca\textsuperscript{2+} channel activation occurs close to resting potential, Ca\textsuperscript{2+} influx is permitted when cells are at rest (Magee & Johnston, 1995) or
in response to subthreshold synaptic inputs (Markram & Sakmann, 1994; Magee & Johnston, 1995). Thus these channels enhance neuronal excitability and contribute to the generation of subthreshold membrane potential oscillations that lead to bursts of Na⁺-dependent action potentials (Huguenard, 1996). Whilst unable to mediate synaptic transmission alone, T-type channels do serve to boost synaptic inputs and potentiate the generation of high-threshold action potentials. Their block would result in an overall reduction in the underlying level of neuronal excitability. This would render the achievement of levels of membrane depolarization at ion channel-activating thresholds less likely, such that action potential production would be reduced. Postsynaptically, NMDA receptor activation would be decreased, as would the consequential development of central sensitization.

In this study, ethosuximide exerted its greatest inhibitory effects upon the input, postdischarge and excess spikes. Each of these electrical measures can be related to a specific part of the nociceptive pathway. The non-potentiated input response can be related to the baseline level of C-fibre mediated synaptic transmission between the central terminals of primary afferents and the neurones of the spinal cord dorsal horn, in the absence of NMDA receptor mediated wind-up events. Thus blockade of T-type channels at this location is likely causing a reduction in the exocytosis of excitatory neurotransmitters by prohibiting the depolarization required for activation of HVA Ca²⁺ channels. Alternatively, ethosuximide could be exerting its effects directly on neurones located early in polysynaptic spinal pathways. Even more susceptible to the actions of ethosuximide were the postsynaptic NMDA receptor-mediated postdischarge and excess spike measurements, indicative of central sensitization and neuronal hyperexcitability. Since T-type Ca²⁺ channels are heavily linked to the level of neuronal excitability it follows that they would have a greater functional role here (see Figure 18).

The results presented here show that after the establishment of neuropathy no difference was detected in the effects of ethosuximide. This appears somewhat surprising especially since neuronal hyperexcitability, in which LVA Ca²⁺ channels are implicated, is a key underlying factor. Interestingly it has been shown that although unilateral cortical ablation causes a 68% increase in T-current measured
from isolated rat thalamic relay neurones, methyl-phenyl-succimide (another related Ca\textsubscript{v}3 VDCC antagonist) was more effective in reducing T-current in normal rats than in axotomized animals (Chung et al., 1993). The authors suggest an injury-induced alteration in the pharmacological properties the Ca\textsubscript{v}3 VDCCs either by de novo synthesis and/or modification. In the present study, it may be that there is indeed no increase in the functional role of LVA Ca\textsuperscript{2+} channels after nerve injury. Alternatively, the specificity and/or potency of ethosuximide may be such that subtle differences in Ca\textsubscript{v}3 VDCC function were not highlighted.

Peripheral nerve injury results in reduced afferent input via L5 and L6 spinal nerves, yet as observed here, the magnitude of neuronal responses recorded was not diminished in comparison to sham and normal rats. Conversely, increased frequency and occurrence of spontaneous activity was observed. This suggests that perhaps compensatory increases in peripheral and/or spinal neuronal activity come into play after neuropathy (see chapter 3). Ectopic C-fibre activity originating within DRG, the nerve injury site, or residual intact afferents provides an ongoing, continual barrage of neuronal activity via primary afferents into the spinal cord (Wall & Devor, 1983; Kajander et al., 1992). Evidence suggests that this is one possible source for the initiation and maintenance of central sensitization and therefore the positive sensory symptoms observed after nerve damage (Devor & Seltzer, 1999). Interestingly, SNL has been shown to increase the prevalence of subthreshold membrane potential oscillations in DRG neurones which augments ectopic discharge (Liu et al., 2000). It does not seem unreasonable to postulate a role for Ca\textsubscript{v}3 VDCCs in this underlying oscillatory behaviour, which may imply increased activity via these channels in the remaining afferents tantamount to a restoration of excitability to that in the non-injured situation. However, the spinal application of ethosuximide used in this study would not inhibit any T-type mediated oscillatory behaviour at the level of the DRG.

Another recent study also indicates a role for peripheral T-type VDCCs in nociceptive pathways (Todorovic et al., 2001). In isolated rat sensory neurones shown to participate in thermal nociception, reducing agents have been demonstrated to augment T-currents, but not HVA Ca\textsuperscript{2+} currents, K\textsuperscript{+} and Na\textsuperscript{+} currents, whereas
oxidizing agents decreased LVA Ca\(^{2+}\) current. A T-type VDCC antagonist mibefradil (Todorovic & Lingle, 1998), the first member of a relatively new class of VDCC antagonists to be clinically approved for the treatment of hypertension, abolished the enhanced DRG T-currents, leaving baseline currents intact. In the same study, this was mirrored in vivo, where peripheral injection of the endogenous reducing agent L-cysteine produced thermal hyperalgesia that was blocked by peripherally applied mibefradil, but not NMDA receptor blockers. Redox sites in sensory neurones could involve any of the many L-cysteine residues present in putative extracellular regions of T-type VDCCs (Cribbs et al., 1998; Perez-Reyes et al., 1998) and could be targets for agents that modify pain perception.

The existence of a neuronal Ca\(^{2+}\) current elicited just above the resting potential was first established in primary sensory neurones (Carbone & Lux, 1984; Bossu et al., 1985; Fedulova et al., 1985; Nowycky et al., 1985), and LVA currents have since been observed in a wide variety of cell types (Huguenard, 1996). The presence of a relatively large T-type current in some superficially located rat spinal dorsal horn neurones is of much interest because this region is involved in processing and integration of sensory information, including pain (Ryu & Randic, 1990). T-type channels are pharmacologically and physiologically heterogeneous (Akaike, 1991; Huguenard, 1996; Tarasenko et al., 1997), which may reflect differential expression of the three known subtypes Ca\(^{3.1}\), Ca\(^{3.2}\) and Ca\(^{3.3}\) (\(\alpha_{1G}\), \(\alpha_{1H}\) and \(\alpha_{1I}\), respectively). The regional and cellular distribution of gene expression for the different Ca\(^{3}\) VDCC family members in the rat central and peripheral nervous systems has recently been determined using in situ hybridization and these channels appear to have unique and relatively non-overlapping distributions (Talley et al., 1999). All three transcripts were detected in sensory areas and in the dorsal horn of the spinal cord where in particular \(\alpha_{1H}\) was mainly restricted to the outermost laminae I and II. In the DRG high levels of \(\alpha_{1H}\) and moderate levels of \(\alpha_{1I}\) mRNA were found, and \(\alpha_{1H}\) has been shown to display the most similar pharmacological profile to native DRG T-type currents (Todorovic et al., 2000). The presence of T-type VDCCs in DRG determined by in situ hybridization appeared to be restricted to small and medium sized neurones, whereas the extremely large cells were not labelled. This correlates with substantial T-type current observed in medium diameter DRG neurones isolated from adult rats that is absent in larger DRG cells.
(Scroggs & Fox, 1992a). Since DRG cell body diameter is correlated to axon conduction velocity and sensory modality (Yaksh & Hammond, 1982), this evidence is indicative of T-type current specifically localized to smaller Aδ- and C-type sensory neurones that convey thermal and nociceptive information. In contrast larger Aβ-type neurones that subserve tactile and proprioceptive pathways do not appear to possess T-type current. In the present study the extent of inhibition observed with the highest dose of ethosuximide was Aδ-fibre > C-fibre > Aβ-fibre, which fits well with these studies. However, the Aδ- and C-fibre responses were not markedly inhibited over the Aβ-fibre response, as one might expect if smaller diameter sensory neurones exhibited substantial LVA Ca\(^{2+}\) current. Additionally, no difference in the extent of inhibition was observed for the evoked responses to innocuous and noxious mechanical and thermal stimuli, which are conveyed to the spinal cord by the different primary afferent fibre types. This may again be explained by the existence of the three different α\(_1\) subunits that comprise the Ca\(_{\text{v}3}\) VDCCs, each showing a unique neuronal distribution.

Collation of these observations suggests different neuronal populations that have alternative patterns of T-type channel localization: cells in which functional channels are localized in the somatic membrane and others in which functional channels are only expressed dendritically. Electrophysiological studies of dissociated neurones allow robust recordings to be made only from cell soma and proximal processes. Immunohistochemistry (Craig et al., 1999), Ca\(^{2+}\) imaging (Christie et al., 1995) and electrophysiological studies (Kavalali et al., 1997; Mouginot et al., 1997) suggest that in certain neurones, LVA channels have a dendritic location. A predominantly dendritic expression of T-type channels has been described for hippocampal neurones (Karst et al., 1993).

Progress in revealing the differential physiological roles of the Ca\(_{\text{v}3}\) family of VDCCs have been impeded by the lack of ligands that distinguish not only between LVA and HVA VDCCs but also between Ca\(_{\text{v}3.1}\), Ca\(_{\text{v}3.2}\) and Ca\(_{\text{v}3.3}\). The Ca\(_{\text{v}3}\) VDCCs are not inhibited by many types of antagonists, and those that do, lack specificity. For example in DRG neurones mibefradil has been shown to be one of the best ligands for T-type channels with an IC\(_{50}\) of 3 μM (Todorovic & Lingle,
However mibebradil also blocks VDCCs of the Ca,1 (Glasser, 1998) and Ca,2 (Bezprozvanny & Tsien, 1995) families. Variable sensitivity of T-type Ca\textsuperscript{2+} channels to dihydropiridines (DHPs) has also been described. This ranges from quite high LVA Ca\textsuperscript{2+} current DHP sensitivity in cerebellar Purkinje (Kaneda et al., 1990), hippocampal (Takahashi & Akaike, 1991) neurones with K_d's of around 10 \mu M, to virtually DHP insensitive in primary sensory neurones (see Kostyuk, 1999).

T-type Ca\textsuperscript{2+} current is also reported to be sensitive to block by volatile anaesthetics (Todorovic & Lingle, 1998; McDowell et al., 1999). It has been shown that isofluorane is 3.5 times more potent than halothane in blocking rat DRG T-current which parallels the observation that isofluorane is more analgesic (Levine et al., 1986) and more effective at inhibiting somatosensory evoked potentials (Peterson et al., 1986) than halothane. Whether anaesthetic effects are in part mediated via T-type VDCCs remains to be determined, however this may be indicative of a potential role for T-type Ca\textsuperscript{2+} channels in sensory transmission.

As regards ethosuximide, its current clinical use for the treatment of epilepsy is via block of T-current in thalamic neurones (Coulter et al., 1989c; Coulter et al., 1989b). In contrast, the T-current in GH3 cells is relatively resistant to block by ethosuximide (Herrington & Lingle, 1992) and in DRG neurones ethosuximide has been shown to be over an order of magnitude less effective (Coulter et al., 1989c; Coulter et al., 1989b). However, the blockade of T-current mediated by ethosuximide observed in DRG is complete, but only partial in thalamic neurones. A preference of ethosuximide for T-type over L-type Ca\textsuperscript{2+} channels in terms of both efficacy and affinity has also been reported for isolated DRG neurones (Kostyuk et al., 1992), specificity (K_d 7 \mu M and 15 \mu M, respectively) being preserved up to concentrations of 1mM. Furthermore, in thalamic neurones ethosuximide has been shown to inhibit T-type currents by about 40% with an EC\textsubscript{50} of 200 \mu M, with no effects on HVA currents (Coulter et al., 1989b). Thus the data suggests that diversity exists between T-currents of different cell types both in terms of kinetics and pharmacological sensitivity (see also Huguenard, 1996). It is not yet known whether ethosuximide blocks all members of the Ca,3 VDCC family equally or whether it displays subtype selectivity. It must be taken into consideration that \alpha_{1G} is the
predominant subtype found in thalamic relay neurones, the target site of ethosuximide for the treatment of absence epilepsy. Therefore the possibility exists that Ca\textsubscript{v}3.1 (α\textsubscript{1G}) is more sensitive to the effects of ethosuximide in comparison to the Ca\textsubscript{v}3.2 (α\textsubscript{1H}) VDCC which is more abundant than α\textsubscript{1G} in the outer lamina of the spinal cord and dorsal root ganglia (Talley et al., 1999). The synthesis of ligands able to distinguish between the LVA Ca\textsuperscript{2+} channels, in particular the α\textsubscript{1H} subtype which anatomically appears to be implicated in nociceptive pathways, is eagerly awaited. Until then the role of T-type Ca\textsuperscript{2+} channels in nociception cannot be truly established. The results from the present study may have underestimated the importance of LVA VDCCs in sensory transmission as ethosuximide may not have been a sensitive enough pharmacological tool. Nevertheless, I deemed ethosuximide the best pharmacological option available, over other known T-type VDCC antagonists, to study the role of LVA Ca\textsuperscript{2+} channels within the pain pathway, due to its reported selectivity for LVA over HVA VDCCs (Coulter et al., 1989b; Kostyuk et al., 1992) and its ability to completely block T-type current in DRG neurones (Todorovic & Lingle, 1998).

An alternative explanation for the limited effect of ethosuximide on evoked dorsal horn neurone responses and the lack of increased effects after nerve injury could be due to the fact that T-type channels are rapidly inactivating and require hyperpolarized membrane potentials for removal of inactivation (Coulter et al., 1989a; Toselli & Taglietti, 1992). Since during repetitive stimulation, postsynaptic dorsal horn neurones would be rapidly depolarized, an effect likely enhanced after nerve injury due to the ensuing hyperexcitability, T-type VDCCs at this location are maybe largely inactivated and thus the inhibitory effects mediated by ethosuximide would be relatively redundant. On a similar note, in 5 - 10 day old rat DRG sensory neurones, PKC activation selectively inhibited low-threshold T-type Ca\textsuperscript{2+} current, present only on a subpopulation of neurones, with no effect on high threshold current (Schroeder et al., 1990). Thus, activation of PKC could diminish excitability of some sensory cells while leaving others unaffected, providing the potential for selective modulation of particular sensations. This is contrast to the Ca\textsuperscript{2+} current-enhancing effects of PKC activation observed through N-type VDCCs (see chapter 4.4). Given the increased role of PKC activity in mediating neuropathic pain behaviours and the antinociceptive effect exerted through its inhibition, it follows that in the presence of
neuropathy T-type VDCCs on some sensory neurones would actually be inhibited. Again this supports my finding of an unaltered role of T-type VDCCs after nerve injury, as observed through the actions of ethosuximide. However, on the basis of the PKC evidence a decreased inhibitory effect of ethosuximide on evoked neuronal responses might have been expected.

Two other licensed anticonvulsants, carbamazepine and gabapentin, have been investigated using the same experimental protocol as the present study (Chapman et al., 1998a). Carbamazepine, a Na\(^+\) channel blocker, and gabapentin, thought to act via Ca\(^{2+}\) channels (see chapter 7), were found to have similar efficacy and range of effectiveness as ethosuximide observed here. Although both gabapentin and ethosuximide were equally effective in sham and neuropathic animals, carbamazepine was only effective in the latter group which could possibly be a consequence of differential regulation of particular Ca\(^{2+}\) and Na\(^+\) channels following nerve injury. To my knowledge the present study is the first to demonstrate a possible role of T-type Ca\(^{2+}\) channels in the spinal processing of sensory information related to pain. Given the parallels between epilepsy and pain, the likelihood of common causal mechanisms and the ability of antiepileptic drugs to be effective in neuropathic pain states, the results indicate that ethosuximide may merit both behavioural testing in animals and human studies.
CHAPTER 6

VOLTAGE-DEPENDENT CALCIUM CHANNELS:

THE CA_{\gamma}1 FAMILY
6.1 INTRODUCTION

High voltage-activated (HVA) Ca\(^{2+}\) channels, of the Ca\(_{v1}\) (L-type) and Ca\(_{v2}\) families (N-, P/Q- and R-types) are activated by relatively strong membrane depolarization and permit Ca\(^{2+}\) influx in response to action potentials. Consequential secondary actions include neurotransmitter release, thus these channels establish a major link between neuronal excitability and synaptic transmission. On the other hand, the low voltage-activated (LVA) Ca\(^{2+}\) channels of the Ca\(_{v3}\) (T-type) family are activated at voltages close to resting potential. These VDCCs permit Ca\(^{2+}\) influx when cells are at rest, thus regulating cell excitability and most likely the depolarization required to activate HVA VDCCs. Extensive behavioural and electrophysiological studies into nociception have demonstrated an important role in general for VDCCs in the processing of pain see chapters 4 and 5 and (Vanegas & Schaible, 2000) for review. The employment of specific Ca\(^{2+}\) channel blockers and antagonists has revealed that the contribution of L-, N- and P/Q-type channels differs dependent on the nature of pain (acute or chronic, inflammatory or neuropathic in origin). Results from the previous chapters, in conjunction with other studies, highlight a predominant pronociceptive role for N-type channels, which is enhanced after neuropathy (Chaplan et al., 1994; Bowersox et al., 1996; Brose et al., 1997; White & Cousins, 1998).

Pre-clinical studies provide varied conclusions regarding the antinociceptive effects of L-type VDCC blockade. The general consensus is that inhibition of Ca\(^{2+}\) influx through these channels has little or no antinociceptive effect. For completeness of this thesis I thought it necessary to establish the role of the L-type VDCCs in the spinal transmission of sensory information and assess alterations that might occur in their functioning following peripheral nerve injury, given the plasticity I discovered in N-type VDCCs at this level and the roles of the P- and T-type channels in sensory events. Of the four members that comprise the family of L-type or Ca\(_{v1}\) VDCCs (Ellis et al., 1988; Williams et al., 1992; Tomlinson et al., 1993; Bech-Hansen et al., 1998) see also chapter 1.4, it is the Ca\(_{v1.2}\) (\(\alpha_{1C}\)) and Ca\(_{v1.3}\) (\(\alpha_{1D}\)) channels that are located in the CNS. In rat brain, the \(\alpha_{1C}\) subunit (first defined in cardiac muscle) and the \(\alpha_{1D}\) subunit (first defined in endocrine cells) are found throughout the brain (Hell et al., 1993), where they are important for regulation of gene expression and the
integration of synaptic inputs (Bean, 1989b; Bean, 1989a; Murphy et al., 1991; Bading et al., 1993; Bito et al., 1996; Deisseroth et al., 1996). Immunohistochemical studies of the rat spinal cord have revealed staining of these channels scattered throughout the entire dorsal horn, localized predominantly to the cell bodies and proximal dendrites of neurones (Ahlijanian et al., 1990; Hell et al., 1993; Westenbroek et al., 1998).

L-type VDCCs are selectively blocked by 1,4-dihydropyridines (DHPs), phenylalkylamines and benzothiazepines (see Tsien et al., 1988; Spedding & Paoletti, 1992). DHPs, such as nifedipine, are a class of synthetic Ca\(^{2+}\) channel blockers used for treatment of hypertension, due to their depressive action on the cardiovascular system. Specifically they prevent Ca\(^{2+}\) entry through VDCCs on vascular smooth muscle, reducing contraction which then leads to vasodilation and thus they lower arterial pressure. The present study uses both naïve and SNL animals to investigate the effects of spinally delivered nifedipine on a wide range of electrically- and naturally-evoked dorsal horn neuronal activity.

**6.2 METHODS**

A total of 12 male adult Sprague-Dawley rats were used in this study, of which 6 were naïve, and 6 were SNL. The animals were prepared in the normal way and the effect of nifedipine (10, 110 and 260 μg), applied directly onto the exposed surface of the spinal cord, was investigated on the evoked dorsal horn neuronal responses. The effects of each dose were followed until the responses plateaued (a minimum of 60 minutes), when the next dose would be applied cumulatively.

**6.3 RESULTS**

**6.3.1 BEHAVIOURAL ASSESSMENT**

Rats subjected to SNL exhibited 'guarding' foot posture ipsilateral to nerve injury and successful replication of the nerve injury model was confirmed by behavioural testing which demonstrated the development of mechanical and cooling allodynia of the injured hindpaw (see chapter 3 for combined behavioural data
obtained from all studies used in this thesis). This was not displayed by the contralateral hindpaw. Upon establishment of a neuropathic state, animals were then used for *in vivo* electrophysiology and the pharmacological study at PO days 14 - 17.

### 6.3.2 Spinal Cord Electrophysiology: Neurone Characterization

The number of dorsal horn neurones recorded from in each group were 6 in SNL rats and 6 in naïve rats. All neurones had a receptive field over the hindpaw ipsilateral to surgery (where performed). No significant differences were found between experimental groups in the mean cell depth of recorded neurones and the mean neuronal responses evoked by electrical and natural stimulation. However, 2 out of the 5 neurones characterised in SNL rats exhibited spontaneous activity at a rate greater than 0.9 Hz whereas none of the neurones characterized from naïve animals showed any spontaneous activity.

### 6.3.3 Spinal Cord Electrophysiology: Effects of Nifedipine

The effect of nifedipine (10 - 260 μg), applied directly onto the spinal cord, on the electrically- and naturally-evoked dorsal horn neuronal responses was tested in SNL and naïve animals. Although the sham-operation is the appropriate control for SNL, results obtained from the naïve, non-operated group and the SNL group show that nifedipine exerted only limited inhibitory effects on the evoked neuronal responses. Furthermore, no difference in the effects of nifedipine was observed between these experimental groups, thus it was deemed unnecessary and wasteful to perform these experiments upon sham-operated rats. This is supported by the results from chapter 5 where no difference in the effects of ethosuximide was observed between sham and naïve groups, nor between SNL and either sham or naïve animals. Nifedipine produced limited inhibitions of the electrically- and naturally-evoked responses in neurones in naïve and SNL rats (Figures 22 - 24), and there was no difference in the magnitude of its effects between groups. For measurements where nifedipine-mediated inhibition was more marked, its effects were dose-related and seen around 60 - 90 minutes. Nifedipine was dissolved in mixture of cremophor, saline and ethanol, due its insolubility in saline alone (see chapter 2). No vehicle control was performed in this series of experiments since it has already been
Figure 22 Comparison of the effect of spinally applied nifedipine on the electrically-evoked dorsal horn neuronal responses recorded from un-operated, naive (n = 6) and SNL (n = 5) rats at PO days 14 - 17. Data are expressed as the mean maximal % inhibition of the pre-drug control values ± S.E.M.
demonstrated to be without effect (Stanfa et al., 1996). For naïve animals the highest
dose of nifedipine used, 260 µg (dictated by its poor solubility), produced
statistically significant inhibitions, compared to pre-drug control values, on the
electrically-evoked responses, postdischarge, excess spikes, input and C-fibre
(Figures 22a - e, respectively; \( P < 0.05; \ n = 6 \)). Additionally, the input response in
naïve animals was also significantly inhibited at the middle dose, 110 µg nifedipine.
In contrast, for the SNL group significant inhibition from pre-drug control value was
only seen after the top dose of nifedipine for the input measurement (\( P < 0.05; \ n = 6 \)). In both groups the different fibre type-evoked responses (Aβ, Aδ and C) were
least affected with mean maximal inhibitions at top dose in the range 6 ± 21\% to 38
± 12\% (Figures 22d - f, respectively). Greater inhibitory effects of nifedipine were
observed on the postdischarge, excess spikes and input measurements (Figures 22a -
c, respectively). At 260 µg, nifedipine maximally inhibited these measures within the
range 32 ± 12\% to 56 ± 12\%. It should also be noted that low doses of nifedipine
mediated facilitation of the electrically-evoked responses in the naïve group (2 out of
6 cells) and in the SNL group (2 out of 5 cells), for the Aδ-fibre, C-fibre,
postdischarge, input and excess spike measurements. This explains the large error
bars displayed on some of the graphs. These excitatory effects were later inhibited at
the top dose, 260 µg nifedipine.

Figure 23 is an example of the effects of nifedipine on the wind-up of an
individual neurone recorded from a SNL rat. This neurone was selected as one
displaying a clear wind-up response i.e. an increasing number of evoked action
potentials (300 ms – 800 ms latency band) observed with increasing electrical
stimulus number, and the drug effects are consistent with the rest of the neurones
tested. It is apparent that nifedipine reduces the wind-up, in a dose-related manner
(evident by a flattening of the curve), with a much less pronounced effect on the
initial C-fibre input (response to stimulus number 1) and no difference was observed
in comparison to neurones recorded from naïve rats.
Figure 23. Example of the inhibitory effect of spinally applied nifedipine on an individual dorsal horn neurone exhibiting wind-up recorded from SNL rat. Data is expressed as the number of evoked action potentials, in the 90 - 800 ms latency band, to each electrical stimulus.

Nifedipine also produced an inhibitory effect on the naturally-evoked neuronal responses (Figure 24). This was only found to be significant in SNL animals at all doses for the noxious heat-evoked response ($P < 0.05$; $n = 5$). No differences in the effects of the drug were apparent between experimental groups and the mean maximal inhibitions established at top dose were in the range $32 \pm 14\%$ to $74 \pm 8\%$. Unfortunately, for the limited amount of cells studied in this investigation, difficulties were encountered in locating dorsal horn neurones in SNL rats that responded to the innocuous von Frey 9 g mechanical stimulation (1 out of 5 cells). The results from this one cell have been displayed on the graph (Figure 24a), since the effects of nifedipine upon the von Frey 9 g evoked response of this neurone were similar to that obtained in the naïve group, which is in keeping with the findings from the rest of the study.
Figure 24 Comparison of the effect of spinally applied nifedipine on the naturally-evoked dorsal horn neuronal responses recorded from un-operated, naive (n = 5) and SNL (n = 5) rats. (Note for SNL rats n = 1 for the von Frey 9 g response). Data are expressed as mean maximal % inhibition of pre-drug control values ± S.E.M.
6.4 DISCUSSION

In this study I have addressed the role of the Ca\textsubscript{v1} family of VDCCs (HVA L-type Ca\textsuperscript{2+} channels) in the spinal processing of sensory information after nerve injury. Spinal nifedipine, a relatively specific L-type VDCC antagonist, mediated a small inhibition of the electrically- and naturally- (innocuous and noxious) evoked rat dorsal horn neuronal responses, significant only at the highest dose used for measures predominantly related to dorsal horn neuronal excitability. These results are suggestive of a limited role of L-type Ca\textsuperscript{2+} channels in sensory transmission at the spinal cord level, in a manner unaltered by nerve injury.

Extensive behavioural and electrophysiological studies into nociception have demonstrated an important role in general for HVA Ca\textsuperscript{2+} channels in the processing of pain see chapters 4 and 5 and (Vanegas & Schaible, 2000) for review. The employment of specific Ca\textsuperscript{2+} channel blockers and antagonists has revealed that the contribution of L-, N- or P/Q- type channels differs dependent on the nature of pain (acute or chronic, inflammatory or neuropathic in origin). A predominant nociceptive role for N-type channels has been established, which is enhanced after neuropathy (Chaplan et al., 1994; Xiao & Bennett, 1995; Bowersox et al., 1996; Brose et al., 1997; White & Cousins, 1998). Upon substantial membrane depolarisation N- and P/Q-type VDCCs mediate the release of excitatory neurotransmitters, such as glutamate, substance P and CGRP, critical for wind-up and central sensitization, in the presence of constant afferent input (Dickenson, 1994). On the other hand, the LVA T-type Ca\textsuperscript{2+} channels are activated close to resting potential. These VDCCs permit Ca\textsuperscript{2+} influx when cells are at rest thus regulating cell excitability and most likely the depolarization required to activate HVA L-, N- and P/Q-type channels, the latter two Ca\textsubscript{v2} VDCCs strongly implicated in neurotransmission.

L-type VDCCs are found within the sensory transmission pathways from periphery to the spinal cord to the brain. Immunohistochemical studies have demonstrated the neuronal presence of α\textsubscript{1C} and α\textsubscript{1D} subunits upon the cell bodies and dendrites in a variety of cell populations, including the spinal cord, where they are distributed throughout the deep dorsal and ventral horns (Ahlijanian et al., 1990; Hell
et al., 1993; Westenbroek et al., 1998). Further evidence comes from the observations that nifedipine reduced the sustained component of HVA Ca\(^{2+}\) current in rat dorsal horn neurones (Ryu & Randic, 1990) and that L-type VDCCs comprise 50% of the whole Ca\(^{2+}\) current of small diameter DRG neurones, much greater than that observed in large diameter cell bodies (Scroggs & Fox, 1992a).

It is, however, generally accepted that the Ca\(_{2,2}\) family of VDCCs are responsible for depolarization-coupled neurotransmitter release (Miljanich & Ramachandran, 1995), with no contribution to this physiological function from the Ca\(_{2,1}\) family. However, some reports have demonstrated that Ca\(^{2+}\) current through L-type channels is linked to the endogenous release of glutamate in rat hippocampus (Terrian et al., 1990) and substance P release from sensory neurones (Perney et al., 1986; Rane et al., 1987). These relatively few studies are somewhat discredited by the overwhelming evidence to the contrary and L-type VDCCs are generally thought not to be directly related to synaptic transmission in the dorsal horn. In most investigations DHPs have no effect on neurotransmitter release (Holz et al., 1988; Kamiya et al., 1988; Takahashi & Momiyama, 1993; Wheeler et al., 1994; Wu & Saggau, 1994). Release of CGRP from rat spinal afferents has been demonstrated not to be mediated by nifedipine-sensitive L-type VDCCs, in contrast to the key roles of ω-conotoxin GVIA sensitive channels (Santicioli et al., 1992). Furthermore, in rat hippocampal slices, nimodipine or nifedipine were found to reduce Ca\(^{2+}\) entry evoked by repetitive electrical stimulation only postsynaptically, and not that occurring presynaptically (Jones & Heinemann, 1987; Igelmund et al., 1996). Taken together these studies indicate that L-type VDCCs are not involved in presynaptic Ca\(^{2+}\) entry, and subsequent neurotransmitter release, but rather to have a postsynaptic location (see also Spedding & Paoletti, 1992). L-type VDCCs are therefore implicated in neurone depolarization and influx of Ca\(^{2+}\) for intracellular mechanisms linked to the activation of signal transduction cascades.

Here I have demonstrated that most susceptible to the effects of nifedipine were the postsynaptic NMDA receptor-mediated postdischarge and excess spikes measurements, indicators of central sensitization and neuronal hyperexcitability. Nifedipine potently inhibits L-type currents in a use-dependent manner. Nifedipine
has a low affinity for L-type VDCCs that are at rest and preferentially binds to the inactivated state of the channel (Bean, 1984). At negative holding potentials DHPs have little effect upon the whole cell Ca\(^{2+}\) current suggestive that L-type VDCCs are minimally active. Upon increasing neuronal depolarization, as might occur during the central sensitization that ensues after peripheral nerve damage or repetitive electrical stimulation, L-type VDCCs may mediate a greater proportion of the Ca\(^{2+}\) current (Bean et al., 1993). The voltage-dependent interaction exhibited by nifedipine would mean that the likelihood of its binding to and blocking L-type VDCCs increases with increased neuronal excitability. It might be expected then that the effectiveness of nifedipine would positively correlate with maintained activation of the channel due to the reported ongoing, continual barrage of neuronal activity via primary afferents into the spinal cord arising from ectopic C-fibre activity that follows nerve injury (Wall & Devor, 1983; Kajander et al., 1992). If a C-fibre afferent barrage results in activation of VDCCs then nifedipine could reduce postsynaptic Ca\(^{2+}\) entry, which also implies that nifedipine would be less effective in the naïve animal. However, as demonstrated here this was not the case. Peripheral nerve injury did not induce plasticity in the events mediated by L-type VDCCs at the level of the spinal cord. Furthermore, equally susceptible to the effects of nifedipine was the non-potentiated input response which can be related to the baseline level of C-fibre mediated synaptic transmission between the central terminals of primary afferents and the neurones of the spinal cord dorsal horn, in the absence of NMDA receptor mediated wind-up events. Even if nerve injury did not induce changes in the functioning of L-type VDCCs, considering the above discussion it might be expected that this measurement would be inhibited less by nifedipine in comparison to postdischarge and excess spikes. It could be that the depolarization mediated by the first electrical stimulus was sufficient to maximally activate L-type VDCCs within the sensory pathway, therefore no difference would be observed in the effects of by L-type VDCC block in the presence or absence of NMDA-mediated wind-up.

There are a few reports that may relate to these observations, where L-type VDCCs are implicated in regenerative Ca\(^{2+}\)-dependent plateau potentials of deep dorsal horn neurones that possibly comprises another critical component of wind-up, operating downstream of the predominant synaptic NMDA-mediated processes (Morisset & Nagy, 1996; Morisset & Nagy, 1998). Electrical stimulation (0.4 – 1
Hz) of high-threshold primary afferent fibres in the dorsal root, induced action potential wind-up and afterdischarge correlating with the production of Ca\(^{2+}\)-dependent plateau potentials, as recorded intracellularly from deep dorsal horn neurones in slice preparation of rat spinal cord. These phenomena were suppressed by nifedipine indicating that Ca\(^{2+}\)-dependent plateau potentials were involved in this form of wind-up (Morisset & Nagy, 2000). Being associated with Ca\(^{2+}\) influx, generation of plateau potentials could be a link between short-term plasticity and the long-term modification of dorsal horn neuronal excitability associated with central sensitization.

In pre-clinical acute nociception studies, L-type VDCC antagonists of the DHP, phenylalkylamine and benzothiazepine classes can block evoked nociceptive responses (Del Pozo et al., 1987; Miranda et al., 1992), although these observations have not been consistently confirmed (Hoffmeister & Tettenborn, 1986; Contreras et al., 1988; Omote et al., 1995). Furthermore, given the marked vascular effects of these antagonists any behavioural study with heat or inflammatory stimuli has to be viewed with caution as changes in blood flow will alter heat transfer across the skin. Intrathecal L-type VDCC antagonists, verapamil and diltiazem, have been demonstrated to have no effect on the 1\(^{st}\), acute phase and produce only modest inhibition of the 2\(^{nd}\), tonic phase of nocifensive behaviours induced by formalin (Malmberg & Yaksh, 1994). In the same experimental model nifedipine was found to mediate some antinociceptive actions, but these were much less effective than the NMDA receptor antagonist MK-801 (Coderre, 1992). Furthermore, intrathecal nifedipine failed to prevent or reverse thermal hyperalgesia or spontaneous behaviours induced by a rodent model of acute arthritis, where N- and P/Q-type VDCC blockade was antinociceptive (Sluka, 1998). This is indicative of no spinal role for L-type Ca\(^{2+}\) channels in the secondary heat hyperalgesia induced by joint inflammation. However, in one model of inflammation intrathecal nifedipine was shown to prevent secondary mechanical hyperalgesia and allodynia developed in response to injection of capsaicin into the hindpaw (Sluka, 1997). In models of neuropathic pain no role for spinal L-type VDCCs has been uncovered. Using the SNL model of neuropathy, administration of L-type VDCC antagonists, diltiazem, verapamil and nimodipine, intrathecally, intravenously and to the site of injury, also had no antinociceptive effects (Chaplan et al., 1994), nor did injection of nifedipine
into the hindpaw in PSL model of peripheral nerve injury (White & Cousins, 1998).

Although there is behavioural evidence regarding the role of L-type VDCCs in neuropathy, only studies investigating acute or inflammatory pain have been executed electrophysiologically. Recordings of single dorsal horn neurones, in a manner identical to that used in this study, demonstrated that verapamil was without effect on responses to formalin injection, in contrast to intrathecal ω-agatoxin IVA and ω-conotoxin GVIA which reduced the $2^{nd}$ phase and additionally the $1^{st}$ phase, respectively (Diaz & Dickenson, 1997). It was, however, demonstrated that L-type VDCCs might be involved in the central sensitization of dorsal horn neurones to mechanical stimuli induced by joint inflammation in the model of acute arthritis, yet the dose administered also reduced the response of unsensitized neurones (Neugebauer et al., 1996).

The data I present here is in keeping with the theme demonstrated by the vast majority of studies surrounding L-type VDCCs and sensory transmission. Unlike predominantly the N-type, and to a lesser extent P/Q-type VDCCs, spinal L-type channels do not appear to be critical to the transmission of nociceptive information observed both behaviourally and electrophysiologically. It could be argued that the lack of effects of nifedipine on the evoked dorsal horn neuronal responses in this study were due to insufficient doses of the drug, which were limited by its solubility. Similar problems have been encountered in other studies using nifedipine where the maximum concentration possible was 1 mM and this required 30% DMSO to enable nifedipine to go into solution (Sluka, 1997; Sluka, 1998). One of these studies reported comparable antinociceptive effects to capsaicin treatment mediated by block of spinal L- or N-type VDCCs, using intrathecal nifedipine at a concentration 100-fold greater than that of ω-conotoxin GVIA (Sluka, 1997). However in a subsequent study in the formalin model the same concentration of nifedipine was without effect (Sluka, 1998). In my study, the maximum concentration of spinally applied nifedipine used (15 mM) was similarly 1000-fold greater than the concentrations of ω-conotoxin GVIA required to exert maximum effects (15 - 20 μM) upon the evoked neuronal responses (see chapter 4). Thus it is unlikely that an increase in dose of nifedipine would cause further inhibitory effects.
Nonetheless, blockade of L-type Ca\(^{2+}\) channels would thus be expected to reduce neuronal activity and reduce the influx of Ca\(^{2+}\), not unlike that observed with block of T-type channels (see chapter 5). Since intracellular Ca\(^{2+}\) can activate second messenger pathways, in addition to acting as a second messenger itself, intracellular events downstream of synaptic transmission might be affected. Maximal effects mediated by nifedipine decreased input, postdischarge and excess spikes measurements by approximately half and it appears that L-type block was the basis for some decrease in neurotransmitter release, and possibly attenuation of second messenger systems activation and sensitization of dorsal horn neurones. It is unlikely however that this would be manifest behaviourally as demonstrated by other studies. The effects of nifedipine upon the different fibre type-evoked deep dorsal horn neuronal responses were not markedly differential to noxious or innocuous afferent input from the periphery. Given the distribution of L-type VDCCs in the deep dorsal horn this it not surprising. At this location Ca\(^{2+}\) influx through these channels would participate in convergent sensory pathways, not specific to nociception in particular.

Given the similarities in the effects of nifedipine on evoked dorsal horn neuronal responses with those mediated by block of T-type VDCCs with ethosuximide (chapter 5), it should be noted that variable sensitivity of T-type Ca\(^{2+}\) channels to DHPs has also been described. However, this ranges from quite high LVA Ca\(^{2+}\) current DHP sensitivity in cerebellar Purkinje (Kaneda et al., 1990) and hippocampal (Takahashi & Akaike, 1991) neurones, to virtually DHP insensitive in primary sensory neurones (see Kostyuk, 1999). Since the latter relates more to the sensory systems investigated here, it may be that LVA VDCCs on dorsal horn neurones within the sensory pathway may also be DHP insensitive. However the fact that ethosuximide inhibitions were greater than those observed for nifedipine, but with a similar profile, means that the possibility that nifedipine-mediated inhibitions were due to inhibition of Ca\(^{2+}\) influx through LVA VDCCs, cannot be ruled out.

Although inhibition of L-type VDCC activation per se does not generally mediate antinociception in experimental conditions (Chaplan et al., 1994; Omote et al., 1995; Sluka, 1998), it has been demonstrated that blockers or antagonists may be useful in the potentiation or restoration of opioid analgesia. This effect seems to rely
upon the establishment of opioid tolerance due to chronic administration, or a pain state that is opioid resistant. A synergistic effect interaction between L-type Ca\(^{2+}\) channels blockers and opioids under either or both of these situations has been consistently reported (Welch & Olson, 1991; Antkiewicz-Michaluk et al., 1993; Diaz et al., 1995). Since DHPs, phenylalkylamines and benzothiazepines are clinically established therapeutic drugs there have been some clinical assessments into their potential use in analgesia. Clinically relevant doses of L-type Ca\(^{2+}\) channel antagonists demonstrate a moderate potentiation of the analgesic effect of opioids (von Bormann et al., 1985; Boldt et al., 1987; Carta et al., 1990; Filos et al., 1993; Santillan et al., 1998), whereas others did not confirm this (Lehmann et al., 1989; Roca et al., 1996; Hasegawa & Zacny, 1997b). In keeping with the hypothesis L-type VDCC block has been shown to correlate with opioid responsiveness (Hasegawa & Zacny, 1997b; Santillan et al., 1998). Findings suggest that chronic blockade of L-type VDCCs prevents some compensatory mechanisms responsible for opioid tolerance and/or expression. Alternatively the vasodilatory effects of DHPs could increase absorption of morphine into the vasculature resulting in increased distribution to its sites of action, and thus increased effectiveness. Radioligand binding studies to assess alterations in L-type channels in the tolerant state have varied in methodology and provide contrasting reports. Chronic morphine treatment has been reported to increase the number of membrane bound L-type VDCCs in the brain (Ramkumar & el-Fakahany, 1988; Zharkovsky et al., 1993) but not in the spinal cord (Bernstein & Welch, 1995).

Thus the consensus from results presented here, in conjunction with pre-clinical behavioural studies is that L-type VDCCs have little role in the spinal processing of sensory information in both acute and chronic pain states, unlike its N-type VDCC counterpart strongly linked to neurotransmitter release and pathological conditions. Results from pre-clinical and clinical investigations do however reveal that in situations of opioid tolerance, L-type VDCC antagonism may be of therapeutic benefit.
CHAPTER 7

GABAPENTIN AND MORPHINE:

A COMBINED APPROACH TO INHIBITION

OF CA\(^{2+}\) INFLUX
7.1 INTRODUCTION

Damage to the peripheral and central nervous system can lead to the development of neuropathic pain where patients often experience a combination of sensory deficits with spontaneous and stimulus-evoked pain (allodynia and hyperalgesia). Due to the multiplicity of causes of neuropathy, ranging from trauma, viral infections to diabetes, and the number of possible resultant symptoms, the underlying dysfunctional mechanisms are likely to be diverse. This may contribute to the problematic clinical management of nerve injury pains. Neuropathic pain and epilepsy both share neuronal hyperexcitability as a common underlying mechanism. There are established antiepileptic drugs that target the generation of neuronal hyperexcitability and some of these have been proven effective in the treatment of various forms of neuropathic pain (Swerdlow & Cundill, 1981; McQuay et al., 1995). Excitability blockers, antidepressants and opioids can be useful therapies but the numbers needed to treat are about three even for the most effective agents (Sindrup & Jensen, 2000) and their use can be limited by unfavourable side-effects (Foley & Inturrisi, 1987). The diverse mechanisms and symptoms of neuropathic pain lends credence to the idea that combination therapy, based on multiple pharmacological targets and low drug doses, could improve both the pain relief and side-effect profiles.

The anticonvulsant gabapentin (GBP) is widely becoming accepted as an alternative treatment for various types of neuropathic pain (Nicholson, 2000) as it provides reasonable efficacy and is well tolerated. Designed as an analogue of the inhibitory neurotransmitter γ-aminobutyric acid (GABA) (Satzinger, 1994) to cross the blood brain barrier, it does not however, clearly modulate GABA receptor function, and the mechanism(s) of its anticonvulsant/analgesic actions remain undetermined. GBP does not bind to any known neurotransmitter receptor but binds to a unique site in the CNS identified as α2δ, a VDCC modulatory accessory subunit (Gee et al., 1996). Animal models have demonstrated the antinociceptive abilities of specific VDCC blockers in line with electrophysiologically observed reductions in spinal cord hyperexcitability (as demonstrated in chapters 4, 5 and 6) and together these highlight the differential role each subtype plays in nociception, often dependent on the nature of the pain state (see Vanegas & Schaible, 2000, for review).
Morphine acts via a number of CNS sites, including the spinal cord where pre- and to a lesser extent, postsynaptic μ opioid receptors mediate the majority of morphine’s effects upon nociceptive transmission (see section 1.3.2.5 for further discussion). Of the four identified opioid receptors (μ, δ, κ and ORL-1), the affinity of morphine for μ opioid receptors is 50 times higher than that for δ opioid receptors, and morphine shows minimal affinity for the κ and ORL-1 receptors (Wick et al., 1994; Kieffer, 1997). The μ opioid receptors (and δ, κ and ORL-1 receptors) are coupled to G-proteins and upon binding of morphine (or the appropriate ligand for the other receptors, see Table 2) to the extracellular domain, a conformational change in the receptor is elicited that subsequently opens K+ channels (μ, δ and ORL1 receptors). The resultant neuronal hyperpolarization leads to a decrease in the opening of VDCCs, and in the case of κ opioid receptor activation this reduction of Ca^{2+} influx is direct. At presynaptic locations a reduction in the release of neurotransmitter from the afferent nerve ensues. This presynaptic action is common to all opioid-mediated inhibitory effects at central and peripheral sites, the net result being a reduction in neuronal excitability. At the less frequent postsynaptic receptors, the same mechanism results in the direct hyperpolarization of the neurone and the attenuation of firing.

Clinically it is generally agreed that morphine and other opioid drugs are less effective in treating neuropathic pain compared to nociceptive or inflammatory pain although the effectiveness of opioids in neuropathic pain as reported in the literature is still controversial (Portenoy et al., 1990; Foley & Portenoy, 1991; Rowbotham et al., 1991; Jadad et al., 1992; Cherny et al., 1994; Kingery, 1997; Dellemijn, 1999). Possible explanations include the many associated underlying pathophysiological mechanisms (Dubner, 1991; Besson et al., 1993), and dose-escalation (Portenoy et al., 1990; Rowbotham et al., 1991). Animal models of neuropathic pain have been used in an attempt to clarify the relative efficacy of opioids in different types of neuropathic pain. Studies have revealed that several factors appear to be accountable for the variability observed in opioid efficacy such as pre-clinical model, route of administration and testing stimuli used (Attal et al., 1991; Lee et al., 1994; Bian et al., 1995; Lee et al., 1995; Ossipov et al., 1995b; Jasmin et al., 1998; Suzuki et al., 1999).
Due to the differing ionic mechanisms of inhibition mentioned above, it could be predicted that morphine and GBP would interact positively, through concomitant decrease of excitation and increase of inhibition. Thus far, there have no \textit{in vivo} electrophysiological studies investigating the role of GBP in conjunction with morphine in the spinal processing of neuropathic pain. This study uses the SNL model, confirmed by behavioural testing, to induce a neuropathic state. Subsequent to this, electrophysiological studies of dorsal horn spinal neurones were made to investigate the effects of low doses of subcutaneously delivered GBP and morphine on a wide range of electrically- and naturally-evoked neuronal activity. Due to the serious difficulties in conducting combination studies in patients with neuropathic pain it is hoped this approach may provide a guide to potential improvements in the treatment of this type of pain.

7.2 \textbf{METHODS}

A total of 62 male adult Sprague-Dawley rats were used in this study, of which 19 were naïve, 19 were sham-operated and 24 were SNL. The animals were prepared in the normal way and the drugs were administered subcutaneously into the scruff of the neck (250 µl volume). In one series of experiments the effects of morphine alone (1 & 4 mg/kg applied cumulatively) was monitored until exertion of maximum effects (a minimum of 50 minutes). In a second series of experiments the effects of a combination of GBP and morphine were investigated. The drug combination protocol consisted of a single dose of GBP (either 10 or 20 mg/kg), monitored for 60 minutes, proceeded by two doses of morphine (1 & 4 mg/kg). Reversal of opiate-mediated effects was assessed by spinally application of 5 µg naloxone.

7.3 \textbf{RESULTS}

7.3.1 \textbf{BEHAVIOURAL ASSESSMENT}

Rats subjected to SNL exhibited 'guarding' foot posture ipsilateral to nerve injury and successful replication of the nerve injury model was confirmed by behavioural testing which demonstrated the development of mechanical and cooling
allodynia of the injured hindpaw (see chapter 3 for combined behavioural data obtained from all studies used in this thesis). This was not displayed by either the contralateral hindpaw, or in the sham-operated rats. Upon establishment of a neuropathic state, animals were then used for in vivo electrophysiology and the pharmacological study at PO days 14 - 17.

7.3.2 Spinal Cord Electrophysiology: Neurone Characterization

The number of dorsal horn neurones recorded from in each group were 24 in SNL rats, 19 in sham-operated rats and 19 in naïve rats. All neurones had a receptive field over the hindpaw ipsilateral to surgery (when performed). No significant differences were found between experimental groups in the mean values of recorded neurone depth or responses evoked by electrical and natural stimulation. However, the mean level of ongoing spontaneous activity recorded from neurones in nerve injured rats was $1.73 \pm 2.53$ Hz which was significantly higher ($P < 0.05$) than that observed in sham and naïve ($0.39 \pm 0.89$ and $0.42 \pm 0.71$ Hz, respectively). It is also worth noting that 71% of neurones characterized in SNL rats exhibited spontaneous activity at a rate greater than 0.1 Hz in comparison to only 37% of characterized neurones in sham-operated rats and 39% of naïve.

7.3.3 Spinal Cord Electrophysiology: Effects of Morphine Alone

The effect of subcutaneously administered morphine (1 and 4 mg/kg applied cumulatively), on the electrically- and naturally-evoked dorsal horn neuronal responses was tested in SNL, sham-operated and naïve animals. Morphine produced a dose-related inhibition of the electrically- and naturally-evoked responses in neurones recorded from naïve and sham-operated animals (Figures 25 - 28), with clear effects seen around 40 - 50 minutes (Figures 25a & 25b). The greatest effect was seen in the sham group (Figures 26 & 27) with maximal inhibitions achieved using 4mg/kg morphine in the range 45 ± 15% to 80 ± 11% (excluding Aβ-fibres). Statistically significant inhibitions ($P < 0.05$) compared to pre-drug control values, of the post-discharge (Figure 26a), input (Figure 26b), excess spikes (Figure 26c) and the C-fibre (Figure 27a) measurements were achieved at 4mg/kg morphine for both naïve ($n = 6$) and sham ($n = 6$) groups. 1 mg/kg morphine significantly inhibited the
excess spikes and heat response in the sham group as did 4mg/kg for von Frey 9 g (Figure 28a) and von Frey 75 g (Figure 28b) and heat (Figure 28c) evoked responses (n = 6, P < 0.05). Morphine was noticeably less effective at inhibiting the evoked responses in nerve injured rats in comparison to naïve and sham-operated rats (Figures 25c & 26 - 28). In fact 2 out of 7 cells showed and increase in their response resulting in and average excitation for the input (Figure 26b) and excess spikes (Figure 26c) measurements at the low dose. The only inhibitions found to be significant (n = 7, P < 0.05) were von Frey 9 g with 1 mg/kg morphine and von Frey 75 g with 4 mg/kg morphine (Figures 28a & 28b). No statistical significance was determined for the direct comparison of the effects of morphine between experimental groups. Morphine mediated inhibitions were reversed with spinally applied naloxone (Figure 25).

7.3.4 Spinal Cord Electrophysiology: Effects of Gabapentin Alone

The effect of subcutaneously administered GBP (10 & 20 mg/kg), on the electrically- and naturally-evoked dorsal horn neuronal responses was tested in SNL, sham-operated and naïve animals. GBP produced a dose-related inhibition of the electrically- and naturally-evoked responses in neurones in SNL and sham-operated animals, with clear effects seen around 40 - 50 minutes. The greatest effect was observed in the neuropathic animals for which statistically significant inhibitions (n = 10, P < 0.05) compared to pre-drug control values, were achieved with 20 mg/kg for all measurements (excluding A-fibres, which were relatively spared) in the range of 24 ± 9% to 56 ± 9% (Figures 26 - 28). The response to noxious heat (Figure 28c) was most susceptible to the effects of GBP with statistically significant inhibitions (P < 0.05) achieved at 10 and 20 mg/kg in nerve injury rats (n = 10) and 20 mg/kg in sham (n = 7). In contrast GBP did not have a constant inhibitory effect on all neurones and measurements recorded from the naïve group. With the exception of the heat response, either 10 or 20 mg/kg GBP (or in some cases both) produced an overall excitation of the neuronal responses. This was most marked for post-discharge (Figure 26a) and excess spikes (Figure 26c) for which 18% ± 29 and 42 ± 28% excitation was achieved, respectively. In the naïve group both doses of GBP elicited excitation in 4 out of 7 neurones. No statistical significance was determined for direct comparison of the effects of GBP between experimental groups.
Figure 25. Time course of the effect of subcutaneously applied morphine, with spinal naloxone reversal, on the evoked response of a typical dorsal horn neurone recorded from (a) naïve, (b) sham and (c) SNL rats. Examples of the effect on the Aβ-fibre, C-fibre and von Frey 75 g measurements are shown with the cumulative dose indicated. Data is expressed as % of the averaged pre-drug control values.
Figure 26. Effect of subcutaneously administered morphine and gabapentin, both individually and in combination, on electrically-evoked dorsal horn neuronal responses recorded from naive, sham-operated, and SNL rats (n = 6 – 10 for each experimental group in each drug testing protocol). Data are expressed as maximal mean % inhibition of the pre-drug values + S.E.M. * denotes statistically significant inhibitory response compared to pre-drug control value; ** denotes a significantly greater inhibitory effect of morphine in the presence of gabapentin compared to morphine alone at a specific dose (P < 0.05).
Figure 27. Effect of subcutaneously administered morphine and gabapentin, both individually and in combination, on electrically-evoked dorsal horn neuronal responses recorded from naive, sham-operated, and SNL rats (n = 6 – 10 for each experimental group in each drug testing protocol). Data are expressed as maximal mean % inhibition of the pre-drug values ± S.E.M. * denotes statistically significant inhibitory response compared to pre-drug control value; ** denotes a significantly greater inhibitory effect of morphine in the presence of gabapentin compared to morphine alone at a specific dose (P < 0.05).
**Figure 28.** Effect of subcutaneously administered morphine and gabapentin, both individually and in combination, on naturally-evoked dorsal horn neuronal responses recorded from naive, sham-operated, and SNL rats (n = 6 – 10 for each experimental group in each drug testing protocol). Data are expressed as maximal mean % inhibition of the pre-drug values + S.E.M. * denotes statistically significant inhibitory response compared to pre-drug control value; ** denotes a significantly greater inhibitory effect of morphine in the presence of gabapentin compared to morphine alone at a specific dose (P < 0.05).
7.3.5 Spinal Cord Electrophysiology: Effect of Morphine in the Presence of Gabapentin

The effect of subcutaneously administered morphine (1 and 4 mg/kg applied cumulatively), 60 minutes after the subcutaneous administration of either 10 or 20 mg/kg GBP, on the electrically- and naturally-evoked dorsal horn neuronal responses was tested in SNL, sham-operated and naïve animals. In combination with GBP, morphine produced a dose-related inhibition of the electrically- and naturally-evoked responses in neurones in naïve, sham-operated and nerve injured animals, with clear effects seen around 40 - 50 minutes, and these were reversed by spinally applied naloxone (Figure 29). Maximal inhibitions within each experimental group were achieved with a combination of 10 mg/kg GBP and 4mg/kg morphine (Figure 26 - 28). The greatest effect was observed in the nerve injury group with maximal inhibitions in the range 57 ± 12% - 92 ± 5%. In the presence of 10 mg/kg GBP the levels of inhibition attained after administration of morphine of all evoked responses in each experimental group were greater than those observed with morphine alone. In comparison to the pre-drug control values, the drug combination inhibitions were found to be statistically significant in: SNL rats for C-fibre, postdischarge and input using 1 mg/kg morphine in conjunction with 10 mg/kg GBP; in SNL and sham for C-fibre using 4 mg/kg morphine with 10 mg/kg GBP; and in SNL, sham and naïve for post-discharge, input and excess spikes using 4 mg/kg morphine with 10 mg/kg GBP (Figures 26 & 27). In the presence of 10 mg/kg GBP both doses of morphine significantly inhibited the von Frey 9 g, von Frey 75 g and heat (Figure 28) evoked responses, in all 3 experimental groups (with the exception of naïve with 4 mg/kg morphine for von Frey 75 g and heat). When morphine was used in combination with 20 mg/kg GBP the levels of inhibition reached were no greater than those achieved in the presence of 10 mg/kg GBP (Figures 26 - 28). Effects of the GBP/morphine combination were fairly comparable between sham and SNL groups. However, in the neuropathic group there was a marked increase in the inhibitions achieved with morphine in the presence of GBP compared to the effects of morphine alone. This was found statistically significant (n = 7 - 10, P < 0.05) for; C-fibre (Figure 27a) and input (Figure 26b) using 4 mg/kg morphine in conjunction with both 10 and 20 mg/kg GBP; post-discharge (Figure 26a) using 4 mg/kg morphine with 10 mg/kg GBP; and von Frey 75 g (Figure 28b) using 1 mg/kg with 20 mg/kg GBP. Since in the sham group the levels of inhibition mediated by morphine alone were greater
than compared to that observed after SNL, the increase observed in the presence of GBP in sham was not as marked. However, statistical significance was found for input (Figure 26b) using 4 mg/kg morphine in conjunction with 20 mg/kg GBP and for heat (Figure 28c) using 1 mg/kg morphine with 20 mg/kg GBP. The neuronal responses of the naïve group were least inhibited by the drug combination (Figures 26 - 28) for which no statistically significant difference was found in the comparison of the effects of morphine in the presence of GBP to morphine alone.

**Figure 29.** Time course of the effect of subcutaneously applied morphine, with spinal naloxone reversal, on the evoked response of a typical dorsal horn neurone recorded from (a) naïve, (b) sham and (c) SNL rats. Examples of the effect on the Aβ-fibre, C-fibre and von Frey 75 g measurements are shown with the cumulative dose indicated. Data is expressed as % of the averaged pre-drug control values.
7.4 DISCUSSION

In this study I have examined the effect of a combination of subcutaneously administered GBP and morphine on the spinal processing of sensory information after nerve injury. In naïve, sham-operated and SNL groups significant inhibitions of the evoked dorsal horn neuronal responses were achieved with a combination of the two drugs at doses that when given alone lacked any effect. The greatest increase in the effectiveness of morphine after GBP treatment occurred after neuropathy, where systemic morphine was almost ineffective.

7.4.1 Effect of Morphine Alone

The present study has shown that subcutaneously administered morphine has a reduced inhibitory effect on the electrically-evoked dorsal horn neuronal responses in rats subject to SNL in comparison to sham-operated and naïve animals. Additionally, morphine dose escalation did not improve the inhibition mediated in nerve injured rats, whereas its effect was dose-related in sham and naïve groups. Doses used here were identical to those demonstrated to have effects in behavioural studies of neuropathy and inflammation (Field et al., 1999b). Within the SNL group the greatest observed morphine mediated inhibitions were on the mechanical von Frey stimuli evoked responses. Across all three experimental groups Aβ-fibre evoked responses were relatively spared. A reduced sensitivity of evoked neuronal responses in SNL rats to systemic morphine has previously been demonstrated in a similar electrophysiological study (Suzuki et al., 1999) and this is in line with a behavioural observation (Kontinen et al., 1998). However, the antiallodynic and antinociceptive abilities of morphine in behavioural studies involving neuropathy is somewhat variable and appears to be dependent on the model of neuropathy employed, behavioural assessment and nature of stimuli utilized, as well as the route of morphine administration. In keeping with my findings a reduced effectiveness of intrathecally administered morphine after neuropathy has been observed for thermal hyperalgesia in the SNL model (Ossipov et al., 1995a; Ossipov et al., 1995b; Nichols et al., 1997; Wegert et al., 1997), the CCI model (Mao et al., 1995) and PSL model (Yamamoto & Sakashita, 1999).
On the other hand, it has been demonstrated that under neuropathic conditions, adopting the systemic route of administration, morphine can be effective in alleviating mechanical allodynia in the SNL model (Bian et al., 1995; Lee et al., 1995; Wegert et al., 1997) see however (Kontinen et al., 1998), for which the spinal route has been proven to be both with (Hwang et al., 2000) and without effect (Bian et al., 1995; Lee et al., 1995; Nichols et al., 1995; Wegert et al., 1997). Likewise, in the CCI model, systemic morphine was shown to be effective against mechanical allodynia (Kayser et al., 1995) and hyperalgesia (Attal et al., 1991; Desmeules et al., 1993; Koch et al., 1996), thermal hyperalgesia (Yamamoto & Yaksh, 1991; Lee et al., 1994; Backonja et al., 1995), cold allodynia (Hedley et al., 1995; Jasmin et al., 1998) and spontaneous pain (Jazat & Guilbaud, 1991). Furthermore, in various experimental conditions an increased sensitivity to morphine after neuropathy has also been observed (Kayser et al., 1995; Suzuki et al., 1999). In an electrophysiological study, Suzuki et al (1999) demonstrated that intrathecal morphine had an enhanced potency on the C-fibre evoked and noxious natural stimuli evoked neuronal response of SNL rats. In the same study however, it was also shown that systemic morphine was almost without inhibitory effect, as I have also demonstrated. Thus, it appears that there is an inconsistency between the inhibitory effects of systemically administered morphine observed behaviourally, where its has a proven effect, in contrast to its lack of mediated inhibition observed electrophysiologically. The majority of behavioural data supports the effectiveness of systemically administered morphine for the relief of neuropathic behaviours. This may possibly reflect a direct action of morphine at supraspinal (Yaksh, 1997; Sohn et al., 2000) or spinal sites (Duggan & North, 1984; Dickenson & Sullivan, 1986), areas abundant in opioid receptors (see Mansour et al., 1995), or an indirect action upon spinal transmission via spinal descending inhibitory controls (see Basbaum & Fields, 1984). With regards to the lack of inhibitory effect observed electrophysiologically here and by Suzuki et al (1999), it should be noted that in behavioural studies only threshold responses are employed, which contrasts to suprathreshold neuronal responses employed electrophysiologically, most likely requiring higher morphine concentrations for inhibition. Indeed, the naturally-evoked neuronal responses to von Frey 9 g in the present study were most susceptible to morphine after neuropathy, which may relate more to behavioural testing. Intravenous versus subcutaneous administration routes may be another issue since in
many of the animal studies intravenous morphine mediated greater effects.

Reduced efficacy of opioids on nerve injury-induced static mechanical allodynia and on hyperalgesia (which implies traditional nociceptive pathways) could be a result of increased excitation of spinal neurones (Woolf, 1983) due to activation of NMDA receptors (Dickenson, 1997), resultant from peripheral nerve injury and suprathreshold stimuli (as already mentioned). This enhanced activity may simply require more opioid drug in order to be controlled at the spinal cord level as demonstrated by the studies already discussed. This is further supported by an observed increase in the efficacy of morphine in neuropathic pain models in the presence of NMDA receptor antagonists (Chapman & Dickenson, 1992; Yamamoto & Yaksh, 1992; Nichols et al., 1997; Wegert et al., 1997; Christensen et al., 1998; Kauppila et al., 1998b). Postsynaptic cell hyperpolarization can produce inhibition of sensory transmission by activation of opioid receptors on dendrites of projection neurones, interneurones and cell bodies of output neurones. It has been demonstrated electrophysiologically comparing control animals to those lacking presynaptic opioid receptors that postsynaptic actions of opioids require higher doses of systemic morphine in comparison to presynaptic-mediated effects (Lombard & Besson, 1989). Thus, the reduced effect of opioids on hyperalgesias could partly be overcome by dose escalation. Indeed, neuropathic states may not be completely resistant to spinal opioids, but rather less sensitive, since it has been reported that intrathecal opioids in a number of animal models can produce effective antinociception, albeit at higher doses than those used in normal rats (Bian et al., 1995; Lee et al., 1995; Ossipov et al., 1995a; deGroot et al., 1997; Abdulla & Smith, 1998; Zurek et al., 2001). It should also be considered, however, that spread of the opioid drug away from the site of injection to the brain might occur with the required increase in dose, which could be responsible for the antinociceptive effects (Zurek et al., 2001).

Another possible mechanism for the occurrence of opioid resistance is that spinal cord opioid mechanisms may be disturbed. As previously discussed (section 1.3.2.5) opioids predominantly act a presynaptic site upon nerve terminals. Autoradiographic and immunohistochemical methods have demonstrated high levels of spinal cord opioid receptors around C-fibre terminal zones in lamina I and the
substantia gelatinosa, and lower levels in deeper laminae (Besse et al., 1990b; Rahman et al., 1998; Zhang et al., 1998). A lack of opioid receptors on the terminals of large diameter, low-threshold A\(\beta\)-fibres that convey non-noxious information, explains the nociception-specific actions of morphine (Dickenson & Suzuki, 1999), also seen in the present study. Opioid receptors are synthesized in the cell bodies of small afferent fibres in the DRG and transported to the peripheral and central terminals, permitting modulation of neurotransmitter release (Zhang et al., 1998). This makes spinal opioid receptors susceptible to the effects of peripheral nerve damage in such a way that nerve section has been shown to cause a substantial reduction in spinal opioid receptors at this level, mostly a result of disturbed axonal transport (Besse et al., 1990b). Immunohistochemistry and in situ hybridization has revealed a down-regulation of \(\mu\) opioid receptors in rat and monkey DRG neurones and in the dorsal horn after complete or partial sciatic nerve injury (Zhang et al., 1998) Autoradiographic binding studies indicate that peripheral nerve injury induces a reduction in the level of opioid receptors in the dorsal horn of the spinal cord (Lombard et al., 1990; Stevens et al., 1991; Besse et al., 1992). Furthermore a reduction of opioid receptors localized to the L6 region, has been observed 7 days after SNL, ipsilateral to nerve injury (Porreca et al., 1998). In the case of less severe peripheral nerve injury where axonal transport is somewhat intact, down-regulation or destruction of opioid receptors could also possibly be mediated by increased production of PKC following activation of NMDA receptors in postsynaptic cells (Mao et al., 1995).

Activation of opioid receptors on output neurones, unlike the other postsynaptic and presynaptic locations would not be selective for noxious transmission, and could explain the small reduction of low-threshold activity with doses that abolish nociceptive C-fibre activity, observed in this study and by others (Chapman & Dickenson, 1992; Suzuki et al., 1999). Thus, minor inhibitions of low threshold inputs by opioids are likely to account for the weak effects of spinal opioids on allodynias.

Another explanation for the reduced efficacy in neuropathy is the antagonism of the inhibitory actions of opioids by the CCK system (see Wiesenfeld-
There are two identified receptors for the peptide CCK, named CCK1 and CCK2 (Alexander, 1998), the latter being the predominant type in the spinal cord of rats, whereas it is CCK1 in primates (Hill et al., 1990; Ghilardi et al., 1992). Under non-pathological conditions primary afferent neurones do not contain the peptide CCK, whereas interneurones in the superficial laminae and laminae IV-VI of the dorsal horn appear to be its primary source. Peripheral nerve injury has been shown to induce an upregulation of CCK mRNA in ipsilateral DRG neurones (Verge et al., 1992; Xu et al., 1993), though not accompanied by an increase in peptide immunoreactivity. Whereas upregulation of the CCK2 receptor mRNA in DRG neurones (Zhang et al., 1993b) is reflected by increases in the receptor protein, particularly in the superficial layers of the lumbar dorsal horn (see Wiesenfeld-Hallin et al., 1999). CCK appears to function as an anti-opioid peptide although its mechanism is not fully understood. It is not due to a direct hyperalgesic effect, since CCK does not alter non-pathological pain thresholds, nor mediated via binding to opioid receptors. CCK receptor antagonists have been found to be antinociceptive against neuropathic pain administered alone and are able to re-establish the effect of morphine lost after neuropathy (see Wiesenfeld-Hallin et al., 1999). Thus, it is suggested that nerve injury leads to increased activity in the CCK system, through either changes in the peptide or its receptors, and this in turn reduces the efficacy of opioids in neuropathic pain which can possibly be overcome by dose escalation.

Inconsistencies in the literature surrounding morphine efficacy in neuropathic situations may also be explained by the method employed to assess nociceptive behaviour, variable in both stimulus nature and intensity and thus likely to be processed via different pathways. For example two types of mechanical allodynia, static and dynamic, are evident following nerve damage, determined by the type of stimuli required for their detection (Koltzenburg et al., 1992; Ochoa & Yarnitsky, 1993; Field et al., 1999a). Static allodynia is evoked by increasing pressure to the skin (such as von Frey stimulation) and in neuropathic pain patients has been shown not to depend on Aβ-fibres, since it survives compression-ischaemia that interrupts conduction in myelinated fibres (Ochoa & Yarnitsky, 1993). Static allodynia is however, dependent upon capsaicin-sensitive Aδ-fibres. Dynamic mechanical allodynia, induced by lightly stroking the surface of the skin appears to be signalled by the large diameter myelinated Aβ-sensory neurones (Kajander et al.,
Morphine has previously been shown to block static alldynia following systemic administration in nerve ligation models of neuropathic pain (Bian et al., 1995; Lee et al., 1995), where however, spinal administration was ineffective. In contrast in the model of diabetes-induced neuropathy both spinal (Calcutt & Chaplan, 1997) and subcutaneously administered morphine (Field et al., 1999b) were shown to be effective at blocking static alldynia, yet the dynamic component was left intact (Field et al., 1999b). The latter study, which also employed identical doses of subcutaneous morphine to those used here, is in accordance with the reductions in the evoked neuronal responses to low von Frey stimuli that I have demonstrated. Furthermore to the effects of morphine upon static but not dynamic alldynia, the responses of dorsal horn neurones to Aδ and C-fibre, but not Aβ-fibre stimulation have also been shown to be blocked by morphine (Le Bars et al., 1976). It has also been demonstrated that stroking of the hindpaw ipsilateral to CCI induced Fos expression (considered an indirect marker of nociceptive processes) at the spinal cord level in superficial (laminae I-II) and deep dorsal horn (laminae V-VI). This was not observed in control animals, and was insensitive to morphine, in contrast to that evoked by noxious heat (Catheline et al., 2001). The lack of effect of morphine on stroking-evoked Fos expression in the dorsal horn supports the hypothesis that tactile allodynia is related to the activation of large primary afferent fibres (Koltzenburg et al., 1994a; Koltzenburg et al., 1994b; Woolf, 1994). This appearance of Fos immunoreactivity in the superficial dorsal horn evoked by low-threshold stimuli possibly results from Aβ-fibre sprouting into laminae I-II (see section 1.3.2.3) or activation of C-fibres with reduced thresholds after nerve injury.

The results of the present study and pre-clinical investigations discussed provide a basis for the difficulties encountered clinically surrounding the efficacy of opioids in the treatment of neuropathic pain where their effectiveness remains controversial, as will be discussed later (Arner & Meyerson, 1988; Portenoy et al., 1990; Foley & Portenoy, 1991; Kupers et al., 1991; Rowbotham et al., 1991; Jadad et al., 1992; Cherny et al., 1994; Moulin et al., 1996; Kingery, 1997; Benedetti et al., 1998; Dellemijn, 1999). Indeed, the route of administration, the test used for pain evaluation (possibly relating to the nature and symptoms of pain experienced in the clinic) and the origin of lesion employed in pre-clinical studies impacts upon the
relative effectiveness of opioids which may translate to it clinical management.

7.4.2 Effect of Gabapentin Alone

It has been demonstrated here that subcutaneously administered GBP, at the highest dose used (20 mg/kg, which was intentionally low for the main focus of the study yet is within the dose range used clinically), had a greater inhibitory effect on the electrically- and naturally-evoked dorsal horn neuronal responses in rats subject to SNL in comparison to sham-operated and naïve animals. Effects of GBP were established around 40 - 50 minutes. The systemic doses used here and time course of effects observed electrophysiologically are in accordance with those seen behaviourally, not associated with any motor deficits (Field et al., 1999b; Kayser & Christensen, 2000).

Behavioural studies show GBP has a negligible effect against physiological sensory nociception to thermal (Hunter et al., 1997), mechanical (Field et al., 1997b) and chemical stimuli (Shimoyama et al., 1997b; Yoon & Yaksh, 1999), yet it is effective in pathophysiological situations involving the presence of central sensitization. Indeed, in various models of nerve injury, inflammation and surgery, GBP has been shown to reduce any resultant mechanical/thermal allodynias and hyperalgesias. Systemically, GBP has been shown to be antinociceptive and antiallodynic in nerve injury models of SNL (Hunter et al., 1997; Abdi et al., 1998; Field et al., 2000), CCI (Hunter et al., 1997; Kayser & Christensen, 2000) and PSL (Pan et al., 1999), and also reduced evoked dorsal horn neuronal responses after SNL (Chapman et al., 1998a). In the nerve injury model of diabetic neuropathy systemic and intrathecal GBP blocked both static and dynamic components of mechanical allodynia, (Field et al., 1999b; Field et al., 2000). GBP prevented only the second phase, which reflects central sensitization (Coderre et al., 1990), of the formalin response when administered intrathecally (Field et al., 1997b; Shimoyama et al., 1997b; Yoon & Yaksh, 1999) and systemically (Field et al., 1997b). Both systemic and intrathecal GBP blocked carrageenan-induced thermal and mechanical hyperalgesia (Field et al., 1997b), which was supported by its systemic ability to block the maintenance of carrageenan-induced sensitization of dorsal horn neurones (Stanfa et al., 1997). Additionally after SNL, subarachnoid GBP relieved tactile
allodynia (Hwang & Yaksh, 1997), systemic GBP prevented the development of mechanical allodynia and hyperalgesia in a model of post-operative pain (Field et al., 1997a), and intrathecal GBP was demonstrated to reverse thermal hyperalgesia (Jun & Yaksh, 1998). GBP also had some inhibitory effect in the experimentally appropriate sham-operated control group, yet had a tendency to increase the neuronal responses of the un-operated naïve group. This is in accordance with other similar studies and may well result from the ability of the drug to act in inflammatory states (Stanfa et al., 1997; Chapman et al., 1998a).

Peripheral nerve damage leads to the generation of ectopic activity at the injury site (Wall & Devor, 1983) or within the DRG (Wall & Devor, 1983; Kajander et al., 1992). This provides an ongoing, continual barrage of neuronal activity via primary afferents into the spinal cord which could lead to central sensitization and hyperexcitability (Gracely et al., 1992; Sheen & Chung, 1993; Yoon et al., 1996). Interestingly it has been demonstrated that GBP inhibits the ectopic discharge activity from injured nerve sites in the PSL model at doses determined therapeutic for tactile allodynia (Pan et al., 1999), yet not after SNL (Abdi et al., 1998). Throughout all these pre-clinical studies, systemic doses of GBP are within a similar range (10 - 300 mg/kg). However required therapeutic doses of GBP utilizing the spinal route of application are much lower (10 - 100 µg per animal) and the positive effects of direct administration indicates GBP has a spinal site of action. As yet mechanism(s) of its anticonvulsant/analgesic effects remain elusive.

GBP was originally designed as an analogue of GABA that would cross the blood brain barrier (Satzinger, 1994), for indication as an anticonvulsant. Nonetheless, GBP does not bind to either GABA_A or GABA_B receptors, it is not converted metabolically into GABA and is neither a substrate nor an inhibitor of GABA transport (Taylor et al., 1998). Some evidence suggests that GBP increases GABA release, thereby enhancing inhibitory neurotransmission (Gotz et al., 1993). More recently it has been shown that the antiallodynic action of GBP is not sensitive to either GABA_A or GABA_B receptor antagonists (Hwang & Yaksh, 1997; Patel et al., 2001), therefore the increase in CNS GABA levels are unlikely to be responsible for the mode of action of GBP in models of pain. In vitro, GBP stimulated the
activity of catabolic enzyme glutamate dehydrogenase at high concentrations implicating effects on glutamate metabolism (Goldlust et al., 1995). However, the onset of these metabolic effects on neurotransmitters would be too slow to solely account for the time course of antinociceptive effects observed as early as 15 minutes after intrathecal GBP administration (Shimoyama et al., 1997a). GBP does not affect ligand binding at a variety of commonly studied drug and neurotransmitter binding sites and voltage-activated ion channels including GABA, glutamate and glycine receptors, as demonstrated by lack of inhibition of [³H]-GBP binding (Taylor, 1995). A range of anticonvulsants including, carbamazepine and ethosuximide, also do not displace bound [³H]-GBP, suggesting a novel pharmacological site to which binding is important for GBP’s antiepileptic activity (Taylor et al., 1998). In vivo behavioural studies have shown that the antinociceptive effects of systemic GBP are prevented by D-serine, a glycine/NMDA receptor agonist (Singh et al., 1996). However, radioligand binding assays have not shown GBP to inhibit strychnine-insensitive [³H]-glycine binding to brain membranes or to influence the binding of [³H]-MK-801 to the NMDA receptor channel, and D-serine does not bind to the novel GBP binding site (Suman-Chauhan et al., 1993). Although many studies have attempted to establish the mechanisms responsible for the actions of GBP, identification of definite cellular or molecular target(s) is still awaited. Given the possibilities, it is fairly probable that different mechanisms account for the anticonvulsant, antinociceptive and anxiolytic actions of GBP. This is supported by the fact that GBP has a delayed anticonvulsant effect after bolus injection in rats (Welty et al., 1993) compared to the analgesic action attained rapidly after intrathecal injection (Field et al., 1997b).

By far the most convincing, and experimentally supported theory for the mode action of GBP, over the other possibilities, is a mechanism involving the inhibition of Ca²⁺ influx via VDCCs, which is of interest to this thesis. The only GBP binding site to be identified was originally purified from pig brain and found to correspond to the α₂δ subunit of VDCCs (Gee et al., 1996). The VDCC α₂δ subunit is expressed in skeletal muscle, brain and heart (Ellis et al., 1988). In vitro studies have shown that the carboxyl δ peptide contains the transmembrane domain that plays a role in anchoring the α₂δ subunit and in stabilizing subunit interactions (Jay et al., 1991; Gurnett et al., 1996). The remaining majority of the protein is
extracellular (Brickley et al., 1995; Gurnett et al., 1996; Wiser et al., 1996) suggesting little interaction with intracellular molecules such as protein kinases and G-proteins. The $\alpha_2\delta$ subunit is likely a rate-limiting factor in VDCC assembly, since coexpression with other VDCC subunits results in enhancement of current amplitude (Mikami et al., 1989; Brust et al., 1993; Gurnett et al., 1996) and increased expression of $\omega$-conotoxin and DHP binding at the cell surface (Singer et al., 1991; Brust et al., 1993). Since $\alpha_2\delta$ is able to modulate VDCC function, and coexpression /assembly with the $\alpha_1$ subunit is required for the physiological activation of $\text{Ca}^{2+}$ currents (Gurnett et al., 1996), interaction of GBP with this subunit may permit the drug to influence neuronal excitability. Indeed, there are several in vitro studies establishing this link. GBP inhibited $\text{Ca}^{2+}$ currents in isolated rat brain neurones (Stefani et al., 1998) and $\text{K}^+$-induced $\text{Ca}^{2+}$ influx in synaptosomes (Ma & Woolf, 1995; Fink et al., 2000; Meder & Dooley, 2000) and subsequent neurotransmitter release from rat neocortical slices (Fink et al., 2000). GBP presynaptically inhibited glutamatergic synaptic transmission in rat spinal cord slices (Shimoyama et al., 2000), predominantly in the superficial lamina where nociceptive primary afferent fibres terminate. Therefore antinociceptive effects of GBP may involve the inhibition of the release of excitatory amino acids from presynaptic terminals. Furthermore GBP inhibited synaptic transmission in the superficial dorsal horn, using spinal cord slices from hyperalgesic, but not normal, rats (Patel et al., 2000). GBP also reduced HVA $\text{Ca}^{2+}$ current amplitude in DRG cells in a use-dependent manner (Alden & Garcia, 2001) over a clinically effective range (10 - 100 $\mu$M) as determined from the serum of human patients (Taylor et al., 1998). A consistent inhibition of HVA $\text{Ca}^{2+}$ conductance, but not $\text{Na}^+$ or glutamate currents and GABA responses, was mediated by GBP in isolated adult rat cortical neurones (Stefani et al., 2001). Similarly GBP inhibited HVA $\text{Ca}^{2+}$ currents in the same neurones in which $\text{Na}^+$ channels were unaffected (Wamil & McLean, 1994). Furthermore, the inactivation steady-state properties of $\text{Ca}^{2+}$ channels were not significant modified by GBP, whilst deactivation gating was slightly modulated, probably through interaction with the $\alpha_2\delta$ subunit. When studied in different expression studies $\alpha_2\delta$ has been shown to be responsible for a substantial increase of the whole-cell $\text{Ca}^{2+}$ current (Stefani et al., 2001). This is probably not only a result of increased $\alpha_1$ subunit in the plasma membrane, but mainly through alterations in the mean open time of the channel itself (Shistik et al., 1995; Gurnett et al., 1996; Saegusa et al., 2000). In contrast, GBP has
been shown to be without effect on Ca^{2+} current effects when tested in cultured nodose and DRG neurones (Rock et al., 1993) and acutely dissociated human hippocampal granule cells (Schumacher et al., 1998), which may be explained by their use of cultured neuronal cells subject to developmental variables.

The \( \alpha_2\delta \) subunit is common to all VDCCs, yet GBP has been demonstrated to specifically target chronic pain with minimal adverse effects (Field et al., 1997a; Field et al., 1997b). The ubiquitous distribution of VDCCs may thus require specific subunit combinations and particular modulatory conditions to allow GBP to exert its inhibitory actions under pathophysiological circumstances. Three different \( \alpha_2\delta \) subtypes (\( \alpha_2\delta-1, 2 \) and 3) and several splice variants with differing distributions have recently been described (Klugbauer et al., 1999; Hobom et al., 2000), and it appears that GBP may only interact with \( \alpha_2\delta-1 \), based on comparative amino acid sequences and mRNA distributions with the GBP-binding protein (Taylor et al., 1998). Expression of a rat DRG \( \alpha_2 \) subunit, unique at least at the post-translational modification level, has also been reported (Luo, 2000). Nerve injury induces marked tissue-specific up-regulation of the DRG VDCC \( \alpha_2\delta \) subunit (Luo et al., 2001). The latter study demonstrated that upregulation of DRG \( \alpha_2\delta \) mRNA and protein was evident in rats with SNL, unilateral sciatic nerve crush, but not dorsal rhizotomy, indicative of a peripheral site of regulation of expression. After SNL a temporal correlation between DRG \( \alpha_2\delta \) subunit upregulation and allodynia was also established. Differential expression of distinct \( \alpha_2\delta \) subunits in spinal cord and DRG indicated that spinal cord \( \alpha_2\delta \) was not synthesized and retrogradely transported from DRG. The reported spinal antihyperalgesic actions of intrathecal GBP could actually result from drug interactions with spinal cord and/or DRG \( \alpha_2\delta \) subunits as other intrathecally delivered agents have been shown to access DRG (Porreca et al., 1999; Lai et al., 2000). Since tissue-specific expression of the DRG \( \alpha_2\delta \) is implicated in models of neuropathic pain, this subunit may be an important component in governing tissue-specific functions of VDCCs and neuroplasticity contributing to allodynia after nerve injury. It is known that the \( \alpha_2\delta \) subunit is common to all VDCCs (Hofmann et al., 1994; Isom et al., 1994) therefore GBP actions may involve more than one type of VDCC. Given the predominant role of N-type VDCCs in neuropathic pain after nerve injury (see chapter 4), in conjunction with the
heterogeneity of α₂δ (Luo, 2000) subunits it is likely that functional and tissue-specific expression of the N-type VDCCs may depend on its subunit composition. This would allow N-type VDCCs to make unique contributions to nerve injury-induced neuropathic pain and also provides GBP with a potential subunit-specific interaction associated with the pain pathway, which may explain its specificity of action.

Some other intriguing evidence supporting the α₂δ subunit as the mediator of GBP’s antihyperalgesic actions comes from studies involving stereoisomers of GBP analogues. To date GBP and (S)-3-isobutylgaba (pregabalin) are the most active compounds that interact with the isolated GBP-binding site, predicted to be α₂δ. Pregabalin stereoselectively inhibits [³H]-GBP binding to brain membranes with the (S)-isomer showing similar affinity as GBP in contrast to the R-isomer which was shown to be 10 times weaker (Taylor et al., 1993). Using the model of diabetes-induced peripheral neuropathy (Field et al., 1999b; Field et al., 2000) and SNL (Field et al., 2000), GBP and pregabalin blocked allodynia with similar potency whereas its (R)-isomer was inactive. These reports are consistent with the involvement of the α₂δ subunit in the mediation of GBP’s antinociceptive effects. Similar stereoselective effects have also been reported in animal models of epilepsy (Bryans et al., 1998), reinforcing the hypothesis that modulation of HVA Ca²⁺ currents is critical (although probably not sufficient) in the putative effect of GBP as an anticonvulsant.

There is an established role for VDCCs in nociception and the contribution of the different channel subtypes appears to alter depending on the nature of the pain (see Vanegas & Schaible, 2000). Chapter 4 clearly demonstrates a predominant nociceptive role for N-type VDCCs, which is enhanced after neuropathy. In contrast, chapter 4 also demonstrates that the P/Q-type channels play a modest role in sensory transmission in a manner that is not altered after nerve injury. Likewise, in chapter 5, inhibition of the T-type channel with the anti-epileptic ethosuximide was also shown to have a limited effect on evoked dorsal horn neuronal responses, again remaining unchanged after neuropathy. Upon substantial membrane depolarization N- and P/Q-type VDCCs mediate the release of excitatory neurotransmitters, such as glutamate,
substance P and CGRP, critical for wind-up and central sensitization, in the presence of constant afferent input (Dickenson, 1994). Since T-type channel activation occurs close to resting potential, they allow Ca\(^{2+}\) influx when cells are at rest thus regulating cell excitability and most likely the depolarization required to activate high-voltage activated N- and P/Q-type channels necessary for neurotransmission. The discussions raised in chapters 4, 5 and 6 suggest that the N-type VDCCs are selectively subject to plasticity after nerve injury and thus could potentially be the main candidate target for GBP, if indeed it does exert its actions through VDCCs.

A few electrophysiological studies have attempted to define the VDCCs inhibited by GBP, but no general consensus has been determined. It has been demonstrated that GBP alters the affinity of DHP for the Ca\(^{2+}\) channel (Stefani et al., 2001). In another study GBP inhibited whole-cell Ca\(^{2+}\) currents recorded from neurones isolated from adult rat brain in a fast and voltage-independent manner. DHPs (nimodipine and nifedipine) prevented the GBP-mediated inhibition, whereas \(\omega\)-conotoxin GVIA and MVIIIC (a P/Q-type VDCC blocker) partially reduced its effect (Stefani et al., 1998). They hypothesize that L-type, in comparison to N- and P/Q-type, are the predominant VDCC target of GBP in these neurones. However this study failed to present data comparing the level of inhibition achieved with the used doses of L-, N- and P/Q-type VDCC blockers alone. It appears that blockade of L-type Ca\(^{2+}\) current produced substantial inhibition of the Ca\(^{2+}\) current leaving very little to be further inhibited by a dose of GBP that did not produce a similar inhibition alone. In contrast, the inhibition produced by the \(\omega\)-conotoxins alone was comparable to that achieved with GBP alone. Additionally, differences in the mode of VDCC antagonism exerted by DHPs and the \(\omega\)-conotoxins might possibly affect the binding of GBP to the \(\alpha_2\delta\) subunit or its subsequent inhibitory mechanism and thus these results should be viewed with caution. Given that L-type VDCCs are not involved in neurotransmitter release and play a minimal role in sensory transmission, not altered by peripheral nerve damage, it seems functionally unlikely that GBP would exert its antinociceptive effects through these channels \textit{in vivo}. Moreover, the critical role of N- and P/Q-type VDCCs in neurotransmission and the similarities in the enhanced role of N-type VDCCs in nociception and antinociceptive actions exerted by GBP after nerve injury highlight these channels as a much more convincing substrate. GBP has been shown to inhibit glutamate and aspartate release.
by 20% from synaptosomes (Fink et al., 2000) and this suggests that GBP inhibited Ca$^{2+}$ influx into glutamatergic terminals by acting on P/Q-type VDCCs, which predominate on synaptosomal terminals (Burke et al., 1993; Luebke et al., 1993). In contrast, other studies GBP has been shown not to significantly affect L-, N-, or T-type Ca$^{2+}$ currents (Rock et al., 1993). However given the diversity, and tissue specific expression of the various VDCC subunits, the potential combination heterogeneity of VDCCs at the structural level is enormous. It is possible that GBP exerts functional effects only with a particular combination of subunits. Moreover, these effects specific to chronic pain states may be observed only under pathophysiological conditions, perhaps dependent upon situations that mimic the neuronal excitability that characterizes epilepsy, GBP's initial clinical target.

Presented with all the pre-clinical and in vitro evidence there is a substantial body of evidence linking an α2δ-mediated GBP mode of action, certainly with respect to chronic pain. It remains to been seen whether the α2δ subunit has an as yet, undiscovered alternative physiological function. Although so far, the interaction of GBP with the α2δ most likely impacts upon the functioning of one or more subtypes of VDCCs.

7.4.3 **Effect of Gabapentin and Morphine Co-administration: Clinical Implications**

The present investigation has convincingly demonstrated that after nerve injury the evoked dorsal horn neuronal responses, shown to be refractory to systemic morphine, become susceptible to its inhibitory actions in a dose-related manner when in the presence of systemic GBP (itself at a dose that alone mediated negligible neuronal inhibitions). These inhibitions were reversed by spinally applied naloxone, which indicates a spinal site of action and provides a way of reversing the effects of the combination if adverse effects arose with the combination in patients. Since the two agents, at ineffective doses alone, interacted in a positive manner, block of the effect of one of the agents abolishes the entire effect. The spinal cord site of interaction of the combination indicates that the substrate acted upon by GBP must reside on circuitry also possessing μ opioid receptors. This would support the ideas
expounded above that tissue-specific, and in this context, spinal $\alpha_2\delta$ subunit events could well mediate the observed effect.

In behavioural studies systemic GBP has been shown to have effects that peak 1 hour after administration, and that are still apparent at 5 hours (Hunter et al., 1997). This indicates that GBP would still be active throughout the entire time-course of an experiment in the present study where morphine was given 1 hour after GBP and then followed for a further 2 hours. Thus, there is great clinical promise for the treatment of poorly opioid-responsive neuropathic pains for which many cases morphine dose-escalation becomes limited by adverse side-effects. The majority of the evoked neuronal responses were significantly inhibited by a combination of GBP and morphine, the greatest effects being seen on the C-fibre, postdischarge, input and von Frey evoked responses. These observations are important since high-threshold C-fibres specifically relay nociceptive information, post-discharge is a measure of spinal cord hyperexcitability and von Frey evoked responses may relate to behaviourally observed mechanical allodynia, all important features of neuropathy. In contrast, the A$\beta$-fibre response was relatively spared, which may indicate that physiological touch sensations would be left intact (see chapter 3).

Other pre-clinical studies have also investigated the use of morphine in conjunction with other drugs. GBP has been shown to enhance the antinociceptive effects of morphine in the rat tail flick test (Shimoyama et al., 1997a). Intrathecal morphine and N-type VDCC blockers have also been proven to mediate additive and synergistic inhibitions in rat models of nociception (Wang et al., 2000a; Pirec et al., 2001), which is supportive of an N-type VDCC mediated inhibitory effects of GBP. After nerve injury synergistic effects of morphine have been observed with clonidine (Ossipov et al., 1997) and with neostigmine (Hwang et al., 2000). However, GBP’s central action does not involve an opiate mechanism, tolerance does not develop and morphine tolerance does not cross-generalize to GBP’s antihyperalgesic effects (Field et al., 1997b).

L-type VDCC blockers have been clinically investigated for their analgesic effects when administered alone and in combination with morphine. In the absence
of a pathophysiological pain state, these studies revealed no L-type VDCC mediated analgesic effects (Hasegawa & Zacny, 1997a). However, nimodipine decreased the required morphine dose in cancer patients with severe pain who had developed morphine tolerance (Santillan et al., 1998), an effect that possibly reflects alterations in L-type VDCCs related to the mechanism of morphine tolerance (Bernstein & Welch, 1995, see also chapter 6). This lends credence to the notion that GBP is unlikely to exert its analgesic effects via L-type VDCCs.

There is an increasing amount of clinical data in support of the control of various neuropathic pains with GBP, such as postherpetic neuralgia, painful diabetic neuropathy, multiple sclerosis and trigeminal neuralgia, to name a few (Nicholson, 2000). Two separate randomized, double-blind studies in postherpetic neuralgia patients (Rowbotham et al., 1998) and patients with diabetic peripheral neuropathy (Backonja et al., 1998) reported significant reductions in pain scores compared to placebo over a period of 8 weeks. Furthermore, improvements on sleep and mood were noted for the GBP-treated patients and tolerance did not appear to develop. Reported adverse effects included somnolence and dizziness, however occurrence of these was minimal and where experienced only mild to moderate. Alongside other studies and case-reports it has been proposed that GBP is a useful first line treatment for chronic neuropathic pain, especially where other therapies fail (see Nicholson, 2000, for review). GBP has substantial advantages concerning pharmacokinetics, safety and tolerability in comparison with established anticonvulsants, as their clinical use is often limited by a small therapeutic range and several drug interactions (Patsalos & Duncan, 1993; Dallocchio et al., 2000). Clinically, GBP is well tolerated and does not show any acute or chronic toxicity, it is excreted by glomerular filtration unmetabolized, and does not present any unwarranted drug interactions (Goa & Sorkin, 1993). However larger long-term controlled trials are needed to determine the place of GBP in relation to other therapeutic options (Hemstreet & Lapointe, 2001).

On the other hand, the clinical use of opioids in neuropathic pain is more complex and varied responsiveness is reported which may be due to the different types of pain, mediated by differing causal dysfunctions. There have been many
randomized, double blind controlled trials conducted in this area (Dellemijn, 1999) and morphine effectiveness ranges from opioid resistant (Arner & Meyerson, 1988) to modest pain relief (Rowbotham et al., 1991) to good pain relief (Likar et al., 1999). It is widely believed that neuropathic pains are less susceptible, but not resistant to systemic morphine (Jadad et al., 1992; Cherny et al., 1994), as observed in the present study. Results from pre-clinical studies indicate that the route of administration, the test used for pain evaluation (possibly relating to the nature of pain experienced in the clinic) and the origin of lesion employed in pre-clinical studies impacts upon the relative effectiveness of opioids which may translate to it clinical management. Although dose-escalation appears to overcome the lack of antinociceptive actions of opioids in animal models of neuropathic pain, clinically this is hindered by side-effects (such as sedation, nausea, vomiting, constipation and respiratory depression) (Foley & Inturrisi, 1987), or the development of tolerance (Portenoy, 1994). As a consequence, opioid monotherapy may result in inadequate analgesia. Because a multiplicity of mechanisms are involved in pain the limitations in the use of opioids can be overcome by combination with one or more non-opioid analgesics to obtain a more favourable balance of analgesia and side-effects.

There are many benefits to a multi-drug regimen. Since neuropathic pain has many causes and resultant symptoms, the underlying dysfunctional mechanisms are likely to be diverse, hence its problematic clinical management. The use of GBP to target the excitatory system and morphine to target the inhibitory system, tackles a sensitized spinal cord from two perspectives. It is also likely to offer a better side-effect profile since, as shown in this study, only low doses of both GBP and morphine, each alone being ineffective, were required to mediate significant effects. The clinical use of GBP in conjunction with morphine may also confer important characteristics of GBP-mediated antinociception, seen in pre-clinical studies, that morphine lacks. In particular its ability to block both static and dynamic components of mechanical allodynia (Field et al., 1999b), and the lack of tolerance development with chronic use (Field et al., 1997b).

Since the undertaking of the present investigation two clinical studies have been published also examining the potential therapeutic potential of a morphine and
GBP combination for analgesia (Caraceni et al., 1999; Eckhardt et al., 2000). Eckhardt et al (2000) investigated in a randomized, placebo-controlled, double-blinded study, the pharmacodynamic and pharmacokinetic interaction of GBP and morphine in 12 healthy volunteers using the cold pressor test. GBP was shown to have no analgesic effect on its own after a single oral dose of 600mg in comparison to placebo, which was not surprising since the cold pressor test is not accompanied by tissue or nerve damage. The same dose of GBP significantly enhanced the analgesic effect of morphine in comparison to morphine alone. Only the pharmacokinetics of GBP and not the pharmacokinetics and metabolism of morphine were significantly altered upon coadministration. Morphine alone led to the expected opioid-mediated side-effects, however these were no different to those observed with the morphine and GBP combination. Caraceni et al (1999) studied the combination of morphine and GBP in patients suffering neuropathic cancer pain where improved analgesia in comparison with morphine alone was demonstrated. These results warrant further controlled trials in patients for the relevance of the combination of morphine and GBP for treating severe pain, where the analgesic effect of GBP should be more pronounced than in healthy volunteers, considering the data derived from animal models of chronic pain.

Pre-clinical and clinical results support the clinical investigation of GBP and morphine in combination for the treatment of chronic pain, which responds poorly to opioids. Although, pre-clinical data is invaluable in elucidating drug effectiveness, caution must be exerted when attempting to extrapolate animal antinociception and neuronal responses to human analgesia where pain perception also involves its intensity and unpleasantness. None the less, the results I present here are generally in accordance with the observed clinical data surrounding the use of morphine and GBP alone in neuropathic pain, and together in the limited clinical studies reported. Alongside the favourable profile of GBP, especially in comparison with other anticonvulsants, the clinical investigation of the additional administration of GBP in patients suffering severe and chronic pain treated with morphine is warranted. The limitations of opioids have led to the concept of combination therapy in the hope of attaining a favourable balance of analgesia and side-effects with a reduction in the dose. I have demonstrated here the potential for the use of low dose morphine and GBP combinational treatment after neuropathy. A GBP/morphine combination
should result both in better pain control, including types of pain that respond poorly to opioids, and a decrease in morphine dose and consequently a decrease in opioid-mediated adverse reactions.
CHAPTER 8

FINAL DISCUSSION
Basic pain research in the preclinical setting has made many advances in recent years in elucidating the neurobiology of neuropathic pain. However, clinical interpretation and medical use of this new-found knowledge has been relatively slow and pharmacological treatment of neuropathic pain remains contentious. In terms of aetiology, anatomical site of nerve lesion and symptoms, neuropathic pain is somewhat heterogeneous. Causes are diverse and range from trauma to metabolic disorders, such as diabetes, to viral infections, such as herpes zoster. Attempts to apply particular treatment regimens have previously been based upon a pathology-based classification, however on the whole, the various clinically used drugs have not clearly been seen to exert conspicuous differences in their effects on various types of neuropathic pain (McQuay et al., 1995; Sindrup & Jensen, 1999). Despite such sources of diversity many neuropathic conditions manifest common clinical phenomena, including sensory deficits, allodynia and hyperalgesia, however these are encountered by patients in various combinations and to differing extents, and thus it may be at the level of experienced symptoms that underlying neuropathic mechanisms differ. This concept is not encompassed by a pathology-based approach to neuropathic pain management and thus may be the source of much of the contention. A fresh perspective in neuropathic pain pharmacotherapy is now suggested to be symptom-based (Woolf et al., 1998). Although this has been put forward as a new approach, already in practice, anticonvulsants are generally used to control the lancinating or stabbing qualities of neuropathic pain whereas constant burning pain components are tackled with antidepressants and Na⁺ channel blockers (MacFarlane et al., 1997). However, this approach still requires a more definitive appreciation of the underlying pathophysiology. Improved knowledge of the changes induced by nerve injury ascertained from pre-clinical studies together with clinical information regarding quantification of specific symptoms and particular drug effectiveness in various neuropathic conditions should permit a judicious approach to effective treatment.

Recent work with animal models has indicated that several key events contribute towards the pathogenesis of abnormal pain states following peripheral nerve injury. Dysfunctions in both the peripheral and central nervous systems are implicated and interaction between the two pathogenic loci is critical. Peripheral nerve damage has been shown to enhance expression and initiate redistribution of
channels including some of the newly discovered $\mathrm{Na}^+$ channels from sensory neurone cell bodies to the injury site (Novakovic et al., 1998). Peripherally, this contributes to the appearance of ectopic discharge in damaged primary afferent axons and the emergence of abnormal evoked responses in intact primary afferent axons within the damaged nerve, and these aberrant signals are communicated to the CNS where further changes ensue (Wall & Devor, 1983). This is corroborated by the observation that evoked pains are sustained after resolution of the ectopic discharge in some animal models of neuropathic pain (Scadding, 1981). Despite the fact that these peripheral mechanisms appear to be the origin of pathophysiological pain states, many of the drugs that act to inhibit peripheral excitability via $\mathrm{Na}^+$ channel block have narrow therapeutic windows. Identification of novel drugs to target the specific $\mathrm{Na}^+$ channels responsible in these locations, that lack side-effects, are awaited. The key role of peripheral mechanisms cannot be understated but the drugs that block $\mathrm{Na}^+$ channels, although shown to exert effects at peripheral sites, will also reduce the excitability of central neurones, and indeed are used exactly for this latter effect in epilepsy.

In the CNS the key pathogenic event seems to be triggered in dorsal horn neurones by C-fibre inputs, although a role for A$\delta$-fibres cannot be excluded. Thus, peripherally driven, high frequency ectopic discharge culminates in the generation of central sensitization. This involves enhanced spinal neurotransmitter release, expanded receptive fields and increased dorsal horn neuronal excitability, which are indicative of the associated central spinal plasticity possibly responsible for the evoked pains of neuropathic conditions. Reorganization of receptive fields is an established characteristic of nerve injury-induced neuronal plasticity, and as discussed in chapter 3, this was reflected by the increased receptive fields of convergent dorsal horn neurones mapped to low von Frey hair stimulation in the animal model of SNL. For any given stimulus it is expected that an increased number of spinal neurones would be recruited in the presence of an expanded receptive field size, such that a greater afferent input and likely enhanced pain transmission would result (Suzuki et al., 2000b). Peripheral nerve damage-induced plasticity of voltage-dependent and ligand-gated ion channels is also apparent at the level of the spinal cord and there are numerous drugs that act upon the various systems to reduce central hyperexcitability. These agents produce reductions in transmission mediated
by modulation of inhibitory systems, such as morphine, or blockade of excitatory
events such as ketamine at the NMDA-receptor complex. Given the convergence of
peripheral afferent input that occurs at the level of the spinal cord, however
appealing the goal of a symptom-based therapy for neuropathic pain, this might not
be attainable through modulation of specific aspects of the pain pathway. It appears
somewhat ambiguous that this would be symptom or modality-specific. More
workable and possibly beneficial would be the targeting of aberrant excitatory or
inhibitory systems at play in neuropathic pain.

Common to all neuropathic pain states is increased dorsal horn neuronal
excitability and at the root of this is Ca$^{2+}$ entry into neurones via N- and P/Q-type
VDCCs, which is ultimately responsible for permitting neurotransmitter release.
Blockade of VDCCs may simultaneously attenuate the release of multiple
neurotransmitters that act at a number of receptor systems that participate in nearly
all states/models of hyperalgesia and allodynia. This offers an advantage over
individual receptor antagonists as they would have a broad mechanism of action that
would not be modality, or possibly symptom-specific. Block of neurotransmitter
actions at their source of release would possibly offer a treatment useful across the
spectrum of neuropathic syndromes. This description insinuates that blockade of
VDCCs is rather heavy-handed, however this approach does offer some beneficial
features, as has been clearly demonstrated and discussed in chapters 4, 5 and 6. Since
these channels are voltage-gated it should be possible to reduce aberrant transmission
leaving more physiological events unchanged. A predominant role for N-, over P/Q-,
T- and L-type VDCCs in sensory transmission has been established at the level of the
dorsal horn, in a manner that is enhanced after peripheral nerve damage, and thus
provides some specificity in mode of action. This is substantiated by numerous
behavioural studies where block of spinal N-type VDCCs is antiallodynic and
antihyperalgesic in models of neuropathy, and in contrast to opioids which may also
mediate a general inhibitory action including prohibiting neurotransmitter release,
block of VDCCs does not appear to generate tolerance (Malmberg & Yaksh, 1995;
Bowersox et al., 1996). Furthermore, immunolocalization has demonstrated N-type
channels to be ideally located in the superficial dorsal horn where nociceptive fibre
afferents terminate. Spinal P/Q-type VDCCs are found primarily on the nerve
terminals of dorsal horn neurones in laminae II-VI, but unlike N-type channels they
are rarely found in neurones that contain substance P (Westenbroek et al., 1998). Intriguingly, this finding may indicate that N-type channels regulate the release of neurotransmitters including glutamate, from peptide-containing C-fibres, whereas P-type could possibly be localized to non-peptide, IB4-positive neurones. The naturally occurring peptides that selectively block N-type VDCCs are unsuitable for clinical application, but the synthetic analogue SNX-111 has shown encouraging analgesic effects in clinical trials. Despite the generation of a potentially new drug for the treatment of neuropathic pain, SNX-111 still requires inconvenient intrathecal administration and is not without unwanted side-effects (Brose et al., 1997).

VDCCs may well be the critical link between the peripheral mechanisms of neuropathic (and also inflammatory) pain to central events. The role of peripheral Na\(^+\) channels is clear in both nerve and tissue damage-related pains. Changes in their activity, distribution and expression may well lead to changes in action potential characteristics. In fact, the TTX-resistant Na\(^+\) channels can have very different biophysical characteristics from the TTX-sensitive channels. Changes in the functional aspects of the combinations of these channels in peripheral nerve terminals could either cause repetitive firing or altered action potential shape such that the plateau phase becomes sustained (Waxman, 2001). Both could cause a greater number of VDCCs to be open.

Recently there has been a reappraisal in the effectiveness of traditional analgesics such as morphine. Historically, neuropathic pain has been labelled unresponsive to opioids, yet now it appears that this can be overcome in some situations by dose escalation (Portenoy et al., 1990; Jadad et al., 1992) and thus the opioids have been redeemed as useful for neuropathic pain control regimens. Unfortunately, they are not always effective in all patients and dose titration can be hindered by unpleasant side-effects. Treatment, more often than not, involves the utilization of other compounds that are not conventionally recognized as analgesics. Compounds originally developed for uses other than pain management, such as the antidepressants and anticonvulsants, have new-found roles as analgesics. The use of meta-analyses of all currently used mainline drugs shows that have almost identical efficacy (Sindrup & Jensen, 1999). Of current interest is the antiepileptic gabapentin,
thought to have a unique interaction with VDCCs through binding to the \( \alpha_2\delta \) subunit, which has been proven to provide relief from neuropathic pains (Backonja et al., 1998; Rowbotham et al., 1998) and is supported by a mounting body of preclinical data. Although the pain relief experienced by neuropathic patients with gabapentin appears to be no greater than that mediated by antidepressants or anticonvulsants, gabapentin is renowned for its minimal side-effects and good tolerability, lack of toxicity and lack of interaction with other drugs. Thus, although perhaps no more efficacious than more established therapies, gabapentin's worthy characteristic profile makes it a preferred analgesic for what can often be a long-term management of neuropathic pain. As yet, gabapentin can not considered a first-line treatment for neuropathic pain (Hemstreet & Lapointe, 2001), however its lack of pharmacokinetic interactions does make it an excellent candidate for add-on therapy where monotherapy is inadequate. Chapter 7 clearly demonstrates the benefits of combination therapy, whereby utilizing low systemic doses of both morphine and gabapentin, that alone mediate negligible inhibitory effects on dorsal horn neuronal responses, together mediated substantial inhibitions. In particular, in the presence of a neuropathic pain state, in contrast to gabapentin the inhibitory effects of morphine alone were reduced in comparison to normal, yet in this pathological scenario in conjunction with gabapentin, the most marked inhibitions were observed. Since gabapentin and morphine are clinically licensed drugs, low doses and convenient routes of administration were used, possibly offering advantageous side-effect profiles, translation of this combined therapy into the clinic deserves investigation for the treatment of neuropathic pain.

Progress in the clinical management of neuropathic pain requires both more detailed descriptions of experience of the clinical symptoms and effects of existing drugs from the clinical setting, alongside improved knowledge of the underlying dysfunctional mechanisms derived from basic scientific research. This should help better define mechanisms and drug targets involved in particular neuropathic pain syndromes. Hopefully this thesis has substantiated or even illuminated potential paths towards this goal.
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Effects of spinally delivered N- and P-type voltage-dependent calcium channel antagonists on dorsal horn neuronal responses in a rat model of neuropathy

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Abstract

Neuropathic pain, due to peripheral nerve damage, can include allodynia (perception of innocuous stimuli as being painful), hyperalgesia (increased sensitivity to noxious stimuli) and spontaneous pain, often accompanied by sensory deficits. Plasticity in transmission and modulatory systems are implicated in the underlying mechanisms. The Kim and Chung rodent model of neuropathy (Kim and Chung, Pain 50 (1992) 355) employed here involves unilateral tight ligation of two (L5 and L6) of the three (L4, L5, and L6) spinal nerves of the sciatic nerve and reproducibly induced mechanical and cold allodynia in the ipsilateral hindpaw over the 14 day post-operative period. In vivo electrophysiological techniques have then been used to record the response of dorsal horn neurones to innocuous and noxious electrical and natural (mechanical and thermal) stimuli after spinal nerve ligation (SNL). Activation of voltage-dependent calcium channels (VDCCs) is critical for neurotransmitter release and neuronal excitability, and antagonists can be antinociceptive. Here, for the first time, the effect of N- and P-type VDCC antagonists (ω-conotoxin-GVIA and ω-agatoxin-IVA, respectively) on the evoked dorsal horn neuronal responses after neuropathy have been investigated. Spinal ω-conotoxin-GVIA (0.1–3.2 μg) produced prolonged inhibitions of both the electrically- and low- and high-intensity naturally-evoked neuronal responses in SNL and control rats. Spinal ω-agatoxin-IVA (0.1–3.2 μg) also had an inhibitory effect but to a lesser extent. After neuropathy the potency of ω-conotoxin-GVIA was increased at lower doses in comparison to control. This indicates an altered role for N-type but not P-type VDCCs in sensory transmission after neuropathy and selective plasticity in these channels after nerve injury. Both pre- and post-synaptic VDCCs appear to be important. © 2001 International Association for the Study of Pain. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

Neuropathic pain arises from injury- or disease-evoked damage to the peripheral or central nervous system and patients often experience sensory deficits, ongoing pain, and stimulus-evoked pain (allodynia and hyperalgesia). Animal models of neuropathy have been critical in elucidating the complex causal mechanisms involving plasticity in nociceptive transmission and modulating systems. The spinal nerve ligation (SNL) model (Kim and Chung, 1992) involves tight ligation of two (L5 and L6) of the three spinal nerves that form the sciatic nerve. Behavioural consequences include thermal hyperalgesia, and mechanical and cooling allodynia (Kim and Chung, 1992; Chaplan et al., 1997).

Sensory neurones express a number of classes of voltage-dependent calcium channels (VDCCs) (L, N, P, Q, R, and T), distinguished by their electrophysiological and pharmacological profiles. N- and P-type VDCCs, sensitive to block by ω-conotoxin-GVIA and ω-agatoxin-IVA, respectively (Olivera et al., 1994), are widely expressed throughout the brain and spinal cord (Kerr et al., 1988; Mintz et al., 1992; Gohil et al., 1994). The N-type channel is concentrated in laminae I and II of the superficial dorsal horn, where nociceptive primary afferents synapse (Kerr et al., 1988; Gohil et al., 1994). In vitro studies have demonstrated the requirement of calcium ion influx through N- and P-type VDCCs for depolarization-coupled neurotransmitter release (Miljanich and Ramachandran, 1995). Spinal release of primary afferent peptides, calcitonin gene-related peptide (CGRP) and substance P, is ω-conotoxin-sensitive (Holz et al., 1988; Maggi et al., 1990; Santoscioli et al., 1992), and glutamate release is both ω-conotoxin- and ω-agatoxin-sensitive.

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Furthemore, calcium influx into neurones can enhance excitability and produce intracellular changes including gene induction.

VDCC antagonists are antinoceptive in models of inflammation, based on behaviour and in vivo electrophysiology, confirming a role for calcium influx into neurones in the spinal processing of nociceptive information. In the formalin test, behavioural antinoception is observed to varying degrees with specific N-, P-, and L-type VDCC blockers (Malmberg and Yaksh, 1994, 1995; Bowersox et al., 1996), and Diaz and Dickenson (1997) reported phase-dependent effects of N- and P-type blockers on neuronal responses. Blockade of N- and P-type VDCCs (Neugebauer et al., 1996; Nebe et al., 1997, 1998; Sluka, 1997) has been shown to reduce enhanced neuronal responses after knee joint inflammation and intradermal capsaicin (Sluka, 1998). Studies in models of neuropathy have been limited; however, calcium chelation (White and Cousins, 1998) and N-type VDCC antagonism have been shown to be effective (Chaplan et al., 1994; Xiao and Bennett, 1995; Bowersox et al., 1996; White and Cousins, 1998). In contrast, L- and P-type antagonists show little effect (Chaplan et al., 1994; White and Cousins, 1998).

Thus far, there are no in vivo electrophysiological studies investigating the role of spinal VDCCs in the processing of neuropathic pain. This study uses the SNL model, confirmed by behavioural testing, to induce a neuropathic state. After the establishment of neuropathy, electrophysiological studies of dorsal horn spinal neurones were made to investigate the effects of spinally delivered ω-conotoxin-GVIA and ω-agatoxin-IVA on a wide range of electrically- and naturally-evoked neuronal activity.

2. Materials and methods

2.1. SNL

Male Sprague–Dawley rats, initially weighing 130–150 g, were used in this study. All experimental procedures were approved by the Home Office and follow the guidelines under the International Association for the Study of Pain (Zimmerman, 1983). Selective tight ligation of spinal nerves L5 and L6, and a sham procedure were performed as first described by Kim and Chung (1992). For details see Chapman et al. (1998).

2.2. Behavioural testing

For 2 weeks following surgery the rats were housed in groups of four in plastic cages under a 12 h day/night cycle and their general health was monitored. Successful reproduction of the neuropathic model was confirmed by behavioural testing (post-operative (PO) days 2, 3, 5, 7, 9, 12, and 14) by assessing the sensitivity of both the ipsilateral and contralateral hindpaws to normally non-noxious punctate mechanical (von Frey filaments) and cooling (acetone) stimuli. Rats were placed in transparent plastic cubicles on a mesh floor and allowed to acclimatize before any tests were initiated. Foot withdrawals to von Frey filaments 1, 5, and 9 g (trials of ten) and acetone (trials of five) were quantified as described in Chapman et al. (1998) and expressed as difference scores = ipsilateral response – contralateral response.

2.3. Spinal cord electrophysiology

Subsequent to behavioural testing (PO days 14–17), the operated rats were used for electrophysiological studies (Dickenson and Sullivan, 1986). Briefly, anaesthesia was induced with 3% halothane in a mixture of 66% N2O and 33% O2 and a cannula was inserted into the trachea. A laminectomy was performed (vertebrae L1–L3) to expose segments L4 and L5 of the spinal cord and the level of halothane was reduced to 1.8%. Extracellular recordings of single convergent neurones located deep within the dorsal horn (>500 μm), receiving input from the toe region ipsilateral to the SNL or sham procedure, were made using a parylene coated tungsten electrode. Neurones selected responded to both noxious (pinch) and non-noxious (touch) stimuli.

2.3.1. Cell characterization

Spontaneous activity exhibited by a neurone was recorded over 10 min. Action potentials evoked by natural stimuli applied constantly over 10 s were quantified by the application of both punctate mechanical (von Frey filaments 9 and 75 g) and thermal (constant water jet at 45°C) stimuli applied to the centre of the neurone's receptive field. The thermal response to 45°C was determined by subtracting the response to 32°C (a non-noxious temperature so as to ascertain any mechanical response evoked by the water jet) from the response to 45°C. All responses to natural stimuli were normalized by the subtraction of any spontaneous activity measured before the application of each stimulus. The response of the neurone to intracutaneous electrical stimulation was established by insertion of two fine needles into the centre of its peripheral receptive field. A test consisted of a train of 16 stimuli (2 ms wide pulse at 0.5 Hz at three times the threshold required to evoke a C-fibre response), and a post-stimulus histogram was constructed. The thresholds were determined by increasing the electrical stimulus from 0 mA until an action potential was evoked in the corresponding latency band. Electrically-evoked action potentials were separated on a latency basis into Aδ-fibres (0–20 ms), Aβ-fibres (20–90 ms), C-fibres (90–300 ms) and post-discharge (300–800 ms). The 'input' (non-potentiated response), and the 'wind-up' (potentiated response, evident by increased neuronal excitability to repeated stimulation) were calculated as follows: input = (action potentials (90–800 ms) evoked by first pulse at three times C-fibre threshold) × total number of pulses (16); wind-up = (total
action potentials (90–800 ms) after 16 train stimulus at three times C-fibre threshold) — input.

2.3.2. Pharmacological studies

The testing protocol, initiated every 15 min, consisted of an electrical test followed by the natural stimuli, as described above. Stabilization of the neuronal responses was at least three consistent pre-drug responses (<10% variation over at least 1 h) for all measures. These values were then averaged to generate pre-drug control values with which to compare the effect of drug administration on subsequent evoked responses. The effect of two different VDCC blockers was studied on these responses. The N-type calcium channel blocker, \( \omega \)-conotoxin-GVIA (Sigma-Aldrich Company Ltd., Poole, Dorset, UK), and the P-type calcium channel blocker, \( \omega \)-agatoxin-IVA (Pepptide Institute, Inc., Scientific Marketing Associates, Barnet, Hertfordshire, UK) (0.1, 0.4, 0.8, 1.2, 2.2, and 3.2 µg), were each dissolved in saline and applied directly onto the spinal cord in 50 µl volumes. The effects of each dose were followed until the responses plateaued (a minimum of 60 min), when the next dose would be applied cumulatively. The results were calculated as maximum percentage changes from the averaged pre-drug value for each neuron and the overall results for each dose were expressed as means ± standard error of the mean (SEM) of the normalized data. Statistical analysis of maximal drug effects at each dose compared to the averaged pre-drug value was determined by a paired t-test on raw data. An unpaired t-test on the normalized data was used for the comparison of drug effects between different experimental groups. The level of significance was taken as \( P < 0.05 \).

3. Results

3.1. Behavioural studies

During the PO period the animals showed normal weight gain and maintained good general health. Rats subjected to SNL exhibited abnormal foot posture ipsilateral to nerve injury whereby toes were held together in a 'guarding' behaviour. This did not occur in either the contralateral hindpaw, or in the sham-operated rats. Successful replication of the nerve injury model was confirmed by behavioural testing which demonstrated the development of the ongoing spontaneous activity which to co-compare the effect of drug administration on subsequent evoked responses. The effect of two different VDCC blockers was studied on these responses. The N-type calcium channel blocker, \( \omega \)-conotoxin-GVIA (Sigma-Aldrich Company Ltd., Poole, Dorset, UK), and the P-type calcium channel blocker, \( \omega \)-agatoxin-IVA (Pepptide Institute, Inc., Scientific Marketing Associates, Barnet, Hertfordshire, UK) (0.1–3.2 µg), applied directly onto the spinal cord, on the electrically- and naturally-evoked dorsal horn neuronal responses was tested in SNL, sham-operated and naive animals. Since the sham-operation is the appropriate control never accompanied by the pain-like behaviours displayed by the lesioned hindpaw of SNL rats.

3.2. Spinal cord electrophysiology

3.2.1. Cell characterization

The number of ipsilateral dorsal horn neurones characterized in each group were 23 in SNL rats, 21 in sham-operated rats, and 37 in naive rats (Table 1). All neurones had a receptive field over the left ipsilateral hindpaw. The level of ongoing spontaneous activity was found to be significantly higher (\( P < 0.05 \)) in neurones in SNL rats compared to sham-operated rats. Furthermore, 71% of neurones characterized in SNL rats exhibited spontaneous activity at a rate greater than 0.1 Hz in comparison to only 20% of characterized neurones in sham-operated rats (\( P = 0.003 \), Fisher's exact test). No significant difference was found between experimental groups in the mean cell depth of recorded neurones and the mean neuronal responses evoked by electrical and natural stimulation.

3.2.2. Pharmacological studies

The effect of \( \omega \)-conotoxin-GVIA and \( \omega \)-agatoxin-IVA (0.1–3.2 µg), applied directly onto the spinal cord, on the electrically- and naturally-evoked dorsal horn neuronal responses was tested in SNL, sham-operated and naive animals. Since the sham-operation is the appropriate control

Fig. 1. Development of mechanical and cooling allodynia in the ipsilateral hindpaw over the 2 week period following SNL. Data are expressed as the mean difference score ± SEM (\( n = 15 \)) in the withdrawal response to punctate mechanical stimuli (von Frey filaments bending forces 1 (•), 5 (△) and 9 g (○)) and cooling stimulus (drop of acetone (●)) applied to the planter surface of the hindpaws (trials of ten for the mechanical and five for the cooling). Difference score = (ipsilateral response frequency) − (contralateral response frequency). No nociceptive behaviour was observed in the sham-operated rats (data not shown).
for SNL, and for clarity, the results obtained from the naive, non-operated group shall not be displayed on the graphs.

3.2.2.1. Effects of ω-conotoxin-GVIA. At the higher doses, ω-conotoxin-GVIA produced inhibitions of all the electrically-evoked responses in neurones in all experimental groups (Fig. 2). Interestingly, in the sham-operated group, low doses tended to facilitate the neuronal responses, especially those induced by electrical stimulation. For each dose (0.1, 0.4, 0.8, 1.2, 2.2, and 3.2 μg) the effects of the toxin were slow in onset with clear effects seen around 40 min, and maximal inhibitions established at 70–90 min (Fig. 3), which were irreversible.

In SNL rats, all doses of ω-conotoxin-GVIA (0.1–3.2 μg) elicited significant effects compared to pre-drug control of the C-fibre, wind-up, input, and post-discharge and AB-fibre responses (Fig. 2a–d) and at 0.4 μg for the AB-fibre response (Fig. 2e). ω-Conotoxin-GVIA also produced an inhibitory effect on the evoked responses to non-noxious mechanical stimulation (von Frey 9 g) and 45°C water jet at 35°C applied to the centre of the cell's receptive field.

After neuropathy the inhibitory effects of ω-conotoxin-GVIA on the electrically-evoked responses appeared to be more marked at the lower end of the dose range in comparison to sham-operated rats. This was statistically significant (P < 0.05, n = 8) for C-fibre, wind-up, input, and post-discharge responses (Fig. 2a–d) and at 0.4 μg for the Aβ-fibre response (Fig. 2e). ω-Conotoxin-GVIA (0.1 and 0.4 μg) had a significantly greater effect (P < 0.05) on the C-fibre response (Fig. 2a) in SNL rats (9 ± 3% and 16 ± 14% inhibitions, respectively, n = 8) compared to sham-operated rats (7 ± 10% and 5 ± 15% facilitations, n = 8), the wind-up response (Fig. 2b) in SNL rats (63 ± 12% and 77 ± 8% inhibitions, respectively, n = 8) compared to sham-operated rats (14 ± 10% and 15 ± 17% inhibitions, n = 8), the input response (Fig. 2a) in SNL rats (30 ± 5% and 54 ± 9% inhibitions, respectively, n = 8) compared to sham-operated rats (19 ± 15% and 14 ± 21% facilitations, n = 7), and the post-discharge (Fig. 2d) in SNL rats (50 ± 13% and 67 ± 10% inhibitions, respectively, n = 8) compared to sham-operated rats (6 ± 11% and 20 ± 16% inhibitions, n = 6).

ω-Conotoxin-GVIA also produced an inhibitory effect on the naturally-evoked neuronal responses (Fig. 4). At 3.2 μg ω-conotoxin-GVIA, the response to non-noxious mechanical stimulation (von Frey 9 g) was inhibited by 95 ± 3% in both SNL (n = 5) and sham-operated rats (n = 5), and by 75 ± 6% in naive rats (n = 10) (Fig. 4a, naive not shown).
The response to noxious mechanical stimulation (von Frey 75 g) was inhibited by 83 ± 5% in SNL rats (n = 7), 78 ± 12% in the control group (n = 6), and 69 ± 5% in naïve rats (n = 10) (Fig. 4b, naïve not shown). The response to noxious thermal stimulation (water jet at 45°C) was inhibited by 89 ± 5% in SNL rats (n = 6), 93 ± 5% in sham-operated rats (n = 5) and 79 ± 9% in naïve rats (n = 8) (Fig. 4c, naïve not shown). After neuropathy, w-conotoxin-GVIA had a greater inhibitory effect on the naturally-evoked neuronal responses at the lower end of the dose range compared to sham-operated animals. This was found to be statistically significant at 0.1 and 0.4 µg w-conotoxin-GVIA for the von Frey 9 g evoked responses (Fig. 4a) (55 ± 6% and 69 ± 7% inhibitions, respectively,
in SNL rats, compared to 6 ± 12% facilitation and 17 ± 11% inhibition, respectively, in sham-operated rats; 
\( P \leq 0.001, n = 8 \), and at 0.4 \( \mu g \) for the von Frey 75 g evoked responses (Fig. 4b) (51 ± 6% inhibition in SNL rats compared to 21 ± 12% inhibition in sham-operated rats; \( P \leq 0.05, n = 8 \)).

### 3.2.2.2. Effects of \( \omega \)-agatoxin-IVA.

\( \omega \)-Agatoxin-IVA inhibited the electrically-evoked neuronal responses measured in all experimental groups, but to a lesser extent than that achieved with \( \omega \)-conotoxin-GVIA (Fig. 5). In SNL rats, at the highest dose, mean maximal inhibitions from pre-drug controls were in the range 37 ± 13% to 60 ± 10% for wind-up, input, post-discharge and A\( \beta \)-fibre (Fig. 5b-e), and only 18% for the C- and A\( \beta \)-fibre (Fig. 5a,f, respectively), at which significance was achieved (\( P \leq 0.05, n = 7 \)). Similar maximal inhibitions were observed in sham-operated rats, and there was no difference in the drug effect at the lower doses compared to SNL animals. \( \omega \)-Agatoxin-IVA had no significant inhibitory effects on the responses in naive animals; however, with the low doses (0.1-0.8 \( \mu g \)) there was a tendency for the electrically-evoked neuronal responses to be facilitated and this was found to be statistically significant (\( P \leq 0.05, n = 5 \)) for the C-fibre response.

The naturally-evoked neuronal responses were also inhibited by \( \omega \)-agatoxin-IVA (Fig. 6). In SNL rats, at the highest dose, mean maximal inhibitions from the pre-drug control were in the range 57 ± 11% to 71 ± 12%, and this was statistically significant for von Frey 9 and 75 g (\( P \leq 0.05, n = 7 \)). Again, no difference in the extent of inhibition was observed between experimental groups.

The spontaneous rate of activity, when present, was clearly inhibited by both toxins. Due to the low pre-drug baseline values and variability between neurones in this ongoing activity, it was not quantified.
4. Discussion

This is the first electrophysiological study addressing the role of VDCCs in the spinal processing of sensory information after nerve injury. Spinal ω-conotoxin-GVIA, an N-type blocker, inhibited the electrically- and naturally-(innocuous and noxious) evoked dorsal horn neuronal responses. The effects of low doses were significantly enhanced after the establishment of neuropathy. In comparison, the P-type antagonist ω-agatoxin-IVA inhibited the evoked neuronal responses to a lesser extent and its profile was unaltered after nerve injury.

Although the concentration of the toxins at their sites of action in vivo obviously cannot be determined, the concentration applied to the surface of the spinal cord at a mid-range dose (0.8 μg) was 5 μM for ω-conotoxin-GVIA and 3...
Fig. 6. Comparison of the effect of spinally applied ω-agatoxin-IVA on the naturally-evoked dorsal horn neuronal responses (see Section 2) recorded from SNL rats (●) and sham-operated rats (▲). (a) innocuous punctate mechanical stimulus (von Frey 9 g); (b) noxious punctate mechanical stimulus (von Frey 75 g); (c) noxious heat stimulus (water jet at 45°C normalized by response to water jet at 32°C). No statistically significant difference in the effects of ω-agatoxin-IVA was observed between SNL rats (n = 6) and sham-operated rats (n = 6). Data are expressed as the maximal mean % inhibition of the pre-drug values ± SEM.

These concentrations are higher than the selective dose ranges for these toxins in vitro (Hofmann et al., 1999), where there is direct application to the membrane. However, the concentration within the spinal cord will be at least three orders of magnitude less and also diffusion of these large peptides into the tissue will be limited as demonstrated by the slow establishment of maximal inhibitions (Fig. 3). The different profile of the toxins suggests that they are acting at the appropriate VDCC.

Unilateral tight ligation of L5 and L6 spinal nerves produced reproducible nociceptive syndromes in the lesioned hindpaw. A clear withdrawal reflex with associated aversive behaviours, indicative of the development of mechanical and cooling alldynia, was produced as previously described by Chapman et al. (1998). Thus, all SNL animals used for the electrophysiology and subsequent pharmacology exhibited neuropathic signs; sham-operated animals did not.

A possible explanation of these results is that if there are increases in the probability and/or frequency of opening of pre-existing VDCCs, the likelihood of a given concentration of ω-conotoxin-GVIA blocking a functionally important channel would be increased. Indeed, it is likely that calcium influx via VDCCs on central terminals of peripheral nerves and neurones, and consequently neurotransmitter release and membrane depolarization (Smith and Augustine, 1988), are increased due to enhanced neuronal activity observed peripherally after neuropathy (Wall and Devor, 1983; Sheen and Chung, 1993). Furthermore, despite reduced afferent input via L5 and L6 spinal nerves, the magnitude of neuronal responses recorded was not diminished after SNL. Conversely, increased frequency and occurrence of spontaneous activity was observed. These points have been recently discussed in more detail (Suzuki et al., 2000a) but the neuronal profiles seen after nerve injury are similar to those previously reported in this model (Chapman et al., 1998). Furthermore, in keeping with these ideas, we have recently shown enlarged receptive fields of these neurones (Suzuki et al., 2000b). This suggests that there are compensatory increases in peripheral and/or spinal neuronal activity after neuropathy leading to a greater functional role of VDCCs in the observed neuronal responses.

Another possibility is that rather than an increase in activity of existing VDCCs there is an increase in VDCC expression. In support of this idea, the expression of the N-type VDCC α1B subunit is increased in small dorsal root ganglion (DRG) cells and in lamina II of the spinal cord after neuropathy (Cizkova et al., 1999). Furthermore, the mRNA and protein for the auxiliary αδ subunit of VDCCs is also upregulated in the ipsilateral DRG and spinal cord of SNL rats (Luo et al., 1999). In addition to its ability to modulate VDCC kinetics (Hobom et al., 2000), αδ can modulate the binding affinity of ω-conotoxins to the N-type VDCC (Brust et al., 1993). Although it is generally believed that the
ω-toxins primarily affect the function of the α1 subunit (Olivera et al., 1994), evidence indicates that they can also bind the α2 component of the α2β subunit (Barbanin et al., 1988). It remains to be determined whether upregulation of α2β is responsible for the selective enhancement of ω-conotoxin-GVIA's inhibitory actions we report here.

Peripheral nerve damage leads to the generation of ectopic activity at the injury site (Wall and Devor, 1983) or within the DRG (Wall and Devor, 1983; Kajander et al., 1992). This provides an ongoing, continual barrage of neuronal activity via primary afferents into the spinal cord which could lead to central sensitization and hyperexcitability (Gracely et al., 1992; Sheen and Chung, 1993; Yoon et al., 1996). Evidence indicates that enhanced nociceptive transmission in neuropathic pain states is mediated, at least in part, by the action of excitatory amino acids, particularly at N-methyl-D-aspartate (NMDA) receptor sites (Dubner and Bennett, 1983; Dickenson and Sullivan, 1987; Codere et al., 1993), and NMDA antagonists have been shown to produce antinociception in patients (Eide et al., 1994; Price et al., 1994). Thus, any increased neuronal depolarization, such as that produced by NMDA receptor events, would increase the likelihood of VDCC activity in spinal neurones since they are voltage-operated ion channels. Indeed events such as these regulate the expression of new channels and receptor subunits.

4.1. Roles of N-type VDCCs

It is evident here that blockade of N-type VDCCs results in a greater inhibition of electrically- and naturally-evoked dorsal horn neuronal responses after SNL, which is indicative of a role for N-type VDCCs in mechanisms of central sensitization of nociceptive dorsal horn neurones after neuropathy. Increased primary afferent activity after peripheral nerve damage could result in enhanced exocytosis of excitatory neurotransmitters, which would in turn increase activation of receptor systems such as those for glutamate (NMDA), substance P and CGRP, which produce excitation of spinal cord neurones. Nerve terminals throughout the dorsal horn have been demonstrated to be immunoreactive for N-type VDCCs, often correlating with the presence of substance P (Westenbroek et al., 1998). Since N-type VDCCs are abundant on nerve terminals and crucial for neurotransmitter release (Smith and Augustin, 1988; Miljanich and Ramachandran, 1995) the simplest explanation for the effects of ω-conotoxin-GVIA is that it blocks pre-synaptic N-type VDCCs and therefore calcium influx and evoked neurotransmitter release. This is supported by the observation that the non-potentiated 'input' response recorded in this study, which would relate to the level of synaptic transmission between the central terminals of primary afferents and the neurones of the spinal cord dorsal horn, is significantly inhibited by the spinal application of the toxin. Small DRG cells express N-type calcium currents (Scroggs and Fox, 1992a,b) and the release of CGRP (Santiago et al., 1992); sensory neuropeptides (Maggi et al., 1990) and substance P in culture (Holz et al., 1988) are all ω-conopeptide-sensitive. As for the control of glutamate release and subsequent NMDA receptor/channel activation, implicated in central hyperexcitability, the role of N-type VDCCs is debatable. Glutamatergic synaptic transmission between DRG cells and spinal cord neurones is inhibited by blockade of pre-synaptic N-type VDCCs (Gruner and Silva, 1994), and ω-conopeptides can cause significant reduction in depolarization-evoked glutamate release (Terrian et al., 1990; Dickie and Davies, 1992). Given the evidence for a role of N-type VDCCs in peptide release it would be expected that a similar situation would exist for glutamate, although other studies have reported that N-type VDCCs have a minimal role in mediating glutamate release (Pocock and Nicholls, 1992; Turner et al., 1992).

It is also likely that ω-conotoxin-GVIA may also inhibit N-type VDCCs at post-synaptic sites (Takahashi and Momiyama, 1993) and thereby reduce excitation of spinal cord neurones. Nociceptive signals arriving at the spinal cord from damaged peripheral nerves may be subject to amplification via interneurones that project onto an output neurone, resulting in the phenomena of wind-up and central sensitization. In support of a possible interneuronal site of action is the fact that NMDA receptors are not considered to be directly post-synaptic to primary afferents (Davies and Watkins, 1983). Also, the greatest inhibitions seen here produced by the low doses of ω-conotoxin-GVIA after SNL were observed on the 'wind-up' and post-discharge responses, indicators of neuronal hyperexcitability, and mediated by post-synaptic events. However, in the latter case, blockade of pre-synaptic transmitter release by the VDCC antagonists could indirectly also prevent post-synaptic hyperexcitability.

4.2. Roles of P-type VDCCs

In contrast to the actions of ω-conotoxin-GVIA, the P-type VDCC antagonist ω-agatoxin-IVA had limited inhibitory effects and no difference was seen between experimental groups. Interestingly, a significant inhibition of wind-up and post-discharge was seen with higher doses in sham-operated and SNL rats, although the maximum effect was lower than after the ω-conotoxin-GVIA. In naive and sham-operated animals, the electrically-evoked responses tended to be facilitated at low doses and similarly in an in vivo electrophysiological study (Nebe et al., 1997), ω-agatoxin-IVA increased neuronal responses in the absence of inflammation but mediated inhibition in its presence. In vitro studies have demonstrated that ω-agatoxin-IVA blocks excitatory (Luebke et al., 1993; Castillo et al., 1994; Yamamoto et al., 1994) as well as inhibitory (Takahashi and Momiyama, 1993) synaptic transmission, which may explain the facilitatory and inhibitory effects observed here. P-type VDCCs may be involved in the release of glutamate, aspartate, dopamine, serotonin, noradrenaline, dopamine, serotonin, noradrenaline,

ω-Agatoxin-IVA given prior to capsaicin into the hindpaw (Sluka, 1997) and carrageenan into the knee joint (Sluka, 1998) prevents the development of hyperalgesia. This indicates that P-type channels may be most important in the initiation of a facilitated pain state although we find a limited role in the maintenance of neuropathy. The P-type channel appears to be linked selectively to NMDA receptor-mediated events as here post-SNL-administered ω-agatoxin-IVA had most marked effects on NMDA receptor-mediated wind-up and post-discharge measurements. This is in accordance with results with the formalin response where ω-agatoxin-IVA only inhibited neuronal responses in the second phase (Diaz and Dickenson, 1997), for which NMDA receptor activity is critical (Haley et al., 1990).

Evidence to support a post-synaptic role for P-type VDCCs is based on the fact that post-synaptic currents in numerous central nervous system neurones are markedly suppressed by ω-agatoxin-IVA and reduced to a lesser extent by ω-conotoxin-GVIA (Takahashi and Momiyama, 1993). Pre-synaptic P-type channels are also present. Interestingly, in the superficial dorsal horn, the distribution of P/Q-type channels complements rather than co-localizes with N-type VDCC-containing neurones. Immunolocalization has demonstrated the presence of P/Q-type VDCCs primarily on the nerve terminals of dorsal horn neurones in laminae II–VI, but unlike N-type channels, rarely in substance P-containing neurones (Westenbroek et al., 1998). Intriguingly, this may indicate that N-type channels regulate the release of neurotransmitters, including glutamate, from peptide-containing C-fibres, whereas P-type channels could possibly be localized to non-peptide, IB4-positive neurones. Furthermore, both N- and P-type VDCCs are found in the deeper laminae of the dorsal horn, suggesting localization on terminals of large fibres and spinal neurones.

4.3. Implications

From this study, in agreement with studies in inflammation, the spinal N-type VDCC is the predominant isofrom involved in the pre- and post-synaptic processing of sensory nociceptive information. This is the first electrophysiological study investigating the role of N- and P-type VDCCs in the transmission of nociception at the level of the spinal cord after nerve injury and it extends results from behavioural studies. Tactile allodynia in the SNL model can result in considerable plasticity in the N-type VDCCs pre-synaptically. N-type VDCCs have been demonstrated to be analgesic in both animal models of acute and chronic pain, and in humans. The doses utilized here are comparable to those used in behavioural studies where no obvious side-effects were noted (Malmberg and Yaksh, 1994, 1995). In a clinical study, SNX-111 delivered intrathecally to chronic pain patients produced some pain relief (Brose et al., 1997). However, pharmacological targeting of these channels with toxin antagonists as a potential therapy for the treatment of chronic pain is hindered by the adverse systemic side-effects (Penn and Paice, 2000) and the inconvenient spinal route of administration.

It is noteworthy that the anti-epileptic drug gabapentin (GBP), with analgesic efficacy in patients with postherpetic neuralgia (Rowbotham et al., 1998) and diabetic polyneuropathy (Backonja et al., 1998), may exert its effects via binding to VDCCs. Despite being a GABA analogue it has been demonstrated to bind to the auxiliary α2-δ subunit of VDCCs (Taylor et al., 1994) where it is assumed to act as an antagonist. GBP may highlight the importance of VDCCs as targets in pain control. It is difficult to envisage how interactions of GBP with ubiquitous α2-δ subunits can result in specific therapeutic effects. However, DRG cells have now been shown to express at least two distinct forms of the α2-δ subunit that differ from those in spinal cord and brain (Luo, 1999). This may allow tissue-specific effects of agents that act on VDCCs.

Whatever the case, the present results, together with a number of previous behavioural reports, emphasize the key roles of VDCCs in sensory events within the spinal cord. Here, we show that N-type VDCCs are the predominant functional type in terms of a wide range of sensory modalities and exhibit plasticity following nerve injury.
The increased ability of ω-conotoxin-GVIA to inhibit evoked neuronal responses to a wide range of stimuli suggests that N-type VDCCs are a key component of increased central excitability that follows nerve injury.

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Effects of ethosuximide, a T-type Ca\(^{2+}\) channel blocker, on dorsal horn neuronal responses in rats

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Abstract

Plasticity in transmission and modulatory systems are implicated in mechanisms of neuropathic pain. Studies demonstrate the importance of high voltage-activated Ca\(^{2+}\) channels in pain transmission, but the role of low voltage-activated, T-type Ca\(^{2+}\) channels in nociception has not been investigated. The Kim and Chung rodent model of neuropathy (Pain 50 (1992) 355) was used to induce mechanical and cold allodynia in the ipsilateral hindpaw. In vivo electrophysiological techniques were used to record the response of dorsal horn neurones to innocuous and noxious electrical and natural (mechanical and thermal) stimuli after spinal nerve ligation. Spinal ethosuximide (5-1055 μg) exerted dose-related inhibitions of both the electrically and low- and high-intensity mechanical and thermal evoked neuronal responses and its profile remained unaltered after neuropathy. Measures of spinal cord hyperexcitability were most susceptible to ethosuximide. This study, for the first time, indicates a possible role for low voltage-activated Ca\(^{2+}\) channels in sensory transmission. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Ca\(^{2+}\) channels, voltage-dependent; Neuropathic pain; T-type channel; Analgesia; Nociception; Dorsal horn

1. Introduction

Neuropathic pain, arising from injury- or disease-evoked damage to the peripheral or central nervous system, often responds poorly to traditional analgesics. Patients often experience sensory deficits, persistent and stimulus-evoked pain (allodynia and hyperalgesia). Peripheral nerve damage provides abnormal input into the central nervous system and then leads to dorsal horn hyperexcitability. Under the influence of both excitatory and inhibitory neurotransmitter systems, the dorsal horn is a site of peripheral input modulation before projection to higher brain centres, thus it controls the stimulus–response relationship. Reduction of this excitability is a possible key to neuropathic pain management.

Animal models of neuropathy have been critical in elucidating its complex causal mechanisms, involving plasticity in nociceptive transmission and modulating systems. The rat spinal nerve ligation model (Kim and Chung, 1992) involves tight ligation of two (L5 and L6) of the three spinal nerves that form the sciatic nerve. Behavioural consequences include thermal hyperalgesia, and mechanical and cooling allodynia (Kim and Chung, 1992; Chaplan et al., 1997).

High voltage-activated Ca\(^{2+}\) channels (L-, N-, P/Q- and R-types), consisting of a pore-forming α1 subunit and modulatory accessory subunits, β, α₂-δ and γ (Walker and De Waard, 1998), are widely expressed throughout the brain and spinal cord (Kerr et al., 1988; Mintz et al., 1992; Gohl et al., 1994). They are activated by relatively strong membrane depolarisation and permit Ca\(^{2+}\) influx in response to action potentials. Consequential secondary actions include neurotransmitter release; thus these channels establish a major link between neuronal excitability and synaptic transmission. For these reasons high voltage-activated Ca\(^{2+}\) channels have been the focus of both acute and persistent pain transmission studies. Animal models have demonstrated the antinociceptive abilities of antagonists specific for L-, N- and P/Q-type Ca\(^{2+}\) channels, highlighting the differential role each subtype plays in...
nociception, often dependent on the nature of the pain state (Vanegas and Schaible, 2000).

In addition to high voltage-activated Ca$^{2+}$ channels, kinetically distinct low voltage-activated Ca$^{2+}$ channels, or T-type channels, also exist both in neuronal and non-neuronal cells. They activate at voltages near the resting membrane potential, inactivate rapidly, deactivate slowly and have a small single channel conductance (Huguenard, 1996). These unique gating properties prohibit T-type channels alone to support neurotransmission, however they permit their involvement in low-amplitude oscillations, neuronal bursting, synaptic signal boosting, Ca$^{2+}$ entry promotion and lowering threshold for high-threshold spike generation. This Ca$^{2+}$ current appears to play an important physiological role in near-threshold phenomena and regulation of neuronal excitability.

Until recently, the low voltage-activated current was considered a single entity. However, α1G, α1H and α1I subunits have now been cloned, showing 30% homology to high voltage-activated channel forming α1 subunits (Cribbs et al., 1998; Perez-Reyes, 1998; Lee et al., 1999) and hallmark native T-type Ca$^{2+}$ channel properties when expressed heterologously. In situ hybridisation studies on the rat brain have shown that these channels have unique distributions, including the dorsal horn of the spinal cord and sensory ganglia (Talley et al., 1999). This is complemented by reported T-type currents in primary sensory neurones (Carbone and Lux, 1984; Kostyuk et al., 1992; Scroggs and Fox, 1992; Todoric and Lingle, 1998) and some superficial rat dorsal horn neurones (Ryu and Randic, 1990), an important site for the processing and integration of sensory information, including pain. Unlike the high voltage-activated Ca$^{2+}$ channels, the involvement of T-type channels in pain-related central sensitisation has been hindered by a scarcity of specific pharmacological agents.

Neuropathic pain and epilepsy both share neuronal hyperexcitability as a common underlying mechanism. There are established antiepileptic drugs that target the generation of neuronal hyperexcitability in the brain and some of these have been proven effective in the treatment of various forms of neuropathic pain (Swerdlow and Cundhul, 1981; McQuay et al., 1995). The succinimide derivative ethosuximide, or 2-ethyl-2-methylsuccirurnide, is an anticonvulsant (Macdonald and McLean, 1986) effective in the treatment of absence epilepsy (Coulter et al., 1989b); a condition characterised by spike-wave rhythm likely generated by T-type Ca$^{2+}$ current. Ethosuximide has been demonstrated to be a relatively specific T-type channel antagonist in thalamic (Coulter et al., 1989a) and dorsal root ganglion neurones (Kostyuk et al., 1992). This study uses the spinal nerve ligation model, confirmed by behavioural testing, to induce a neuropathic state, subsequent to which electrophysiological studies of dorsal horn spinal neurones were made to investigate the effects of spatially delivered ethosuximide on a wide range of electrical and natural-evoked neuronal activity.

2. Materials and methods

2.1. Spinal nerve ligation

Male Sprague-Dawley rats, initially weighing 130–150 g, were used in this study. All experimental procedures were approved by the Home Office and follow the guidelines under the International Association for the study of Pain (Zimmermann, 1983). Selective tight ligation of spinal nerves L5 and L6, and a sham procedure were performed as first described by Kim and Chung (1992). For details, see Chapman et al. (1998).

2.2. Behavioural testing

For 2 weeks following surgery, the rats were housed in groups of 4, in plastic cages under a 12/12 h day/night cycle and their general health monitored. Successful reproduction of the neuropathic model was confirmed by behavioural testing (post-operative days 2, 3, 5, 7, 9, 12 and 14) assessing the sensitivity of both the ipsilateral and contralateral hindpaws to normally non-noxious punctate mechanical (von Frey filaments) and cooling (acetone) stimuli. Rats were placed in transparent plastic cubicles on a mesh floor and allowed to acclimatise before initiating any tests. Foot withdrawals to von Frey filaments 1, 5 and 9 g (trials of 10) and acetone (trials of 5) were quantified as described in Chapman et al. (1998) and expressed as Difference Scores = Ipsilateral response – contralateral response.

2.3. Spinal cord electrophysiology

Subsequent to behavioural testing (post-operative days 14–17), the operated rats were used for electrophysiological studies (Dickenson and Sullivan, 1986). Briefly, anaesthesia was induced with 3% halothane in a mixture of 66% N$_2$O and 33% O$_2$ and a cannula inserted into the trachea. A laminectomy was performed (vertebrae L1–L3) to expose segments L4–L5 of the spinal cord and the level of halothane was reduced to 1.8%. Extracellular recordings of single convergent neurones, located deep within the dorsal horn (> 500 μm), receiving input from the toe region ipsilateral to the spinal nerve ligation or sham procedure, were made using a parylene coated tungsten electrode. Neurones selected responded to both noxious (pinch) and non-noxious (touch) stimuli.

2.3.1. Cell characterisation

Spontaneous activity exhibited by a neurone was recorded over 10 min. Action potentials evoked by natural stimuli applied constantly over 10 s were quantified by the application of both punctate mechanical (von Frey fila-
significance was taken as P < 0.05. The level of normalised data was used for the comparison of drug effects between different experimental groups. The level of significance was taken as P ≤ 0.05.

3. Results

3.1. Behavioural studies

During the post-operative period the animals showed normal weight gain and maintained good general health. Rats subjected to spinal nerve ligation exhibited abnormal foot posture ipsilateral to nerve injury whereby toes were held together in a 'guarding' behaviour. This did not occur in either the contralateral hindpaw, or in the sham-operated rats. Successful replication of the nerve injury model was confirmed by behavioural testing which demonstrated the development of mechanical and cooling allodynia of the injured hindpaw of spinal nerve ligated rats. Evoked allodynia, in response to innocuous mechanical (von Frey filaments bending force 1–9 g) and cooling (acetone) stimuli, was displayed as a brisk withdrawal, accompanied in some cases by shaking and licking of the foot ipsilateral to spinal nerve ligation. This was evident at post-operative day 2, reached maximum at days 7–12 and still maintained at day 14 (Fig. 1). Consistent withdrawal responses were never exhibited by the control group or by the contralateral hindpaw of the experimental group, and when present were never accompanied by the pain-like behaviours displayed by the lesioned hindpaw of spinal nerve ligated rats.

3.2. Spinal cord electrophysiology

3.2.1. Cell characterisation

The numbers of ipsilateral dorsal horn neurones characterised in each group were 11 in spinal nerve ligated rats, 6

![Fig. 1. Development of mechanical and cooling allodynia in the ipsilateral hindpaw over the 2-week period following spinal nerve ligation. Data is presented as the mean difference score ± S.E.M. (n = 11) in the withdrawal response to punctate mechanical stimuli (von Frey filaments) and cooling stimulus (drop of acetone) applied to the plantar surface of the hindpaws (trials of 10 for the mechanical and 5 for the cooling). Difference score = (ipsilateral response frequency) — (contralateral response frequency). No nociceptive behaviour was observed in the sham-operated rats (data not shown).](image-url)
in sham-operated rats and 8 in naïve rats. All neurones had a receptive field over the left ipsilateral hindpaw. No significant differences were found between experimental groups in the mean values of recorded neurone depth, responses evoked by electrical and natural stimulation, and level of ongoing spontaneous activity. It is worth noting that 45% of neurones characterised in spinal nerve ligated rats exhibited spontaneous activity at a rate greater than 0.1 Hz in comparison to only 17% of characterised neurones in sham-operated rats and 25% of naïve.

3.2.2. Pharmacological studies

The effect of ethosuximide (5–1055 µg), applied directly onto the spinal cord, on the electrically and naturally evoked dorsal hom neuronal responses was tested in spinal nerve ligated, sham-operated and naïve animals. Since the sham operation is the appropriate control for spinal nerve ligation, and for clarity, the results obtained from the naïve, non-operated, group shall not be displayed on the graphs. However, no difference in the effects of ethosuximide was observed between sham and naïve groups.

Ethosuximide produced a dose-related inhibition of the electrically and naturally evoked responses in neurones in all experimental groups (Figs. 2, 3 and 4) and there was no difference in the extent of its effects between groups. Clear effects were seen around 40 min, with maximal inhibitions established at around 60 min. For sham and spinal nerve ligated groups, all doses of ethosuximide (5–1055 µg) elicited statistically significant inhibitions of the electrically evoked responses, compared to pre-drug control (Fig. 2; *P* ≤ 0.05; *n* = 6–11). For naïve animals, statistically significant inhibitions were elicited by ethosuximide: at all doses (5–1055 µg) for the Aβ-fibre, Aδ-fibre, post-discharge and excess spikes measurements; at 55–1055 µg ethosuximide for the C-fibre response; and at 555 and 1055 µg for the input response (*P* ≤ 0.05; *n* = 7). In all three groups, the Aβ-fibre response was least affected with mean maximal inhibitions at top dose ranging from 17 ± 6% to 26 ± 8% (Fig. 2F). In all three groups, the C-fibre and Aδ-fibre responses reached similar mean maximal inhibitions at top dose ranging from 33 ± 13% to 47 ± 17% (Fig. 2D and E, respectively). Greater inhibitory effects

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**Fig. 2.** Effect of spinally applied ethosuximide on the electrically evoked dorsal hom neuronal responses (see Materials and methods) recorded from spinal nerve ligated (*n* = 6–11) and sham-operated (*n* = 6) rats at post-operative days 14–17. Data is expressed as maximal mean % inhibition of the pre-drug values ± S.E.M.
over the entire ethosuximide concentration range were observed on the input, post-discharge and excess spikes measurements (Fig. 2A, B and C, respectively). At 1055 μg, ethosuximide maximally inhibited the input and excess spikes to within the range 48 ± 8% to 61 ± 14%. The greatest effect was observed on post-discharge, which was maximally inhibited in spinal nerve ligated, sham and naïve rats ranging from 58 ± 10% to 75 ± 13%.

Fig. 3A and B shows examples of the effects of ethosuximide on the wind-up of an individual neurone recorded from a spinal nerve ligated and sham rat, respectively. It is clear that ethosuximide produces a dose-dependent inhibition from the control response of both the wind-up (evident by a flattening of the curve) and to a lesser extent, the input, with little difference seen between experimental animal groups.

Ethosuximide also produced an inhibitory effect on the naturally evoked neuronal responses (Fig. 4). In spinal nerve ligated animals this was significant (P ≤ 0.05; n = 6–11) for all concentrations of ethosuximide employed in this study on the response to non-noxious mechanical stimulation (von Frey 9g, Fig. 4A), noxious mechanical stimulation (von Frey 75g, Fig. 4B) and noxious thermal stimulation (water jet at 45°C, Fig. 4C). In sham rats the von Frey 9g and heat responses were significantly inhibited at all concentrations of ethosuximide, and the von Frey 75g response was inhibited by 555 and 1055 μg (P ≤ 0.05; n = 5–6). In naïve animals responses to both von Frey hairs were significantly inhibited by all concentrations of ethosuximide, and inhibition of the heat response reached significance at 555 and 1055 μg (P ≤ 0.05; n = 5–7). No differences in the effects of the drug were
apparent between experimental groups and the mean maximal inhibitions established at top dose were in the range 61 ± 11% to 77 ± 10%.

4. Discussion

Unilateral tight ligation of L5 and L6 spinal nerves produced reproducible nociceptive syndromes in the lesioned hindpaw. A clear withdrawal reflex with associated aversive behaviours, indicative of the development of mechanical and chemical allodynia, was produced as previously described by Chapman et al. (1998). Thus, all spinal nerve ligated animals used for the electrophysiology and subsequent pharmacology exhibited neuropathic signs; sham-operated did not. This is the first electrophysiological study addressing the role of low voltage-activated T-type Ca\(^{2+}\) channels in the spinal processing of sensory information after nerve injury. Spinal ethosuximide, a relatively specific T-type channel antagonist, mediated significant inhibition of the electrical and natural (innocuous and noxious) evoked rat dorsal horn neuronal responses, suggesting some role for T-type Ca\(^{2+}\) channels in sensory transmission.

Extensive behavioural and electrophysiological nociceptive studies have demonstrated an important role for high voltage-activated Ca\(^{2+}\) channels in the processing of pain (Vanegas and Schaible, 2000). The contribution of N-, P/Q- or L-type channels appears to alter depending on the nature of the pain (acute or chronic, inflammatory or neuropathic in origin), and this has been established by the use of specific channel antagonists. A predominant nociceptive role for N-type...channels...has...been...established, which is enhanced after neuropathy (Chapman et al., 1994; Xiao and Bennett, 1995; Bowersox et al., 1996; Brose et al., 1997; White and Cousins, 1998; Matthews and Dickenson, 2000). Upon substantial membrane depolarisation N- and P-type voltage-activated Ca\(^{2+}\) channels mediate the release of excitatory neurotransmitters, such as glutamate, substance P and calcitonin gene-related peptide, critical for wind-up and central sensitisation, in the presence of constant afferent input (Dickenson, 1994). This study highlights the role of Ca\(^{2+}\) influx via T-type channels in the nociception pathway, within which ethosuximide may be exerting its effects at a number of sites. The biophysical characteristics of the T-type channel have led to its implication mainly in the regulation of cell excitability. The broad action of ethosuximide, with no marked selectivity for a particular modality or evoked response, is in keeping with an action on postsynaptically located Ca\(^{2+}\) channels. Since T-type channel activation occurs close to resting potential, they allow Ca\(^{2+}\) influx when cells are at rest (Magee and Johnston, 1995) or in response to subthreshold synaptic inputs (Markram and Sakmann, 1994; Magee and Johnston, 1995). Thus, these channels enhance neuronal excitability and contribute to the generation of subthreshold membrane potential oscillations that lead to bursts of sodium dependent action potentials (Huguenard, 1996). Whilst unable to mediate synaptic transmission alone, T-type channels do serve to boost synaptic inputs and lower threshold for high-threshold spike generation. Their block would result in an overall reduction in the underlying level of neuronal excitability, rendering the achievement of threshold levels of membrane depolarisation less likely. Postsynaptically, NMDA receptor activation would be reduced as would the consequential development of central sensitisation.

In this study, ethosuximide exerted its greatest inhibitory effects upon the input, post-discharge and excess spikes. Each of these electrical measures can be related to a specific part of the nociceptive pathway. The non-potentiated input response can be related to the level of synaptic transmission between the central terminals of primary afferents and the neurones of the spinal cord dorsal horn. Thus, blockade of T-type channels at this location is likely causing a reduction in the exocytosis of excitatory neurotransmitters by prohibiting the depolarisation required to activate high voltage-activated Ca\(^{2+}\) channels. Alternatively, ethosuximide could be exerting its effects directly on neurones located early in polysynaptic pathways. Even more susceptible to the actions of ethosuximide were the postsynaptic NMDA receptor-mediated post-discharge and excess spike measurements. These are indicative of central sensitisation and neuronal hyperexcitability, and since T-type Ca\(^{2+}\) channels are heavily linked to the level of neuronal excitability it follows that they would have a greater functional role here.

What is surprising is that we saw no difference in the effects of ethosuximide after the establishment of neuropathy, especially since neuronal hyperexcitability is a key underlying factor. Interestingly it has been shown that although unilateral cortical ablation causes a 68% increase in T-current measured from isolated rat thalamic relay neurones, a-methyl-\(\beta\)-phenylsuccimide (another related T-type channel antagonist) was more effective in reducing T-current in normal rats compared to axotomised animals (Chung et al., 1993). The authors suggest an injury induced alteration in the pharmacological properties the T-type channels either by de novo synthesis and/or modification. In this study, it may be that there is indeed no increase in the functional role of T-type Ca\(^{2+}\) channels after nerve injury. Alternatively, the specificity and/or potency of ethosuximide may be such that subtle differences in T-type channel function were not highlighted.

Peripheral nerve injury results in reduced afferent input via L5 and L6 spinal nerves, yet as observed here, the magnitude of neuronal responses recorded was not diminished in comparison to sham and normal rats. Conversely, increased frequency and occurrence of spontaneous activity was observed. This suggests that perhaps compensatory increases in peripheral and/or spinal neuronal activity are in play after neuropathy. Ectopic C-fibre activity originat-
The existence of a neuronal Ca$$^{2+}$$ current elicited just above the resting potential was first established in primary sensory neurones (Carbone and Lux, 1984; Bossu et al., 1985; Fedulova et al., 1985; Nowycky et al., 1985), and low voltage-activated current has since been observed in a wide variety of cell types (Huguenard, 1996). The presence of a relatively large T-type current in some superficially located rat spinal dorsal horn neurones is of much interest because this region is involved in processing and integration of sensory information, including pain (Ryu and Randic; 1990). T-type channels are pharmacologically and physiologically heterogeneous (Akaike, 1991; Huguenard, 1996; Tarasenko et al., 1997), which may reflect differential expression of the three known subtypes ($$\alpha_1$$G, H and I). The regional and cellular distribution of gene expression for the different T-type Ca$$^{2+}$$ channel family members in the rat central and peripheral nervous systems has recently been determined using in situ hybridisation (Talley et al., 1999). All three transcripts were detected in sensory areas and in the dorsal horn of the spinal cord where in particular $$\alpha_1$$H was mainly restricted to the outermost laminae I and II. In the dorsal root ganglion high levels of $$\alpha_1$$H and moderate levels of $$\alpha_1$$I mRNA were found restricted to small and medium sized neurones, whereas the extremely large were not labelled. This correlates with substantial T-type current observed in medium-diameter dorsal root ganglion neurones isolated from adult rats that is absent in larger dorsal root ganglion cells (Scruggs and Fox, 1992). Since dorsal root ganglion cell body diameter is correlated to axon conduction velocity and sensory modality (Yaksh and Hammond, 1982), this evidence is indicative of T-type current specifically localised to smaller A$$\delta$$- and C-type sensory neurones that convey thermal and nociceptive information and not to larger A$$\beta$$-type neurones that subserve tactile and proprioceptive pathways. In the present study the extent of inhibition observed with the highest dose of ethosuximide was A$$\delta$$- fibre > C-fibre > A$$\beta$$- fibre, which fits well with these studies. However, the A$$\delta$$- and C-fibre responses were not markedly inhibited over the A$$\beta$$- fibre response, as one might expect if smaller diameter sensory neurones exhibited substantial low voltage-activated Ca$$^{2+}$$ current. Also no difference in the extent of inhibition was observed for the evoked responses to innocuous and noxious mechanical and thermal stimuli. This may again be explained by the existence of the three $$\alpha_1$$ subunits, each with a unique distribution, encoding for T-type Ca$$^{2+}$$ channels. Data suggests that diversity exists between T-currents of different cell types both in terms of kinetics and pharmacological sensitivity (Huguenard, 1996). For example, the current clinical use of ethosuximide to treat epilepsy is via block of T-current in thalamic neurones (Coulter et al., 1989a,b). However, T-current in GH3 cells is relatively resistant to block by ethosuximide (Herrington and Lingle, 1992) and in dorsal root ganglion neurones ethosuximide is over an order of magnitude less effective (Coulter et al., 1989a,b). Furthermore, the blockade is complete in dorsal root ganglion but only partial (40%) in thalamic neurones. $$\alpha_1$$G is the predominant subtype found in thalamic relay neurones, and therefore may be more sensitive to the effects of ethosuximide in comparison to the $$\alpha_1$$H T-type channel which is more abundant than $$\alpha_1$$G in the outer lamina of the spinal cord and dorsal root ganglia (Talley et al., 1999).

Two other licensed anticonvulsants, carbamazepine and gabapentin, have been investigated using the same experimental protocol as the present study (Chapman et al., 1998). Carbamazepine, a sodium channel blocker, and gabapentin, thought to act via Ca$$^{2+}$$ channels (see Matthews and Dickenson, 2000) were found to have similar efficacy and range of effectiveness as ethosuximide. Although both gabapentin and ethosuximide were equally effective in shamm and neuropathic animals, carbamazepine was only effective in the latter group and this could possibly be a consequence of differential regulation of Ca$$^{2+}$$ and sodium channels following nerve injury. To our knowledge the present study is the first to demonstrate a possible role of T-type Ca$$^{2+}$$ channels in the spinal processing of sensory information related to pain. Given the parallels between epilepsy and pain, the likelihood of common causal mechanisms and the ability of antiepileptic drugs to be effective in neuropathic pain states, the results indicate that ethosuximide may merit both behavioural testing in animals and human studies.

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