THE EFFECT OF NERVE INJURY
ON THE SPINAL AND PERIPHERAL ACTIONS OF
GALANIN AND INTERLEUKIN-6
ON SENSORY PROCESSING

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ABSTRACT

Neuropathic pain, subsequent to nerve damage, is a complex condition to alleviate and leads to many chronic pain patients receiving inadequate relief from their symptoms with the current treatments available. Nerve injury results in a number of anatomical, physiological and pharmacological changes. These include an upregulation of galanin, a 29-amino acid neuropeptide, and interleukin-6 (IL-6), a neuropoietic cytokine, in the dorsal root ganglion (DRG) and in the spinal cord. The aim of this thesis is to establish what role galanin and interleukin-6 play in sensory processing spinally or in the periphery in naive and spinal nerve ligated (SNL) rats. The effects of galanin and interleukin-6 were examined *in vivo* and *in vitro* using behavioural techniques and electrophysiology to record from convergent dorsal horn neurones and single nociceptive fibres. Spinal exogenous galanin profoundly inhibited neuronal responses in SNL rats, yet facilitated the same responses in naive rats. Peripheral galanin administration inhibited responses in a majority of nociceptive C-fibres and spinal neurones and facilitated responses in the remaining fibres and neurones. Following nerve injury, the proportion of neurones inhibited by galanin was increased. Spinal IL-6 administration inhibited neuronal hyperexcitability and responses to C-fibre and mechanical stimulation in SNL rats, but had no effect in naive rats. Peripheral IL-6 administration inhibited spinal neuronal responses *in vivo*, heat responses of nociceptive fibres *in vitro* and had a behavioural anti-nociceptive effect in naive rats. In SNL rats, only spinal neuronal responses to heat were inhibited by peripheral IL-6 administration. Overall these studies show that nerve injury changes the effects of galanin and interleukin-6 and suggests these systems could be potential novel targets for the treatment of neuropathic pain.
DEDICATION

For my beloved parents, without you this would not have been possible, and is written in hope of an ultimate aim:

To feel alright down both sides, not just one.
...And if the night runs over
And if the day won’t last
And if your way should falter
Along the stony pass
It’s just a moment
This time will pass
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First and foremost, sincere thanks to the Almighty for comfort, guidance, and strength.

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Many thanks to my academic supervisor Tony for invaluable advice over nearly 7 years at UCL, as admissions tutor - to come to UCL for BSc Pharmacology, as graduate advisor - to join your lab for a PhD and throughout my PhD. For your support, enthusiasm, superb diagrams and an excellent start to my career, I will be eternally grateful. Thanks to the girls of G35, for numerous things: Louise (scientific explanations and comedy sharp intakes of breath - the cell was slipping!), Rie (neuropathic stuff and fantastic support during the marathon), Katie (clinical answers and company on late night/weekend shifts), Liz (lessons in Chung and Word, but NOT tequila!) and Idil (double wammy assistance and salsa). Many thanks also to the old boys of F13, Mark for chat and cups of green tea, Vesa for good advice and company in San Diego and Lars - for making conferences such amusing alcoholic fun!

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Someone told me when I was at 19 miles of the 2001 London marathon that my PhD would be really easy compared to running the marathon. They lied. I may well run another marathon, but I will never ever write up another thesis. Admittedly there was a similar desire at about two-thirds the way through both my Phd and marathon ......... ....to run to the nearest pub!!

SJLF, 19th March 2002
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1.1 Clinical aspects of neuropathic pain

Pain can be broadly divided into two categories, acute pain and chronic pain, which differ in their duration and pathophysiology. Acute pain arises from noxious stimulation caused by tissue or nerve damage, but more often the former, and subsides within days or a few weeks. Chronic pain persists for months or years and can be inflammatory (arising from tissue damage) or neuropathic (arising from nerve damage) in its nature, or a combination of both of these. Neuropathic pain, the focus of this thesis, has been defined as 'pain initiated or caused by a primary lesion or dysfunction in the peripheral or central nervous system' by The International Association for the Study of Pain (IASP). Conditions in which neuropathic pain can occur are usually categorised as either peripheral or central neuropathies. Peripheral neuropathies could result from many different situations including traumatic nerve injury, nerve entrapment or compression, amputation resulting in stump or phantom pain, and cancer i.e. nerve damage as a result of neural invasion or tissue compressing a nerve as a result of a tumour. Other causes include viral infections such as HIV and herpes zoster i.e. postherpetic neuralgia and the effects of chemotherapy, radiation or surgery. Painful neuropathies can also result from metabolic disorders such as diabetes. Central neuropathies may appear following spinal cord injury, stroke or multiple sclerosis. These conditions do not necessarily result in neuropathic pain for all patients with these conditions and for many of these clinical situations it is not known what percentage of patients suffer neuropathic pain. However, it is estimated that 5% of patients (Sunderland 1993) with traumatic nerve injury, 8% of stroke patients (Andersen et al. 1995) and 28% patients with multiple sclerosis (Boivie 1999) suffer with neuropathic/central pain.

Neuropathic pain is characterised by several symptoms and clinical phenomena that often include sensory deficits, ongoing pain, allodynia (touch-evoked pain) and hyperalgesia (abnormal painful response to a noxious stimulus). The pain experienced by patients varies in its nature and can be continuous, spontaneous, paroxysmal, evoked abnormally e.g. allodynia, or a combination of these. Paroxysmal pain is episodic and is often described as a stabbing, electrical shock-like pain or 'sharp attacks'. Patients with
neuropathic pain have described their pain with terms including burning, crawling, aching and cramping pain. Allodynia is defined as pain evoked in response to a normally innocuous stimulus usually a light mechanical dynamic stimulus e.g. brush or even contact with clothes (mechanical allodynia) but it can manifest as an enhanced sensitivity to cold (cold allodynia). In addition, a static mechanical allodynia can been evoked by punctate non-noxious stimulus (Jensen 1996).

Areas of sensory deficits are a characteristic of neuropathic pain where there is a complete or partial loss of afferent sensory function often to noxious and thermal stimuli. These can lead to negative symptoms e.g. numbness or muscle weakness – where impaired conduction in efferent nerves leads to sensory and motor deficits. In contrast, patients have also described positive symptoms, which can manifest as tingling sensations or a heightened recognition of sensation which can be without pain (hyperesthesias) or painful (dysesthesias). One example of abnormal evoked pain seen in neuropathic pain conditions and quite different to allodynia is hyperpathia, where pain thresholds are higher than normal but once the pain threshold is reached the pain intensity rapidly increases resulting in an explosive pain response. Abnormal summation or wind-up-like pain is the progressive heightening of pain evoked by repetitive stimulation with a mechanical or thermal mildly noxious stimulus e.g. pinprick and is a feature of neuropathic pain which can result in after-sensations and a persistent pain, which lasts long after the painful stimulus has stopped. The main pharmacological treatments for neuropathic pain will be described later (see section 1.5), following the description of the mechanisms of sensory processing and the changes which occur following nerve injury.

1.2 Peripheral mechanisms of sensory processing

Neuropathic pain arises from changes in the peripheral sensory neurone that in turn impact upon the CNS. The primary afferent neurone is essentially a cell body (dorsal root ganglion) with a stem process that bifurcates into a central process that enters the
dorsal horn and a peripheral process, which extends out to its target organ in the periphery. Here, I will describe the events between transduction of a stimulus from the peripheral endings through to the spinal cord, and consider the different sensory receptors, afferent fibres and dorsal root ganglion (DRG).

1.2.1 Sensory receptors

The energy of an external stimulus can change the permeability of sensory membranes to ions, and action potentials are generated. This electrical signal is then conveyed to the spinal cord. The nature of this signal depends on the type of receptors activated, which is dependent on the modality of the initial stimulus. The sensory receptors of the skin are broadly categorised in terms of the stimuli that they respond to, giving rise to three categories, mechanoreceptors, thermoreceptors and nociceptors. Mechano- and thermoreceptors respond to innocuous mechanical and innocuous thermal stimulation, respectively. Nociceptors respond to noxious stimuli (thermal, mechanical or chemical) that threaten or cause tissue damage.

Mechanoreceptors

The mechanoreceptors respond to innocuous or low threshold mechanical stimulation and not thermal or chemical stimuli. They generally consist of an afferent fibre terminal that is linked to a specially adapted end organ. There are 3 broad classes of cutaneous mechanoreceptors, slowly adapting, rapidly adapting and Pacinian afferents (Fox 1999; Johnson 2001). Slowly adapting receptors are split into two categories, SA I/Type I and SA II/Type II. SA I are present in glabrous skin and these receptors are known as Type I in hairy skin. The transduction is through Merkel cells of the epidermis that are innervated by Aβ-fibres. SA I/Type I receptors respond to sustained indentation of the skin with a sustained slowly adapting discharge that is related to the indentation depth (Johnson 2001). SA II receptors are found in glabrous skin and termed Type II receptors in hairy skin. They are also innervated by Aβ-fibres that end in Ruffini corpuscles found in the connective tissue of the dermis and thus are mainly sensitive to stretch of the skin. SA II have larger receptive fields than SA I (Johnson 2001).
Rapidly adapting receptors of glabrous skin are responsible for the detection and discrimination of low frequency stimulation. They consist of Aβ-fibres ending in Meissner corpuscles of the dermal ridges that lie just below the epidermis (Johnson 2001). In hairy skin, rapidly adapting receptors are associated with the follicles of guard (G), tylotrich (T) or down (D) hairs (Fox 1999). D-hair receptors have only been reported in animals, are highly responsive to hair movement and are innervated by Aδ-fibres (Burgess et al. 1968; Lynn and Carpenter 1982). G- and T-hair receptors innervate Aβ-fibres and respond to movement of longer somatic hairs (Burgess et al. 1968). Finally, Pacinian afferents are innervated by Aβ-fibres, which end in Pacinian corpuscles that are found in the dermis and deeper tissues. These mechanosensitive transducers are extremely sensitive to skin indentation and are most responsive to high frequency vibratory stimulation.

Thermoreceptors

Two types of sensory receptors are found in mammalian skin that respond to innocuous changes in temperature; warm receptors and cold receptors. Warm receptors are thought to have free nerve terminals that are situated within the dermis and most are innervated by C-fibres. The temperature range that the warm receptors fire in is somewhat approximate, but they usually show a steady discharge somewhere between 30°C - 46°C and this activity can increase as the temperature increases (Raja et al. 1997; Fox 1999). However, characteristically, the discharge from warm receptors ceases completely with cooling or when noxious heat beyond their temperature range is applied.

Cold receptors are innervated by C- or Aδ-fibres and these receptors display a background discharge at normal skin temperatures and as the skin is cooled their firing increases. The proportion of sensory receptors responding to cold, like heat, appears to depend on the temperature of the cold stimulus. 4% of the C-fibre units (Lynn and Carpenter 1982) and 30% of the A-fibre units (Simone and Kajander 1997) examined in the rat saphenous nerve were classified as cold receptors using a 0°C stimulus. However,
it has also been reported that all of the A- and C-fibre nociceptors examined responded to temperatures below 0°C (Simone and Kajander 1996; Simone and Kajander 1997) suggesting that the number of cutaneous nociceptors excited by noxious cold is very high. This year, a breakthrough has been made in the understanding of cold sensation, with the identification of a receptor that responds to cool (8-28°C) temperatures and menthol (McKemy et al. 2002; Peier et al. 2002). This receptor is a member of the transient receptor potential (TRP) family of ion channels and has been named CMR1 (cold- and menthol-sensitive receptor) (McKemy et al. 2002) and TRPM8 (Peier et al. 2002). Both groups find that CMR1/TRPM8 is expressed on small DRG neurones, but not on large DRG neurones and suggest that a small proportion (10-20%) of C-fibres/Aδ-fibres express this receptor.

**Nociceptors**

The nociceptors are the sensory receptors of the skin that respond to noxious stimulation and have been most closely studied experimentally, both in humans (Van Hees and Gybels 1972) and animals (Bessou and Perl 1969; Lynn and Carpenter 1982). Single unit recording *in vivo* (Lynn and Carpenter 1982) and *in vitro* (Reeh 1986) has shown that four classes of cutaneous nociceptors exist, HTMs, HMs, polymodal and silent nociceptors. HTM nociceptors are so-called because they respond only to high threshold mechanical, not heat or chemical, stimuli and are innervated by Aδ-fibres (Aδ-HTM) or C-fibres (C-HTM). Studies using rat skin found approximately 20% of Aδ-fibres examined were Aδ-HTMs and 10-15% of C-fibres examined were C-HTMs (Lynn and Carpenter 1982; Seno and Dray 1993). Ultrastructural studies have examined Aδ-HTMs to find they have fine endings covered with Schwann cells, which are situated in the epidermis (Kruger et al. 1981). Aδ-HTMs have complex receptive fields with multiple sites of sensitivity (Lynn and Carpenter 1982) and whilst they do not characteristically respond to noxious heat, a small number have been reported to respond to heat following repeated applications (Fitzgerald and Lynn 1977; Lynn and Shakhanbeh 1988).
HM nociceptors (some references use the term MH nociceptors) respond to both noxious mechanical and noxious heat stimuli and are innervated by A\(\delta\)-fibres (A\(\delta\)-HM) or C-fibres (C-HM) (Bessou and Perl 1969). Although in more recent years, HM nociceptors have been found to frequently respond to chemical stimulation and thus are referred to as polymodal nociceptors (see below). C-HMs are found in glabrous and hairy skin whereas A\(\delta\)-HMs predominant in hairy skin (Raja et al. 1997). However, some authors have classed nociceptors slightly differently from others. For example, A\(\delta\)-HTMs (see Fox 1999) have also been termed Type I AHMs on account of them responding to mechanical stimuli and temperatures exceeding 52 °C (Raja et al. 1997). A\(\delta\)-HMs have also been termed Type II AHMs as they respond to lower temperatures and are mechanically sensitive (Raja et al. 1997).

Polymodal nociceptors respond to noxious mechanical, heat and chemical stimulation and may be innervated by A\(\delta\)-fibres or C-fibres. A number of in vitro studies with rat skin report that the majority of polymodal nociceptors display C-fibre conduction velocities (see 1.2.2) with a much smaller proportion innervated by A\(\delta\)-fibres (Szolcsanyi et al. 1988; Lang et al. 1990; Seno and Dray 1993). This may suggest that C-fibres are more likely than A\(\delta\)-fibres to display polymodal characteristics. 73% of the C-fibre units examined in the rat saphenous nerve were found to be polymodal nociceptors. C polymodal nociceptors have single spot receptive fields of less than 2mm\(^2\) (Bessou and Perl 1969; Lynn and Carpenter 1982), yet the morphology of these receptive terminals is unclear at present. Polymodal nociceptors are often characterised by their ability to respond to capsaicin as mechanoreceptors, thermoreceptors, HTMs and HMs do not respond to this chemical stimulus (Szolcsanyi et al. 1988; Lang et al. 1990). Both A\(\delta\)-HTMs and C polymodal/ C-HMs nociceptors have been reported to be sensitised following noxious heat (Fitzgerald and Lynn 1977; Lynn 1979; Lynn and Shakhanbeh 1988). However others have found that C polymodal/ C-HMs nociceptors in glabrous skin can become less responsive following repetitive heat stimulation (see section 3.3) (Raja et al. 1997). An important issue relates to the polymodal nature of many nociceptors - how do they respond to both chemical and other stimuli? The answer
seems to be that many receptor proteins exist on nociceptors, some of which respond to thermal/mechanical stimuli and others chemical stimuli, whereas others respond to both chemical and thermal/mechanical stimuli (see below).

The fourth and final class of nociceptors is the 'silent' nociceptors. This class acquired its name because they do not normally respond to acute noxious stimuli. However, following inflammation and tissue injury these nociceptors can be sensitized and will then respond to chemical mediators e.g. bradykinin (Lang et al. 1990). Silent nociceptors are innervated by C-fibres and their recruitment under pathological conditions would enhance the level of excitation that reaches the spinal cord (Millan 1999).

*Endogenous heat-activated ion channels*

A discussion about sensory receptors that respond to heat would perhaps be incomplete without referring to the recent identification of the putative heat sensor, the vanilloid receptor, VR1 (Caterina et al. 1997). VR1 receptors are found on C-fibre afferents and are activated by capsaicin (a response which is potentiated by protons, reduced pH) and temperatures exceeding 43°C (Caterina et al. 1997). The VR1 cDNA encodes for an 838 amino acid protein with six proposed transmembrane regions and VR1 receptors belong to the TRP family of ion channels. VR1 knockout mice did not show capsaicin-evoked pain behaviour but did show longer behavioural latencies to 50-58°C temperatures than wild-type mice (Caterina et al. 2000). Using the skin-nerve preparation (see section 2.6), the heat responses of nociceptive fibres in VR1 knockout mice were reduced but not abolished (Caterina et al. 2000). This indicates that the VR1 receptor is not solely responsible for responses to heat. Caterina and colleagues have also identified a capsaicin-receptor homologue, VRL-1, which is only activated by high temperatures (~52°C) and does not respond to capsaicin (Caterina et al. 1999). Therefore at the molecular level, perhaps VR1 and VRL-1 receptors, and potentially others awaiting identification are responsible for the ability to detect a range of heat intensities.
There are a whole host of other receptors in the periphery that respond to various chemical mediators including P2X\textsubscript{2,3}, P2Y\textsubscript{4} (respond to adenosine triphosphate, ATP), B\textsubscript{2} (bradykinin), NK\textsubscript{1} (substance P), EP\textsubscript{2,4} (prostaglandins), H\textsubscript{1/2} (histamine), CGRP\textsubscript{1,2}, A\textsubscript{2A} (adenosine), 5-HT\textsubscript{2A,3,4,7}, ASICs (acid sensing ion channels, protons), trkA (nerve growth factor, NGF), trkB (brain derived neurotrophic factor, BDNF, neurotrophin 4/5, NT-4/5), trkC (neurotrophin 3, NT-3) and p75 (NGF, BDNF, NT-4/5, NT-3) (Millan 1999).

1.2.2 Sensory afferents

Sensory information is transmitted from the periphery to the spinal cord via three different types of sensory afferent fibres, A\textbeta-fibres, A\delta-fibres and C-fibres. Under normal conditions, A\textbeta-fibres transmit innocuous messages, A\delta-fibres transmit both innocuous and noxious whereas C-fibres predominantly transmit noxious messages. Thus there are two types of A\delta-fibres, one group responding to high threshold and another to lower threshold stimuli (Raja et al. 1997). In general, it is thought that A\delta-fibres elicit a swift 'sharp' first phase of pain whereas C-fibres evoke a second wave of 'dull' pain following a noxious stimulus to the skin (Millan 1999). Interestingly, it has been quantified that high rates of noxious skin heating activate A\delta-fibres and low rates of skin heating activate C-fibres (Yeomans et al. 1996; Zachariou et al. 1997).

Typically, about 70% of the sensory fibres in the skin are C-fibres, 10% A\delta-fibres and 20% A\textbeta-fibres, (Millan 1999). A\textbeta-fibres and A\delta-fibres are myelinated whereas C-fibres are unmyelinated. Myelin is interrupted along the axon forming nodes of Ranvier, which facilitate the propagation of electrical signals along the axon. Thus the conduction velocity of fibres is dependent on the myelination and diameter of the fibre; resulting in fast conduction of non-nociceptive information (A\textbeta-fibres) and slower conduction of nociceptive information (A\delta-fibres and C-fibres), see Table 1.2.2. C-fibres predominantly terminate in the lamina II outer, with a few terminations in lamina I (Sorkin and Carlton 1997). A\delta-fibres terminate mainly in lamina I, although this is
dependent upon where the Aδ-fibres have originated from. The innocuous Aδ-fibres arising from D-hair receptors terminate mainly in lamina III with a small contribution to lamina IIi, whereas Aδ-HTMs terminate in laminae I, IIo and V (Sorkin and Carlton 1997). Aδ-fibres from deep tissues such as muscle and joints either distribute to lamina I only, or lamina IV/V with some contribution to lamina I (Mense and Prabhakar 1986). Finally, the large myelinated Aβ-fibres terminate profusely in laminae III/IV with some termination in laminae V from SA I mechanoreceptors (Sorkin and Carlton 1997).

Table 1.2.2 Characteristics of sensory fibres in the rat. Taken from (Millan 1999), (Sorkin and Carlton 1997).

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<th>Conduction Velocity</th>
<th>Fibre Diameter</th>
<th>Main Lamina Innervated</th>
<th>Cell size in DRG</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-fibres</td>
<td>0.5 - 2 m/s</td>
<td>0.4 - 1.2 μm</td>
<td>IIo</td>
<td>Small &lt; 30 μm</td>
</tr>
<tr>
<td>Aδ-fibres</td>
<td>2 - 7 m/s</td>
<td>2 - 6 μm</td>
<td>I &amp; V</td>
<td>Medium 30-40 μm</td>
</tr>
<tr>
<td>Aβ-fibres</td>
<td>7 - 75 m/s</td>
<td>&gt; 10 μm</td>
<td>III / IV</td>
<td>Large 40 - 75 μm</td>
</tr>
</tbody>
</table>

1.2.3 Dorsal root ganglion

The DRG is the source of proteins such as receptors, channels and transporters, which once made, can be transported bi-directionally out towards the central and peripheral terminals of the neurone. DRG cells can be divided into three biochemically-defined populations that only fractionally overlap (McMahon et al. 1997; Boucher and McMahon 2001). Firstly, the ‘large light’ DRG cell population have myelinated axons (Aβ-fibres) and are distinguished by their expression of neurofilament. These cells constitute approximately 30% of DRG cells and express p75, trkB and trkC neurotrophin receptors. A second population (~40% of DRG cells) of DRG cells expresses CGRP and substance P and are mostly small in size with unmyelinated axons. This neuropeptide-expression population is dependent on NGF and mainly expresses VR1, p75 and trkA neurotrophin receptors. The third class of DRG cells comprises
roughly 30% of the total DRG cell population and can be identified by their non-peptide nature and the binding of the lectin IB4. These small DRG cells with unmyelinated axons are dependent upon the trophic factor, glial cell line-derived neurotrophic factor (GDNF) and express VR1, P2X_{3}, RET (GDNF signal transducer) and GFRα1/2 (GDNF receptor) (McMahon et al. 1997; Boucher and McMahon 2001).

1.3 Central mechanisms of sensory processing

This section will outline the anatomy, neurophysiology and pharmacology of the spinal cord. The dynamic properties of the inhibitory and excitatory spinal systems under normal physiological conditions will be discussed, and how these systems converge and then relay painful messages to the brain. This thesis focuses on the effects of drugs on spinal neurones and thus the ascending and descending pathways to and from the brain will be outlined, however the anatomy and neurophysiology of the brain will not be discussed.

1.3.1 Anatomy of the spinal cord

Afferent sensory fibres and efferent motor fibres enter the spinal cord through the dorsal root and to a minor extent, through the ventral root, respectively. A small proportion of afferent fibres enter through the ventral root and terminate in lamina I (marginal zone) and lamina II (substantia gelatinosa) (Light and Metz 1978). As primary afferents enter the spinal cord they project into the dorsal horn at the segment of entry and form collaterals which project in a rostral and caudal direction. Collaterals containing large myelinated afferents, presumably Aβ-fibres, project into the dorsal columns both directly and also via the postsynaptic dorsal column pathway (PSDC). Collaterals containing small myelinated and unmyelinated afferents, presumably Aδ-fibres and C-fibres, travel in a bundle known as Lissauier's tract. These collaterals then may re-enter the dorsal horn at other segments and make connections with neurons that reside outside of the initial point of entry.
The spinal cord consists of white matter containing axons and a core of gray matter containing neuronal cell bodies. The gray matter is divided into 10 laminae. This division of the gray matter was first described in the early 1950's in cats and was based on the local cyroarchitecture i.e. the different types, myelination and density of cells (Rexed 1952). Thereafter, these laminae were also shown in the rat (Wall 1967; Molander et al. 1984). These laminae are present throughout the spinal cord from sacral to cervical segments with the exception of lamina VI, which is absence in thoracic spinal cord (Rexed 1952). Laminae I-VI constitute the dorsal horn, laminae VII-IX the ventral horn and lamina X is the gray matter surrounding the central canal, also known as the substantia grisea centralis. The main terminations of sensory afferents are shown in Table 1.2.2 and Figure 1.3.1 illustrates the laminar and afferent terminal organization of the spinal cord.

Lamina I, or marginal zone, forms a thin layer around the top of the dorsal horn and this extends around and down the side of the dorsal horn to the level of lamina III. Lamina I contains large marginal cells of Waldeyer, which have long horizontal dendrites that usually pass over the surface of the dorsal horn but sometimes terminate in deeper layers. Small neurones are also found in this layer and these neurones project to the brain.

Lamina II is also known as the substantia gelatinosa (SG), due to its clear gelatinous appearance (see Cervero and Iggo 1980) and is divided into two sections, lamina II outer (IIo) and lamina II inner (IIi). Lamina II outer contains densely packed cells whereas lamina II inner is less compact and more ventral in its location. Two predominant types of cells are found in lamina II, stalk cells and islet cells, but arboreal, border and spiny cells are also present. The cell bodies of stalk cells reside in IIo with dendrites projecting into deeper laminae (Light and Kavookjian 1988). Stalk cells relay transmission from the superficial to deeper laminae and are thought to be excitatory (Gobel et al. 1980), however enkephalin has been found in some stalk cells (Bennett et al. 1980). Islet cells are mainly found in IIi and their dendrites stretch out within lamina II in a mediolateral
plane (Gobel 1978). Islet cells are thought to be inhibitory cells (Gobel et al. 1980) as cells with a similar morphology contain GABA, also the dendrites of these cells contain vesicles and form dendroaxonic/dendrodendritic synapses (Gobel 1978).

The cell bodies in lamina III are on average larger and less densely packed compared to the substantia gelatinosa (Gobel 1978). Branches of lamina III axons terminate in lamina I, IV, V and VI or add to ascending tracts to the brain (Matsushita and Ikeda 1970; Mannen and Sugiura 1976). Lamina IV forms the base of the head of the dorsal horn and the cells are less densely packed than seen in lamina III due to increased numbers of nerve fibres passing through this space (Sorkin and Carlton 1997). Lamina V crosses the narrowest section of the dorsal horn and the cells found here project their dendrites dorsally towards superficial laminae. Thus, lamina V cells can receive input from C-fibres via their dendrites situated in lamina II where C-fibres terminate (Fitzgerald and Wall 1980). Lamina VI comprises the base of the dorsal horn and is present in cervical and lumbar segments of the spinal cord only. The majority of cells in lamina VI are thought to be propriospinal neurones and the dendrites of lamina VI cells do not extend as far as laminae I and II.
Figure 1.3.1 Laminar organisation of the spinal cord with termination patterns of afferent sensory C-, Aδ- and Aβ-fibres.
Types of dorsal horn sensory neurones

There are three categories of dorsal horn neurones, interneurones, propriospinal neurones and projection neurones. These classes of neurones process sensory input which enables the organism to make appropriate and graded responses. The vast majority of intrinsic dorsal horn neurones are local interneurones. These neurones are typically small cells, either excitatory or inhibitory, and relay information between different laminae of the spinal cord. Propriospinal neurones have longer axons than interneurones and transfer information between spinal segments and so participate in heterosegmental reflexes. Projection neurones transfer sensory information from the spinal cord to the brain, yet they constitute a small number of the total number of cells in the spinal cord (Sorkin and Carlton 1997).

In addition to this classification of neurones based on their projections, neurones have also been grouped in terms of their responses to natural peripheral stimuli. This intensity-dependent categorisation has resulted in four main classes of cells, low-threshold (LT), multireceptive, high threshold (HT), and deep cells. LT cells (class 1) respond to light touch, pressure or hair movement and can be subdivided into neurones that respond to touch or hair movement (class 1A) and touch-pressure neurones (class 1B). These neurones do not usually respond to noxious heat or chemical stimuli and their firing frequency cannot be increased with noxious mechanical stimulation. LT cells are mainly innervated by Aβ-fibres and are found in laminae III and IV of the dorsal horn. Multireceptive (class 2) cells are also known as wide dynamic range (WDR) or convergent dorsal horn neurones. These names describe both low- and high-threshold inputs that converge onto these cells from Aβ-, Aδ- and C-fibres and also that these neurones respond to both innocuous and noxious mechanical and thermal stimulation. Moreover, the firing frequency of these neurones increases as stimulus intensity increases; this is demonstrated in chapter 3 with the characterisation of the convergent dorsal horn neurones that I have recorded from laminae V. Smaller populations of wide dynamic neurones are found in lamina I and X. High threshold (HT), or nociceptive-specific neurones (class 3) are also subdivided; class 3A-neurones that only respond to
noxious mechanical stimuli and are mainly innervated by Aδ-fibres and class 3B-neurones that respond to noxious mechanical/thermal stimulation and are innervated by Aδ- and C-fibres. HT cells are mainly found in lamina I, with lower concentrations in lamina V and X. Finally, deep cells (class 4) response to stimulation of joints or muscles and are found in the laminae V & VI of the dorsal horn.

*Ascending pathways from spinal cord to brain*

Following processing in the dorsal horn, sensory messages are transmitted to supraspinal sites via several ascending tracts that terminate in different areas of the brain (Sorkin and Carlton 1997; Millan 1999). I will describe these tracts in terms of their origin, destination and cell type – low threshold (LT), wide dynamic range (WDR) and high threshold (HT) and hence the type of message these pathways transmit as described previously. The most important of these appears to be the spinothalamic tract (STT) which projects from the spinal cord to various thalamic sites including the ventral posterior nucleus (VPL) and the central lateral nucleus. STT neurones are chiefly WDR and HT and they originate mainly from lamina I, but also from laminae IV, V and VII. The spinoreticular tract (SRT) refers to spinal neurones predominantly from laminae V and VI, with some from lamina I, that project to the reticular formation of the brain stem. The SRT neurones are largely HT, with some WDR and even fewer LT neurones. The spinomesencephalic tract (SMT) consists of several discrete pathways that project from the spinal cord to the parabrachial area in the midbrain. The lamina I projection, containing HT neurones, of the SMT terminates in the lateral periaqueductal gray (PAG) whereas as the smaller spinal projections, of LT and WDR neurones, from deeper laminae (IV-VIII) terminate in the lateral and medial PAG. The spinohypothalamic tract (SHT) mainly consists of HT and WDR neurones which transmit information from the laminae I and V to the hypothalamus. Neurones of the spinocervical tract (SCT) are mostly of the LT type with a few WDR neurones. The SCT predominantly starts in laminae III/IV with much smaller inputs from laminae I/V and projects to the lateral cervical nucleus. The postsynaptic dorsal column pathway (PSDC) chiefly originates from laminae III-V, projecting to the dorsal column nuclei and consists of LT, WDR and
HT neurones. The spinoparabrachio-amygdaloid and spinoparabrachio-hypothalamic tracts both project to the parabrachial nucleus then onto the amygdala and hypothalamus, respectively. Both these tracts originate primarily in lamina I, with a small contribution from lamina II and they consist of LT neurones alone (Sorkin and Carlton 1997; Millan 1999).

1.3.2 Pharmacology of excitatory pain transmission

The stimulation of primary afferents results in the excitation of dorsal horn neurones. Excitatory transmitters in the spinal cord include glutamate, aspartate, substance P, calcitonin gene-related peptide (CGRP) and neurokinin A. There are also other excitatory mediators, adenosine triphosphate (ATP), prostaglandins and nitric oxide that are produced and released on demand. I will elaborate on the two main excitatory transmitters, glutamate and substance P, in normal conditions.

Glutamate

Glutamate was first shown to depolarize and excite neurones in the dorsal horn of cats (Curtis et al. 1959). This amino acid is found in rat DRG neurones (Battaglia and Rustioni 1988), dorsal roots (Westlund et al. 1989), neurones and terminals of the superficial dorsal horn (Miller et al. 1988; Kai-Kai and Howe 1991). The quantity of glutamate-immunoreactive terminals in the rat spinal cord was more than 10-fold higher than terminals containing aspartate and five-fold higher than any other transmitter (Merighi et al. 1991). Evidence for glutamate release following noxious stimulation has been demonstrated both in vitro and in vivo. An increase in glutamate release from rat primary afferent neurones in culture (Jeftinija et al. 1991) and rat spinal dorsal horn slices (Kangrga and Randic 1991) was seen following the peripheral application of capsaicin. Also an intradermal injection of 5% formalin in vivo, caused marked increases in the extracellular concentration of glutamate in the dorsal horn of rats (Skilling et al. 1988). However, glutamate is not only associated with nociceptive fibres, it is found in some large DRG neurones (Battaglia and Rustioni 1988) and selective stimulation of
low threshold Aβ-fibres does evoke an increase in glutamate release (Kangrga and Randic 1991).

The receptors that are activated by glutamate are classified into two major subtypes, ionotrophic glutamate receptors - coupled directly to cation-permeable ion channels and metabotropic glutamate receptors (mGluRs) – coupled via G-proteins to soluble second messengers. The ionotrophic receptors are divided into N-methyl-D-aspartate (NMDA) receptors and non-NMDA receptors. The non-NMDA receptors can be further divided into α-amino-2, 3-dihydro-5-methyl-3-oxo-4-isoxazolepropanic acid (AMPA) receptors and kainate receptors originally on the basis of the preferential binding of agonists, AMPA and kainate, but more recently from molecular approaches.

AMPA receptors consist of GluR1-4 subunits and the majority are calcium impermeable (determined by the GluR2 subunit). Kainate receptors are made from GluR5-7 and KA1-2 subunits and are calcium permeable (Hollmann and Heinemann 1994). In both AMPA and kainate receptors these respective subunits form homomeric or heteromeric pentamers (Hollmann and Heinemann 1994). AMPA and kainate subunits are expressed in the DRG (Sato et al. 1993). GluR1-4 mRNA is expressed throughout the dorsal horn, with high levels of GluR2 mRNA and moderate levels of GluR1 mRNA in the superficial dorsal horn (Furuyama et al. 1993; Tolle et al. 1993). AMPA receptors are present pre- and post-synaptically in the spinal cord and are responsible for rapid excitatory transmission in the CNS (Carlton and Hayes 1990; Carpenter and Dickenson 2001). GluR6 mRNA is not detectable in the dorsal horn whereas mRNA encoding the other kainate subunits is present, but at lower levels compared to AMPA subunits (Furuyama et al. 1993). There is evidence to suggest that kainate receptors consisting of GluR5 subunits are expressed presynaptically (Agrawal and Evans 1986; Partin et al. 1993), with some electrophysiological evidence of a postsynaptic expression (Stanfa and Dickenson 1999).
NM DA receptors are involved in responses of dorsal horn neurones to intense noxious stimulation (see 1.3.3) and NM DA receptor activation results in slower more sustained EPSPs, which can last for a few minutes. The NM DA receptor is composed of subunits in that NR1 combines with NR2A, NR2B, NR2C or NR2D. In the dorsal horn of adult rats, the NM DA receptor is thought to be a tetramer and often consists of 2 NR1 subunits plus a permutation of NR2A/B, as little to no expression of NR2C and NR2D is seen in adult spinal cord. These subunits dictate characteristics of the receptors e.g. the kinetics of desensitization, ligand binding at regulatory sites and the magnesium block (Hollmann and Heinemann 1994). NM DA receptors are located on the post-synaptic membranes of dorsal horn neurones throughout the dorsal horn (Wilcox and Seybold 1997), although there appears to be some evidence for a presynaptic location (Liu et al. 1994; Ma and Hargreaves 2000).

So far, eight types of mGluRs have been identified that mediate slower modulatory events and they are split into three groups. Group I, mGluR1 and mGluR5, couple to Gq-protein activating phospholipase C (PLC). Group II, mGluR2 & mGluR3, and group III, mGluR4, 6-8 couple to Gi-protein to inhibit adenyl cyclase and increase potassium conductance. Group I mGluRs agonists have produced pro-nociceptive effects in normal animals (Stanfa and Dickenson 1998; Dolan and Nolan 2000). In contrast group, II mGluRs are thought to play an anti-nociceptive role in normal animals (Stanfa and Dickenson 1998; Dolan and Nolan 2000). The role of group III mGluRs remains to be elucidated, due to the lack of selective pharmacological agents.

Substance P
Substance P-containing neurones are found in the DRG and superficial dorsal horn (Gibson et al. 1981). Approximately, 30% of C-fibres and 10% of Aδ-fibres in rats display substance P-like immunoreactivity (McCarthy and Lawson 1989). Substance P extensively co-localises with glutamate in rat DRG neurones (Battaglia and Rustioni 1988), neuronal terminals in the rat dorsal horn and even in the same axonal boutons (Merighi et al. 1991). Furthermore, the DRG neurones that contain substance P are
small, with 90% of these also containing glutamate (Battaglia and Rustioni 1988). As most small DRG neurones are C-fibres, this strongly suggests that a noxious stimulus results in the co-release of glutamate and substance P, potentially resulting in wind-up (see 1.3.3). Substance P can also co-localize with CGRP, CCK, somatostatin, bombesin, VIP, dynorphin or enkephalin (Dickenson and Reeve 1999). A basal release of substance P in the laminae II, V and VI of the cat spinal cord has been demonstrated (Duggan and Hendry 1986). Moreover, noxious thermal, mechanical and chemical peripheral stimuli increased substance P release in the lamina II region, yet non-noxious thermal and mechanical stimulation did not alter substance P release (Duggan et al. 1988). Substance P preferentially binds to NK1 receptors, which are located postsynaptically in relatively high amounts in the superficial dorsal horn and to a lesser extent in lamina X (Kar and Quirion 1995). Exogenous administration of substance P produces pain-like behaviours e.g. scratching, biting and licking (Seybold et al. 1982) and hyperalgesia (Yasphal et al. 1982). Although, NK1 antagonists produced antinociceptive effects in animal models (Cahill and Coderre 2002), they did not prove to be effective in human pain states (for overview see Hill 2000).

1.3.3 Wind-up

The phenomenon of wind-up was first described in the mid 1960's, as a frequency-dependent facilitation of spinal cord neuronal responses mediated by afferent C-fibres (Mendell 1966). In other words, the repetition of a C-fibre stimulus (at an identical intensity) induces a sudden marked increase of certain spinal neurones, illustrations of this phenomenon are shown in figures 4.4.3 and 5.3.3 (p131 & p160). Wind-up is only observed at frequencies above 0.3Hz, and frequencies above 20Hz result in a wind-down of the response (Schouenborg 1984). The physiological and pharmacological data on wind-up and the preparations in which wind-up has been demonstrated has been extensively reviewed elsewhere (Herrero et al. 2000). Here, I aim to describe what is generally accepted as the mechanism underlining wind-up. The NMDA receptors are inactivate under normal physiological conditions because the ion channel of this receptor
is blocked by magnesium, Mg²⁺ (Mayer et al. 1984). Therefore, for NMDA receptor activation this magnesium block has to be removed and this is not achieved by the binding of glutamate and NMDA receptor co-agonist glycine. In the spinal cord, both glutamate and substance P and CGRP are released from presynaptic terminals. Substance P binds to NK₁ receptors on the postsynaptic membrane resulting in a slow summating depolarisation of this membrane. As the membrane potential becomes more positive the affinity of Mg²⁺ for the ion channel of the NMDA receptor is lessened and the Mg²⁺ block is removed. This results in a large increase in sodium and calcium influx into the postsynaptic membrane and high firing frequency of action potentials. In normal animals, wind-up can only be evoked by the activation of C-fibres and not Aβ-fibres (Schouenborg and Sjolund 1983). This is likely to be a consequence of substance P release, as noxious stimulation (C-fibre mediated) and not innocuous stimulation (Aβ-fibre mediated) elicits substance P release (Kuraishi et al. 1985a).

1.3.4 Pharmacology of inhibitory modulation

Inhibitory transmitters are also present in the spinal cord and these substances play an important modulatory role in suppressing nociceptive messages. Many transmitters produce antinociceptive effects including γ-aminobutyric acid (GABA), opioids, glycine, noradrenaline, adenosine, serotonin, neuropeptide Y (NPY) and somatostatin. I will elaborate on two of the endogenous inhibitory transmitters, GABA and the endogenous opioids.

γ-aminobutyric acid - GABA

GABA has been widely established as the main inhibitory neurotransmitter in the CNS (Dickenson et al. 1997b). GABAergic neurones have been found throughout the dorsal horn of rats and monkeys (Barber et al. 1982; Carlton and Hayes 1990) with GABA-immunoreactive profiles principally concentrated in laminae I-III (Magoul et al. 1987). In lamina II of the rat dorsal horn, presynaptic GABAergic terminals have been found to be found (Todd and Lochhead 1990), although there is also evidence of postsynaptic
GABA-immunoreactive dendrites in laminae I-III of the monkey spinal cord (Carlton and Hayes 1990). Within the rat dorsal horn, GABA has also been shown to co-exist with glycine (Todd and Sullivan 1990), met-enkephalin (Todd et al. 1992) and galanin (Simmons et al. 1995). Three GABA receptors have been identified, GABA_A, GABA_B and GABA_C. GABA_C receptors are not thought to play a role in sensory processing. GABA_A receptors consist of four different subunits, which form a chloride-permeable ion channel and thus GABA_A receptor activation results in hyperpolarisation. GABA_B receptors are G-protein coupled and when activated inhibit adenyl cyclase, decrease Ca^{2+} current and increase K^+ current. Iontophoretic application of GABA was shown to depress spinal neurones (Curtis et al. 1959). More recently baclofen, a GABA_B agonist, has been shown in electrophysiological studies to elicit inhibitory effects on primary afferents (Henry 1982; Dickenson et al. 1985) and is thought to work presynaptically, inhibiting transmitter release.

**Endogenous opioids**

Four opioid receptors and endogenous ligands for these receptors have been identified. The µ opioid receptor responds to endogenous β-endorphin and/or endomorphins. δ and κ opioid receptors are activated by endogenous enkephalins and dynorphins, respectively (Dickenson 1994). Most recently, a fourth opioid orphan (ORL-1) receptor was found, and can be activated by orphanin FQ also known as nociceptin (Meunier et al. 1995; Reinscheid et al. 1995). Opioid receptors are predominantly present on presynaptic terminals and their activation opens potassium channels (µ, δ and ORL-1) or closes calcium channels (κ receptors). This results in antinociceptive effects by hyperpolarising neurones and inhibiting neurotransmitter release (Dickenson 1994; Dickenson 1999). Cholecystokinin (CCK) can exert an anti-opioid effect via CCK_B receptors mobilizing calcium from intracellular stores. This would counteract an opioid-induced inhibition of calcium influx, thus reducing the opioid-induced inhibition of neurotransmitter release. The effects of CCK are somewhat selective, opioid analgesia mediated by δ-receptors is unaffected by CCK, whereas morphine analgesia is enhanced in the presence of a CCK_B antagonist (Dickenson 1999).
1.3.5 Inhibitory descending controls

As previously discussed (see 1.3.1), there are several ascending tracts that transmit nociceptive information from the spinal cord to the brain. The information that the majority of these nociceptive pathways transmit is in turn controlled by bulbospinal projections from brainstem nuclei that descend in the dorsolateral funiculus to terminate in the spinal cord (Dickenson et al. 1997a). Generally two descending modulatory pathways exist, one arises from the locus coeruleus and lateral tegmentum cells systems containing noradrenaline (Kwiat and Basbaum 1992) and the other arises from the rostroventromedial medulla (RVM) containing serotonin also known as 5-hydroxytryptamine, 5-HT (Azmitia and Gannon 1986). The periaqueductal gray (PAG) directly influences both origins of these pathways and the PAG is under the control of higher brain areas, (i.e. hypothalamus and frontal cortex) that can also have an additional direct effect on the RVM (Mantyh and Peschanski 1982; Holstege 1987). This basic nociceptive circuitry is completed with inputs from the dorsal horn to the thalamus, PAG and RVM by the STT, SMT and SRT ascending tracts, respectively (Sorkin and Carlton 1997; Millan 1999).

Further evidence of the descending modulatory role of noradrenaline is that noradrenaline is only released in response to high frequency afferent stimulation (Tyce and Yaksh 1981) and an intrathecal noradrenergic antagonist attenuated the antinociception induced by opiate administration into the PAG (Camarata and Yaksh 1985). In addition, noradrenaline-containing terminals in the spinal cord arise from supraspinal sites; no noradrenaline positive intrinsic spinal neurones have been found (Sorkin and Carlton 1997). Two adrenergic receptors have been identified in the spinal cord (Nicholas et al. 1993), presynaptic $\alpha_2c$ and postsynaptic $\alpha_2a$ receptors, which are thought to be coupled to $G_{i/o}$-protein and $K^+$ channels, respectively (Sorkin and Carlton 1997). The activation of these receptors inhibits transmitter release from primary afferent neurones (Kuraishi et al. 1985b; Pang and Vasko 1986; Holz et al. 1989) and dorsal horn neuronal responses to noxious stimulation (Fleetwood-Walker et al. 1985).
Like noradrenaline, 5-HT is also released following high but not low frequency afferent stimulation (Tyce and Yaksh 1981). Also intrathecal 5-HT antagonists attenuated the antinociception induced by electrical stimulation of the nucleus raphe magnus and nuclei of the reticular formation (areas within the RVM) (Hammond and Yaksh 1984). Unlike noradrenaline, 5-HT containing neurones have been found in laminae I, II and X of the spinal cord, however these are small in number and the majority of spinal cord 5-HT arises from supraspinal sites (Sorkin and Carlton 1997).

The effect of 5-HT in the spinal cord can be contrasting; for instance, 5-HT release induced by electrical stimulation of the nucleus raphe magnus caused both inhibition (Giesler et al. 1979) and excitation (Clatworthy et al. 1988). This is most likely due to the existence of many different 5-HT receptor subtypes, 5-HT_{1A,D}, 5-HT_{2A,C} and 5-HT_{3}. Descending inhibition is thought to occur via the activation of 5-HT_{1B} (5-HT_{1D} in humans) receptors (Alhaider and Wilcox 1993). This may be the substrate for the analgesic effects of tricyclic antidepressants and SSRIs (see 1.5) which block the reuptake of 5-HT (Kehl and Wilcox 1984; Hwang and Wilcox 1987). In contrast, 5-HT can also result in descending facilitation through the activation of 5-HT_{3} receptors (Millan 1997). In relation to this thesis, galanin has been reported to hyperpolarize noradrenaline and 5-HT neurones and also to enhance the inhibitory effect of 5-HT in these pathways (Hokfelt et al. 1998).

1.4 Mechanisms of sensory processing following nerve injury

Peripheral nerve injury results in many changes both peripherally and centrally, which can be anatomical or physiological in their nature. This section will firstly discuss how injury to the sciatic nerve of rats can induce neuropathic pain experimentally. From this, I will describe the consequences of such an injury in terms of; anatomical changes of the peripheral nerve, altered expression of ion channels and transmitters, involvement of the sympathetic nervous system and central changes in response to injured peripheral nerves.
1.4.1 Models of neuropathic pain

A wide variety of animal models of neuropathic pain have been developed including models of post herpetic neuralgia (Fleetwood-Walker et al. 1999), central pain (Xu et al. 1992) and diabetic neuropathy (Ahlgren and Levine 1993). I will describe in detail the models that involve an injury to the sciatic nerve. The first widely used of these models was axotomy (Wall et al. 1979), also referred to as complete sciatic nerve transection (CSNT). The sciatic nerve is exposed, cut and a complete section of the nerve removed. This procedure results in animals displaying autotomy, self-mutilation of the ipsilateral paw (Wall et al. 1979). Since humans do not display this symptom it may have limited relevance to human states. In a variation of this model the sciatic nerve is crushed and is often used to examine nerve regeneration (Devor et al. 1979). The three more commonly used models of peripheral nerve injury, the Bennett (chronic constriction injury), Seltzer (partial sciatic ligation) and Chung (spinal nerve ligation) models, involve some form of sciatic nerve ligation. The hyperalgesia and the effect of sympathectomy in these rat models of neuropathic pain have been directly compared in the same study (Kim et al. 1997) and the findings will be discussed below.

Chronic constriction injury - Bennett model

The CCI or Bennett model uses four loose ligatures of chromic gut tied around the sciatic nerve at mid thigh level (Bennett and Xie 1988). These rats develop mechanical and cold allodynia and also a persistent thermal hyperalgesia (Bennett and Xie 1988). However, some authors find that CCI does not produce allodynia in all operated rats (Gazelius et al. 1996; Cui et al. 2000). It has been suggested that the thermal hyperalgesia produced in this model may be partly due to the use of chromic gut, as similar operations with plain gut or silk produced slightly lesser degrees of thermal hyperalgesia (Maves et al. 1993). However, there was no difference in the degree of mechanical allodynia produced in the rats with chromic gut ligations compared to the rats with plain gut or silk ligation (Maves et al. 1993). CCI rats show the lowest response frequency to mechanical stimuli compared to PSL and SNL rats and their
evoked pain behaviours are reduced less after surgical lumbar sympathectomy than in PSL and SNL models (Kim et al. 1997).

Partial sciatic ligation - Seltzer model
In the PSL or Seltzer model, the dorsal third to half of the sciatic nerve is tightly ligated with silk thread (Seltzer et al. 1990). These rats develop mechanical allodynia and thermal hyperalgesia, but not autotomy (Seltzer et al. 1990). Like CCI, authors report that not all rats develop allodynia following PSL (Cui et al. 2000). PSL rats develop a more pronounced mechanical hyperalgesia than CCI rats, which is evident day 1 following surgery (Kim et al. 1997). Sympathectomy has a greater effect on the reversal of evoked pain behaviours induced by PSL than CCI (Kim et al. 1997).

Spinal nerve ligation - Chung model
In the SNL or Chung model, the L5 and L6 spinal nerves are isolated and tightly ligated with silk thread, leaving the L4 spinal nerve undamaged (Kim and Chung 1992). The idea here is to mimic nerve constriction whilst leaving an undamaged nerve to serve as a control. SNL rats develop both mechanical and cold allodynia, but not autotomy (see Chapter 3) (Kim and Chung 1992). Thermal hyperalgesia has been shown in SNL rats (Kim and Chung 1992); however it is not marked and other authors have not found any thermal hyperalgesia in rats following SNL (Kontinen et al. 1998). Compared to PSL and CCI rats, SNL rats show highest response frequency to mechanical stimuli. The effect of sympathectomy is controversial since it has been reported to cause a marked reversal of evoked pain behaviours in SNL rats compared to PSL and CCI rats (Kim et al. 1997). Although, other authors report that sympathectomy does not reverse mechanical hyperalgesia in SNL rats (Ringkamp et al. 1999). Therefore, to rank these three models in terms of their neuropathic pain characteristics would result in from the highest to the lowest; mechanical sensitivity SNL ≥ PSL > CCI and decrease in evoked pain behaviours following sympathectomy SNL > PSL > CCI. Cold allodynia was similar in magnitude for SNL, PSL and CCI rats (Kim et al. 1997).
Spared nerve injury

The spared nerve injury (SNI) model has been recently developed, involving axotomy and ligation of the common peroneal and tibial nerves leaving the sural nerve intact (Decosterd and Woolf 2000). This operation induces a marked mechanical and cold allodynia, in the lateral plantar and lateral dorsal parts of the ipsilateral paw, which are innervated by the sural nerve. Mechanical hypersensitivity was also produced in the saphenous nerve territory, medial plantar paw, although this was not as marked as that seen in the sural nerve territory. Thermal hyperalgesia and autotomy were not observed in this model. This model permits the testing of non-injured skin territories that are next to denervated areas in the same paw. However this model is not dissimilar to the SNL model, which also leaves an uninjured branch of the sciatic nerve. The advantages of this model as opposed to the SNL model are not clear. Models of sciatic nerve ligation are shown in figure 1.4.1, page 35.

Other models of sciatic nerve injury

Sciatic cryoneurolysis (SCN) model involves freezing the proximal sciatic nerve using a cryoprobe cooled to -60 degrees C in a 30/5/30 sec freeze-thaw-freeze sequence (DeLeo et al. 1994). Initially this operation results in a dysfunctional limb and foot oedema. As the sensation returns to the ipsilateral paw, autotomy was typically displayed and a prolonged bilateral mechanical allodynia, but not thermal hyperalgesia was observed. Sympathectomy did not reverse the mechanical allodynia produced in this model (Willenbring et al. 1995). The Gazelius model involves an ischaemic lesion of the sciatic nerve induced photochemically employing a low powered laser (Gazelius et al. 1996). These rats display mechanical allodynia, which is reported to occur with a greater incidence than CCI rats, although responses to thermal stimulation were similar in Gazelius and CCI rats (Gazelius et al. 1996). Generally speaking these two models have not been widely used to date.
Figure 1.4.1 Models of nerve injury involving a ligation of the sciatic nerve. This figure illustrates the four models described in the text, namely, the spinal nerve ligation (SNL) model, the partial sciatic ligation (PSL) model, the chronic constriction injury (CCI) model and the spared nerve injury (SNI) model.
1.4.2 Influence of the sympathetic nervous system

Despite extensive investigation, the contribution of the sympathetic nervous system to neuropathic pain is not yet fully understood. Under normal conditions, the sympathetic nervous system does not influence primary afferents. In contrast following nerve injury, the neuroma and DRG become sensitive to sympathetic stimulation and adrenergic agents such as noradrenaline, as a consequence of sympathetic axons sprouting to injured afferents. The mechanisms underlying such sensitivity to noradrenaline are unclear, potentially via $\alpha_2$-adrenoreceptors (for reviews see Millan 1999; Ramer et al. 1999). The effect of surgical sympathectomy has been shown to be effective in reversing neuropathic pain behaviours in rat models of peripheral neuropathy as previously discussed (see section 1.4.1). In animal models of nerve injury, sympathetic-primary afferent coupling can occur at the nerve lesion site or some distance away from the injury site (Janig and Baron 2001). In addition, following nerve injury sympathetic afferents sprout into the DRG and form basket-like structures around cell bodies (Ramer et al. 1999). The function of these sympathetic baskets is unknown and they are thought to grow in response to some factor(s) that are induced in the DRG following nerve injury (Ramer et al. 1999). Evidence suggests that two potential sprouting factors are NGF, as anti-NGF treatment reduces basket formation (Ramer and Bisby 1999) and IL-6, because basket formation is impaired in IL-6 knockout mice (Ramer et al. 1998).

1.4.3 Peripheral changes following neuropathy

Following injury to a peripheral nerve, most axons will regrow and re-establish synaptic connections. This process of regeneration involves the removal of debris from the injury site and the formation of growth cones (Garry and Tanelian 1997). However, some nerve fibres fail to regenerate and the axonal membrane fuses ‘sealing off’ the injured site forming a neuroma, or end bulb swelling (Garry and Tanelian 1997). The endings of injured sensory axons are depleted of myelin but enveloped by Schwann cell processes and neuroma formation is thought to arise from the continuing axonal transport carrying membrane-bound organelles to peripheral neuronal terminals (Fried et al. 1991). This
demyelination is thought to be caused by a process known as Wallerian degeneration, where macrophages infiltrate the site of injury and phagocytose myelin from damaged axons (Garry and Tanelian 1997; Rutkowski et al. 1999). The degree of Wallerian degeneration seems to be dependent on nerve blood flow (Myers et al. 1993) and the location of the nerve lesion; i.e. dorsal root lesion does not produce Wallerian degeneration (Koltzenburg and Scadding 2001).

Peripheral nerve injury reduces the number of sensory axons, especially large myelinated fibres (Garry and Tanelian 1997). CCI is reported to produce a near complete loss of large myelinated fibres distal to the ligatures (Basbaum et al. 1991). Furthermore, electrophysiologically an 85%, 55% and 9% loss of A\(\beta\)-, A\(\delta\)- and C-fibres has been reported following CCI (Kajander and Bennett 1992). Interestingly following CCI, A-fibres displayed increased spontaneous activity, although the majority of these did not conduct through the injury site (Kajander and Bennett 1992). Peripheral nerve injury can also affect uninjured neighbouring nerves in a phenomenon known as collateral sprouting. This refers to neighbouring intact nerves e.g. saphenous, branching into the area innervated by the damaged nerve, e.g. sciatic (Devor et al. 1979; Brenan 1986; Pertovaara 1988). In addition, the collateral sprouting of the saphenous nerve, following sciatic nerve crush, has been shown to be responsible for cutaneous reinnervation, but once the sciatic nerve has regenerated the saphenous nerve returns to its original location (Devor et al. 1979). This process is thought to be due to increased levels of NGF in the skin, as anti-NGF treatment inhibits this process (Boucher et al. 2000a).

**Ectopic activity**

Nerve injury causes changes in the activity of peripheral neurones, which manifests as ectopic discharges and ongoing spontaneous activity (Garry and Tanelian 1997). A small proportion of DRGs exhibit spontaneous activity, which markedly increased following nerve injury (Devor and Wall 1990). Ectopic discharges can originate from the DRG or the neuroma (Wall and Devor 1983) and the type of nerve injury appears to
influence the amount of ectopic discharges originating from the DRG (Garry and Tanelian 1997). Ectopic discharges from the DRG occur at a slow rate and in an irregular fashion (Wall and Devor 1983; Study and Kral 1996). In comparison, spontaneous activity from injured afferents occurs continuously at a fast rate, although not all injured afferents display such activity (Burchiel 1984; Garry and Tanelian 1997). Certain factors have been found to influence the development of spontaneous activity in injured peripheral fibres including fibre type and time (Garry and Tanelian 1997). With respect to fibre type spontaneous discharges were observed in 35% of A beta fibers, 15% of A delta fibers, and only 3% of C-fibers of rats following CCI injury (Kajander and Bennett 1992). Spontaneous discharges following axotomy take a few days to develop (Govrin-Lippmann and Devor 1978) and are seen in A-fibres before C-fibres (Garry and Tanelian 1997).

**Ephatic transmission**

Normal intact nerves transmit electrical impulses through channels which are insulated from neighbouring nerve fibres by myelin or in the case of unmyelinated C-fibres by Schwann cell processes (Garry and Tanelian 1997; Suzuki and Dickenson 2000). Nerve injury causes a disruption in this insulation and consequently impulses carried in one nerve fibre can be transmitted to a neighbouring nerve fibre in a phenomenon known as ephatic transmission or ‘cross-talk’ (Seltzer and Devor 1979; Devor and Wall 1990). Ephatic transmission has been reported to occur immediately after nerve injury and then dissipates (Devor 1991), but is more commonly reported to occur between axon endings several weeks following nerve injury (Seltzer and Devor 1979; Blumberg and Janig 1982; Lisney and Pover 1983). This form of communication does not occur through specific anatomical structures, but through the close proximity of membranes (Blumberg and Janig 1982; Devor and Bernstein 1982).
1.4.4 Expression of ion channels

In the previous section, one of the most evident changes in the periphery following nerve injury is the development of ectopic activity. Since action potential generation is dependent upon ion channel activity interest in the changes in the expression of ion channels arose. Perhaps one of the first indications that the expression of ion channels changed following nerve injury was observed by Meiri et al. (1981). They reported that the membrane properties at the regenerating tip of injured axons were different from normal membrane properties and there was an increase in the Na⁺, Ca²⁺ and K⁺ conductance of the resting membrane (Meiri et al. 1981).

*Sodium channels*

Voltage-gated sodium channels elicit an inward transmembrane current, which depolarises the cell membrane and therefore are vital to action potential generation. To date, ten distinct sodium channels have been identified and different genes encode them. All sodium channel α-subunits consist of four homologous domains, which form a single, voltage-gated aqueous pore and are associated with β-subunits that can modify channel properties and interact with extracellular matrix proteins (Wood and Baker 2001). DRG neurones have been shown to have multiple different sodium currents that can be distinguished by their various voltage dependencies, kinetics and sensitivity to tetrodotoxin (TTX), providing evidence for the existence of several types of VGSCs on DRG neurones (Black et al. 2001). In fact, 8 of the 10 sodium channels are detectable in DRG neurones (see Table 1.4.3). Furthermore, DRG and trigeminal neurones are the only neuronal cell types to express significant mRNA levels of three sodium channels: Nav1.7/PN1 channels are expressed on almost all DRG neurones, Nav1.8/SNS (sensory-neurone-specific)/PN3 are preferentially expressed in small and medium DRG neurones and Nav1.9/NaN sodium channels are mainly found on small DRG neurones for (references see Black et al. 2001).
Table 1.4.3 Voltage-gated Na⁺ channel α-subunits of sensory neurones – adapted from (Wood and Baker 2001) TTX-s, TTX sensitive; TTX-r, TTX resistant

<table>
<thead>
<tr>
<th>Channel</th>
<th>Previous Name</th>
<th>TTX sensitivity</th>
<th>Abundance in adult DRG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nav1.1</td>
<td>Type I</td>
<td>TTX-s</td>
<td>Present</td>
</tr>
<tr>
<td>Nav1.2</td>
<td>Type II</td>
<td>TTX-s</td>
<td>Present</td>
</tr>
<tr>
<td>Nav1.3</td>
<td>Type III</td>
<td>TTX-s</td>
<td>Present</td>
</tr>
<tr>
<td>Nav1.4</td>
<td>SkM</td>
<td>TTX-s</td>
<td>Absent</td>
</tr>
<tr>
<td>Nav1.5</td>
<td>Cardiac</td>
<td>TTX-r</td>
<td>Absent</td>
</tr>
<tr>
<td>Nav1.6</td>
<td>NaCh6</td>
<td>TTX-s</td>
<td>Abundant</td>
</tr>
<tr>
<td>Nav1.7</td>
<td>PN1</td>
<td>TTX-s</td>
<td>Abundant</td>
</tr>
<tr>
<td>Nav1.8</td>
<td>SNS/PN3</td>
<td>TTX-r</td>
<td>Abundant</td>
</tr>
<tr>
<td>Nav1.9</td>
<td>NaN</td>
<td>TTX-r</td>
<td>Abundant</td>
</tr>
<tr>
<td>Nax</td>
<td>NaG</td>
<td>?</td>
<td>Present</td>
</tr>
</tbody>
</table>

Nerve injury causes the disruption of axonal transport, thus abnormal accumulations of sodium channels have been found at the tips of injured axons (Devor et al. 1989; England et al. 1994; England et al. 1996) and found to be Nav1.3/Type III and Nav1.8/SNS (Novakovic et al. 1998; Black et al. 1999). This accumulation of sodium channels is thought to cause a decrease in the action potential threshold and hence spontaneous ectopic activity. Following nerve injury, both Nav1.8/SNS mRNA and Nav 1.9/NaN mRNA are downregulated in the DRG (Dib-Hajj et al. 1996; Dib-Hajj et al. 1998; Dib-Hajj et al. 1999). Furthermore, Nav1.3/Type III mRNA, which is normally only expressed developmentally, is upregulated following nerve injury (Waxman et al. 1994; Dib-Hajj et al. 1999). As yet there are no specific antagonists are available for the sodium channel subtypes, however selective knock-down of Nav1.8/SNS reversed allodynia in SNL rats (Porreca et al. 1999).
Calcium channels

Several calcium channel subtypes have been identified; low-voltage activated T-type and high-voltage activated N-, L-, P/Q- and R-types. High-voltage activated calcium channels consist of an $\alpha_I$ subunit that forms the pore of the channel with $\alpha_\delta$, $\beta$ and $\gamma$ subunits that modulate the $\alpha_I$ subunit (Walker and De Waard 1998). Alternative splicing of the $\alpha_{1A}$ gene generates P- and Q-type channels, $\alpha_{1B}$ encodes N-type, $\alpha_{1C}$, $\alpha_{1D}$ and $\alpha_{1F}$ encode L-type channels and $\alpha_{1G}$, $\alpha_{1H}$ and $\alpha_{1I}$ form T-type channels. The gene responsible for R-type channels may be $\alpha_{1E}$ but this has not been firmly established (Snutch et al. 2001). L-type calcium channels do not have a major role in nociception and L-type channel blockers are used in the treatment of cardiovascular disease (Snutch et al. 2001). T-type calcium channels have been found in the dorsal horn and sensory ganglia (Talley et al. 1999) but currently lack specific pharmacological agents. However, an anticonvulsant ethosuximide, is a relatively specific T-type antagonist and was shown to inhibit dorsal horn neurones in a similar manner both before and after nerve injury (Matthews and Dickenson 2001a). P/Q-type channels are found in the dorsal horn and have been implicated in inflammation (Sluka 1998). However, selective block of P/Q-type channels by $\omega$-agatoxin IVA had the same effect in normal and neuropathic rats (Matthews and Dickenson 2001b). The most influential calcium channel following neuropathy appears to be the N-type calcium channel (Dickenson et al. 2001). N-type channels are found in the superficial dorsal horn and following nerve injury the $\alpha_{1B}$ and $\alpha_\delta$, subunits are upregulated in the DRG and spinal cord (Cizkova et al. 1999; Luo et al. 1999b). N-type channel blockers (e.g. $\omega$-conotoxin GVIA) have been shown to inhibit thermal hyperalgesia, mechanical allodynia and post-surgical pain (Vanegas and Schaible 2000). In electrophysiological studies, N-type channel blockers have an enhanced inhibitory effect on neuronal responses in neuropathic rats compared to sham-operated rats (Matthews and Dickenson 2001b).
Potassium channels

There are many subtypes of voltage-gated potassium channels (Kv), but yet the molecular identity of Kv channels that regulate neuronal activity is not clear. However, the Kv1 are composed of 4 transmembrane α subunits (Jan and Jan 1997) and up to 4 modulatory cytoplasmic Kvβ subunits (Trimmer 1998). A recent study examined Kv1 subunit expression in normal DRG neurones to find that Kv1.4 was mainly expressed on small DRG neurones and Kv1.1/Kv1.2 were expressed on larger diameter DRG neurones. Of the Kvβ subunits, only Kvβ2.1 was found, mostly on larger DRG neurones (Rasband et al. 2001). These authors also report that following SNL there is a 50% reduction in Kv1 subunit expression and a 25% reduction in Kvβ2.1 expression.

An earlier study examined the effect of axotomy on specific Kv subunit expression in the DRG (Ishikawa et al. 1999). They report large decreases in Kv1.2 and 2.1 immunoreactivity and smaller reductions in Kv1.1 and 1.3 expression. Kv1.4, Kv1.5 and Kv1.6 expression were unaffected by axotomy. Potassium channel blockers have been shown to increase the firing of sensory neurones (Burchiel and Russell 1985) and induce activity in previously silent A-fibres (Kajander et al. 1992) following nerve injury.

1.4.5 Neurochemical changes

Nerve injury causes changes in the expression of transmitters and modulators. I will focus on the alterations in neuropeptide expression (VIP, NPY, SP and CGRP) and the roles of neurotrophins and nitric oxide (NO) in neuropathy.

Neuropeptide upregulation

Following nerve injury, there is a large increase in galanin expression (for details see section 1.8.3). Vasoactive intestinal peptide (VIP) is expressed at very low levels in the lumbar section of the spinal cord in normal animals (Sorkin and Carlton 1997) and afferent stimulation elicits VIP release in the rat spinal cord (Yaksh et al. 1982; Takano et al. 1993). VIP expression is upregulated in rat DRG following CCI (Nahin et al. 2001).
and sciatic nerve crush, predominantly in small DRG neurones (Kashiba et al. 1992b). Similarly, in the dorsal horn increased VIP expression is evident following sciatic nerve crush and axotomy (McGregor et al. 1984; Sorkin and Carlton 1997). Intrathecal VIP has been reported to increase spinal cord excitability in normal and axotomised rats in electrophysiological studies of the spinal flexor reflex (Wiesenfeld-Hallin 1987; Wiesenfeld-Hallin 1989). These apparent facilitatory central effects of VIP, which have also been demonstrated on behaviour nociceptive measures in uninjured rats (Cridland and Henry 1988), appear to be accompanied by a facilitatory role of VIP in neuronal regeneration (Garry and Tanelian 1997).

Neuropeptide Y (NPY) is found in terminals and fibres of lamina I-III, V and VI of the spinal cord in normal rats (Sorkin and Carlton 1997). NPY is not normally expressed in rat DRG (Landry et al. 2000). However, NPY expression has been demonstrated in rat DRG following a variety of nerve injuries including axotomy (Wakisaka et al. 1992; Landry et al. 2000), nerve crush (Wakisaka et al. 1992), SNL (Marchand et al. 1999) and CCI (Wakisaka et al. 1992; Nahin et al. 1994). The majority of this expression was found in medium-large DRG neurones (Wakisaka et al. 1992; Marchand et al. 1999). Similar peripheral nerve injuries result in NPY upregulation in laminae III/IV of the rat spinal cord (Wakisaka et al. 1992; Munglani et al. 1995). NPY receptors, mainly of the Y2 type, are found in DRG and the spinal cord of rats (Zhang et al. 1995c) and following nerve injury, there is a marked increase in Y2 receptors (Zhang et al. 1995c; Marchand et al. 1999; Landry et al. 2000). The effects of NPY in neuropathic pain states is unclear at present, but antinociceptive effects of NPY have been demonstrated on thermal, but not mechanical, behavioural measures in normal rats (Hua et al. 1991).

Neuropeptide downregulation

In contrast to the upregulation of VIP and NPY following nerve injury, is the downregulation of two other neuropeptides, substance P (SP) and CGRP (Garry and Tanelian 1997). The effects and expression of substance P under normal conditions has been previously described in section 1.3.2. SP levels are reported to decrease in the rat
DRG and the spinal cord following different nerve injuries including axotomy (Jessell et al. 1979; Villar et al. 1989), nerve crush (Villar et al. 1991), SCN (Fromm et al. 1993) and CCI (Nahin et al. 1994; Kajander and Xu 1995). The decrease in SP expression following nerve injury is reflected by a decrease in the substance P precursor, preprotachykinin (PPT) mRNA, in the DRG (Nielsch et al. 1987; Noguchi et al. 1989). The reduction in PPT gene expression and SP-immunoreactivity following axotomy was more recently reported to be restricted to small DRG neurones (Noguchi et al. 1994). Moreover, SP and PPT mRNA was reported to be induced in medium and large DRG neurones (Noguchi et al. 1994; Millan 1999). The SP receptor, NK₁, expression is also affected by axotomy and was shown to be upregulated in the dorsal horn (Abbadie et al. 1996).

In normal rats, CGRP-containing fibres and terminals are found in laminae I, II, and V of the dorsal horn (McNeill et al. 1988). Both myelinated and unmyelinated fibres express CGRP and the CGRP terminals in the spinal cord originate from primary afferents (Sorkin and Carlton 1997). In behavioural studies on uninjured rats, CGRP has been shown to be excitatory, producing hyperalgesia (Cridland and Henry 1988) and also potentiating SP-induced hyperalgesia (Wiesenfeld-Hallin et al. 1984). Like substance P, CGRP levels have been shown to decrease in rat DRG and spinal cord following different nerve injuries including axotomy (Doughty et al. 1991; Kajander and Xu 1995), CCI (Nahin et al. 1994; Kajander and Xu 1995), and nerve crush (Villar et al. 1991). Also similar to substance P expression, an increase in CGRP expression in medium to large DRG neurones projecting to the gracile nucleus following peripheral nerve injury has been reported (Miki et al. 1998). The effect of nerve injury on CGRP receptors is unclear.
Neurotrophic factors

Multiple neurotrophic factors exist in the nervous system and discussion of all factors and their roles in pain and inflammation is beyond the scope of this thesis. Therefore, I will briefly describe the roles of the three most important factors in the context of nerve injury: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF).

NGF selectively binds to trkA receptors and to p75 receptors with a lower affinity. It is produced in small amounts in the periphery, then retrogradely transported to small DRG cells that express trkA receptors (see section1.2.3) (McMahon et al. 1997). NGF is essential for the development of small DRG neurones, demonstrated by the large loss of DRG neurones in NGF or trkA knockout mice (Crowley et al. 1994; Smeyne et al. 1994). Following nerve injury, the retrograde transport of NGF is decreased (McMahon et al. 1997), as is the expression of trkA and p75 receptors in the DRG (Verge et al. 1992). This lack of NGF following nerve injury is thought to be responsible for some of the electrophysiological and neurochemical changes in the dorsal horn and DRG (Fitzgerald et al. 1985; Verge et al. 1995). Hence the upregulation of VIP, NPY, CCK and galanin and the downregulation of SP and CGRP following axotomy was counteracted by an intrathecal infusion of NGF (Verge et al. 1995).

The lack of NGF from the periphery following nerve injury appears to be slightly compensated as NGF mRNA is upregulated in the DRG (Zhou et al. 1999) and p75 receptor expression is increased in the damaged nerve and satellite cells surrounding the DRG (Boucher et al. 2000a). In addition following nerve injury, NGF release occurs from Schwann cells in the periphery, as a result of Wallerian degeneration where IL-1β is released macrophages (among other substances) (Heumann et al. 1987). There are conflicting reports of the effects of peripherally administered (NGF does not cross the blood brain barrier) exogenous NGF to the ligation site in CCI rats. One study reports that NGF reversed thermal and mechanical hyperalgesia (Ren et al. 1995), and another
describes the delayed occurrence of hyperalgesia following application of anti-serum to NGF (Herzberg et al. 1997).

BDNF also binds with low affinity to p75 receptors and selectively binds with high affinity to trkB receptors (McMahon et al. 1997). In contrast to NGF, levels of BDNF are increased following nerve injury in large diameter afferents and in the deep dorsal horn (Boucher et al. 2000a). BDNF has been shown to potentiate NMDA-induced depolarization in an isolated spinal cord preparation, which suggests that BDNF may facilitate central sensitisation in chronic pain states (Boucher et al. 2000a). GDNF activates a receptor complex of RET (a transmembrane tyrosine kinase signal-transducing domain) and GFRα1 receptor. Two weeks after axotomy, the expression of RET and GFRα1 is markedly increased in large DRG cells (Boucher et al. 2000a). Moreover, continuous intrathecal infusion of GDNF, reversed and prevented the development of mechanical and thermal hyperalgesia in rats, following partial sciatic and spinal nerve ligations (Boucher et al. 2000b).

Nitric oxide
The expression of nitric oxide (NO) is examined indirectly by staining for nitric oxide synthase (NOS), the enzyme that generates NO from L-arginine (Moncada et al. 1991). NOS-like immunoreactivity is found in a few small/medium sized DRG neurones and in the dorsal horn of normal rats, mainly in inner lamina II (Zhang et al. 1993c). Following axotomy in rats, NOS expression was markedly increased in the DRG, predominantly in small/medium neurones and also throughout lamina II of the dorsal horn (Zhang et al. 1993c). The action of NO is predominantly a facilitatory one, resulting in the activation of NMDA receptors see (Wilcox and Seybold 1997) such that the inhibition of NO synthesis blocks behavioral hyperalgesia elicited via NMDA receptors i.e. the formalin test (Meller et al. 1992; Malmberg and Yaksh 1993). The upregulation of neuronal NOS (nNOS) is not responsible for the development or maintenance of allodynia following nerve injury (Luo et al. 1999a). These authors demonstrated that the inhibition of nNOS did not prevent or reverse allodynia induced by spinal nerve ligation and nNOS levels
remained elevated in SNL rats several months following injury even though allodynia had subsided (Luo et al. 1999a).

1.4.6 Central changes following neuropathy

In concert to the plasticity displayed in the periphery following nerve injury, there are also marked alterations in the circuitry of the spinal cord including central sensitisation, loss of inhibitory neurotransmission and the possible sprouting of Aβ-fibres into the superficial dorsal horn. In addition, there is evidence of changes in supraspinal mechanisms following nerve injury, which I will briefly describe.

Central sensitisation

Central sensitisation is essentially a term used to describe the increase in the excitability of spinal neurones triggered by increased nociceptive input from the periphery (e.g. during inflammation) or peripheral nerve injury. The first evidence for such central spinal hypersensitivity showed that a C-fibre activating conditioning stimulus produced a sustained flexion withdrawal reflex in spinal-decerebrate rats (Woolf 1983).

Characteristics of central sensitisation are a reduction in the stimulation threshold required for spinal neurones to fire, increase in the spontaneous activity of spinal neurones and the enlargement of peripheral receptive fields of spinal neurones. Such characteristics have been reported in rats following SNL (Chapman et al. 1998; Suzuki et al. 2000), CCI (Laird and Bennett 1993; Cumberbatch et al. 1998) and PSL (Behbehani and Dollberg-Stolik 1994).

NMDA receptor activation is involved in the underlying mechanism of central sensitisation, as it is in the mechanism of wind-up (see section 1.3.3). The induction of wind-up by afferent input may also be sufficient to cause central sensitisation, however wind-up is not essential for central sensitisation (Woolf 1996). In fact, central sensitisation has been induced in the absence of wind-up in visceral pathways. Spinal neurones with visceral input display the characteristics of central sensitisation described
above (Roza et al. 1998), however electrical stimulation of visceral afferent C-fibres does not evoke wind-up in these neurones (Alarcon and Cervero 1990). The maintenance of central sensitisation depends upon persistent afferent input and spinal inhibitory controls. Hence, following nerve injury central sensitisation persists due to ectopic activity and decreases in spinal GABA (see below).

**Loss of inhibitory neurotransmission**

Spinal levels of the main inhibitory neurotransmitter GABA decrease following nerve injury (Castro-Lopes et al. 1993; Ralston et al. 1997), as does GABA$_A$ and GABA$_B$ receptor binding (Castro-Lopes et al. 1995). This loss of inhibitory control effectively enhances the effects of the excitatory activity received from the periphery in the spinal cord, potentiating central sensitisation. Recently, the spinal administration of midazolam, a benzodiazepine (these drugs enhance the effects of GABA by binding to GABA$_A$ receptors, resulting in allosteric modulation which increases the opening frequency of the chloride channel) in SNL rats reduced evoked C-fibre and wind-up responses (Kontinen and Dickenson 2000).

**Aβ-fibre sprouting**

It has been reported that following nerve injury, Aβ-fibres sprout from laminae III/IV, their normal destination, into lamina II where C-fibres terminate (Woolf et al. 1992; Woolf et al. 1995). Using cholera toxin-conjugated horseradish peroxidase (CB-HRP), which labels myelinated afferent terminals in the spinal cord, CB-HRP labeling was found extending from lamina III to lamina II following axotomy (Woolf et al. 1992), CCI (Nakamura and Myers 1999) and SNL (Lekan et al. 1996). These reports lead to the hypothesis that Aβ-fibres could connect to pain transmission neurones of lamina II that normally receive noxious information from C-fibres and hence an innocuous stimulus transmitted by Aβ-fibres would be perceived as painful, touch-evoked allodynia. Exogenous NGF and GDNF, but not BDNF, have been reported to prevent Aβ-fibre sprouting following axotomy (Boucher et al. 2000a). Evidence has been
reported showing that CB-HRP labeling is not a reliable method of establishing whether A\(\beta\)-fibres sprout following nerve injury (Tong et al. 1999). These authors demonstrate an increase in CB-HRP labeling in DRG neurones after axotomy, predominantly in small DRG neurones, whereas this marker labeled large DRG neurones in normal animals. This study was undertaken in rats and monkeys and shows that following axotomy CB-HRP labeling does not distinguish between small and large DRG cells. Thus, by extension, CB-HRP labeling may also not be able to distinguish between myelinated A\(\beta\)-fibres and unmyelinated C-fibres in the dorsal horn after axotomy, which may account for the reports of A\(\beta\)-fibre sprouting following peripheral nerve injury (Tong et al. 1999).

**Supraspinal mechanisms**

As previously described there are descending inhibitory and facilitatory pathways from the brain to the spinal cord (see section 1.3.5). It is thought that following nerve injury, there is an increase in descending facilitation and a decrease in descending inhibition to the spinal cord, which may maintain neuropathic pain behaviours (Millan 1999). For example, the RVM and PAG appear to play a role in mechanical allodynia as microinjections of lidocaine into the RVM and PAG have antiallodynic effects (Pertovaara et al. 1996). The mechanisms underlying these putative changes in descending modulation are unclear (Millan 1999; Ossipov et al. 2001). There is also some evidence to suggest that nerve injury causes changes in the thalamus and cortex. For example, rat thalamic and S1 neurones displayed increased sensitivity to mechanical stimulation of their receptive fields following CCI but it is not known whether these effects are independent of changes in the spinal cord following nerve injury (Guilbaud et al. 1992).
1.5 Current pharmacological treatments of neuropathic pain

Although the understanding of the mechanisms underlying neuropathic pain has significantly improved over the last twenty years, many neuropathic pain patients still have little relief from their symptoms. The following section aims to overview the currently prescribed drugs for the treatment of neuropathic pain.

Opiates

Neuropathic pain is not always as responsive to chronic opioid therapy as other pain states such as inflammatory pain (McQuay and Moore 1997). One reason is that higher doses of morphine maybe required to provide relief from neuropathic pain compared to nociceptive pain and this increase in dose can result in an increased chance of developing side-effects such as nausea and constipation at best and at worse respiratory depression (McQuay and Moore 1997). However, even though the use of opiate analgesics for chronic neuropathic pain is controversial in some areas of the world, some patients do receive good pain relief.

Opioids have been reported to have no effect on alldynia in humans in one study (Arner and Meyerson 1988) whereas more recent studies have shown opiates to have significant effects on mechanical alldynia (Max et al. 1995; Leung et al. 2001). Intravenous fentanyl has been shown to have a significant effect on neuropathic pain in a double-blind placebo controlled trial (Dellermijn and Vanneste 1997). In 1992, Zenz et al. reported that of 100 patients with back pain and neuropathic pain that were treated for up to 224 days with opioids, 51 patients reported good pain relief and 28 patients reported partial pain relief (Zenz et al. 1992). Opioids have also been shown to produce significant pain relief in patients with phantom limb pain (Urban et al. 1986), postherpetic neuralgia (Watson and Babul 1998) and diabetic neuropathy (Harati et al. 1998; Sindrup and Jensen 1999). The mechanisms of action of opioids are given in section 1.3.4.
Antidepressants

Tricyclic antidepressants (TCAs) inhibit the re-uptake of monoamines and by increasing their synaptic levels enhance the effects of noradrenaline and serotonin (5-HT) in modulating transmission of sensory events in the CNS. Amitriptyline is the most widely prescribed TCA for the treatment of chronic pain and has been shown to be more effective than placebo in the treatment of nerve injury pain (Kalso et al. 1996), diabetic neuropathy (Max et al. 1987) and postherpetic neuralgia (Max et al. 1988). A systematic review of the randomised controlled trials using antidepressants for the treatment of neuropathic pain concluded that compared to placebo, antidepressants had a clear analgesic effect (McQuay et al. 1996). Moreover, of 100 neuropathic pain patients given antidepressants, 30 will report more than 50% pain relief (McQuay et al. 1996).

Sodium channel blockers

These drugs work in a use-dependent fashion entering sodium channels when they are in an open state and block the channels from the inside, thus preventing the generation of action potentials. Lidocaine is inconvenient for chronic treatment as it can only be administered locally or intravenously and not orally. Subanaesthetic doses of systemic lidocaine have been reported to produce pain relief in diabetic neuropathy (Kastrup et al. 1987), postherpetic neuralgia (Rowbotham et al. 1991) and neuropathic pain (Glazer and Portenoy 1991). Topical lidocaine has also proved effective in the treatment of postherpetic neuralgia (Rowbotham et al. 1996). Mexiletine, an oral analogue of lidocaine, has been examined in clinical trials of diabetic neuropathy without a consistent outcome. Some of these studies have shown mexiletine to be more effective than placebo (Dejgard et al. 1988; Oskarsson et al. 1997), yet others have reported little effect (Stracke et al. 1992; Wright et al. 1997). In addition, mexiletine has been reported to provide pain relief following peripheral nerve injury (Chabal et al. 1992) but not after spinal cord injury (Chiou-Tan et al. 1996). Anticonvulsants such as phenytoin, carbamazepine and lamotrigine also reduce neuronal excitability via actions on sodium channels. Carbamazepine is the treatment of choice for trigeminal neuralgia and is reported to produce reductions in pain paroxysms and pain intensity (Campbell et al. 1996).
Carbamazepine has also been shown to be effective in diabetic neuralgia (Rull et al. 1969) and phantom limb pain (Patterson 1988). Lamotrigine, a relatively new anticonvulsant is well-tolerated and shows promise as a potential treatment for neuropathic pain, but evidence of these effects are only from case reports in patients with neuropathic pain (di Vadi and Hamann 1998) and central pain (Canavero and Bonicalzi 1996). Although a double-blind placebo-controlled study showed a dose of lamotrigine to have no effect in neuropathic pain, it was shown from preliminary studies that higher doses may be required for analgesic effects (McCleane 1999).

**GABAergic agents**

These drugs mimic or enhance the effects of the inhibitory transmitter, γ-aminobutyric acid (GABA) in the CNS. Baclofen is a GABA$_B$ agonist and acts at presynaptic GABA$_B$ receptors in the spinal cord inhibiting the release of excitatory neurotransmitters. Baclofen has been found to be more effective than placebo in the pain relief of trigeminal neuralgia (Fromm et al. 1984), but this is an isolated usage and the drug is not widely used. Valproate is thought to increase GABA levels by increasing postsynaptic GABA activity or inhibiting the breakdown of GABA. Clinically, it has been shown to relieve chronic central pain caused by spinal cord injury (Drewes et al. 1994), but studies on its effects on other types of neuropathic pain have not been reported.

**Gabapentin**

This drug was originally developed for the control of epileptic seizures and was synthesized to be an active GABA analogue. However, it does not interact with GABA receptors or sodium channels. At present its mechanism of action is unknown, although it does bind to the α$_2$δ subunit of high-voltage activated calcium channels (Gee et al. 1996) (see 1.4.4). In randomised, double blind, placebo-controlled clinical trials, gabapentin produced a significant reduction in pain and an improvement in the quality of life of patients with postherpetic neuralgia (Rowbotham et al. 1998; Rice and Maton 2001) and diabetic neuropathy (Backonja et al. 1994).
**NMDA antagonists**

These drugs, such as ketamine, dextromethorphan, memantine and amantadine, act by limiting the recruitment of NMDA receptors and so prevent mechanisms of wind-up and thus decrease central sensitization (see sections 1.3.3 & 1.4.6). These drugs can have unpleasant side effects including sedation, psychological disturbances and hallucinogenic effects, which has proved to be problematic in clinical trials. However, some double blind, placebo controlled studies have shown intravenous ketamine, at tolerable doses, to significantly reduce neuropathic pain and allodynia (Max et al. 1995) (Leung et al. 2001). Ketamine is also reported to produce analgesic effects in patients suffering from postherpetic neuralgia (Eide et al. 1994), neuropathic cancer pain (Mercadante et al. 1995) and phantom pain (Nikolajsen et al. 1996). Clinical trials with oral dextromethorphan have shown it to be effective in diabetic neuropathy (Nelson et al. 1997) but not postherpetic neuralgia (Nelson et al. 1997) or a mixed population of patients with peripheral neuropathies (McQuay et al. 1994). Finally, intravenous amantadine had a significant inhibitory effect on surgical neuropathic pain in cancer patients (Pud et al. 1998).

**Sympathetic agents**

Neuropathic pain patients can be divided into two groups in regard to their response to a therapeutic block of sympathetic nerves. Patients that such treatment is effective are considered to suffer sympathetically maintained pain (SMP) and patients that do not respond to such treatment suffer sympathetically independent pain (SIP).

Therapeutically, sympathetic nerves are blocked by injections of local anaesthetic around sympathetic ganglia that project to the affected body part. Alternatively, a regional intravenous injection of guanethidine, bretylium or reserpine is given which will deplete the postganglionic axon of noradrenaline. The efficacy of these treatments is difficult to evaluate due to the lack of placebo-controlled clinical trials (Janig and Baron 2001).
Capsaicin

Topically administered capsaicin, an alkaloid derived from chillis, produces burning pain and hyperalgesia on initial application but with repeated application it inactivates the nociceptors (Bjerring et al. 1990). The problem with these capsaicin preparations is that the burning sensation they produce can prove intolerable for the patient so they have been given in conjunction with local anaesthetics. Capsaicin based creams have been shown to be beneficial compared to placebo creams for patients with nerve injury pain (Watson and Evans 1992), postherpetic neuralgia (Bernstein et al. 1989) and diabetic neuropathy (Tandan et al. 1992).

1.6 Cytokines

Cytokines are a heterogenous group of polypeptide mediators associated with inflammatory responses and immune system activation (Hopkins and Rothwell 1995; Rothwell and Hopkins 1995). They have a diverse range of action and are hence are termed pleotrophic. Several cytokine ‘families’ have been identified on not necessarily logical criteria and many cytokines could be included in more than one of these families on the basis of their name or function. Importantly, the effect of one cytokine of a particular family does not necessarily mean the other members produce similar effects. Cytokine families include: interleukins, chemokines, tumour necrosis factors, interferons, colony stimulating factors, growth factors, neurotrophins and neuropoietins (Hopkins and Rothwell 1995).

One of the subjects of this thesis, interleukin-6 (IL-6) is a member of the neuropoietic cytokine family (neuropoietic because these cytokines elicit effects in the nervous and hematopoietic systems), also known as the family of IL-6-like cytokines. This family has six other members, ciliary neurotrophic factor (CNTF), oncostatin M, leukemia inhibitory factor (LIF), growth promoting activity (GPA), interleukin-11 (IL-11) and cardiotrophin-1 (CT-1). All members have a molecular weight between 20-24 kDa in their non-glycosylated form and the amino acid homology between members is less than
30%. Yet they are grouped as a family because their tertiary structure, which consists of 4 antiparallel α-helices, forming a very similar 3D shape. Like IL-6, LIF has also been implicated in pain, as it is upregulated within the sciatic nerve following nerve injury, but not in the DRG (Sun and Zigmond 1996). Exogenous LIF has elicited anti-nociceptive (Banner et al. 1998) and hyperalgesic effects (Thompson et al. 1996). Studies with LIF knockout mice have also produced contrasting results, with reports of anti-inflammatory effects of LIF (Banner et al. 1998) and pro-inflammatory effects (Sugiura et al. 2000).

1.7 Interleukin-6 (IL-6)

Interleukin-6 (IL-6) is a pleotropic, pro-inflammatory cytokine and was cloned and purified by several different groups and known by 10 different names, each of which reflected a different characteristic of the protein. It was first identified as an interferon, IFNβ, when attempts were made to clone interferon-β cDNA from human fibroblasts (Weissenbach et al. 1980). I chose this particular cytokine to investigate because it is upregulated following nerve injury (see 1.7.4) and unlike other cytokines, e.g. TNFα and IL-1β, few studies existed regarding the role of IL-6 in chronic pain.

1.7.1 Immunological functions of IL-6

I will now give a brief overview of the, mainly immunological, functions of IL-6 that are not related to the nervous system. IL-6 plays an important role in hematopoiesis, the formation and development of blood cells from a pluripotent stem cells to red (erythocytes and thrombocytes) and white (e.g. macrophages, neutrophils, mast cells) blood cells. Stem cells are dormant and reside in the G0 phase of the cell cycle. IL-6 stimulates the proliferation of hematopoietic stem cells, in conjunction with IL-3, by reducing the G0 residence time of hematopoietic stem cells (Ikebuchi et al. 1987). IL-6 has been shown to be essential in B-cell differentiation for activated B-cells to become immunoglobulin-secretating plasma cells (Kishimoto 1989). The proliferation of plasmacytomas and myelomas, cancers of B-cell origin, are enhanced by IL-6 (see (Van
IL-6 stimulates the proliferation of peripheral and thymic T-cells (Uyttenhove et al. 1988) and synergises with IL-1 to control the initial steps in T-cell activation leading to the formation of cytolytic T-cells (Renauld et al. 1989). The acute phase response is a complex reaction that occurs in response to infection, trauma or injury resulting in fever, increased vascular permeability, leukocytosis, changes in plasma steroid concentrations and an increase in the synthesis of acute phase proteins e.g. hemopexin, α2-macroglobulin and C-reactive protein (only in humans). IL-6 induces acute phase proteins in vivo (Geiger et al. 1988) and increases in core temperature (Rothwell et al. 1991). Administration of lipopolysaccharide (LPS; the active fragment of endotoxin from gram-negative bacteria) induces fever and IL-6 production in vivo (Shalaby et al. 1989). LPS has been shown to increase IL-6 release from medial basal hypothalami (MBH) (Spangelo et al. 1990). Such local IL-6 production in the medial hypothalamus activates the hypothalamic-pituitary-adrenocortical (HPA) axis, which regulates several central effects including fever and sleep (for review, see Schobitz et al. 1994). The essential role of IL-6 in fever is perhaps most clearly demonstrated by IL-6 deficient mice in which LPS failed to induce fever (Chai et al. 1996).

IL-6 has been reported to have a role in bone metabolism with a significant correlation found between bone mineral density and polymorphic variations of the IL-6 gene in humans (Murray et al. 1997). It has been suggested that the dysregulation of IL-6 actions in bone cells may lead to osteoporosis and Paget’s disease (Stein and Sutherland 1998). Elevated levels of IL-6 have also been found in the patients with other diseases including rheumatoid arthritis (Houssiau et al. 1988) and HIV (Laurenzi et al. 1990).
1.7.2 Neurological functions of IL-6

Other than the role of IL-6 in nociception (1.7.6), there is evidence that IL-6 has a role in neuronal survival, neuroprotection and neuronal differentiation. *In vitro* IL-6 has been shown to enhance the survival of catecholaminergic neurons in the mesencephalon and cholinergic neurones in the septum and basal forebrain in postnatal rats (Hama et al. 1989; Hama et al. 1991). In the spinal cord of embryonic (E15) rats, IL-6 enhanced the survival of acetylcholinesterase (AChE)-positive neurones and increased the number of neuron-like cells in a dose-dependent manner (Kushima and Hatanaka 1992). More recently, the addition of both IL-6 and the soluble IL-6 receptor (see section 1.7.5) promoted the survival of newborn rat dorsal root ganglion (Thier et al. 1999). The first study to report neuroprotective effects of IL-6 *in vivo* demonstrated that IL-6 attenuated the NMDA-induced neurotoxic effects on rat striatal cholinergic neurones but not on GABAergic neurones (Toulmond et al. 1992). Since then, IL-6 has been shown to protect certain neuronal subpopulations against other neuronal insults such as glutamate-induced cell death in hippocampal neurones (Yamada and Hatanaka 1994), 1-methyl-4-phenyl pyridinium (MPP⁺) neurotoxicity in fetal dopaminergic neurones (experimental model of Parkinson’s disease) (Akaneya et al. 1995) and axotomy in developing spinal motoneurons (Ikeda et al. 1996).

IL-6 was reported to induce neurite outgrowth in PC12 (pheochromocytoma) cells (Satoh et al. 1988) however others demonstrate that IL-6 will only trigger such neuronal differentiation in PC12 cells in the presence of its soluble receptor (Marz et al. 1997). These discrepancies have been suggested to be a result of differences in the number of IL-6 receptors expressed on the PC12 subclones (Gadient and Otten 1997). This neuronal differentiation effect of IL-6 has also been observed in cultured hippocampal neurones from the fetal rat brain (Sarder et al. 1996).
1.7.3 IL-6 in the brain

Elevated levels of IL-6 occur in many CNS diseases, for instance IL-6 levels are increased in the cerebro-spinal fluid (CSF) of patients with bacterial meningitis (Waage et al. 1989), Alzheimer’s disease and Parkinson’s disease (Blum-Degen et al. 1995). Furthermore, significantly higher levels of IL-6 protein has been found in the dopaminergic, striatal regions of parkinsonian patients than those in control subjects (Mogi et al. 1994). Similarly, IL-6 protein was more frequently detectable in the temporal cortex of patients with Alzheimer’s disease than control brains (Wood et al. 1993). Traumatic brain injury also results in increased IL-6 levels (Kossmann et al. 1996). Although there is an upregulation of IL-6 levels, the role of IL-6 in the pathologic process of diseases such as Alzheimer’s and Parkinson’s remains to be defined.

The majority of animal studies have examined the levels of IL-6 in the brain in terms of mRNA. IL-6 mRNA is evident in a number of brain regions including the striatum, neocortex, cerebellum, brain stem and hippocampus, with a tendency for higher levels of IL-6 mRNA in forebrain structures compared to more caudal regions (Gadient and Otten 1995). Many neuronal types throughout the brain express IL-6 mRNA including neurones of the dorsomedial (periventricular), ventromedial, medial preoptic nucleus of the hypothalamus and habenular nucleus, pyramidal and granular neurones of the hippocampus, cerebellar granular neurones, pyramidal neurones of the cerebral cortex (for review, see Schobitz et al. 1994) and cerebellar Purkinje neurones (Gadient and Otten 1994).

IL-6 receptor (IL-6R) mRNA is usually coexpressed with IL-6 mRNA. The coexpression of IL-6 mRNA and IL-6R mRNA also occurs at low levels within the white matter of fibre tracts in the forebrain e.g. the corpus callosum, lateral olfactory tract (Yan et al. 1992). This suggests that oligodendrocytes, responsible for the myelination of axons of the CNS neurones that comprise these tracts, express IL-6 and IL-6R. The most abundant cell type in the CNS, astrocytes, which provide neuronal support also express IL-6 and IL-6R mRNA. Incidentally, IL-6 has been shown to stimulate astrocyte
proliferation *in vivo* using mice which displayed a cerebral overexpression of IL-6 (Campbell et al. 1993). This wide-ranging expression of IL-6 mRNA and IL-6R mRNA shows that several different cell types have the potential to express IL-6 or respond to IL-6 released or perhaps synthesize IL-6 and respond to IL-6. Whether this means that IL-6 is involved in cell-to-cell signaling remains to be established, as is the functional purpose of such potential pathways.

Levels of IL-6 in the CNS are low or undetectable under normal physiological conditions, which suggests that although many different cells are capable of IL-6 synthesis, relatively little constitutive biosynthesis actually occurs. However, following CNS disease (as mentioned previously), inflammation and injury there is increased synthesis of IL-6. Sources of IL-6 in these conditions include astrocytes, microglia, endothelial cells of the vascular system, T cells, macrophages, endothelial cells of the blood brain barrier (BBB), neurones and oligodendrocytes (for review see (Gruol and Nelson 1997). In terms of motoneuron injury for instance, the transection of the facial nerve causes a rapid upregulation of IL-6 mRNA in the facial nucleus (Kiefer et al. 1993). With such a wide distribution of IL-6 and IL-6R mRNA the functional roles of IL-6 in the central nervous system will perhaps increase in the future. At present, as previously discussed, the literature indicates that IL-6 has roles in fever, regulation of the HPA axis, neuronal differentiation, survival and protection.

**1.7.4 IL-6 expression in the spinal cord and periphery**

In humans, IL-6-like immunoreactivity (IR) has been found in juvenile and foetal dorsal root ganglion (DRG) (Nordlind et al. 2000) and nerve explants have been shown to secrete IL-6 from Schwann cells (Rutkowski et al. 1999). Peripheral nerve-like structures also exhibit IL-6-like IR in normal human skin and to a greater extent in inflamed skin (Nordlind et al. 1996).
IL-6 mRNA is expressed in sympathetic and sensory ganglia of adult rats (Gadient and Otten 1996). Many animal studies have examined the effect of nerve injury on IL-6 expression. Complete rat sciatic nerve transection resulted in a large increase in IL-6 mRNA in the sciatic nerve within hours of this insult (Zhong and Heumann 1995; Bourde et al. 1996); this increase was transient returning to control levels within 24 hours. In mice, a persistent upregulation of IL-6 mRNA in the sciatic nerve is seen following nerve injury and is present 21 days post-injury (Reichert et al. 1996).

Following sciatic nerve crush, plasma levels of IL-6 increase (Wells et al. 1992) and IL-6 mRNA is induced in Schwann cells at the site of injury (Bolin et al. 1995; Grothe et al. 2000). These changes are also transient returning to control levels within 24 hours.

Comparing sciatic crush injury and axotomy showed the same increase (35-fold) and timecourse of IL-6 mRNA expression (Ito et al. 1998). In the Bennett (CCI), Seltzer (PSL) and Gazelius (photochemically induced ischaemic lesion) models of neuropathic pain, an increase in IL-6 positive cells in the sciatic nerve has been reported (Cui et al. 2000). In addition, this study also reports a highly significant increase in the number of IL-6-positive cells in alldynic Bennett/Seltzer rats compared to non-alldynic Bennett/Seltzer rats. CCI induced a significant increase in IL-6 mRNA in the sciatic nerve after 7 days (Okamoto et al. 2001) and in the ipsilateral DRG (Murphy et al. 1999b). Axotomy resulted in upregulation of IL-6 mRNA peaking at 2-4 days after injury in rat DRG neurones (Murphy et al. 1995). Nerve crush and spinal nerve root transection also induce, to a lesser extent, IL-6 mRNA in DRG neurones (Murphy et al. 1999a). A bilateral elevation of IL-6 protein is seen in the infraorbital nerve and brainstem 3-10 days following constriction of this nerve (Anderson and Rao 2001). All the above studies report little to no expression of IL-6 mRNA under normal conditions.

In terms of the autonomic nervous system, cultured rat sympathetic neurones express IL-6 mRNA and have been shown to secrete biologically active IL-6 (Marz et al. 1998).

In the contused spinal cord there is a 50-fold increase in IL-6 mRNA production six hours after injury, levels returning back to near normal levels after 24 hours (Streit et al. 2001).
1998). Tight ligation of the L5 spinal nerve resulted in a significant increase in IL-6 mRNA after 7 days in the adjacent superficial laminae of the dorsal horn and this expression was found to be limited to small and medium-sized neurones (Arruda et al. 1998). IL-6 mRNA upregulation is also observed in the superficial dorsal horn following the induction of chronic monoarthritis by complete Freud’s adjuvant (Berthele et al. 2000). In addition, L5 spinal nerve transection, L5 nerve root injury and sciatic cryoneurolysis also resulted in a significant increase in spinal IL-6 mRNA and in spinal IL-6 protein (DeLeo et al. 1996; Winkelstein et al. 2001). In summary, IL-6 is expressed at very low levels under normal conditions, following a variety of nerve injuries IL-6 levels are dramatically increased in the injured nerve, DRG and spinal cord for between 1 -7 days.

1.7.5 IL-6 receptor and gp130

When IL-6 binds to its specific (cytokine type 1) receptor (IL-6R), the IL-6/receptor complex then associates with two gp130 (~130kD transmembrane glycoprotein) molecules (Hirano et al. 1994). The dimerization of gp130 triggers the activation of the associated tyrosine kinases JAK1, JAK2 and TYK2 of the Janus kinase family. Activated JAKs then, phosphorylate at tyrosine a series of substrates including gp130 and trigger a sequence of events in which phosphorylated gp130 recruits the signal transducer and activator of transcription-3 (STAT3). STAT3 then becomes phosphorylated and translocates into the nucleus where it activates transcription.

An alternative pathway can be initiated which involves the G-protein Ras this leads to downstream activation of mitogen-activated protein kinases (MAPKs) which phosphorylate another set of transcription factors at threonine or serine residues. In addition to the regulation of transcription by IL-6, it has also been thought IL-6 may affect pre- and/or postsynaptic neurotransmission via phosphorylation of synaptic substrates that have a role in synaptic transmission, evidence for this has been found in the rat cerebral cortex (D’Arcangelo et al. 2000). These mechanisms will be discussed
further in relation to the results of my experiments with exogenously administered IL-6 (Chapter 4).

The IL-6 receptor is soluble and can be easily removed from the cell surface by proteolysis (Rose-John and Heinrich 1994). The soluble IL-6 receptor (sIL-6R) still binds highly efficiently to IL-6 molecules, in this state. The complex formed by the soluble IL-6 receptor and the IL-6 molecule can still interact with gp130 on target cells and so trigger the signal transduction pathway. Therefore, cells that do not express the specific IL-6R will not respond to IL-6 alone, but will respond to IL-6/sIL-6R complexes. This process is known as the trans-signalling (Mullberg et al. 1999). As a consequence of this there are several ways in which IL-6 could initiate an effect (see Fig. 1.7, p64). IL-6 could act directly on a neurone by binding to neuronal gp130 molecules via membrane bound IL-6Rs or sIL-6Rs. Alternatively, IL-6 could bind to gp130 molecules in non-neuronal cells e.g. microglia or astrocytes via membrane bound IL-6Rs or sIL-6Rs causing them to generate and release secondary mediators. Such secondary mediators could potentially be either neurotrophic, defending neurones against injury or neurotoxic, resulting in neurodegeneration. These potential mechanisms of action could provide some explanation as to how this cytokine can have such a variety of somewhat contrasting functions within the nervous system.

Cellular responsiveness is dependent on the ratio between the IL-6R and gp130 molecules (Mullberg et al. 1999). The expression of gp130 is thought to be ubiquitous, however the extent of IL-6R expression on the surfaces of target cells can be variable. This results in varying effects on a target cell's response to the presence of the soluble IL-6R, according to the ratio of IL-6R to gp130. In the case of PC12 cells (Marz et al. 1997) and cultured sympathetic neurones (Marz et al. 1998), there appear to be few IL-6 receptors on their surface, as there was little response to IL-6 alone but were stimulated by the soluble IL-6/IL-6R complex. By contrast, cells e.g. hepatocytes that express less IL-6 receptors than gp130 molecules will respond to IL-6 alone and their response could be enhanced by soluble IL-6 receptors (Mullberg et al. 1999). As yet it is not possible to
distinguish between membrane-bound and soluble forms of the receptor in the detection of IL-6R mRNA. Thus when IL-6R mRNA is identified it is not possible to know whether both forms of the receptor are present and if so in what proportions. As an extension of this it is not possible to know if the functional role of the soluble IL-6 receptor is different from the role of the membrane bound receptor. Thus potentially the relative levels of membrane bound to soluble forms IL-6 receptors may also have an effect on the actions of IL-6.

In terms of sensory processing, IL-6 receptor (IL-6R) mRNA is expressed in the sympathetic and sensory ganglia of adult rats (Gradent and Otten 1996). IL-6R mRNA is found in vascular endothelial cells and Schwann cells of the intact sciatic nerve and is upregulated at the site of injury following nerve crush (Ito et al. 1998; Grothe et al. 2000). IL-6R mRNA expression peaks two days after sciatic nerve crush (8-fold increase) and transection (35-fold increase) and gradually decreases after 28 days (Ito et al. 1998). Intact nerves display low levels of gp130 mRNA, yet following nerve injury there is also a marked increase in gp130 mRNA which is present for up to 21 days (Ito et al. 1998).
Figure 1.7 Potential mechanisms of action of interleukin-6 involve IL-6 binding to membrane bound or soluble IL-6 receptors found on neurones and/or glia. Both involve gp 130 binding to produce the effects of the cytokine (adapted from Gadient and Otten 1997).
1.7.6 Role of IL-6 in nociception

Patients with persistent sciatic pain display elevated IL-6 levels in the blood (Geiss et al. 1997). Considering the abundant literature referring to the upregulation of IL-6 after nerve injury in humans and animals, there are few studies that have investigated the role of this cytokine in nociception or effects of exogenously administered IL-6. Of particular interest to my thesis, are the reports that intraneural IL-6 administered to the uninjured sciatic nerve caused a significant increase in galanin-like IR (Thompson et al. 1998) and intrathecal IL-6 infusion induced galanin mRNA in DRG neurones (Murphy et al. 1999b). Higher plasma levels of IL-6 have been observed in rats with formalin-induced pain (Aloisi et al. 1995). Intracerebroventricular injection of IL-6 decreased hot-plate withdrawal latencies in naive rats (Oka et al. 1995) whereas intrathecal IL-6 had no effect on thermal withdrawal latencies in uninjured rats (DeLeo et al. 1996). In the same study, after nerve lesion (sciatic cryoneurolysis) rats displayed thermal hyperalgesia following a intrathecal IL-6, but only on the contralateral paw. DeLeo et al (1996) also report intrathecal IL-6 produced touch evoked allodynia in normal rats, however this effect was only described and no data was shown to result in such a conclusion. Intraplantar IL-6 increased mechanical thresholds of inflamed rat hindpaws but had little effect in normal conditions (Czlonkowski et al. 1993). Others have reported intraplantar IL-6 in normal rats induced mechanical hyperalgesia although this study lacks formal statistical analysis (Cunha et al. 1992). In vitro, IL-6 only increased the heat evoked CGRP release from nociceptors in rat skin in the presence of its soluble receptor, sIL-6R (Opree and Kress 2000).

1.7.7 IL-6 knockout mice

Studies using IL-6 deficient mice have not been conclusive, reporting varying effects of endogenous IL-6 in nociception. Two studies found no difference in the thermal and mechanical withdrawal latencies of IL-6 knockout mice (KO) and wild-type (WT) mice (Bianchi et al. 1999; Murphy et al. 1999b). However, it has been reported that IL-6 KOs have increased front paw thermal thresholds compared to normal animals (Zhong et al.
1999). In contrast, others report IL-6 KOs to have lower thermal and mechanical
thresholds than WT mice (Xu et al. 1997). The development of thermal hyperalgesia
following an L5 spinal nerve ligation (SNL) was similar in IL-6 KOs and WT mice but
the development of mechanical allodynia was delayed in KOs and not as marked as that
seen in WT mice (Ramer et al. 1998). Following CCI, thermal and mechanical
withdrawal latencies were significantly reduced in IL-6 KOs compared to WT mice
(Murphy et al. 1999b). The reasons for the different outcomes of these studies is
unclear, although the ‘knockout’ of IL-6 appears to result in compensatory effects such
as a three-fold increase in TNFα levels (Fattori et al. 1994).

1.8 Galanin

Galanin is a neuropeptide consisting of 29 amino acids (30 in humans) originally
isolated from porcine gut (Tatemoto et al. 1983). Galanin is cleaved from a 124 amino
acid precursor known as preprogalanin, along with a signal peptide and a 60-amino acid
galanin mRNA-associated peptide (GMAP) (Kaplan et al. 1988). The functions of
GMAP remain to be fully elicited, however GMAP has been shown to be upregulated
following nerve injury (Xu et al. 1995a) and partially alleviated mechanical allodynia
(Hao et al. 1999). Galanin is unrelated to other known families of regulatory peptides.
Although, a 60 amino acid protein from the porcine hypothalamus was recently purified
on the basis of its binding to specific galanin receptor expressing cells. This sequenced
protein included an internal region identical to the N-terminal 13 residues of galanin and
was thus designated galanin-like peptide (GALP).

1.8.1 Functions of galanin

Apart from galanin’s role in nociception, which will be discussed (1.8.5) and explored
later in this thesis, galanin has roles in other regulatory functions such as insulin release,
learning, memory, feeding and endocrine modulation (for reviews see (Bartfai et al.
1993; Bedecs et al. 1995). Hyperglycaemia was the first reported induced effect of
galanin following intravenous administration in dogs (Tatemoto et al. 1983) this led to
the discovery that galanin inhibits insulin release from pancreatic cells (for review see Ahren and Lindskog 1992). Central galanin administration has inhibitory actions in several rodent learning and memory tasks such as the Morris water task (McDonald et al. 1998; Ogren et al. 1998). There is also a body of evidence linking galanin to Alzheimer's disease (for review, see Crawley 1996). For example, in humans the most consistent marker of neuronal loss in Alzheimer's disease is reported to be the decrease in the number of cholinergic neurones of the basal nucleus of Meynert (Coyle et al. 1983). Postmortem studies show that galanin concentrations in the nucleus basalis of Meynert of Alzheimer's victims are almost double those of samples from non-diseased brains of the same age (Beal et al. 1990). Galanin has a significant role in feeding demonstrated by galanin injections into the hypothalamus of rats which caused a marked (300%) increase in food intake (Leibowitz 1989; Crawley et al. 1990). Galanin has also been identified as a potential drug target for the treatment of obesity (Halford 2001). Another function of galanin is endocrine modulation for instance it has been reported that intravenous galanin administration in humans stimulates growth hormone release (Bauer et al. 1986). Galanin administration has also been shown to affect the release of prolactin and corticosterone via actions in the hippocampus and hypothalamus (see Bedecs et al. 1995).

1.8.2 Galanin in the brain

Galanin is widely distributed throughout the CNS and this distribution in the rat has been extensively reviewed (Merchenthaler et al. 1993). Galanin-like immunoreactive (IR) neurones and fibres were found throughout the brain in almost all structures. To summarise, moderate to high levels of galanin-IR are found in the cerebral cortex and the following forebrain structures - lateral septal nucleus, diagonal band of Broca's, bed nucleus of the stria terminalis, basal nucleus of Meynert, amygdala, hippocampus and fibre tracts. Similar levels of galanin-IR are found in the parafascicular and periventricular nuclei of the thalamus and all areas of the hypothalamus with the exceptions of the mamillary body and the supramammillary and ventromedial nuclei.
which express low levels of galanin-IR. In the midbrain, medium levels of galanin-IR are found in the locus coeruleus and dorsal raphe with low levels found in other areas e.g. the periaqueductal gray (PAG). In the pons and medulla oblongata areas of the rat brain, medium to high levels of galanin-IR are found in the locus coeruleus, dorsal and median raphe, nucleus raphe pallidus and obscurus, nucleus of the solitary tract, dorsal vagal complex, caudal spinal trigeminal nucleus and the spinal tract of the trigeminal nucleus (Merchenthaler et al. 1993).

Recently, the distribution of galaninergic immunoreactivity was elegantly examined in the brain of the mouse (Perez et al. 2001). This study is of particular importance in light of the putative role galanin plays in Alzheimer’s disease and recent developments of both galanin knockout (Wynick et al. 1998) and galanin overexpressing mice (Blakeman et al. 2001). The most relevant, to this thesis, of the studies that have examined the effects of galanin in the brain are those which have investigated the effects of galanin on hippocampal hyperexcitability (Zini et al. 1993a; Zini et al. 1993b). Using rat hippocampal slices, galanin was shown to reduce the ischaemia-induced release of glutamate (Zini et al. 1993a). Moreover, galanin produced a 50-60% presynaptic inhibition of endogenous glutamate and aspartate release evoked by potassium depolarization (Zini et al. 1993b). In addition, galanin has been shown to inhibit long-term potentiation (LTP) at Schaffer collateral-CA1 synapses in guinea-pig hippocampal slices (Sakurai et al. 1996). These data along with the observation of an anticonvulsant effect following galanin administration into the brain (Mazarati et al. 1992) pointed to a role for galanin in epilepsy. Mazarati and colleagues then went on to find that status epilepticus induced a depletion of galanin from the rat hippocampus and galanin administration could prevent the induction of self-sustaining status epilepticus in rats (Mazarati et al. 1998). Recently, evidence has been presented to suggest that epilepsy may have a genetic component (Mazarati et al. 2000). Galanin knockout mice were more likely to develop status epilepticus with more severe convulsions whereas galanin overexpressing mice had an increased resistance to seizure induction. In addition, LTP was
enhanced in the knockout animals, whilst a reduction in LTP was observed in galanin over-expressing mice (Mazarati et al. 2000).

1.8.3 Galanin expression in the spinal cord and periphery

In the spinal cord, under normal circumstances, low levels of galanin-IR are found the ventral horn, high levels of galanin-IR are found in the intermediolateral column and lamina X and very high levels of galanin-IR are found in the dorsal horn of the rat spinal cord (Merchenthaler et al. 1993). The galanin-IR neurones around the central canal (lamina VII & X) also contain cholecystokinin (CCK) and project to thalamic structures (Ju et al. 1987). Galanin-IR is localized to fibres and cell bodies and the distribution of galanin-IR fibres and terminals was similar in the cervical, thoracic, lumbar and sacral segments of the rat spinal cord (Ch'ng et al. 1985). Ultrastructural studies of laminae I and II of the dorsal horn of lumbar 4/5 segments of the rat spinal cord found galanin-IR in a large proportion of primary afferent terminals, yet the level of galanin-like immunoreactivity (-LI) in each terminal appeared to be low (Zhang et al. 1993b).

Galanin has been shown to co-exist with CGRP and substance P in large dense core vesicles (LDCVs) of primary afferent terminals, in fact 50% of CGRP-positive terminals in lamina II contain low levels of galanin-LI (Zhang et al. 1995d). This would suggest that galanin can be released with substance P and CGRP under normal conditions although in much smaller quantities. Several dorsal horn neurones mainly in lamina II express galanin-LI which is co-localizes with neuropeptide Y, enkephalin, and GABA (Simmons et al. 1995; Zhang et al. 1995d). Moving out into the periphery under normal conditions, galanin is expressed in few sensory neurones (2-3%) in the DRG of rats (Skofitsch and Jacobowitz 1985) and a slightly higher expression (17%) is seen in monkeys (Zhang et al. 1993a). Also galanin-like immunoreactivity has been found in uninjured skin of rats (Ji et al. 1995) and humans (Johansson et al. 1988). In addition to the widespread expression of galanin in the nervous system, it has been demonstrated in vivo that there is a basal release of IR-galanin in the superficial dorsal horn (lamina II
region) of naive cats (Morton and Hutchison 1989) and rats (Hope et al. 1994; Colvin et al. 1997). Interestingly, neither innocuous nor noxious peripheral stimulation affected this release of IR-galanin. This observation along with the very low levels of galanin in the DRG suggests that the basal release of IR-galanin in naive animals is unlikely to be from primary afferents.

Inflammation and nerve damage affects the expression of galanin and its receptors (see 1.8.4). Carrageenan-induced inflammation resulted in an increase in galanin mRNA levels, yet no alteration in galanin-LI, in the superficial dorsal horn. Conversely both galanin mRNA and galanin-LI were decreased in the DRG (Ji et al. 1995). Chronic inflammation in the form of polyarthritis resulted in a persistent upregulation of galanin in the DRG, which was present at 21 and 79 days following induction (Calza et al. 2000). Nerve injury causes a dramatic increase in galanin levels the specifics of which will be discussed shortly. However, upregulation of galanin expression in primary sensory neurones has also been reported following; a low dose of vinblastine, a cancer drug which can block axonal transport without causing neuronal damage (Kashiba et al. 1992a), resiniferatoxin, a potent capsaicin analogue (Farkas-Szallasi et al. 1995) and herpes simplex virus (Henken and Martin 1992).

Of most relevance to this thesis, are the changes in galanin expression observed following nerve injury. The dramatic upregulation in galanin-LI following nerve injury was first observed by Hokfelt and colleagues in rat DRGs following sciatic nerve transection (Hokfelt et al. 1987). This upregulation was quantified as a 120-fold increase in galanin levels in the DRG at 3 and 14 days following nerve transection (Villar et al. 1989). This increase reflected an increased synthesis, as an increase of galanin mRNA was seen 24 hours after injury in the DRG. 50% of the remaining cells in the DRG following transection, exhibit increased levels of galanin-LI (Villar et al. 1989), however it has been shown that nerve transection causes a 15-30% cell loss in the DRG (Arvidsson et al. 1986). This upregulation in galanin levels in the DRG is maintained up to a year following axotomy (Villar et al. 1989; Hu and McLachlan 2001). In the
primary afferents terminating in the superficial dorsal horn there is also an increase in galanin-like immunoreactivity following axotomy (Zhang et al. 1998). Although there is a marked upregulation in galanin mRNA and protein, no significant change was seen in the total number of LDCVs (large dense core vesicles) containing galanin-LI per terminal. However, the number of galanin-positive LDCVs was practically doubled, whereas the number of CGRP/substance P-positive, galanin-negative LDCVs is markedly decreased which suggests that galanin actually replaces substance P and CGRP in primary afferent terminals after axotomy (Zhang et al. 1995a; Carlton and Coggeshall 1996). The marked upregulation of galanin mRNA and protein in the DRG has been shown to result in increased out-transport of galanin into central and peripheral branches of primary sensory neurones (Villar et al. 1991). Moreover, Colvin et al. (1997) showed a spontaneous increased release of galanin in lamina II area following peripheral nerve injury (CCI), this release is likely to be from primary afferents. These authors also report that high threshold electrical stimulation, but not Aβ-fibre stimulation, enhanced this release of galanin (Colvin and Duggan 1998).

Like axotomy, the upregulation of galanin has been demonstrated in a variety of other models of nerve injury. Nerve crush (Villar et al. 1989), CCI (Nahin et al. 1994; Ma and Bisby 1997; Shi et al. 1999), photochemical nerve injury (Shi et al. 1999) and partial sciatic nerve transection (PSNT) (Ma and Bisby 1997; Ma and Bisby 1999; Shi et al. 1999) caused an increase in galanin levels in the DRG and spinal cord. Tight spinal nerve ligation also resulted in an increase in galanin-LI in the superficial dorsal horn, the DRG were not examined (Carlton and Coggeshall 1996). PSL (partial sciatic nerve ligation) has been shown to produce galanin upregulation in a small proportion (8-10%) of spared (uninjured) neurones (Ma and Bisby 1999). Of more interest, are the comparisons that have been drawn between the effect of these different nerve injuries on galanin expression in rats (Ma and Bisby 1997; Shi et al. 1999). Ma and Bisby (1997) showed an increase in galanin-LI in laminae I-V of the spinal cord following CCI and PSNT injuries whereas following axotomy an upregulation was only seen in the superficial dorsal horn (laminae I&II). Furthermore, the degree of galanin upregulation
was significantly lower in rats displaying allodynia following CCI (Bennett) and PSL (Seltzer) injuries than non-allodynic Bennett and Seltzer rats (Shi et al. 1999).

1.8.4 Galanin receptors

So far, three galanin receptors subtypes (for review, see Branchek et al. 2000) have been cloned and are known as GalR1, GalR2 and GalR3. GalR1 has been cloned from human Bowes melanoma cells (Habert-Ortholi et al. 1994), rat insulinoma cells (Parker et al. 1995), rat brain (Burgevin et al. 1995), human colon (Lorimer and Benya 1996), and mouse brain (Wang et al. 1997c). GalR2 has been cloned from rat hypothalamus (Fathi et al. 1997; Howard et al. 1997; Smith et al. 1997; Wang et al. 1997a), rat dorsal root ganglion (Ahmad et al. 1998), mouse brain (Pang et al. 1998), human heart (Borowsky et al. 1998) and human placenta (Bloomquist et al. 1998). GalR3 was cloned from rat hypothalamus (Wang et al. 1997b; Smith et al. 1998), human placenta (Smith et al. 1998), and human DNA library (Kolakowski et al. 1998). Rat GalR1 shows 40% and 36% homology with rat GalR2 and GalR3, respectively, and rat GalR2 and GalR3 share 54% homology (see Branchek et al. 2000). There also is approximately 90% homology between the human and rat galanin receptors and galanin has a similar affinity for each of the receptors (Branchek et al. 2000). Activation of GalR1 and GalR3 receptors expressed in cell lines leads to inhibition of cAMP levels via coupling to Gi-protein (Smith et al. 1998; Wang et al. 1998). In contrast, GalR2 has been shown to be excitatory, activating phospholipase C and increasing phosphoinositol and intracellular calcium levels via coupling to Go/Gq-proteins (Wang et al. 1998).

The expression of these receptor subtypes has been examined at the mRNA level in rats (O'Donnell et al. 1999; Waters and Krause 2000). Some disparities in the levels of expression have been found between different studies, which is thought to be the result of the type of assay used, I will mainly describe the findings by Waters and Krause (2000) using the reverse transcription-polymerase chain reaction (RT-PCR). Whilst GalR1 mRNA is found only in the peripheral and central nervous systems. GalR2
mRNA and GalR3 mRNA are widespread in both central and peripheral tissues. In the rat brain, GalR1-3 mRNA were found in the hippocampus, hypothalamus, cortex and amygdala (Waters and Krause 2000). A comparison of the levels of GalR1 and GalR2 mRNA in areas of the rat CNS has been reported (O'Donnell et al. 1999). They showed that in the ventral hippocampus, GalR1 mRNA was expressed but not GalR2 mRNA, which is likely to explain the inhibitory effect of galanin in this region demonstrated by Zini et al. (1993) and Sakurai et al. (1996), previously discussed in 1.8.2. Of importance to sensory processing, GalR1 and GalR2 mRNAs have been shown to be present in the adult rat spinal cord and DRG (Sten Shi et al. 1997; O'Donnell et al. 1999). GalR1 mRNA expression is somewhat limited to the superficial dorsal horn and in the DRG is predominantly expressed on large DRG neurones, whilst GalR2 mRNA is expressed at lower levels throughout the dorsal horn and is mainly present on small and medium DRG neurones (O'Donnell et al. 1999). GalR3 mRNA is expressed at lower levels in the spinal cord and DRG (Waters and Krause 2000).

Before the identification of the galanin receptor subtypes, it was reported that peripheral axotomy had no effect on galanin binding in the rat DRG and dorsal horn. (Zhang et al. 1995b), whereas others report a decrease in galanin binding in the superficial spinal cord following axotomy (Kar and Quirion 1994). Since then in situ hybridization has shown following axotomy, GalR1 and GalR2 mRNA levels are decreased in the DRG (Xu et al. 1996; Sten Shi et al. 1997). GalR2 mRNA was downregulated in the DRG, at 3, 7, 14, and most pronounced 28 days following axotomy (Sten Shi et al. 1997). Inflammation induces a decrease in GalR1 mRNA levels in DRG neurones (Xu et al. 1996), but an upregulation in GalR2 mRNA (Sten Shi et al. 1997). The effect of nerve injury and inflammation on GalR3 expression and GalR1/2 expression in the spinal cord is unknown at present.
1.8.5 Role of galanin in nociception

In previous studies galanin has been shown to have both facilitatory and inhibitory effects on normal animals. In electrophysiological studies, facilitatory effects of galanin have been shown with the depolarisation of cultured DRG neurones (Puttick et al. 1994) and the facilitation of electrically evoked responses of spinal neurones (Reeve et al. 2000). Behaviourally, mechanical hyperalgesia has been demonstrated following intrathecal galanin (Kuraishi et al. 1991b; Kerr et al. 2000; Liu et al. 2001). In contrast, electrophysiologically, galanin depressed the capsaicin induced nociceptive reflex (Yanagisawa et al. 1986) and reduced the ventral root reflex in isolated rat spinal cord preparations (Yanagisawa et al. 1986; Nussbaumer et al. 1989). In addition, the discharge frequency of wide-dynamic range neurones was inhibited by galanin (Yu et al. 2001). In thermal behavioural testing, intrathecal galanin produced increases in hot plate and tail flick withdrawal latencies in rats and mice (Cridland and Henry 1988; Post et al. 1988; Wiesenfeld-Hallin et al. 1993). Furthermore, galanin was reported to inhibit the pain-induced behaviours of substance P (Kuraishi et al. 1991a) and a thyrotrophin-releasing hormone (TRH) analogue (Fone and Dixon 1991). Finally, biphasic effects of galanin, facilitation at low doses followed by inhibition at high doses, have been reported in normal animals (Wiesenfeld-Hallin et al. 1989a; Xu et al. 1991) and peripheral galanin produces mixed effects on afferent nerve fibres in normal rat knee joints (Heppelmann et al. 2000).

Previous studies have tried to identify the role of galanin in neuropathic and inflammatory pain models. Galanin has been reported to produce an inhibitory effect on the flexor reflex following inflammation (Xu et al. 1998a) and carrageenan-induced hyperalgesia was reversed by an anti-galanin antibody (Kuraishi et al. 1991b; Satoh et al. 1992). Following nerve injury, electrophysiological studies have shown intrathecal galanin to elicit inhibitory effects on evoked neuronal responses and discharge frequency (Wiesenfeld-Hallin et al. 1989b; Xu et al. 2000a). In behavioural studies, this peptide alleviated mechanical and cold allodynia in photochemically nerve-injured rats.
(Hao et al. 1999) and in chronic constriction injury (CCI) rats (Yu et al. 1999; Liu and Hokfelt 2000). Recently, it has been reported that a selective GalR2 agonist had no effect on allodynia whilst a GalR1/GalR2 agonist increased the mechanical threshold in allodynic rats (Liu et al. 2001). Table 1.8.5 (p76) summarises the effect of spinal galanin administration in electrophysiological and behavioural studies of nociception.
Table 1.8.5. - Summary of the effects of spinal galanin on nociception in naive, inflamed and nerve-injured animals. EP - Electrophysiological, B - Behavioural in rats unless stated otherwise. Inflamm- inflammation, N.I. - nerve injury.

<table>
<thead>
<tr>
<th>Type of rat - study</th>
<th>Preparation / Test</th>
<th>Dose or concentration of galanin</th>
<th>Inhibition ↓ Facilitation ↑ Biphasic ↑↓</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naive - EP</td>
<td>in vivo, spinal neurones</td>
<td>0.15-15 nmol</td>
<td>↑</td>
<td>(Reeve et al. 2000)</td>
</tr>
<tr>
<td>Naive - B</td>
<td>Mechanical- Paw Pressure</td>
<td>0.1-1 nmol</td>
<td>↓</td>
<td>(Kuraishi et al. 1991b)</td>
</tr>
<tr>
<td>Naive - B</td>
<td>Mechanical - von Frey filaments</td>
<td>25ng/hour for 2 weeks</td>
<td>↓</td>
<td>(Kerr et al. 2000), (Liu et al. 2001)</td>
</tr>
<tr>
<td>Naive - EP</td>
<td>in vitro newborn rat tail-spinal cord</td>
<td>0.3 - 2μM</td>
<td>↓</td>
<td>(Yanagisawa et al. 1986)/(Nussbaumer et al. 1989)</td>
</tr>
<tr>
<td>Naive - EP</td>
<td>in vivo, spinal neurons</td>
<td>0.5 - 1 nmol</td>
<td>↓</td>
<td>(Yu et al. 2001)</td>
</tr>
<tr>
<td>Naive - B</td>
<td>tail flick / hot plate in rat/mice</td>
<td>0.6-6.5 nmols /1 - 10μg</td>
<td>↓</td>
<td>(Cridland and Henry 1988) / (Post et al. 1988)</td>
</tr>
<tr>
<td>Naive - B</td>
<td>tail flick &amp; paw pressure</td>
<td>1 - 10μg</td>
<td>↓</td>
<td>(Wiesenfeld-Hallin et al. 1993)</td>
</tr>
<tr>
<td>Naive - EP</td>
<td>in vivo, spinal flexor reflex</td>
<td>10ng - 10μg</td>
<td>↑↓</td>
<td>(Wiesenfeld-Hallin et al. 1989a)</td>
</tr>
<tr>
<td>Inflam - EP</td>
<td>in vivo, spinal flexor reflex</td>
<td>100ng - 10μg</td>
<td>↓</td>
<td>(Xu et al. 1998a)</td>
</tr>
<tr>
<td>N.I. - EP</td>
<td>in vivo, spinal flexor reflex</td>
<td>1ng - 1μg</td>
<td>↓</td>
<td>(Wiesenfeld-Hallin et al. 1989b)</td>
</tr>
<tr>
<td>N.I. - EP</td>
<td>in vivo, spinal neurones</td>
<td>0.1-1 nmol</td>
<td>↓</td>
<td>(Xu et al. 2000a)</td>
</tr>
<tr>
<td>N.I. - B</td>
<td>mechanical and cold allodynia in Gazelius model</td>
<td>cumulative 30μg</td>
<td>↓</td>
<td>(Hao et al. 1999)</td>
</tr>
<tr>
<td>N.I. - B</td>
<td>mechanical allodynia in CCI</td>
<td>Up to 30μg</td>
<td>↓</td>
<td>(Liu and Hokfelt 2000)/(Yu et al.1999)</td>
</tr>
</tbody>
</table>
1.8.6 Galanin antagonists

Structure-activity studies of galanin established that the N-terminal portion of the peptide (amino acids 1-16) was the part required for receptor recognition, whilst the C-terminal fragment (amino acids 17-29) is not recognised by spinal, hippocampal hypothalamic and pancreatic receptors (Fisone et al. 1989; Bartfai et al. 1992). Thus the recognition by the receptor for galanin depends upon an intact N-terminal fragment and the bent shape of the galanin molecule. From this chimeric bireceptor recognising peptides, M15 also called galantide and M35, were made consisting of the N-terminal fragment of galanin with the C-terminal portion of substance P or bradykinin, respectively. Prior to the identification of GalR1-3, M-15 showed promising antagonistic properties in rats blocking galanin-mediated. Reports included the inhibition of insulin release (Lindskog et al. 1992), inhibition of evoked acetylcholine release in the hippocampus in vivo, facilitation of the spinal flexor reflex in vivo and hyperpolarisation of locus coeruleus neurones in vitro (Bartfai et al. 1991). M35 showed similar effects, antagonising the galanin-induced facilitation of the spinal flexor reflex and displacing galanin from the rat spinal cord (Wiesenfeld-Hallin et al. 1992). However, M35 displayed agonist properties usually at higher doses in the locus coeruleus (Xu et al. 1998b), rat striatum (Ogren et al. 1998) and rat pancreatic cells (Kask et al. 1995). Other putative galanin antagonists namely, C7, M32, M38, M40 and most recently M242, were developed and displayed similar mixed agonist/antagonist effects depending on the dose and experimental conditions employed (Xu et al. 1995b; Floren et al. 2000). Of most importance is the observation that all of these chimeric peptides act as agonists at the cloned GalR1, GalR2 and GalR3 receptors and therefore none of them are used as standard galanin antagonists or even as selective agonists (Floren et al. 2000). In recent years, an antisense peptide nucleic acid (PNA) complementary to the rat GalR1 mRNA has been developed (Pooga et al. 1998) with some ability to block galanin induced effects (Rezaei et al. 2000). This potentially offers new resources to establish the role that GalR1-3 mediate, in the many functions of galanin.
1.8.7 Galanin transgenic mice

Galanin knockout (Gal KO) mice have been generated (Wynick et al. 1998) and used to shed further light on the role of galanin in nociception (Holmes et al. 2000; Kerr et al. 2000; Kerr et al. 2001a; Kerr et al. 2001b). These mice grow normally and are able to reproduce, although lactation was attenuated (Wynick et al. 1998). The lack of galanin throughout development does appear to result in some differences within the nervous system when compared to wild-type (WT) mice (Holmes et al. 2000). There is a small (13%) significant decrease in the total number of DRG cells, most of these were small peptidergic neurones with a 24% and 15% decrease in the number of DRG cells expressing substance P and CGRP, respectively. This cell loss appears to occur at postnatal days 3 and 4, where there is about a 2.5 fold higher rate of apoptosis than in WT animals (Holmes et al. 2000). This study also thoroughly examined the ability of damaged nerves to regenerate following sciatic nerve crush injury. The regeneration distance was significantly reduced in Gal KOs compared WT mice in the ‘sensory pinch test’, which depends on the retrograde conduction along sensory neurones and in GAP43 staining for growth cones. Functional recovery was tested using the toe spreading index to find that Gal KOs required 2 more weeks than WT mice for the full recovery of sensory innervation. Similarly, in vitro cultured DRG cells from Gal KOs produced fewer and shorter neurites than WT mice (Holmes et al. 2000).

The mechanical and thermal sensitivities of a substantial number (n=15) of Gal KO and WT mice were compared to find that Gal KO mice have slightly lower mechanical and thermal thresholds (Kerr et al. 2000). Both behavioural phases of the formalin response were attenuated in Gal KO mice compared WT mice, although the expression of c-fos in the spinal cord was similar in both animal groups (Kerr et al. 2001a). Following carrageenan inflammation, the spinal hyperexcitability displayed electrophysiologically in Gal KO mice was diminished compared to WT mice, which could suggest that endogenous galanin is necessary for central sensitization (Kerr et al. 2001a). However exogenous galanin was found to inhibit spinal hyperexcitability in the same spinal flexor reflex preparation (Xu et al. 1998a), perhaps suggesting that exogenous galanin
produces different effects to the endogenous peptide (see Kerr et al. 2001a). Following nerve injury (complete sciatic nerve transection), characteristic self-mutilation behaviour (autotomy) was observed in all WT mice examined whereas only one in nine of Gal KO mice developed similar behaviour (Kerr et al. 2000). Both WT and Gal KO mice develop mechanical allodynia and thermal hyperalgesia following partial sciatic nerve ligation, however the degree of mechanical and thermal sensitivities less pronounced in the Gal KO mice (Kerr et al. 2000). Axotomised Gal KO mice displayed significantly less wind-up in the spinal flexor reflex preparation than WT mice, although the baseline reflex activity was similar in both groups (Kerr et al. 2001b). These results could suggest that endogenous galanin is required for a complete wind-up profile following nerve injury. However, as there is a 25% decrease in substance P in the DRG of Gal KO mice (Holmes et al. 2000) the attenuated wind-up observed could perhaps be a result of decreased NK₁ activation in the spinal cord (see 1.3.3).

More recently, mice overexpressing galanin (Gal OE) have been generated (Blakeman et al. 2001) with up to 8-fold higher levels of galanin in the cerebral cortex and hippocampus of these mice (Kokaia et al. 2001). As previously, mentioned, Gal OE mice show a suppression in the development of seizures (Mazarati et al. 2000; Kokaia et al. 2001). In nociceptive behavioural testing, Gal OE mice showed increased paw and tail withdrawal latencies to heat stimulation, but similar responses to mechanical and cold stimulation, compared to WT mice (Blakeman et al. 2001).
1.9 Aims of thesis

The initial aim of this thesis was to establish the effect of interleukin-6 and galanin on sensory processing in naive rats. This was examined from two perspectives, firstly how did the peripheral actions of these two drugs on rudimentary units of nociceptive transmission i.e. the single nociceptive fibre, compare to their effects on complex nociceptive processing units i.e. the convergent dorsal horn neurone or behavioural reflex responses. From a different perspective, I examined whether the route of administration (peripheral versus spinal) of interleukin-6 or galanin in vivo, altered the effects of the agents on spinal neuronal responses or behavioural measures of nociception. It is well established that following nerve injury, levels of interleukin-6 and galanin are markedly increased in nociceptive pathways. However, the functions of the enhanced levels of this cytokine and neuropeptide in neuropathic pain states are not yet understood. Therefore, the broader aim of this thesis was to investigate the peripheral and spinal actions of interleukin-6 and galanin following spinal nerve ligation, comparing these findings to those from naive and sham-operated rats. This was done as in naive rats, by measuring the effects of these drugs, on the spinal neuronal responses to electrical and natural stimulation, also on behavioural nociceptive responses, in neuropathic rats.
Chapter 2

Methods
In all the techniques described below, male Sprague-Dawley rats were used and housed in groups of five in plastic cages with artificial lighting that had a fixed 12-hour light-dark cycle. Food and water were available ad libitum. Guidelines for animal research by U.K. Home Office and the International Association for the Study of Pain (Zimmermann 1983) were adhered to throughout all experiments employed.

2.1 Spinal nerve ligation (SNL) surgery

The study of the changes after nerve injury requires a rat model of neuropathy. These are described and discussed in section 1.4.1. The spinal nerve ligation, SNL, model of neuropathic pain first described by Kim and Chung (1992) was used in my studies. Rats, weighing between 130-150g, were anaesthetised with 3.5% halothane in a 50% O₂, 50% N₂O gaseous mixture. Then once the animal was unconscious and areflexia had been produced, anaesthesia was maintained at 1.5 % halothane. A small piece of left paravertebral muscle between the spinous processes L4 - S2 was removed. Then using small rongeurs, part of the left L6 transverse process was removed to expose the L4, L5 & L6 spinal nerves. The L5 and L6 spinal nerves were isolated and tightly ligated a few millimetres up from the point where the spinal nerve joins (towards the spinal cord) using 6-0 silk, leaving the L4 spinal nerve uninjured. Haemostasis was ensured with pressure applied using a cotton bud, before the muscle and the adjacent fascia were closed with sutures (silk 3.0). The skin was then closed with wound clips. The surgery for the sham-operated rats was identical to the SNL rats with the exception of the ligation of L5 and L6 spinal nerves. Following surgery, the rats were monitored while they recovered from the anaesthetic and their posture/gait were examined to ensure that the surgery had not compromised motor function on the operated (ipsilateral) hindpaw.

2.2 Behavioural monitoring in SNL rats

The following procedure was employed for both SNL and sham rats that were to be used in the electrophysiological studies. Following surgery, the animals were monitored for 2 weeks post-surgery and during this post-operative period none of the rats displayed autotomy. All rats displayed normal weight gain (7-9g per day) and levels of grooming
indicative of good health. Behavioural testing was performed on days 2, 4, 7, 9, 11 and 14 following surgery to assess the responses of the SNL and sham-operated rats to innocuous mechanical and cold stimuli. The rats were placed in individual boxes with a metal mesh floor and allowed to acclimatise. Von Frey hairs with bending forces of 1g, 5g and 9g were applied to the plantar surface of both paws in ascending order of force. Each hair was applied 10 times with 5-second intervals. Behavioural signs of cold sensitivity were assessed by the application of acetone to the plantar surface of both paws. Acetone was applied a total of 5 times. To allow the acetone to evaporate and hence elicit a cooling effect several minutes were left before reapplying acetone to the same paw. A withdrawal response was observed as a shaking, flicking or licking of the paw following acetone application. The outcome of both these behavioural tests is expressed as a difference score, number of contralateral withdrawals minus number of ipsilateral withdrawals, (see section 3.1).

2.3 Behavioural testing of mechanical thresholds

The following procedure was used to examine the effect of a spinal administration of galanin on the mechanical thresholds of naive and SNL rats, the results of which are presented in section 5.4. In the experiment using SNL rats, the surgery was performed over a period of two days. Mechanical thresholds were determined prior to surgery (day - 1), then on days 3/4, 7/8, 10/11 and 17/18 following surgery to assess the changes in mechanical threshold induced by spinal nerve ligation surgery (results in section 3.1).

Rats (200-250g) were then divided into groups of six in the naive study and groups of five in the SNL study. The rats (one group at a time) were placed in individual boxes with a metal mesh floor and allowed to acclimatise for 5-10 minutes. Von Frey hairs with bending forces of 1g, 1.2g, 1.66g, 2.75g, 3.63g, 4.57g, 5.5g, 7.58g, 8.51g, 12.6g, 15.1g and 20.9g were applied to the plantar surface of both paws in ascending order of force. Von Frey hairs with higher bending forces were not used as they were so broad that they lifted the paw. Thus magnitudes of force higher than 20.9g could not be used. Each hair
was applied 10 times to each hindpaw; if the animal withdrew from the stimulus 6 out of the 10 trials then this was taken as the mechanical threshold for that hindpaw.

In the experiment with naive rats, the mechanical threshold for each hindpaw was determined twice prior to galanin administration and these readings were averaged to obtain the predose readings of each group of rats. Galanin (0.5μg, 5μg, 50μg dissolved in saline) or saline was administered intrathecally in a volume of 10μl, under anaesthesia. Mechanical thresholds were then determined 30 minutes and 90 minutes following galanin/saline administration. As for the experiments in naive rats, SNL rats received a 10μl intrathecal injection of galanin (0.5μg, 5μg, 50μg dissolved in saline) or saline under anaesthesia. Following galanin/saline administration, mechanical thresholds were then determined 30 minutes and 90 minutes later.

2.4 Behavioural testing of thermal withdrawal latencies

This technique was employed to examine the effect of IL-6 on the thermal thresholds of naive rats, when administered peripherally (for results see section 4.3) or spinally (for results see section 4.5). Rats (200-250g) were randomly assigned to groups of six. Nociceptive responses to a noxious thermal stimulus were examined by measuring the latency to withdrawal of the hindpaws from a focused beam of radiant heat to the plantar surface using a Ugo Basile Plantar Test apparatus. Animals (one group at a time) were placed in 6 perspex transparent chambers (dimensions - 18cm x 29cm x 13cm) with a thin glass floor and allowed to acclimatise for 5-10 minutes before withdrawal latencies were measured on both hindpaws. With a cut-off of 31.8 seconds, predose withdrawal latencies were measured and then again, one and three hours following a 10μl intraplantar injection of either IL-6 (0.01μg, 0.1μg, 1μg dissolved in saline) or saline. In separate experiments, naive rats received a 10μl intrathecal injection of either IL-6 (100ng, 250ng or 500ng dissolved in saline) or saline and withdrawal latencies were determined at the same timepoints. At each time point, both hindpaws of each rat were tested twice and then an average of these two readings was taken. To avoid sensitisation of the paws, several minutes was left before a paw was tested for the second time.
Thermal responses were also measured to examine the effect of spinal galanin in SNL rats (for results see section 5.4). The surgery of 24 rats was performed over two days. SNL rats were divided into groups of six and the experiment took place on post operative day 14/15. The same protocol of testing was followed as described above. The exceptions were that the SNL rats received a 10μl intrathecal injection of either galanin (0.5μg, 5μg, 50μg dissolved in saline) or saline. Thermal withdrawal latencies were measured 30 and 90 minutes post galanin/saline administration (akin to the mechanical behavioural testing galanin experiment).

2.5 In vivo electrophysiology

Experiments were performed 14-18 days after the surgery for the SNL and sham rats and on uninjured (naive) animals of a similar weight (225-270g) as previously described (Chapman et al. 1998). Rats were initially anaesthetised in a closed box with 3.5% halothane in a 33% O₂, 66% N₂O gaseous mixture until the animal was unconscious and areflexia was produced. Anaesthesia was then delivered via a nose cone, whilst the trachea was cannulated with a piece of fine bore polythene tubing (1.57mm diameter). Thereafter anaesthesia was supplied via this cannula. Rats were secured in a stereotaxic frame with ear bars and a laminectomy was performed to expose segments L4-L5 of the spinal cord. The dura of the exposed spinal cord was removed and the spine was held rigid by clamps caudal and rostral to the laminectomy that were attached to a frame to maintain stability during electrophysiological recordings. The halothane concentration was lowered (2.5-2.8%) whilst the surgery was performed, and then held at 1.9-2.2% for the duration of the experiment. This level of anaesthesia maintained areflexia. The rats breathed spontaneously at a rate of on average 120 breaths per minute and their circulatory system was not compromised as gauged by the rose pink colour of his feet. Throughout the experiment the core body temperature of the rat was monitored and maintained (36.5-37°C) by means of a heating blanket connected to a rectal thermal probe via an automatic
feedback control unit. At the end of the experiment, rats were killed with an overdose of halothane.

2.5.1 Isolation of a neurone

A parylene-coated tungsten electrode (A-M systems Inc. USA) connected to a headstage (held in an electrode holder) was lowered into the spinal cord manually, enabling the extracellular recording of convergent dorsal horn neurones. Once the electrode had penetrated the spinal cord, fine movements (10μm at a time) were made using a SCAT microdrive (Digitimer UK), in a dorso-ventral direction to find and isolate a single neurone. This device enabled the depth of the neurone (always greater than 500μm) from the surface of the dorsal horn of the spinal cord to be measured. In SNL and sham-operated animals neurones ipsilateral to the surgery were recorded, whereas in naive rats neurones could be recorded from either side of the cord. All neurones were of a depth between 500 and 1000μm; correlating to lamina V of the spinal cord (Wall 1967). As the electrode was lowered into the spinal cord, the hindpaw was tapped with a finger, which enabled the identification of a neurone that had a receptive field in the region of the toes. A single cell was identified by the identical amplitude of the action potentials evoked, which could be clearly differentiated from the background signal. Extracellular recordings were made from single convergent dorsal horn neurones responding to both innocuous (brush) and noxious (pinch) stimuli applied to the receptive fields in the plantar region of the hind paw provided the action potentials elicited could be clearly counted above the background signal. To assess whether the convergent dorsal horn neurone isolated received C-fibre input; the receptive field was electrically stimulated. Two small fine needles were inserted into the centre of the receptive field and the needles were connected to a stimulus isolator box (NL800 Neurolog System). This transcutaneous electrical stimulation allowed the determination of the C-fibre threshold and hence the means to evoke neuronal responses for the duration of the experiment (see 2.5.3).
2.5.2 Recording equipment

Neurolog (Digitimer, UK) equipment was used to record neuronal events and electrically stimulate the receptive fields (see 2.5.3). A diagram of the recording equipment is shown in Figure 2.5.2 (p88). The electrode was held in a headstage (NL100AK) from which there were 3 leads, one connecting the electrode to the headstage and then the preamplifier, the earth lead and the B lead. The earth lead ran from the headstage to the stereotaxic frame and steel table grounding the headstage. The B lead was attached to the skin of the rat with a crocodile clip, which transmitted the signal from the surrounding electrical noise. The A. C. preamp was set on differential recording so (A-B) so that the signal from the surroundings (B) was subtracted from the signal from the electrode (A). This resultant signal (A-B) was then amplified (AC-DC amp, NL106 module) and filtered (filters, NL125 module) to a loudspeaker and oscilloscope (via the audio amp NL120 module).

The window height (spike trig. NL201 module) was adjustable, so that neurones could be differentially recorded according to their amplitude (height of spikes). This could be seen on the oscilloscope as dots above the height of the spikes and this signal relayed to a C.E.D. 1401 interface, which in turn relayed the captured data to a computer. Spike 2 software (Cambridge Electronic Design, UK) was used then to quantify the number of action potentials evoked.
This system provides low-noise AC recording with fully adjustable filters, spike discrimination and audio monitoring.

The NL100AK provides high-impedance differential pre-amplification.

The NL104A has a high Common Mode rejection and converts the differential signal to single-ended one. It amplifies the signal between x100 and x20,000.

The optional NL106 provides a continuous variation of gain up to x100.

The NL125 (use a NL126 for 60Hz mains) offers continuous LP and HP filters as well as a mains rejection Notch filter.

The NL120 provides the audio amplification and control. With its two inputs, either the signal or the detected spikes can be monitored.

In this configuration, only one lead is needed! That is between the NL125 and the NL120. All the other connections are via the Mother board of the NL900.

The connections shown in grey are via the rear panel. The connections shown in black are actual cables.

NL985 Loudspeaker
2.5.3 Electrical stimulation

The C-fibre threshold current was determined by applying single electrical pulses to hindpaw receptive field of the neurone through a pair of stainless steel needles inserted in the centre of the receptive field of that neurone. Increasing the amplitude of this electrical current (to a maximum 10mA, 2 msec width, 0.5Hz) until a consistent response of the neurone in the C-fibre latency range (see below) was evoked. Electrical stimulation was then applied to the receptive field at three times the C-fibre threshold current. This did not damage the hindpaw (no oedema was produced or skin damage), even at the maximal current of 9.9mA. An electrical test comprised of 16 consecutive electrical impulses applied to the receptive field. A switch on the ‘counter’ Neurolog module (NL603), which also sets the number of pulses (16) generated, initiated an electrical test.

The electrical stimulation is set by another series of Neurolog modules, which produces a square shaped electrical signal to the stimulus isolator box. The period generator module (NL304) sets the frequency of electrical stimulation (0.5Hz). The digital width module (NL401) sets the duration of each electrical pulse (2msecs). Finally, the pulse buffer module (NL510) controls the amplitude of the current generated (3 x the C-fibre threshold for the neurone). Two further Neurolog modules count the number of action potentials evoked between 90 and 800msecs post stimulus. The ‘delay width’ module (NL403) sets the period (90-800msecs) following the electrical impulse and the ‘latch counter’ module (NL606) counts the number of action potentials that occur between 90-800msecs post-stimulus. These numbers enable the calculation of the wind-up responses (see below).

From the 16 electrical impulses, a post-stimulus time histogram (PSTH, see Fig. 2.5.5, p94) was constructed using a C.E.D. 1401 interface and Spike 2 software. This enabled Aβ-fibre, Aδ-fibre and C fibre-evoked responses to be separated and quantified by latency following electrical stimulation (0-20 msecs, 20-90 msecs and 90-300 msecs respectively). The remaining neuronal response (300-800ms post-stimulus) is known as the post-discharge response and is a measure of neuronal hyperexcitability. The initial C-fibre response (non-potentiated response) is the number of action potentials evoked between 90 and 800 msecs in response to the first electrical impulse, prior to any wind-up of the
neurone. Wind-up was calculated as the difference between the total number of action potentials (90-800 msecs) produced by the train of 16 electrical stimuli and the non-potentiated response produced by the train of 16 electrical stimuli (initial C-fibre response multiplied by 16). Electrical stimulation was only used in experiments using spinal drug administration. The circuitry of the Neurolog system is shown in Fig.2.5.3, p91.

2.5.4 Natural stimulation

Responses of convergent dorsal horn neurones were recorded following defined innocuous and noxious mechanical stimulation of the hindpaw receptive field using von Frey hairs with bending forces of 8.51g and 28.84g respectively. Each hair (8.51g hair followed by the 28.84g hair) was applied for 15 seconds and the number of action potentials this stimulation generated was recorded. The Neurolog recording equipment was identical to that previously described in section 2.5.2 and illustrated in Figure 2.5.2/3. Spike 2 software was also used to generate a rate histogram, which continually counts the action potentials and plots the events against time. The spontaneous activity of the neurone was taken into account by measuring the number of action potentials over a 15-second period prior to any stimulation. This ‘background measurement’ was then subtracted from the response to each of the von Frey hairs. Following mechanical stimulation, a constant jet of 32°C water was applied to the receptive field for 15 seconds using a hypodermic needle and large syringe. This was repeated with a constant jet of 45°C water for 15 seconds. The number of action potentials evoked by the innocuous 32°C water jet was then subtracted from the number of action potentials evoked by the 45°C water jet to determine the response of the neurone to noxious heat. The purpose of this is to account for the action potentials evoked due to the mechanical stimulus caused by the impact of the water jet.
Figure 2.5.3 Schematic diagram of data capture in electrophysiological experiments
2.5.5 Drug administration

Two different routes of drug administration were employed to investigate the effects of galanin /IL-6 in sensory processing, spinal administration and peripheral administration. This resulted in two different experimental protocols. Figure 2.5.5 simply illustrates these two protocols, along with examples of a PSTH and rate histogram trace (p94).

Spinal administration

Control tests of the neurone, using electrical stimulation were then followed by natural stimulation, were made at 10-minute intervals until the responses did not differ by more than 15%. The neuronal responses from the last three control tests were averaged to comprise the pre-drug control responses. IL-6 (100ng, 250ng and 500ng dissolved in 50μl saline) or galanin (0.5μg, 5μg, 50μg dissolved in saline) was then administered spinaly, in a cumulative manner, onto the exposed spinal cord (akin to the intrathecal route). The laminectomy results in the formation of a small well around the electrode. A 50μl Hamilton syringe was then used to carefully drop the dose of drug into this well to create a small pool of drug solution on top of the exposed spinal cord. Tests were performed every 10 minutes for a time course of 90 minutes, following each dose of IL-6/galanin. This resulted in a 270-minute total time course of IL-6/galanin exposure to the spinal cord. Effects of IL-6/galanin were expressed as percentages of the pre-drug control value for each evoked response, allowing each neurone to serve as its own control.

Neuronal responses to electrical stimulation are expressed as the total number of action potentials evoked by the 16 electrical stimuli divided into the Aβ-fibre, Aδ-fibre, C-fibre, post-discharge, initial C-fibre and wind-up responses as described in section 2.5.3. For the natural stimulation, the total numbers of action potentials evoked by the 15-second stimulus application were counted. The results of the spinal IL-6 and galanin electrophysiological experiments are shown in sections 4.4 and 5.3, respectively.
Peripheral administration

Responses to mechanical and thermal stimulation were determined at 10-minute intervals until the number of action potentials evoked to each stimulus did not differ by more than 15%. The last four tests were then averaged for each stimulus to comprise the pre-drug control response. IL-6 (40ng and 100ng dissolved in 20µl saline) or galanin (0.1µg, 1µg, 10µg and 100µg dissolved in 20µl saline) was then injected, with cumulative doses, into the toe containing the receptive field of the spinal neurone. Tests were then performed every 10 minutes over a 120-minute time course following each dose of IL-6. This resulted in a 4-hour total time course of IL-6 exposure to the peripheral receptive field of the spinal neurone. In the galanin experiments, tests were also performed every 10 minutes over a 60 minute time course following each dose of galanin. This also resulted in a 4-hour total time course of galanin exposure to the peripheral receptive field of the spinal neurone. Effects of IL-6/galanin were expressed as percentages of the pre-drug control value for each evoked response, allowing each neurone to act as its own control. The results of peripheral IL-6 and galanin electrophysiological experiments are shown in sections 4.2 and 5.2, respectively. A similar protocol was followed in separate experiments to assess the effect of saline injections on the naturally-evoked neuronal responses. For results see section 4.2.
Figure 2.5.5 Electrophysiological studies on spinal neurones. The figure shows examples of the various electrical and natural responses of the neurones and depicts the routes of drug administration used in these studies. Post stimulus time histograms and rate recordings were used to quantify the neuronal responses and drug effects thereon.
2.6 *In vitro skin-nerve preparation*

This technique was first described by Reeh (1986) and illustrated in Fig. 2.6 (p97). Rats (160-215g) were sacrificed by CO\textsubscript{2} overdose and the skin of one hindpaw with attached saphenous nerve was dissected. The saphenous nerve was cut proximally to the epigastric artery and dissected free from the thigh muscle. A piece of muscle adjacent to the nerve, near the ankle was also dissected as it enters the skin to ensure that any small branches of the nerve were not damaged as the skin was dissected from the ankle. The saphenous nerve branches that innervate the tibial skin were cut and the hairy skin was carefully dissected from the foot. The edge of the skin preparation started where the nerve entered the skin and ran along both edges of the paw, finishing at the base of the toes (see Figure 2.6, p90). The skin was pinned 'hairy side down' to expose the corium side and the end of the nerve threaded through a hole (2mm diameter) into a separate recording chamber and laid on a mirror. The skin chamber was perfused (15ml/min) with a physiological salt solution (PSS) of the following composition (in mM): NaCl 138.6; KCl 3.5; CaCl\textsubscript{2} 1.5; MgCl\textsubscript{2} 1.2; NaHCO\textsubscript{3} 21.0; NaH\textsubscript{2}PO\textsubscript{4} 0.58; glucose 10.0, which was perfused with 95% O\textsubscript{2} and 5% CO\textsubscript{2} and maintained at 33°C. The aqueous solution in the recording chamber was overlaid by paraffin oil, the epineurium was then pulled back to allow the dissection of fine filaments which were placed on a single platinum wire in the paraffin layer to record single unit activity. Single units were identified following electrical stimulation of the nerve trunk via a bipolar platinum electrode, which enabled the measurement of the conduction velocity of the nociceptive fibres (distance between stimulating electrode and recording electrode divided by time taken for action potential propagation). Electrical stimulation was generated using a simplified version of the Neurolog system previously described in section 2.5.3 and illustrated in Figure 2.5.3 (p91). The recording equipment used to capture and record the action potentials generated was the same as previously described in section 2.5.2 and illustrated in Figure 2.5.2 (p88). Receptive fields were located by gently prodding the skin with a blunt glass rod and the mechanical thresholds determined with calibrated von Frey hairs. A hollow metal cylinder (8mm diameter) was placed on the receptive field and perfused with PSS at a rate of 5ml/min with the temperature regulated by an electrical continuous flow heater (Peltier device) which was under the influence of a
Marlow temperature controller. A thermocouple placed in the receptive field cylinder recorded the temperature of PSS at the skin. Using the Marlow device, the temperature of solution at the skin was increased to 49°C and maintained for 30 seconds then cooled back to 35°C. The resultant heat ramp and the action potentials elicited were recorded using Spike 2 software via a CED 1401 interface, which enabled different fibres to be distinguished and their responses to be quantified. This was done by monitoring the waveform (comparing back to a template of the shape of the action potential) and amplitude of the fibres. To examine the effect of repetitive noxious heat alone, the receptive fields of 16 nociceptive fibres were exposed to noxiously heated PSS three times with 10-minute intervals. The effects of noxious heat alone were then compared to the effect of IL-6 or galanin on the heat responses of nociceptive fibres.

Following a control thermal stimulation, galanin (1μM in PSS) was perfused onto the receptive field for 10 minutes and thermal stimulation was performed in the continued presence of galanin. The thermal response was then repeated following a 10-minute washout period. Galanin was only applied once to each skin preparation and the results of these experiments with sample traces are presented in section 5.1. Similarly, following a control thermal stimulation, IL-6 (20ng/ml or 50ng/ml, dissolved in PSS) was perfused onto the receptive field for 10 minutes and thermal stimulation was performed in the continued presence of IL-6. The thermal response was then repeated following a 10-minute washout period. Thus as in the galanin experiments, nociceptive fibre responses to heat were measured before IL-6 (to act as pre-drug control), in the presence of IL-6 and 10 minutes post IL-6 application. In addition, the effect of the soluble IL-6 receptor (shIL-6R) was investigated by perfusing shIL-6R solution (25ng/ml dissolved in PSS) for 10 minutes at 35°C, prior to exposure to 20ng/ml IL-6. IL-6 (and shIL-6R) was only applied once to each skin preparation and the results of these experiments with sample traces are presented in section 4.1.
Figure 2.5.2 Recording Equipment Figure 2.5.3

Figure 2.6 In vitro skin-nerve preparation
- adapted from drawing by L. Urban, (Novartis)
2.7 Statistics

ANOVA was used to test for differences in control neuronal responses prior to spinal/peripheral drug administration for the naive, sham and SNL rat groups. ANOVA was also used to test for differences in conduction velocity, mechanical threshold and first heat response between nociceptive fibre groups, e.g. between the heat alone, 20ng IL-6, 20ng IL-6+sIL-6R and 50ngIL-6 fibre groups in the IL-6 study. Paired t-tests were used to test the differences between the control responses and maximal effects following spinal/peripheral drug application in vivo and also to test the effect of galanin or IL-6+sIL-6R on nociceptive fibres compared to the first (control) heat response. Unpaired t-tests were used to compare the drug effects between neuronal populations e.g. the effect of galanin on wind-up in SNL rats compared to naive rats. Unpaired t-tests were also used to compare the effects of different treatments on the heat responses of nociceptive fibres e.g. the effect of IL-6+sIL-6R on nociceptive fibres compared to the effect of heat alone.

ANOVA (for repeated measures) followed by Tukey’s HSD test were used to compare behavioural withdrawal latencies after drug administration to predose values. All tests used a 5% level of significance, p < 0.05. When a significant difference has been found in my results the statistical test used is stated in brackets accordingly. Statistical analysis was aided by the use of Statview4.5 and InStat 2.01 computer packages.
2.8 Drugs

Human recombinant interleukin-6 (IL-6) was obtained from Sigma-Aldrich Company Ltd., U.K. The cytokine was expressed in *E. coli* and also contained the carrier protein, bovine serum albumin (BSA). The cytokine was dissolved in 0.9% saline, with the exception of the skin-nerve experiments where it was dissolved in distilled water. The resultant solutions were stored in aliquots at -20°C.

Soluble human recombinant interleukin-6 receptor (shIL-6R) was obtained from R&D Systems, U.K. The receptor was dissolved in distilled water and stored in aliquots at -20°C.

Porcine galanin was obtained from Bachem Ltd., U.K. The peptide was dissolved in 0.9% saline, with the exception of the skin-nerve experiments where it was dissolved in distilled water. The resultant solutions were stored in aliquots at -20°C.
Chapter 3

Behavioural measures and electrophysiological characterisation of neurones
3. Characterisation

This chapter presents the behavioural characteristics of pain-related responses of rats induced by spinal nerve ligation (SNL). In addition, based on the electrophysiological studies, the response characteristics of convergent dorsal horn neurones and single nociceptive fibres are presented.

3.1 SNL Behaviour

The rats used in the electrophysiological studies were monitored for 2 weeks post-surgery and behavioural testing was performed on post-operative days 2, 4, 7, 9, 11 and 14 to assess the sensitivity of the SNL and sham-operated rats to innocuous mechanical and cold stimuli (acetone). SNL rats developed mechanical and cold allodynia compared to sham rats (Fig. 3.1.1, p102). A withdrawal response from innocuous mechanical or cold stimuli was rapid and often accompanied with shaking, licking and/or pulling of the ipsilateral paw. Similar behaviours were extremely rarely observed in the contralateral paw of SNL rats or in sham rats (see Fig 3.1.1, p102) and such rare observations were of a rapid withdrawal without any licking, pulling or sustained elevation of the paw. The ipsilateral paw of SNL rats exhibited an abnormal foot posture where the toes were raised and held together in a ‘guarding’ behaviour. All rats displayed normal weight gain, nail growth and levels of grooming indicative of good health. Also none of the rats displayed autotomy (self-mutilation) or signs of distress during this post-operative period.

In addition to the timecourse of neuropathic pain behaviours (Fig.3.1.1, p102), I examined the mechanical thresholds of SNL rats over a similar period of time (Fig. 3.1.2, p103) prior to investigating the effect of spinal galanin on mechanical thresholds (see section 5.4). SNL produced an average decrease in the mechanical threshold of the ipsilateral paw of 10g compared to the mechanical threshold before surgery (postoperative day -1) and the contralateral paw (Fig. 3.1.2, p103). This decrease was highly significant, p < 0.0001 (ANOVA for repeated measures).
Figure 3.1.1. Behavioural effects of spinal nerve ligation compared to sham operation. These rats were used for the electrophysiological studies. Plots show the mean ± SEM in the difference score (number of contralateral withdrawals minus number of ipsilateral withdrawals), $n = 63$ for SNL rats and $n = 47$ for sham rats. Von Frey filaments were applied to each hindpaw 10 times and acetone was applied 5 times.
Figure 3.1.2. Timecourse of the effect of spinal nerve ligation on the mechanical thresholds of the ipsilateral and contralateral paw. Plots show the mean ± SEM in the mechanical threshold in grams, n = 20. Mechanical thresholds were measured before surgery (day -1) and on post-operative days 3/4, 7/8, 10/11 and 17/18 (as the surgery of 20 rats occurred over a period of two days). SNL produced a marked significant decrease in the mechanical thresholds of the ipsilateral paw (p < 0.0001, ANOVA).
3.2 Spinal neuronal responses to electrical and natural stimuli

A total of 194 convergent dorsal horn neurones were recorded, on which the data presented in chapters 4 and 5 are based. 98 of these neurones were from naive rats, 46 from sham rats and 50 from SNL rats. Neurones were of a depth between 500 and 1000µm and Table 3.2 shows a similar mean depth and mean C-fibre threshold of the neurones recorded from naive, sham and SNL rats. Neurones were stimulated differently depending on the particular study. In general, when spinal administration was used, the receptive fields were stimulated both electrically and by natural stimuli. In studies where the drug was administered into the peripheral receptive fields only natural stimulation was used. Neuronal responses to electrical stimulation (3 x C-fibre threshold) are expressed as the total number of action potentials (APs) evoked by the 16 electrical stimuli. For the natural stimulation (mechanical and thermal), the total number of action potentials (APs) evoked by the 15-second stimulus application is given.

Table 3.2 - Spinal neuronal characteristics of naive, sham and SNL rats

<table>
<thead>
<tr>
<th>Neuronal Characteristic</th>
<th>Neurones From Naive Rats</th>
<th>Neurones From Sham Rats</th>
<th>Neurones From SNL Rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Depth of Neuron (µm)</td>
<td>648 ± 17</td>
<td>713 ± 24</td>
<td>661 ± 21</td>
</tr>
<tr>
<td>C-fibre threshold (mA)</td>
<td>2.09 ± 0.24</td>
<td>2.1 ± 0.19</td>
<td>1.99 ± 0.15</td>
</tr>
<tr>
<td>C-Fibres (APs)</td>
<td>266 ± 14</td>
<td>287 ± 19</td>
<td>258 ± 11</td>
</tr>
<tr>
<td>Aδ-Fibres (APs)</td>
<td>55 ± 10</td>
<td>61 ± 9</td>
<td>56 ± 13</td>
</tr>
<tr>
<td>Aβ-Fibres (APs)</td>
<td>91 ± 9</td>
<td>93 ± 6</td>
<td>96 ± 7</td>
</tr>
<tr>
<td>Initial C-fibre (APs)</td>
<td>15 ± 2</td>
<td>17 ± 4</td>
<td>16 ± 2</td>
</tr>
<tr>
<td>Post discharge (APs)</td>
<td>147 ± 21</td>
<td>188 ± 35</td>
<td>163 ± 29</td>
</tr>
<tr>
<td>Wind-up (APs)</td>
<td>181 ± 32</td>
<td>226 ± 40</td>
<td>211 ± 51</td>
</tr>
<tr>
<td>Innocuous mechanical</td>
<td>146 ± 31</td>
<td>181 ± 28</td>
<td>163 ± 42</td>
</tr>
<tr>
<td>(von Frey 8.5g, APs)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Noxious mechanical</td>
<td>506 ±58</td>
<td>575 ± 44</td>
<td>517 ± 40</td>
</tr>
<tr>
<td>(von Frey 28.84g, APs)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Noxious Heat (APs)</td>
<td>410 ± 75</td>
<td>378 ± 63</td>
<td>389 ± 68</td>
</tr>
</tbody>
</table>

No significant differences were found between the three animal groups (ANOVA) for any of the neuronal characteristics listed in Table 3.2. This shows that despite two-thirds of the input from the peripheral sciatic nerve into the lumbar segments of the spinal cord being practically obliterated in SNL rats, the neuronal response to various stimuli were of a
similar magnitude to neuronal responses in naive and sham rats. Furthermore, there is a decrease (~35%) in the afferent input to the lumbar segment that is mainly innervated by the undamaged L4 spinal nerve because of the loss of the rostral unmyelinated fibre innervations from L5/6 spinal nerves (Besse et al. 1991). The lack of change in the neuronal responses of SNL rats compared to naive rats suggests that spinal excitability is increased following nerve injury to compensate for the reduction in the input reaching the spinal cord from the periphery. Indeed, there are electrophysiological characteristics to demonstrate that following nerve injury such compensations in the nervous system exist.

As previously mentioned (sections 1.4.2 & 1.4.6), it is widely agreed that following nerve injury, spinal neurones display increased spontaneous firing (Laird and Bennett 1993; Behbehani and Dollberg-Stolik 1994; Pertovaara et al. 1997; Chapman et al. 1998; Tabo et al. 1999). This spontaneous activity strongly suggests that there is an ongoing release of neurotransmitters in the spinal cord, which could then result in NMDA receptor activation, enhancing spinal hyperexcitability. Evidence for some degree of ongoing NMDA receptor activation following nerve injury, in the absence of intense peripheral stimulation, has been shown in behavioural and electrophysiological studies. Behaviourally, NMDA receptor antagonists e.g. MK801 have been shown to relieve pain behaviours in rats following neuropathy, but have no effect in uninjured rats (Davar et al. 1991; Mao et al. 1992; Yamamoto and Yaksh 1992). Similarly, in electrophysiological studies, NMDA receptor antagonists were shown to produce greater inhibitory effects on neuronal responses of SNL rats compared to sham-operated rats (Suzuki et al. 2001). In addition as previously mentioned, there appears to be plasticity in the expression of calcium channels (which are essential in neurotransmitter release) with a reported upregulation of N-type channels, following nerve injury (Cizkova et al. 1999). Moreover, N-type blockers have been shown to have an enhanced anti-nociceptive effect following nerve injury (Xiao and Bennett 1995; Matthews and Dickenson 2001b).

Another widely recognised consequence of nerve injury is expanded peripheral receptive fields (Behbehani and Dollberg-Stolik 1994; Takaishi et al. 1996; Cumberbatch et al. 2001).
1998; Tabo et al. 1999; Suzuki et al. 2000). This means that peripheral stimulation, which prior to nerve injury did not evoke a response in spinal neurones because it occurred outside the receptive field or was insufficiently noxious, results in action potential generation in the spinal cord. Also this increase in peripheral receptive fields means that for any given stimulus, a larger number of spinal neurones will respond, potentially enhancing pain transmission to the brain.

In other studies following nerve injury, the responses of spinal neurones have been compared to sham-operated rats. Following SNL, neuronal responses to electrical and natural stimuli have been shown to be largely similar to neuronal responses in sham and naive rats (Chapman et al. 1998; Matthews and Dickenson 2001b; Suzuki et al. 2001). In contrast, other authors report increased neuronal responses to mechanical stimulation in SNL rats compared to sham rats (Leem et al. 1995; Pertovaara et al. 1997). CCI induced mechanical allodynia but did not alter the mean mechanical threshold of spinal neurones, although there appeared to be a lack of neurones which responded to low mechanical stimulation in CCI rats (Laird and Bennett 1993). Electrophysiological studies have also examined the responses of spinal neurones in injured animals compared to sham-operated animals in relation to changes in behavioral thermal nociception. Tabo et al. (1999) reported thermal hypoalgesia following dorsal root constriction, but no change in thermally evoked responses of spinal (WDR) neurones in injured rats compared to sham rats. Other authors report rats to exhibit thermal hyperalgesia following CCI (Laird and Bennett 1993) and PSL (Takaishi et al. 1996), yet also find no change in the thermal responses of spinal (WDR) neurones.

Despite the neuronal responses in SNL rats being similar to sham and naive rats, there are marked differences in the drug effects on a neurone from a SNL rat compared to a neurone from a naive rat. Examples of which have been previously mentioned and this will be demonstrated further in chapter 4 and 5. Therefore the study of convergent dorsal horn neurones, as performed in a large part of this thesis, in the assessment of potential analgesic drugs is advantageous for several reasons. Firstly, these neurones receive inputs
from both A- and C-fibres; thus differential effects of drugs on fibre type can be elucidated. Secondly, activation of convergent dorsal horn (WDR) neurones, as opposed to neurones of the superficial spinal cord, results in human pain perception (Mayer et al. 1975; Price and Mayer 1975). These studies report that the electrical thresholds and refractory periods of convergent dorsal horn neurones closely parallel those of a neuronal population in the anterolateral quadrant (ALQ) of the human spinal cord that when stimulated produce pain. Thirdly, convergent dorsal horn neurones also comprise a major part of the spinothalamic tract (STT) (Sorkin and Carlton 1997). This major ascending pathway to the brain is essential for the transmission of nociceptive information from peripheral receptive fields (Sorkin and Carlton 1997). Finally, convergent dorsal horn neurones were shown to be more accurate than nociceptive-specific (high-threshold) neurones in the perception of changes in the intensities of stimulation (Maixner et al. 1986). Finally, wind-up is most pronounced in convergent dorsal horn neurones compared to low-threshold (Class 1) or nociceptive-specific (high-threshold, Class 3), which show little wind-up (Schouenborg and Sjolund 1983).
3.3 Characteristics of nociceptive fibres

A total of 67 nociceptive fibres were recorded from and characterised in terms of their conduction velocity and mechanical threshold. Measurement of the conduction velocities of these 67 fibres, resulted in their classification as 3 Aδ-fibres with a mean conduction velocity of 2.67m/s ± 0.36m/s and 64 C-fibres with a mean conduction velocity of 0.75m/s ± 0.03m/s. There was no clear difference between the mechanical threshold of the Aδ-fibres, 2.02g ± 0.36g and the C-fibres, 1.91g ± 0.11g.

Following characterisation, the responses of these nociceptive fibres to repetitive noxious heat were measured in the presence of IL-6 (see section 4.1) or galanin (see section 5.1) or in the absence of both. 16 fibres (all conducting in the C-fibre range) were exposed to repetitive noxious heat alone. The effect of repetitive noxious heat on the number of action potentials elicited and the threshold at which the firing response commenced is shown in Figure 3.3.1 (p109). There is a small but significant (p < 0.05, paired t-test) decrease in the number of action potentials (decrease of 28 action potentials or 37% decrease compared to first heat response) evoked to noxious heat at the third heat exposure compared to the first heat exposure. This desensitisation or ‘fatigue’ in the response of nociceptive fibres to noxious heat has been previously reported elsewhere (Raja et al. 1997). There also appears to be a slight drop in the threshold of the response, although this is not significant.
Figure 3.3.1. The effect of repetitive noxious heat on the A) magnitude and B) threshold of the responses evoked by a population of C-fibres, n = 16. These fibres were recorded in vitro from the skin of naive rats. *p < 0.05 (paired t-test) significantly different response from heat response 1.
In nociceptive DRG neurones, repetitive heat stimulation at similar temperatures 43-47°C to those I used, also induced tachyphylaxis of the heat current, which was found to occur independently of extra- and intracellular calcium (Schwarz et al. 2000). The molecular mechanisms responsible for this desensitisation or adaptation are not clear. Presumably, VR1 receptors (Caterina et al. 1997) are involved in this process, as VR1 receptors are directly activated by heat in single channel recordings from excised membrane patches (Tominaga et al. 1998). Furthermore capsazepine (vanilloid antagonist) blocks heat-evoked currents in transfected cells (Tominaga et al. 1998). The desensitisation of the heat response is unlikely to be caused by changes in the primary structure of VR1 receptors as the temperature range used here is noxious, but is perhaps unlikely to be high enough to cause protein denaturation. As previously mentioned, the detection of noxious heat was only impaired in VR1 knockout mice (Caterina et al. 2000), suggesting that mechanisms not involving VR1 receptors are also responsible for the transduction of noxious heat. Recently, it has been shown that exposure to very high temperatures (55-61°C) exert sustained changes in the membrane currents of nociceptive rat DRG neurones (Lyfenko et al. 2002). The deactivation kinetics of the membrane current was slowed down and the temperature threshold was markedly decreased. However, the heat current of these nociceptive DRG neurones only altered with temperatures exceeding 50°C (Lyfenko et al. 2002). The change in membrane current kinetics at temperatures exceeding 50°C were suggested to be responsible for earlier reports of the sensitisation of C-fibres following a 60°C stimulus (Lynn 1979). Although, in the rat, sensitisation of C-fibre units following suprathreshold heating was found not to be as common as in other species (Lynn and Carpenter 1982).

In conclusion, the reduction in heat-evoked activity that I have observed in nociceptive fibres, was sufficiently small not to compromise subsequent pharmacology and allowed the drug effects to be compared to the appropriate response in the absence of the drug.
Chapter 4

Effects of Interleukin-6 on Sensory Processing in Naive and Neuropathic Rats
4. Introduction

Many animal studies have examined the effect of nerve injury on IL-6 expression (see Chapter 1). In short, studies report little to no expression of IL-6 mRNA and protein under normal conditions. Following a variety of nerve injuries, IL-6 mRNA has been shown to be consistently upregulated in the injured sciatic nerve (Zhong and Heumann 1995), in Schwann cells at the site of injury (Bolin et al. 1995), in the DRG (Murphy et al. 1995) and in the spinal cord (Arruda et al. 1998; Winkelstein et al. 2001). IL-6 binds to a specific cell surface (cytokine type 1) receptor and for signal transduction to occur, the IL-6/receptor complex then associates with two gp130 molecules (Hirano et al. 1994; Mullberg et al. 1999). IL-6 receptor (IL-6R) mRNA is found in vascular endothelial cells and Schwann cells of the intact sciatic nerve and is upregulated following sciatic nerve crush (Grothe et al. 2000) and transection (Ito et al. 1998). Intact nerves display low levels of gp130 mRNA, but following nerve injury, like the ligand IL6, there is also a marked increase in gp130 mRNA (Ito et al. 1998).

Little is known on the effects of exogenously administered IL-6 on nociceptive transmission. Intracerebroventricular injection of IL-6 decreased hot-plate withdrawal latencies in naive rats (Oka et al. 1995). Intrathecal IL-6 had no effect on thermal withdrawal latencies in naive rats but decreased the contralateral thermal withdrawal latencies in animals with sciatic cryoneurolysis (SCN) rats (DeLeo et al. 1996). Intraplantar IL-6 is reported to cause mechanical hyperalgesia, in both paws within 30 minutes of administration in naive rats (Cunha et al. 1992). In contrast, intraplantar IL-6 had little effect in normal conditions but increased mechanical thresholds of in rats with inflammation (Czlonkowski et al. 1993).

Therefore, the aim of these studies is to investigate the effect of spinal and peripheral IL-6 on nociceptive transmission in naive and neuropathic states. I will present the effect of exogenous IL-6 given peripherally, both in vitro and in vivo, using three different techniques. Firstly, employing the in vitro skin-nerve preparation (Reeh 1986), I will show the effect of IL-6 and its soluble receptor (sIL-6R) on the responses of single nociceptive
fibres. Secondly, the *in vivo* effect of peripheral IL-6 administration on naturally evoked responses of convergent dorsal horn neurones will be presented and the effect of nerve injury on this action. Finally, I will demonstrate the behavioural effects of peripheral IL-6 administration on thermal withdrawal latencies, in naive rats. The effect of IL-6 at the spinal level has been investigated using *in vivo* electrophysiology, to examine the effects of spinal IL-6 administration on the responses of convergent dorsal horn neurones in naive and neuropathic rats. Finally, I will show the behavioural effects of exogenous spinal IL-6 in naive rats.

### 4.1 Effects of IL-6 on nociceptive fibres in vitro

Responses were obtained from a total of 47 nociceptive fibres which were classified as 45 C-fibres and 2 A\(\delta\)-fibres with mean conduction velocities of 0.79 ± 0.03m/s and 2.8 ± 0.13m/s, respectively. There was no significant difference between the mean conduction velocity or mean mechanical threshold for the four fibre groups, heat alone, 20ng/ml IL-6, 50ng/ml IL-6 and 20ng/ml IL-6 + sIL-6R (ANOVA, Table 4.1). However the number of action potentials elicited in the control response for the 50ng IL-6 fibre group and the 20ng + sIL-6R group was significantly higher than in the control response of the heat alone group (p < 0.05, unpaired t-test, Table 4.1). Therefore, the effects of these drug treatments cannot be directly compared to effect of heat alone but will be compared to their own pre-drug control response.

| Table 4.1 - Characteristics of the control responses of the nociceptive fibres in the various experimental groups of naive rats used in the skin-nerve studies. |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| **Fibre population**           | **Heat Alone**  | **20ng IL-6**   | **20ng IL-6 + sIL-6R** | **50ng IL-6**   |
| No. of action potentials       | 78 ±12          | 72 ± 14         | 145 ± 22         | 134 ± 35        |
| Conduction Velocity (m/s)      | 0.73 ± 0.04     | 1.16 ± 0.28     | 0.72 ± 0.04      | 0.95 ± 0.1      |
| Mechanical Threshold (g)       | 2.18 ± 0.28     | 1.96 ± 0.21     | 1.69 ± 0.27      | 1.83 ± 0.37     |
|                                  |                 |                 |                  |                 |

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Figure 4.1.1 (p115) shows the effect of IL-6 on the heat responses of nociceptive fibres. This figure shows that 20ng IL-6 (Fig. 4.1.1A) had little effect on firing elicited in response to noxious heat (spikes that occurred within the heat ramp). In comparison, 50ng IL-6 (Fig. 4.1.1B) induced a marked inhibition of firing elicited in response to noxious heat. A comparison of the four experimental groups (Fig. 4.1.2, p116) clearly demonstrates, firstly, that 20ng IL-6 (n =11) had no significant effect on the heat response compared to its pre-drug control and also compared to the heat alone group (n =16). Secondly, 50ng IL-6 (n =11) produced a significant mean 60% inhibition of the heat response compared to the pre-drug control response of that fibre group (p < 0.01, paired t-test). This inhibition by 50ng IL-6 persisted following washout (p < 0.05, paired t-test) however, a similar significant degree of inhibition was seen in the fibres exposed to repetitive heat alone (p < 0.05, paired t-test). This shows that the inhibition induced by 50ng IL-6 was reversible following washout and the post-IL-6 heat response in these fibres was what would have been expected following three exposures to noxious heat in the absence of IL-6. However, there was no significant difference between the number of action potentials elicited in the second heat response compared to those in the first heat response for the heat alone fibre group. This shows that the inhibition shown in Fig. 4.1.2 (p116) by different drug treatments, is a result of a pharmacological action and not due to repetitive noxious heat application. Interestingly, the combined exposure of 20ng IL-6 and sIL-6R (n = 9) resulted in a significant 46% inhibition of the heat response compared to its pre-drug control (p < 0.01, paired t-test). This would suggest that 20ng/ml IL-6 is not a high enough concentration of IL-6 to activate sufficient IL-6 receptors that are already present in the skin but the addition of more IL-6 receptors in the form of sIL-6R means that 20ng IL-6 can induce an effect. Probably by the IL-6 ligand and soluble receptors binding together and then binding to cells which have gp130 but lacking IL-6 receptors thus resulting in an effect that was observed only at higher concentrations of IL-6 acting on receptors in the skin. This inhibition was not reversed by washout, increasing to a 79% inhibition of this response (p < 0.01, paired t-test).
Figure 4.1.1 Single nociceptive fibres firing in response to noxious heat before IL-6 (control), in the presence of IL-6 (20ng or 50ng IL-6) and after 10 minutes wash-out (post IL-6). A) An example of the mild facilitatory effect of 20ng IL-6 on the firing induced by noxious heat. B) An example of the marked inhibitory effect of 50ng IL-6 on the firing induced by noxious heat.
Figure 4.1.2. Effect of IL-6 and its soluble receptor, sIL-6R, on single nociceptive fibres recorded from skins of naive rats. Number of action potentials elicited during the heat ramp for each fibre were expressed as a percentage of the control response. Plots show the mean ± SEM for each fibre population, n = 9-16. * p < 0.05, ‡ p < 0.01 (paired t-test) mean response is significantly different from control (pre-drug) response.
Repetitive heat caused a small but significant decrease in the magnitude of response of single C-fibres, the third heat response being significantly smaller than the first heat response \((p < 0.01, \text{ Fig. } 4.1.2, \text{ p116})\). This desensitisation or ‘fatigue’ in the response of nociceptive fibres to noxious heat has been previously observed and reported (Raja et al. 1997). IL-6 had dose-dependent effects on the responses of single nociceptive fibres to noxious heat. Furthermore, the prior exposure to the soluble IL-6 receptor, sIL-6R, resulted in the previously ineffective dose of 20ng IL-6 significantly inhibiting the heat responses of nociceptive fibres to almost the same extent as the inhibition induced by 50ng IL-6. Additionally, the inhibition induced by 20ng IL-6 and sIL-6R persisted and increased following washout whereas the inhibition by 50ng IL-6 was largely reversed. The prolonged effect of peripheral IL-6 suggests IL-6 could have a long half-life (unlikely as it is washed out) or more likely persistent receptor binding. Similar effects of IL-6 and its receptor have been described on heat evoked CGRP release from the rat skin; 20ng IL-6 and noxious heat did not evoke significant CGRP release but noxious heat plus 20ng IL-6 and sIL-6R significantly increased CGRP release from nociceptors (Opree and Kress 2000). These authors concluded that normal rat skin does not express IL-6 receptors but did express gp130; hence a significant effect was only seen following the addition of sIL-6R. However, I found a significant effect was induced by a higher IL-6 concentration. Therefore I would suggest that IL-6 receptors are present in normal rat skin but their expression is too low for 20ng IL-6 to produce a significant effect. In agreement with Opree et al. (2000), I observe a significant inhibition of the heat response following 20ng IL-6 and sIL-6R demonstrating the expression of gp130 in normal skin.
4.2 Effects of peripheral IL-6 on spinal neurones

There was no significant difference between the mean depths of the neuronal populations from naive, sham and SNL or in their responses to the mechanical and thermal stimuli prior to galanin administration (Table 4.2 - expressed as number of action potentials, APs, evoked). Prior to electrophysiological study, the SNL rats displayed behavioural mechanical and cold allodynia, whereas the sham rats did not (see section 3.2).

Table 4.2 - Mean depth and pre-drug control responses of neurones exposed to peripheral IL-6

<table>
<thead>
<tr>
<th>Neuronal Characteristic</th>
<th>Neurones from Naive Rats ( n = 10 )</th>
<th>Neurones from Sham Rats ( n = 9 )</th>
<th>Neurones from SNL Rats ( n = 9 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Depth of Neurone (( \mu m ))</td>
<td>748 ± 48</td>
<td>708 ± 43</td>
<td>636 ± 16</td>
</tr>
<tr>
<td>Innocuous mechanical (von Frey 8.5g, APs)</td>
<td>169 ± 53</td>
<td>153 ± 32</td>
<td>178 ± 48</td>
</tr>
<tr>
<td>Noxious mechanical (von Frey 28.84g, APs)</td>
<td>498 ± 99</td>
<td>582 ± 93</td>
<td>488 ± 98</td>
</tr>
<tr>
<td>Noxious Heat (APs)</td>
<td>418 ± 72</td>
<td>357 ± 40</td>
<td>421 ± 75</td>
</tr>
</tbody>
</table>

The effects on spinal neuronal responses to innocuous and noxious mechanical and noxious heat stimulation following IL-6 injections into their peripheral receptive fields are illustrated in Fig. 4.2.1A-C (p120). In naive rats, peripheral IL-6 produced a marked significant inhibition of the neuronal response to stimulation by von Frey 8.51g (p < 0.05, paired t-test) resulting in a maximal 70% inhibition of the pre-drug control response following 100ng IL-6 (Fig. 4.2.1A). There was no effect of peripheral IL-6 on this neuronal response to an innocuous mechanical stimulus in sham or SNL rats although in sham rats there is a tendency towards inhibition at 100ng IL-6 (Fig. 4.2.1A).

Similarly, peripheral IL-6 elicits a significant dose-related inhibition (p < 0.05 40ng, p < 0.01 100ng, paired t-test) of the neuronal response to noxious mechanical stimulation in naive rats, with a maximal inhibition of 57% of the pre-drug control response at 100ng IL-6 (Fig. 4.2.1B, p120). A significant inhibition (p < 0.05, paired t-test) of this response to von Frey 28.84g is also observed in sham rats following 40ng and 100ng IL-6, (26% and 38% inhibition respectively). However, similar to the neuronal response to von Frey 8.51g,
peripheral IL-6 has no significant effect on the neuronal response to von Frey 28.84g in SNL rats, although a marginal tendency towards inhibition could be suggested (Fig. 4.2.1B).

In contrast, to group dependent effects of peripheral IL-6 on neuronal responses to mechanical stimulation (Fig. 4.2.1A&B, p120) are the effects of peripheral IL-6 on neuronal responses to noxious heat (Fig. 4.2.1C). Here I find that peripheral IL-6 elicits a significant inhibition (p < 0.05, paired t-test) in naive, sham and SNL rats and to a similar degree (41% - 46% inhibition). In addition, no dose-related effects of peripheral IL-6 on the neuronal response to heat are seen in naive, sham or SNL rats. Comparing between animal groups, the effect of 100ng IL-6 on the response to innocuous mechanical stimulation was significantly different (p < 0.01, unpaired t-test) in SNL rats compared to naive rats.
Figure 4.2.1. Effect of peripheral IL-6 on neuronal responses to 8.51 g von Frey mechanical stimulation (A), 28.84 g von Frey mechanical stimulation (B) and noxious heat (C). Changes in the responses following peripheral IL-6 administration are expressed as a percentage of the control response. A) - C) show the mean ± SEM of the maximum effect which occurred for the neurone population over the 120-minute time-course, n = 7 - 15. * p < 0.05, † ‡ p < 0.01 (paired t-test) when maximum effect is significantly different from the control response prior to peripheral IL-6 administration. In C) effects in all three animal groups are significantly different from their control responses p < 0.05.
Thus in SNL rats, peripheral IL-6 inhibited the heat responses but had no effect on the mechanical responses. The reason for this seemingly modality-specific inhibition is unclear. One possibility would simply be the magnitude of the neuronal response evoked by the stimuli. It is not possible to compare whether the 45°C heat stimulus is more or less noxious than the 28.84g von Frey stimulus, but the heat response elicited a larger number of action potentials than the 8.51g von Frey stimulus yet was inhibited (see Table 4.2, p118). Another explanation would be the location of the receptor on different peripheral fibres. The expression of IL-6 receptors in the periphery has not been examined. Perhaps under normal conditions IL-6 receptors are expressed in the periphery predominantly on C-fibres with a limited expression on A-fibres. Following nerve injury, maybe a decrease in the number of peripheral IL-6 receptors which means exogenous IL-6 can no longer have an effect on the predominantly A-fibre driven response to von Frey 8.51g but can affect the predominantly C-fibre driven response. However, these results indicate that the ability of peripheral IL-6 to reduce innocuous responses is attenuated by nerve injury.

Separate experiments examined the effect of peripheral saline injections on spinal neuronal responses to mechanical (Fig.4.2.2A&B, p122) and thermal stimulation (Fig. 4.2.3, p123) in naive rats. This was done to show that the inhibitory effects induced by peripheral injections of IL-6 are due to IL-6 itself and do not occur as a result of the presence of the vehicle (saline) or the actual injection itself.
Figure 4.2.2. Timecourse of the effect of IL-6/saline on mechanical responses of neuronal populations in naive rats. The two responses shown are A) von Frey 8.51g, n = 8-11. B) von Frey 28.84g, n = 10-15. The responses are expressed as a percentage of the control response to mechanical stimulation prior to injection. The plots show the mean ± SEM of the responses over the four hour timecourse.
Figure 4.2.3. Timecourse of the effect of IL-6/saline on neuronal responses to noxious heat of separate neuronal populations from naive rats, n = 3-9. Responses are expressed as a percentage of the control response to noxious heat stimulation prior to either an injection of saline or IL-6. The plots show the mean ± SEM of the responses for the timecourse.
Saline facilitated the neuronal response to innocuous mechanical stimulation especially following the first injection (Fig. 4.2.2A, p122). Although the facilitation of this response was not as marked over the latter states of the timecourse following the second injection it is still clearly different from the inhibitory effect of peripheral IL-6 injections. Saline also facilitated, to a lesser extent than in Fig. 4.2.2A, the neuronal responses to noxious mechanical stimulation (Fig. 4.2.2B, p122). Expressing the effects of saline in terms of a maximum effect and comparing between neuronal populations, as done in Fig. 4.2.1A-C, shows that the effects of saline on neuronal responses to mechanical stimulation is significantly different to the effects of IL-6 in naive rats (p < 0.05, unpaired t-test). Facilitatory effects of saline have also been reported in behavioural testing (Xu et al. 1997) and in previous electrophysiological studies (Carpenter et al. 2000). The effect of saline on the neuronal response to noxious heat was slight (Fig. 4.2.3, p123) and not significantly different from this response following peripheral IL-6. It was not possible to complete a full 4-hour time course on the effect of saline on the neuronal response to noxious heat.

In conclusion, the facilitatory effect of saline on mechanically evoked neuronal responses could potentially offset the inhibitory activity of IL-6, such that the degree of inhibition observed is an underestimate. The ability of IL-6 to inhibit mechanical-evoked neuronal responses was attenuated by nerve injury, yet heat responses were inhibited to similar degrees following nerve injury as was observed in naive rats.
4.3 Behavioural effects of peripheral IL-6

Intraplantar administration of IL-6 produced a significant increase in the withdrawal latencies of the ipsilateral paw (Fig. 4.3.1A, p 126). 0.01μg IL-6 caused a significant increase at 1 hour post administration (p < 0.01, ANOVA for repeated measures, Tukey’s HSD test) and at 3 hours post administration (p < 0.01). 0.1μg IL-6 and 1μg IL-6 also caused a significant increase in the ipsilateral withdrawal latency at 3 hours post administration (p < 0.05). Contralateral withdrawal latencies (Fig. 4.3.1B, p 126) were unaffected by IL-6, with the exception of the highest dose at three hours following administration (p < 0.01). The vehicle (saline) group showed no significant difference in the withdrawal latencies of either the ipsilateral or the contralateral paw over the time course of the experiment. This experiment clearly shows that the inhibitory effect of peripheral IL-6 on neuronal responses (Section 4.2.) had a clear behavioural counterpart in naive rats. The contralateral effect observed after three hours with the top dose of IL-6 (1μg) could suggest that perhaps IL-6 is producing at this late stage, via systemic actions, central changes. This is unlikely as spinal (100ng-500ng) IL-6 had little effect on the electrically evoked neuronal responses (Section 4.4). This contralateral effect was observed with a dose two-fold higher than that used in the electrophysiological study and the neuronal responses were evoked by a supra-threshold stimulus. Saline had no effect on the withdrawal latency of either paw for the time course of the experiment. Similarly, peripheral saline administration into the receptive fields of convergent dorsal horn neurones had no significant effect on neuronal responses to heat (Fig. 4.2.3, p 123). So to conclude peripheral IL-6 produced anti-nociceptive effects which, I expected in view of the effects of peripheral IL-6 on the neuronal responses to heat observed electrophysiologically. An earlier study reports that intraplantar IL-6 (0.001ng-1ng) induced mechanical hyperalgesia in normal rats (Cunha et al. 1992). However, this study used a 100μl intraplantar injection without a vehicle control group and lacks formal statistical tests. Also the mechanical hyperalgesia reported is present in both paws, 30 mins post administration whereas I only observe a contralateral effect after three hours at a dose one-thousand fold higher, than the top dose used in this study.
Figure 4.3.1. Effect of intraplantar IL-6 on paw withdrawal latencies to a focused heat stimulus for the A) ipsilateral paw and B) contralateral paw of naive rats. Each column shows the mean withdrawal latency ± SEM for each group of six rats. *p-value < 0.05, † p-value < 0.01 (ANOVA followed by Tukey’s HSD test) significant increase in latency compared to group’s predose reading.
### 4.4 Effects of spinal IL-6 on spinal neurones

All the SNL rats displayed behavioural mechanical and cold allodynia whereas the sham rats did not (see section 3.2). There was no significant difference (ANOVA) in the depth of neurones recorded (Table 4.4) or in the pre-drug control neuronal responses obtained prior to IL-6 administration (Table 4.4 - expressed as number of action potentials, APs).

#### Table 4.4- Mean depth and pre-drug control responses of neurones exposed to spinal IL-6

<table>
<thead>
<tr>
<th>Neuronal Characteristic</th>
<th>Neurones from Naive Rats ( n = 11 )</th>
<th>Neurones from Sham Rats ( n = 10 )</th>
<th>Neurones from SNL Rats ( n = 9 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Depth of Neurone (( \mu m ))</td>
<td>793 ± 45</td>
<td>724 ± 34</td>
<td>624 ± 30</td>
</tr>
<tr>
<td>C-Fibres (APs)</td>
<td>255 ± 30</td>
<td>268 ± 42</td>
<td>229 ± 26</td>
</tr>
<tr>
<td>A( \delta )-Fibres (APs)</td>
<td>38 ± 9</td>
<td>67 ± 17</td>
<td>60 ± 16</td>
</tr>
<tr>
<td>A( \beta )-Fibres (APs)</td>
<td>96 ± 13</td>
<td>84 ± 7</td>
<td>87 ± 13</td>
</tr>
<tr>
<td>Initial C-fibre (APs)</td>
<td>13 ± 2</td>
<td>19 ± 3</td>
<td>18 ± 3</td>
</tr>
<tr>
<td>Post discharge (APs)</td>
<td>125 ± 17</td>
<td>202 ± 48</td>
<td>142 ± 26</td>
</tr>
<tr>
<td>Wind-up (APs)</td>
<td>208 ± 32</td>
<td>201 ± 41</td>
<td>184 ± 32</td>
</tr>
<tr>
<td>Innocuous mechanical (von Frey 8.5g, APs)</td>
<td>-</td>
<td>231 ± 46</td>
<td>125 ± 41</td>
</tr>
<tr>
<td>Noxious mechanical (von Frey 28.84g, APs)</td>
<td>-</td>
<td>586 ± 96</td>
<td>357 ± 88</td>
</tr>
<tr>
<td>Noxious Heat (APs)</td>
<td>-</td>
<td>343 ± 93</td>
<td>228 ± 58</td>
</tr>
</tbody>
</table>

Figure 4.4.1A-C (p128) illustrates the effects of spinal IL-6 administration on the neuronal responses to C-fibre, A\( \delta \)-fibre and A\( \beta \)-fibre electrical stimulation. Spinal IL-6 did not significantly affect the evoked C-fibre response in naive and sham rats (Fig. 4.4.1A). However in SNL rats, spinal IL-6 induced a significant dose-related inhibition of the C-fibre response (250ng & 500ng \( p < 0.01 \), paired t-test) with a maximal 47% inhibition of the pre-drug control response after 500ng IL-6. There was no significant effect of spinal IL-6 on the evoked A\( \delta \)-fibre response (Fig. 4.4.1B) or the evoked A\( \beta \)-fibre response (Fig. 4.4.1C) at any dose, for either of the animal groups.
Figure 4.4.1. Effect of spinal IL-6 on the electrically-evoked A) C-fibre, B) Aδ-fibre and C) Aβ-fibre neuronal responses. Changes in the responses following IL-6 administration are expressed as a percentage of the control response. A) - C) show the mean ± SEM of the maximum effect which occurred for the neurone population over the 90-minute time course, n = 6 - 10. ‡ p < 0.01 (paired t-test) maximum effect is significantly different from the control response prior to spinal IL-6 administration for the animal group in question.
Fig. 4.4.2A-C (p130) illustrates the effects of spinal IL-6 administration on the electrically-evoked initial C-fibre, post discharge and wind-up neuronal responses. Spinal IL-6 significantly inhibited the initial C-fibre response, in sham and SNL rats, following 250ng and 500ng IL-6 (p < 0.05, paired t-test, Fig.4.4.2C). The initial C-fibre response in naive rats was inhibited to a similar extent (40% inhibition) following 500ng IL-6 as that seen in the sham and SNL groups but this effect was not significant (p = 0.175, paired t-test). Spinal IL-6 has a pronounced dose-related inhibitory effect on the post discharge response in SNL rats (p < 0.05, paired t-test) resulting in a 70% inhibition of the pre-drug control response after 500ng IL-6 (Fig. 4.4.2B). A similar effect is seen in sham rats although the IL-6-induced inhibition was only significant at 500ng IL-6 (p < 0.01, paired t-test). In contrast, spinal IL-6 did not produce any significant effects on the post discharge response in naive rats, however 100ng IL-6 tended to produce a facilitation of this response.

Effects of spinal IL-6 on wind-up are expressed in terms of maximal effects (Fig.4.4.2C) and comparing the wind-up graphs of neurones from a naive, sham and SNL rat before and after IL-6 administration (Fig. 4.4.3 p131). Spinal IL-6 had no effect on wind-up responses in naive rats. Spinal IL-6 inhibited wind-up responses in SNL and sham rats with a maximal effect of 83% inhibition and 67% inhibition, respectively, following 500ng IL-6 (Fig. 4.4.3A). The inhibitory effects induced by 500ng IL-6 were significantly different in SNL rats compared to sham rats (p < 0.05 unpaired t-test).
Figure 4.4.2. Effect of spinal IL-6 on the electrically-evoked A) Initial C-fibre, B) post discharge and C) wind-up neuronal responses. Changes in the responses following IL-6 administration are expressed as a percentage of the control response. A) - C) show the mean ± SEM of the maximum effect which occurred for the neurone population over the 90-minute time-course, n = 6 -10. * p < 0.05 ‡ p < 0.01 (paired t-test) maximum effect is significantly different from the control response prior to spinal IL-6 administration for the animal group in question.
Figure 4.4.3. Examples of the effect of exogenous spinal IL-6 on the wind-up response of a neurone in a A) Naive, B) Sham and C) SNL rat.
The initial C-fibre response is the number of action potentials evoked in response to the first electrical impulse prior to the generation of wind-up and thus is indicative of pre-synaptic mechanisms whereas post discharge and wind-up are predominantly post-synaptic events leading to neuronal hyperexcitability (Chapman and Dickenson 1992). Therefore the inhibition of responses in SNL rats suggests that spinal IL-6 acts both pre- and post-synaptically following nerve injury and perhaps also, to lesser extent following tissue injury, considering the effects in sham-operated rats. It has been suggested that IL-6, in addition to the regulation of transcription, has a more direct role in synaptic transmission in the brain (Li et al. 1997; D'Arcangelo et al. 2000). More specifically, it has been reported that IL-6 inhibits the spread of excitation and evoked glutamate release in the cerebral cortex via pre- and post-synaptic mechanisms (D'Arcangelo et al. 2000).

In SNL rats, neuronal responses to 8.51g and 28.84g mechanical stimulation (Fig.4.4.4A&B, p133) were markedly inhibited by spinal IL-6 (p < 0.05) with a maximal 81% and 67% inhibition at 500ng IL-6, respectively. In sham rats, 100ng IL-6 elicited a significant 53% facilitation of the neuronal response to von Frey 8.51g in sham rats (p < 0.05). No significant effect was seen on neuronal responses to innocuous (8.51g) and noxious (28.84g) von Frey stimulation at higher doses of spinal IL-6, yet 500ng IL-6 tended to cause an inhibition of these responses. Statistical comparisons across the animal groups show that 100ng spinal IL-6 produced significantly different responses to innocuous mechanical stimulation in sham rats compared to SNL rats. There was also a significant difference in the neuronal response to noxious mechanical stimulation in sham rats compared to the same response in SNL rats following 250ng spinal IL-6. The effect of spinal IL-6 on neuronal responses to noxious heat in SNL and sham rats was also examined (Fig.4.4.4C, p133). There was no significant or consistent effect of spinal IL-6 on the neuronal responses to noxious heat for either SNL or sham rats at any dose.
Figure 4.4.4. Effect of spinal IL-6 on neuronal responses to A) 8.51g von Frey mechanical, B) 28.84g von Frey mechanical and C) noxious heat stimulation. Changes in the responses following IL-6 administration are expressed as a percentage of the control response. A) - C) show the mean ± SEM of the maximum effect which occurred for the neurone population over the 90-minute time-course, n = 6 - 9. * p < 0.05 ‡ p < 0.01 (paired t-test) maximum effect is significantly different from the control response prior to spinal IL-6 administration for the animal group in question.
The contrasting effects of spinal IL-6 in sham and SNL rats were more pronounced on mechanically evoked neuronal responses than the electrically-evoked responses. The profound inhibition of the neuronal responses to 8.51g von Frey stimulation seen here in SNL rats could suggest that intrathecal IL-6 may produce anti-allodynic effects. Behavioural studies have reported that a low dose of anti-IL-6 antibody attenuated 2g von Frey measurement of mechanical allodynia but higher doses of this antibody had no effect (Arruda et al. 2000). However in the same study, assessment of allodynia using a 12g von Frey filament was unaltered by intrathecal anti-IL-6 antibody. Interestingly, more IL-6 positive cells are found in the sciatic nerve of alldynic rats than in non-alldynic rats following peripheral nerve injury (Cui et al. 2000) suggesting some putative role of IL-6 in alldynia. We found spinal IL-6 to have no effect on the neuronal (recording from ipsilateral neurones) responses to heat in SNL rats. A similar lack of effect has been shown on the ipsilateral thermal withdrawal latencies in nerve-injured rats following intrathecal IL-6, although these authors report a decrease in contralateral withdrawal latencies in the same rats (DeLeo et al. 1996).

The data presented here shows spinal IL-6 to have no effect on dorsal horn neuronal responses in naive rats but a marked dose-related inhibitory effect in SNL rats on the majority of the neuronal responses examined. Therefore, what changes might have occurred after nerve injury to produce in this inhibitory effect? It is known that both IL-6R mRNA and gp130 mRNA are markedly upregulated in the sciatic nerve following nerve injury and this upregulation persists for 3-4 weeks after the injury (Ito et al. 1998; Grothe et al. 2000). Therefore, perhaps there is also an increase in IL-6R and gp130 expression at the spinal level following peripheral nerve injury but there are as yet no studies on this. If this is the case then the exogenous spinal IL-6 administered in SNL rats has access to a larger receptor/gp130 population, which results in the observed inhibitory effects. By extension, under normal conditions perhaps the levels of IL-6 receptor and gp130 in the spinal cord are too low for exogenous administered IL-6 to induce a significant effect on the neuronal responses.
4.5 Behavioural effects of spinal IL-6

Spinal administration of IL-6 (100ng-500ng) did not affect either left or right hindpaw withdrawal latencies to a focused heat stimulus (Fig.4.5.1A&B, p136). Statistical analysis (ANOVA for repeated measures, Tukey’s HSD) showed there was no significant difference in the withdrawal latencies at 1 hour or 3 hours post IL-6 administration compared to predose readings. DeLeo et al. (1996)(DeLeo et al. 1996) also found that an intrathecal administration of 100ng IL-6 had no effect on the withdrawal latencies of normal rats to a thermal stimulus. This shows that spinal IL-6 does not produce behavioural anti-nociceptive effects in naive rats unlike the anti-nociceptive effects observed following peripheral IL-6 administration (see section 4.3). Therefore, spinal IL-6 did not affect either behavioural or electrophysiological measures of nociception in naive rats.
Figure 4.5.1. Effect of spinal IL-6 on paw withdrawal latencies to a focused heat stimulus for the A) right paw and B) left paw of naive rats. Each column shows the mean withdrawal latency ± SEM for each group of six rats. No significant changes in withdrawal latencies compared to predose readings in either paw.
4.6 Summary

This chapter presents a profile of the functional effects of spinal and peripheral interleukin-6 (IL-6) on nociceptive transmission, in naive and neuropathic states using different electrophysiological techniques. In addition, I have shown that the effects of IL-6 seen electrophysiologically can be mirrored with similar behavioural IL-6 effects.

The results that can be achieved from behavioural experiments compared to in vivo electrophysiological experiments are different but are equally as useful in the search for new drug targets. From in vivo electrophysiology it can potentially be established how a drug elicits its effects e.g. does it inhibit C-fibre function as opposed to A-fibres? Does it facilitate neuronal hyperexcitability? Whereas behavioural experiments will only show whether a drug produces analgesia and does not give necessarily indicate how this effect is induced. However, it is not possible to show from in vivo electrophysiology whether a drug will have sedative effects for example, as the animals are anaesthetised. Thus using a combination of these techniques together it is possible to establish quite a comprehensive profile of a drug’s potential analgesia properties.

An interesting aspect of these experiments was how the different routes of IL-6 administration produced different resultant effects on neuronal responses. This was particularly obvious in SNL rats on their neuronal responses to mechanical stimulation, as spinal IL-6 markedly inhibited these responses whereas peripheral IL-6 did not. Similarly, in naive rats, spinal IL-6 had little effect on neuronal responses and withdrawal latencies, but a marked inhibitory effect when administered peripherally. Possible explanations for these effects could include levels of receptor expression and access of exogenous IL-6 to its receptors. For instance in SNL rats, more IL-6 receptors are expressed in the spinal cord compared to the periphery, so spinal IL-6 elicits greater effects than peripheral IL-6. Alternatively, perhaps in naive rats exogenous IL-6 has easier access to its receptors in the periphery than in the spinal cord.

In summary, I have find that spinal administration of interleukin-6 had no effect in vivo on electrically evoked responses in the anaesthetised naive rat or on withdrawal latencies in
the conscious naive rat. In contrast, peripheral administration of IL-6 produced a significant inhibition of spinal neuronal responses \textit{in vivo}, heat responses of nociceptive fibres \textit{in vitro}, as well as a behavioural anti-nociceptive effect in naive rats.

In sham rats, higher doses of spinal IL-6 inhibited neuronal hyperexcitability and the initial C-fibre response but did not affect A\(\beta\)-fibre, A\(\delta\)-fibre, C-fibre responses or naturally evoked neuronal responses. Peripheral IL-6 administration in sham rats resulted in the inhibition of the naturally evoked, mechanical and thermal neuronal responses.

Spinal IL-6 administration induced a marked inhibition of evoked C-fibre responses, neuronal hyperexcitability and mechanical neuronal responses in neuropathic rats. Peripheral IL-6 administration in neuropathic rats induced contrasting effects, with the inhibition of spinal neuronal responses to noxious heat but not mechanical neuronal responses.

In conclusion, these studies suggest that under normal conditions increased levels of IL-6 in the periphery will result in an attenuation of nociception. In contrast, these results suggest that following nerve injury increased spinal levels of IL-6 will inhibit nociceptive transmission. The lack of effect of spinal IL-6 in naive rats compared to the pronounced effects of spinal IL-6 following nerve injury suggest that spinally administered IL-6 could prove an effective treatment for neuropathic pain. Especially as spinal IL-6 has inhibitory effects on C-fibres but does not affect A\(\beta\)-fibres and also inhibits neuronal hyperexcitability which is thought to be one of the main problems in chronic pain states. Whether these effects seen electrophysiologically will translate to pronounced behavioural anti-nociceptive effects in neuropathic rats, as I have observed the experiments on naive rats, remains to be seen.
Chapter 5

Effects of Galanin on Sensory Processing in
Naive and Neuropathic Rats
5. Introduction

Galanin is a 29-amino acid neuropeptide widely distributed in the central and peripheral nervous system (Ch'ng et al. 1985; Merchenthaler et al. 1993). The concept that galanin had a role in nociception was initially based on the finding that, following peripheral nerve injury there is a 120-fold increase in galanin levels in the rat DRG (Villar et al. 1989) and; this role has been recently reviewed by (Xu et al. 2000b). The upregulation of galanin following nerve injury has been established in several models of nerve injury, such as nerve crush (Villar et al. 1989), chronic constriction injury (CCI)(Nahin et al. 1994; Ma and Bisby 1997), partial nerve transection (Ma and Bisby 1997), and nerve ligation (Carlton and Coggeshall 1996).

Three galanin receptor subtypes have been identified and termed GalR1, GalR2 and GalR3. In isolated cell lines, GalR1 and GalR3 have been found to be inhibitory (Smith et al. 1998; Wang et al. 1998) and GalR2 excitatory (Wang et al. 1998). GalR1 and GalR2 mRNA has been found in the rat spinal cord and DRG (Sten Shi et al. 1997; O'Donnell et al. 1999). Following axotomy GalR1 and GalR2 mRNA levels are decreased in the DRG (Xu et al. 1996; Sten Shi et al. 1997). GalR3 mRNA is expressed at lower levels in the spinal cord and DRG (Waters and Krause 2000) and the effect of nerve injury on GalR3 expression is unknown at present.

The aim of the following studies is to examine the effects of exogenous galanin administered spinally and peripherally on sensory processing and whether these galanin-induced effects alter following nerve injury. The effects of peripheral galanin are investigated both in vitro and in vivo using electrophysiological techniques. Specifically, the effect of galanin on the responses of single C-fibres to noxious heat and the effect of peripherally administered galanin on the mechanical and thermal responses of convergent dorsal horn neurones in naive, sham and neuropathic rats are demonstrated. The effects of spinal galanin administration are also examined in vivo using electrophysiological and behavioural techniques. The effects of exogenous spinal galanin on the responses of convergent dorsal horn neurones to electrical, mechanical and thermal stimulation in anaesthetised normal, sham and SNL rats are shown. In behavioural tests, the actions of
spinal galanin on the mechanical thresholds and thermal withdrawal latencies of neuropathic rats are also examined.

5.1. Effect of galanin on nociceptive fibres

Responses were obtained from 36 C-fibres with a mean conduction velocity of 0.74 ± 0.06 m/s and a mean mechanical threshold of 1.96 ± 0.15 g (see Table 5.1). 1μM galanin inhibited responses to noxious heat in 13 of the 20 (65%) fibres responses to noxious heat whilst heat responses in the remaining 7 fibres (35%) showed a slight facilitation. Figure 5.1.1 (p142) shows sample traces of fibre responses to noxious heat and clearly illustrates the distinct inhibitory (Fig 5.1.1A) and facilitatory (Fig 5.1.1B) effects of galanin on single nociceptive fibres. The fibres shown in Fig. 5.1.1 fire in response to noxious heat at different temperatures on the heat ramp. There was no trend in the fibres examined, to suggest that galanin produced either magnitudes or directions of effects that were dependent on the type of heat response of fibre. In other words, fibres that fired in response to warm temperatures (e.g. 38°C-43°C) were inhibited or facilitated following galanin application in similar proportions to fibres that only fired in response to noxious heat (e.g. 44°C-49°C). As a result of this, the galanin treated group was split into two populations, those that were inhibited and those that facilitated. There was no significant difference between the first heat response, mean conduction velocity and mean mechanical threshold for the three fibre populations (ANOVA, see Table 5.1).

Table 5.1- Characteristics of the control responses of the nociceptive fibres in the various experimental groups of naive rats used in the skin-nerve studies.

<table>
<thead>
<tr>
<th>Fibre population</th>
<th>Heat Alone</th>
<th>Galanin Inhibitions</th>
<th>Galanin Facilitations</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of action potentials</td>
<td>78 ±12</td>
<td>81 ± 12</td>
<td>79 ± 25</td>
</tr>
<tr>
<td>Conduction Velocity (m/s)</td>
<td>0.73 ± 0.04</td>
<td>0.6 ± 0.03</td>
<td>1.02 ± 0.28</td>
</tr>
<tr>
<td>Mechanical Threshold (g)</td>
<td>2.18 ± 0.28</td>
<td>1.74 ± 0.17</td>
<td>1.88 ± 0.24</td>
</tr>
</tbody>
</table>

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Figure 5.1.1 Single nociceptive fibres firing in response to noxious heat before galanin (control), in the presence of galanin (1 μM galanin) and after 10 minutes wash-out (post galanin). A) An example of galanin-induced facilitation of firing induced by noxious heat. B) An example of galanin-induced inhibition of firing induced by noxious heat. The heat ramps for this experiment were the same as shown in A).
Fig. 5.1.2 (p144) shows the effect of galanin on single nociceptive fibres in terms of those that were facilitated and those that were inhibited. The galanin-induced inhibition (26% ± 8% of pre-drug control values) was pronounced and highly significant compared to pre-drug control responses (p < 0.001, paired t-test) and this inhibition persisted in these fibres following washout (p < 0.001). The galanin-induced facilitation was more variable with an increase to 175% ± 41% of pre-drug control values. Repetitive heat caused a small but significant decrease in the magnitude of response of single C-fibres, the third heat response being significantly smaller than the first heat response (Fig. 5.1.2, p < 0.01). However, there is no significant difference between the number of action potentials elicited in the second heat response compared to those in the first heat response. This allows the effects of galanin to be argued without the problem of the fibres displaying desensitisation or ‘fatigue’ (Raja et al. 1997) as a result of repetitive exposure to noxious heat. Thus, the presence of galanin produced a significant decrease in response to noxious heat compared to the response to noxious heat in the absence of galanin (p < 0.01, unpaired t-test). The galanin-induced facilitation of the heat response was not significantly different to the heat response with physiological salt solution.
Figure 5.1.2. Effect of 1μM galanin on single nociceptive fibres, n = 7-16, recorded from skins of naive rats. Plots show the mean ± SEM of the number of action potentials elicited during the heat ramp for each fibre population. * p-value < 0.05 comparing response to control responses (paired t-test). ‡ p < 0.01 significant difference between the inhibitions following galanin compared to the same response in the absence of galanin (unpaired t-test).
Similar findings have been reported for afferent fibres supplying the knee joint (Heppelmann et al. 2000). Responses of 20 articular afferents to noxious movements in naive knee joints were examined before and after an injection of galanin with three different outcomes. 40% of the afferents were inhibited following galanin, 15% were facilitated and the remaining 45% of afferents examined were unaffected by galanin. This is broadly similar to our findings here, where 65% of the nociceptive fibres examined were inhibited and the remaining 35% facilitated (to varying extents) following galanin application. A difference is that I did not observe a population of fibres that were unaffected by following galanin administration in the fibres I examined, which may have occurred by chance or potentially may reflect differences between joint and cutaneous afferents in their expression of galanin receptors. Galanin-like immunoreactivity has been found in uninjured skin of rats (Ji et al. 1995) and humans (Johansson et al. 1988). In addition, galanin binding sites have been identified in rat skin (Ji et al. 1995) although the specific galanin receptor subtype of these binding sites has not been established. Therefore, the inhibition of the heat response in one population of nociceptive fibres may well be a result of a predominant GalR1/GalR3 receptor location and subsequent activation. By extension, the facilitation of this same response in another nociceptive fibre population may result from predominant GalR2 receptor activation.
5.2. Effects of peripheral galanin on spinal neurones

There was no significant difference between the mean depths of the neuronal populations from naive, sham and SNL or in their responses to the mechanical and thermal stimuli prior to galanin administration (Table 5.2 - expressed as number of action potentials, APs, evoked). All the SNL rats displayed behavioural mechanical and cold allodynia whereas the sham rats did not (see section 3.2).

Table 5.2 - Mean depth and pre-drug control responses of neurones exposed to peripheral galanin

<table>
<thead>
<tr>
<th>Neuronal Characteristic</th>
<th>Neurones from Naive Rats n = 11</th>
<th>Neurones from Sham Rats n = 10</th>
<th>Neurones from SNL Rats n = 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Depth of neurone (µm)</td>
<td>661 ± 31</td>
<td>722 ± 46</td>
<td>699 ± 32</td>
</tr>
<tr>
<td>Innocuous mechanical, von Frey 8.51g (APs)</td>
<td>140 ± 27</td>
<td>129 ± 34</td>
<td>119 ± 24</td>
</tr>
<tr>
<td>Noxious mechanical, von Frey 28.84g (APs)</td>
<td>498 ± 57</td>
<td>581 ± 101</td>
<td>454 ± 112</td>
</tr>
<tr>
<td>Noxious Heat (APs)</td>
<td>437 ± 63</td>
<td>399 ± 75</td>
<td>356 ± 77</td>
</tr>
</tbody>
</table>

Galanin elicited a facilitation of the neuronal responses in some experiments, but an inhibition of the neuronal responses in others. This mirrors the effects seen in the single peripheral C-fibres described in the previous section. We did not observe any bi-phasic effects of galanin on the neuronal responses for any of the neurones tested in any of the animal groups unlike previously reported studies (Wiesenfeld-Hallin et al. 1989a; Wiesenfeld-Hallin et al. 1989b). Thus if a neuronal response to heat was inhibited following 0.1µg galanin, the response was also inhibited after 1µg, 10µg and 100µg galanin for the neurone in question. This is illustrated in Fig. 5.2.1 (p147), where the maximal effect of each dose of galanin is shown for each neurone examined. This shows that the inhibition or facilitation produced following peripheral galanin is distinct.
Figure 5.2.1. Responses of individual neurones to noxious heat following peripheral galanin administration in A) Naive, B) Sham and C) SNL rats, illustrating the distinct inhibitory and facilitatory neuronal populations.
This resulted in the classification of the effects of galanin on the neurones as either facilitatory or inhibitory terms of the effect on responses to innocuous mechanical, noxious mechanical and noxious heat stimulation. The proportions of neurones inhibited by galanin and facilitated in naive, sham and SNL rats are illustrated in Figure 5.2.2 (p149). In naive animals, approximately equal numbers of neurones show inhibitory and facilitatory responses to galanin, with no differences between the three stimuli. However, there is a clear increase in the proportion of neurones inhibited in SNL rats. Sham rats also show a slight increase in the number of neurones inhibited by galanin. For example, in naive rats 5 out of the 11 neurones (45%) were inhibited in their response to noxious mechanical, von Frey 28.84g, stimulation following exogenous galanin. In contrast in SNL rats, 8 out of the 10 neurones (80%) were inhibited in their response to von Frey 28.84g stimulation. In experiments involving peripheral injections of saline, the neuronal responses to innocuous mechanical, noxious mechanical and noxious heat stimuli were, without exception, facilitated (to varying extents) following saline administration. The effect of peripheral saline injections on the neuronal responses of spinal neurones has been previously discussed in Chapter 4, section 4.2, Figures 4.2.2 & 4.2.3 (pp122 &123).
Figure 5.2.2. Neuronal populations from naive, sham and SNL rats whose responses to von Frey 8.51g, von Frey 28.84g and noxious heat stimulation were inhibited or facilitated following peripheral galanin application. A higher proportion of the neuronal population was inhibited after galanin in SNL rats compared to a similar neuronal population in naive rats.
I will now discuss the degree of inhibition produced following peripheral galanin administration in the inhibitory neuronal sub-populations for each animal group. In inhibitory neuronal populations, the neuronal responses to von Frey 8.51g stimulation (Fig. 5.2.3A, p151) were significantly inhibited by galanin compared to pre-drug control responses ($p < 0.05$, paired t-test). The exception was in SNL rats following 0.1µg galanin. Overall these neuronal responses were inhibited 50-70% and no clear dose-dependent effects were seen. In Fig. 5.2.3B (p151), the neuronal responses of the inhibitory neuronal populations to von Frey 28.84g stimulation are illustrated. Each dose of galanin, with the exception of 0.1µg in sham rats, produced a statistically significant inhibition ($p < 0.05$, paired t-test) of this response in naive, sham and SNL rats compared to pre-drug control responses. A slightly lesser degree of inhibition was elicited on this response compared to fig. 5.2.3A and was on average 49%. The neuronal responses of the inhibitory neuronal populations to noxious heat (Fig. 5.2.3C, p151) are without exception significantly inhibited following galanin administration at each dose for naive, sham and SNL rats compared to pre-drug controls ($p < 0.05$, paired t-test). These neuronal responses were inhibited on average 68% and again there was no obvious dose-response relationship.
Figure 5.2.3. Degree of inhibition of the response to A) von Frey 8.51g, B) von Frey 28.84g and C) heat stimulation elicited by each dose of galanin for the inhibitory neuronal populations from naive, sham and SNL rats. A) - C) show the mean ± SEM of the maximum effect which occurred for the neurone population over the 60-minute time-course, n = 4 - 9. All neuronal responses following each dose of galanin were significantly inhibited compared to pre-drug control responses (p < 0.05 paired t-test) with two exceptions, 0.1 µg galanin on neuronal responses to von Frey 8.51g stimulation in SNL rats and 0.1 µg galanin on neuronal responses to von Frey 28.84g stimulation in sham rats.
The degree of facilitation in the facilitatory neuronal populations of the naive, sham and SNL rats varied considerably in their magnitude (Fig. 5.2.4, p 153) and overall fewer neurones were facilitated following peripheral galanin administration. Peripheral galanin induced 120-140% facilitation of the pre-drug control neuronal responses to von Frey 8.51g stimulation which was statistically significant for each dose of galanin (p < 0.05 paired t-test). Neuronal responses to innocuous mechanical stimulation were also markedly facilitated in sham and SNL rats but this was only observed in 1 neurone from SNL rats and 2 neurones from sham rats making statistical tests largely impractical. The 33-67% facilitation of facilitatory neuronal responses to von Frey 28.84g stimulation following peripheral galanin in naive rats was not as marked as seen with neuronal responses to von Frey 8.51g but still significant at each dose (p < 0.05, paired t-test). 10μg and 100μg peripheral galanin significantly facilitated neuronal responses to von Frey 28.84g in the two facilitatory neurones from SNL rats (p < 0.05, paired t-test). The facilitations (on average 30%) of this response in sham rats were smaller in magnitude and not significant. The facilitation of neuronal responses to noxious heat in this neuronal population from naive rats was significant at each dose (p < 0.05, paired t-test) of peripherally administered galanin with a maximal 160% facilitation at 10μg galanin. Similarly, in sham rats the corresponding neuronal population was significantly facilitated following each dose of peripheral galanin (p < 0.05, paired t-test) with a maximal 117% facilitation. One neurone in SNL rats was facilitated by 189% following peripheral galanin administration.
Figure 5.2.4. Degree of facilitation of the response to A) von Frey 8.51g, B) von Frey 28.84g and C) heat stimulation elicited by each dose of galanin for the facilitatory neuronal populations from naive, sham and SNL rats. A) - C) show the mean ± SEM of the maximum effect which occurred for the neurone population over the 60-minute time-course, n =1 - 6. * p < 0.05 (paired t-test) neuronal responses were significantly facilitated compared to control responses prior to peripheral galanin administration.
Following nerve injury, there was an increase in the number of neurones showing inhibitory responses to peripheral galanin. This shift towards inhibitory galanin-induced effects was evident for the neuronal responses to innocuous mechanical, noxious mechanical and noxious heat. These results could suggest changes in the galanin receptor population in the periphery following nerve injury. Immunohistochemical studies on galanin receptor subtype density in the periphery and possible changes following nerve injury have not been examined. However, it has been found that following axotomy GalR1 and GalR2 mRNA levels are decreased in the DRG (Xu et al. 1996; Sten Shi et al. 1997). Thus, a possible explanation for the increase in inhibitory effects of galanin in SNL rats seen here is that GalR3, present in low levels in the DRG (Waters and Krause 2000), is upregulated after nerve ligation. Then transported to the periphery during the two-week period prior to the electrophysiological study. This would result in an increase in inhibitory galanin receptors in the periphery. A recent study used selective agonists to elucidate which galanin receptor subtype is responsible for these observed changes in neuropathic states (Liu et al. 2001). The authors compared the effects of a high affinity GalR1/GalR2 agonist (AR-M961) and a selective GalR2 agonist (AR-M1896) in allodynic rats following CCI and found that AR-M961 dose-dependently increased the mechanical threshold in these rats whereas AR-M1896 had no effect (Liu et al. 2001). This suggests similar conclusions to the data presented here, that an inhibitory galanin receptor, albeit GalR1 or GalR3, is responsible for the galanin induced-inhibition seen following nerve injury.
5.3. Effects of spinal galanin on dorsal horn neurones

All the SNL rats displayed behavioural mechanical and cold allodynia whereas the sham rats did not (see section 3.2). There was no significant difference (ANOVA) in the depth of neurones recorded (Table 5.3) or in the pre-drug control neuronal responses obtained prior to galanin administration (Table 5.3 - expressed as number of action potentials, APs). Since the baseline neuronal responses for each stimulus were similar for the three animal groups, a valid comparison of the effects of a particular dose of galanin on the neuronal responses between rat groups can be made.

Table 5.3 - Mean depth and pre-drug control responses of neurones exposed to spinal galanin

<table>
<thead>
<tr>
<th>Neuronal Characteristic</th>
<th>Neurones from Naive Rats n = 9</th>
<th>Neurones from Sham Rats n = 9</th>
<th>Neurones from SNL Rats n = 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Depth of neurone (µm)</td>
<td>735 ± 40</td>
<td>693 ± 42</td>
<td>683 ± 34</td>
</tr>
<tr>
<td>C-Fibres (APs)</td>
<td>281 ± 46</td>
<td>314 ± 41</td>
<td>251 ± 25</td>
</tr>
<tr>
<td>Aδ-Fibres (APs)</td>
<td>76 ± 20</td>
<td>60 ± 9</td>
<td>56 ± 6</td>
</tr>
<tr>
<td>Aβ-Fibres (APs)</td>
<td>93 ± 19</td>
<td>99 ± 8</td>
<td>111 ± 4</td>
</tr>
<tr>
<td>Initial C-fibre (APs)</td>
<td>19 ± 3</td>
<td>18 ± 4</td>
<td>16 ± 2</td>
</tr>
<tr>
<td>Post discharge (APs)</td>
<td>97 ± 24</td>
<td>199 ± 52</td>
<td>183 ± 46</td>
</tr>
<tr>
<td>Wind-up (APs)</td>
<td>133 ± 26</td>
<td>292 ± 84</td>
<td>249 ± 78</td>
</tr>
<tr>
<td>Innocuous mechanical (von Frey 8.51g, APs)</td>
<td>130 ± 20</td>
<td>204 ± 43</td>
<td>150 ± 96</td>
</tr>
<tr>
<td>Noxious mechanical (von Frey 28.84g, APs)</td>
<td>659 ± 108</td>
<td>498 ± 116</td>
<td>306 ± 94</td>
</tr>
<tr>
<td>Noxious Heat (APs)</td>
<td>451 ± 121</td>
<td>415 ± 68</td>
<td>465 ± 140</td>
</tr>
</tbody>
</table>

Figure 5.3.1.A-C (p157) illustrates the effects of spinal galanin administration on the neuronal responses to C-fibre, Aδ-fibre and Aβ-fibre. In naive rats, the electrically evoked C-fibre response was slightly facilitated whilst in sham-operated rats this response was significantly inhibited by 50µg galanin. However, in SNL rats the C-fibre response was significantly inhibited by all three doses of galanin compared to control readings (p < 0.05, p < 0.01 paired t-test) with a maximal 47% inhibition evoked by 50µg galanin. Statistical comparisons between groups showed significant differences in the neuronal responses of
SNL rats and naive rats at all doses (p < 0.05, unpaired t-test), and between SNL and sham rats at 0.5μg and 50μg doses (p < 0.05, unpaired t-test). The effects of the peptide on electrically-evoked Aδ-fibre neuronal response for naive, sham and SNL rats followed a similar pattern to the C-fibre evoked responses (Fig. 5.3.1B, p157). In SNL rats, the Aδ-fibre response was significantly inhibited at all three doses of galanin (p < 0.05, p < 0.01 paired t-test). In naive rats, the Aδ-fibre response was slightly facilitated by the 0.5μg dose and in sham rats was inhibited by the 50μg dose, although these effects were not significant. The electrically evoked Aβ-fibre neuronal response (fig. 5.3.1C, p157) was largely unaltered following exogenous spinal galanin administration in naive, sham and SNL rats. Between group comparisons also showed no significant difference in the effect of galanin on the evoked Aβ-fibre response for the three rat groups.
Figure 5.3.1. Effect of spinal galanin on electrically-evoked A) C-fibre, B) Aδ-fibre and C) Aβ-fibre neuronal responses. Changes in the responses following galanin administration are expressed as a percentage of the control response. A) - C) show the mean ± SEM of the maximum effect which occurred for the neurone population over the 90-minute time-course, n = 7 - 10. * p < 0.05 ‡ p < 0.01 (paired t-test) maximum effect is significantly different from the control response prior to spinal galanin administration for the animal group in question.
The initial C-fibre neuronal response was facilitated by galanin in naive rats (p < 0.05, 0.5μg galanin, paired t-test) although this was not dose-related (fig. 5.3.2A, p159) and again 50μg galanin significantly inhibited this response in sham animals (p < 0.05, paired t-test). In SNL rats there was a pronounced dose-related inhibition of the initial C-fibre response by galanin with a maximal 66% inhibition following 50μg galanin (p < 0.01, 5μg and 50μg galanin, paired t-test). Unpaired t-tests between animal groups showed significant differences in the effects of galanin on the initial C-fibre responses of SNL rats (p < 0.01) and naive rats at all galanin doses, also between the responses of SNL and sham rats following 50μg galanin (p < 0.05).

Post-discharge, a measure of neuronal hyperexcitability was studied in all three groups. In naive animals, there was a tendency towards facilitation following galanin administration although this was not significant. In contrast, approximately 50% and 80% inhibition (p < 0.05, p < 0.01 paired t-test) of the post discharge response was seen in both sham and SNL rats following 5μg and 50μg doses of galanin, respectively. There was a significant difference, p < 0.05, in post discharge responses between naive and SNL rats at 50μg galanin. Fig. 5.3.2B (p159) illustrates the effect of exogenous galanin on the electrically evoked post discharge response.

Fig.5.3.2C (p159) shows the maximal effects of exogenous galanin on wind-up responses. Each dose of galanin produced a similar degree of inhibition, 70-80% in SNL rats. Following higher doses of exogenous galanin, the wind-up response in sham rats was also inhibited to a similar extent as seen in the SNL rats. In naive rats, no significant effects were observed on wind-up responses following galanin administration. Fig. 5.3.3 (p160) illustrates the effects of exogenous galanin (50μg) on wind-up, with a comparison of the wind-up graphs of neurones from a naive, sham and SNL rat before and after galanin administration. Therefore, following nerve injury and to a lesser extent tissue injury, spinal galanin markedly inhibits measures of neuronal hyperexcitability, post discharge and wind-up.
Figure 5.3.2. Effect of spinal galanin on the electrically-evoked A) Initial C-fibre, B) post discharge and C) wind-up neuronal responses. Changes in the responses following galanin administration are expressed as a percentage of the control response. A) - C) show the mean ± SEM of the maximum effect which occurred for the neurone population over the 90-minute time-course, n = 6 - 10. * p < 0.05 ‡ p < 0.01 (paired t-test) maximum effect is significantly different from the control response prior to spinal galanin administration for the animal group in question.
Figure 5.3.3. Examples of the effect of exogenous spinal galanin on the wind-up response of a neurone in A) Naive, B) Sham and C) SNL rat.
Fig. 5.3.4A-C (p162) depicts the effects of galanin on the neuronal responses to natural stimulation. Spinal galanin resulted in a two-fold facilitation of the neuronal response to von Frey 8.51g stimulation in naive rats (p< 0.01, paired t-test, Fig. 5.3.4A). In marked contrast, this response was significantly inhibited following 5µg and 50µg galanin doses in sham rats (60% inhibition, p< 0.05 and 76% inhibition, p< 0.01 respectively, paired t-test) and to a slightly greater extent (73% and 91% inhibition respectively, p< 0.01) in SNL rats. Comparing across the groups, the effect of galanin on the response to 8.51g von Frey stimulation was significantly different (p< 0.01, unpaired t-test) in SNL rats compared to naive rats at all three doses. Galanin, 0.5µg and 50µg, had a significantly greater effect on this response in SNL rats compared to sham rats (p< 0.05, unpaired t-test). Finally, there was a significant difference (p< 0.01, unpaired t-test) in the effect of 5µg and 50µg doses of galanin between sham and naive rats. This suggests tissue injury and to a greater extent nerve injury, causes changes that switch the effect of spinal galanin on innocuous mechanical neuronal responses from facilitation to inhibition.

A similar profile was seen for the effect of galanin on the noxious mechanical, von Frey 28.84g, stimulus (fig. 5.3.4B). In naive rats, galanin tended to produce facilitation but these effects were not significant. In sham rats, the noxious mechanical response was inhibited by 64% following 50µg galanin (p < 0.05, paired t-test), whilst in SNL rats 65% and 80% inhibition was observed following 5µg and 50µg galanin, respectively (p < 0.01, paired t-test). Comparing the three animal groups, there is a significant difference in drug effects between SNL rats and naive rats for all three doses (p < 0.01, unpaired t-test). This is also evident in sham rats compared to naive rats (p < 0.05, unpaired t-test). In addition, the effects of galanin on noxious mechanical simulation in SNL rats are significantly different to these responses in sham rats at 0.5µg galanin (p < 0.05, unpaired t-test).

Galanin had no significant effect on the neuronal responses to noxious heat in naive and sham rats (fig. 5.3.4C). In contrast, the neuronal responses to noxious heat in SNL rats were significantly inhibited by 80% at all three galanin doses (p < 0.05, paired t-test); these effects are also significantly different to those in naive rats to noxious heat following galanin administration (p < 0.05, unpaired t-test).
Figure 5.3.4. Effect of spinal galanin on neuronal responses to A) 8.51g von Frey mechanical, B) 28.84g von Frey mechanical and C) noxious heat stimulation. Changes in the responses following galanin administration are expressed as a percentage of the control response. A) - C) show the mean ± SEM of the maximum effect which occurred for the neurone population over the 90-minute time-course, n = 6 - 9. * p < 0.05 ¥ p < 0.01 (paired t-test) maximum effect is significantly different from the control response prior to spinal galanin administration for the animal group in question.
These experiments show that galanin has markedly contrasting effects on spinal nociceptive neurones in naive and neuropathic rats. In naive animals, exogenous galanin produced mild facilitatory effects on dorsal horn neuronal responses to electrical and natural stimulation of peripheral receptive fields to varying extents, with the exception of the unaffected electrically evoked Aβ-fibre response. This facilitation was most pronounced for the neuronal responses to innocuous and noxious mechanical stimuli, the electrically evoked post discharge and initial C-fibre responses. Reeve et al. (2000) also reported similar findings \textit{in vivo} with exogenous galanin producing facilitation of the initial C-fibre and post discharge responses. Spinal galanin administration facilitated the neuronal responses to mechanical stimulation especially von Frey 8.51g stimulation, yet had no significant effect on responses to noxious heat. Similar effects have been demonstrated behaviourally (Kuraishi et al. 1991b), where intrathecal galanin administration produced mechanical hyperalgesia, but not thermal hyperalgesia, in naive rats. More recently, it was reported that chronic intrathecal galanin administration to naive rats caused a significant decrease in mechanical thresholds but had no effect on thermal withdrawal latencies (Kerr et al. 2000). In another study, intrathecal galanin increased sensitivity to both mechanical and cold stimuli in normal rats (Liu et al. 2001).

The facilitation seen here could conceivably result from activation of receptors located pre- and post-synaptically. Galanin has been shown to depolarise cultured DRG neurones (Puttick et al. 1994), which could be a pre-synaptic basis for the facilitation of spinal neuronal responses following galanin. Whilst the receptor type responsible is unknown, GalR2 receptors have been shown to have excitatory effects in isolated cell lines resulting in an increase in intracellular calcium (Smith et al. 1997). GalR2 receptors are found both on DRG neurones and in the superficial layers of the dorsal horn and are less abundant than the inhibitory GalR1 receptors in the rat dorsal horn (O'Donnell et al. 1999). Another possibility is the activation of inhibitory galanin receptors, presumably GalR1, expressed on inhibitory neurones, so that exogenous galanin produces the observed facilitations through disinhibition.
Remarkably, in SNL rats exogenous galanin produced a pronounced inhibition of all the neuronal responses with the only exception being the electrically evoked Aβ-fibre responses. Electrically evoked C-fibre, Aδ-fibre, initial C-fibre and post discharge responses were inhibited in a dose-related manner, as were the neuronal responses to both innocuous and noxious mechanical stimuli. The wind-up response and the responses of the neurones to noxious heat stimulation were inhibited to the same extent with each dose of galanin. The effects of galanin in SNL rats are in agreement with a recent study, where galanin inhibited the spontaneous discharge of dorsal horn neurones in the CCI model of neuropathic pain (Xu et al. 2000a). An earlier study, (Wiesenfeld-Hallin et al. 1989b) found that intrathecal galanin elicited a greater inhibitory effect on the rat flexor reflex in axotomised rats compared to naive rats. Their findings agree with ours, in that the inhibitory effects of galanin seen following nerve injury occur at lower doses. My results extend these limited measures of spontaneous and reflex responses to show that galanin is able to suppress noxious evoked activity mediated through Aδ-fibre and C-fibre inputs and to modulate central hyperexcitability such as wind-up. Furthermore, the peptide reduced both mechanical and thermal responses.

The mechanisms underlying the marked shift to an inhibitory action of galanin following spinal nerve ligation are unknown. It has been reported that following axotomy both GalR1 mRNA and GalR2 mRNA are downregulated in DRG neurones (Xu et al. 1996; Sten Shi et al. 1997), although no marked change in the expression of these receptors in the dorsal horn has been reported (Zhang et al. 1998). So it is probably unlikely that selective plasticity in terms of GalR1 and GalR2 receptor activation is a possible basis for this inhibitory effect. However it is unknown at present if the marked upregulation of galanin following nerve injury has a knock-on effect on the level of GalR3 expression. Potentially, inhibitory GalR3 receptors, which are present at low levels in the spinal cord under normal conditions (Waters and Krause 2000), could be upregulated resulting in the profound inhibitory effects of galanin in SNL rats. Interestingly, neuropathic pain-related behaviour involves genes that map close to the GalR3 receptor (Seltzer et al. 2001). Additionally, as previously discussed in section 5.2, Liu et al. (2001) also suggested that
inhibitory galanin receptor activation was responsible for anti-allodynic effects. It has been shown that all galanin-containing neurones in the superficial dorsal horn are GABAergic (Simmons et al. 1995). Since nerve injury, in this case sciatic nerve ligation produces complex changes in the roles of GABA and its receptors after nerve injury (Kontinen et al. 2001), it is quite conceivable that the marked shift to inhibitions results from changes in these inhibitory neuronal populations.

The sham operation is an essential control experiment for the SNL procedure, and may also represent a state of post-surgical tissue damage with an inflammatory component. It is unlikely that any post-operative inflammation is present two weeks following the surgery and the sham-operated animals did not show any behavioural mechanical hyperalgesia (see section 3.1). However, it could be postulated that enduring sub-threshold inflammatory processes could be occurring following the surgery, or the initial inflammatory insult could have induced long-lasting changes in galanin receptor expression in the spinal cord. Kuraishi et al. (1991b) found mechanical, but not thermal hyperalgesia induced by carrageenan inflammation was reversed by intrathecal administration of anti-galanin antiserum. This finding suggests that galanin is responsible for the generation of certain hyperalgesias seen after inflammation.

I have shown that galanin inhibits neuronal responses to mechanical stimulation but does not significantly alter neuronal responses to heat. Inflammation has been shown to cause a decrease in GalR1 mRNA (Xu et al. 1996) and an increase in GalR2 mRNA (Sten Shi et al. 1997) in DRG neurones. If these changes have functional importance, a facilitation of the neuronal responses in sham rats could possibly result from GalR2 receptor activation. However, we find other neuronal responses (von Frey 8.51g and post-discharge) in sham rats to be inhibited to the same extent as in SNL rats. A possible explanation for these modality-selective effects is that the inhibition seen in sham rats arises as a result of upregulation of the putative inhibitory GalR3. Perhaps there are regional distributions of the galanin receptors on different neuronal structures that allow these selective effects of galanin.
5.4. Behavioural effects of spinal galanin

Intrathecal galanin (0.5μg-50μg) did not affect the mechanical thresholds of naive rats in either paw (Fig. 5.4.1, p167). Statistical analysis (ANOVA for repeated measures, Tukey’s HSD) showed no significant difference in the mechanical thresholds at 30 minutes or 90 minutes post galanin administration compared to predose readings. 5μg and 50μg galanin did appear to have a tendency to decrease the mechanical threshold. Two other studies have examined the effect of a chronic intrathecal infusion of galanin (25ng/hr) on the mechanical thresholds of normal rats (Kerr et al. 2000; Liu et al. 2001). These authors collectively report significant decreases in the mechanical thresholds of these rats 2-14 days post surgery, but not on post operative day 1 (Kerr et al. 2000).

Intrathecal galanin (0.5μg-50μg) also did not significantly affect the mechanical thresholds of the ipsilateral or contralateral paw of SNL rats, 30 and 90 minutes post administration compared to predose readings (Fig. 5.4.2, ANOVA for repeated measures, Tukey’s HSD, p168). A previous study reported that 20μg galanin administered intrathecally significantly increased the von Frey threshold in the photochemical model of neuropathy (Hao et al. 1999). Also in alldynic CCI rats, an intrathecal cumulative dose of 30μg galanin was shown to significantly increase the mechanical threshold by 4-5 grams (Liu and Hokfelt 2000). I observed a similar increase (6g) in the mechanical threshold of the ipsilateral paw at 30 minutes following 5μg galanin yet this was not significant with the statistical analysis I employed. This would perhaps be more convincing if there had not been a slight increase in the mechanical threshold of the ipsilateral paw in the saline group.

Withdrawal latencies to a focused heat stimulus were also examined in SNL rats prior to and following intrathecal galanin (0.5μg-50μg) administration (Fig. 5.4.3, p169). There was no significant difference in the withdrawal latencies of either paw at 30 or 90 minutes post galanin administration compared to predose readings (ANOVA for repeated measures, Tukey’s HSD).
Figure 5.4.1. Effect of spinal galanin on the mechanical thresholds of the A) left paw and B) right paw of naive rats. Each column shows the mean withdrawal latency ± SEM for each group of six rats. No significant changes in the mechanical thresholds in either paw compared to predose readings.
Figure 5.4.2. Effect of spinal galanin on the mechanical thresholds of the A) ipsilateral paw and B) contralateral paw of SNL rats. Each column shows the mean withdrawal latency ± SEM for each group of five rats. No significant changes in the mechanical thresholds in either paw compared to predose readings.
Figure 5.4.3. Effect of intrathecal galanin on paw withdrawal latencies to a focused heat stimulus for the A) ipsilateral paw and B) contralateral paw of SNL rats. Each column shows the mean withdrawal latency ± SEM for each group of six rats. No significant changes in withdrawal latencies compared to predose readings in either paw.
The electrophysiological effects of spinal galanin were not reflected in this behavioural analysis. The degree of thermal hyperalgesia produced following spinal nerve ligation (L5 and L6) is variable and not as marked as the sensitivity to mechanical stimuli (Kim and Chung 1992), also another study reports the absence of thermal hyperalgesia following SNL (Kontinen et al. 1998). I have found a slightly higher degree of thermal hyperalgesia in SNL rats, at post-operative day 14, compared to Kim and Chung (1992). Expressing my data as a difference score (latency of ipsilateral paw - latency of contralateral paw) results in scores ranging from -4 to -9 for the four treatment groups compared to the previously reported (Kim and Chung 1992) score of -3. Despite a lesser degree of hyperalgesia compared to the mechanical assessment, there was still potentially a large enough window (cut-off was 31.8 seconds) to observe anti-nociceptive effects of intrathecal galanin, which I felt justified this investigation.

The dramatically different effects of exogenous spinal galanin on the neuronal responses to innocuous mechanical (von Frey 8.51g) and noxious heat stimulation lead to the further investigation of the behavioural effects of exogenous spinal galanin. From the electrophysiological data, I would have predicted intrathecal galanin administration would have resulted in mechanical hyperalgesia in naive rats because the neuronal responses to innocuous mechanical stimulation were markedly facilitated. In SNL rats, there was a marked inhibition of the neuronal responses to von Frey 8.51g and noxious heat, so I would have expected to observe a reversal of mechanical allodynia and thermal hyperalgesia in behavioural testing. However, my expectations were not realised which could for reasons that I will now elaborate upon.

The number of action potentials evoked in response to the innocuous mechanical, noxious mechanical and noxious heat stimulation were similar for naive, sham and SNL rats (see Table 5.3, p155) as previously published (Chapman et al. 1998). So despite two-thirds of the input into the L4/L5 section of the spinal cord being damaged in SNL rats the neuronal response to these stimuli is similar to neurones from naive rats. Thus it is unclear how exactly behavioural hyperalgesia and allodynia relates to action potential generation in the
spinal cord. In the electrophysiological studies the neuronal responses are evoked by suprathreshold stimulation, as the receptive field is stimulated for 15 seconds and does not end due to a withdrawal from the stimulation as seen in the behavioural studies. In addition, in behavioural testing a von Frey hair is not applied to the hindpaw for 15 seconds. So perhaps spinal galanin will reduce the response to suprathreshold stimulation, but have no effect on threshold responses. With this thought in mind, if only ten action potentials may be needed for a withdrawal response from von Frey 8.51g in behavioural testing with SNL rats. Yet 100 action potentials are evoked in response to von Frey 8.51g in electrophysiological testing with SNL rats. Galanin could then inhibit the response by 89% in electrophysiological testing but the remaining action potentials are still evoked and so a behavioural withdrawal response from this stimulus is still observed.

5.5. Summary

This chapter demonstrates the functional effects of peripheral and spinal galanin on sensory processing in naive and neuropathic states using different electrophysiological techniques.

In summary in this chapter, I have investigated the effects of exogenous galanin on nociceptive transmission in the periphery employing in vitro and in vivo electrophysiology. Peripheral galanin produced inhibitory effects in a larger proportion of the nociceptive fibres and spinal neurones examined and a more variable facilitatory effect in the remaining fibres and neurones. Following nerve injury the proportion of neurones inhibited by galanin was increased.

I have shown that galanin has markedly contrasting effects on spinal nociceptive neurones in naive and neuropathic rats. Spinal galanin administration caused a facilitation of the neuronal responses to electrical and natural stimulation in naive rats. In sham rats, galanin at high doses caused an inhibition of their neuronal responses. Exogenous spinal galanin elicits a marked inhibition of all neuronal responses, electrically evoked, mechanical and
thermal responses in neuropathic rats. Yet spinal galanin did not produce behavioural antinociceptive effects in neuropathic rats.

Spinal nerve ligation induces changes in the effect of spinal galanin so that the marked inhibitory effects of spinally administered galanin are seen compared to mild facilitatory effects produced in naive rats. This shift towards inhibition after nerve injury is again present following peripheral administration of galanin. However, it is less dramatic than that seen following spinal galanin administration. There appears to be two distinct populations of peripheral C-fibres in terms of their responses to galanin and these dual effects of galanin are reflected perfectly at the central spinal level irrespective of what must be considerable convergence. These bi-directional effects of galanin could be regulated by the integrity of the peripheral nerves and may result from differential expression of the three galanin receptor subtypes on populations of afferent fibres.

Selective GalR1 and GalR3 agonists, or selective galanin receptor knockout mice, will be required to shed more light on the role of this peptide in normal and neuropathic states. Nevertheless, whatever the mechanisms underlying these changes in the effects of galanin after peripheral nerve injury, these results suggest that galanin and/or its receptors may be a useful target for the treatment of neuropathic pain.
Chapter 6

Final Discussion
This thesis has explored the effect of nerve injury on the spinal and peripheral actions of the cytokine, interleukin-6 (IL-6) and the neuropeptide, galanin. Both these substances are markedly upregulated following peripheral nerve injury, both in the DRG and in the spinal cord and this can give rise to possible interpretations of their putative roles, in terms of neuropathic pain. The first is that IL-6 and/or galanin upregulation is a causal factor in the generation of neuropathic pain, or secondly, that their marked upregulation is part of the repertoire of the nervous system as an attempt to decrease the extensive hyperexcitability induced by nerve injury. Full answers to these questions can only be produced when selective antagonists are available, but in the meanwhile, the effects of IL-6 and galanin on sensory processing under normal physiological conditions have to be established.

I found that peripheral IL-6 administration had inhibitory effects in naive rats, seen both in vitro on responses of nociceptive fibres and in vivo on spinal neuronal responses and on behavioural nociceptive responses. Spinal IL-6 administration also had no effect on neuronal responses or behavioural nociceptive responses in naive rats. Exogenous galanin had quite different effects to IL-6 in naive rats. Two distinct populations of nociceptive fibres and spinal neurones were revealed in terms of their responses to peripheral galanin administration, one that was inhibited and the other facilitated. This suggests that inhibitory galanin receptors, GalR1/3 and excitatory galanin receptors, GalR2, are expressed in the periphery. Spinal administration of galanin facilitated many of the neuronal responses to electrical and natural stimulation, but did not affect behavioural measures of nociception. It is not known how much of an increase in the firing of spinal neurones is required to result in behavioural hyperalgesia. This lack of a behavioural effect of spinal galanin in naive rats could suggest that the facilitation of spinal neurones is not marked enough to result in behavioural hyperalgesia.

Spinal nerve ligation (SNL) had profound consequences for the actions of peripheral and spinal IL-6 and galanin on sensory processing. Peripheral IL-6 administration had no effect on mechanical responses of spinal neurones in neuropathic rats, yet inhibited the heat
responses of these neurones, to a similar extent as heat responses in naive rats. Spinal IL-6 markedly inhibited the majority of spinal neuronal responses examined in neuropathic rats. Peripheral galanin administration in neuropathic rats again produced two directions of effects on the neuronal responses. However, compared to naive animals a greater proportion of neurones was inhibited following peripheral galanin in neuropathic rats compared to those in naive rats. Perhaps the most contrasting effects produced in neuropathic rats compared to naive rats, were those that were induced by spinal galanin in the electrophysiological experiments. In neuropathic rats, spinal galanin markedly inhibited all the neuronal responses evoked by nociceptive fibres compared to the facilitation of similar responses in naive rats. These marked inhibitory effects of galanin following nerve injury were not observed in behavioural experiments, where spinal galanin had no effect on nociceptive responses. As previously discussed (see section 5.4), this could potentially reflect an ability of galanin to inhibit the majority (~70-80%) of the response to a suprathreshold stimulus in electrophysiology, but not the remaining (~20-30%) that could be responsible for threshold responses in behavioural testing.

Therefore, broadly speaking, nerve injury induces plasticity in the mechanisms of sensory processing, which shifts the effects of exogenous galanin towards inhibition. Perhaps the most likely reason for this shift is an increase in the expression of inhibitory galanin receptors, GalR1 and/or GalR3. As previously discussed in chapter 5, evidence for an upregulation of GalR1 receptors following nerve injury comes from the recent investigation of more selective receptor agonists in allodynic rats (Liu et al. 2001). Alternatively, GalR3 upregulation may explain these inhibitory effects of galanin following SNL, considering the reported downregulation of GalR1 and GalR2 mRNA in the DRG following axotomy (Xu et al. 1996; Sten Shi et al. 1997) provided a similar downregulation was also induced by SNL. Selective GalR1 and GalR3 agonists or selective galanin receptor knockout animals will be required to shed more light on the role of this peptide in neuropathic pain.
The effects of nerve injury on the spinal and peripheral actions of IL-6 appear to be more complex, with the cytokine inducing inhibitory effects when administered spinaly compared to normal conditions, yet when administered peripherally produced reduced inhibitory effects compared to those seen in naive rats. As both IL-6R mRNA and gp130 mRNA are markedly and persistently upregulated in the sciatic nerve following its injury (Ito et al. 1998; Grothe et al. 2000). The inhibitory effects of spinal IL-6 in neuropathic rats may well arise as a consequence of an increased expression of the IL-6/gp130 complex in the spinal cord. The reasons for reduced inhibitory effects of peripheral IL-6 following nerve injury, on neuronal responses evoked by innocuous, but not noxious, stimuli is less apparent. Potentially, the expression of IL-6 receptors may differ between C- and A-fibres following nerve injury such that C-fibres express higher levels of IL-6 receptors compared to A-fibres, due to a potential loss of these receptors from A-fibres.

As previously discussed (section 1.7.7), studies using IL-6 knockout mice have not conclusively established the role of IL-6 in nociception and the ‘knockout’ of IL-6 is not without its compensations such as the marked upregulation of TNFα (Fattori et al. 1994). The use of a selective IL-6 receptor antagonist or knockout mice may prove beneficial in further elucidating the role of IL-6 in neuropathic pain. However, studies with IL-6 knockout mice report contrasting effects in terms of nociception and nerve injury (see section 1.7.7). The antagonism or knockout of the gp130 transduction molecule is potentially unadvisable as a related cytokine, LIF (see section 1.6), like IL-6 requires gp130 to elicit its actions and is also upregulated following nerve injury (Sun and Zigmond 1996). Thus differentiating whether the effects of gp130 blockade are due to an IL-6 or a LIF action, or both, could become problematic.

An attractive avenue to further the research undertaken in this thesis would be to investigate the synergy between galanin and IL-6 in neuropathic pain. Data suggesting such an integrative relationship between this neuropeptide and the cytokine has been previously published. Firstly, intraneural injection of IL-6 in the intact sciatic nerve of rats produced a
significant increase in galanin expression in the DRG (Thompson et al. 1998). These authors also reported that continuous delivery of a gp130 antibody to the axotomised stump of the sciatic nerve attenuated the axotomy-induced upregulation of galanin. Suggesting that cytokines which mediate their effects via gp130 molecules i.e. IL-6 of LIF, are partially responsible for the upregulation of galanin following nerve injury. Cloning of the rat galanin gene revealed the presence of a STAT (signal transducer and activator of transcription) binding site (Corness et al. 1997), which forms part of the IL-6 signal transduction pathway (see section 1.7.5). Galanin upregulation in IL-6 knockout (KO) mice was attenuated compared to that in wild-type (WT) mice (Murphy et al. 1999b). Specifically, no increase in galanin levels were seen in laminae III/IV of the dorsal horn of IL-6 KOs and the increase in the nucleus gracilis was 50% of that seen in WT mice. However, galanin mRNA was induced in a similar number of DRG neurones (~one-third) in IL-6 KO and WT mice (Murphy et al. 1999b). This suggests that endogenous IL-6 is required for the full central upregulation of galanin following nerve injury, but is not required for the upregulation of galanin in the DRG. Although as previously mentioned the knockout of IL-6 may have resulted in the increased production of other cytokines such as LIF, which has been shown to play a role in galanin upregulation following nerve injury (Corness et al. 1996).

To further investigate the synergy between IL-6 and galanin using the results of this thesis as a starting point, a number of angles could be examined. A first approach would be to observe the effects of co-administration of galanin and IL-6 together. Here a combination of low dose galanin followed by a low dose of IL-6, given at the peripheral and/or spinal level might be expected to produce synergistic inhibitory effects. However, this sort of study would only reveal interactions between the exogenous peptide and cytokine; clearly it would be of interest to know how if the endogenous mediators would interact in the same way but as discussed previously the absence of good antagonists makes this difficult.

Interestingly, I have shown that both spinal IL-6 and galanin administration produced similar effects on the majority of spinal neuronal responses examined in neuropathic rats.
A dose-related marked inhibition was observed in SNL rats, following spinal IL-6 and galanin administration in 6 out of the 9 spinal neuronal responses examined namely; the electrically evoked C-fibre, initial C-fibre, post discharge and wind-up responses and neuronal responses to innocuous and noxious mechanical stimulation. In relation to neuropathic pain the inhibition of these responses shows the selective inhibitory action of IL-6 and galanin on reception of pain in the spinal cord and the spinal hyperexcitability produced in response to pain from the periphery. In addition the inhibition of innocuous mechanical stimulation suggests potential inhibitory effects on allodynia.

As discussed in section 1.5, there are a number of agents that currently used to treat neuropathic pain which include opiates, antidepressants, sodium channel blockers, GABAergic agents, gabapentin, NMDA antagonists, sympathetic agents and capsaicin. Based on the evidence for a number of peripheral and central mechanisms that appear to underlie the symptoms of neuropathic pain (see section 1.4), the concept of using multiple agents to treat patients would appear to be logical and also advantageous in decreasing the incidence of side-effects. From a pharmacological point of view, a combination of agents, which worked in a contrasting manner, would perhaps prove most effective. Examples could be a drug which decreases neuronal excitability e.g. gabapentin, carbamazepine administered in combination with a drug which increased inhibitory controls e.g. amitryptiline, morphine. The idea would be that the combination of agents would allow more than a single pharmacological target to be manipulated that could improve pain control of patients. Any combination of the drugs above would be of interest as would a combination of these agents with galanin or IL-6. To take a single example, the ability of an opioid such as morphine to reduce transmitter release would be expected to interact in a positive manner with spinal galanin which I have shown to have marked inhibitory effects on measures of post-synaptic neuronal hyperexcitability such as post discharge and wind-up. Furthermore, the ability of galanin to attenuate measures of allodynia would also be of benefit in conjunction with opioids that tend to produce weak effects on these low threshold mechanical measures.
In conclusion, the marked upregulation of galanin and IL-6 following nerve injury appears to be part of the solution of the injured nervous system, produced to potentially dampen down the abnormal spinal hyperexcitability induced. In the search for new drug treatments, it is important to consider the route of administration; the results presented here show that the analgesic effects of IL-6 and galanin are most marked following spinal administration. Equally important for patient compliance is the need for a convenient painless drug administration. Therefore to achieve an oral dose of galanin in particular will require the generation of a non-peptide analogue. Aside from the possible pharmacokinetic issues, the anti-nociceptive effect of exogenous spinal galanin and IL-6 in neuropathic rats, but not in naive rats, suggests that these systems warrant further investigation and may prove beneficial novel targets for the treatment of neuropathic pain.


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