Characteristics, Plasticity and Pharmacology of Lamina I Neurones in the Rat Spinal Cord: An Electrophysiological Study

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Abstract

The response characteristics to peripheral stimulation and the pharmacological properties of lamina I superficial dorsal horn neurones, have been assessed and analysed in this study. Lamina I neuronal responses to electrical, mechanical and thermal stimuli applied to the peripheral receptive field area were quantified. From this it was found that the majority of lamina I neurones (50-71%) were nociceptive specific (NS) in response characteristics in all animal models tested. Further characterisations of lamina I neuronal responses were made in neuropathic animals and after ablation of lamina I/Ill NK1 receptor expressing neurones, where only small increases in neuronal responses were seen.

The role of ionotropic glutamate receptors in lamina I neuronal responses to peripheral electrical stimuli were evaluated using spinally administered selective receptor antagonists for AMPA (NBQX), AMPA/ kainite (CNQX), kainate (LY293558), NMDA (APV) and NR2B (Ifenprodil and ACEA-1244) receptors. AMPA and kainate receptors play a major role in lamina I neuronal responses (NBQX, 50μg/ 50μl, 53-98% reduction, Aδ-fibre, C-fibre, Post-Discharge and XS-Spike responses), yet the NMDA receptors have comparatively smaller roles as compared with deeper dorsal horn neurones which have been previously characterised (APV, 500μg/ 50μl, 19% reduction in lamina I neurones). The majority of these excitatory amino acid receptor antagonists were also assessed in lamina I neurones recorded from neuropathic rat models, to evaluate any potential changes arising from peripheral nerve damage of which only minor differences were evident.

The role of the ionotropic GABAergic receptor was investigated using a spinally administered GABA receptor antagonist (Bicuculline) on lamina I neuronal responses. GABA receptors appear to exert inhibitory influences on both electrical and mechanical response characteristics (50μg/ 50μl, 277±69% of Aδ-fibre response & 308±115, 277±96 von Frey 9, 30g mechanical responses respectively). Following nerve injury, their role was less pronounced in the mechanically evoked response, yet the facilitation evoked by Bicuculline of the A-δ fibre response was similar. The role of the glycine receptor in lamina I neuronal responses in normal models was also similarly investigated using a spinally administered glycine receptor antagonist (Strychnine). However glycine receptors appeared to play a smaller although noticeable role in lamina I neuronal response properties.

Finally the role of the 5HT3 receptor (using the 5HT3 receptor antagonist Ondansetron) in lamina I neuronal responses to electrical and naturally evoked stimuli was assessed in a model whereby neurones containing the NK1 receptor were selectively ablated as well as a control model. Reductions in the electrically evoked responses were seen in both models, however larger reductions were more common in the control group (10-100μg/ 50μl Ondansetron, 26-82% reduction of all electrically evoked responses).

The results provide a detailed account of the response properties of lamina I neurones in the rat and suggest that their high threshold properties may result from a combination of a lack of a major NMDA component to their responses and an active GABAergic receptor inhibition.
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<th>Abbreviation</th>
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<td>Dopamine</td>
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<td>Heat Pinch Cold</td>
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<td>HPC*</td>
<td>Hippocampal Pyramidal Cells</td>
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<td>HTM</td>
<td>High Threshold Mechanoreceptor</td>
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<td>HVA</td>
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<td>iGluR</td>
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<td>Inhibitory Interneurone</td>
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<td>LTP</td>
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<td>Messenger Ribonucleic Acid</td>
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<td>Multiple Sclerosis</td>
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<td>MCTGF</td>
<td>Mitogenic Cytokine Transforming Growth Factor</td>
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<td>Na⁺</td>
<td>Sodium Ion</td>
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<td>Sodium Channel 6</td>
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<td>Noradrenaline</td>
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<td>NADPH</td>
<td>Nicotinamide Adenine Dinucleotide Phosphate</td>
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<td>NaG Channel</td>
<td>Voltage Gated 'Glia' Sodium Channel</td>
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<td>NaN</td>
<td>Neuronal Sodium Channel</td>
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<td>NGF</td>
<td>Nerve Growth Factor</td>
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<td>NMDA</td>
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<td>Nitric Oxide Synthase</td>
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<td>NMDA Receptor Subunit 2 A-D</td>
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<td>NMDA Receptor Subunit 3</td>
</tr>
<tr>
<td>NRM</td>
<td>Nucleus Raphe Magnus</td>
</tr>
<tr>
<td>NR-PN</td>
<td>Nodes of Ranvier- Peripheral Nerve</td>
</tr>
<tr>
<td>NR-PN*</td>
<td>Nodes of Ranvier – Rat Sciatic Nerve Only</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
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<td>--------------------------------------</td>
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<tr>
<td>VPI</td>
<td>Ventral Posterior Inferior Nucleus</td>
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<tr>
<td>VR1</td>
<td>Vanilloid Receptor-1</td>
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<tr>
<td>VVP</td>
<td>Ventroposterior Complex</td>
</tr>
<tr>
<td>WDR</td>
<td>Wide Dynamic Range</td>
</tr>
<tr>
<td>Zn$^{2+}$</td>
<td>Zinc Ion</td>
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1.0 Introduction

1.1 Peripheral Processes of Pain

Sensory receptors within the mammalian peripheral nervous system detect and transmit mechanical, thermal and chemical stimuli via electrical signals (Chery-Croze 1983; Lynn et al. 1995; Treede 1995; Perl 1996; Treede 1999). These signals, called action potentials, are delivered to the spinal cord via distinct sensory nerve fibres, where they are integrated into the central nervous system, perceived and reacted upon (Chery-Croze 1983; Fox 1999). Cutaneous administration of non-noxious mechanical, heat and cold stimuli is sufficient to activate mechanoreceptors and thermoreceptors located in the skin (Reeh et al. 1987; Leem et al. 1993; Levine et al. 1993; Koltzenburg and Handwerker 1994; Lynn et al. 1995; Lawson 1996; Fox 1999). However noxious thermal, mechanical and chemical stimuli, which may be potentially harmful, activate a distinct set of cutaneous sensory receptors called nociceptors e.g. polymodal nociceptors and high threshold mechanical nociceptors (HTM) (Fox 1999). These respond primarily to high threshold stimuli (Reeh et al. 1987; Leem et al. 1993; Levine et al. 1993; Koltzenburg and Handwerker 1994; Perl 1996).

Mechanoreceptors, found in mammalian skin, specifically respond to low threshold innocuous mechanical stimuli (Fox 1999). They can be separated into 3 distinct groups, all if which are innervated by large myelinated A-β fibres (Kruger et al. 1981; Leem et al. 1993; Fox 1999):

- Slowly Adapting (SA1/ Type I & SAII/ Type II)
- Rapidly Adapting
- Pacinian Afferents

Thermoreceptors, can be sub-categorised as warm (30°C - 46°C) and cold receptors. Interestingly, although these receptors conduct low threshold innocuous stimuli, they are both innervated by small diameter unmyelinated C-fibre afferents (Chery-Croze 1983; Fox 1999).
Furthermore, thinly myelinated A-δ fibre afferents innervate cold receptors as well (Fox 1999). Polymodal nociceptors, innervated by both A-δ- and to a larger extent, C-fibre afferents, are named so, on account of their ability to respond to high threshold mechanical, thermal and chemical stimuli (Szolcsanyi et al. 1988; Lang et al. 1990; Seno and Dray 1993). Interestingly, in vitro studies have revealed that polymodal C-fibre nociceptors may also be activated by capsaicin, quite unlike other categories of cutaneous nociceptor (Szolcsanyi et al. 1988; Lang et al. 1990; Seno and Dray 1993).

High threshold mechano-heat sensitive units are nociceptors, which respond to both high threshold heat and mechanical stimuli. They are often separated according to the type of fibre that innervate them, e.g. A-δ-HTMs and C-HTMs (Campbell et al. 1989; Raja et al. 1998; Fox 1999). Lastly, upon tissue damage another class of nociceptor is activated, which is normally silent. These have been named accordingly. Thus ‘silent nociceptors’ (innervated by unmyelinated C-fibre afferents) form a final category of high threshold receptors which have been shown to respond to bradykinin and other inflammatory mediators, allowing the mammalian system to recognise tissue damage in the periphery (Lang et al. 1990).

Interestingly, there has been much interest surrounding the identification of a heat activated ion channel, located on mechano-heat sensitive C-fibre nociceptors in the periphery (Szolcsanyi et al. 1988). Transient receptor potentiated (TRP) channels were first identified in the drosophila fly (Clapham 2003). In mammals they are putative six transmembrane polypeptide subunits that form tetrameric complexes (Clapham 2003). They are believed to decipher sweet, bitter, umami, warmth, heat and cold sensations (Clapham 2003). There are numerous subfamilies of TRP channels (listed below) (Clapham 2003):

- Melastatin (TRPM)
- Canonical (TRPC)
- Polycystin (TRPP)
- Mucolipin (TRPML)
- Vanilloid osm9-like (TRPV)
However, the TRPV subfamily is thought to be primarily involved in thermal sensory transduction (Clapham 2003). TRPV1-4, formerly known as VR1, VRL-1, 2 and 3, are four different TRP channels belonging to this subfamily, which are known to code thermal stimuli (Smith et al. 2002; Clapham 2003). Two more channels comprise this TRPV family. TRPV5-6, formerly known as ECAC1 & 2, are constitutively active channels located predominantly in transporting epithelia of the kidney and intestine and won't be discussed in this section (Caterina et al. 1997; Gunthorpe et al. 2002; Clapham 2003).

<table>
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<tr>
<th>TRPV Channel Subtype</th>
<th>Temperature Sensitivity</th>
<th>Location</th>
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<tr>
<td>TRPV1 (VR1)</td>
<td>43°C</td>
<td>Mechano-heat sensitive C-fibre Nociceptors (Periphery)</td>
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<tr>
<td>TRPV2 (VRL-1)</td>
<td>≥52°C</td>
<td>Type 1 Aδ-fibres &amp; Capsaicin insensitive DRG Neurones</td>
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<tr>
<td>TRPV3 (VRL-2)</td>
<td>≥31°C</td>
<td>Tongue, Skin &amp; Nervous System</td>
</tr>
<tr>
<td>TRPV4 (VRL-3)</td>
<td>≥25°C</td>
<td>Tongue, Skin &amp; Nervous System</td>
</tr>
<tr>
<td>TRPV5 (ECAC1)</td>
<td>?</td>
<td>Transporting Epithelia (Kidney &amp; Intestine)</td>
</tr>
<tr>
<td>TRPV6 (ECAC2)</td>
<td>?</td>
<td>Transporting Epithelia (Kidney &amp; Intestine)</td>
</tr>
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Table 1.1.a illustrating the thermal sensitivities of the TRPV subfamily of heat-activated ion channels (Caterina et al. 1997)

The TRPV1 channel, activated by both capsaicin, the hot component of chilli peppers, and noxious thermal stimuli (43°C) is involved in both acute thermal nociception and thermal hyperalgesia, which arises following tissue damage (Caterina et al. 1997; Gunthorpe et al. 2002; Clapham 2003). Block of the TRPV1 receptors, using the selective capsaicin antagonist capsazepine, prevents activation of peripheral afferent fibres containing the capsaicin receptor and therefore reduces thermal and chemically induced nociception exposure (Seno and Dray 1993; Garcia-Martinez et al. 2002). Furthermore, vanilloids fail to evoke nociceptive, inflammatory and hypothermic behaviours in TRPV1-/- mice (Seno and Dray 1993; Caterina et al. 1997; Gunthorpe et
However activation of TRPV1 receptors, using capsaicin, can also be used as a model for chemically induced thermal hyperalgesia due to capsaicin's neurotoxic effects following prolonged exposure (Seno and Dray 1993; Garcia-Martinez et al. 2002). The TRPV2 receptor channel is thought to respond to high threshold thermal stimuli (>52°C) and it is believed that these channels are located predominantly on type 1 Aδ-fibres, as well as capsaicin insensitive DRG neurones in vitro (Caterina et al. 1997; Gunthorpe et al. 2002; Clapham 2003). TRPV3 and TRPV4 channels encode warm stimuli (>31 & >25°C respectively) and are found primarily in the tongue, skin and nervous system (Caterina et al. 1997; Gunthorpe et al. 2002; Clapham 2003). Overall these channels act to decipher both innocuous and noxious thermal stimuli, which allow us to discriminate temperature changes in the external environment. Table 1.1.b illustrates the vast majority of other chemical mediators and their receptors, with pronociceptive roles.

<table>
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<td>Histamine</td>
<td>H 1/2</td>
</tr>
<tr>
<td>Protons</td>
<td>ASICs</td>
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<tr>
<td>Neurotrophins (NGF, BDNF)</td>
<td>p75</td>
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<td>Neurotrophins (NT-4/5)</td>
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<td>Neurotrophins (NGF)</td>
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<td>5-HT</td>
<td>5-HT 2A/3/4/7</td>
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<td>CGRP</td>
<td>CGRP 1/2</td>
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<td>Substance P</td>
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<tr>
<td>Prostaglandins</td>
<td>EP 2-4</td>
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*Table 1.1.b (Millan 1999). Other classes of chemical mediators, with pronociceptive roles (Millan 1999)*

Thus it is clear that a polymodal nociceptor is not simply a single sensor but a number of receptors and channels that can transduce noxious stimuli. Some appear to be relatively selective for a single mediator whereas others such as the TRPV1 receptor respond to a number of stimuli and so may integrate several modalities.
There are predominantly three types of sensory neurone, which transmit different types of sensory information from the periphery to the spinal cord. These are the myelinated A fibres (Aβ and Aδ) and the unmyelinated C-fibres (Millan 1999). The myelinated A β-fibres are large diameter fibres (>10 μM) and are able to transmit innocuous information in a fast (30-100 m sec\(^{-1}\)) and efficient manner (Millan 1999). A β-fibres are thought to terminate predominantly in lamina III-VI of the dorsal horn (as well as having weak projections to V/VI, II, and I) and make up 20% of the primary afferent fibres that innervate the skin (Millan 1999). A δ-fibres conduct both innocuous and high threshold information, these fibres are thinly myelinated and medium in size (2-6 μM) with slightly slower conduction velocities than A β-fibres (12-30 m sec\(^{-1}\)) (Millan 1999).

Cutaneous A δ-fibres, originating largely from A δ-HTMs in the periphery, appear to terminate largely in the confines of lamina I which, as discussed later, forms one of the major ascending output from the spinal cord to higher centres of the brain (Sorkin et al. 1997; Millan 1999). However, it is suggested that A δ-fibres may also terminate in lamina II\(_o\) and X, as well as in lamina II, III and V depending on the peripheral location the fibre is derived from (Sorkin et al. 1997; Millan 1999). Interestingly, A δ-fibres comprise the smallest (10%) population of primary afferent fibre innervating the skin (Millan 1999).

Lastly, C-fibre primary afferents are far smaller than A-fibres (0.4-1.2 μM). They transmit noxious information from the periphery to the spinal cord far more slowly (0.5-2.0 m sec\(^{-1}\)), due to their distinct lack of myelination (Campbell et al. 1989; Raja et al. 1998; Millan 1999). C-fibres form the largest population of fibres innervating the skin (70%) and terminate largely in lamina II\(_o\) of the dorsal horn, as well as lamina I to a lesser extent (Millan 1999) (See Diagram 1.1.a (Fitzgerald 1989)). Cutaneous C-fibre afferents also project to deeper lamina IV/V and X (Millan 1999). It is widely hypothesised that the distinct fast ‘first pain’ elicited by a noxious stimulus is therefore produced by A δ-fibre transmission and the ‘second pain’ is a result of the more slowly conducting c-fibre afferents (Millan 1999).

CGRP, TrkA and IB4 are neurochemical markers for sensory neurones within the nervous system (Bennett et al. 1996). Nerve growth factor (NGF), located on both
developing and adult sensory neurones, acts on TrkA receptors. Therefore sensory neurones containing NGF will also express TrkA receptors (Bennett et al. 1996). It is known that 40-50% of sensory neurones express the TrkA receptor and this is therefore often a useful marker. However, both TrkA and CGRP are expressed predominantly in visceral afferents, rather than cutaneous afferents (75% & 69%, compared to 43% & 51% respectively) (Bennett et al. 1996). IB4 seems to be a more accurate marker for cutaneous afferent fibres (43%) than visceral afferents (29%) (Bennett et al. 1996).
1 - C Cold Mechanical Nociceptor
2 - A-delta High Threshold Mechanoceptor
3 - C Polymodal Nociceptor
4 - Rapidly Adapting Mechanoceptor
5 - Slowly Adapting Mechanoceptor
6 - Hair Follicle Afferent

Interestingly, the origin of all the transmitters, receptors, ion channels and other such proteins which comprise these sensory nerves is the dorsal root ganglia (DRG), which is a collection of cell bodies located on the nerve fibre (McMahon 1997). From the DRG proteins can be transported to their appropriate destinations, either in the direction of the periphery, or towards the spinal cord and the central terminals of the neurone (McMahon 1997). There are three broad types of DRG cells, classified essentially, by their biochemical composition (McMahon 1997; Boucher and McMahon 2001) listed below:

- **'Large Light' DRG's** – comprise 30% total population, neurofilament & neurotrophin receptor expression and myelinated Aβ-fibre axons (McMahon 1997; Boucher and McMahon 2001).
- **Peptide Expressing DRG's** – 40% total population, CGRP & substance P expression, unmyelinated c-fibre axons (McMahon 1997; Boucher and McMahon 2001).
- **Non-Peptide small DRG's** – 30% total population, RET, GFRα1/2, VR1, P2X3 channel expression, binds lectin (IB4), GDNF dependant and has unmyelinated C-fibre axons (McMahon 1997; Boucher and McMahon 2001).

### 1.2 Spinal Cord Anatomy

The spinal cord is protected by three meningeal layers, which act to cushion and safeguard, as well as aid the supply of vital nutrients (Everett 1971). These three meningeal layers are termed the pia mater, which surrounds the spinal cord, the arachnoid mater, which is the middlemost layer and the dura mater, which is the outermost layer (Everett 1971).

The spinal cord is separated into gray and white columns, of which further subdivisions can be made. It is essentially comprised of an intricate network of neurones and their associated axons, cell bodies and dendrites (Everett 1971). It is important to mention two main neuronal cell types, the internuncial and tract cells, which have cell bodies situated in the posterior gray columns (Everett 1971). Internuncial cells form spinal reflex arcs within the spinal cord and in addition, dorsal root fibre axons, which project to internuncial cells, extend to many other areas in the gray matter thus transmitting important sensory activity within the spinal cord (Everett 1971). Internuncial cell axons also project to other areas of the gray matter, particularly the anterior gray columns,
where they form additional reflex arcs (Everett 1971). Peripheral neurones terminate near tract cells, and thus also transmit important sensory information, which is then also largely modulated within the spinal cord (Everett 1971). Spinal dorsal root processes may send axon projections within the gray matter or alternatively to the white matter, which may be the basis for ascending and descending projections towards much higher centres (as will be discussed later) (Everett 1971). These neurones provide an important link between peripherally derived sensory information and motor reflexes in the spinal cord and supraspinal processing, homeostasis and emotional characteristics attributed to higher neuronal centres (Everett 1971). The white matter is separated into three main funiculi, based on the position these different areas inhabit within the spinal cord. These are the posterior, lateral and anterior funiculi which are each comprised of various different fiber tracts. Interestingly, within each funiculi, the fasciculus propius is in direct contact to the gray matter of the spinal cord and are composed of all the ascending and descending internuncial cell projections (Everett 1971).

1.3 Spinal Cord Dorsal Horn and Lamina Segmentation

The dorsal horn can be separated into six distinct laminae I-VI (a further 3 lamina, VII-IX, comprise the ventral horn). However it is more broadly separated into 5 general areas based on its anatomical image (Sorkin and Carlton 1998). These are listed below:

- Marginal Layer
- Substantia Gelatinosa
- Nucleus Propius
- Central Canal & surrounding Area
- Ventral Horn

The marginal layer is the most dorsal layer of gray matter within the spinal cord and along with the substantia gelatinosa, forms the superficial dorsal horn (Sorkin and Carlton 1998).
The marginal layer is comprised mainly of small neurones and classically, large disk shaped Waldeyer cells with protracted dendrites, extending throughout the rostrocaudal plane (Sorkin and Carlton 1998). The preponderance of lamina I neuronal dendrites project to other lamina I neurones within the spinal cord, however some do penetrate deeper lamina layers and in humans, terminal arbors can be recognised in lamina II as well (Sorkin and Carlton 1998). It is now clear that lamina I is one of the main ascending output centres in the spinal cord and projects to many different areas of the brain (Gauriau and Bernard 2002). Such ascending neuronal pathways stemming from the marginal layer, project to these different areas in the brain via 5 main ascending pathways (Sorkin and Carlton 1998):

- Spinothalamic tract
- Spinoreticular tract
- Spinoparabrachial tract
- Spinomesencephalic tract
- Spinocervical tract

Lamina II is a densely packed neuronal band, which lies directly beneath lamina I and is commonly separated into two distinct layers, lamina II inner and lamina II outer (Sorkin and Carlton 1998). The substantia gelatinosa is comprised mainly of small neurones, with unmyelinated axons. The two main neuronal cell types being islet and stalked cells, however other cell types have been described within this layer including arboreal, II-III border and spiny cells, which appear to arborise in deeper dorsal horn laminae (Sorkin and Carlton 1998). Stalk cells, which project into lamina I, are found predominantly in lamina II outer. Islet cells, which unlike stalk cells have axons and dendrites confined to the lamina II region, are located predominantly within the inner lamina II area (Sorkin and Carlton 1998). Studies have since suggested that these islet cells may possess the inhibitory amino acid γ-aminobutyric acid (GABA) and therefore exercise an inhibitory role in the dorsal horn (Sorkin and Carlton 1998). Unlike lamina I, lamina II has few, if any, projection neurones reaching higher supraspinal areas (Gauriau and Bernard 2002).

Lamina III, partly comprising the nucleus propius, is a thick neuronal layer, which lies directly beneath lamina II. Axonal projections and the dendritic arrangements in lamina
Ill are very similar to that of lamina II, however this layer is composed of larger cells with myelinated axons and is far less condensed. Interestingly, spinocervical tract (SCT) and postsynaptic dorsal column pathways (PDSC) involved in the transmission of painful stimuli, are believed to emanate from lamina III (Sorkin and Carlton 1998). Interestingly, the cells that give rise to postsynaptic dorsal column pathways, are believed to project to the superficial dorsal horn, whereby small diameter primary afferent terminal fibres synapse and therefore transmit nociceptive information (Sorkin and Carlton 1998).

Lamina IV to VI forms the deep dorsal horn. Lamina IV is the least densely packed layer in the dorsal horn and it has emerged that this layer also contains spinothalamic tract (STT) cell somas (Sorkin and Carlton 1998). Lamina V contains a far more varied selection of neuronal cell types compared to the lamina IV, and the majority of these extend up towards the superficial lamina layers. Interestingly, some of the cell bodies located within lamina V form STT, as well as SCT and PSDC ascending pathways to a lesser extent, and thus contribute to spinal nociceptive transmission (Sorkin and Carlton 1998). Lamina VI is comprised of a slightly smaller and a more orderly arranged population of neurones, which are thought to have principally propriospinal functions within the dorsal horn, thus separate from monosynaptic inputs originating from primary afferent terminals in the more superficial laminae (Sorkin and Carlton 1998).

Interestingly, laminae VII to VIII, which belong to the ventral horn, contribute to nociceptive transmission as they are comprised, partly, of cells which form the spinoreticular, spinomesencephalic and medial STT ascending pathways (Wall 1989; Sorkin and Carlton 1998). Furthermore, laminae X which is often included as a component of the dorsal horn (Wall 1989) and situated in the areas surrounding the central canal, is thought to be involved in propriospinal systems within the central nervous system (Sorkin and Carlton 1998). This layer contains both fusiform and pyramidal cells, as well as stellate cells system (Sorkin and Carlton 1998).

Essentially, there are three broad classes of neurones (which have already been mentioned in this section, but which need emphasising) located in the dorsal horn. These neuronal types each enable the transduction and modification of spinal sensory input, in order to appropriately convey individual stimuli from the periphery to higher
centres and exhibit the necessary responses (Light et al. 1979). Furthermore, there are often differences in the species used in studies, so that unveiling the termination patterns of primary afferent neurones in different dorsal horn laminae becomes complex. However, staining studies comparing cat, monkey and rat models have illustrated that such termination patterns in the dorsal horn are remarkably similar (Light and Perl 1979).

- Interneurones – small cells, comprise the majority of fundamental dorsal horn neurones, excitatory/ inhibitory, trans-lamina projections and arborisations
- Propriospinal neurones – contribute to heterosegmental reflexes, long elongated axons
- Projection neurones – project from spinal cord to brain, minor population of dorsal horn neurones.

1.4 Lamina I of the Superficial Dorsal Horn

1.4.1 Lamina I Cell Morphology

Early intracellular staining studies in cats conducted by (Light et al. 1979) used horseradish peroxidase to examine the morphological features of superficial lamina I & II neurones, within the spinal cord. Such studies substantiated evidence that thinly myelinated and unmyelinated afferents, which conduct mechanically evoked stimuli, terminate in the superficial regions of the dorsal horn. The morphology of cells that receive primary afferent neuronal terminations was also revealed (Light et al. 1979). Myelinated nociceptive fibres terminate predominantly on cells with significant dendritic branching throughout lamina I, yet unmyelinated nociceptive fibres excite a population of neurones with extensive branching in lamina II (Light et al. 1979). Lamina I and II clearly represent a crucial centre for the termination of nociceptive primary afferent fibres transmitting biologically threatening noxious information from the periphery (Kumazawa and Perl 1978; Light et al. 1979).

Further studies, using golgi preparations, have investigated the distribution of primary afferents in the marginal zone in monkey and cat models (Beal and Bicknell 1981). These studies have confirmed that small diameter fibres, which terminate in lamina I, originate from the dorsolateral fasciculus of Lissauer (Beal and Bicknell 1981). These fibres form a mass which traverses the most superficial lamina I region of the dorsal
horn, and thickens in bulk in the lateral and widest most section (Beal and Bicknell 1981). Other lamina I afferents located less superficially may arborise with neurones located within lamina II$_o$ (Beal and Bicknell 1981).

Interestingly, lamina I afferent fibres (which often produce small oval disc shaped boutons terminaux) traverse the entire marginal zone layer and lie adjacent to lamina I cell dendrites (Beal and Bicknell 1981). Different classes of spinothalamic lamina I neurones have been examined using retrograde labelling of cat STT neurones (Zhang et al. 1996). Such studies originally concluded that there were three morphologically distinct cell types (Zhang et al. 1996):

- **Fusiform Cells** – Vertically positioned bipolar dendrites and small spindle-like somata (Zhang et al. 1996)
- **Pyramidal Cells** – Predominantly vertical positioned dendrites and three dendritic branches, triangle-shaped somata (Zhang et al. 1996)
- **Multipolar Cells** – Four longitudinal and mediolateral positioned dendritic branches and big multiangular somata (Zhang et al. 1996)

However, the authors did recognise that there were slight variations in multipolar cell morphology, which may give rise to subclasses and in addition there were a small population of cells with changing shapes and unrecognisable soma which therefore remained uncategorised (Zhang et al. 1996). Although the relative distribution of each cell type did vary along the dorsal horn, it appeared as though, from the total population, 36% of all marginal cells were pyramidal, 34% fusiform, 25% multipolar and a small percentage (5%) were left uncategorised (Zhang et al. 1996). Interestingly, further retrograde labelling studies in spinothalamic tract neurones have revealed that similar cell types exist in monkeys, however authors noted both larger divergence and further subtypes (Zhang and Craig 1997). There were a larger overall proportion of fusiform cells in monkey models (47%), less pyramidal cell types (27%) and similar numbers of both multipolar and uncategorised cell groups (22%) (Zhang and Craig 1997). Studies in rat models have shown that there are four distinctive cell types localised in lamina I (Prescott and De Koninck 2002). These are distinguished according to their morphology, yet they also have rather characteristic membrane properties (Prescott and De Koninck 2002). Whole cell patch clamp recordings in adults rat models have
unveiled the presence of four distinct lamina I dorsal horn cell types, each with different response properties to an injected somatic current (Prescott and De Koninck 2002). These distinct populations have been correlated with the previously identified morphologically distinct lamina I cell populations, based on the unambiguous similarities between them (Prescott and De Koninck 2002).

- **Tonic cells** – Continuous slow firing during stimulation, typically fusiform cells (Prescott and De Koninck 2002)
- **Phasic cells** – High frequency bursts with inconsistent firing duration, typically pyramidal cells (Prescott and De Koninck 2002)
- **Delayed onset cells** – Irregular, delayed firing, typically multipolar cells (Prescott and De Koninck 2002)
- **Single spike cells** – One action potential evoked by stimulus application, typically multipolar cells (Prescott and De Koninck 2002)

It may be noted that variations in specie types may account for some differences in lamina I cell morphology accounted in these studies.

### 1.4.2 Lamina I Neurones and the NK1 receptor

A large proportion of lamina I neurones studied in the rat, express the NK1 receptor (80% lamina I projection neurones) and interestingly, these NK1 positive neurones receive large inputs from primary afferent fibres containing substance P (Todd 2002). Interestingly, over 80% of pyramidal cells contain the substance P NK1 receptor (Todd et al. 2002). Such NK1 expressing neurones in the rat can generally be separated into two groups based on differences in both soma and dendritic size, extent, projections and orientation (Cheunsuang and Morris 2000).

Group 1 comprises a population of densely packed NK1 expressing, large neurones with corresponding soma (Cheunsuang and Morris 2000). Such neurones have a single axon and 3-5 foremost, spine free, dendrite branches, each with small amounts of subbranching (Cheunsuang and Morris 2000). These sparsely distributed dendrites protract throughout the mediolateral and rostrocaudal plane (Cheunsuang and Morris 2000). The axon associated with these neurones extend to deeper laminae, with few branches.
Group 2 are less densely packed with NK1 receptors and have protracted spiny dendrites extending predominantly in the rostrocaudal plane (Cheunsuang and Morris 2000). The soma within this group of neurones is fusiform shaped and small (Cheunsuang and Morris 2000).

It is widely believed that NK1 containing neurones receive strong, opioid sensitive, C-fibre inputs (Cheunsuang et al. 2002). In addition, they are activated by noxious stimuli and are associated with the development of hyperalgesia (Littlewood et al. 1995; Cheunsuang et al. 2002; Todd et al. 2002). Interestingly, thermal hyperalgesia is significantly hampered following ablation of NK1 containing neurones in the superficial dorsal horn of rats and further studies have also shown that VR1-positive afferents form synapses onto lamina I spinoparabrachial NK1 positive fibres (Mantyh et al. 1997; Hwang et al. 2003). As mentioned later, Fos immunostaining studies have elucidated that noxious signals emanating from the periphery via evoked stimuli, inflammatory insult or nerve injury are regulated via substance p release (Doyle and Hunt 1999b). Such studies have concluded that NK1 lamina I neurones observed in rat models are distinct in their ability to code the intensity of nociceptive stimuli (Doyle and Hunt 1999b).

Interestingly, very little is known about non-NKI containing neurones and it is therefore believed that NK1 containing neurones underlie the majority of the nociceptive processing attributed to lamina I neuronal functions. Non-NK1 containing neurones in the rat, appear to be comprised of large gephyrin (the glycine receptor-associated protein) containing cells. These cells appear to respond to acute high-threshold stimuli and project predominantly to the parabrachial area, although they only comprise 2% of lamina I spinoparabrachial cells (Puskar et al. 2001; Todd 2002). Furthermore, it is suspected that gephyrin rich neurones are extensively modulated by adjacent inhibitory GABAergic neurones, confirmed by the presence of glutamate decarboxylase on neighbouring axons (Puskar et al. 2001).

1.4.3 Physiological Response Properties of Lamina I Neurones

Early studies reported that within lamina I, there were distinct groups of neurones that should be classified according to their response characteristics, as well as their morphological properties (Christensen and Perl 1970). These studies were the first of a
long line to characterise nociceptive specific (NS) cells, which were responsive to high-threshold mechanical and heat stimuli. Investigations of the response properties of spinthalamic lamina I neurones in the monkey revealed that the majority of STT lamina I neurones were unable to code cutaneous innocuous mechanical, and to some extent, thermal stimuli (Ferrington et al. 1987). However, these neurones could adequately signal cutaneous high threshold mechanical and thermal stimuli, which supported the hypothesis that lamina I neurones are largely composed of nociceptive specific neurones (Ferrington et al. 1987). In addition, early studies recognised the presence of thermoreceptive cells, specifically activated by cutaneous cooling, as well as a subset also responding to other such high-threshold stimuli thus termed polymodal nociceptive (HPC) in recent literature (Christensen and Perl 1970; Craig and Dostrovsky 2001). Interestingly, thermoreceptive cold-specific lamina I neurones in the cat, which project to the thalamus, have long since been reported (Craig and Hunsley 1991; Craig and Bushnell 1994). Recent literature has confirmed the existence of cooling specific lamina I neurones in the monkey, with direct projections to the ventral medial nucleus (VMpo) of the thalamus (Dostrovsky and Craig 1996).

Overall, physiological studies focussing on the response properties of lamina I neurones (particularly spinothalamic tract lamina I neurones in the cat and monkey) have identified four main neuronal populations. These are categorised according to their modality response profiles, thalamic terminations and axonal conduction velocities (Ferrington et al. 1987; Craig and Hunsley 1991; Craig and Bushnell 1994; Craig and Serrano 1994; Dostrovsky and Craig 1996; Han et al. 1998):

- Nociceptive-specific (NS) cells
- Thermoreceptive-specific (COLD) cells
- Polymodal-specific (HPC) cells
- Wide Dynamic Range (WDR) cells

Wide dynamic range cells have also been identified in the monkey and rat (Ferrington et al. 1987; Dostrovsky and Craig 1996; Dickenson et al. 1998). Interestingly, recent studies have suggested that these distinct neuronal populations are anatomically different (Han et al. 1998). In cat studies, fusiform cells were all nociceptive specific neurones, with unmyelinated axons (Han et al. 1998). Pyramidal cells were primarily
thermoreceptive-specific in their response characteristics with myelinated axons, therefore exhibiting faster conduction velocities compared to nociceptive specific neurones (Han et al. 1998). Lastly, multipolar cells appeared to be largely correlated with HPC cells, although only 60% of the multipolar cell population actually exhibited these response characteristics (Han et al. 1998). However, the converse was not always the same, for example out of the nociceptive specific neuronal population, only 67% were fusiform in morphology, the remaining were multipolar (22%) and unclassified (11%) (Han et al. 1998). Interestingly, in this report 47% of the spinothalamic neuronal population characterised were nociceptive specific, 34% were COLD cells and 18% were HPC cells (Han et al. 1998). Furthermore, the population of nociceptive specific neurones could be further categorised into those responding to noxious heat and pinch (44% of NS neurones or 21% of the whole neuronal population), and those merely responding to pinch (56% of NS neurones or 26% of the whole population) (Han et al. 1998). The authors also commented on small receptive field areas of all lamina I neurones recorded (Han et al. 1998).

Other studies have reported further distinct and detailed response characteristics of spinothalamic lamina I neurones. Following application of a maintained high threshold mechanical stimulus, NS cells in the cat exhibit large response properties (Andrew and Craig 2002b). Furthermore, significantly less adaptation to the stimulus and clear coding of the stimulus intensity (as well as the stimulus area size/size of the probe) (Andrew and Craig 2002a), compared to HPC cells is also evident (Andrew and Craig 2002b). ‘Maintained cells’ are cells whereby the evoked responses to maintained mechanical stimuli stay above 50% of the initial response throughout stimulation. These results illustrated that ‘maintained cells’ were comprised largely of NS cells (77% were NS cells) (Andrew and Craig 2002b). ‘Adapting cells’ were predominantly comprised of HPC cells (79% were HPC cells) due to the fast reduction in response magnitude (<50%) following the initial stimulus, a characteristic of these neurones (Andrew and Craig 2002b). Interestingly, in other studies the response characteristics of NS cells after application of graded mechanical stimuli, largely mimics much of the human mechanical pain sensations, thus implicating NS neurones in the transmission of mechanically based nociceptive information (Andrew and Craig 2002a). Much investigations surrounding the response properties of spinothalamic lamina I neurones in cats have also led to the recognition of innocuous warming sensitive superficial dorsal horn neurones with
thresholds between 35-37°C, although these comprise only 2% of a large population studied (474 neurones) (Andrew and Craig 2001a). Furthermore, studies in human models, which elicit a 'first' sharp pain and a stronger 'second' burning pain by application of a heat stimulus (Craig and Andrew 2002), have suggested that these different sensations and their timing correlate with the response properties of NS and HPC cells respectively (Craig and Andrew 2002). Iontophoretic histamine has also been shown to evoke responses in a sub-class of spinothalamic lamina I neurones, thus implicating a small population of STT lamina I neurones in the transmission of itch sensations (Andrew and Craig 2001b).

Although the majority of the work on lamina I neurones have focussed on the response characteristics of STT lamina I neurones in the cat and monkey, recent work has also investigated the physiological properties of lamina I spinoparabrachial neurones in the rat (Bester et al. 2000). This study demonstrated that lamina I spinoparabrachial (spino-PB) neurones had small receptive field areas similar to that reported in STT neurones, had relatively little spontaneous activity and had thinly myelinated axons with conduction velocities between 2.8-27.8 m/s (Bester et al. 2000). Application of electrical stimuli to the rat hindpaw activated unmyelinated and thinly myelinated C- and A-fibre activity in spino-PB neurones, and furthermore these neurones were efficient at coding the intensity of the evoked stimuli (Bester et al. 2000). Interestingly, spino-PB neurones were comprised predominantly of NS cells (75%) which is significantly more than the proportion of STT NS cells (23%) reported in previous studies (Craig and Dostrovsky 2001). These studies did not classify HPC neurones. The rest of the spino-PB neurones characterised, responded to both noxious stimuli and innocuous stimuli (to a lesser extent), suggesting a small proportion of wide dynamic range neurones (Bester et al. 2000). The vast majority (92%) of these spino-PB neurones responded to both mechanical and heat stimuli and a smaller proportion (35%) of the total population also exhibited responses to noxious cold, suggesting the presence of HPC neurones within this population (Bester et al. 2000).

Interestingly, a minor population (8%) of spino-PB neurones could selectively be activated by noxious heat, with no response to evoked innocuous heat, or innocuous and noxious mechanical stimuli (Bester et al. 2000). One of the most intriguing finds was that compared to deep dorsal horn neurones, which classically exhibit a 'wind-up'
response to repetitive noxious stimuli, lamina I spino-PB neurones failed to exhibit ‘wind-up’ (Dickenson and Sullivan 1987; Dickenson 1990; Dickenson 1995b; Dickenson 1995a; Dickenson et al. 1997; Bester et al. 2000). Wind-up is a sharp increase in response to a constant stimulus. These spino-PB lamina I neurones demonstrated a reduced ‘wind-up’ effect which appeared to be characteristic of this neuronal population (Bester et al. 2000).

1.4.4 Lamina I Ascending Projections

Lamina I of the superficial dorsal horn is occupied by a large number of projection neurones (Todd 2002). It has emerged that there are indeed multiple pain pathways projecting from the spinal cord, to the brainstem and to the forebrain (Hunt and Mantyh 2001) (See Diagram 1.4.4.a (Craig 2003b; Craig 2003a)). For a longtime it was assumed that the spinothalamic tract was the predominant pain pathway for most dorsal horn neurones in the spinal cord (Fitzgerald 1989; Willis 1989; Willis and Westlund 1997; Sorkin and Carlton 1998). This assumption was based partly on evidence that thalamic lesions inhibit pain-related behaviours and sensations (Fitzgerald 1989; Willis 1989; Willis and Westlund 1997; Sorkin and Carlton 1998). In addition, following electrical stimulation of the thalamus, pain sensations are significantly enhanced which also strongly implicated this area in pronociception and pain control (Fitzgerald 1989; Willis 1989; Willis and Westlund 1997; Sorkin and Carlton 1998). As lamina I is comprised primarily of projection neurones, the thalamus was therefore implicated as their main projection target. This led to numerous studies assessing the proportion of lamina I neurones projecting to various supraspinal sites involved in pain processing and control, which will be discussed in this section. In the thoracolumbar spinal cord, lamina I neurones project largely to the sympathetic cell columns, before terminating in supraspinal sites thought to be involved in homeostatic functions (Craig 2002).
Diagram 1.4.4.a. Lamina I Projections in the Primate. Taken and adapted from A.D. (Bud) Craig, 2003
Generally, the thalamus, the periaqueductal grey matter (PAG), the lateral parabrachial area (PB), the medullary reticular formation and the nucleus of the solitary tract are the principal areas thought to receive lamina I neuronal projections, as seen in the rat (Menetrey et al. 1980; Cechetto et al. 1985; Tavares et al. 1993; Gamboa-Esteves et al. 2001a; Gamboa-Esteves et al. 2001b; Todd 2002; Todd et al. 2002). Such lamina I pathways are thought to be primarily contralateral (Todd 2002).

Interestingly, it is evident that over half of the projections received by both the thalamus and the brainstem from the spinal cord, are that of lamina I projection neurones confirming that these areas are in fact, major projection targets for lamina I neurones (Craig 2002). However, additional studies and species variations complicate this somewhat simplistic view. Injection of retrograde tracers e.g. cholera toxin B (CTb), into the nuclei of cell bodies has helped clearly unveil the projection sites and their proportions of lamina I neurones (Todd 2002). Such techniques have been performed in rat models, whereby CTb is injected into different areas of the brain, and retrogradely labels contralateral lamina I spinal neurones (Todd 2002). This provides a good estimate of the varying proportions of lamina I projections in these injected brain areas. As a result, a large proportion of labelled neurones are observed following CTb application in the caudal ventrolateral medulla (CVLM) and the lateral parabrachial area (7.8-11 cells/ 70 μm and 8.4-9.8 cells/ 70 μm transverse section respectively) (Todd 2002). Less dense labelling is also observed following injections to the PAG and lumbar enlargement of the thalamus (1.6-3.8 and 0.3 cells per / 70 μm transverse section respectively) (Todd 2002). These studies suggest that overall, lamina I spinothalamic tract neurones make up a small proportion of lamina I projection neurones in rat models. It is also evident that spinomedullary and spinoparabrachial lamina I projection pathways predominate (Todd 2002).

Further studies have confirmed that a very large number of lamina I projection neurones converge bilaterally in the superficial region of the parabrachial area. This is seen in both cat and rat models (Burton et al. 1979; Cechetto et al. 1985; Hylden et al. 1985; Swett et al. 1985; Swett and Woolf 1985; Bernard et al. 1989; Blomqvist et al. 1989; Kitamura et al. 1993; Bernard et al. 1995; Saper 1995).
This pathway is therefore largely implicated in nociception and pain processing (Blomqvist et al. 1989; Hunt and Mantyh 2001). Interestingly, the neurones that comprise this spinoparabrachial pathway are thought to be primarily NK1 containing lamina I neurones (Hunt and Mantyh 2001). The parabrachial area is composed of many neurones with projections to the medullary reticular formation and pons, therefore exhibiting a role in homeostatic control. Furthermore the numerous PB neurones which project to the hypothalamus, amygdala and thalamus also largely implicate a homeostatic role for PB neurones in pain processing (Willis 1989; Craig and Dostrovsky 1998; Craig 2003b; Craig 2003a).

The use of Phaseolus vulgaris-leucoagglutinin (PHA-L) injections in rats, have demonstrated less dense, yet extremely prominent lamina I projections in the ventral segment of the lateral quadrant of the PAG (via the spinomesencephalic tract) in similar retrograde tracing studies. Such studies therefore support the findings mentioned above (Bernard et al. 1989; Bernard et al. 1995). Indeed, it has been suggested that the PAG receives almost three times the number of projection neurones which converge in the thalamus of cat models. The PAG therefore comprises a major projection pathway originating from lamina I, for pain transmission and sensory processing (Mouton and Holstege 1998). It is important to note that the PAG is a major integration area for both limbic motor output and homeostatic processing (Craig and Dostrovsky 1998).

Lamina I spinomedullary projection neurones in the cat have been characterised and these lamina I neurones appear to project specifically to the ventrolateral medulla and the A1 noradrenergic cell populations, which are involved in both the function of the cardiorespiratory system and neuroendocrine responses respectively (Andrew et al. 2003). A large proportion of the neuronal projections from the spinal cord terminating in this area of the medulla, originate from lamina I and are quite distinct from the extensively studied spinothalamic tract neurones (Andrew et al. 2003). Interestingly, lesions made in the medullary dorsal reticular nucleus have been shown to reduce painful responses and is thus also an area implicated in pain control, as is the ventrolateral medulla in rat studies discussed above (Gauriau and Bernard 2002; Lima and Almeida 2002; Todd 2002). These findings therefore support a clear role of lamina I projection neurones in homeostatic regulation of such organisms (Andrew et al. 2003; Craig 2003b).
Much work has been done to unravel the projection sites of lamina I spinothalamic and trigeminothalamic projection neurones in the cat using antidromic activation of such neurones (Craig and Dostrovsky 2001). This is despite evidence that the spinothalamic tract may not be the principal ‘pain’ pathway that originates from superficial lamina I neurones. Intriguingly though, it has been proposed that different physiological classes of lamina I projection neurones, may have distinct projection sites within the thalamus (Craig and Dostrovsky 1998).

The submedius nucleus (Sm), the ventral aspect of the ventroposterior complex (vVP) and the dorsomedial aspect of the ventroposterior complex (dmVPM) have long been implicated as main projection targets within the thalamic region of the brain in cat models (Craig and Dostrovsky 1998; Craig and Dostrovsky 2001). Interestingly, thermoreceptive cells appear to have relatively equal projections to all three thalamic regions (20/23, 21/23, 17/23 to dmVPM, vVP and Sm respectively) (Craig and Dostrovsky 2001). Yet nociceptive specific (NS) neurones project largely to the vVP (9/9) and Sm (9/9) regions and to a far less extent to the dmVPM (1/9) (Craig and Dostrovsky 2001). HPC cells project predominantly to the vVP and Sm (7/8 and 6/8 respectively) and not to the dmVPM (0/8) (Craig and Dostrovsky 2001), implicating distinct differences in the projection targets of physiologically different lamina I projection neurones (Craig and Dostrovsky 2001). In addition, it may suggest that although the thalamus is not the main projection target of lamina I neurones, it may serve some specific function in distinguishing the neuronal response characteristics of lamina I cells.

In monkeys lamina I neurones have been shown to terminate in the ventral posterior lateral nucleus of the thalamus (Willis et al. 2001). The ventral medial nucleus (VMpo) of the posterior thalamus also receives projections from NS and thermo-receptive specific lamina I neurones. These lamina I neurones are thought to originate from the medulla and spinal dorsal horn, and terminate in the VMpo, which is an area strongly implicated in both thermal and pain sensations (Craig et al. 1994; Blomqvist et al. 1996; Zhang and Craig 1997; Craig et al. 1999; Blomqvist et al. 2000). Furthermore, the VMpo as well as the ventrocaudal mediodorsal nucleus (MDvc) and the ventral posterior inferior nucleus (VPI), project to specific cerebral cortical areas strongly stimulated by both noxious and heat stimuli, as illustrated in human imaging studies (Friedman and Murray 1986; Jones 1991; Casey et al. 1994; Craig et al. 1994; Craig 1995; Craig et al. 1996;
Blomqvist et al. 2000). The complicated pathways and the intricate web of neural connections between such spinal and supraspinal lamina I projection targets and other areas of the brain, mean that the processing of noxious stimuli from the periphery via lamina I is complex and worth a lot of thought. The unpleasant sensations and displeasing ‘feelings’ following application of a painful or noxious stimuli may implicate these lamina I central projection areas associated with homeostasis and emotional conditions (Craig 2002; Craig 2003b). However, once again one must be extremely cautious with regard to species variations. Also, little is known about potential changes in lamina I after pathology such as nerve injury.

1.5 What is Neuropathic Pain?

Neuropathic pain is the term used to describe the wide range of sensory abnormalities resulting from dysfunction or damage to the peripheral or central nervous system, caused by a diverse heterogeneous range of conditions. It has been classically defined as ‘Pain initiated or caused by a primary lesion or dysfunction in the nervous system’ and affects approximately 1% of the UK population (Bowsher 1991). Both the aetiology and topography of neuropathic pain are diverse and remain poorly understood by both scientists and clinicians, consequently the broad range of symptoms arising from this condition are still hard to treat. The problem is not just based on the unpleasant sensations experienced by patients, but also associated anger, depression and sexual problems (Monga et al. 1998; Greenwood et al. 2003), as well as significant impacts on the sufferers daily life and work performance (Blyth et al. 2003). Studies have observed the coping ability of patients with chronic pain and have also evaluated the use of individual pain coping assessments, but are as yet inconclusive and demonstrate little correlation between coping responses and any adjustment by the sufferer, to neuropathic pain (Tan et al. 2001).

The assortment and anatomical origin of lesions that result in neuropathic pain are numerous. Clinically, neuropathic pain syndromes can be categorised according to their anatomy or aetiology (Jensen et al. 2001b), however other classification schemes have been proposed based on underlying mechanisms (Woolf et al. 1998) or treatments (Sindrup and Jensen 1999; Sindou and Mertens 2000).
Peripheral Spinal Brain

Neuropathies Multiple sclerosis Stroke
Herpes Zoster Spinal cord injury Multiple sclerosis
Nerve Injuries Arachnoiditis Neoplasms
Amputations Neoplasms Syringomyelia
Plexopathies Syringomyelia Parkinson's disease?
Radiculopathies Spinal stroke Epilepsy?
Avulsions
Neoplasms
Trigeminal neuralgia

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Table 1.5. a. Classification of neuropathic pain, based on the location of neuronal damage and pathology. Taken from (Jensen et al. 2001b).

It is clear from the above classification scheme that the underlying origins of neuropathic pain are multiple (Devor 1991; Bennett 1998; Chinyanga et al. 2000; Baheti 2001; Bouhassira 2001; Bridges et al. 2001; Koltzenburg and Scadding 2001; England and Gould 2002). The variety of neuronal insults that can lead to neuropathic pain can be categorised (Devor 1991; Bennett 1998; Chinyanga et al. 2000; Baheti 2001; Bouhassira 2001; Bridges et al. 2001; Koltzenburg and Scadding 2001; England and Gould 2002). Neuropathic pain may stem from a variety of conditions, e.g. accidental damage (injury, burns and trauma), chemical damage (chemotherapy and toxins), viral damage (HIV, herpes zoster) (Devor 1991; Bennett 1998; Baheti 2001; Bouhassira 2001; Bridges et al. 2001; Koltzenburg and Scadding 2001; England and Gould 2002). Furthermore, neurological conditions (Parkinson's disease), cancers (tumour compression), polyneuropathic conditions and vascular diseases (diabetes, stroke-induced ischaemia) can also result in neuropathic pain syndromes (Devor 1991; Bennett 1998; Chinyanga et al. 2000; Baheti 2001; Bouhassira 2001; Bridges et al. 2001; Koltzenburg and Scadding 2001; England and Gould 2002). Whatever the cause, neuronal insult can be focal or widespread, depending on the extent, progression and source of neuronal damage within the nervous system. Degenerative autoimmune diseases e.g. diabetic neuropathy and multiple sclerosis (MS) can often result in escalating and ongoing damage to the nervous system (Devor 1991; Bennett 1998; Chinyanga et al. 2000; Baheti 2001; Bouhassira 2001; Bridges et al. 2001; Koltzenburg and Scadding 2001; England and Gould 2002).
In general, the syndromes underlying neuropathic pain states can be separated into positive and negative symptoms. Sensory loss and apoptosis of neurones, both central and peripheral, following nerve damage (Gillardon et al. 1996; Azkue et al. 1998; Zimmermann 2001) may explain the negative symptoms (sensory deficits) consistently reported by patients. The degree of sensory loss may vary according to the extent and type of damage and it may also involve some or all sensory modalities (thermal, mechanical etc). Interestingly, some studies have reported that spinothalamic activity is critical to the maintenance of sensory function and loss of spinothalamic activity will therefore result in sensory deficits (Jensen and Lenz 1995). Paradoxically, excessive and spontaneous neuronal activity, following damage, contribute towards identification of the positive symptoms (dysesthesia, paresthesia, hyperpathia, paroxysms, referred pain, allodynia and hyperalgesia) of neuropathic pain (Chong and Bajwa 2003).

Clinically, spontaneous ongoing pain distinguishes neuropathic pain types from other pain categories (Jensen et al. 2001b). Such spontaneous pain conditions may be termed stimulus independent or stimulus dependant (Jensen et al. 2001b). Stimulus independent type pain describes the continuous or paroxysmal shock-like, cramping or burning sensations prevalent in entrapment and amputation based neuropathies (Nikolajsen and Staehelin Jensen 2000; Jensen et al. 2001b). More specifically, paroxysmal spontaneous pain, often described as 'shooting electric pulse like' in nature, is characteristically episodic and shorter lasting than continuous pain. Continuous spontaneous pain is a more common persistent ongoing pain experienced by patients suffering from neuropathic syndromes (Jensen et al. 2001b). Continuous pain is often concentrated around the area innervated by the damaged nerve, and consequently such burning, stabbing, aching, smarting and cramping sensations are described by affected patients in these areas (Jensen et al. 2001b). The mechanisms underlying the development of such stimulus independent pain types will be discussed later, however hyperexcitability appears to play a key role in such processes (Jensen et al. 2001b). Stimulus dependant type pain, as the name suggests, is categorised based on the type of stimulus that evokes it i.e. mechanical or thermal and may sometimes only occur in the presence of the stimuli (Jensen et al. 2001b). Nevertheless, resulting symptoms are often confusing.
Allodynia is the perception of pain resulting from a normally innocuous stimulus (of whatever nature). For this reason, normal innocuous tactile sensations such as wearing clothes and gentle brushing, is now very painful for these patients and represents a qualitative change in such pain perception. Disturbances in the neurochemistry of the spinal cord have been implicated in the development of such neuropathic pain based symptoms and a variety of studies have investigated such theories. Alterations in GABA, glycine and glutamate have been suggested in the development of allodynia-like responses following nerve damage (Onaka et al. 1996). However, more importantly, alterations in the neuroanatomy of the spinal cord have also been given implicated in the development of such neuropathic pain symptoms. It has been suggested that low threshold A-β fibres mediate allodynia (Ossipov et al. 1999), and there has been debate as to whether A-fibre sprouting into lamina II of the dorsal horn, which receives input from nociceptive C-fibres, is responsible for tactile allodynia (Mannion et al. 1996; Mannion et al. 1998). This will be discussed later in more detail.

Studies using a partial sciatic nerve ligation model (see section 1.6.2.), have suggested that allodynia may be associated with heightened neuronal mechanosensitivity, leading to chronic pain. (Takaishi et al. 1996). Other studies, using spinal nerve ligation models of neuropathy, have documented modified firing properties of dorsal root ganglion neurones following nerve damage (Liu et al. 2000a). This indicates that central sensitisation evoked by ectopic activity in damaged myelinated afferent fibres, may be partially responsible for observed tactile allodynia (Liu et al. 2000a). This is supported by studies performed in nerve transection models (refer to section 1.6.), whereby ectopic mechanosensitivity and spontaneous discharge are observed in a range of myelinated afferent fibres following nerve insult, to varying extents (Tal et al. 1999). Most intriguingly is the finding that following peripheral neuropathy, the development of mechanical allodynia is more strongly established in older rats (16 – 18 months old), compared to young adult rats (7-8 weeks old) (Kim et al. 1995). However, there was no detected difference in the development of thermal allodynia.

The proposal that following axonal injury, modifications in electrophysiological properties of primary sensory neurones may be responsible for neuropathic pain type symptoms, led to further electrophysiological experiments using spinal nerve transection models (refer to section 1.6.) (Kim et al. 1999b).
Following S1 spinal nerve transaction, rats exhibiting cold allodynia, showed alterations in A-δ type DRG cells after intracellular recordings were made from excised S1 dorsal root ganglia (Kim et al. 1999b). It was suggested that the generation of cold allodynia may be due to changes in the membrane properties of A-δ cell somata and dorsal root axons (Kim et al. 1999b). Furthermore, tactile allodynia may be predominantly dependant on activation of descending facilitation's by supraspinal sites via the dorsolateral funiculus, rather than spinal sites (Ossipov et al. 2000). Other studies have also examined the function of the supraspinal sites in the development of allodynia. Spinal dorsal columns as well as areas of neuronal termination, including the nucleus gracilis, following spinal nerve ligation, have been implicated in the development of tactile allodynia (Sun et al. 2001).

Hyperalgesia (whether thermal or mechanical) describes the heightened intensity of pain perceived following application of a suprathreshold stimulus and demonstrates augmented pain sensitivity to noxious stimuli in a quantitative fashion. The mechanisms responsible for hyperalgesia appear to be different from those causing allodynia (described above). Thus studies have proposed that increased neuronal sensitivity, a key factor in the development of tactile allodynia following nerve damage, is not responsible for thermal hyperalgesia. (Takaishi et al. 1996). The other terms mentioned above, including paraesthesias and dysesthesias describe the wide variety of other abnormal sensations experienced by a patient suffering from neuropathic pain, and are described in more detail in clinically based papers. Abnormal sensations resulting from nerve damage are not always painful, but often unpleasant, atypical and novel for the previously healthy individual, therefore resulting in a perception of ill health.

Treatment is as difficult to prescribe as is the aetiology and symptoms of neuropathic pain to interpret. In most cases, the type and extent of neuronal damage, and the resulting symptoms decide what form of treatment the clinician prescribes. Most commonly, treatment can be classified as non-invasive and invasive (Chong and Bajwa 2003).
Non-invasive therapies, used to manage neuropathic syndromes, range from the traditional transcutaneous nerve stimulation (TENS) devised in the 1960's, to more modern pharmacological interventions such as antiepileptics and ion channel blockers (carbamazepine, phenytoin, lidocaine), antiarrhythmics (mexilitine, lidocaine), corticosteroids, antidepressants (amitriptyline, desipramine) and GABA<sub>B</sub> agonists (baclofen) (Chong and Bajwa 2003). Invasive treatments such as nerve modulation and neural ablation (dorsal rhizotomy, thalamotomies, frontal lobotomies and spinal dorsal root entry zone lesions) are less commonly utilised in the treatment of neuropathic pain syndromes due to the increased risk involved in enhancing the initial problem (Sindou and Mertens 2000; Chong and Bajwa 2003). These treatment methods are described in section 1.8. in more detail.

As a result of the vast causes, underlying mechanisms and symptoms of neuropathic pain, and the difficulty in finding an effective and specific treatment to control these reported painful conditions, many animal models have been devised and studies been performed to provide a better understanding in this most confounding field.

1.6 Animal Models of Neuropathic Pain

There are now various animal models of neuropathic pain used to study the anatomical, neurochemical, molecular and behavioural changes that arise following nerve injury evoked by various mechanisms and which contribute to the development of neuropathic pain related sensory abnormalities and symptoms. Animal models can be separated into those evoking peripheral and central nerve injuries. These models can also be distinguished by the causal processes which underlie the development of neuropathic pain i.e. diabetic neuropathy, spinal cord injury and post-herpetic neuralgia (Ahlgren and Levine 1993; Fleetwood-Walker et al. 1999; Vierck et al. 2000). However the focus of this PhD is peripheral nerve injury, in particular sciatic nerve damage which is widely
1 - Spinal Nerve Ligation Model (SNL)
2 - Partial Sciatic Ligation (PSL)
3 - Chronic Constriction Injury (CCI)
4 - Spared Nerve Injury (SNI)

A - Common Peroneal  B - Tibial  C - Sural

used and has been shown to induce a wide variety of abnormal continuous and induced pain conditions (Kim et al. 1997a) (See Diagram 1.6.a for the more popular animal models used (Flatters 2002)).

The most popular animal models of peripheral nerve injury models include:

- Chronic Constriction Injury (Bennett & Xie, 1988)
- Partial Sciatic Nerve Injury (Seltzer, 1990)
- Spared Nerve Injury Models (Decosterd and Woolf 2000)
- Segmental Spinal Nerve Ligation (Kim and Chung 1992)
- Sciatic Cryoneurolysis (SCN) model (DeLeo et al. 1994)
- Photochemical Sciatic Nerve Ischeamic Lesion Model (Gazelius et al. 1996)
- Experimental Neuritis Models (Elia et al. 1999)
- Partial Sciatic Nerve Transection (Lindenlaub and Sommer 2000)
- Clip Compression Injury (Bruce et al. 2002)
- Photochemical Induced Ischemia of Sciatic Nerve (Mouse Model) (Hao et al. 2000)
- Sciatic Inflammatory Neuritis Models (Chacur et al. 2001)
- Tibial Nerve Injury (Hofmann et al. 2003)

1.6.1 Chronic Constriction Injury (CCI) Model

This is informally known as the Bennett model, after Bennett and Xie who first developed the model having felt that previous animal models did not exhibit behavioural symptoms common in neuropathic pain patients in the clinic (Bennett and Xie 1988). Loose constrictive ligations are positioned around the common sciatic nerve of the rat during anaesthesia. This has been shown to result in the development of certain behaviours therefore suggesting the occurance of neuropathic pain (Bennett and Xie 1988). Interestingly, the authors reported early development of thermal hyperalgesia, mechanical allodynia as well as spontaneous pain, inferred by repetitive impulsive nocifensive responses and a distinct lack of appetite in numerous rats (Bennett and Xie 1988). Since the development of this model almost 15 years ago, there have been reports that the Bennett and Xie model does not evoke allodynia in 100% rats, which have undergone the procedure (Gazelius et al. 1996; Cui et al. 2000). In comparison to other animal models such as spinal nerve ligation and partial sciatic ligation, as yet to be
discussed, the CCI animal models developed less mechanical allodynia. However, ongoing pain characterised and quantified by 'cold stress exacerbated ongoing pain and spontaneous pain' tests first described by (Choi et al. 1994), suggest that the Bennett and Xie procedure is the most efficient at producing ongoing pain characteristics in rat models (Kim et al. 1997a).

1.6.2 Partial Sciatic Nerve Ligation (PSL) Model

First described thirteen years ago by (Seltzer et al. 1990), the partial sciatic nerve injury model of neuropathic pain represented a novel model of partial injury known to cause causalgia in humans (Seltzer et al. 1990). Half of the rat sciatic nerve is unilaterally ligated, and consequent licking and guarding is observed in the affected rat hindpaw thereafter (Seltzer et al. 1990). Both thermal and mechanical hyperalgesia and mechanical allodynia is evident in these rats, as well as spontaneous burning pains and causalgia (Seltzer et al. 1990). However, contradictory studies claim that PSL does not always result in the development of mechanical allodynia and may not be an entirely efficient model of neuropathy (Cui et al. 2000). However, when compared to the Bennett model described above, PSL animal models appear to exert more conspicuous mechanical hyperalgesia, noticeable almost immediately after surgery has taken place (Kim et al. 1997a).

1.6.3 Spared Nerve Injury (SNI) Model

Spared Nerve Injury (SNI), one of the most recently developed models described in this section, is an alternative to partial denervation whereby the tibial and common peroneal nerve terminal branches of the sciatic nerve are lesioned (Decosterd and Woolf 2000). In this model, the sural branch of the sciatic nerve is left undamaged. Interestingly, the attraction of this model is that association of degenerating axons with unscathed distal axons is limited, compared to other animal models of peripheral nerve injury (Decosterd and Woolf 2000). Thus, undamaged cutaneous receptive field areas lying in close proximity to denervated patches on the rat hindpaw, can be tested and compared (Decosterd and Woolf 2000).
Operated animals exhibited both short and long term behavioural changes, as well as mechanical allodynia which was characterised by lowering thresholds to mechanical von Frey stimuli in the sural receptive field area of the affected hindpaw (Decosterd and Woolf 2000). Interestingly, no change was seen in the heat thresholds upon application of thermal stimuli, yet responses to such stimuli were generally increased in the ipsilateral hindpaw areas, innervated by the sural nerve (Decosterd and Woolf 2000). Such changes were less obvious upon stimulation of the saphenous nerve receptive field areas in the ipsilateral affected hindpaw (Decosterd and Woolf 2000).

1.6.4 Spinal Nerve Ligation (SNL) Model

Probably the most frequently used model for studying nerve injury and resulting neuropathic pain, is the Spinal Nerve Ligation (SNL), or Chung model of neuropathy (Kim and Chung 1992). There are two subtle variations of this model, both of which have been used in experimental studies. Tight ligation of both L5 and L6 spinal nerves or just the L5 spinal nerve is performed, however both models refrain from exerting any damage to the adjacent L4 spinal nerve (Kim and Chung 1992). Persistent thermal hyperalgesia, as well as mechanical allodynia is evident in both models in the ipsilateral hindpaw, lasting between 5 – 10 weeks following surgery (Kim and Chung 1992). However more recent studies suggest that although mechanical and cold allodynia are apparent, thermal hyperalgesia is absent following spinal nerve ligation of L5 and L6 spinal nerves (Kontinen et al. 1998). Licking and clenching deformities of the hindpaw are witnessed in this model and such behaviour indicates that spontaneous pain, representing that seen in human models of causalgia, develops (Kim and Chung 1992). More recent electrophysiological studies have also found that deep dorsal horn neurones become spontaneously active following spinal nerve ligation of L5 and L6, and the receptive field size on the ipsilateral hindpaw is significantly larger in response to innocuous mechanical stimuli (Suzuki et al. 2000). This may suggest both allodynia and central sensitisation arise following such neuronal insults (Suzuki et al. 2000). The type of sensory abnormalities and behavioural changes evoked in both forms of SNL model are similar, however ligation of L5 alone appears to result in behavioural and sensory changes of a noticeably smaller magnitude compared to L5 and L6 SNL models (Kim and Chung 1992).
Interestingly, when compared to the Bennett and Seltzer models of nerve injury, the SNL model appears to result in the most developed mechanical allodynia, however cold allodynia appears to be of similar intensity in all the models tested (Kim et al. 1997a). Following lumbar sympathectomy, mechanical allodynia and other evoked pain conditions reported in these models were most profoundly depressed in the SNL model described here, compared to Bennett and Seltzer models (Kim et al. 1997a). More recent studies report that following spinal nerve ligation of L5, rats retain their increased sensitivity to noxious or non-noxious mechanical stimuli (Ringkamp et al. 1999). Such differences may be due to the subtle variation of the SNL model used, or that the SNL model simply is not a predictable and fail-safe model of sympathetically maintained pain as suggested by (Ringkamp et al. 1999).

### 1.6.5 Sciatic Nerve Injury – other models

A wide variety of other animal models of nerve injury have also been developed, but are less widely used. Neuropathic pain can be mimicked in animals using a variety of novel techniques such as ischaemic lesions (the Gazelius model) or freezing of the sciatic nerve (Sciatic Cryoneurolysis (SC) model), demonstrated by the emergence of mechanical allodynia and other behavioural changes (DeLeo et al. 1994; Gazelius et al. 1996). More recently, another model has been developed to reproduce inflammation of damaged peripheral nerves and the ensuing changes which result in painful neuropathies (Eliav et al. 1999). Experimental Neuritis is a technique used to study such peripheral nerve damage evoked by inflammation, and this involves surrounding the sciatic nerve with hemostatic oxidised cellulose bathed in either carageenan (CARRA) or Freunds adjuvant (CFA) (Eliav et al. 1999). In the ipsilateral hindpaw, such animals demonstrate both heat and mechanical hyperalgesia, as well as mechanical and cold allodynia (Eliav et al. 1999). Partial Sciatic Nerve Transection (PST) developed by (Lindenlaub and Sommer 2000) is an animal model based on that developed by (Seltzer et al. 1990). However this involves complete cut of the sciatic nerve in female rats (Lindenlaub and Sommer 2000). These rats develop both mechanical allodynia and thermal hyperalgesia in the affected hindpaw region, similar to other animal models. The PST model is thought to be superior in comparison, as it does not require the addition of foreign substances to the nervous system in order to evoke nerve injury (Lindenlaub and Sommer 2000). Foreign substances may result in inflammation,
hypersensitivity and resulting neuronal plasticity, which is often difficult to distinguish behaviourally, from that caused by the developmental nerve injury (Lindenlaub and Sommer 2000).

Many other models, as listed above, have also been developed to mimic both peripheral (most commonly via insult to the sciatic nerve) or central neuropathic behavioural changes experienced by humans in the clinic. All the models mentioned above have pros and cons, and the best animal model to use in order to study nerve injury induced neuronal changes, is frequently a topic of much debate in the scientific world.

1.7 Mechanisms of Neuropathic Pain

1.7.1 Anatomical Changes following Peripheral Nerve Damage

Damage to a peripheral nerve, often results in damage to the surrounding axon. The consequence of any axon damage is the loss of axoplasm, normally contained within the axonal membrane, before the axon membrane is able to repair (Garry and Tanelian 1998). Axoplasm is important for the shape of the axon and also forms a pathway for vital cellular components and organelles between the cell body and the rest of the axon, termed ‘axonal transport’ (Garry and Tanelian 1998). ‘Neuroma endbulbs’ are common on segments of intact axons surrounding the area of damage (Fried et al. 1991; Garry and Tanelian 1998). Neuromas, which are a swelling of the axon terminals, seem to be a consequence of the relentless axonal transport system vital to the neurones survival and this is confirmed by the presence of a dense population of membrane bound organelles (Fried et al. 1991). However, neuromas do not contain myelin, unlike the original axon segment, yet ultrastructural studies support the theory that following some forms of nerve damage, Schwann cell cytoplasm encompasses these demyelinated endbulb swellings (Carlton et al. 1991; Fried et al. 1991). Interestingly, recent studies have quashed previous theories regarding the mechanism of demyelination, and have lent support to the theory that macrophages, which pervade areas of neuronal damage, aid the destruction of myelin on injured axons (Fried et al. 1991; Stoll et al. 1993a; Stoll et al. 1993b). Phagocytosis of myelin fragments prevents replenishment of myelin on axon terminals, as myelin debris is required for its synthesis (Stoll et al. 1993a; Stoll et al. 1993b). Interestingly, immunocytochemical studies have confirmed the presence and
production of tumour necrosis factor –alpha (TNF-α) in macrophages (Stoll et al. 1993a; Stoll et al. 1993b). Such studies observed that following demyelination, occurring after nerve transection, macrophages travelling in and out of nerves no longer produced TNF-α (Stoll et al. 1993a; Stoll et al. 1993b). Yet, macrophages detected inside damaged nerve fibers still contained TNF-α (Stoll et al. 1993b). This indicates that following axotomy, TNF-α synthesised by macrophages, assists myelin degradation (Stoll et al. 1993b). In the chronic nerve compression (CCI) model, extensive apoptosis of Schwann cells along with an increase in Schwann cell numbers, arising in the absence of axon degeneration and swelling has been shown (Gupta and Steward 2003). These studies implicate alterations in Schwann cell turnover with limited damage to the axon. Such studies also proposed that application of a mechanical stimulus aided this turnover effect (Gupta and Steward 2003).

Interestingly, there is considerable anatomical evidence that loss of A-β and A-δ fibre populations (55% - 89%) incurs following axotomy (Garry and Tanelian 1998). Furthermore, after nerve damage has insued, spontaneous activity in myelinated A-fibres and the emergence of abnormal pain responses (e.g. hyperalgesia and allostynia, common in neuropathic pain patients) simultaneously develops (Garry and Tanelian 1998). Interestingly, cell death within the DRG itself has been observed in some separate cases, however such observations appeared to be more pronounced in the neonatal rat as opposed to the adult rat (Schmalbruch 1987; Schmalbruch 1988). Interestingly, the depleted supply of target-derived trophic factors following peripheral nerve damage may underlie the death of these sensory neurones (Garry and Tanelian 1998). More recent studies have illustrated apoptosis in neurones and oligodendrocytes following spinal cord injury, which is initiated by TNF-α acting as an external signal (Yune et al. 2003).

After spinal cord injury, such TNF-α induced apoptosis may be regulated by iNOS (Yune et al. 2003). iNOS is up-regulated following SCI, and this results in increased nitric oxide production and thus increased TNF-α levels (Yune et al. 2003). The dorsal root ganglia also appears to be anatomically altered after nerve damage, following a small decrease in the size of the DRG, there is an increase in the DRG cell body size (Garry and Tanelian 1998). As well as alterations in DRG size, there are also reported aberrations in the nucleus of DRG neurones, possibly due to metabolic changes within the dorsal
root ganglia as a whole (Garry and Tanelian 1998). Not only are there alterations in the axon segments of peripherally damaged nerves and dorsal root ganglia cell death, there is substantial evidence that in the CCI model, degeneration of cutaneous nerve terminals occurs (Lin et al. 2001). Immunocytochemical staining with pan-axonal markers have shown partial denervation of the epidermis and it appears that this anatomical alteration encourages the development of thermal hyperalgesia, and thus neuropathic pain (Lin et al. 2001).

Axonal and collateral sprouting and regeneration represent distinct central anatomical changes that arise following spinal cord injury (Hill et al. 2001). It is clear now that descending spinal axons are able to regenerate in axotomy models of spinal cord injury (Hill et al. 2001). Previous studies have observed lengthening and sprouting of brainstem and propriospinal axons, following schwann cell and olfactory ensheathing glia transplantation into axotomised areas (Xu et al. 1995; Chen et al. 1996; Menei et al. 1998; Ramon-Cueto et al. 1998; Hill et al. 2001). Similarly, administration of neurotrophins and reductions in inhibitory neurotransmitter systems encourage sprouting of corticospinal tract axons in the spinal cord, in some injury models (Schnell and Schwab 1993; Schnell et al. 1994; Bregman et al. 1997; Hill et al. 2001). Interestingly, in one study, application of brain-derived neurotrophic factor, neurotrophins -3, and -4 as well as ciliary-derived neurotrophic factor resulted in augmented ingrowth of serotonergic, noradrenergic and corticospinal axons into areas where such neurotrophins were administered (Bregman et al. 1997). Although solitary administration of CDNF alone failed to induce such axonal sprouting (Bregman et al. 1997).

Contusive Spinal Cord Injury models (SCI) of neuropathy have been utilised to study axonal sprouting, as the development of chronic lesion cavities in the spinal cord, which interestingly have a spared fibre surrounding, are a common phenomenon in such models (Hill et al. 2001). Axons project from this spared fibre surrounding to the cavity nucleus via tissue bridges, and thus separate the centre of the cavity into various sections called trabeculae (Hill et al. 2001). Between 1 day – 8 months following injury, neurones in the corticospinal tract progressively begin to ‘dieback’ and retraction bulbs also appear (Hill et al. 2001). Between 3 and 12 weeks, such corticospinal axons then begin to sprout, eventually making connections with the lesion site (Hill et al. 2001). After a 3 month period, reticulospinal fibres then collateralise and also make connections
in similar areas (Hill et al. 2001). These studies firmly indicate that brainstem and cortically derived axons are able to regenerate and collateralise into damaged spinal cord areas after spinal cord injury (Hill et al. 2001).

1.7.2 Neurochemical Changes

Neurotrophins are a subset of neurotrophic factors, which help growth, support and are vital for the existence of neurones in the central and peripheral nervous system (Boucher and McMahon 2001). Nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 and neurotrophins-4/5 are all vital for the maintenance and also the role that primary sensory neurones play, within the PNS (Snider and Silos-Santiago 1996; Snider and Wright 1996; Carroll et al. 1998; Patel et al. 2000). Not only do these neurotrophins appear to have vital roles in neuronal survival, but the growth-derived neurotrophin factors (GDNF family e.g. neurturin, GDNF, persephin and artemin) also play a distinct role in neuronal existence (Boucher and McMahon 2001). Such is the role of GDNF in the nervous system, that depletion is a factor in the emergence of some sensory abnormalities (Boucher and McMahon 2001). Administration of GDNF following peripheral nerve injury appears to prevent development of both mechanical and thermal based hyperalgesia and thus exerts substantial analgesia in these rat models (Boucher and McMahon 2001). Following peripheral nerve injury, it is believed that NGF is retrogradely transported to damaged spinal nerves as studies have reported raised levels of NGF compared to control groups (Oh et al. 2000). However, in the DRG, NGF levels remain the same as non-injured groups, suggesting NGF synthesis occurs within the DRG following peripheral nerve damage (Oh et al. 2000).

Overall there appears to be some distinct changes in the level of certain neurotrophic factors after nerve injury, and so far application of key neurotrophic factors may influence some neuropathic sensory abnormalities (Zhou et al. 2000). Studies whereby neurotrophic factors are neutralised with antibodies administered to injured DRG's, suggest a role for neurotrophic factors in the development of aldynia following nerve injury (Zhou et al. 2000). Removal of NGF as well as NT-3 and BDNF appears to reduce allodynia-like behaviours following nerve injury, at various time course following the initial insult (Zhou et al. 2000). Confirming this effect, direct application of BDNF or NGF to damaged DRGs promotes the development of a prolonged mechanical aldynia (Zhou
et al. 2000). These studies, as a whole, implicate neurotrophic factors in the development of neuropathic pain. Other neurochemical changes also appear to take place following nerve injury. Studies have observed increases in the levels of cyclooxygenase (COX)2 within macrophages and other cell types, in areas of peripheral nerve injury (Ma and Eisenach 2002). Interestingly, these same studies also identified increases in COX1 levels in the ipsilateral hindpaw epidermis as well. Nonselective COX inhibitors hamper the development of alldynia when applied to the injury site or alternatively to the affected hindpaw region (Ma and Eisenach 2002). Upregulation of COX1 and 2 may therefore result in the over-production of prostaglandins. Upregulation of peripheral prostaglandins thus appear to have a distinct role in the development of alldynia, these studies suggest that prostaglandins are involved in sensitisation of peripheral nociceptors and central sensitisation at the spinal cord level (Ma and Eisenach 2002). Ever more intriguing is the changes in neuropeptide levels following peripheral nerve injury. Raised levels of vasointestinal peptide (VIP) and neuropeptide Y are observed in ipsilateral DRG following both complete sciatic nerve transection and chronic constriction injury (Ma and Bisby 1998). Raised levels of NPY occurred predominantly in medium and large neurones, and increases in axonal nerve fibres were observed in lamina I –IV (Ma and Bisby 1998). Raised levels of VIP was only seen in axonal fibres in the superficial dorsal horn (Ma and Bisby 1998). Interestingly, following partial nerve injury, raised levels of VIP were also detected in lamina V of the dorsal horn implicating larger levels of nerve injury induced changes in partial, compared to complete, peripheral nerve damage (Ma and Bisby 1998).
Primary sensory neurones transmit nociceptive information from the periphery to the spinal cord. Following nerve injury, alterations in the excitability of neurones, suggest changes in the expression and density of sodium channels within the cell body and neuronal dendrites (Waxman 1999; Waxman et al. 1999a; Waxman et al. 1999b; Waxman et al. 2000; Waxman 2001). Voltage-gated sodium channels (VGSC’s) are responsible for the inward membrane current in the nervous system, required for the production of the continuously evoked action potentials vital for neuronal transmission of nociceptive and other sensory information (Waxman et al. 1999b). VGSC’s are thought to be a possible molecular target for novel pain treatment (Waxman et al. 1999b; Waxman et al. 2000). They are not only expressed abundantly on primary sensory neurones, but can also be separated into a large family of distinct sodium channel subtypes encoded by a large variety of different genes within the DRG (Waxman et al. 1999b; Waxman et al. 2000). Not only are they expressed as an abundant array of subtypes, they are also found to actively alter their expression throughout both development and disease (Waxman et al. 1999b; Waxman et al. 2000). Some sodium channels are specifically confined to sensory neuron populations as opposed to other neural systems (Waxman 1999; Waxman et al. 1999a; Waxman et al. 1999b; Waxman et al. 2000; Waxman 2001). VGSC’s are heteromeric protein complexes, composed of large α- (260kDa) and smaller β- subunits (33-45kDa). Each protein complex contains one α- subunit, forming the ion channel pore and two β-subunits, named β-1,2 and 3 (Lai et al. 2003). Originally, there were thought to be eight separate VGSC’s within the nervous system, all of which were encoded by 8 different genes (Waxman et al. 1999a). However more recent accounts specify nine α-subunit isoforms named Na⁺1.1-1.9 using modern nomenclature (Lai et al. 2003). Within the peripheral nervous system, a number of VGSC’s have been found, including Na⁺1.6, 1.7, 1.8 and 1.9. These VGSC’s are located on different nerve fibre types and as such, their distribution may alter following neuronal insult (Lai et al. 2003). As interest, both industrially and academically, has risen exponentially over the past few years towards the development of VGSC blockers as neuropathic pain drugs, so has nomenclature changed for a better understanding and a more uniform recognition of the large number of detected VGSC’s now cloned (see Table 1.7.3.a. below).
Previously, in situ hybridizations and reverse transcription –PCR techniques, which reveal the mRNA’s encoding distinct sodium channels, revealed that within the DRG there were only 6 different sodium channel transcripts expressed, namely the Na\textsubscript{1.1} and Na\textsubscript{1.6} channels. These sodium channels were found mostly in medium and large DRG cell types in large quantities. However, these sodium channels, which elicit sodium channel currents, are all found to be expressed at varying levels in other neuronal cell types (Waxman et al. 1999b; Waxman et al. 2000; Lai et al. 2003).

The DRG also expresses sodium channels exclusive to the primary sensory neurones, as opposed to other neuronal populations, these were the Na\textsubscript{1.7}, Na\textsubscript{1.8}, Na\textsubscript{1.9} and Na\textsubscript{X} channels (Waxman et al. 1999b; Waxman et al. 2000; Lai et al. 2003). All of these sodium channels could be divided into TTX-sensitive and TTX-resistant VGSC
categories, based on their sensitivity to tetrodotoxin, a non-competitive voltage-gated sodium channel blocker (Waxman et al. 1999b; Lai et al. 2003). The TTX-sensitive sodium channels include Na\textsubscript{v}1.1, Na\textsubscript{v}1.2, Na\textsubscript{v}1.3, Na\textsubscript{v}1.4, Na\textsubscript{v}1.6 and Na\textsubscript{v}1.7 channels, the rest of which are TTX-resistant sodium channel types (Lai et al. 2003). Na\textsubscript{v}1.7 sodium channels transcripts are also located within the terminals of the majority of DRG neurones (Lai et al. 2003). The Na\textsubscript{v}1.8 sodium channel, as well as Na\textsubscript{v}1.9 channels, are found in trigeminal neurones as well as small diameter C-type dorsal root ganglion. It has emerged that Na\textsubscript{v}1.9 is also present in human embryonic kidney (HEK) cells as well (Akopian et al. 1996; Sangameswaran et al. 1997; Waxman 1999; Waxman et al. 1999a; Waxman et al. 1999b; Waxman et al. 2000; Waxman et al. 2002). Na\textsubscript{v}1.8 and Na\textsubscript{v}1.9, which are TTX resistant, have evoked much interest as the majority of nociceptive neurones make up small DRG neurones. Furthermore, it is known from more recent studies that Nav1.8 activates a TTX-resistant current within C-fibre populations, exhibiting slow-activating and inactivating, as well as rapid repriming kinetics (Lai et al. 2003) (Rush et al. 1998). It is therefore evident that VGSC’s are prevalent and participate in the conduction of nociceptive information. Not only is the development of action potentials in small DRG neurones largely diminished in Nav1.8 null mutant mice, but hypoalgesia also develops in response to application of varying forms of noxious stimuli (Waxman 1999; Waxman et al. 1999a; Waxman et al. 1999b; Waxman et al. 2000; Waxman et al. 2002; Lai et al. 2003). Nav1.9 is thought to be active at the resting membrane potential in small DRG cells and it has therefore been suggested that Nav1.9 regulates the resting membrane potential of nociceptive C-fibres. Recent findings have located Nav1.9 in the hippocampus, and therefore lay doubt to the initial hypothesis that Nav1.9 is restricted to the DRG (Blum et al. 2002).

Much interest has evolved around the concept of sodium ion channels and their targets as prospective analgesics following nerve injury, because it is now apparent that the type of channels expressed following neuronal insult is altered (Waxman et al. 1999a). Recently, development of antisense oligonucleotides which disturb Na\textsubscript{v}1.8 synthesis have been used to ‘knock-down’ Na\textsubscript{v}1.8 VGSC’s and therefore result in a 50% reduction in the expression of these VGSC’s (Lai et al. 2002; Lai et al. 2003). Interestingly, following spinal nerve ligation, ‘knock-down’ has been seen to quash neuropathic pain and its associated reductions in thermal and mechanical sensory thresholds (Lai et al. 2002; Lai et al. 2003). The same treatment in normal models has no such effect on the
sensory threshold values to peripherally applied noxious thermal stimuli (Lai et al. 2002; Lai et al. 2003). These ‘knock-down’ studies have not found any effect on thermal or mechanical hypersensitivity in neuropathic animals following attenuation of Nav1.9 synthesis. This is consistent with findings in sham operated animal groups (Porreca et al. 1999).

Following neuronal axotomy, other studies have revealed that there is both an upregulation of the α-III sodium channel and a downregulation of Na+,1.8 and Na+,1.9 gene expression within DRG neurones (Waxman et al. 1999a; Dib-Hajj et al. 2002). Down-regulation of Na+,1.8 channel proteins in more recent studies following spinal nerve ligation of L5/L6, have specified that Na+,1.8 is reduced in the injured DRG cell bodies and thus Na+,1.8 channel functioning is unlikely to be in damaged nerve fibres (Decosterd et al. 2002; Lai et al. 2002; Gold et al. 2003). Such down-regulation possibly prevents electrical conductivity in damaged C-fibre populations (Renganathan et al. 2001; Lai et al. 2002; Lai et al. 2003). This is despite original theories, based on the role of Nav1.8 protein channels in DRG cell conductance, that following nerve injury alterations in the activity of Nav1.8 protein channels could be responsible for spontaneous activity and repetitive firing of DRG cells (Black et al. 1999; Waxman et al. 1999b). Interestingly, not only does such down-regulation result in a reduction of TTX-resistant sodium currents within the DRG neurones, but also the development of a ‘rapidly conducting repriming’ current within the TTX-sensitive sodium currents in DRG neurones (Black et al. 1999; Waxman et al. 1999b). This is thought to be due to the upregulation of the previously silent Na+,1.3 sodium channel. (Black et al. 1999; Waxman et al. 1999a). Patch clamping studies have revealed that kinetically slow TTX-resistant sodium channel currents are almost abolished following axotomy (Rizzo et al. 1995).

Using the SNL model of neuropathy, these studies have also shown that the levels of Nav1.8 are unaltered in the uninjured L4 ganglia, yet upon immunoreactivity analysis of the sciatic nerve the levels of Nav1.8 are dramatically increased (Gold et al. 2003). In the neuropathic group, TTX was unable to block (>40%) the compound action potentials (CAP) at C-fibre latencies, compared to sham operated rats whose CAP remained sensitive (>90%) to TTX (100 μm) (Gold et al. 2003). These studies strongly emphasised the possible role of Nav1.8 blocking agents in the reversal of neuropathic pain conditions (Gold et al. 2003). Following Nav1.8 knock-down studies in the sciatic
nerve, it has become evident that Nav1.9 channels may be responsible for the observed residual TTXr currents in the C-fibres population (Gold et al. 2003). However, it is clear that the presence of Nav1.9 channels along the nerve axon is undisturbed by nerve damage, as differences in the density of voltage-gated sodium channel currents in neuropathic and sham operated animals is minimal (Gold et al. 2003). Such Nav1.8 knock-down of uninjured neurones, have also proven to be both anti-allodynic and anti-hyperalgesic in relevant animal models (Gold et al. 2003). It is feasible that such alterations in sodium channel expression as well as changes in sodium channel densities within DRG neurones, may underlie the spontaneous firing and lower thresholds which are responsible for such abnormal firing after axotomy and nerve damage (Matzner and Devor 1992; Matzner and Devor 1994). It is also possible that the development of spontaneous activity is a pre-requisite of hypersensitive allodynic and hyperalgesic states that characterise neuropathic conditions, along with the actual insult to the peripheral nerves (Lai et al. 2003).

Interestingly, it has been shown that not only does the alteration in sodium channel expression result in altered conduction properties in neuropathic models, but also alterations in the production and role of neurotrophins at neuronal sites, may alter sodium channel expression (Waxman et al. 1999a). Many studies have looked at the role of brain-derived growth factor (BDNF), glial-derived growth factor and NGF in the modulation of sodium channel expression. Apart from a study which observed a regulatory role of glial-derived growth factor in the expression of Nav1.9 sodium channels in small DRG neurones (Fjell et al. 1999), none except for those based on the role of NGF following axotomy, have shown any profound function of neurotrophins in sodium channel expression (Waxman et al. 1999a).

However, other studies have found a role of BDNF in GABA_A-receptor induced conductances following axotomy (Oyelese et al. 1997). Studies have implicated NGF as having a distinct role in the alteration of some sodium ion channel transcripts, with α-III down-regulated upon direct application of NGF to DRG cell bodies (Black et al. 1997) and Nav1.8 expression within the DRG as profuse following nerve damage (Black et al. 1999). These studies are supported by other studies, which reveal an up-regulation in TTX-resistant sodium channel currents in small DRG, as well as Nav1.8 channel currents (mentioned above) following direct application of exogenous NGF to the
proximal nerve stump. A reduction in the reservoir of neurotrophic factors or a loss of their influence, may therefore reveal a valid argument for the contradictory down-regulations of such sodium channel currents, following nerve injury (Dib-Hajj et al. 1996; Waxman et al. 1999a).

Overall, the findings discussed above have implicated certain distinct sodium channels in the longsearch for neuropathic pain treatments, as well as a firm understanding of the abnormal sensory activities that classify neuropathic pain syndrome and that occur following nerve damage or deafferentation. The widely researched changes in Nav1.8, as well as Nav1.9, protein channel expression has provided a basis for the development of novel blockers, which may prove valuable analgesic drugs. Specific antisense oligodeoxynucleotides can be used to ‘knock-down’ DRG PN3/Nav1.8 proteins and have intriguingly been shown to stop the development of injury-induced allodynia and hyperalgesia (Porreca et al. 1999). Unfortunately, Nav1.9 protein ‘knock-down’ has failed to replicate such encouraging results (Porreca et al. 1999). However, this general outcome has led the way for the development of PN3/Nav1.8 specific sodium channel blockers, ever more attractive due to their restriction on sensory neuron populations (Porreca et al. 1999). Furthermore, recent research has extended these animals studies to human pain models and found both Nav1.8 and Nav1.9 sodium channels on peripheral nerves, using selective antibodies, in neurogenic pain patients (Coward et al. 2000). Immunolocalization enabled the location of such sodium channels within patients that had brachial plexus injury, to be discovered. This study revealed that following spinal cord root avulsion, Nav1.8 and Nav1.9 sodium channel expression in neuronal cell bodies is decreased in human models.

However, as with previous animal studies, following peripheral axotomy sodium channels already synthesised can still be transported to the area of nerve damage and amass in at these sites, which may be essential for the development of hypersensitive states (Coward et al. 2000). Interestingly, those patients suffering chronic local hyperalgesia had increased levels of Nav1.8 immunoreactivity in neuroma nerve terminal and skin samples, again linking the Nav1.8 sodium channel with chronic abnormal and hypersensitive pain symptoms, underlying neuropathic pain (Coward et al. 2000). Also, the majority of patients with brachial plexus injury also had related positive symptoms
and demonstrated a positive Tinel's sign, fuelling belief that block of Nav1.8 voltage-gated sodium channels could aid chronic local hypersensitivity in neuropathic patients (Coward et al. 2000). Indeed, recent studies looking at the effect of novel sodium channel blockers in neuropathic and inflammatory pain, as well as their anti-allodynic effects, reveal distinct anti-nociceptive properties (Erichsen et al. 2003; Veneroni et al. 2003).

1.7.3.2 Voltage dependent Calcium Channels

Not only are there alterations in the distribution and number of certain VGSC subtypes expressed, following nerve injury, there are also reported changes in the expression and role of calcium channels. High voltage-activated Ca\(^{2+}\) channels, activated by large membrane deolplarisations, are now thought to be extremely important in pain pathways. There are a number of different types of high voltage Ca\(^{2+}\) channel expressed in the nervous system, which include the four L-, N-, P/Q- and R-type Ca\(^{2+}\) channels (Matthews and Dickenson 2001b; Matthews 2001). These voltage activated Ca\(^{2+}\) -channels (also referred to as voltage dependent calcium channels, VDCCs) are all composed of an \(\alpha_{1}\) subunit, which forms the Ca\(^{2+}\) channel pore, and related \(\beta\), \(\alpha_{2-\delta}\) and \(\gamma\) subunit proteins (Walker et al. 1998). Recent studies have now implicated low-voltage T-type channels, found both in and out of the nervous system, in pain processes (Matthews and Dickenson 2001a). Using the T-type Ca\(^{2+}\) channel blocker, ethosuximide it is evident that T-type Ca\(^{2+}\) channels are involved in both noxious electrical, mechanical and thermally induced neuronal responses (Matthews and Dickenson 2001a). However, following spinal nerve ligation the role of these T-type Ca\(^{2+}\) channels remains the same (Matthews and Dickenson 2001a).

Following nerve injury, electrophysiological studies have used intrathecal omega-conotoxin-GVIA which blocks the N-type VDCC, and results confirm significant reductions in noxious electrically and naturally induced dorsal horn neuronal responses and thus also prove N-type VDCC's play a distinct role in nociceptive transmission before and after peripheral nerve ligation (Matthews and Dickenson 2001b). However, at the lower dose range, block of N-type VDCC's caused a more pronounced inhibition in neuropathic animals, suggesting a small upregulation of N-type VDCCs following peripheral nerve ligation (Matthews and Dickenson 2001b). Confirming these reports
are studies performed on mutant mice lacking the N-type VDCC, which display less neuropathic pain related symptoms compared to control groups (Saegusa et al. 2001). The P-type VDCC antagonist omega-agatoxin-IVA did not have as large an effect as the N-type blocker and thus plays a smaller role in electrical and naturally evoked neuronal transmission (Matthews and Dickenson 2001b).

Furthermore, monoclonal antibody studies and western blot techniques have also recently suggested an upregulation of α2δ-1 subunits and increased gabapentin sensitivity following diabetic based and mechanical (i.e. Sciatic Nerve Injury and CCI models mentioned in section 1.6) neuropathies. This may therefore implicate a small role in the development of allodynia in these systems (Luo et al. 2002). Overall a variety of studies have signified the involvement of VDCCs, particularly N-type VDCC's in the development of neuropathic pain related behaviours and could thus suggest a future role of N-type VDCC antagonists in the treatment of neuropathic pain symptoms. Recently the nomenclature for calcium channels has been updated and table 1.7.3.b demonstrates both the new nomenclature as well as the location and function of classified calcium channels. The table was kindly taken and adapted with permission, from (Matthews 2001).
<table>
<thead>
<tr>
<th>VDCC type</th>
<th>Current type</th>
<th>α1 subunit</th>
<th>Tissue localization</th>
<th>Subcellular localization</th>
<th>Spinal cord distribution</th>
<th>Specific blocker</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca1.1</td>
<td>L (HVA)</td>
<td>α1S</td>
<td>Skeletal muscle</td>
<td>T-tubules</td>
<td>×</td>
<td>DHPs</td>
<td>Excitation-contraction coupling</td>
</tr>
<tr>
<td>Ca1.2</td>
<td>L (HVA)</td>
<td>α1C</td>
<td>Cardiac muscle</td>
<td>Membrane surface, t-tubule sarclemma</td>
<td>✓ deep dorsal and ventral horns</td>
<td>DHPs</td>
<td>Excitation-contraction coupling, hormone secretion, gene regulation</td>
</tr>
<tr>
<td>Ca1.3</td>
<td>L (HVA)</td>
<td>α1D</td>
<td>Smooth muscle, Neurones</td>
<td>Membrane surface, t-tubule sarclemma, Cell soma,</td>
<td>✓ deep dorsal and ventral horns</td>
<td>DHPs</td>
<td>Hormone secretion, gene regulation</td>
</tr>
<tr>
<td>Ca1.4</td>
<td>L (HVA)</td>
<td>α1F</td>
<td>Pancreas Endocrine tissue Neurones</td>
<td>Cell soma and larger dendrites of neurones, Retina</td>
<td>✓</td>
<td>None</td>
<td>Tonic release of neurotransmitters</td>
</tr>
<tr>
<td>Ca2.1</td>
<td>P/Q (HVA)</td>
<td>α1A</td>
<td>Neurones</td>
<td>Nerve terminals and dendrites</td>
<td>✓ throughout</td>
<td>αo-agatoxin IVA</td>
<td>Neurotransmitter release</td>
</tr>
<tr>
<td>Ca2.2</td>
<td>N (HVA)</td>
<td>α1B</td>
<td>Neurones</td>
<td>Nerve terminals and dendrites</td>
<td>✓ mostly concentrated in superficial laminae</td>
<td>αo-conotoxin GVIA</td>
<td>Neurotransmitter release</td>
</tr>
<tr>
<td>Ca2.3</td>
<td>R (HVA)</td>
<td>α1E</td>
<td>Neurones</td>
<td>Soma, dendrites and terminals</td>
<td>✓</td>
<td>None</td>
<td>Neurotransmitter release</td>
</tr>
<tr>
<td>Ca3.1</td>
<td>T (LVA)</td>
<td>α1G</td>
<td>Neurones</td>
<td>Soma and dendrites</td>
<td>✓ low levels throughout</td>
<td>None</td>
<td>Pacemaking, gradual depolarization for multiple APs and oscillatory behaviour, depolarizes cells to threshold for other channels</td>
</tr>
<tr>
<td>Ca3.2</td>
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<td>α1H</td>
<td>Cardiac muscle</td>
<td>Neurones</td>
<td>✓ mostly restricted to superficial laminae</td>
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<td></td>
</tr>
<tr>
<td>Ca3.3</td>
<td>T (LVA)</td>
<td>α1I</td>
<td>Cardiac muscle</td>
<td>Soma and dendrites</td>
<td>✓ throughout, mainly in laminae III-IV</td>
<td>None</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: VDCC (voltage-dependent calcium channel); HVA (high voltage-activated); LVA (low voltage-activated); DHPs (dihydropiridines); APs (action potentials) (Matthews, 2001)
1.7.4 Ectopic Discharge

Development of aberrant sensory activity and thus abnormal pain behaviours and symptoms following neuropathic pain has been frequently reported. Indeed, following chronic DRG compression, recordings taken from neurones with undamaged small and large peripheral fibres illustrated a large increase (0.96% to 8.61%) in spontaneous activity originating from large myelinated A-fibres in the DRG (Song et al. 1999). Furthermore, parasthesias, dyasthesias and other intense, as well as distressing, pains can now be explained by the induction of unusual afferent discharge and spontaneous activity at ectopic areas of damaged primary sensory neurones (Amir et al. 1999).

In addition, it is believed that ectopic activity in damaged primary sensory neurones may prompt central sensitisation within the central nervous system and thus contribute towards the development of hyperexcitable states underlying the development of allodynia and ongoing pain, also common in neuropathic pain patients (Amir et al. 1999). More specifically, it has been proposed that a bombardment of neuronal firing in unmyelinated C-fibre afferents is responsible for such central hyperexcitability (Woolf 1996; Woolf and Mannion 1999; Wu et al. 2001). Interestingly, more recent studies recording from L5 dorsal roots following L5 nerve injury, have suggested that spontaneous activity occurs primarily in A-fibre afferents as opposed to C-fibre afferents (Boucher et al. 2000; Liu et al. 2000b). These findings thus contradict the theory that central sensitisation is a product of ectopic firing of injured C-fibre afferents terminating in the spinal cord (Wu et al. 2001). However, uninjured intact C-fibre afferents appear to establish ectopic impulses and spontaneous activity if neighbouring L6 spinal nerves are damaged (Ali et al. 1999).

Single nerve fibre recordings in L4 spinal nerves, have now revealed that following L5 spinal nerve injury, intact C-fibre afferent fibres develop early onset, distinct spontaneous activity. Such is the case, these findings have now illustrated that ectopic activity in adjacent intact nociceptive afferents may be the underlying cause of central sensitization and thus hyperalgesia as commonly witnessed in neuropathic pain models (Wu et al. 2001). Interestingly, loose ligation of the sciatic nerve has been shown to result in the development of post-tetanic potentiation of A-fibre activity within the sciatic nerve, persisting for several weeks following the initial insult.
Furthermore, thermal hyperalgesia that also develops following such neuronal insult, only appears to last for several weeks after ligation and corresponds to the depletion of such prolonged post-tetanic A-fibre activity in these neurones (Draganic et al. 2001). The underlying origin of ectopic discharge has been subject to debate, however more recent in vitro recordings from rat DRG have illustrated that following nerve injury there were a larger proportion of cells exhibiting voltage sensitive sub-threshold oscillations in their membrane potential (Amir et al. 1999). Thus the development of action potentials was achieved when the membrane potential reached threshold and the generation of sustained discharge patterns at both resting and depolarised membrane potentials was made possible in a larger proportion of neurones within the DRG (Amir et al. 1999). Administration of TTX and lidocaine abolished ectopic activity thus favouring a TTX-sensitive Na+ channel in the development of such aberrant spontaneous activity following nerve injury (Amir et al. 1999). It is known that the Na1.3 channel mediates a TTX-sensitive, fast activating and inactivating voltage gated current in the nervous system (Lai et al. 2003). It is this Na1.3 channel that is thought to underlie the TTX-sensitive current implicated in the generation of spontaneous activity, particularly in axotomised small DRG neurones (Cummins and Waxman 1997; Cummins et al. 2001). Following chronic constriction and transection of the sciatic nerve, as well as spinal nerve ligation, Na1.3 channels are promptly upregulated in the DRG of primary sensory neurones despite low levels of expression under normal conditions (Waxman et al. 1994; Black et al. 1999; Dib-Hajj et al. 1999; Boucher et al. 2000; Kim et al. 2001a; Lai et al. 2003). However, links are yet to be established between the upregulation and increased prevalence of such Na1.3 channels following axotomy and the generation of spontaneous activity and ectopic discharges in injured and intact nerve fibres (Lai et al. 2003).

### 1.7.5 Ephatic Transmission (Cross Talk)

Primary sensory neurones within the peripheral nervous system transmit important tactile and nociceptive information from the periphery to the central nervous system (Amir and Devor 2000). These primary sensory neurones are entirely independent of one another and only form synaptic contacts and thus transmit electrical impulses within the spinal cord and higher centres of the CNS (Amir and Devor 2000). However, following axotomy this is no longer the case.
Exposed axons in the vicinity, now form close connections with injured neurones thus allowing both ephatic and chemical cross-excitation of these peripheral processes (Seltzer and Devor 1979; Devor and Janig 1981; Lisney and Devor 1987; Devor and Wall 1990; Devor 1991; Fried et al. 1993). Teased nerve fibre recordings in the rat sciatic nerve have been extremely informative with regards to cross-talk in such peripheral primary sensory neurones. Following sciatic nerve injury there is a large increase in the number of DRG primary afferent neurones exhibiting spontaneous activity (Devor and Wall 1990). Furthermore the same studies have illustrated that not only can such spontaneous activity be enhanced with administration of tetanic stimulation, but that cross excitation will only develop in neurones sharing the same DRG (Devor and Wall 1990). Such cross-excitation is called 'DRG Crossed Afterdischarge' (Devor and Wall 1990). Interestingly, out of all the spontaneously active neurones, the majority of neurones (83.5%) exhibiting such cross-talk were those containing myelinated large A-fibre axons (Devor and Wall 1990). Far less (4.4%) were neurones with unmyelinated C-fibre axons (Devor and Wall 1990). It was also possible to evoke activity in a small proportion (3.1%) of previously quiescent A-fibre neurones upon stimulation of adjacent fibres (Devor and Wall 1990). These studies also revealed that the magnitude of afterdischarge arose and also kept increasing within the first 500ms, in response to stimulation. Furthermore, as the magnitude of the stimuli increased, so did the cross afterdischarge in these neurones, in response (Devor and Wall 1990). It was also clear that the development of cross-talk was not just limited to damaged DRG, but also emerged in uninjured DRG neurones (Devor and Wall 1990).

Despite the fact that these studies reveal cross-excitation merely in large A-fibre neuronal populations, more recent in vitro electrophysiological recordings have been made in primary afferent neurones containing nociceptive unmyelinated C-fibre axons following L4 and L5 excision (Amir and Devor 2000). These studies have revealed that neuronal activity can be evoked in a large proportion of C-fibre axons, following peripheral stimulation of activity in neurones containing large A-fibre axons within the same DRG (Amir and Devor 2000). These results thus provide a rationale for the generation of nociceptive activity, by activation of low-threshold mechanoreceptors and the development of abnormal pain behaviours such as allodynia (Amir and Devor 2000).
1.7.6 A-β fibre sprouting

The spinal cord dorsal horn is composed of distinct laminae, separated based on their cytoarchitecture. Lamina I and II comprise the superficial dorsal horn, which conveys both noxious and thermal information to other areas within the dorsal horn and higher centres (Fitzgerald 1989). A-δ fibres from the periphery, predominantly terminate in the outermost area of the superficial dorsal horn; lamina I, with a small number of terminal arbors reaching the outer lamina II area (Sorkin and Carlton 1998; Fitzgerald, 1989). However many studies suggest that A-δ fibres also penetrate the deep dorsal horn layers, with some collateral projections from these deeper laminae reaching lamina I (Fitzgerald 1989; Sorkin and Carlton 1998). Unmyelinated C-fibres terminate predominantly in lamina II, yet C-fibre terminals have also been located in lamina I and some polymodal nociceptor terminals have also been found in deeper dorsal horn layers (Sorkin and Carlton 1998). Large myelinated A-fibres, conveying innocuous mechanical information, transcend deeper into the dorsal horn with dense aborisations located in laminae III-IV (Sorkin and Carlton 1998).

Studies investigating termination patterns of peripheral nerve fibres within the dorsal horn of the spinal cord commonly used axonal markers to identify neuronal anatomical alterations following nerve injury. Cholera toxin β-subunit-horseradish peroxidase or the B subunit of cholera toxin were previously popular axonal markers in many studies surrounding the theoretical sprouting of A-β fibres in lamina II of the dorsal horn. CTB and CTB-HRP, in normal conditions, is transported by myelinated A-fibres which possess GM1 ganglioside on their axonal membranes and enables staining of lamina I, III-V of the dorsal horn (Woolf et al. 1992; Mannion et al. 1996; Mannion et al. 1998). This method was used after nerve injury and it was believed it would allow identification of any reorganisation arising as a result. More specifically, it unveiled the first of many theories, surrounding the sprouting of mechanoreceptive A-β fibres into lamina II, that was thought to occur following peripheral nerve crush (Woolf et al. 1992). After which, numerous reports of the A-fibre sprouting phenomena were abundantly described in many previous studies (Woolf et al. 1992; Doubell et al. 1997; Krenz and Weaver 1998; Ma et al. 2000; Ma and Tian 2001).
The idea then emerged that A-fibre sprouting may therefore be a contributing factor to the occurrence of abnormal sensory abnormalities, frequently reported following nerve injury. (Woolf et al. 1992). Studies focussed on the link between A-fibre sprouting following chronic constriction injury (CCI) to the rat sciatic nerve and the development of mechanical allodynia in the rat hindpaw (Nakamura and Myers 1999). Such studies, again using cholera toxin β-subunit-horseradish peroxidase or the B subunit of cholera toxin retrograde axonal tracings, suggested that myelinated A-fibre sprouting into lamina II was a probable cause of chronic mechanical allodynia.

This was explained by the replacement of nociceptive C-fibre inputs within lamina II in normal models, by innocuous A-fibres afferents (which normally convey touch and non-noxious mechanical information) following nerve damage (Nakamura and Myers 1999). Further studies investigating this link, using a partial dorsal root ganglion injury model, suggested the same association between A-fibre sprouting and the pathogenesis of mechanical allodynia (Nakamura and Myers 2000). However, confusingly, these studies failed to explain the contradictory time courses between the development of each phenomenon. Nakamura and Myers reported that 3 days following injury, mechanical allodynia was observed in the correlating hindpaw, yet myelinated afferents, identified by the normal tracing techniques, were not seen to sprout into lamina II of the dorsal horn until 2-4 weeks after injury was induced (Nakamura and Myers 2000).

Such conflicting studies and the discrepancies in developmental time courses of A-fibre sprouting and mechanical allodynia rendered the original speculations surrounding the link between dorsal horn reorganization and subsequent abnormal pain related behaviours an ambiguous subject, to be approached with caution and worth further investigation. However the observation that A-fibres sprout into lamina II was an all too tempting theory behind such abnormal pain states. The mechanisms behind dorsal horn re-organisation were intensely researched. It was deemed likely that after peripheral nerve crush, C-fibre terminals within lamina II of the dorsal horn degenerate. This in turn was thought to allow A-β fibres to replace any unoccupied synaptic sites within the superficial dorsal horn layer (Mannion et al. 1998).
Studies suggested an upregulation of proteins such as GAP-43, actin and tubulin following peripheral nerve injury, which are normally involved in growth and the structural composition of nerve fibres, enabling sprouting. Interestingly, up-regulated levels of GAP-43 are not present in A-β fibre axons, but are present in other axon projections to lamina II of the dorsal horn, (Skene and Shooter 1983; Doubell and Woolf 1997) which was believed to aid A-β fibre sprouting.

Following the initial findings that A-β fibres sprout into lamina II of the dorsal horn, (suspected from Golgi studies as early as 1958) other studies investigated further whether such sprouting indeed requires both C-fibre degeneration and A-fibre conditioning, as suggested previously (Mannion et al. 1996). This was tested by selectively damaging the C-fibre population, using capsaicin applied to the sciatic nerve of rats (Mannion et al. 1996). The resulting effect of capsaicin treatment, was the degeneration of C-fibre terminals within lamina II (Mannion et al. 1996). One could then compare the A-β fibre terminal aborisions after peripheral nerve injury to untreated examples. Interestingly using the same axonal staining technique described above, such studies showed that staining between groups treated with capsaicin and those that are not was virtually the same, confirming that injury to C-fibres allowed the collateral sprouting of A-fibre terminals within lamina II (Mannion et al. 1996). Other such studies also observed sprouting of sciatic nerve A-fibres into areas of lamina II, inhabited by C-fibres of axotomised neighbouring nerves, providing the C-fibre terminals of such nearby nerves are situated dorsal to the sciatic A-fibres within lamina II (Doubell et al. 1997). These studies were supported by whole-cell recordings made from substantia gelatinosa neurones in sciatic-nerve transected rats, which when compared to sham operated animals demonstrated significantly increased numbers of neurones exhibiting EPSCs following A-β fibre stimulation (Okamoto et al. 2001). It became evident that the basis of A-fibre innervation of the lamina II layer is not a result of denervation or damage to A-fibres, but was assumed that collateral as opposed to tangential sprouting induced by deafferentation of peripheral nerve fibres was the cause (Doubell et al. 1997).

Additional studies then investigated whether dorsal L5 rhizotomy, which results in denervated vacant areas in lamina I and II, was actually enough to influence the sprouting of uninjured A-fibres into the superficial dorsal horn from nearby intact dorsal root ganglia and interestingly contradicted those previous studies discussed above.
Neither denervated or uninjured peripheral A-fibres were seen to sprout into lamina II, following dorsal L5 rhizotomy, and furthermore such sprouting was only evident in areas of lamina II inhabited by C-fibre terminals damaged in the periphery (Mannion et al. 1998). These studies therefore implicated that the presence of vacated synapses was not sufficient to induce such A-β fibre sprouting, but sprouting in areas of damaged C-fibre terminals suggested that these C-fibres may be releasing chemo-attractants tempting these A-fibre afferents into lamina II (Mannion et al. 1998).

Despite some conflicting studies, newer investigations into this field questioned in more detail what instigated the frequently observed central sprouting of A-fibres within lamina II. Studies using sciatic nerve transection concluded that it was a result of nerve injury rather than the loss of target tissues (Ma et al. 2000). Removal of the A-fibre terminals from lamina II after a period of 6-8 months following nerve damage and then resection of the previously injured sciatic nerve resulted in further sprouting (Ma et al. 2000). This meant that the likelihood of target deprivation being an instigator of A-fibre sprouting was low (Ma et al. 2000).

However, doubts as to the mechanisms and cause of A-fibre sprouting did not merely focus on the physiological and anatomical disparities between studies, but also on the techniques used to uncover the process of dorsal horn reorganisation following nerve damage. These doubts and further revelations regarding the techniques used have almost dispelled all the previous studies findings regarding the observation that A-fibres sprout into lamina II and its links to the development of allodynia (Tong et al. 1999). Such studies have shed new light onto the limitations of the neuronal tracer cholera toxin B subunit (CTB), as well as CTB-HRP, which are both widely used as anterograde and transganglionic tracers in unveiling the terminal fields of primary afferent neurones with the dorsal horn (Tong et al. 1999). It has emerged that CTB is not entirely specific to sprouting myelinated A-fibres following peripheral nerve transection, and can also label unmyelinated C-fibre afferents in lamina II as well as A-δ fibre afferents in lamina I (Bao et al. 2002). This is despite previously documented reports that in the rat, CTB and CTB-HRP are taken up by large DRG neurones, which develop into A-β fibres (Tong et al. 1999). Bao et al suggested that A-fibre sprouting within lamina II is much less pronounced than previously documented, confined to a limited number of A-fibre terminals reaching the inner lamina II region.
This study revealed that reorganisation of the dorsal horn circuitry was therefore minimal compared to earlier reports. Furthermore, it was found that large neuropeptide Y (NPY) DRG neurones comprised the majority of these sprouts and innervated the inner lamina II region that is densely occupied by Y1 receptors (Bao et al. 2002). Correlating with these findings, (Tong et al. 1999) also found that in rat and monkey models, CTB labelling was not restricted to large DRG neurones as seen prior to nerve transection, and uptake by small DRG neurones following sciatic nerve cut dramatically increased from 11-73% in L5 DRG neurones. These changes could be a result of marked alterations in the expression of a variety of neurotransmitters, receptors and possibly neurotrophic factors (Tong et al. 1999). Such hypotheses are supported by very recent studies, which have implicated up-regulation of vasoactive intestinal peptide (VIP) in small afferents in the superficial dorsal horn, which may act as a attraction for CTB neuronal uptake (Shehab et al. 2003).

Overall, the widely accounted theory that A-β fibre afferents sprout into lamina II following nerve injury (an area inhabited by nociceptive C-fibre afferents in normal conditions) provided an attractive story as to the emergence of allodynia in neuropathic models. Much speculation and hype meant that numerous studies intensely researched the mechanisms and stimulus for such anatomical re-organisation in the dorsal horn. However, results were somewhat dubious and the time course for the materialization of allodynia and sprouting was mis-matched. Further suspicions emerged when it was evident that the techniques commonly utilised for such investigations were not as reliable as first thought. The only real evidence thus being whole cell recordings which provided evidence for Aβ-fibre afferent activity within the substantia gelatinosa following sciatic nerve transection (Okamoto et al. 2001). However, for now, theories surrounding A-β-fibre sprouting and the development of allodynia, as a result, have been more or less discarded.

1.7.7 Sympathetic System

Following peripheral nerve injury, there are numerous reports that sprouting of sympathetic post-ganglionic nerve fibres occurs in the damaged nerve and associated dorsal root ganglia (Kim et al. 1999a). Anatomical studies have suggested that such sprouting arises via interactions between substance P positive sensory fibres and
sympathetic afferents (Ruocco et al. 2000). However, previously it has emerged that involvement of neuropeptides (i.e. substance P, CGRP, galanin and tyrosine) are not necessary for sympathetic afferent sprouting induced neuropathic pain, and these sympathetic axons terminate on DRG based sensory neurones that do not contain such neuropeptides (Ramer and Bisby 1998). Interestingly, there has been some noted discrepancies in the importance of sympathetic nerve sprouting in relation to neuropathic pain development between various different animals models (Lee et al. 1998).

In the SNL model, such sprouting is now thought to play a much larger role than in CCI and PSL models, so this subject and its relation to the clinic should be approached with some care (Lee et al. 1998). Within the DRG, these sympathetic nerve sprouts appear to form varicose baskets surrounding large diameter A-fibre neurones via interactions with reactive satellite cells within the injured DRG (Zhou et al. 1996; Zhou et al. 1999; Deng et al. 2000b). These anatomical changes are often related to increases in sympathetic activity and neuropathic pain related behaviours observed in such models (Treede et al. 1992; Kim et al. 1999a). However, such sympathetic sprouting does not always produce neuropathic sympathetic pain dependence in a parallel fashion (Kim et al. 1998) yet, it has emerged recently that the degree of such sympathetic sprouting is clearly relative to the quantity and magnitude of nerve injury (Kim et al. 2001b). Furthermore, sprouting does not always induce adrenosensitivity in damaged peripheral nerves (Rubin et al. 1997; Kim et al. 1999a). Interestingly, recent immuno-histochemical staining studies using rats models with inferior and superior caudal trunk unilateral transections between S1-S2 of the spinal nerves, have examined whether there is any correlation between sprouting in DRG's and the generation of neuropathic pain and its related symptoms (Kim et al. 1999a).

Using tyrosine hydroxylase antibodies for the transected S1 peripheral DRGs, these staining studies clearly demonstrated that sprouting of sympathetic nerves into these DRGs was consistent. This was regardless of the degree of pain related behaviours (i.e. cold, mechanical and thermal alldynia) exhibited by these animals, thus contradicting previous suggestions that sympathetic sprouting may underlie the development of neuropathic pain related behaviours (Kim et al. 1999a). Interestingly, within the DRG upregulation of certain chemicals (i.e. mitogenic cytokine transforming growth factor MCTGF and MCTGF-\(\alpha\) receptor) and the synthesis of certain neurotrophic factors (Deng
et al. 2000a; Zhou et al. 2000), which is confirmed by the absence of sympathetic sprouting and reduced allodynia after removal of these neurotrophins, suggest a role of these factors in prompting sympathetic sprouting (Xian and Zhou 1999; Deng et al. 2000a; Deng et al. 2000b).

Using similar immuno-histochemical studies, mentioned above, recent studies have now revealed that following L5 spinal nerve transection sympathetic sprouting and related basket formation is dramatically decreased compared to control groups, when neurotrophic factors (NGF, BDNF & NT3) were neutralised with sheep antisera (Deng et al. 2000b). Overall it appears as though sympathetic nerve sprouting may play a role in the development and the sympathetic dependency of neuropathic pain related behaviours, and such sprouting is stimulated by increase synthesis of neurotrophic factors within injured DRG regions.

1.7.8 Nitric Oxide and Nitric Oxide Synthase

The molecule nitric oxide is synthesised from the amino acid L-arginine, by nitric oxide synthase (NOS) activation. It is found throughout the central and peripheral nervous system, however its distribution also extends to the cardiovascular and immune systems. Nitric oxide is a short lived and unstable free radical, with a variety of functions in the nervous system. Nitric oxide can function as an inter- and intra-cellular messenger, in both normal and pathological conditions, examples of which are diverse but include nerve injury and synaptic plasticity. It can also exert cytotoxic actions throughout all the above systems (Gonzalez-Hernandez and Rustioni 1999). NOS is found in three different isoforms.

These include the endothelial isoform (eNOS) located in endothelial cells of the cardiovascular system, the inducible isoform (iNOS), located in glial cells and macrophages of the immune system and the neuronal isoform (nNOS), located in different neuronal populations. All three isoforms depend on the same electron donor reduced nicotinamide adenine dinucleotide phosphate (NADPH) for their function. Many studies have investigated the role of nitric oxide and its activating enzyme (NOS) following neuronal damage. Nitric oxide is believed to play a role in transmission of nociceptive information and also the development of abnormal pain conditions in chronic
pain models (Meller and Gebhart 1993). One study investigated the effect of intrathecal application of L-arginine or NMDA in the tail-flick behavioural model of nociception, whereby it was demonstrated that an increase in the tail-flick reflex occurred following L-arginine application.

However, prior application of drugs such as the NOS inhibitor N omega-nitro-L-arginine methyl ester (L-NAME), methylene blue or the NMDA receptor antagonist DL-5-aminophosphonovaleric acid (AP5) eliminated any NMDA mediated magnification of the tail-flick reflex (Meller et al. 1992a). Such behavioural results imply that NMDA induced amplification of a thermal nociceptive reflex is due to activation of an NMDA receptor that facilitates endogenous nitric oxide production and in turn, increases the amount of soluble guanylate cyclase in the lumbar spinal cord (Meller et al. 1992a). Interestingly, there is other behavioural evidence for the involvement of NMDA/NO in the production of thermal hyperalgesia following both inflammatory conditions, evoked by application of carrageenan and nerve injury evoked by the CCI model (Yamamoto and Yaksh 1991; Meller et al. 1992b; Yamamoto and Yaksh 1992a; Meller and Gebhart 1994). Furthermore, inhibition of NOS by L-NAME may also reduce mechanical allodynia, as seen by the heightened vocalisation thresholds to such stimuli following spinal cord lesions (Hao et al. 1994). Such results are confirmed by the inhibition by L-NAME (>50uM/kg) of behavioural changes, resulting from mechanical allodynia using von Frey filaments applied to the plantar surface of the rat hindpaw, following SNL (Yoon et al. 1998).

Immunocytochemical and histochemical techniques have studied the regenerative role of NOS in peripheral nerves, as well as the spinal cord and DRG following tight sciatic ligation (Gonzalez-Hernandez and Rustioni 1999). These investigations have concluded that all three types of NOS are upregulated following peripheral nerve damage (Gonzalez-Hernandez and Rustioni 1999). It is also believed that upregulation of all three NOS isoforms has a beneficial role in the regeneration of peripheral nerves following nerve damage (Gonzalez-Hernandez and Rustioni 1999). This is contradictory to the detrimental effect of the upregulation of NOS isoforms in cerebral ischemia, whereby large amounts of NO actually contributes to existing damage (Gonzalez-Hernandez and Rustioni 1999). Following peripheral nerve injury, nNOS production is upregulated in DRG neurones and also transferred to growing axons, whereby it is
thought to maintain NO levels required for the activation of guanylyl cyclase (Gonzalez-Hernandez and Rustioni 1999). This in turn augments the production of cGMP required for axon outgrowth within the nervous system (Gonzalez-Hernandez and Rustioni 1999). Upregulation of eNOS in vasa nervorum, following tight sciatic nerve ligation is thought to be beneficial in the repair of peripheral nerves via a number of mechanisms (Gonzalez-Hernandez and Rustioni 1999). These include its vasodilatatory, anti-aggregating and angiogenic roles, whereby increased blood flow to regenerating neurones and the inhibition of platelet aggregation aids repair (Gonzalez-Hernandez and Rustioni 1999).

Upregulation of iNOS occurs in macrophage populations gathered to the site of nerve injury, following peripheral nerve damage (Levy and Zochodne 1998; Gonzalez-Hernandez and Rustioni 1999). Interestingly, NO is produced in huge quantities by the activation of iNOS and inturn its synthesis contributes to the cytotoxic actions of NO in immune conditions (Gonzalez-Hernandez and Rustioni 1999). It is feasible that following peripheral nerve injury, cytotoxic actions of NO results in the diminished mRNA content, which is required for synthesis of important myelin proteins in Schwann cells under normal conditions (Gonzalez-Hernandez and Rustioni 1999). Both mechanisms, will therefore assist neuronal regeneration by aiding destruction of myelin and axon materials, necessary for the removal of damaged nerves (Gonzalez-Hernandez and Rustioni 1999).

1.7.9 Central Plasticity

Nerve injury is normally associated with a loss of sensory nerve fibres (Suzuki and Dickenson 2000). However, the extent of nerve fibre destruction differs depending on the severity of the initial insult (Suzuki and Dickenson 2000). The consequence of neuronal destruction, is the loss of sensory input within the central nervous system. Furthermore, studies have reported that nerve injury induced apoptosis within the central nervous system, is often confined to inhibitory spinal neurones (Zimmermann 2001). Interestingly, recordings from spinal neurones have demonstrated marked central plasticity, which results in increased receptive field areas and different firing patterns following peripheral nerve ligation (Suzuki and Dickenson 2000; Suzuki et al. 2000). However, the evoked responses of spinal neurones following peripheral nerve injury are
often smaller than one would expect given the degree of sensory loss (Chapman et al. 1998; Suzuki and Dickenson 2000). Increased spinal responses to certain evoked stimuli may be a consequence of increased central hyperexcitability and central sensitisation, resulting from nerve injury induced ectopic activity and ion channel plasticity (Suzuki and Dickenson 2000). However, other pharmacological studies have also suggested a raised GABAergic inhibitory influence on spinal neurones, following peripheral nerve damage (Kontinen et al. 2001). It is believed that this increased inhibitory tone compensates for the heightened central hyperexcitability and can therefore dampen down neuronal responses to certain evoked stimuli (Kontinen et al. 2001).

1.8 Treatment options in Neuropathic Pain Syndromes

At present, treatment for neuropathic pain can be hard to generalise, partly due to the numerous heterogeneous conditions and symptoms responsible for the universally used term 'neuropathic pain' (Jensen et al. 2001a; Jensen et al. 2001b). Differences in both the aetiology and location of neuropathic pain conditions, mean that it is hard to treat all symptoms reported with just one drug – or at least as yet (Jensen et al. 2001a; Jensen et al. 2001b). As a result of the abundant array of disease types, injuries and other such disorders classically resulting in neuropathic pain conditions, the location of the offending lesion can be anywhere in the nervous system.

The more frequently documented neuropathic pain syndromes develop from peripheral, spinal, plexi, brain and dorsal nerve root insults and despite the vastly varying areas that nerve injury and thus neuropathic pain can stem from, often the same treatments can be used for the different symptoms (Jensen et al. 2001a; Jensen et al. 2001b). Consequently, it has been realised that despite the disparate aetiology and topography, many nerve injury induced syndromes develop from common processes (Jensen et al. 2001a; Jensen et al. 2001b). At present there are a wide variety of available analgesics suitable for neuropathic pain related syndromes, as well as novel targets and new drugs undergoing development. The clinician often evaluates the patients history, along with an assessment of the sensory abnormalities and a diagnostic clinical examination observing the patients response to mechanical, thermal and chemical stimuli, as well as determination of sympathetic activity and hypersensitive states (Jensen et al. 2001a;
Jensen et al. 2001b). After which evaluation of available pharmacological remedies can be performed, so that the most suitable treatment is given to the neuropathic pain patient (Jensen et al. 2001a; Jensen et al. 2001b). The voltage gated calcium channel blockers gabapentin and pregablin have been shown to suppress tactile allodynia in neuropathic models (Wallin et al. 2002), as have 5-HT$_{1A}$ receptor agonists (Deseure et al. 2002), and nerve growth factor receptor antagonists (Owolabi et al. 1999).

Furthermore, combined intrathecal application of the A$_1$-receptor agonist R-phenylisopropyladenosine and morphine demonstrated a distinct suppression of mechanical allodynia in the rat, following spinal nerve injury. This implicates adenosine in the development of neuropathic pain related behaviour, making it an interesting target for suppression of abnormal behavioural sensations following nerve damage (von Heijne et al. 2000). Contradictory to this study, the opioid receptor agonist buprenorphine have been shown to have antinociceptive and anti-hyperalgesic effects in behavioural tests in neuropathic animals (Kouya et al. 2002). Other studies illustrate the importance of excitatory amino acid antagonists in the inhibition of alldynia-like responses (Hao and Xu 1996; Hao et al. 1998). N-methyl-D-aspartate (NMDA) and NMDA glycine site antagonists both suppress thermal and mechanical alldynia in rat models of peripheral nerve damage (Kim et al. 1997b; Quartaroli et al. 2001). Although motor impairment and increased spontaneous activity have proven to be of hindrance in the clinical usefulness of these drugs.

NBQX, the competitive $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptor antagonist also relieves mechanical alldynia, yet sedation and reduced muscle tone, mean that the effectiveness of AMPA receptor antagonists are also restricted (Hao and Xu 1996). At present the main categories of pharmacological treatments include (Jensen et al. 2001a; Jensen et al. 2001b):

- **Antidepressants** – Tricyclic Antidepressants’s and Selective Serotonin Reuptake Inhibitor’s
- **Anticonvulsants** - Gabapentin, Carbamazepine, Lamotrigine, and phenytoin
- **Systemic Local Anesthetics** – Lidocaine and Mexilitine
- **Sympatholytic Agents** – Phentolamine, Phenoxybenzamine, Clonidine and Tizanidine
- **NMDA Receptor Antagonists** – Ketamine and Dextromethorphan
Antidepressant drugs are frequently used for the treatment of neuropathic syndromes, namely painful polyneuropathy. Tricyclic antidepressants (TCA’s) which inhibit the presynaptic reuptake of serotonin and noradrenaline, have remained the most popular antidepressant used for such conditions, and have remained so for nearly 30 years (Sindrup and Jensen 1999; Chong and Bajwa 2003; Sindrup et al. 2003). However, intolerable side-effects have meant that the usefulness of such drugs are restricted (Sindrup and Jensen 1999; Sindrup et al. 2003). Newer anti-depressants such as the selective serotonin reuptake inhibitors (SSRI’s) and the second generation non-TCA’s are now implicated as novel and more effective treatments, despite SSRI’s having previously been shown as ineffective in treating painful polyneuropathies (Sindrup and Jensen 1999; Sindrup et al. 2003). Recent studies have shown that the SSRI venlafaxine exhibits analgesic actions when administered to both male and female patients, suffering from painful polyneuropathies (Sindrup and Jensen 1999; Sindrup et al. 2003). Imipramine, a classic TCA often prescribed to neuropathic patients, exhibited some analgesic effects. However, comparison to venlafaxine revealed the SSRI to be a more effective treatment option (Sindrup and Jensen 1999; Sindrup et al. 2003).

Furthermore, bupropion, which is a second generation non-TCA that acts by inhibiting neuronal noradrenaline re-uptake and weakly inhibiting dopamine re-uptake, has been shown to significantly decrease neuropathic pain in affected non-depressed patients, in a randomised, double-blind, placebo-controlled, crossover study (Semenchuk et al. 2001). However, insomnia, dry mouth, dizziness, constipation and headache were among the non dose-limiting side effects experienced by most patients. Interestingly, dopamine agonists have also long been implicated as analgesics, from studies which demonstrated that dopamine agonists blocked noxious inputs to the spinal cord (Jensen and Yaksh 1984). The dopamine precursor levodopa has also been shown to have analgesic actions within patients suffering acute herpes zoster, as well as diabetic neuropathy, therefore implicating such drugs as effective analgesics in neuropathic pain.
syndromes. However side effects may be limit the development of such drugs (Sindrup and Jensen 1999).

1.8.2 Anticonvulsants

Anticonvulsants were first used in the treatment of neuropathic pain syndromes following observation that the pathophysiological processes underlying epilepsy and that of neuropathic pain are very similar (Dickenson et al. 2002). Following nerve injury, NMDA mediated wind-up is largely responsible for the development of pain sensations. Interestingly, NMDA mediated neuronal kindling in the hippocampus, which occurs in epilepsy has distinct similarities to the wind-up phenomena resulting from nerve damage (Backonja 2002). Therefore the usefulness of sodium channel blockers in the treatment of epilepsy, can be extended to that of the increased neuronal excitability exhibited by damaged peripheral nerves in neuropathic pain conditions (Backonja 2002; Dickenson et al. 2002). Carbamazepine is frequently used in neuropathic conditions, and its frequency dependence makes it ideal in spontaneously active small diameter nerve fibres transmitting noxious information from the periphery to central processes (Backonja 2002). Carbamazepine is more popular in the treatment of trigeminal neuralgia and painful diabetic neuropathic conditions (Backonja 2002).

However, carbamazepine is not without adverse effects, and many patients have reported somnolence, gait disorders and dizziness following treatment as well as agranulocytosis immediately after treatment commences (Backonja 2002). Phenytoin has also been tested as a neuropathic pain treatment in diabetic neuropathies, however conflicting studies have meant that it is used cautiously and perhaps further investigations into its usefulness are performed (Backonja 2002). Gabapentin, is no longer thought to have any effect on GABAergic uptake, metabolism or receptor action. However it is thought to block α-2-δ calcium channels, and therefore exhibit analgesic effects via action at these ion channels (Backonja 2002). However, as yet Gabapentin's analgesic effects in neuropathic pain are still not fully elucidated. Numerous studies have confirmed that gabapentin is a good analgesic in patients suffering from painful diabetic neuropathies and postherpetic neuralgia (Rowbotham et al. 1998; Backonja 2000; Backonja 2002; Backonja 2003). Interestingly, the side-effect profile of gabapentin is very similar to that of other anti-convulsants (Backonja 2000; Backonja
Electrophysiological studies using a combination of morphine and gabapentin have illustrated increased effectiveness of morphine when administered with small doses of gabapentin, where morphine previously exerted reduced effectiveness following nerve injury (Matthews and Dickenson 2002). These studies implicate combination treatment as a feasible choice of treatment therapies in some neuropathic-based pains.

Lamotrigine blocks voltage-gated sodium channels and is a novel treatment for epilepsy, interestingly it also inhibits glutamate release (Backonja 2000; Backonja 2002; Backonja 2003). Lamotrigine has seen to be effective in patients suffering from trigeminal neuralgia, HIV–associated neuropathies and central post-stroke neuralgia (Backonja 2000; Vestergaard et al. 2001; Backonja 2002; Backonja 2003). However, such optimistic results aren’t without contradictory studies demonstrating no such effective analgesic actions of lamotrigine in a wide-range of neuropathic pain syndromes at slightly lower dose ranges (Backonja 2000; Backonja 2002; Backonja 2003). Again, similar side-effect profiles could restrict the usefulness of this novel anti-convulsant drug (Backonja 2000; Backonja 2002; Backonja 2003). Lastly, other anticonvulsants such as zonisamide, lorazepam, valproate, topiramide and tiagabine have also been tested as potentials neuropathic pain analgesics with varying success.

Zonisamide is a new anticonvulsant, with mixed beneficial effects. Zonisamide acts by blocking voltage–gated sodium channels as well as the T-type calcium channels. Interestingly, it also increases release of the inhibitory amino acid GABA (Backonja 2000; Backonja 2002; Backonja 2003). Zonisamide appears to have analgesic actions in patients with neuropathic pain syndromes, ranging from diabetic neuropathy, peripheral neuropathy, radiculopathy, post-laminectomy syndrome and reflex sympathetic dystrophy. Furthermore, much of this pain relief is experienced by patients who have never previously obtained beneficial pain relief from other pharmacological agents (Backonja 2000; Backonja 2002; Backonja 2003). Finally, the anticonvulsant retigabine, a KCNQ channel opener has also been shown to have anti-nociceptive effects in spared nerve and chronic constriction injury (CCI) models of neuropathic pain (Blackburn-Munro and Jensen 2003). In both models mechanical hypersensitivity in the injured rat hindpaw was significantly reduced, however this was only the case with pinprick stimuli as opposed to von Frey stimuli (Blackburn-Munro and Jensen 2003).
Cold allodynia was reduced in the CCI model, but this effect was not observed in the spared nerve model and no effect was seen in the response to noxious thermal stimuli in either model (Blackburn-Munro and Jensen 2003). Interestingly, the retigabine reduced flinching behaviour previously noted in the formalin test in the second phase (Blackburn-Munro and Jensen 2003). In general, the results strongly implicate KCNQ channel openers as future potential analgesics in neuropathic pain syndromes. Overall, studies looking at the beneficial properties of a variety of anticonvulsants in painful neuropathic conditions have been encouraging, yet side-effects limit the effectiveness of many of these agents. The search for more selective ion channel blockers should therefore continue.

1.8.3 Systemic Local Anesthetics

Systemic local anaesthetics have been used in the treatment of pain conditions, namely diabetic neuropathy, postherpetic neuralgia, Dercum’s disease and other neuropathic pain conditions related to peripheral nerve injury over the past few years. It has been proposed in many related studies that a reduced plasma concentration of systemic local anaesthetic is more effective in treating painful neuropathies compared to that required for analgesic effectiveness in ordinary pain conditions (Fields et al. 1997).

In neuropathic pain models, the abnormal ectopic action potential firing means that systemically administered local anaesthetics are more able to reach their target sites (Fields et al. 1997). Intravenous lidocaine and oral local anaesthetic anti-arrhythmics such as mexilitine and flecanide have been tested as probable neuropathic pain treatments. Both drug types have been shown to be effective in painful neuropathies, yet cardiac based side-effects are often limiting.

1.8.4 Sympatholytic Agents

Alpha-2 adrenoceptor agonists have often been used in the treatment of some pain conditions due to their analgesic and anxiolysis effects (Khan et al. 1999). Drugs such as clonidine, dexmedetomidine and mivazerol are not specific alpha-2 adrenoceptor agonists, however, like rilmenidine and moxonidine, they also act on the nonadrenergic imidazoline receptors (Khan et al. 1999). Alpha-2 adrenoceptor agonists have been
studied in relation to their effects in chronic pain conditions (Khan et al. 1999). Both clonidine and the NMDA receptor antagonist MK-801 have both been shown to eliminate neuropathic pain in rat models of peripheral nerve injury (Lee and Yaksh 1995). So far studies have suggested a clear benefit for epidural administration of clonidine in neuropathy as well as topical administration, which alleviates hyperalgesia, in sympathetically maintained pain (Davis et al. 1991; Carroll et al. 1993; Khan et al. 1999).

1.8.5 NMDA Receptor Antagonists

NMDA receptor mediated central plasticity may underlie the developmental increase in synaptic excitability as well as the development of abnormal pain syndromes and behaviours, such as allodynia and hyperalgesia (Parsons 2001). As a consequence, the use of NMDA receptor antagonists as prospective analgesics in neuropathic pain conditions has been widely tested and implicated (Parsons 2001; Suzuki et al. 2001). Following spinal nerve ligation of L5/L6 in a rat model of mononeuropathy, intravenously administered ketamine has been shown to produce profound attenuation of wind-up and post-discharge (or neuronal excitability) in response to peripherally applied noxious electrical stimuli. Ketamine also caused reductions in the peripherally applied thermal and mechanically evoked responses in these neuropathic rats (Suzuki et al. 2001). Subcutaneously administered memantine also produced similar effects. However, MK-801, another NMDA receptor antagonist, produced comparable reductions to electrical and naturally evoked stimuli, in neuropathic and sham control groups (Suzuki et al. 2001).

Block of NMDA receptor mediated transmission can be achieved via numerous target sites, these include the glycine, polyamine and phencyclidine site all of which are pharmacologically utilised. However, as the NMDA receptor is widely located it is inevitable that side effects will emerge through complete block of these receptors (Parsons 2001). Recent studies have focussed on subunit selective antagonists, namely the NR2B selective blockers and glycine receptor channel blockers, which may have improved therapeutic profiles. Interestingly, topical administration of ketamine has also been demonstrated to improve post-herpetic neuralgia and sympathetically mediated pain in patients suffering from herpes zoster (Shingles). Topical application therefore
target peripheral NMDA receptors, reducing the side effect profile of centrally administered NMDA receptor antagonists that cross the blood brain barrier and therefore evoke CNS side effect profiles (Qian et al. 1996; Parsons 2001).

1.8.6 Opioids

The majority of clinically available opioids for treating pain are μ-opioid selective agonists, such as morphine. Studies have long suggested the effectiveness of μ-opioid selective agonists in the treatment of pain conditions (Dickenson et al. 1981; Dickenson and Le Bars 1987; Dickenson 1995a; Dickenson 1995b). κ-opioid selective agonists are also utilised in the clinic (e.g. butorphanol and nalbuphine), however these agonists are not widely used due to the distinct central side effect profiles which result in limited dose ranges (Martin and Eisenach 2001). This is also seen to some extent with the μ-opioid selective agonists (Martin and Eisenach 2001). The opioids have long been used as systemic treatments for mild, moderate and severe pain conditions however frequent and concerning debates regarding the side effects, dependence and addiction profiles associated with these pharmacological agents, have meant that the quest for a more modern drug is prevalent in many research laboratories.

Furthermore, the reduced responsiveness by neuropathic pain patients to opioids and their noticable tolerance to even the strongest of μ-opioid selective agonists is worrying, suggesting central and peripheral plasticity and altered expression following nerve injury (Martin and Eisenach 2001). It has been suggested that opioid receptors in dorsal horn C-fibre terminals may be destroyed following peripheral nerve insult (Dickenson 1994), it is also supposed that there are increases in morphine-3-glucoronide within the nervous system which may meddle with the analgesic actions of the opioid agonists (Dickenson 1994). Furthermore, it is possible that the function and systemic quantities of the neuropeptides cholecystokinin, F8Fa, or the opioid peptide dynorphin, may alter and thus interfere with the analgesic effect of opioid agonists (Dickenson 1994). Central sensitisation, resulting primarily from increased activation of the NMDA receptor, may also contribute to reduced opioid effectiveness (Dickenson 1994). However electrophysiological studies have indicated that low dose spinal application of morphine is far more effective at attenuating both noxious electrical and noxious mechanical and heat evoked neuronal responses in an SNL model of peripheral nerve injury, compared
to control models (Suzuki et al. 1999). This suggests that perhaps the mechanism of administration and/or mechanisms at the sites of opioid receptor action may be altered.

### 1.8.7 Topical Agents

Studies investigating treatments for post-herpetic neuralgia have shown that subcutaneous infiltration of local anaesthetic agents (i.e. lidocaine) into the most painful areas is highly effective and exhibits good analgesic actions. Topical lidocaine, applied to patients suffering from post-herpetic neuralgia is highly effective in dampening neuropathic pain in areas most affected, as demonstrated in double-blind vehicle controlled studies (Rowbotham et al. 1995; Rowbotham and Fields 1996). Interestingly, studies have demonstrated that the site of action of local anaesthetics topically applied at affected areas of such patients, is at the cutaneous afferents of the damaged or injured sensory nerves (Rowbotham et al. 1995; Rowbotham and Fields 1996). Overall, these studies implicate that local anaesthetic and anti-arrhythmic agents are useful in neuropathic pain conditions and their method of administration can determine the effectiveness of such agents in different neuropathic syndromes.

### 1.8.8 Neurotrophic Factors

During the search for novel and more efficient therapeutic interventions to treat neuropathic pain conditions neurotrophic factors have been unveiled as a potential new category of neuropathic pain analgesics (Sah et al. 2003). Neurotrophic factors (as described earlier) are natural proteins, which assist the growth, plasticity, structural integrity and existence of certain populations of neurones in the peripheral and central nervous system (Sah et al. 2003). Four main neurotrophic factors, which are implicated as potential treatments in painful peripheral neuropathic pain conditions, are listed below. These particular neurotrophins have been broadly studied due to their down-regulation following nerve injury and therefore their supposed role in the development of neuropathic syndromes (Sah et al. 2003).

- Nerve Growth Factor (NGF)
- Brain-derived neurotrophic factor (BDNF)
- Neurotrophin-3 (NT-3)
Glial cell line-derived Neurotrophic Factor (GDNF)

Interestingly, the use of neurotrophic factors as a treatment for neuropathic pain syndromes would not only reduce the sensation of painful neuropathies presented in patients but would also treat the underlying condition resulting in abnormal painful sensations. Most drugs available at present do little to fix the underlying mechanisms evoking such abnormal pain sensations, and therefore have a palliative role whilst the pathophysiological condition not only endures, but continues to develop (Sah et al. 2003).

1.8.9 Invasive and Non-Invasive Surgical Techniques

1.8.9.1 Invasive Treatments

Nerve modulation and neural ablation are two neurosurgical techniques that may be used to treat neuropathic pain syndromes (Chong and Bajwa 2003). Other neurosurgical techniques used to ablate selected neurones can also be utilised, examples of these are listed below:

- Nerve Avulsion
- Dorsal Rhizotomy
- Spinal Dorsal Root Entry Zone Lesions
- Spinothalamic Tractotomies
- Thalamotomies
- Cingulotomy
- Frontal Lobotomy
- Primary Sensory Cortex Destruction

(Chong and Bajwa 2003)

All of these techniques look and sound brutal and may often result in more neural damage and thus further development or exasperation of the original neuropathic pain condition (Chong and Bajwa 2003). However, these methods have been employed in the past and, rarely, even the present day, which demonstrates the lengths some patients and clinicians are prepared to go to treat what must be severely debilitating neuropathic conditions experienced by neuropathic pain sufferers (Chong and Bajwa 2003).
2003). Out of all the above surgical procedures, none have consistently and successfully cured neuropathies in all affected patients, however dorsal column stimulation (DCS) is though to be the most successful surgical technique in reducing neuropathic pain (Chong and Bajwa 2003). DCS treatment is thought to modulate spinal rostral nociceptive transmission via stimulation of Aβ-fibres (Chong and Bajwa 2003). However, efficacy of this treatment is dependant on the type of patients and their underlying neuropathies, therefore such success is still variable (Chong and Bajwa 2003).

1.8.9.2 Non-Invasive Treatments

Non-invasive treatments such as transcutaneous electrical nerve stimulation (TENS) is one of the more basic, yet effective treatments for neuropathic pain conditions. For over 40 years this method has been utilised, since the ‘Gate Control Theory’ of pain transmission was proposed by Melzack & Wall. TENS takes advantage of the convergence of both small C-fibre and Aδ-fibre populations and large Aβ-fibre populations on spinal dorsal horn neurones and their interaction prior to ascending projections to higher centrally based areas (Chong and Bajwa 2003). A small number of studies have also implicated alternative therapies such as acupuncture as beneficial treatments in chronic pain patients (Chong and Bajwa 2003).

1.9 Spinal Transmission of Pain and Neurotransmitter Systems

1.9.1 Excitatory Amino Acids

Glutamate is a major excitatory neurotransmitter, found abundantly throughout the mammalian CNS. It has been shown to contribute towards synaptic plasticity, which is related to learning and memory, as well as pain and sensory transmission. It may also exert excitotoxic effects following excessive activity, and may therefore be implicated in the development of neurodegenerative disease states (Ozawa et al. 1998). Glutamate acts on a wide range of receptors and these are subdivided into two main groups, ionotropic and metabotropic receptors.
The ionotropic receptor (iGluR) group can be further subdivided into smaller categories (See Diagram 1.9.1.a (Bleakman and Lodge 1998)) (Ozawa et al. 1998):

- $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors
- Kainate receptors
- N-methyl-D-aspartate (NMDA) receptors

Ionotropic glutamate receptors contain cation-specific ion channels (Ozawa et al. 1998). However, the metabotropic receptors (mGluRs) are G-protein linked receptors, coupled to GTP-binding proteins and therefore modulate intracellular cell signalling via certain messenger systems (Ozawa et al. 1998).
Diagram 1.9.1.a. Glutamate Release and Glutamate Receptors. Taken and adapted from Bleakman & Hodge, 1998
There is an entire family of mGluR G-protein linked receptors (mGluR1-8), which have now been identified (Ozawa et al. 1998). These are all large proteins, with similar homology and they have been shown to exert both inhibitory and excitatory actions on both pre and postsynaptic sites (Ozawa et al. 1998). Interestingly, mGluR2/3 and 5 receptor subunits are expressed widely in the superficial dorsal horn and thus serve to modulate sensory transmission at the spinal level.

The focus of this section is the ionotropic glutamate receptors, as these will be discussed and investigated in significantly more detail, in later chapters. The three main iGluR receptors are named according to their agonist specificities, although it is important to note that although kainate and AMPA receptors (non-NMDA receptors) are structurally and functionally different, their respective agonists share the same affinity for the other receptor (Ozawa et al. 1998). Molecular and expression cloning have identified the cDNA's encoding subunits within each of the three ionotropic receptor groups (Ozawa et al. 1998). See below (Ozawa et al. 1998):

- AMPA – GluR 1-4, similar size, 68-73% sequence homology (Ozawa et al. 1998).
- Kainate – GluR5,7, similar size, 75-80% sequence homology. KA1 & KA2 are larger than GluR5-7. KA1 & KA2 share 70% sequence homology (Ozawa et al. 1998).
- NMDA – NR1 (8 splice variants reported) & NR2A-D, low sequence homology (<18%), 40-50% between NR2 subunits (Ozawa et al. 1998). NR3A may be a modulatory subunit, important in development of synaptic elements (Das et al. 1998).

Overall, iGluR receptors are comprised of four hydrophobic membrane segments (M1-M4), with a bulky extracellular N-terminus and an intracellular C-terminal domain. Interestingly, iGluRs are thought to have only three transmembrane segments, which explain the intracellular based C-terminus, whereby M2 does not traverse the membrane but merely lies as a hairpin bend located in the intracellular side of the membrane (Ozawa et al. 1998).
Diagram 1.9.1.b. The Heteromeric AMPA Receptor Complex
The AMPA receptor is thought to be a major glutamate receptor, mediating fast excitatory transmission throughout the mammalian CNS (See Diagram 1.9.1.b (Song and Huganir 2002)) (Ozawa et al. 1998). AMPA receptor subunits present as either flip and flop alternate splice variants, depending on a 38 amino acid exon residue (Tolle et al. 1995). AMPA receptors are permeable to both Na+ and K+ ions, and relatively impermeable to Ca2+ ions. However, it has emerged from expression studies that AMPA receptors, which express anything but the GluR2 subunit (i.e. GluR1, 3, & 4) are indeed permeable to Ca2+ ions (Ozawa et al. 1998). Studies in the rat have shown that AMPA receptors are located throughout the central nervous system, particularly the hippocampus (primarily CA1 & CA3 and pyramidal cell layers), superficial cerebral cortex and the molecular layer of the dentate gyrus (Ozawa et al. 1998).

The caudate putamen and deep layer cortex also contain significant amounts of AMPA receptor sites based on [H3] AMPA binding studies, as do the diencephalon, midbrain and brainstem in smaller quantities (Ozawa et al. 1998). In addition, immunocytochemical staining studies have suggested the presence of iGluR1-4 receptor subunits in lamina I-III of the spinal cord, largely implicating a role for AMPA receptors in sensory transmission at the spinal cord level (Furuyama et al. 1993; Yung 1998). Interestingly, GluR2 and GluR3 AMPA receptor subunits are thought to be involved in 5-HT-dependent activation of silent synapses which is important for the development of synaptic potentiation of sensory information and therefore the development of chronic pain conditions (Li et al. 1999b). This is thought to be via an interaction between GluR2 and GluR3 cytoplasmic C-termini and the glutamate receptor interacting protein (GRIP) among other PDZ-domain-containing proteins (Li et al. 1999b).

The kainate receptor is widely distributed throughout the entire central nervous system, although very little is known regarding its function compared to the AMPA receptor. GluR5-7 subunits have been shown to form homomeric kainate receptors (Chittajallu et al. 1999). However KA1 and KA2 subunits, although unable to form homomeric subunits alone, can form heteromeric complexes with the GluR5-7 subunits (Chittajallu et al. 1999). It is important to note that differences in the expression of kainate receptor subunits, have large effects on the functionality and properties of the ion channel receptor protein (Chittajallu et al. 1999).
Consistent with the AMPA receptor, kainate receptors are permeable to Ca\(^{2+}\) ions and the GluR6 subunit has been shown to exert substantial Ca\(^{2+}\) permeability in expression studies (Ozawa et al. 1998). Radioligand binding studies in the rat have revealed dense labelling for \([H^3] \text{Kainate}\) throughout the hippocampus, CA3 and granular layer of the cerebellum (Ozawa et al. 1998). Kainate receptor subunits (iGluR5-7) are also distributed within lamina I-III of the spinal cord (Furuyama et al. 1993; Yung 1998). DRG neurones are also believed to contain functional kainate receptors, strongly implicating expression of kainate receptors on primary afferent neurones in the superficial dorsal horn (Kerchner et al. 2001b). Indeed, it is believed that presynaptic kainate receptors serve to regulate sensory, particularly nociceptive, transmission in the superficial dorsal horn (Kerchner et al. 2001b).

The NMDA receptor mediates excitatory neurotransmission in the CNS (See Diagram 1.9.1.c) (Sucher et al. 1996). However the role of the NMDA receptor is extremely diverse and is implicated in neuronal plasticity, gene expression induction, as well as neuronal growth and survival within the central nervous system (Sucher et al. 1996). The NMDA receptor has unique properties compared to the AMPA and kainate receptors. NMDA receptors are blocked by Mg\(^{2+}\) in a voltage dependant manner, this infers that at normal resting membrane potentials the NMDA receptor does not allow the passage of ions through its ion channel pore (Ozawa et al. 1998). Interestingly, NR1-NR2A and NR1-NR2B subunits appear to be blocked by Mg\(^{2+}\) ions more efficiently than the other heteromeric complexes, which form the NMDA receptor (Kuner and Schoepfer 1996). Furthermore, the NMDA receptor exerts slow gating kinetics, as well as being highly permeable to Ca\(^{2+}\), Na\(^+\) and K\(^+\) ions vital for electrical transmission (Ozawa et al. 1998).

Based on expression studies, the NR1 subunits can form homomeric receptors in Xenopus oocytes (unlike NR2). Yet within the central nervous system, it is thought that NMDA receptor subunits form heteromeric receptor complexes consisting of four subunits (tetrameric) with larger and more appropriate current response amplitudes than that of NR1 homomers (Sucher et al. 1996; Ozawa et al. 1998). It is therefore widely believed that NR1 NMDA receptor subunits are co-expressed with NR2 subunits, forming functional NMDA receptors.
Diagram 1.9.1.c. The NMDA Receptor

Glycine

7-CK Mrz 2/571 Mrz 2/579 (Antagonists)

Glutamate

APV (Antagonist)

Mg^{2+}

Ketamine MK-801 (Blockers)

Ca^{2+} Na^{+}

H^{+} Zn^{2+}

Polyamines

7-CK Mrz 2/571 Mrz 2/579 (Antagonists)
Combination of the NR1 subunit with different NR2 subunits (A-D) form a variety of functional NMDA receptor subtypes but with varying properties, it is thus clear that NR2 subunits are primarily modulatory subunits and differ somewhat in their functionality (Ozawa et al. 1998).

Interestingly, studies in rat brains have revealed that the NMDA receptor subunits are expressed in distinct areas of the central nervous system in varying quantities (Ozawa et al. 1998). NMDA receptors are largely distributed in the hippocampal CA1 region, as well as other areas of the brain (predominantly the forebrain). The NR1 subunit, consistent with these findings, is also found abundantly throughout the entire central nervous system. However, the regional distributions of the NR2 subunits are relatively distinct, as listed below (Ozawa et al. 1998):

- NR2A - Cerebral Cortex, Hippocampus and Cerebellum (Ozawa et al. 1998).
- NR2B - Cerebral Cortex, Hippocampus, Septum, Caudate Putamen and Olfactory Bulb (Ozawa et al. 1998).
- NR2C - Granule Layer of Cerebellum, Olfactory Bulb, Thalamus.
- NR2D - Hippocampal Interneurones.

Immunocytochemical studies in the rat have revealed that within the dorsal horn of the spinal cord NMDA2B subunits are found predominantly in lamina I-III, as are NMDA1 subunits (Yung 1998). Other reports suggest that NR2D subunits can also be located in the superficial dorsal horn (Yaksh and Aimone 1983). Interestingly, NMDA2A and NMDA2B subunits were not found in the dorsal horn, suggesting these receptors play a small role in nociceptive transmission at the spinal cord level (Furuyama et al. 1993; Yung 1998).

It is clear from the literature that both glutamate and the amino acid glycine are required to activate the NMDA receptor channel, and such findings suggest glycine is a co-agonist in the activation and function of NMDA receptor activity (Wood 1995). It is broadly assumed that glycine is abundant in the nervous system, and therefore has no controlling influence on NMDA receptor function. However, many studies have challenged this view and feel that the role of glycine as an NMDA receptor co-agonist requires more in depth investigation (Wood 1995).
Furthermore, the NMDA receptor structure also contains modulatory sites for polyamines, protons, redox agents and Zn\(^{2+}\) suggesting that other agents may affect the activity of NMDA receptors, alongside glycine (Sucher et al. 1996). Interestingly, these sites can also be distinguished and modulated pharmacologically, as will be discussed in more detail in later chapters.

1.9.1.1 NMDA Receptor mediated Neuronal Response Properties

NMDA receptors, as already mentioned, have a variety of distinct properties. (Dickenson 1990). One of the most intriguing properties is the 'wind-up' effect consistently attributed to NMDA receptor channels, following repetitive high threshold stimuli of nociceptive neurones (Dickenson 1990). 'Wind-up' describes the sharp increase in response following repetitive constant stimuli and this effect is thought to underlie the mechanisms for acute pain transmission and central sensitisation in the central nervous system (Dickenson 1995a; Li et al. 1999a). Central sensitization frequently arises following tissue injury or neuronal insult (Li et al. 1999a). Such damage often evokes increased excitability of dorsal horn neurones, increased receptive field sizes, and heightened response properties in nociceptive and sensory nerve fibres (Li et al. 1999a).

In electrophysiological studies, wind-up can be evoked following repetitive electrical stimuli administered at C-fibre thresholds (Dickenson 1995a). Repetitive noxious stimuli prompts peptide released from pre-synaptic C-fibre afferents within the superficial laminae and this, in turn, results in the depolarisation required to remove the voltage dependant Mg\(^{2+}\) block of NMDA receptors. The recruitment of NMDA receptors in the neuronal response to repetitive noxious stimuli is also enhanced by nitric oxide production and eventually gene induction within central neurones (Dickenson 1995a; Stanfa et al. 1996). Wind-up is the result of progressive increases in neuronal response properties, particularly that of C-fibres, which are no longer proportional to the original evoked stimulus. This effect is thus attributed to the activation of NMDA receptors. Electrophysiological studies whereby the wind-up response is significantly hampered following application of NMDA receptor antagonists thus confirm such a role of NMDA receptors in the wind-up response (Suzuki et al. 2001).
Also attributed to the distinct NMDA receptor characteristics, is long term potentiation (LTP) and long term depression (LTD), whereby NMDA receptors underlie a persistent change in synaptic strength and functionality (Hrabetova and Sacktor 1997). Interestingly, NMDA receptors with different subunit composition underlie these two relatively distinct alterations in synaptic strength (Hrabetova and Sacktor 1997). Following nerve injury, central plasticity and changes in the normal transmission of sensory information, result in various abnormal pain related behaviours (Devor 1991; Wall 1991; Woolf and Mannion 1999; Orza et al. 2000; Attal 2001; Baheti 2001; Bouhassira 2001; Bridges et al. 2001; Koltzenburg and Scadding 2001; Meyer-Rosberg et al. 2001; Taylor 2001; Cabaleiro 2002; England and Gould 2002; Hansson 2002; Max 2002; Wilson 2002; Chong and Bajwa 2003). It is therefore possible that alterations in both the expression and location of excitatory amino acid receptors, known to play such a large role in sensory transmission, may contribute to the occurrence of such conditions. It is known that excitatory amino acids acting at the NMDA receptor, contribute to the development of central sensitisation (Coderre and Melzack 1992). There are distinct upregulations in the expression of excitatory amino acid receptors in the ipsilateral superficial laminae of the lumbar spinal cord following nerve injury, suggesting that excitatory amino acid receptors contribute towards the abnormal pain conditions mentioned above e.g. hyperalgesia (Harris et al. 1996). In later chapters, the role of the excitatory amino acid receptors in normal and neuropathic systems will therefore be more fully analysed and discussed.

1.9.2 Inhibitory Amino Acids

GABA is both a crucial and an abundant neurotransmitter and the most well recognised inhibitory amino acid within the CNS (See Diagram 1.9.2.a, (Carlson and Olsen 1998)) (Malcangio and Bowery 1996). It is also believed to control over 40% of the inhibitory events within the CNS (Malcangio and Bowery 1993; Malcangio and Bowery 1996). GABA is a product of the Krebs cycle and relies on the presence of glutamic acid decarboxylase (GAD) for its production (Malcangio and Bowery 1996). Once produced, GABA is stored in vesicles within the nerve terminal whereby, it is only released upon raised intracellular calcium concentrations, which arise following neuronal activation (Malcangio and Bowery 1996).
Within the mammalian spinal cord GABA is generally found throughout the dorsal horn, particularly the superficial laminae (I-III) (McLaughlin et al. 1975; Hunt et al. 1981; Todd and McKenzie 1989; Malcangio and Bowery 1996). In the superficial dorsal horn GABA and GAD are located within cell bodies and neuronal axons (Barber et al. 1978; Barber et al. 1982; Todd and McKenzie 1989). Using antiserum for GABA, 24-33% of the entire lamina I-III neuronal population have been shown to comprise this inhibitory amino acid (Todd and McKenzie 1989).

The ventral horn as well as the surrounding white matter also contains small amounts of GABA (Malcangio and Bowery 1996). Within the superficial dorsal horn, interneurones, cell bodies and neuronal axon terminals contain large amounts of GABA, interestingly these GABAergic interneurones are predominantly lamina II islet cells and may project onto primary afferent terminals within lamina I and II exerting inhibitory effects (Malcangio and Bowery 1996). More specifically, it is evident that GABAergic axons terminate presynaptically on HTM afferents or their post-synaptic neurones in lamina I and II of cat and monkey spinal cords, thus hampering the transduction of nociceptive mechanical stimuli (Alvarez et al. 1992). Electrophysiological studies whereby A-δ-fibre and noxious mechanical responses are facilitated in deep dorsal horn neurones following bicuculline mediated block of GABA_A receptors, confirm these effects (Kontinen et al. 2001). Ensuing studies have all agreed with these findings, and intriguingly there appears to be an association between nociceptive inputs in the superficial dorsal horn and nitric oxide containing neurones (Bernardi et al. 1995).

Within the CNS, GABA acts on three main receptor subtypes:

- **GABA_A** – *Ligand gated ion channel (Cl-)* (Malcangio and Bowery 1996).
- **GABA_B** – *G-protein coupled receptor, associated with Ca^{2+} and K^{+} channels* (Malcangio and Bowery 1996).
- **GABA_C** – *Ligand gated ion channel (Cl-)* (Malcangio and Bowery 1996).

(*Ligand gated ion channels allow the influx of Cl- which sustains the resting membrane potential following activation of excitatory receptors, Cl- permeability thus results in membrane depolarisation or hyperpolarisation (Malcangio and Bowery 1996)).
GABA_{A} receptors form pentameric receptor structures and are heteromeric assemblies of three main subunits, $\alpha$- $\beta$- $\gamma$, although four main subunits have been identified e.g. $\alpha$- $\beta$- $\delta$- $\gamma$- subunits (Malcangio and Bowery 1996; Pirker et al. 2000). Multiple isoforms of each receptor subunit have been revealed and interestingly, 6 $\alpha$ subunit isoforms ($\alpha_{1}$-$\alpha_{6}$), 3 $\beta$-subunits ($\beta_{1}$-$\beta_{3}$), and finally 3 $\gamma$-subunits ($\gamma_{1}$-$\gamma_{3}$) have been identified in the rat CNS (Pirker et al. 2000). However, the diversity of these GABAergic receptors does not stop here, $\rho$-subunits ($\rho_{1}$-$\rho_{3}$), $\varepsilon$-subunits $\theta$-subunits and $\pi$-subunits have also been identified in retinal GABA_{C}, rat brain and peripheral tissues respectively (Pirker et al. 2000). Immunocytochemical studies have identified GABA receptor subunits throughout the brain, including cerebellum, thalamus, forebrain and cortical regions (Pirker et al. 2000). GABA_{A} receptors are found throughout the dorsal horn in the spinal cord. However, GABA_{B} receptors are largely confined to lamina I and II (Malcangio and Bowery 1996; Chery and De Koninck 2000). Interestingly, both receptor types are located presynaptically, thus mediating the release of neurotransmitters from dorsal horn neurones and regulating neuronal firing via the presynaptic depolarisation of nociceptive primary afferent nerve fibres (Malcangio and Bowery 1996). Following nerve injury, alterations in the GABAergic influence of normal spinal sensory processing appears to alter the response properties to a variety of evoked peripheral stimuli (Kontinen et al. 2001). The resulting effect is likely to induce behavioural changes, which may underlie chronic pain conditions such as allodynia and hyperalgesia associated with nerve injury. Such plasticity in nerve injured systems will be further discussed in later sections.

Glycine is another major inhibitory neurotransmitter, although far less is known regarding its actions and effects. Glycine is thought to act on specific glycine receptors, which are ligand gated Cl- ion channels (Todd et al. 1996). Two subunits, $\alpha$ and $\beta$ (which act as ligand binding and structural subunits respectively) and the peripheral membrane protein gephyrin comprise glycine receptors (Todd et al. 1996). Gephyrin is crucial in its role in attaching the glycine receptor to synaptic membranes (Todd et al. 1996). Glycine, much like GABA, is thought to act as a major inhibitor of spinal dorsal horn neurones. However, electrophysiological studies which record from deep dorsal horn neurones have failed to find comparable inhibitory effects on neuronal response properties, following both electrical and naturally evoked thermal and mechanical stimuli (Kontinen et al. 2001). It is thus evident that more work is necessary to unravel the role of glycine in the spinal cord and unveil its role in both sensory and nociceptive transmission.
1.9.3 Peptides

There are a wide variety of neuropeptide transmitters within the central nervous system, which enable the transfer of nociceptive information across synapses. Substance P (an 11 amino acid peptide) is located within and released (along with glutamate) from C-fibre afferents in the spinal cord (See Diagram 1.9.3.a, (Malcangio and Bowery 1999)). It is part of the tachykinin/ neurokinin family and is thought to have a major role in pain and hyperalgesia (Dickenson 1995a; Dickenson 1995b; Juranek and Lembeck 1997). Substance P acts on G-protein-linked tachykinin (Neurokinin NK) receptors, which have a rhodopsin-like membrane structure, and consist of three subtypes NK 1,2 & 3 (Harrison and Geppetti 2001). Substance P acts preferentially on the NK1 receptor, and the other tachykinins, NK A and B act on NK 2 & 3 subtype receptors respectively (Harrison and Geppetti 2001). It is thought that the majority of substance P receptors are located on post-synaptic neurones in the dorsal horn, particularly large neurones in lamina III and IV (Todd et al. 2000). However, recent studies have shed light upon a distinct population of NK1 autoreceptors, which enable the modulation of neurotransmitter release from the pre-synaptic primary afferent terminal within the dorsal horn (Malcangio and Bowery 1999). It is believed that upon noxious stimulation in the periphery, substance P is released into the dorsal horn and targets these NK1 receptors (Duggan and Hendry 1986; Lawson et al. 1997). Interestingly, upon activation of the NK1 receptor the receptor is often also internalised by the dorsal horn neurone (Mantyh et al. 1995a; Mantyh et al. 1995b).

Although substance P also plays a distinct role in the peripheral nervous system, including neurogenic inflammation and peripheral sensitisation, I shall be focussing on its role in the central nervous system. Fos immunostaining studies have revealed that NK-1 receptors localised on the superficial lamina I neurones are concerned with the coding of the intensity of noxious stimuli from the periphery (Doyle and Hunt 1999b).
Diagram 1.9.3.a Production and Release of Substance P in the Dorsal Horn. Taken and adapted from Malcangio & Bowery, 1999
Intriguingly, deep dorsal horn NK1 expressing neurones are concerned primarily, with the stimulus modality type and its spatial localisation (Doyle and Hunt 1999b). Interestingly, the vast majority (>90%) of substance P containing nociceptive primary afferents have been shown to project to the contralateral lateral reticular nucleus, in the brainstem as well as the lateral parabrachial nucleus (>60%) and the caudal medulla (Todd et al. 2000). Such studies thus implicate NK1 containing nociceptive dorsal horn neurones as comprising a large proportion of spinal ascending pathways (Todd et al. 2000). Knock-out mice are commonly used to study substance P and the NK1 receptor. However, in recent years the development of a conjugate of substance P and the ribosome-inactivating protein saporin, which is internalised in substance P expressing neurones in lamina I, have allowed the study substance P containing and thus NK1 expressing lamina I neurones and their contribution towards nociceptive transmission (Mantyh et al. 1997).

Calcitonin gene-related peptide (CGRP) is released from primary afferent neurones within the dorsal horn of the spinal cord (Dickenson 1995b). Recent advances have meant that a CGRP receptor antagonist (CGRPs-a?) is now available, however prior to this little was known as to the role of CGRP and its target receptor within the CNS. It has been suggested that the presence of CGRP, alongside substance P, in primary afferent terminals enables the wider release of substance P within the dorsal horn and thus augments the actions of substance P during nociceptive transmission (Dickenson 1995b). It is also believed that CGRP receptor activation is involved in the development of peripheral inflammatory and neuropathic conditions (Bennett et al. 2000). Intriguingly, in rat models of central neuropathic pain, CGRP 8-37 has been shown to reduce the development of both mechanical and thermal allodynia (Bennett et al. 2000).

Other neuropeptides are found in the dorsal horn, and are thought to be involved the transmission of sensory information from the periphery to the spinal cord. Somatostatin is an inhibitory peptide transmitter located within C-fibres and small diameter DRG cells (Dickenson 1995b). It is released upon application of peripherally administered noxious heat, however the result of such release is an ensuing dampening of the spontaneous evoked activity (Dickenson 1995b).
Galanin is also found within nociceptive afferents and is often located in conjunction with CGRP and substance P (Dickenson 1995b). Galanin is believed to have both excitatory and inhibitory actions within the spinal cord, however it has been shown that following peripheral nerve ligation, exogenous galanin exerts primarily inhibitory effects on spinal dorsal horn neuronal response properties (Flatters et al. 2002).

1.9.4 5-HT/Noradrenaline

Serotonin (5-HT) is a widely distributed neurotransmitter within the central nervous system and has distinct effects on nociceptive transmission (Maxwell et al. 2003). There are multiple subtypes of the 5HT receptor, all of which are expressed in mRNA within DRG cells (Millan 1999). These 5HT receptors are 5HT1A,B,E,D,F, 5HT2A-C, 5HT3 & 5HT5A,B, 5HT6.7 subtypes. Interestingly, 5HT1A,1B, 2A, 3, 7 are largely found in the dorsal horn and dorsal root ganglia whereas the other subtypes are found largely in the intermediolateral cell column, the sympathetic ganglion and the ventral horn (Millan 1993; Millan 1994). Serotonergic inputs onto lamina I (and lamina II) via projection neurones, as well as interneurones from lamina II largely influence activity within the spinal cord, however studies have failed to locate serotonergic activity within primary afferent neurones which terminate in the superficial dorsal horn (Millan 1993). It was believed for a long time, that the transmission of nociceptive information from the periphery to the central nervous system may be modulated via descending inhibitory serotonergic fibres thus exhibiting an endogenous analgesic role (Basbaum and Fields 1984). However, other studies have contradicted these beliefs in a variety of ways. For example, nucleus raphe magnus (NRM) is an area contributing to descending 5HT pathways, stimulation of this region has been shown to raise levels of noradrenaline as opposed to 5HT in the dorsal horn, which is largely mediated via α2-adrenoceptors (Millan 1993). Furthermore, 5HT has not been localised in any regulatory ‘On or Off’ cells within the CNS and interestingly, upon application of noxious stimuli, 5HT neurones have not been shown to respond (Millan 1993).
Much interest into the serotonergic system has been focussed on the 5HT$_3$ receptor system, which acts via increasing gCa$^{2+}$ and gNa$^+$ as well as PLC, and is thought to have an antinociceptive role via excitatory actions at GABAergic and other inhibitory sites (Millan 1993). Experimental techniques have allowed the selective ablation of NK1 receptor containing neurones in the superficial dorsal horn (Mantyh et al. 1997). Such experiments have shown that evoked changes in mechanical and thermal coding, receptive field areas and central sensitization are mimicked via block of 5HT$_3$ receptors. From such studies, it is suggested that the 5HT$_3$ receptor system may form part of a descending 5HT facilitatory pathway (Suzuki et al. 2002). In addition it is known that 5HT$_3$ receptors are located within the superficial dorsal horn and it is also suggested that unmyelinated primary afferents may also contain 5HT$_3$ receptors (Millan 1993). Such results strongly implicate the 5HT$_3$ receptor system in the modulation of nociceptive transmission within the spinal cord. It is thus worth further investigation.

Whilst mentioning 5HT it is also important to mention noradrenaline, as this also comprises many of the descending pathways which may exert a modulatory effect in pain transmission. Noradrenaline pathways originate predominantly from A5 and A6 clusters in the pons and project to the spinal cord (Millan 1993; Millan 1999; Millan 2002). It is thought that noradrenaline has direct inhibitory effects on projection neurones, primary afferent terminals and interneurones, particularly in the superficial dorsal horn. Evidence partly stems from the presence of $\alpha_2$ receptors in these aforementioned neuronal populations (Millan 1993; Millan 1999; Millan 2002). Indeed, $\alpha_2$ receptors are located throughout the superficial dorsal horn in large numbers and also in the deep dorsal horn layers (Millan 1993; Millan 1999; Millan 2002). Interestingly, there is some clear evidence emanating from in vitro studies that noradrenaline may also exert excitatory effects on both projection neurones and primary afferents in superficial laminae. Such findings have more recently been confirmed in vivo (Millar and Williams 1989; Millan 1993; Millan 1999; Millan 2002). These reports and more detailed studies largely implicate noradrenaline as a major transmitter in descending pathways involved in the control and modulation of pain transmission.
1.10 Aims

The aims of this study are to investigate the electrophysiological characterisations of lamina I spinal neurones, and any resulting changes following neuropathy or selective ablation of NK1 containing neurones and compare this to well documented electrophysiological characterisations of lamina V deep dorsal horn neurones. It is also my aim to investigate the role of excitatory and inhibitory neurotransmitter systems within lamina before and after spinal nerve injury. I shall be focussing on AMPA, Kainate, NMDA (including the NR2B expressing NMDA receptors) as well as the inhibitory GABA_A and glycine receptor systems in lamina I. Furthermore, I also wish to look at the role of neuropeptides, particularly CGRP, in lamina I within normal rat models. As an extension to this study I will investigate the 5HT_3 receptor systems in lamina I dorsal horn neurones following selective ablation of their NK1 containing neurones. It will be interesting to compare this to a previous studies performed by (Suzuki et al. 2002) whereby distinct and fascinating changes were observed in lamina V dorsal horn neurones.
CHAPTER 2

METHODS
2.0 Materials and Methods

2.1 Anaesthesia and surgery

Intact anaesthetised male Sprague-Dawley rats (200-250g) were used to perform these experiments, in concordance with the U.K. Animals (Scientific Procedures) Act (1986) and International Association for the Study of Pain (IASP) guidelines. The rats were anaesthetised with 3% halothane in 33% O₂ and 66% N₂O until the righting reflex was lost and areflexia was achieved. At this point the anaesthetic was reduced to 2% halothane for the ensuing surgery. The rats body temperature was regulated using a thermostatically controlled heating blanket, maintaining the core temperature of 37 °C. Tracheal cannulation using polyethylene tubing was used to maintain anaesthesia throughout the experiment, and the rat was then secured with ear bars in a stereotaxic frame. The vertebrate column was exposed by removal of surrounding connective tissue and muscle and metal clamps were then placed rostral and caudal to the column to secure it. The spinal cord was then exposed via a laminectomy at vertebrae L1-L3 and the outermost dura mater was removed using fine forceps. The level of anaesthesia was then reduced to approximately 1.6%, which was deemed sufficient to retain a state of complete areflexia. Following this, electrophysiological recording began.

2.2 Electrophysiological Recording

A parylene coated tungsten recording electrode attached to a headstage monitor placed next to the stereotaxic frame was inserted into the exposed spinal cord. The recording equipment, stereotaxic frame and the animal itself is grounded prior to recordings. The signal recorded from the spinal cord is delivered to the data capture 'Neurolog' system, which is fitted with spike discrimination and audio monitoring equipment.
Diagram 2.2.a. Data Capture Process in the Electrophysiological Studies
This signal can then be both filtered and amplified and eventually transduced to the audio speaker and oscilloscope. Evoked action potentials above pre-set amplitudes (described later) can be recognised and captured by CED 1401 interface and spike 2 software which enables the evoked data to be fed into a computer, where it is both quantified and analysed (See Diagram 2.2.a).

2.2.1 Isolating a neurone

The parylene coated tungsten electrode was manually lowered into the spinal cord until it just penetrated the surface. At this point the electrode was retracted to the surface until light tapping of the rat hindpaw/ receptive field no longer evoked any neuronal activity. A SCAT microdrive was then used to lower the electrode through the dorsal horn of the spinal cord in 10µm increments. This allowed accurate analyses of the depth of the electrode in the spinal cord and thus an approximation of the lamina layer in which the electrode was positioned. However, as a large proportion of lamina I neurones were located using a bent electrode (discussed below), the SCAT microdrive could not always accurately perform this function. Manual analyses of depth was often used. Unfortunately lack of histological staining meant that the location and depth of the isolated neurones recorded in this study, could not be confirmed.

Light tapping of the receptive field area evoked activity within the spinal cord. The electrode could be moved rostrally and caudally, along the length of the central vessel, on either side of the spinal cord until a suitable single neurone was located. Action potentials elicited by this neurone in response to peripheral stimulation could be differentiated from background neuronal noise by a window discriminator.
Neurones utilised for drug experiments had to evoke responses of sufficient magnitude to allow appropriate analysis of drug effect. The nature of lamina I neurones recorded meant that these values were lower than previously recorded lamina V neurones in similar and related studies utilising these experimental techniques. It was thus not always feasible to use all responses elicited by lamina I neurones when performing pharmacological studies. More often than not, one or more electrically or naturally evoked responses were not large enough to define a drug effect, these neurones were thus characterised only. 50 action potentials was taken as a response when categorising neurones. However 100 action potentials was a sufficient response for C-fibre evoked activity, > 10 action potentials for input and A-δ fibre responses and >20 action potentials for XS-spike and post-discharge responses.

2.2.2 Characterisation of isolated neurone

Lamina I neurones were located in the superficial marginal layer of the spinal cord (0 – 250 μm) using a parylene-coated tungsten electrode, which then enabled single-unit extracellular recordings to be made. The electrode was positioned to enter the spinal cord at an angle of 45° (using a protractor to measure the angle) enabling it to penetrate the spinal cord at an angle that allowed a large cross-sectional area of the superficial layer to be searched for neuronal activity. Using simple trigonometry depths could be corrected using the cosine equation (cos45° * neuronal depth). A population of lamina V cells were also recorded in order to compare response characteristics. However, the majority of the lamina V neuronal responses presented in this study were taken, with permission, from data compiled by Rie Suzuki and Kate Carpenter over the course of their respective PhD and post-doctoral studies. A variety of innocuous and noxious stimuli were used to characterise the neuronal responses using an assortment of hot,
warm and cold water jets, brush and von Frey hairs. Temperatures of 4, 32, 35, 40, 42, 45, 48, 50, 52°C were applied for 10 seconds, via a water jet in strict ascending order. Temperatures of 32°C were indicative of any mechanical activity elicited by the force of the water jet and could therefore be evaluated as a neutral response when classifying lamina I neurones into various physiological categories. Von Frey filaments (Scientific Marketing Associates, 189/191 High Street, Barnet, Herts, EN5 5SU. Cat No. 18011) were also applied in ascending order 1, 5, 9, 12, 15, 30, 75 and 125g for 10 seconds. Lastly, a fine brush was applied in gentle stroking patterns to the receptive field area for 10 seconds. A time interval of 2 minutes was left between each thermal and mechanical stimulation to prevent desensitisation of the neurone, and 20 minutes was left between each set of tests. All spontaneous activity of the recorded neurone (always low – approximately 0.1-1Hz), was subtracted from the neuronal response to each stimulus. When testing drug effect temperatures of 4, 35, 40, 45, 48, and 50°C were applied for 10 seconds each, via a water jet in strict ascending order, much the same way. In this case 35°C was loosely used as an indicator of mechanical activity evoked by the water jet. The same von Frey filaments were utilised, as well as the brush response. However for one study, the effect of 32 and 45°C, and von Frey 9 and 30g were used to observe drug effects purely to assess the neuronal response to an innocuous and noxious thermal and mechanical stimulus.

The response of these neurones to electrical stimuli was also measured by delivering electrical stimuli through two stimulating 27-gauge needle electrodes into the neuronal peripheral receptive field area, located on the ipsilateral hindpaw. The C-fibre threshold of the neurone was detected and a train of 16 electrical stimuli (0.5 HZ, 2 ms pulse) applied at three times this level was delivered. The threshold was taken as the
necessary current to elicit action potential firing of C-fibre latency (90-300 milliseconds post-stimulus) in the neurone. Following each ‘test’ the responses of A-fibres and C-fibres were captured and plotted as a post-stimulus histogram (spike2 software CED Cambridge, UK). The responses were quantified by separating the action potential firing on a latency basis (0-20 and 20-90 milliseconds respectively, A (β and δ)). As well as the responses attributed to different fibre types, post-discharge could be measured. Post-discharge is the result of repeated C-fibre activity which induces ‘wind-up’, as seen in deep dorsal horn neurones (Dickenson and Sullivan 1987; Dickenson 1990; Dickenson and Sullivan 1990). This then leads to neuronal activity beyond the C-fibre latency band (300-800ms). However, many lamina I neurones did not exhibit a ‘wind-up’ response, merely a small increase in action potential firing as the stimulus was repeated. This was termed ‘reduced wind-up’ and seemed to be a characteristic of lamina I cells. ‘Reduced wind-up’ was presented in the same way as ‘wind-up’ is in studies performed on deep dorsal horn neurones in that the response to each stimulus was plotted against the stimulus number (Dickenson and Sullivan 1990).

As well as post-discharge, excess spikes and the input response were also measured. The input was taken as being the number of action potentials at C-fibre latencies following the first electrical stimuli in the train of 16. Excess spikes was calculated from the input response multiplied by 16, and is a measurement of the amount of action potentials fired beyond what is expected if each stimuli evoked the same number of action potentials. Excess spikes are an indication of wind-up, therefore with most lamina I cells with reduced wind-up, this response was negligible. The receptive field area, based on the pinch response of each lamina I neurone, was mapped on a standard paper template of the projected area of the plantar surface of the rat hindpaw. If a neuronal response of 0.5Hz was seen then this was believed to be within the receptive
field area. Mean % changes in this receptive field area were mapped post-drug application at a 40-minute time point, at which effects of the drug were deemed maximal. The marked area on the diagrams were carefully cut out and weighed. Therefore, the receptive field areas were calculated as the weight of each receptive field area on plain paper always obtained from my lab book. The lamina I neuronal receptive fields were illustrated as a percentage of the mean weight of 20 control diagrams (consistent with measurements by earlier studies) of the whole hindpaw template (75 ±0.4 mg) (Suzuki et al. 2000).

2.2.3 Drug Administration

Neuronal control testing was conducted until stabilisation of the responses were observed (<10% variation with all responses recorded) and a minimum of 3 pre-drug controls performed before drug application proceeded. The effects of the drugs were observed and tests performed at 10-minute intervals. The effects of electrical, von Frey and thermal stimuli were tested following application of certain drugs, this meant that a 20 minute interval between tests was necessary to allow sufficient time for these tests to be elicited. Maximal effects were typically seen between 20-40 minutes-post drug application. Therefore an interval of 40-60 minutes was normally left between cumulative dosing to be consistent with and based on previous similar electrophysiological studies. The effect of different drugs applied directly onto the spinal cord on lamina I neuronal responses were observed. All drugs were administered in 50μl volumes, using a 50μl measured Hamilton syringe. Doses were selected based on previous electrophysiological pharmacological studies, to allow efficient comparison between data samples.
2.3 Drugs Employed

ACEA-1244, IUPAC name 1-[2-(4-Hydroxy-phenoxy)-ethyl]-4-(4-methyl-benzyl)-pi-peridin-4-ol. Kindly provided by Parke-Davis. The Drug was dissolved in ethanol, Cremaphor was added and finally saline was added after the solution had been sonicated for 30 minutes. Proportions were 33% Cremaphor, 7% Ethanol and 60% Saline.

AP-5 Lithium Salt (DL-2-Amino-5-Phosphonovaleric Acid) was obtained from Sigma-Aldrich Company Ltd., Poole, Dorset, U.K. The drug was dissolved in 0.9% saline and stored in glass vials at 4°C. Doses used were 50, 100, 500 µg/50 µl.

(-)-Bicuculline methiodide (C$_{21}$H$_{20}$NO$_{6}$) was obtained from Sigma-Aldrich Company Ltd., Poole, Dorset, U.K. The drug was dissolved in 0.9% saline and stored in glass vials at 4°C. The vials were protected from light using aluminium wrapping. Doses used were 0.5, 5, 50 µg/50 µl.

CGRP 8-37 (rat) Calcitonin gene related peptide 8-37 was obtained from Tocris Cookson Ltd., Northpoint, Fourth Way, Avonmouth BS11 8TA, U.K. The drug was dissolved in saline 0.9% and stored in separate aliquots at -20°C.

CNQX (6-Cyano-7-nitroquinoxaline-2,3-dione) was obtained from from Tocris Cookson Ltd., Northpoint, Fourth Way, Avonmouth BS11 8TA, U.K. The drug was dissolved in saline 0.9% and stored in vials at room temperature.
Ifenprodil Tartrate Salt, (α-[4-Hydroxyphenyl]-β-methyl-4-benzyl-1-piperidineethanol) was obtained from Sigma-Aldrich Company Ltd., Poole, Dorset, U.K. The drug was dissolved in 0.9% saline and stored in glass vials stored at 4°C.

LY293558C was given by Eli Lilly and Company, Lilly Corporate Center, Indianapolis, IN 46285. The drug was dissolved in 0.9% saline and stored in glass vials at 4°C.

NBQX disodium salt (2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide disodium salt) was obtained from from Tocris Cookson Ltd., Northpoint, Fourth Way, Avonmouth BS11 8TA, U.K. The drug was dissolved in 0.9% saline and stored in glass vials stored at -20°C. Doses used were 0.5, 5, 50 μg/50 μl.

Ondansetron (Zofran) Felxi-amp injections (i.v./i.m.) Glaxowellcome, U.K. The drug was dissolved in 0.9% saline and stored in glass vials stored at 4°C.

Strychnine (C_{21}H_{22}N_{2}O_{2}. HCl) Hydrochloride Crystalline was obtained from Sigma-Aldrich Company Ltd., Poole, Dorset, U.K. The drug was dissolved in 0.9% saline and stored in glass vials stored at room temperature, protected from light using aluminium wrapping.

2.4 Analysis of Results

All graphs were presented using CA-Cricket Graph III software packages and then transferred to Canvas™ 3.5.4. alias. Data was displayed on Microsoft Excel Software, whereby mean % controls and standard errors could be calculated. The effect of each drug on the different lamina I neuronal responses recorded in each experiment was only
analysed and included in the results presented below, if the control value was sufficient to demonstrate an effect (e.g. ≥100 spikes C-fibre & Aβ-fibre response, ≥10 Spikes Aδ-fibre & Input response and ≥20-30 spikes Post Discharge and XS spike response). The nature of lamina I neurones was such that responses, particularly in the innocuous stimulus range, were small. Occasionally a neurone was used which generally exerted a good response range, yet one or a couple of responses recorded were too small to establish a clear and definitive drug effect. Lamina I neurones were hard to locate due to the thin layer that they inhabit. Such was the dilemma that when a lamina I neurone with a good response profile overall was found and characterised, rather than risk not obtaining any data for that day, it was normally followed despite not always exerting the full range of responses. It was thus procedure to not analyse drug effect unless satisfactory controls were obtained for that response type.

2.4.1 Statistical analysis of results

The means of all pre-drug controls were used to calculate maximal % changes following drug application. The maximal effects were shown as means ± standard errors of the means. Statistical analyses of these values were performed using a paired t-test on the maximal drug effects compared to pre-drug controls, using the raw data. This was based on the sufficient 'n' numbers achieved in the studies performed and the normal distribution of the data sets. A 95% confidence interval was used for significance. Bonferroni corrections for post hoc analyses of data were made when repeated comparisons were made to the original control values, as tabulated within each chapter. This prevented an increase in the probability of finding significance in the data sets, when repeated measures to the same control data were made. Statistical analyses of group data, whereby comparisons were made between animal groups were performed
using unpaired t-tests, using the raw data. ANOVA (one-way) factorial statistics were used in the characterisation studies to compare neuronal populations. All statistical tests were performed using a Statview package, Abacus Concepts, 1992-1995. Due to time restrictions, time-matched vehicle controls were not performed in these studies. Therefore no statistical comparisons were made between the drug effects and vehicle controls.

2.5 Spinal Nerve Ligation Model of Neuropathy

Spinal nerve ligation (SNL) was conducted using male Sprague-Dawley rats weighing between 120 to 150g (University College London Biological Services). Prior to such procedures being conducted for this study Home Office approval was obtained and guidelines in the International Association for the study of Pain were closely referred to. (Zimmerman, 1983).

Selective tight ligation of spinal nerves L5 and L6 were performed as described by Kim and Chung (1992), as well as a control sham procedure. Anaesthesia was induced by placing the rat into a sealed container and maintained by placing the rat in a prone position attached to a nose cone. A mixture of halothane (3.5% induction, 1.5% for maintenance) was used for this purpose with a flow ratio 1:1 of N₂O:O₂. An incision (2cm long) was made at the midline using a scalpel blade (size 15), from L4-S2, to enable the left paraspinal muscles to be parted from the spinous processes. The L6 transverse process and the sacrum was exposed by removing surrounding ligaments with forceps.
A small segment of the L6 transverse process was then removed using rongeours, to gain a clear view of the underlying L4 and L5 spinal nerves. The L6 could be carefully exposed using curved glass rods, underneath the sacrum. L5 and L6 spinal nerves, proximal to their conjunction to the sciatic nerve and distal to their dorsal root ganglion, could be tightly ligated using 6-0 silk thread. Following completion of this procedure, wounds were sutured using 3-0 silk thread, after confirmation of hemostasis. The animal was allowed to recover from anaesthesia in a ventilated comfortable area. Control groups were also used in this study, sham operated animals. This involved following the same surgical technique described above without contact with or ligation of the L5 and L6 spinal nerves. It is important to mention that when these animals were used for the electrophysiological studies, only the ipsilateral side of the spinal cord was searched for neurones.

2.5.1 Behavioural Testing

After surgery was performed rats were kept in groups of 3/4, in plastic cage's within a Government supervised animal house. The rats were monitored under a 12/12 h day/night cycle, where food and water was in plentiful supply and health and well-being was ensured. Confirmation of neuropathy was obtained via behavioural testing of these rats 2, 4, 7, 9, 11 and 14 postoperatively. Rats were placed in individual transparent perspex boxes, with a wire mesh floor and allowed to acclimatise to their surroundings for at least 15 mins prior to testing.
2.5.1.1 Sensitivity to Punctate Mechanical Stimuli

Von Frey filaments were used to test the sensitivity of the operated animals to punctate innocuous mechanical stimuli for the purpose of confirming neuropathy induced mechanical allodynia. Hindpaw withdrawals to a series of von Frey filaments with increasing bending forces (1.5, 9 grams: 9.9, 49.5 and 89.1 mN respectively) were measured. These are considered low intensity stimuli when applied in normal animal trials (Suzuki et al. 2000). Each von Frey filament was applied to the plantar surface of the rat hindpaw contralaterally and ipsilaterally, 10 times for 3 seconds, through a wire mesh floor. A small period of time (2-3 mins) was left prior to commencing each test. The number of withdrawals (out of 10) from each von Frey application was recorded (Refer Graphs 2.5.1.1.i, ii, iii, & iv).

2.5.1.2 Sensitivity to Cooling Stimuli

A small amount of acetone was applied via a blunt syringe to the plantar surface of both ipsilateral and contralateral hindpaws to confirm neuropathy induced cold allodynia in these post-operated rats. Application was gentle so to avoid mechanical stimulation of the hindpaw. Each test consisted of acetone being squirted through the mesh flooring 5 times, at 4-5 minute intervals. Again, the number of hindpaw withdrawals was recorded (out of 5) (Refer Graphs 2.5.1.1.i, ii, iii, & iv).
2.6 Ablation of Lamina I Spinal Neurons Expressing the Substance P Receptor Model

This study involved the use of 20 male Sprague Dawley rats (Biological Services, UCL, U.K). UK Home Office approval was met for all experimental procedures and guidelines for the International Association for the Study of Pain (IASP) were met. As previously performed by Mantyh 1997, intrathecal administration of the ribosome-inactivating protein saporin conjugated to substance P was used for the selective ablation of substance P receptor expressing lamina I spinal neurones. This was based on the evidence that such a conjugate is internalized and is cytotoxic to substance P receptor expressing lamina I neurones, this toxin is able to kill NK1 expressing cells via the block of their protein synthesis (Mantyh et al. 1997; Suzuki et al. 2002). Following this procedure the animals were allowed to recover in a comfortable area. These procedures were kindly performed by Dr Rie Suzuki, Dr Wendy Rahman, Dr Lars Rygh & Dr. Idil Maie. Rats were typically 350-400g at the time electrophysiology was performed.

2.6.1 Administration of Intrathecal Drugs

The methods described in this section are the same as those described by (Mantyh et al. 1997; Suzuki et al. 2002). Using both intraperitoneal diazepam (2.5 mg/kg.i.p., Phoenix Pharmaceuticals, UK) and intramuscular hypnorm (0.3ml/kg.i.m., Janssen Animal Health, Belgium) rats (130-150g) were anaesthetised (Suzuki et al. 2002). An incision was made (2mm) in the atlanto-occipital membrane to allow insertion of a cannula to the subarachnoid space (Suzuki et al. 2002). The cannula was guided gently to the L4-5 region of the spinal cord. An intrathecal catheter connected to a Hamilton Syringe was used to inject either SP-SAP (10^{-6}M Advanced targeting systems, San Diego, California)
or SAP (10⁶M Advanced Targeting Systems, San Diego, California), then flushed with 5 μl of saline at the L4-5 region (Suzuki et al. 2002). The cannula was then removed from the spinal cord area and the wound sutured using 3-0 surgical sutures (Sherwood Davis & Geck, St. Louis, Missouri) and wound clips, when hemostasis was evident. Electrophysiology was performed on these rats as described above, 28 days after the intrathecal injections.
Graph 2.5.1.1.i. The response of the rat ipsilateral hindpaw to punctate mechanical stimuli up to 14 days post spinal nerve ligation (L5/L6). Number of withdrawals per 10 stimuli, n=20.
Graph 2.5.1.1.ii. The response of the rat contralateral hindpaw to punctate mechanical and cold stimuli up to 14 days post spinal nerve ligation (L5/L6). Number of withdrawals per 10 stimuli, n=20.

Graph 2.5.1.1.iv. The response of the rat contralateral hindpaw to punctate mechanical and cold stimuli up to 14 days post sham procedure. Number of withdrawals per 10 stimuli, n =20.
Graph 2.5.1.1.iii. The response of the rat ipsilateral hindpaw to punctate mechanical stimuli up to 14 days post sham procedure. Number of withdrawals per 10 stimuli, n=20.
CHAPTER 3

CHARACTERISATION OF LAMINA I NEURONES IN THE SUPERFICIAL DORSAL HORN OF THE RAT SPINAL CORD AND THEIR PLASTICITY
3.0 Characterisation of Lamina I Neurones in the Superficial Dorsal Horn of the Rat Spinal Cord and their Plasticity

3.1 Introduction

The initial synapse in the pathways of noxious information from the periphery to the brain is in the superficial dorsal horn of the spinal cord, comprised of lamina I and II. Lamina I is an intrinsic constituent of the central representation of both pain and temperature responsiveness and has a large ascending output to higher centres. Much work has been done to identify the physiological properties of both spinothalamic (STT) and spinoparabrachial (spino-PB) lamina I neurones in monkey, cat and rat models (Bester et al. 2000; Andrew and Craig 2001b; Andrew and Craig 2001a; Andrew and Craig 2002a; Craig and Andrew 2002). The majority of work performed on these STT neurones in the cat have classified lamina I neurones as either nociceptive specific (NS) or polymodal nociceptive (HPC), both of which respond to mechanical and heat stimuli in the noxious range. HPC neurones also appear to respond to cold stimuli (Andrew and Craig 2002b).

Analysis of the responses of these neurones to maintained and graded mechanical stimuli have demonstrated further differences in these two physiological populations, which were discussed in section 1.4.3 but will be reiterated here (Andrew and Craig 2002b; Andrew and Craig 2002a). HPC neurones exhibit smaller response properties, more adaptation and poorer coding of stimulus intensity compared to NS neurones following application of a maintained mechanical stimulus (Andrew and Craig 2002b). They exert responses that quickly adapt to such stimuli and tend to fall below 50% of the initial response value within a two minute time period. HPC neurones are thus referred to as "Adapting" cells (Andrew and Craig 2002b). NS neurones code the area and intensity of a stimulus more efficiently than HPC neurones, in experiments using graded mechanical stimuli (Andrew and Craig 2002a). The majority of NS neurones can also sustain their response to a maintained noxious mechanical stimulus and are referred to as "Maintained" cells (Andrew and Craig 2002b). It is also thought that HPC and NS populations have inputs from different fibre types (Andrew and Craig 2002a). NS neurones receive large A-δ fibre inputs and HPC cells receive large C-fibre inputs within the superficial dorsal horn (Andrew and Craig 2002a). This suggests that NS neurones possess better mechanosensitivity than HPC neurones (Andrew and Craig 2002a).
In the cat NS and HPC lamina I STT neurones are thought to be responsible for first (sharp) and second (burning) pain sensations, following noxious stimuli (Craig and Andrew 2002). Interestingly a small population of lamina I neurones (2%) are thought to respond to warm stimuli and insinuate a possible involvement of STT lamina I neurones in thermoregulation (Andrew and Craig 2001a). Furthermore spinothalamic lamina I neurones are also responsive to histamine when administered iontophoretically and suggest an involvement in the itch response as mentioned in section 1.4.3. (Andrew and Craig 2001b).

Studies have quantified the proportion of NS neurones within rat spinoparabrachial lamina I neurones as discussed in section 1.4.3 and have suggested NS neurones comprise 75% of the total population, corresponding with the large A-δ fibre inputs within lamina I (Bester et al. 2000). HPC neurones were not distinguished from NS neurones in this study, despite the fact 12% of the total population appeared to respond to noxious cold (Bester et al. 2000). The parabrachial area is thought to be involved in cold perception, substantiating evidence that spinoparabrachial lamina I neurones exhibit a cold response (Menendez et al. 1996). These lamina I spinoparabrachial neurones had a low incidence of spontaneous activity, small excitatory receptive fields and a distinct lack of 'wind-up' as seen in lamina V neuronal responses (Dickenson and Sullivan 1987; Dickenson 1990; Dickenson and Sullivan 1991; Bester et al. 2000). In vitro whole cell patch clamp recordings have revealed that superficial dorsal horn neurones have different electrophysiological properties than deeper dorsal horn neurones (Ruscheweyh and Sandkuhler 2002a). Lamina I neurones exhibit distinct firing properties (as described in 1.4.1), which could be classified and are not seen in other laminae (Ruscheweyh and Sandkuhler 2002a). The majority (69%) of lamina I neurones display a delayed or tonic firing pattern whereas lamina II and lamina III-VI contain a far smaller population of neurones with such firing patterns (Ruscheweyh and Sandkuhler 2002a). Lamina I neurones, in general, display broader action potentials compared to lamina V neurones, as well as lower firing frequencies upon depolarisation of the tonic-firing neuronal population within each lamina (Ruscheweyh and Sandkuhler 2002a). In general, these differences in discharge properties between dorsal horn lamina, may go someway to explain why lamina I neuronal characteristics are so distinct.

Following nerve injury, electrical alterations in the firing and evoked response properties of both central and peripheral nerves arise (Suzuki and Dickenson 2000).
Spontaneous activity and augmented neuronal firing within the damaged neurone are commonly reported (Suzuki and Dickenson 2000).

Primary afferent fibres terminating within the dorsal horn transmit ectopic activity, which arises from the damaged region of the DRG in the injured nerve (Suzuki and Dickenson 2000). Electrophysiological studies from dorsal horn neurones in the rat have frequently demonstrated a marked plasticity following peripheral nerve damage. C-fibre thresholds in response to a nociceptive conditioning stimuli are reduced in neuronal populations in SNL animal models compared to sham control groups (Rygh et al. 2000). Interestingly, LTP evoked in WDR neurones after a nociceptive conditioning stimuli under normal conditions, is also reduced in neuropathic rats (Rygh et al. 2000). Analogous studies have demonstrated marked increases in spontaneous activity in primary afferent fibres and in turn dorsal horn neurones, following nerve injury (Tabo et al. 1999; Suzuki et al. 2000; Abdulla et al. 2003).

Further characterisations of rat dorsal horn neurones, have revealed a variety of distinct changes in the response properties of neuropathic groups compared to control groups (Chapman et al. 1998). Not only do a larger population of neurones exhibit spontaneous activity in neuropathic animals, but the frequency of spontaneous activity recorded is also much higher as well (Chapman et al. 1998). It is proposed that these changes may be due to alterations in both the expression and the properties of Na⁺, K⁺ and Ca²⁺ channels within the peripheral and central nervous system (Abdulla et al. 2003). However the most interesting finds are the changes in magnitude of the responses exhibited by the neurones in this particular study over a 14 day time course (Chapman et al. 1998). A larger proportion of neurones exert responses to innocuous brush stimuli in neuropathic animals compared to control groups, in the early post-operative days. However a reduced population of neurones in SNL neuropathic groups exert responses to noxious prod compared to sham controls in the later post-operative period (Chapman et al. 1998). Furthermore in the later post-operative stage (14-17 days) neuronal responses to brush, prod and mechanical stimuli are reduced compared to sham control groups, as are the C-fibre evoked responses (Chapman et al. 1998). Interestingly, prior to this, no distinct differences in these response modalities have been identified (Chapman et al. 1998). In studies whereby electrophysiological recordings were made from WDR neurones in the lumbar dorsal horn of rats with loose ligation of L4-L6 dorsal roots demonstrate no significant changes in the response to graded noxious heat stimuli (Tabo et al. 1999). Yet upon behavioural analysis performed prior to electrophysiological recordings, reductions in the withdrawal thresholds to mechanical stimuli are
observed in the ipsilateral hindpaw of neuropathic rats (Tabo et al. 1999). Confusingly there is an increase in withdrawal time to thermal stimuli immediately post ligation, in behavioural tests (Tabo et al. 1999). Paradoxically, following vincristine induced allodynia and hyperalgesia in rats, not only are marked increases in after-discharge and spontaneous activity evident in WDR neurones, but also increased A- and C-fibre responses. Marked changes in the electrically evoked wind-up response are also observed (Weng et al. 2003).

Following spinal nerve ligation of rat L5/L6 nerves, the receptive field size of dorsal horn neurones recorded two weeks post-ligation, appears to be increased (Suzuki et al. 2000). This is evident when the receptive field is mapped with an innocuous punctate mechanical stimulus (von Frey filaments 9g). However only small changes are seen when mapped with noxious mechanical stimuli (von Frey filaments 15 & 75g). However, enlargement of the receptive field area has been suggested to be a slow process and most electrophysiological studies have demonstrated changes only after 5 weeks post-nerve ligation (Tabo et al. 1999). In related studies, an enlargement of the peripheral receptive field area is recorded following partial sciatic nerve ligation, however this is only seen in the 16th postoperative week and is evident in both ipsilateral and contralateral hindpaws (Takaishi et al. 1996). It is hard to reconcile these late changes with the rapid behavioural changes that are stable by 2 weeks. The Suzuki study supports findings that illustrate that distinct central sensitisation develops following peripheral nerve ligation – hyperexcitable neurones are now able to respond to inputs normally too weak to elicit activity and hence the receptive field expands (Suzuki et al. 2000).

Interestingly, following nerve injury, a certain degree of plasticity within lamina I neurones of the superficial dorsal horn, results in the development of hyperalgesia (Ikeda et al. 2003). It is believed that the neurones responsible for the manifestation of hyperalgesia in models of neuropathic pain, are those containing the NK1 receptor for substance P (Ikeda et al. 2003). It is believed that substance P, synthesised in dorsal root ganglia, is released from primary afferent neurones upon noxious stimulation and interacts with lamina I dorsal horn neurones containing NK1 receptors (Jessell 1982; Helke et al. 1986; De Koninck and Henry 1991; Duggan et al. 1991; Brown et al. 1995; Littlewood et al. 1995; Abbadie et al. 1996; Mantyh et al. 1997). It is clear that NK1 expressing neurones are involved in the transmission of noxious stimuli and can efficiently code the intensity of such nociceptive activity, which has been subsequently confirmed in c-Fos studies of both lamina I and lamina
V neurones (Doyle and Hunt 1999b). Interestingly, a large proportion of lamina I neurones, which project to the thalamus and parabrachial area, express the substance P receptor (SPR) (Ding et al. 1996; Mantyh et al. 1997). However SPR-containing neurones comprise as little as 10% of the total lamina I neuronal population (Brown et al. 1995; Littlewood et al. 1995; Mantyh et al. 1997). It is thus supposed that lamina I neurones containing the NK1 receptor for substance P (SP) must play a large role in nociceptive sensory transmission as well as the transmission of nociceptive information to higher centres (Mantyh et al. 1997). Ablation of lamina I NK1 containing neurones has also allowed their contribution in nociceptive transmission to be assessed. After release, substance P binds to the NK1 receptor and is consequently internalised within the neurone (Mantyh et al. 1997). Mantyh et al (1997) utilised this property and developed a model whereby the ribosome-inactivating protein toxin saporin (SAP) could be conjugated to SP. Internalisation of substance P-saporin (SP-SAP) conjugates results in the death of the NK1 containing neurones within the superficial dorsal horn, as protein synthesis is consequently hampered (Mantyh et al. 1997). A 100% decrease in substance P receptor immunoreactive neurones were demonstrated 10 days after SP-SAP treatment in neonatal rat spinal cord primary cultures, such changes were not evident following SAP, SP or saline treatment alone (Mantyh et al. 1997). Following immunofluorescence labelling, reductions in neuronal populations were only seen in lamina I cells in the lumbar segments where the toxin was administered (Mantyh et al. 1997). This may be due to degradation of the substance P molecule conjugated to SAP, by endogenous proteases, upon penetration into deeper lamina layers within the spinal dorsal horn (Mantyh et al. 1997). 28 days after SP-SAP treatment, 85% of lamina I SPR immunofluorescent cells were destroyed leaving the remaining 15% with shrunken cell bodies and smaller dendritic processes (Mantyh et al. 1997).

It was clear that the majority of SP receptors no longer remain on the plasma membrane, and are instead distributed within the lamina I cell cytoplasm (Mantyh et al. 1997). It is clear from these studies that distinct reductions in capsaicin induced mechanical and thermal hyperalgesia (between 60-85% reductions) as well as nocifensive behaviour, is a result of ablation of NK1-expressing neurones in lamina I (Mantyh et al. 1997). This is despite the fact that SPR-containing neurones make up only a small proportion of the spinal lamina I neuronal population (Brown et al. 1995; Littlewood et al. 1995; Mantyh et al. 1997). It is thus clear that treatment with SP-SAP destroys spinoparabrachial and spinothalamic lamina I neuronal populations, which appear to express SPR to a large extent. SP-SAP thus results in the
destruction of these defined neurones involved in the transmission of nociceptive information to higher centres, responsible for both pain perception and the development of abnormal pain related behaviours (Littlewood et al. 1995; Mantyh et al. 1997; Suzuki et al. 2002). Furthermore recordings made from lamina V WDR spinal neurones illustrate that following selective ablation of NK1-expressing lamina I neurones, distinct changes can be seen in their response properties. SP-SAP treatment reduced both WDR neuronal excitability and their peripheral receptive field size, as well as causing marked changes in the ability of these neurones to code mechanical and heat stimuli. This study went on to illustrate that ablation of these NK1-expressing lamina I neurones interfere with descending pathways which regulate nociceptive transmission (Suzuki et al. 2002). It was thus the aim of this chapter to assess the characteristics of normal lamina I spinal neurones, and compare these properties to lamina I neurones in models of peripheral nerve injury and lamina I neurones in SP-SAP treated normal animals whereby NK1-expressing neurones have been selectively ablated.

3.2 Methods

All rats were anaesthetised prior to exposure of the lumbar spinal cord and electrophysiological testing. Between 5 - 8 lamina I neurones were identified in each experiment using depth analysis and then characterised. On occasions, where different drugs were also being used to test the pharmacological properties of lamina I neurones, only 3-4 neurones were fully characterised prior to drug application. Approximately 30 minutes was left between locating and characterising each lamina I neurone. Lamina I neurones were located in the superficial marginal layer of the spinal cord using a parylene-coated tungsten electrode, which then enabled single-unit extracellular recordings to be made. The electrode was positioned to enter the spinal cord at an angle of 45° horizontal to the spinal cord. The electrode could therefore penetrate the spinal cord at an angle, which enabled a large cross-sectional area of the superficial dorsal horn to be searched for neuronal activity. A depth of 0-250 μm from the surface of the spinal cord (where no neuronal activity could be detected) was deemed to be the lamina I layer.

Therefore the depth of each neurone obtained using a bent electrode (45°) was corrected, using simple trigonometry. On the basis that the electrode projectory was 45°, a simple right angle triangle could be used to calculate the correction using the cosine equation (cos45° * electrode depth). Depths of up to 450μm could therefore
be considered to be within the lamina I layer. The mean depths of the lamina I neurones used were 293±18 µm (using bent electrodes and cosine depth correction). Innocuous and noxious stimuli were used to characterise lamina I neuronal responses. Hot, warm and cold water jets as well as brush and von Frey hairs were utilised for this purpose. Temperatures of 4, 32, 35, 40, 42, 45, 48, 50, 52°C were applied for 10 seconds, via a water jet in strict ascending order. Temperatures of 32°C were indicative of any mechanical activity elicited by the force of the water jet and could therefore be evaluated as a neutral response when classifying lamina I neurones into various physiological categories. Von Frey filaments were also applied in ascending order 1, 5, 9, 12, 15, 30, 75 and 125g for 10 seconds. Lastly, a fine brush was applied in gentle stroking patterns to the receptive field area for 10 seconds. Spontaneous activity of each neurone recorded (always low – approx 0.1-1Hz), was subtracted from the neuronal response to each stimulus. For the purpose of this study, 42°C and above was taken as a noxious thermal stimulus, as defined by a similar electrophysiological in vivo characterisation study in the rat (Urch et al. 2003). Von Frey 12g and above was perceived as a noxious mechanical stimulus in this study. Although Urch et al (2003) defined von Frey 9g as noxious, Suzuki (2002) defined more intense von Frey stimuli as mildly noxious. Therefore for the purpose of this study 12g was perceived as a high threshold mechanical stimulus. Responses were defined as neuronal firing of 50 action potentials and above, to the evoked stimulus in a 10 second time period. Lamina I neurones were characterised in normal animals. Animals that had undergone spinal nerve ligation of L5/L6 peripheral nerves surgery as described by (Kim and Chung 1992) as well as sham operated controls, were used to characterise lamina I neuronal responses following peripheral nerve damage. Lamina I neurones were also characterised in animals that had undergone selective ablation of lamina I neurones as previously performed and described by (Mantyh et al. 1997; Suzuki et al. 2002).

Intrathecal administration of the ribosome-inactivating protein saporin conjugated to substance P was used for the selective ablation of NK1 receptor expressing lamina I spinal neurones in this group of animals. These animals were kindly prepared by Dr Rie Suzuki, Dr Lars Rygh, Dr Wendy Rahman & Dr. Idil Maie. Ablation of lamina I NK1 containing neurones allowed the characterisation of non-NK1 lamina I neurones within the superficial dorsal horn. A group whereby saporin was administered, without having been conjugated to substance P, were used as controls. A separate population of lamina V wide dynamic range deep convergent dorsal horn neurones
were used and their electrical and naturally evoked neuronal response characteristics were compared to that of lamina I neurones in normal rats. Lamina V neurones have frequently been used in similar electrophysiological recordings and their pharmacological properties and receptor distribution have been assessed. Data for lamina V neurones were taken from Rie Suzuki, with her permission, to draw these comparisons, on the basis that Rie Suzuki had studied a large population of lamina V neurones for their electrical, mechanical and thermal response properties. The mean depths of these lamina V neurones (n= 69), was 791±24μm (using straight electrodes).

Anova factorial statistics were performed to compare lamina I responses in the different animal models tested. Statistics could not be performed to compare lamina I and lamina V responses as raw data values for lamina V neurones used to compare lamina I responses were not available, only the grouped data for the overall population presented in this study.

3.3 Results

Unfortunately lack of histological confirmation, (attempts to pass current through these electrodes located superficially in the spinal cord was unsuccessful) meant it was impossible to classify all neurones recorded as definite lamina I neurones. For future reference lamina I neurones are deemed ‘probable’ lamina I neurones and lamina V neurones may also be classified as ‘probable’ lamina V neurones as histological confirmation was not performed on these neurones either. Lamina I neurones were regularly double spiked in all animal models tested and so appeared very different to lamina V neurones in the normal rat model, which were typically single spiked (Diagram 3.3.a).
Fig. 3.3.a. Post-stimulus Histogram for a Lamina V (a) and Lamina I (b) neurone in the rat dorsal horn. Lamina I double spike (inset).
In naïve rats, lamina I neurones had larger C-fibre threshold values (2.5mA±0.5) than lamina V neurones (1.35±0.15mA), as well as smaller response magnitudes, particularly C-fibre, post-discharge and XS spike responses (Graph 3.3.i). Interestingly, almost all lamina V neurones exhibited a wind-up response (Graph 3.3.ii). Only 14% of lamina I neurones exhibited a wind-up response in normal animals, the rest exhibited a reduced 'wind-up' response (Graph 3.3.ii) described as a slow incremental potentiation (27% of neurones) or no augmented response at all (59% of neurones). The majority of lamina V neurones were reportedly WDR (79% of neurones) as compared to 28% in lamina I neurones.

Overall, the electrically evoked lamina I neuronal responses appeared to be similar in sham operated and SNL animal groups, with a tendency for larger C-fibre responses in sham operated animals (181±18 spikes), compared to SNL animal groups (Graph 3.3.iii). The A-δ fibre response appeared to be marginally larger in SNL animal populations compared to sham control groups. C-fibre threshold values and XS-spike responses were very similar in sham and SNL animal groups. Anova One-way statistical analysis found no significant differences between sham and SNL groups. There were small differences in the electrically evoked responses in SAP control groups and SP-SAP treated animal groups (Graph 3.3.iv). Lamina I neurones in SAP control groups generally had lower C-fibre threshold values compared to lamina I neurones in SP-SAP treated animals. Slight increases in A-β fibre and post-discharge responses were evident in lamina I neurones in SP-SAP treated animals compared to those recorded in SAP control groups. However, no statistically significant differences were found between these groups.

When comparing the responses of lamina I neurones to a variety of punctate mechanical stimuli, evoked by von Frey filaments it was evident that lamina I neuronal responses were smaller than lamina V neurones (Graph 3.3.v). Overall lamina I neuronal responses were between 39-61% that of the lamina V neuronal responses evoked by punctate mechanical stimuli at 9g and above. The response to a 5g stimulus was almost the same. When comparing the mechanically evoked lamina I neuronal responses between the different animal models used in this study it is clear that there were distinct differences to be noted. Overall the responses in sham operated and SNL animal groups were similar (Graph 3.3.vi). The mechanically evoked responses in SAP and SP-SAP treated animal models were also very similar, although SP-SAP responses were slightly larger following noxious von Frey 12-125g (SP-SAP responses 102-155% that of than SAP animals) (Graph 3.3.vii). The brush
response was smaller in SP-SAP treated animals than in SAP treated groups. Quite similar results were seen in SNL and sham groups.

The response to noxious thermal stimuli in lamina I neurones in naïve rats was far smaller than that recorded in lamina V neurones (Graph 3.3.viii), with the largest differences evident in the noxious range (lamina I neuronal responses 50-85% of lamina V neuronal responses at 42°C and above). Overall the responses to thermal stimuli appeared to be slightly larger in SNL animals compared to shams (SNL lamina I responses at 4°C; 181%, 32°C; 115%, 40°C; 118%, 45°C; 138% that of sham lamina I responses) (Graph 3.3.ix). The smaller responses to 4°C cold stimuli in sham groups was particularly evident, compared to SNL animals. No statistically significant effects were seen between these animal groups.

The lamina I neuronal response to noxious thermal stimuli appeared to be slightly increased in SP-SAP treated animals compared to lamina I neuronal response values in SAP control groups (48°C; 424±58 in SP-SAP & 50°C; 547±75 in SP-SAP, responses 113-129% that of SAP lamina I responses) (Graph 3.3.x). Interestingly the response to innocuous thermal stimuli, 32 & 35°C which is largely indicative of mechanical stimuli elicited by the water jet, appeared to be smaller in SAP animals compared to SP-SAP treated animal models (82-89% of SP-SAP response values). No statistically significant effects were seen between these animal groups.

The peripheral receptive field area of lamina I neurones recorded was mapped using pinch, on account of the fact that the majority of lamina I neurones recorded were high threshold to punctate mechanical stimuli (Table 3.3.d). Overall the peripheral receptive fields mapped from lamina I neurones were very similar, with little difference seen between animal models. No such statistical differences were seen either.

The neuronal populations were then further categorised according to the classification adopted by (Bester et al. 2000; Andrew and Craig 2001a; Craig et al. 2001; Andrew and Craig 2002b; Andrew and Craig 2002a) and discussed in section 3.1 whereby lamina I neurones were either nociceptive specific (NS), polymodal nociceptive (HPC), COLD or wide dynamic range (WDR).
As discussed in section 3.2 neurones with mechanically evoked responses of von Frey 12g or above and heat evoked responses of 42°C and above, were denoted nociceptive specific. Those with an additional response to noxious cold stimuli (4°C) however, were deemed polymodal nociceptive (HPC). In previous papers defining HPC neurones, temperature ranges between +20 and −10 °C are described as noxious cold (Bester et al. 2000).

Overall there were small differences in the physiological properties of lamina I neurones recorded in this study (Table 3.3.e). The proportion of NS neurones in naive, SNL and SP-SAP animals were similar, yet sham controls had a smaller proportion of NS neurones compared to SNL groups. SAP controls generally had a larger proportion of NS neurones, compared to SP-SAP groups overall. Sham groups had a larger number of HPC neurones than SNL groups, as did SP-SAP compared to SAP groups. The proportion of WDR neurones recorded in each animal group tended to be similar. It must be noted that a large proportion of lamina I neurones exhibited a brush response, often despite the fact that these neurones appeared to be high threshold to punctate mechanical and thermal stimuli. As (Andrew and Craig 2001a; Craig et al. 2001; Andrew and Craig 2002b; Andrew and Craig 2002a) did not use brush stimuli when classifying their neurones we therefore kept the classifications based on their response properties. Contradictory to this, but in agreement with my results (Bester et al. 2000) did demonstrate lamina I neurones with a brush response which were all classified as WDR. However in addition the majority of these neurones had both low pressure and low heat thresholds (≤ 5 Ncm⁻² & 38-40°C respectively) inconsistent with the findings of this study (Table 3.2.f). It is therefore acknowledged that the majority of NS and HPC neurones did exhibit a response to low threshold brush stimuli, yet were classified as NS or HPC if their responses to punctate mechanical and thermal stimuli was limited to the noxious range.

The neurones classified as WDR, exhibited the largest brush responses compared to NS and HPC neurones in all animal groups. NS neurones exhibited a larger brush response than HPC neurones overall. It was evident that WDR lamina I neurones in sham animal models evoked the largest brush responses, compared to WDR lamina I neurones recorded in SNL. SAP and SP-SAP WDR lamina I neurones were similar, with slightly larger brush responses seen in SP-SAP animal groups. The brush response in HPC lamina I neurones was larger in sham groups, than HPC neurones
in the SNL animal models tested. The brush response recorded from NS neurones was smaller in SNL lamina I neurones compared to shams. As was the SP-SAP brush response compared to SAP NS neurones (Table 3.3.f). Overall, a larger proportion of WDR neurones evoked a brush response compared to NS and HPC neurones in all animal models except SAP groups (Table 3.3.f – refer to numbers in brackets), and HPC neurones had the smallest proportion of neurones with a brush response overall.

Furthermore, it was also possible to assess the mechanically and thermally evoked response properties of these individually classified NS, HPC and WDR lamina I neurones, used to categorise them initially. This was performed in the different animal models tested. Differences in firing properties were not seen, as described in section 1.4.1, however attempts to follow the firing pattern of neurones from each physiological class were not done in this study. However, in naïve rats larger neuronal responses were evident for WDR neurones compared to the NS and HPC neurones, following both peripheral mechanical and thermal stimuli. In response to mechanical stimuli, (Graph 3.3.xi) NS and HPC neurones have similar response properties. HPC neurones tended to have smaller responses to brush stimuli, compared to NS neurones (52% of NS brush response). WDR neurones tended to have the largest brush response (168-256% of NS and HPC brush response).

Significant differences in the response of NS and WDR neurones (⊙ P=<0.05, ⊙⊙ P=<0.01 see Graphs 3.3xi and 3.3xii) were seen at von Frey 5-125g (5g: $F_{1,80}=7.22, P=0.0088, 9g; F_{1,56}=19.94, P=0.0001, 12g; F_{1,56}=19.85, P=0.0001, 15g; F_{1,56}=18.66, P=0.0001, 30g; F_{1,41}=15.78, P=0.0003, 75g; F_{1,56}=14, P=0.0004, 125g; F_{1,56}=13.98, P=0.0004). Significant differences in the response of WDR and HPC neurones (● P=≤0.05, ● ● P=≤ 0.01, see Graphs 3.3xi and 3.3xii) were also evident at von Frey stimuli 5, 9, 12, 15 & 125g (5g; $F_{1,24}=7.06, P=0.0138, 9g; F_{1,24}=6.98, P=0.0122, 12g; F_{1,24}=7.34, P=0.0143, 15g; F_{1,24}=5.87, P=0.0233, 125g; F_{1,24}=4.83, P=0.0378). The response of lamina I neurones to thermal stimuli in normal animals (Graph 3.3.xii) was larger in WDR neurones than in HPC neurones (WDR 84-325% that of HPC neurones, particularly in response to innocuous thermal stimuli between 32-42 °C). It was also larger than that of NS neurones (WDR 76-628% response of NS neurones, where the largest difference seen in the response to innocuous thermal stimuli.
Significant differences were seen between the response of NS and WDR neurones to 4, 35, 40, 42, 45 and 48°C (4°C; F_{1,60}=17.48, P=0.0001, 35°C; F_{1,54}=19.42, P=0.0001, 40°C; F_{1,54}=24.67, P=0.0001, 42°C; F_{1,54}=23.53, P=0.0001, 45°C; F_{1,54}=6.31, P=0.0151, 48°C; F_{1,54}=7.43, P=0.0086). Differences observed between the WDR and HPC neurones, particularly at 35, 40 & 42°C were found to be statistically significant (35°C; F_{1,23}=8.21, P=0.0087, 40°C; F_{1,23}=7.58, P=0.0113, 42°C; F_{1,24}=9.64, P=0.0048). WDR neurones and HPC neurones had very similar responses to noxious cold stimuli 4°C. However, HPC neurones had notably larger responses to noxious cold stimuli than NS neurones (HPC neurones 241% of NS neurones). See Graphs 3.3xi & 3.3xii.

In sham and SNL animals, the responses of NS, HPC and WDR to mechanically and thermally evoked responses of lamina I neurones were analysed (Refer to Graphs 3.3xiii – 3.3 xvi). WDR neurones in SNL animal groups tended to be larger than that of NS neurones following punctate mechanical stimulation of the peripheral hindpaw (WDR neurones 261-906% larger than NS neurones to von Frey 1-125g) (Graph 3.3.xiii). Interestingly HPC neurones had larger responses than NS neurones in SNL animal models, particularly in the noxious range of von Frey stimuli (overall HPC neurones 231-588% of NS neurones responses at von Frey 1-125g). WDR neurones also appeared to have larger responses than HPC neurones in the innocuous range of von Frey stimuli (WDR neurones 107-339% of HPC neurone responses at von Frey 1-125g). HPC neurones exhibited the largest brush response 342±91 spikes (157% that of WDR response to brush). NS neurones appeared to have the smallest response to brush stimuli (93±18 spikes).

Significant differences between WDR and NS responses to von Frey 5, 30, 75 and 125g were evident (5g; F_{1,19}=13.62, P=0.0016, 30g; F_{1,20}=8.19, P=0.0097, 75g; F_{1,20}=21, P=0.0002, 125g; F_{1,19}=19.4, P=0.0003). In sham animals WDR neurones had larger responses to punctate mechanical stimuli compared to NS and HPC neurones (Graph 3.3.xiv). WDR neurones exhibited responses 213-789% of the response of NS neurones to von Frey stimuli, the largest difference was evident at von Frey 1-30g in the less noxious range. WDR neurones exhibited responses 222-364% of HPC neuronal responses, with the large increases evident at von Freys 9-12g. However these large differences are largely due to the very small responses exerted by HPC neurones between von Frey 1-15g (5-25 spikes). HPC and NS neurones had very similar response properties. Significant differences were
observed between WDR and NS neurones at von Frey 5, 30, 75 and 125g (5g; $F_{1,13}=9.8$, $P=0.008$, 12g; $F_{1,12}=10.5$, $P=0.0071$, 15g; $F_{1,13}=14.2$, $P=0.0023$, 30g; $F_{1,13}=14.1$, $P=0.002$). Significant differences were observed between HPC and WDR neurones at von Frey 5, 12 and 15g (5g; $F_{1,8}=8.08$, $P=0.0217$, 12g; $F_{1,8}=15.13$, $P=0.0046$, 15g; $F_{1,8}=8.2$, $P=0.0209$). The brush response of WDR neurones (307±84 spikes) was notably larger than that of HPC (133±62 spikes) and NS neurones (145±27 spikes), calculated as 212-231% of NS and HPC brush response properties (See Graphs 3.3xiii-3.3xvi).

When comparing the responses of sham and SNL NS, HPC and WDR neurones to punctate mechanical stimuli, it is clear that sham NS neurones exert larger responses than SNL NS neurones overall (von Frey 1g & 5g sham NS 150 & 194% of SNL NS responses, respectively – refer to Table 3.2(g) in section 3.5). The brush response of NS neurones was also notably larger in sham animal models (156% that of SNL NS neurones). SNL HPC neuronal responses tended to be larger than those of sham HPC lamina I neurones, particularly following innocuous mechanical stimuli (von Frey 1-12g, 200-1700%). The brush response in SNL HPC neurones was larger than that of sham HPC neurones (257%). No statistical differences were observed between the individual neuronal populations recorded in sham and SNL animal groups. When looking at the response to thermal stimuli in sham and SNL animals it was evident that in SNL animals the WDR and HPC neuronal responses to thermal stimuli were surprisingly similar (Graph 3.3.xv and 3.3.xvi). WDR neuronal responses to 42, 45, 48 & 50°C were larger than NS neuronal responses in SNL groups (173-456%). In SNL animals WDR exhibited larger responses to noxious cold stimuli (237±41 spikes) than that of HPC neurones (122±76 spikes), and NS neurones (161±21 spikes) exhibited larger responses than HPC neurones. This is probably a result of the very small number of HPC neurones recorded in this group (n=2). In sham groups, HPC neurones tended to have larger cold responses than NS neurones as would be expected (Graph 3.3.xvi). This was similar to that seen with WDR neurones. Interestingly, HPC neurones exhibited larger responses to highly noxious heat stimuli (48 & 50°C) than NS neurones (HPC neuronal responses were 212-349% that of NS neurones at 48-50°C). WDR neurones had larger responses to all thermal stimuli compared to NS and HPC neuronal responses, however at 50°C HPC neurones (642±125 spikes) exerted very similar response properties to WDR neurones (633±154 spikes). The response to cold stimuli (4°C) was notably different between NS, HPC and WDR neurones. HPC neurones
(149±22 spikes) exerted responses 331% that of NS neurones. However the HPC
and WDR neuronal responses to 4°C were very similar (WDR; 127±54 spikes).

When comparing the responses of sham and SNL WDR neuronal responses to
thermal stimuli, it is evident that sham WDR neuronal responses were larger than
that of SNL WDR neuronal responses, particularly at 35°C (sham WDR neuronal
response 181% that of SNL WDR neuronal response). The SNL WDR response to
noxious cold stimuli (237±41 spikes) was notably larger (187%) than that of sham
WDR cold responses (127±54 spikes). HPC SNL neuronal responses were larger
than that of sham HPC neurones following peripheral thermal stimuli. This was
evident particularly at 32, 40, 42, 45 and 48°C (SNL HPC neuronal responses 331%,
176%, 416%, 673% and 214% respectively of sham HPC neuronal responses).
Furthermore, SNL NS neuronal responses were also larger than sham NS neuronal
responses at 32, 35, 40, 45 and 50°C (SNL NS neuronal responses 142%, 202%,
145%, 123%, 163% and 182% respectively, than that of sham NS neuronal
responses). SNL NS neurones had larger responses (161±21 spikes) to noxious cold
stimuli compared to sham NS neuronal responses (45±11 spikes). In comparison,
the responses of HPC neurones to cold stimuli in these two animal models was
similar (122±76 & 149±22 spikes in SNL & sham animals, respectively). No
statistical difference was noted, between the individual neuronal populations in these
two animal groups.

Finally the responses of NS, HPC and WDR neurones were analysed in the SAP and
SP-SAP treated animal groups. In saporin treated animals, the response to punctate
mechanical stimuli was also assessed in the different neuronal populations (Graph
3.3.xvii). WDR, again exerted larger responses to von Frey stimuli than NS and HPC
neurones. NS neurones exerted larger responses to the noxious von Frey filaments
(30-125g) than HPC neurones. Overall WDR responses were between 217-474%
that of NS neuronal responses and 585-1477% that of HPC neuronal responses,
following noxious mechanical stimuli of 30g and greater. Interestingly the responses
of WDR neurones were also much larger than NS neurones in the innocuous range
as well, and overall WDR neurones responses were 217-1092% that of NS neurones
following mechanical stimuli of 5-125g, with the largest difference evident at von Frey
9g and the smallest at 125g. Significant differences were evident between WDR and
NS neurones following von Frey stimuli 5-125g (5g; F₁,37=42.77, P=0.0001, 9g;
F₁,25=31.3, P=0.0001, 12g; F₁,38=9.19, P=0.0001, 15g; F₁,37=41.1, P=0.0001).
In substance P saporin treated animal groups NS and HPC neurones exerted similar responses to mechanical stimuli (Graph 3.3.xviii). WDR neurones had larger responses overall, particularly from 5-125g (WDR responses 174-457% that of NS neurones and 147-463% that of HPC neurones), as well as brush (WDR neurones; 265±63 spikes compared to 99±47 spikes in HPC neurones and 142±24 spikes in NS neurones). Significant differences between WDR and NS neurones at von Frey 5, 9 & 30g were evident (5g; F_{1,41}=63.9, P=0.0001, 9g; F_{1,41}=52.89, P=0.0001, 30g; F_{1,41}=7.93, P=0.0074). Significant differences were also found between WDR and HPC neurones in these substance P saporin treated animals following von Frey 5,9,12 &30g (5g; F_{1,17}=8.54, P=0.0095, 9g; F_{1,17}=9.08, P=0.0078, 12g; F_{1,17}=9.41, P=0.007, 30g; F_{1,17}=20.25, P=0.0004).

The response to cold stimuli in HPC, WDR and NS neurones were dissimilar in SAP treated animals (Graph 3.3.xix), with HPC neurones exerting responses 210% of NS neuronal responses and 161% that of WDR neurones. NS and HPC neurones exerted similar responses to innocuous thermal stimuli (32-42°C), however NS neurones had much greater responses following more noxious thermal stimuli (45-50°C) than that of HPC neurones (NS neuronal responses were 209-556% of HPC neuronal responses. WDR neurones exerted larger responses to 32-50°C thermal stimuli, than HPC and NS neurones.

Interestingly, HPC neurones exerted very small responses across the thermal range (however it must be noted that due to time restrictions there were only 2 HPC neurones recorded in this group and therefore these results can't possibly fully reflect the overall response properties of HPC neurones recorded in SAP treated animals). Significant differences were noted between NS and WDR neurones in the SAP treated animals in the more noxious range of thermal stimuli 45,48,50°C (45°C; F_{1,35}=12.67, P=0.011, 48°C; F_{1,35}=6.93, P=0.0125, 50°CF_{1,35}=7.11; P=0.0115).

In SP-SAP treated animals the response to noxious cold stimuli was similar between HPC (141±24 spikes) and NS neurones (81±24 spikes), yet a larger response was evident in HPC neurones (Graph 3.3.xx). The WDR neurones exerted far greater responses to cold stimuli, almost double that of HPC neurones and triple that of NS neurones (254±84 spikes, 180% that of HPC neuronal response and 314% that of NS neuronal response to cold stimuli). These differences in responses to noxious...
cold stimuli, between WDR and NS neurones, were significant ($F_{1,37}=6.83$, $P=0.0129$). WDR neurones had much larger responses than NS and HPC neurones overall, following thermal stimuli. WDR neurones tended to be between 205-294% that of NS neurones in SP-SAP animals, following innocuous thermal stimuli (32-42°C) and 271-654% that of HPC neurones. Significant differences were also evident between WDR and NS neurones following these innocuous thermal stimuli (32°C; $F_{1,40}=9$, $P=0.0046$, 35°C; $F_{1,39}=12.58$, $P=0.001$, 40°C; $F_{1,41}=10.62$, $P=0.0023$, 42°C; $F_{1,54}=23.53$, $P=0.0001$). Significant differences were also noted between WDR and HPC neurones (32°C; $F_{1,17}=6.58$, $P=0.02$, 35°C; $F_{1,15}=6.52$, $P=0.024$, 40°C; $F_{1,17}=4.3$, $P=0.0537$, 42°C; $F_{1,24}=9.64$, $P=0.0048$). Interestingly HPC neurones appeared to code noxious heat (45-50°C) more effectively in SP-SAP animal models (responses were 166-790% that of SAP animal HPC neurones) than SAP animal groups. The responses of NS neurones were very similar to thermal stimuli between NS neurones in the two animal models, as were WDR neuronal response properties. No statistically significant differences were found between individual neuronal populations in these two animal groups.
Graph 3.3.i. Mean lamina I and lamina V neuronal responses to a train of 16 peripheral electrical stimuli. No statistical analysis for comparison between data sets were performed as raw data values for lamina V responses were unavailable.
Graph 3.3.ii. The 'wind-up' effect in one lamina V neurone and the 'reduced wind-up' as seen in one lamina I neurone. The data shown is largely representative of the differences in 'wind-up' seen between lamina I and lamina V neurones in response to a train of 16 peripherally applied electrical stimuli.
Graph 3.3.iii. Mean lamina I neuronal responses to peripheral electrical stimuli in naive, sham-operated and SNL animal models. Refer to Tables in section 3.5 for n numbers.
Graph 3.3.iv. Mean lamina I neuronal response to peripheral electrical stimuli in SAP and SP-SAP animal models
Graph 3.3.v. Mean lamina I and lamina V neuronal response to punctate mechanical stimuli in naive rat models. Graph illustrates the number of Action Potentials fired in a 10 second period of mechanical stimulation. Values for von Frey 125 and Brush were not available for lamina V neurones.
Graph 3.3.vi. Mean lamina I neuronal responses to peripheral punctate mechanical stimuli in naive, sham and SNL animal models.
Graph 3.3.vii. Mean lamina I neuronal responses to punctate mechanical stimuli in SAP and SP-SAP animal models.
Graph 3.3.viii. Mean lamina I and lamina V neuronal responses to peripheral thermal stimuli in normal rat models. The response to cold stimuli 4°C was not measured in lamina V neurones.
Graph 3.3. ix. Mean lamina I neuronal response to peripheral thermal stimuli in normal, sham-operated and SNL animal groups. Cold Response represents the response to 4°C.
Graph 3.3.x. Mean lamina I neuronal response to peripheral thermal stimuli in SAP and SP-SAP treated animal models.
Graph 3.3.xi. Mean neuronal response of lamina I neurones in normal animal models separated into physiological response categories following peripheral punctate mechanical stimuli. *P≤0.05, **P≤0.01 illustrated significance between WDR and HPC neuronal populations. ⊙ P≤0.05, ⊙⊙, P≤0.01 illustrated significance between NS and WDR neurones
Graph 3.3.xii. Mean neuronal response of lamina I neurones in naive animal models separated into physiological response categories following peripheral thermal stimuli. *P≤0.05, ** P≤0.01 illustrated significance between WDR and HPC neuronal populations. ° P≤0.05, °°, P≤0.01 illustrated significance between NS and WDR neurones.
Graph 3.3.xiii. Mean neuronal response of lamina I neurones in SNL operated animal models separated into physiological response categories following peripheral punctate mechanical stimuli. ⊙ P=≤0.05, ⊙⊙, P=≥0.01 illustrated significance between NS and WDR neurones
Graph 3.3.xiv. Mean neuronal response of lamina I neurones in sham operated animal models separated into physiological response categories following peripheral punctate mechanical stimuli. *P=≤0.05, ** P=≤ 0.01 illustrated significance between WDR and HPC neuronal populations. ⊙ P=≤0.05, ⋄ ⋄, P=≤0.01 illustrated significance between NS and WDR neurones
Graph 3.3.xv. Mean neuronal response of lamina I neurones in SNL operated animal models separated into physiological response categories following peripheral thermal stimuli
Graph 3.3.xvi. Mean neuronal response of lamina I neurones in sham operated animal models separated into physiological response categories following peripheral thermal stimuli.
Graph 3.3.xvii. Mean neuronal response of lamina I neurones in SAP treated animal models separated into physiological response categories following peripheral punctate mechanical stimuli. ⊙ P=≤0.05, ⊙⊙, P=≤0.01 illustrated significance between NS and WDR neurones
Graph 3.3.xviii. Mean neuronal response of lamina I neurones in substance P saporin treated animal models separated into physiological response categories following peripheral punctate mechanical stimuli. *P≤0.05, **P≤ 0.01 illustrated significance between WDR and HPC neuronal populations. ○ P≤0.05, ○○, P≤0.01 illustrated significance between NS and WDR neurones.
Graph 3.3.xix. Mean neuronal response of lamina I neurones in SAP treated animal models separated into physiological response categories following peripheral thermal stimuli. ○ P≤0.05, ☉☉, P≤0.01 illustrated significance between NS and WDR neurones
Graph 3.3.xx. Mean neuronal response of lamina I neurones in substance P saporin treated animal models separated into physiological response categories following peripheral thermal stimuli. *P=≤0.05, **P=≤0.01 illustrated significance between WDR and HPC neuronal populations. ○ P=≤0.05, ◯○, P=≤0.01 illustrated significance between NS and WDR neurones.
3.4 Discussion

Overall lamina I neurones appeared to be noticeably different to deeper dorsal horn neurones in their biophysical properties and physiological responses to mechanical, thermal and electrically evoked stimuli. Almost all lamina I neurones had a characteristic double spike whereas lamina V neurones had a characteristic single spike following peripheral stimuli. This may be due to the anatomical arrangement of cells within the thin lamina I layer of the superficial dorsal horn (Beal and Bicknell 1981; Woolf and Fitzgerald 1983; Zhang and Craig 1997). Golgi preparations have shown that primary afferent fibres form a transverse plexus of fibres traversing across the marginal layer (Beal and Bicknell 1981). These spikes may be action potentials recorded from both cell bodies and dendrites of primary afferent neurones running parallel to the surface of the dorsal horn, or alternatively from large Waldeyer cells which comprise the marginal layer (Light et al. 1979; Beal and Bicknell 1981; Molony et al. 1981; Bowsher and Abdel-Maguid 1984). Either way, previous studies have commented on difficulties in obtaining unitary activity from extracellular recordings in lamina I and on more careful analysis one can see double spike activity in some extracellular recordings obtained following evoked activity (Light et al. 1979). These authors explained that thick and extended dendritic processes, numerous projecting axons as well as small cell bodies, could be an explanation as to why unitary activity was difficult to obtain (Light et al. 1979).

The majority of neurones recorded in lamina I (86%) did not exhibit a wind-up response characteristic of deep convergent dorsal horn neurones in both animal and human models (Hanai 1998; Dickenson and Sullivan 1987). Interestingly in a study assessing the physiological properties of spinoparabrachial lamina I neurones, a substantial lack of 'wind-up' was noted in response to electrical stimuli (Bester et al. 2000), consistent with the findings reported in this chapter. Bester (2000) reported a 'reduced wind-up' similar to that seen in 27% of the lamina I neurones characterised in normal animal models in this study. It is possible that peripheral inputs in lamina I are not sufficient to depolarise and activate functional NMDA receptors in lamina I and therefore potentiate existing responses to peripherally applied noxious stimuli (Dickenson and Sullivan 1990). This theory is consistent with the reduced ability of NMDA receptor blockers to inhibit both C-fibre, post-discharge and wind-up responses in lamina I compared to lamina V neurones in previous electrophysiological studies (Dickenson and Sullivan 1990). Furthermore, lamina I
neurones had smaller electrically evoked responses, particularly C-fibre, post-discharge and XS-spike responses compared to lamina V neurones. In fitting with previous reports the majority of lamina V neurones were WDR in response characteristic (Dickenson et al. 1998). However only a small proportion (28%) of lamina I neurones were WDR in normal animals.

It is possible these smaller response properties represent the fact that small unmyelinated C-fibre afferents predominantly terminate in lamina II of the superficial dorsal horn and therefore lamina I receives smaller C-fibre afferent projections compared to deeper laminae (Fitzgerald 1989; Wall 1989). However, there were no distinct differences in A-β fibre and A-δ fibre neuronal response properties between these two laminae. A-δ fibre neurones are thought to terminate in lamina I. Thus it is feasible that lamina I and lamina V may receive similar inputs from these thinly myelinated afferents (Cervero et al. 1979; Gobel et al. 1981). However A-β fibres tend to terminate more deeply (Cervero et al. 1979; Gobel et al. 1981). It is possible that these fibres send afferent projections from deeper laminae to the superficial dorsal horn, so that lamina I and V receive similar inputs from this myelinated A-fibre population (Gobel et al. 1981).

Little difference was evident in the electrically evoked responses of lamina I neurones recorded in sham and SNL operated animal groups. However a substantial input, A-β fibre and post-discharge response was evident in sham and SNL animal groups overall, representing substantial pre-synaptic activity, innocuous inputs and hyperexcitability in these two populations of lamina I neurones. A slight tendency for a larger A-δ fibre response in SNL animals (Graph 3.3.iii) suggests nerve injury induced plastic changes in the role of these thinly myelinated fibres in noxious transmission, perhaps due to either an upregulation in receptor populations or increased primary afferent input. A-δ fibres appear to respond pre-dominantly to high-threshold mechanical stimuli, this may contribute to the development of mechanical allodynia and other abnormal pain responses following nerve injury (Kontinen et al. 2001). Following peripheral axotomy sensory transmission in the form of action potentials in both A-β and A-δ fibre neurones is altered (Stebbing et al. 1999). Intracellular recordings have identified wider action potentials with inflections occurring during the repolarisation phase which may contribute towards altered excitability in lamina I neurones following peripheral nerve injury (Stebbing et al. 1999).
Indeed alterations in conduction velocity and conduction block, which is dependent on neuronal activity, is evident in A-δ fibre primary afferent neurones following nerve injury (Won et al. 1997; Won et al. 2000). Such studies have suggested changes in activity dependent hypoexcitability following nerve injury in these thinly myelinated primary afferents which may account for alterations in neuronal excitability (Won et al. 1997; Won et al. 2000). Studies recording from dorsal root primary afferent axons have reported increases in the proportion of A-β and A-δ fibres, as well as C-fibres, which conduct sensory stimuli following stimulation of the injured sciatic nerve, as well as increases in spontaneous activity in such fibres (Kajander and Bennett 1992). This further supports distinct plasticity in A-δ fibre populations following peripheral nerve injury particularly in primary afferent neurones, which are known to terminate in lamina I.

Lamina I neurones in SP-SAP animal models, whereby NK-1 containing lamina I neurones had been selectively ablated, were characteristically different to the NK1 containing neurones recorded in normal animals. However comparisons between naive and SP-SAP groups have not been made due to differences in weight and age in these two animal groups and the fact that SAP animals represent the control group. Lamina I neurones in the SP-SAP model had a slight tendency for larger A-β fibre and post-discharge responses than those recorded in SAP animals. Interestingly, SAP control groups had a tendency to exhibit larger C-fibre responses than that of SP-SAP animals. NK1 neurones are both morphologically and physiologically diverse (Cheunsuang and Morris 2000; Cheunsuang et al. 2002). It is thought that the majority (80%) of projection neurones, which are abundant in the spinal lamina I layer, express NK1 receptors and in turn respond to high threshold nociceptive stimuli (Todd et al. 2002). However, only a total of 5% of lamina I neurones are thought to express NK1 receptors overall (Spike et al. 2003). This is despite the fact that earlier studies have demonstrated as many as 45% of the total lamina I population express NK1 receptors (Todd et al. 1998). Immunocytochemical studies have revealed that noxious stimuli (such as that evoked by formalin) stimulates c-Fos expression in a greater majority of NK1 containing projection neurones than non-NK1 projection neurones in lamina I (Todd et al. 2002). It is therefore evident that spinal neurones need NK1 receptors for central sensitisation that arises in a variety of chronic pain states (Khasabov et al. 2002). It is clear from immunocytochemical studies that non-NK1 expressing neurones tend to have a high
density of gephyrin puncta on their cell bodies and dendrites and are more often than not large Waldeyer cells or giant flattened neurones (Lima and Coimbra 1983; Lima and Coimbra 1986; Lima et al. 1993; Puskar et al. 2001). Most of these neurones neighbour glutamate decarboxylase rich axons and it is assumed that such neurones therefore have distinct GABAergic inputs and hence inhibitory controls (Puskar et al. 2001). One may assume that removal of NK1 containing neurones would increase the amount of neurones with inhibitory inputs, however this is clearly not evident. Previous studies observing the response properties of lamina I neurones in spinothalamic and spinoparabrachial projection neurones, which are thought to be predominantly NK1 expressing neurones, have found that the majority of these neurones are nociceptive specific and have generally lower responses to evoked stimuli (Craig and Kniffki 1985; Bester et al. 2000). It would therefore remain probable that gephyrin rich neurones are not lower responding, as would be predicted of neurones with large inhibitory inputs. The increase in A-β fibres and post-discharge responses may be due to central compensatory mechanisms, counteracting loss of neuronal excitability. Only minor changes were evident in the mechanical coding of lamina I neurones in SP-SAP animals compared to SAP controls. The responses to noxious thermal stimuli were slightly larger in SP-SAP animal groups.

Interestingly, similar studies whereby responses of lamina V neurones have been recorded, have illustrated distinct reductions in both non-potentiated input responses as well as wind-up responses in SP-SAP animal models (Suzuki et al. 2002). Lamina V neurones in SP-SAP animals have clear deficits in both their mechanical and thermal coding properties (Suzuki et al. 2002). Reductions in receptive field sizes are also evident in deep WDR neurones, which is not seen in lamina I neurones. It is thought removal of NK1 lamina I neurones may reduce the transmission of nociceptive information from the spinal cord to brainstem sites. This in turn may reduce inputs from facilitatory serotonergic descending controls, responsible for the coding of sensory information (Suzuki et al. 2002). It may be that lamina I neurones undergo plastic changes following ablation of NK1 expressing neurones, which develop in order to replace these pronociceptive inputs into the spinal cord. Alternatively, removal of facilitatory descending controls, as (Suzuki et al. 2002) suggest, may reduce the excitatory input on gephyrin rich neurones with predominantly inhibitory inputs, resulting in increased lamina I neuronal responses via disinhibitory mechanisms.
A large proportion of lamina I neurones exhibited a brush response, although they were high threshold to punctate mechanical and thermal stimuli. Bester et al (2000) have demonstrated lamina I neurones with a brush response although these are all classified as WDR, however as mentioned, the majority of these neurones have low pressure and low heat thresholds ($\leq 5 \text{ Ncm}^{-2}$ & 38-40°C respectively). The vast majority of NS and HPC neurones in this study exhibited a response to low threshold brush stimuli, yet were classified as NS or HPC if their responses to punctate mechanical and thermal stimuli was limited to the noxious range. It is unclear why this may be, although it is interesting that lamina I neurones respond to both noxious stimuli and stroking sensations (often perceived as pleasant) and that these findings are consistent with studies by Bester et al, who focus on spinoparabrachial lamina I neurones. It is strongly believed that the parabrachial nucleus is associated with the emotional and affective aspects of sensory stimuli (Bester et al. 2000).

The lamina I neurones within each animal model were also separated based on their responses to mechanical and thermal stimuli applied to the peripheral hindpaw receptive field area. In normal animal models, WDR neurones appear to have larger responses to mechanical and thermal stimuli compared to nociceptive specific and polymodal nociceptive neurones as classified by (Andrew and Greenspan 1999; Andrew and Craig 2001a; Craig et al. 2001; Andrew and Craig 2002a; Andrew and Craig 2002b). HPC neurones classified based on their response to noxious thermal, noxious cold and mechanical stimuli, exerted small responses to brush stimuli compared to NS and WDR neurones. However, WDR and HPC neurones appeared to have characteristically larger noxious cold responses. Overall, NS and HPC neurones exerted similar response properties to mechanical and thermal stimuli in normal animal models. In sham and SNL operated animal models, again WDR neurones had characteristically larger responses to all stimulus modalities. In sham operated animals HPC and NS appeared to have similar responses to mechanical stimuli. HPC neurones had characteristically larger responses to noxious cold and thermal stimuli, however the responses to less noxious thermal stimuli were similar between these two neurones. In SNL animals, HPC neurones exerted larger responses than NS neurones predominantly following noxious mechanical stimuli. HPC neurones also had large responses to brush stimuli. Following thermal stimuli NS and HPC had similar responses, except to noxious cold. However, an n=2 HPC neurones in the SNL group, made this comparison hard to determine.
Overall, following nerve injury NS neurones recorded in SNL animals had smaller responses to mechanical stimuli, than that of NS neurones in sham operated animals. The brush response of NS neurones was smaller. HPC neurones in SNL animals had larger responses to mechanical and brush stimuli than HPC neurones in sham animals altogether suggesting a switch from larger responding NS neurones, to NS neurones with far smaller responses and smaller responding HPC neurones to suddenly much larger responding HPC neurones. Evidence of some degree of plasticity following peripheral nerve injury. In SNL animals, WDR neurones appear to exert larger responses to noxious cold stimuli. Similarly, HPC neurones in neuropathic animals have greater responses to heat stimuli as compared to HPC neurones in sham controls. However, interestingly NS neurones have greater responses to thermal stimuli in neuropathic animals as well, compared to sham operated control groups. This again suggests increases in responses in all neuronal subtypes to thermal stimuli following peripheral nerve injury.

In substance P saporin treated animal groups NS and HPC neurones had similar responses to mechanical stimuli, however HPC neurones had slightly larger responses to noxious cold stimuli. WDR neurones had greater responses overall, particularly following innocuous thermal stimuli. In saporin animal groups WDR neurones generally had larger responses overall. NS neurones had larger responses to noxious mechanical and thermal stimuli as compared with HPC neurones. HPC neurones had characteristically larger cold responses than both NS and WDR neurones. Overall HPC neurones in SAP animals appeared to code noxious heat far better than HPC neurones in SP-SAP treated groups, suggesting that removal of NK1 expressing neurones may remove the coding of noxious thermal stimuli in these neurones. Ablation of lamina I NK1 expressing neurones is likely to leave behind a large proportion of gephyrin-rich neurones with distinct innervation by inhibitory neurones as discussed in sections 1.4.2 and 3.1 (Puskar et al. 2001). One would therefore presume that lamina I neurones recorded in SP-SAP animals have smaller response properties than lamina I neurones in SAP animals. However this is not the case, although one cannot discount the possibility that compensatory changes arise and GABAergic neurones do not play such a large modulatory role following ablation of NK1 expressing neurones.

Furthermore, it may suggest that NK1 neurones do not indeed have larger responses than these gephyrin rich neurones purely because they are innervated by GABAergic neurones. Behavioural studies performed in SP-SAP treated animals have shown that operant escape mechanisms are attenuated in these animals following noxious
thermal stimuli, supporting the fact that loss of NK1 expressing neurones can remove the sensitivity of these animals to thermal stimuli (Vierck et al. 2003). Again, the small number of HPC neurones recorded in this study, suggest more recordings would be required to make a definitive hypothesis. However NS and WDR neurones had similar responses, suggesting no preference to NK1 receptors in these neuronal categories. Interestingly this is not the first time that alterations in the ratio of NS, HPC and WDR neurones have been reported in an in vivo electrophysiological study performed in lamina I neurones. In a bone cancer model of pain, it is reported that alterations in the ratio of NS and WDR neurones occurred, as well as increased responses of WDR neurones to all stimulus modalities in the bone cancer model (Urch et al. 2003).

In conclusion, these studies describe a distinct population of neurones located in the superficial dorsal horn that are mainly high threshold nociceptive- which may be partly due to their lack of the typical NMDA mediated wind-up response seen in deeper dorsal horn neurones. This suggests that information relayed to areas, such as the parabrachial area in the rat, will be dominated by noxious inputs and remain constant throughout repeated stimuli. This may allow the emotional and autonomic areas of the brain to be sent different information from that ascending in the spinothalamic tract, mostly arising from deep wide dynamic range neurones that possess quite different physiological and pharmacological properties. Following nerve injury, only small changes arise in lamina I neuronal response properties, consistent with only limited plasticity in the superficial dorsal horn. Overall, following ablation of NK1 expressing lamina I neurones, very small differences are evident, consistent with similar response properties between NK1 and non-NK1 expressing neurones.
### 3.5 Tables

#### Table 3.3 (a) Lamina I and lamina V mean neuronal responses (No. of Action Potentials ±SE) following peripheral electrical stimuli, in different animal groups.

<table>
<thead>
<tr>
<th>Neuronal Characteristic</th>
<th>Lamina V Naïve n=69</th>
<th>Lamina I Naïve n=216</th>
<th>Lamina I Sham n=38</th>
<th>Lamina I SNL n=49</th>
<th>Lamina I SAP n=41</th>
<th>Lamina I SP-SAP n=55</th>
</tr>
</thead>
<tbody>
<tr>
<td>Depth of Neurone (um)</td>
<td>79±124</td>
<td>293±18</td>
<td>422±52</td>
<td>388±30</td>
<td>237±31</td>
<td>256±64</td>
</tr>
<tr>
<td>C-fibre Threshold (mA)</td>
<td>1.35±0.15</td>
<td>2.5±0.5</td>
<td>2.43±0.15</td>
<td>2.14±0.33</td>
<td>1.64±0.14</td>
<td>2.65±0.43</td>
</tr>
<tr>
<td>C-fibres (AP's)</td>
<td>315±14</td>
<td>138±7</td>
<td>181±18</td>
<td>158±15</td>
<td>237±31</td>
<td>225±28</td>
</tr>
<tr>
<td>A-delta fibres (AP's)</td>
<td>58±4</td>
<td>34±2</td>
<td>35±6</td>
<td>44±4</td>
<td>60±9</td>
<td>53±7</td>
</tr>
<tr>
<td>A-beta fibres (AP's)</td>
<td>100±4</td>
<td>132±4</td>
<td>143±10</td>
<td>139±13</td>
<td>160±12</td>
<td></td>
</tr>
<tr>
<td>Initial C-fibre Input</td>
<td>11±1</td>
<td>17±3</td>
<td>15±2</td>
<td>19±3</td>
<td>21±3</td>
<td></td>
</tr>
<tr>
<td>Post Discharge XS-Spike</td>
<td>183±19</td>
<td>103±3</td>
<td>122±21</td>
<td>135±19</td>
<td>167±26</td>
<td>192±27</td>
</tr>
<tr>
<td></td>
<td>28±30</td>
<td>11±11</td>
<td>116±21</td>
<td>114±24</td>
<td>221±46</td>
<td>204±34</td>
</tr>
</tbody>
</table>

#### Table 3.3 (b) Lamina I and lamina V mean neuronal responses (No. of Action Potentials ±SE) following application of peripheral punctate mechanical stimuli.

<table>
<thead>
<tr>
<th>Neuronal Characteristic</th>
<th>Lamina V Naïve n=69</th>
<th>Lamina I Naïve n=65</th>
<th>Lamina I Sham n=20</th>
<th>Lamina I SNL n=24</th>
<th>Lamina I SAP n=41</th>
<th>Lamina I SP-SAP n=55</th>
</tr>
</thead>
<tbody>
<tr>
<td>1g</td>
<td>32±7</td>
<td>27±9</td>
<td>12±3</td>
<td>14±3</td>
<td>15±2</td>
<td></td>
</tr>
<tr>
<td>5g</td>
<td>52±10</td>
<td>66±19</td>
<td>56±17</td>
<td>46±9</td>
<td>40±5</td>
<td></td>
</tr>
<tr>
<td>9g</td>
<td>115±22</td>
<td>73±24</td>
<td>85±25</td>
<td>51±12</td>
<td>45±6</td>
<td></td>
</tr>
<tr>
<td>12g</td>
<td>133±21</td>
<td>90±26</td>
<td>84±24</td>
<td>55±10</td>
<td>85±12</td>
<td></td>
</tr>
<tr>
<td>15g</td>
<td>239±35</td>
<td>114±27</td>
<td>116±41</td>
<td>110±21</td>
<td>129±18</td>
<td></td>
</tr>
<tr>
<td>30g</td>
<td>365±52</td>
<td>151±31</td>
<td>193±44</td>
<td>168±29</td>
<td>172±24</td>
<td></td>
</tr>
<tr>
<td>75g</td>
<td>655±74</td>
<td>259±42</td>
<td>258±36</td>
<td>288±40</td>
<td>288±40</td>
<td></td>
</tr>
<tr>
<td>Brush</td>
<td>231±20</td>
<td>201±35</td>
<td>178±19</td>
<td>207±30</td>
<td>175±24</td>
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</tbody>
</table>

#### Table 3.3 (c) Lamina I and lamina V mean neuronal responses (No. of Action Potentials ±SE) following application of peripheral thermal stimuli.

<table>
<thead>
<tr>
<th>Neuronal Characteristic</th>
<th>Lamina V Naïve n=69</th>
<th>Lamina I Naïve n=65</th>
<th>Lamina I Sham n=20</th>
<th>Lamina I SNL n=24</th>
<th>Lamina I SAP n=41</th>
<th>Lamina I SP-SAP n=55</th>
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<tbody>
<tr>
<td>32°C</td>
<td>158±39</td>
<td>115±37</td>
<td>132±29</td>
<td>121±21</td>
<td>136±19</td>
<td></td>
</tr>
<tr>
<td>35°C</td>
<td>212±50</td>
<td>109±34</td>
<td>101±22</td>
<td>115±24</td>
<td>140±19</td>
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<tr>
<td>40°C</td>
<td>206±41</td>
<td>146±39</td>
<td>173±44</td>
<td>172±31</td>
<td>153±21</td>
<td></td>
</tr>
<tr>
<td>42°C</td>
<td>221±43</td>
<td>146±40</td>
<td>145±47</td>
<td>126±29</td>
<td>144±20</td>
<td></td>
</tr>
<tr>
<td>45°C</td>
<td>289±44</td>
<td>209±50</td>
<td>289±45</td>
<td>294±47</td>
<td>224±31</td>
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<tr>
<td>48°C</td>
<td>439±59</td>
<td>363±85</td>
<td>379±52</td>
<td>375±53</td>
<td>424±58</td>
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<tr>
<td>50°C</td>
<td>833±92</td>
<td>422±77</td>
<td>438±41</td>
<td>423±62</td>
<td>547±75</td>
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<tr>
<td>COLD (4°C)</td>
<td>159±23</td>
<td>93±19</td>
<td>168±33</td>
<td>117±21</td>
<td>135±19</td>
<td></td>
</tr>
<tr>
<td>Animal Model</td>
<td>Mean Weight (g)</td>
<td>% Hindpaw Area</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>--------------</td>
<td>----------------</td>
<td>----------------</td>
<td></td>
<td></td>
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<tr>
<td>Normal</td>
<td>31.68±2.77</td>
<td>42.24±0.04</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>27.12±4.63</td>
<td>36.17±0.06</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>SNL</td>
<td>30.78±6.05</td>
<td>41.04±0.08</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SAP</td>
<td>28.17±4.53</td>
<td>37.56±0.06</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SP-SAP</td>
<td>26.86±2.79</td>
<td>35.84±0.04</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

Table 3.3 (d) Receptive field areas (±SE) for lamina I neurones to peripheral pinch stimuli.

<table>
<thead>
<tr>
<th>% Population</th>
<th>NS</th>
<th>HPC</th>
<th>WDR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naïve n=65</td>
<td>60</td>
<td>12</td>
<td>28</td>
</tr>
<tr>
<td>Sham n=20</td>
<td>50</td>
<td>20</td>
<td>30</td>
</tr>
<tr>
<td>SNL n=24</td>
<td>63</td>
<td>8</td>
<td>29</td>
</tr>
<tr>
<td>SAP n=41</td>
<td>71</td>
<td>6</td>
<td>23</td>
</tr>
<tr>
<td>SP-SAP n=55</td>
<td>62</td>
<td>14</td>
<td>24</td>
</tr>
</tbody>
</table>

Table 3.3 (e) Proportion of lamina I physiological neuronal type in different animal groups tested.

<table>
<thead>
<tr>
<th>Neurone Type</th>
<th>Sham</th>
<th>SNL</th>
<th>SAP</th>
<th>SP-SAP</th>
</tr>
</thead>
<tbody>
<tr>
<td>NS</td>
<td>198±20 (91.4%)</td>
<td>186±42 (67%)</td>
<td>161±21 (92.9%)</td>
<td>192±34 (82%)</td>
</tr>
<tr>
<td>WDR</td>
<td>315±16 (100%)</td>
<td>307±74 (100%)</td>
<td>237±44 (92.9%)</td>
<td>230±53 (75%)</td>
</tr>
<tr>
<td>HPC</td>
<td>130±31 (85.7%)</td>
<td>133±12 (50%)</td>
<td>122±76 (50%)</td>
<td>101±81 (50%)</td>
</tr>
</tbody>
</table>

Table 3.2 (f) Mean brush responses (±SE). In brackets () are the proportion of neurones in each physiological category with a brush response (no. of action potentials >50).

Table 3.2.g. A summary of the mechanical and thermal response properties of NS, HPC and WDR neurones in sham and SNL animal models.
CHAPTER 4

THE ROLE OF AMPA/KAINATE RECEPTORS IN LAMINA I OF THE RAT SUPERFICIAL DORSAL HORN
4.0 The role of AMPA/ Kainate Receptors in Lamina I of the Rat Superficial Dorsal Horn

4.1 Introduction

The major excitatory amino acid glutamate is present in copious amounts throughout the CNS (Fletcher and Lodge 1996). AMPA and kainate receptors are widely distributed throughout the nervous system and are implicated in fast excitatory transmission and synaptic transduction of both sensory and noxious information, from the periphery to higher supraspinal centres of the brain (Cumberbatch et al. 1994; Li et al. 1999b). It is therefore important to understand the role that excitatory amino acid receptors play in sensory transduction and nociception, as well as the potential changes that arise during pathological conditions. Neuropathic pain, resulting from peripheral or centrally evoked neuronal damage, is a widely recognised and poorly treated pain condition (Devor 1991; Wall 1991; Woolf and Mannion 1999; Orza et al. 2000; Attal 2001; Baheti 2001; Bouhassira 2001; Bridges et al. 2001; Koltzenburg and Scadding 2001; Meyer-Rosberg et al. 2001; Taylor 2001; Cabaleiro 2002; England and Gould 2002; Hansson 2002; Max 2002; Wilson 2002; Chong and Bajwa 2003). It is crucial that the alterations and ensuing plasticity, following nerve damage is investigated. This means that changes in the distribution and function of the major neurotransmitters within the nervous system and their associated receptor systems are also intensely studied. In this chapter we discuss the roles of the AMPA and kainate receptors in nociception, before and after neuronal insult and the available pharmacological tools used to elucidate their functions.

The differences in AMPA and kainate receptor function have only recently been unravelled and paradoxically, kainate receptor actions are still yet fully clarified (Wilding and Huettner 1996; Lerma 1997; Lerma et al. 1997; Paternain et al. 1998). This is due to only recent advances in the development of specific AMPA and kainate selective antagonists, as well as novel molecular cloning methods for specific AMPA and kainate receptor subunits (Wilding and Huettner 1996; Lerma 1997; Lerma et al. 1997; Paternain et al. 1998). Previous in vitro studies, using whole cell recordings, have attempted to disclose the distinct AMPA and kainate receptor antagonist pharmacology to help unravel the specific functions of these two glutamate receptor categories (Wilding and Huettner 1996).
Interestingly, following application of several AMPA/kainate antagonists (listed below) 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo(f)quinoxaline (NBQX) exhibited the most selectivity for AMPA receptors, as opposed to kainate receptors.

- **Quinoxaline Derivatives** – CNQX, NBQX, ACEA-1011, ACEA-1021, QX, 6CIQX, 6,7diCIQX, 5NQX, and HQXCA (Wilding and Huettner 1996).
- **Other antagonists** – NS-102 (weak kainate receptor antagonist (Bleakman and Lodge 1998)), 5,6diCLKyn, 8CIDDHB, GAMS (Wilding and Huettner 1996).

5-chloro-7-trifluoromethyl-2,3-quinoxalinedione (ACEA-1011), another agent tested, was shown to be most potent at kainate receptor subtypes (Wilding and Huettner 1996). 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX) exhibited equal selectivity for both AMPA and kainate receptor subtypes (Wilding and Huettner 1996). However, more recent studies have reported that the AMPA/kainate receptor antagonists CNQX and NBQX, exhibit inadequate selectivity at the AMPA receptors compared to kainate receptors (Ruscheweyh and Sandkuhler 2002b). Although NBQX (IC$_{50}$ = 60nM) is considerably more selective than CNQX (IC$_{50}$ = 400nM) when tested on heteromeric receptors of recombinant GluR1/2 and GluR2/4 (Stein et al. 1992). Such poor selectivity restricts their use as fully selective AMPA receptor antagonists (Bleakman and Lodge 1998).

Interestingly the classical decahydroxyisoquinoline carboxylate kainate receptor antagonists LY 294486 and LY 293558, exhibit equal affinity at all kainate receptor subunits and little selectivity for AMPA receptors (Ruscheweyh and Sandkuhler 2002b). Although, contradictory to this proposal, it is also suggested LY 293558 is an AMPA receptor antagonist with some action on GluR5 kainate receptors (pK$_{Da}$ = 602) (Bleakman et al. 1996). LY293558 is thus thought to be a mixed AMPA/kainate receptor antagonist and used for this purpose. Another decahydroxyisoquinoline carboxylate LY 382884, is selective for the GluR5, as opposed to the GluR6, kainate receptor subunits and may be utilised to unravel specific GluR5 receptor function (Ruscheweyh and Sandkuhler 2002b).

Both AMPA and kainate receptors are present on small diameter nociceptive primary afferent fibres, which terminate in the superficial dorsal horn (Procter et al. 1998). Some kainate receptor subunits, most notably GluR5, are present in small and intermediate sized dorsal root ganglia (Huettner 1990; Bleakman and Lodge 1998; Ruscheweyh and Sandkuhler 2002b).
Furthermore, both AMPA and kainate receptors are present on presynaptic primary afferent fibres (Lu et al. 2002; Hwang et al. 2001; Kerchner et al. 2001b), as well as postsynaptic dorsal horn neurones in variable proportions (Jonas and Spruston 1994; Bonnot et al. 1996; Engelman et al. 1999). Immunocytochemical studies have localised GluR1, GluR2, GluR2/3 and GluR4 as well as the kainate receptor subunits GluR5-7 in lamina I-III of the dorsal horn (Furuyama et al. 1993; Yung 1998). Simultaneous double-immunofluorescence and confocal microscopy studies have also clearly shown that GluR1 and GluR2 AMPA preferring receptor subunits are expressed predominantly in lamina I-III. However, these studies also revealed that GluR1 receptor subunits are preferentially expressed on inhibitory neurones whilst GluR2 subunits favour excitatory neurones (Kerr et al. 1998). Studies using postembedding immunogold electron microscopy have identified two types of primary afferent terminations, localised within synaptic glomerulus, in the superficial dorsal horn (Popratiloff et al. 1996b). C1 terminals originate from unmyelinated primary afferents, C2 terminals arise from thinly myelinated Aδ afferents (Popratiloff et al. 1996b). Interestingly, both C1 and C2 terminals project to post-synaptic AMPA receptors, yet whereas the C1 terminals favour GluR1 subtypes, C2 terminals are preferentially positioned presynaptic to GluR2/3 receptor subunits (Popratiloff et al. 1996b). It is thus possible that selective block of calcium permeable GluR1 subunits preferentially reduces C-fibre evoked activity (Popratiloff et al. 1996b). These results suggest that different AMPA and kainate receptor subtypes exert variable functions within the dorsal horn, thus pharmacological tools selective for different subunit containing receptors, may have very different effects.

Original 'in vivo' electrophysiological studies demonstrated that intrathecal administration of the non-specific glutamate receptor antagonist γ-D-glutamylglycine (DGG) reduced both A-fibre and C-fibre neuronal responses following peripheral noxious stimulation in superficial, intermediate and deep dorsal horn neurones (Dickenson and Sullivan 1990). Interestingly, by comparison with a selective NMDA receptor antagonist APV, these results indicated that whilst APV resulted in block of C-fibre activity and ensuing post-discharge in deep and intermediate dorsal horn neurones, non-NMDA receptor antagonists are clearly involved in innocuous and noxious transmission throughout the dorsal horn (Dickenson and Sullivan 1990).
This is substantiated by more recent studies using the same methods, whereby both NBQX and the GluR5 selective kainate antagonist LY 382884 exerted distinct inhibitory effects on the C-fibre, post-discharge and wind-up responses of deep dorsal horn neurones (Stanfa and Dickenson 1999).

Further in vivo electrophysiological recordings from wide dynamic range neurones in the dorsal horn, as well as 'in vitro' dorsal root recordings, have also demonstrated a role for AMPA/ kainate receptors in nociception (Procter et al. 1998). Whilst GluR5 receptor antagonists (LY294486 & LY382884) exhibit inhibitory effects on noxious transmission, the non-competitive AMPA receptor antagonist GYKI 53655 has been shown to exhibit distinctly larger analgesic effects (Procter et al. 1998). Furthermore, both the competitive AMPA receptor antagonist NBQX and the non-competitive blocker GYKI 53655 are able to decrease responses of dorsal horn WDR neurones in anaesthetised animals, to peripherally applied noxious thermal and innocuous mechanical stimuli (Cumberbatch et al. 1994). More recent studies have implied that synergistic AMPA receptor block and µ-opioid receptor activation can exhibit pronounced analgesic effects, particularly to acute noxious heat stimuli (>52.5°C) further proving these receptors play a distinct role in nociception, as well as broad sensory transmission (Nishiyama et al. 1998). Such results have even implicated AMPA and kainate receptor blockers in the search for more effective analgesics than the present and commonly used opioids and other such pharmacological agents (Brennan 1998).

In vitro studies, whereby electrophysiological recordings are made from spinal cord slices, have suggested that low affinity kainate receptors are able to modulate NMDA mediated currents in the superficial dorsal horn (Sequeira and Nasstrom 1998). Long term depression within the substantia gelatinosa, can be evoked via low frequency stimulation of A-δ-fibre primary afferents in rat dorsal root preparations (Sandkuhler et al. 1997). Such intracellular recordings of transverse slices illustrate that LTD is blocked by application of the NMDA receptor antagonist D-2-amino-5-phosphonovaleric acid (APV), yet unaffected by GABA_A and glycine receptor block. (Sandkuhler et al. 1997). Sequeira and Nasstrom (1998) suggest that kainate receptors are able to facilitate long term depression (LTD) in the superficial dorsal horn via the prolonged depression of NMDA induced excitatory currents (Sequeira and Nasstrom 1998) and thus exert an antinociceptive effect.
However, in the hippocampus long-term potentiation (LTP), that is independent of NMDA receptor activity, is reduced following kainate receptor antagonism (Bortolotto et al. 1999). The role kainate receptors appear to play in LTD and LTP, based on these studies, suggest kainate receptors are involved in long term modification at the synaptic level of nociceptive transmission (Ruscheweyh and Sandkuhler 2002b).

Activation of kainate receptors situated on presynaptic primary afferent fibres, appears to hamper the release of glutamate within the superficial dorsal horn (Kerchner et al. 2001b). Such theories are based on studies whereby kainate and the GluR5 selective kainate receptor agonist ATPA have exerted inhibitory effects on dorsal horn neurones in culture (Kerchner et al. 2001b). These studies also indicate an analgesic role for kainate receptors within the spinal cord. However, the proposal that kainate receptors play an inhibitory role in the dorsal horn are contradicted by suggestions that excessive agonist concentrations may competitively block kainate receptors in studies whereby kainate and the GluR5 selective kainate agonist ATPA exert these inhibitory effects (Ruscheweyh and Sandkuhler 2002b). It is also possible that the reduction in neurotransmitter release is not from primary afferent nerve terminals, but also inhibitory neurotransmitters from interneurones, as opposed to primary afferent excitatory amino acid transmitters which mediate fast synaptic transmission (Ruscheweyh and Sandkuhler 2002b). This is based on evidence that kainate receptors may be selectively localised on presynaptic inhibitory dorsal horn neurones (Kerchner et al. 2001a; Kerchner et al. 2001b; Kerchner et al. 2002). Interestingly, application of kainate receptor antagonists have been shown to reduce mechanically evoked pain thresholds in vivo, as well as exerting analgesic effects in hot plate and tail-flick tests supporting evidence that kainate receptors are preferentially pronociceptive (Li et al. 1999c; Sutton et al. 1999).

The function of AMPA and kainate receptors in the CNS, in both sensory and nociceptive transmission is thus clear. Block of both kainate and AMPA receptors clearly exert predominantly inhibitory effects on both innocuous and noxious sensory transmission, therefore both AMPA and kainate receptors act to facilitate sensory transmission under normal circumstances. However, it is clear that both AMPA and kainate receptors exert a role in sensory transmission under abnormal conditions. Following peripheral inflammation both AMPA and kainate receptors have been shown to play an augmented function in facilitating nociceptive transmission in 'in vivo' electrophysiological studies performed in adult rats (Stanfa and Dickenson 1999).
Both AMPA receptor and GluR5 kainate receptor antagonists (NBQX & LY 382884 respectively) are more efficient at reducing deep dorsal horn neuronal responses, particularly wind-up, following peripheral carrageenan injection (Stanfa and Dickenson 1999).

Following nerve injury, which frequently results in neuropathic pain syndromes, alterations in normal sensory transmission arise. The result is the development of various abnormal pain related behaviours such as allodynia and hyperalgesia, which are both challenging to treat and as yet, poorly managed (Wall 1991; Woolf and Mannion 1999; Attal 2001; Koltzenburg and Scadding 2001; Meyer-Rosberg et al. 2001; Hansson 2002; Max 2002; Wilson 2002). Evidence suggests that AMPA and kainate receptors may be distinctly involved in the development of these abnormal pain conditions. Indeed, peripheral nerve damage induced by cold-freeze injury results in distinct thermal hyperalgesia and mechanical allodynia, both of which are effectively reduced following application of the kainate receptor antagonist SYM-2081 (Ta et al. 2000). This effect is replicated following chronic constriction injury (Sutton et al. 1999; Ruscheweyh and Sandkuhler 2002b). Furthermore, mechanical allodynia and thermal hyperalgesia can be evoked by intradermal capsaicin in human volunteers (Sang et al. 1996). In such studies the unpleasantness associated with pain, as well as pain intensity and the peripheral receptive field area whereby mechanical allodynia is associated, can be effectively dampened by intravenous application of LY 293558 in these patients (Sang et al. 1998).

Block of AMPA mediated excitatory transmission also exerts an antinociceptive role following neuronal damage, and NBQX has been shown to reduce mechanical allodynia resulting from transient ischaemic damage within the spinal cord (Xu et al. 1993). This role is further supported by the reduction observed in mechanical allodynia, induced by peripherally applied mild thermal burn, following Joro spider toxin (JSTX) block of Ca\(^{2+}\) permeable AMPA receptors in the dorsal horn (Sorkin et al. 1999). It is therefore evident that alterations in the expression and function of both AMPA and possibly kainate receptors, partly underlie the development of abnormal pain related behaviours. Interestingly, immunostaining studies following deafferentation of five lumbosacral dorsal roots, demonstrate that staining of GluR1 and GluR2/3 AMPA receptor subunits is significantly reduced in lamina I-II, yet GluR1 staining was more dense in lamina III, IV and V (Carlton et al. 1998).
In a normal rat model it was shown that immunostaining of GluR1 and GluR2/3 AMPA receptor subunits was prominent in the superficial dorsal horn and less so in deeper laminae (Carlton et al. 1998). Following deafferentation of the sciatic nerve in the spinal cord of adult rats, AMPA receptors are downregulated initially, after which levels of GluR2/3 are completely restored and GluR1 levels significantly improved, consistent with the development of hyperalgesia (Helgren et al. 1999). Intriguingly, following loose ligation of the sciatic nerve AMPA receptors are upregulated in the dorsal horn of the spinal cord and this is in perfect timing with the onset of hyperalgesia (Harris et al. 1996; Helgren et al. 1999). Other immunofluorescence studies, performed in rat substantia gelatinosa following sciatic nerve lesion, propose an increase in GluR2/3 AMPA receptors at C-fibre synapses terminating within lamina II (Popratiloff et al. 1998).

Overall, we have seen that AMPA and kainate receptors are involved in the transduction of sensory and nociceptive transmission in the dorsal horn. It is also likely that AMPA and kainate receptors are subject to plasticity following nerve injury and are also involved in the development of some abnormal pain related behaviours following neuronal insult. Furthermore, the localisation and distribution of AMPA and kainate receptor subtypes within the spinal cord is evident from immunostaining techniques, as well as plasticity and alterations in distribution following neuronal insult. However, apart from in vitro studies there is no electrophysiological data elucidating the individual roles AMPA and kainate receptors exert in lamina I neurones in the superficial dorsal horn. The first synapses in the transduction of painful information from the point of noxious stimulation, to the brain is the superficial dorsal horn of the spinal cord, comprised of lamina I and II (the marginal zone and substantia gelatinosa, respectively). The marginal layer is an intrinsic constituent of the central representation of both pain and temperature responsiveness and has a large ascending output to higher centres. It is thus essential that the role of both AMPA and kainate receptors, before and after peripheral nerve injury, in the response properties of lamina I dorsal horn neurones is unravelled in vivo. This may also help to clarify the role of AMPA/ Kainate receptors in the processing of afferent information to higher centres, involved in the emotional aspects of pain (Craig 2003b). In this section the effect of two predominantly AMPA receptor antagonists (NBQX and CNQX) and an AMPA/ Kainate receptor blocker (LY293558) on the neuronal response properties in an in vivo electrophysiological model of nociception before and after peripheral nerve damage will be evaluated.
4.2 Methods

Electrophysiological recordings of anaesthetised rats, following tracheal cannulation and a laminectomy exposing L3-L4 regions of the spinal cord, were performed. Isolation of one neurone within the superficial dorsal horn (0-250 μm), enabled single recordings to be made of lamina I dorsal horn neurones at 3 x the C-fibre threshold value. Bending of the electrode at a 45° angle from the spinal horizontal plane, allowed deeper penetration and a larger cross-sectional area for lamina I neurones to be found.

Once the neuronal responses were stable, all drugs were applied spinally and responses to electrical stimuli were recorded for 40-60 minutes. Tests were performed every 10 minutes. To demonstrate the effect of each spinally applied excitatory amino acid receptor antagonist, maximal effects were taken within each dose range, for each response type. These maximal effects were calculated as a % of the control value and averaged. Receptive fields were calculated via weight analysis, as described in the methods section 2.0 and were mapped on a diagram of the hindpaw. Changes following each drug dose were mapped 30 minutes after drug application, at which the maximal effect of each dose of drug was normally achieved. All drugs were tested in normal animal models, however NBQX and LY293558 was also tested in animals following spinal nerve ligation of L5 and L6 nerves as described by Kim and Chung (Kim and Chung 1992) as well as sham operated control models.

Paired t-tests were used to test the statistical significance of the dose related effects of all three drugs used and unpaired t-tests were used to compare statistically significant differences between drug effect on different animal groups. Bonferroni corrections for post-hoc statistical analysis were used when performing paired t-tests to account for multiple comparisons made to the pre-drug control values.
4.2.1 Quinoxaline Derivatives

NBQX (2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide disodium salt) and CNQX (6-Cyano-7-nitroquinoxaline-2,3-dione) were both used in these experiments. CNQX and NBQX are two AMPA receptor antagonists with actions at Kainate receptors as well, both of which dissolved in saline 0.9% and stored in vials at room temperature and 20°C respectively.

4.2.1 Decahydroxyisoquinoline Carboxylate Agents

LY293558C is a mixed AMPA/Kainate antagonist which is more selective kainate receptor antagonist, with some action at AMPA receptor sites. The drug was dissolved in 0.9% saline and stored in glass vials at 4°C. Cumulative dosing of 0.5, 5 and 50 μg/50 μl were used for each drug. Once the maximal effects across each response type was achieved and stabilised, the next dose was applied spinally.

4.3 Results

The mean depth of lamina I neurones recorded for the NBQX study were 358±48μm in normal animals, 118±63 μm in sham animals and 197±152 μm in SNL operated animal groups using a bent electrode (45°). Using simple trigonometry described in section 2.2 this meant depths of approximately 253±34, 71±45 and 140±108 μm were recorded from in these different animal models, respectively. The mean C-fibre threshold value was 2.91±0.3mA in normal animals, 3.3±0mA in sham animals and 2.6±0.7mA in SNL operated animal groups. In the CNQX study, mean depths were 204±142μm (145±101μm) and mean thresholds 2.1±0.4mA. In the LY 293 558 the mean depth of neurones used were 395±34, 495±38 and 332±95μm in normal, sham and SNL groups respectively. Using simple trigonometry described in section 2.2 this meant mean depths of approximately 280±24, 351±27 and 236±67μm corresponded to these lamina I neurones. Mean C-fibre threshold values were 3±0.2, 2±0.4, 2.8±0.3mA in normal, sham and SNL animals respectively.
4.3.1 Quinoxaline Derivatives

Two Quinoxaline derivatives were studied in this experiment (NBQX and CNQX). NBQX and CNQX (0.5, 5 and 50 μg/50 μl) were applied spinally and the responses of lamina I dorsal horn neurones were observed. Only one complete experiment was performed in each animal, which meant the effects of each drug, were tested on one individual lamina I neurone.

The effect of spinal NBQX was studied in naive rats. However, NBQX was also tested in rats following ligation of L5 and L6 spinal nerves, two weeks prior to electrophysiological recordings. SNL (spinal nerve ligation) is a procedure described by Kim and Chung (Kim and Chung 1992) which produces peripheral nerve injury and is thus a suitable model for neuropathic pain. Behavioural testing confirmed the development of neuropathic pain related behaviours (see section 2.5.1). The same electrophysiological procedures and ensuing pharmacology were repeated in control sham operated rats. To evaluate the effect of NBQX, a total of 16 lamina I neurones were studied in naive rats, 10 lamina I neurones in sham operated rats and 9 lamina I neurones in SNL rats. Due to the nature of lamina I neuronal response properties, the effect of each drug on all responses from one lamina I neurone was not always feasible. Lamina I neurones tended to have low C-fibre responses, a low wind-up response (often these neurones exhibited no wind-up effect at all), low post-discharge responses and frequently a very low A-δ fibre and input response as well (see chapter 3.0). Furthermore, peripheral receptive field areas of lamina I neurones tended to be smaller and harder to map. As a pre-requisite, all neurones used in each experiment had to have a C-fibre response >100 action potentials, when electrically stimulating the peripheral receptive field area at 3 x C-fibre threshold. Often, some lamina I cells did not exhibit sufficient responses in one or more of the following: post-discharge, XS-spike, A-δ fibre and input responses and thus this response was excluded and not used in the analysis of the drug effect. As a result large n numbers are seen, yet per response, these varied from 6 – 16, as a minimum of n=6 was deemed necessary for accurate clarification of the drug effect. Although lamina I neuronal responses were low, the large n numbers, as well as prior selection of sufficient control values for each electrically evoked neuronal response meant that a satisfactory analysis of drug effect was possible.
On occasion an experiment may have been terminated before all doses were applied, due to loss of the neurone or premature death of the animal.

NBQX caused a dose related inhibition of A-δ fibre responses in normal animals (50μg/50μl; 31±13% pre-drug control; p=0.0038) (See Graph 4.3.1.iii & refer to Table 4.3.1.a). C-fibre responses were also dose relatedly reduced, following spinal NBQX application, (normal animals 50μg/50μl; 38±17% pre-drug controls; p=0.012, (See Graph 4.3.1.iv). A small reduction in the input response was observed in normal groups (See Graph 4.3.1.i). Post Discharge and XS-spike (XS-spike response; normals; 5μg/50μl; 34±16%; p=0.0062) responses were dose relatedly inhibited following spinal application of NBQX (See Graphs 4.3.1.ii & 4.3.1.iii). The XS-Spike response in normal animal groups was generally dose relatedly inhibited, as reflected in the reduction of the reduced wind-up response (Graph 4.3.1.viii). In these normal animals, the significant reduction in the XS-spike response was seen in the mid dose range. NBQX also resulted in a dose related reduction of receptive field areas (See Graph 4.3.1.vii). Receptive field areas was reduced by spinal NBQX to between 34-44% pre-drug control values (5 and 50 μg) in normal models.

NBQX caused a dose related inhibition of A-δ fibre responses in sham (0.5μg/50μl; 49±12% pre-drug control; p=0.0005, 5μg/50μl; 35±13% pre-drug control; p=0.0027, 50μg/50μl; 29±11% pre-drug control; p=0.0005) and to a similar extent, significantly, in SNL groups (66-71% reduction; 5μg/50μl & 50μg/50μl) (See Graph 4.3.1.iii & refer to Table 4.3.1.a). C-fibre responses were also dose relatedly reduced, following spinal NBQX application, (shams animals 0.5μg/50μl; 58±10% pre-drug controls; p=0.0037 and SNL animals 0.5μg/50μl; 79±9%; p=0.0234; 5μg/50μl; 52±11%; p=0.002; 50μg/50μl; 33±11% pre-drug controls; p=0.0003) (See Graph 4.3.1.iv). There was therefore no clear difference between the sham operated and SNL animal groups. A small reduction in the input response was seen in sham groups, yet statistical analysis showed no significance (See Graph 4.3.1.i). Interestingly this was not seen following nerve injury. Post discharge and XS-spike (XS-spike response; SNL groups; 0.5μg/50μl; 27±11% pre-drug controls; p=0.001) responses were dose relatedly inhibited following spinal application of NBQX in both sham and SNL animal groups (See Graphs 4.3.1.v & 4.3.1.vi). There was a tendency for larger reductions in post-discharge in SNL animal groups and a larger reduction in the XS-spike response was also evident in SNL groups. The XS-Spike response in sham-operated and SNL groups were dose relatedly inhibited, as reflected in the reduction
of the reduced wind-up response. In SNL groups, significant reduction in the XS-spike response was seen in the lowest dose range. In sham operated animals a dose related reduction in receptive field area to between 25-53% pre-drug controls was observed (See Graph 4.3.1.vii). Interestingly in SNL operated animals, less pronounced reductions in receptive field size were seen (to between 59-90% of pre-drug controls). No statistical significant reductions in receptive field size were seen in these two animal groups. Overall very small differences between animal groups was observed, and unpaired t-tests demonstrated no statistical significance.

CNQX another quinoxaline derivative, was tested in a total of 8 lamina I neurones and its effects were observed in normal rat models alone, due to time restrictions. CNQX resulted in dose related reductions in input responses (50μg/50μl; 31±12% pre-drug controls; P=0.0064) (See Graph 4.3.1.xi & refer Table 4.3.1.b). Small reductions in A-β fibre, post-discharge and A-δ fibres were also evident (See Graphs 4.3.1.xi & xii). CNQX also caused a large reduction in C-fibre at the largest dose (50μg/50μl; P=0.0031) and XS-spike responses, which is not reflected in the reduction in the reduced wind-up response (See Graph 4.3.1.xii). There was no significant reduction in receptive field area following spinal application of CNQX, although there was a tendency for a reduction in size upon CNQX application (See Graph 4.3.1.xiii).
Graph 4.3.1.i. The effect of NBQX on the input response in lamina I neurones in the superficial rat dorsal horn in naive, sham and SNL animal models.
Graph 4.3.1.ii. The effect of NBQX on the A-beta fibre response of lamina I neurones in the superficial rat dorsal horn in naive, sham and SNL animal groups.
Graph 4.3.1.iii. The effect of NBQX on the A-delta response in lamina I neurones in the superficial rat dorsal horn in naive, sham and SNL models.
Results mean % control. Paired t-test analysis for dose related effects.
\*P < 0.05, **P < 0.01, paired t-test analysis for dose related effects.
Bonferroni corrected values, whereby a/n (n=no. of tests) sets the significance of post-drug values.
Graph 4.3.1.iv. The effect of NBQX on the C-fibre response in lamina I neurones in the superficial rat dorsal horn in naive, sham and SNL models.

Results mean % control. Paired t-test analysis for dose related effects.

*P < 0.05, **P < 0.01, paired t-test analysis for dose related effects.

Bonferroni corrected values, whereby a/n (n=no. of tests) sets the significance of post-drug values.
Graph 4.3.1.v. The effect of NBQX on the post-discharge response in lamina I neurones recorded from naive, sham and SNL operated animals.
Graph 4.3.1.vi. The effect of NBQX on the XS-spike response in lamina I neurones of the superficial rat dorsal horn in naive, sham and SNL animal models. Results mean % control. Paired t-test analysis for dose related effects.

*P < 0.05, **P < 0.01, paired t-test analysis for dose related effects.

Bonferroni corrected values, whereby a/n (n=no. of tests) sets the significance of post-drug values.
Graph 4.3.1.vii. Graph demonstrating the tendency for inhibition exhibited by NBQX on the receptive field area of lamina I neurones in the rat superficial dorsal horn of naive, sham and SNL animal models.
Graph 4.3.1.viii. The effect of NBQX on the 'reduced wind-up' response of one lamina I neurones of the superficial dorsal horn in a naive rat model.
Graph 4.3.1.ix. The effect of NBQX on the 'reduced wind-up' response in one lamina I neurone of the superficial dorsal horn in a sham operated rat.
Graph 4.3.1.x. The effect of NBQX on the 'reduced wind-up' response in one lamina I neurone recorded in the superficial dorsal horn of a spinally nerve (L5/ L6) ligated rat model.
Graph 4.3.1.xi. The effect of CNQX on the electrical responses of lamina neurones in the rat superficial dorsal horn. Results mean % control. Paired t-test analysis for dose related effects. *P < 0.05, **P < 0.01, paired t-test analysis for dose related effects. Bonferroni corrected values, whereby a/n (n=no. of tests) sets the significance of post-drug values.
Graph 4.3.1.xii. The effect of CNQX on the electrical response of lamina I neurones in the rat superficial dorsal horn. Results mean % control. Paired t-test analysis for dose related effects. *P < 0.05, **P < 0.01, paired t-test analysis for dose related effects. Bonferroni corrected values, whereby a/n (n=no. of tests) sets the significance of post-drug values.
Graph 4.3.1.xiii. The effect of CNQX on the peripheral receptive field area of lamina I neurones in the rat superficial dorsal horn.
Graph 4.3.1.xiv. The effect of CNQX on the reduced wind-up response of one lamina I neurones in the rat superficial dorsal horn.
4.3.2 Decahydroxyisoquinoline Carboxylate Agents

LY293558 was tested in a total of 18 lamina I neurones. As mentioned above, not all control responses were deemed suitable for post-drug analysis, therefore n numbers vary between neuronal response types before and after drug application. As with NBQX, there were no statistically significant variations between the responses of naïve, sham and SNL groups, based on unpaired t-test analysis. In naïve rats LY293558 caused statistically significant dose related reductions in the input response (Normal; 50µg/50µl; 0±0% pre-drug control; P=0.001) (See Graph 4.3.2.i & refer to Table 4.3.2.a). There were large reductions in the A-δ fibre responses of normal animal models (50µg/50µl; 17±12% pre-drug controls; p=0.0026) (See Graph 4.3.2.iii). C-fibre responses were dose relatedly inhibited (Naïve’s; 0.5µg/50µl; 65±11%; p=0.0106, 5µg/50µl; 19±6%; p=0.0001 & 50µg/50µl; 13±8% pre-drug control; p=0.0001) (See Graph 4.3.2.iv). Reductions in post-discharge were observed in normal animals, particularly at the top doses (50µg/50µl; 53% reduction) and less so for XS-spike responses (5 & 50µg/50µl; 31% & 16% reduction respectively). However, no statistically significant effects were seen. Interestingly, reduced wind-up response graphs supported the small reductions observed in the XS spike response, in naïve rats. Dose related reductions in the receptive field area were also observed in naïve rats (see Graph 4.3.2.vii), but no statistically significant reduction was found (50µg/50µl; 34±11% pre-drug control).

When looking at the effects of LY293558 in sham and SNL groups large reductions in the A-δ fibre responses of sham animal groups were found compared to SNL groups (shams; 50µg/50µl; 29±18% pre-drug controls) (See Graph 4.3.2.iii). C-fibre responses were dose relatedly inhibited, in both animal models to very similar extents (Shams; 50µg/50µl; 18±6% pre-drug controls; p=0.0003 and SNL; 0.5µg/50µl; 75±6%; p=0.0013, 5µg/50µl; 40±10%; p=0.0001 & 50µg/50µl; 8±4% pre-drug control; p=0.0001) (See Graph 4.3.2.iv). Very little difference was noted between the animal groups. LY293558 resulted in a reduction of the post-discharge response in the sham and SNL groups (See Graph 4.3.2.v). Such reductions were observed in these sham (5µg/50µl; 17±11% pre-drug controls & 50µg/50µl; 16±11% pre-drug controls) and SNL groups (50µg/50µl; 5±2% pre-drug controls), particularly at the largest dose. Finally, large reductions were seen in the XS-spike response in sham (5µg/50µl; 17±12% pre-drug controls; p=0.0067) and SNL models (0.5µg/50µl; 33±11% pre-drug controls; p=0.0046), largely emulating that seen on the post-
discharge response following LY293558 spinal application (See Graph 4.3.2.vi). Reduced wind-up response graphs supported the reductions observed in the XS spike response, in both animal models (Graphs 4.3.2.ix & 4.3.2.x). Interestingly, dose related reductions in the receptive field area were also observed in sham and SNL animal groups (see Graph 4.3.2.vii), with a tendency for a larger reduction in sham groups.
Graph 4.3.2.i. The effect of LY293 558 on the input response in lamina I neurones in the rat superficial dorsal horn. Results mean % control. Paired t-test analysis for dose related effects. *P < 0.05, **P < 0.01, paired t-test analysis for dose related effects. Bonferroni corrected values, whereby a/n (n=no. of tests) sets the significance of post-drug values.
Graph 4.3.2.ii. The effect of LY293558 on the A-beta fibre response in lamina I neurones of the rat superficial dorsal horn of naive, sham and SNL animal groups. Results mean % control. Paired t-test analysis for dose related effects. *P < 0.05, **P < 0.01, paired t-test analysis for dose related effects. Bonferroni corrected values, whereby a/n (n=no. of tests) sets the significance of post-drug values.
Graph 4.3.2.iii. The effect of LY 293 558 on the A-delta fibre response in lamina I neurones of the rat superficial dorsal horn of naive, sham and SNL groups.

Results mean % control. Paired t-test analysis for dose related effects.

*P < 0.05, **P < 0.01, paired t-test analysis for dose related effects.

Bonferroni corrected values, whereby a/n (n=no. of tests) sets the significance of post-drug values.
Graph 4.3.2.iv. The effect of LY293 558 on the C-fibre response in lamina I neurones in the rat superficial dorsal horn in naive, sham and SNL animal groups. Results mean % control. Paired t-test analysis for dose related effects.

*P < 0.05, **P < 0.01, paired t-test analysis for dose related effects.

Bonferroni corrected values, whereby a/n (n=no. of tests) sets the significance of post-drug values.
Graph 4.3.2. The effect of LY 293 558 on the post-discharge response in lamina I neurones of the rat superficial dorsal horn in naive, sham and SNL animal groups.
Graph 4.3.2.vi. The effect of LY293558 on the XS-spike response in lamina I neurones of the rat superficial dorsal horn in naive, sham and SNL animal groups. Results mean % control. Paired t-test analysis for dose related effects.

*P < 0.05, **P < 0.01, paired t-test analysis for dose related effects.

Bonferroni corrected values, whereby a/n (n=no. of tests) sets the significance of post-drug values.
Graph 4.3.2.vii. The effect of LY293558 on the receptive field area of lamina I neurones in the rat superficial dorsal horn in naïve, sham and SNL animal groups.
Graph 4.3.2.viii. The effect of LY29358 on the reduced wind-up response in one lamina I neurone recorded from the superficial dorsal horn of a naive rat.
Graph 4.3.2.ix. The effect of LY29358 on the reduced wind-up response in one lamina I neurone recorded from the superficial dorsal horn of a sham-operated rat model.
Graph 4.3.2.x. The effect of LY29358 on the 'reduced wind-up' response in one lamina I neurone recorded from the superficial dorsal horn of an spinal nerve (L5/ L6) ligated rat model.
4.4 Discussion

This chapter examined the role of the excitatory amino acid glutamate at AMPA/kainate receptors in lamina I neuronal responses. NBQX is thought to have mixed AMPA/kainate receptor selectivity (Wilding et al. 1998), therefore to some extent, the effects of NBQX suggest AMPA/kainate receptors are present in the superficial dorsal horn and play a distinct role in nociceptive transmission. However, the majority of studies suggest that NBQX has predominantly AMPA receptor affinity and merely recognise the micromolar selectivity for kainate receptor subunits (Bleakman and Lodge 1998; Stanfa and Dickenson 1999). We may therefore assume that the effects of NBQX are largely due to AMPA receptor blockade (Fletcher and Lodge 1996; Stanfa and Dickenson 1999; Ruscheweyh and Sandkuhler 2002b). NBQX had marked inhibitory effects on the receptive field areas of lamina I neurones and their electrically evoked responses in normal, sham-operated and SNL groups suggesting that afferent inputs from the periphery, which terminate in lamina I, produce AMPA receptor mediated transmission into lamina I. Such an effect confirms the large role AMPA receptors play in the transmission of sensory information from the periphery to the spinal cord and higher centres.

Although there was only a minor reduction in the A-β and input evoked responses, the A-δ, C-fibre, post-discharge and XS spikes were all largely inhibited (50μg/50μl; 62-82% reduction) by NBQX in normal animal models. All effects appeared to be predominantly dose related, and no statistically significant difference was observed between sham and SNL animals, suggesting that changes in AMPA receptors in the superficial dorsal horn do not arise following nerve injury. However some slight differences were evident, for example, input appeared to be reduced in sham animals, yet following SNL no effect was observed. The reductions in post-discharge response and the XS-spike response tended to be larger following nerve injury, suggesting that AMPA receptors may play an enhanced role in nociceptive processing in neuropathic animal models. The characteristic ‘reduced wind-up’ response in such neurones was also clearly inhibited in all animal models used, as reflected by the XS spike measure.
These results indicate that the majority of synaptic transmission from afferents onto lamina I neurones is AMPA receptor mediated. Previous studies have located Ca$_{2+}$-permeable AMPA receptors in subsets of superficial dorsal horn neurones and thus confirm their presence in lamina I (Engelman et al. 1999). It was hypothesised that these AMPA receptors are located on many lamina I projection neurones to mediate nociceptive transmission (Engelman et al. 1999). Therefore, our studies can both conclude and confirm that AMPA receptors are likely to be important in the transmission of sensory information in this superficial layer. Interestingly, NBQX has previously been used in the exact same experimental model, but recording from deep dorsal horn neurones (Stanfa and Dickenson 1999). Reductions in C-fibre and input responses across the dose range were observed in lamina V neurones, yet reductions in post-discharge (42±19% reduction) and XS-spike responses were only observed following application of the top dose. This suggests that AMPA receptors in the deep dorsal horn exert a smaller role in XS-spike and post-discharge responses. This was also true of A-δ fibre responses. Interestingly, NBQX exerted a larger effect on the input response in deep dorsal horn neuronal responses than that seen in lamina I dorsal horn responses.

It is clear from these effects that the involvement of AMPA receptors in A-β fibre responses, and therefore tactile low threshold stimuli is minimal, which is surprising given that previous studies have largely implicated glutamate activity at AMPA receptors in non-noxious and noxious transmission (Cumberbatch et al. 1994). Furthermore, immunostaining studies have confirmed the presence of non-NMDA glutamate receptors on myelinated fibres (A-β and A-δ) (Coggeshall and Carlton 1998). However, it is also feasible that A-β fibre responses following electrical stimuli at 3 x C-fibre threshold are largely resistant to competitive AMPA receptor blockade of that magnitude.

Other studies looking at the effects of iontophoretically administered glutamate receptor antagonists in wide dynamic range (WDR) rat dorsal horn neurones following peripheral nerve ligation observed that the nerve injury induced enhanced brush response was significantly reduced by NBQX (Leem et al. 1996). These results indicate that AMPA receptors mediate innocuous mechanical transmission, which is thought to be transmitted primarily from A fibres, following nerve injury. Indeed NBQX significantly reduced the A-δ fibre response in naïve rats. This was
also seen in sham operated animal models, with a tendency for reduction in SNL animal groups as well.

Overall following spinal nerve ligation the role of AMPA receptors in sensory transmission is not very different from that of sham animals, indicating that changes in AMPA receptor distribution and function are largely unaffected by peripheral neuronal insult. Indeed it is has been shown that behavioural responses associated with nerve injury, such as mechanically evoked allodynia are largely unaffected by the AMPA receptor antagonist NBQX (Hao and Xu 1996). Although following nerve injury, increases in immunoreactive labelling of AMPA receptors within the superficial dorsal horn of the lumbar spinal cord have been observed, ipsilateral to the injury site (Harris et al. 1996). These observations implicate AMPA receptors in the development of chronic pain symptoms such as allostynia and hyperalgesia. However functional changes in the superficial spinal cord are not evident (Harris et al. 1996; Helgren et al. 1999).

There is a small but notable change in the involvement of AMPA receptors in the non-potentiated ‘input’ response following nerve injury as demonstrated by the effect of NBQX. This suggests that following nerve injury the role of presynaptic AMPA receptors is reduced compared to sham rats. AMPA receptors are expressed on myelinated and unmyelinated presynaptic primary afferent nerve terminals and some results have implicated AMPA receptors as pre-synaptic in origin (Lu et al. 2002). Nevertheless, as the effects of NBQX on input were considerably less than the effect of NBQX on other measures, we would conclude that post-synaptic AMPA receptors dominate the responses of these cells. Nerve damage may prevent the transport of AMPA receptor subunit proteins from the DRG to the primary afferent terminal or may also result in down regulation of presynaptic AMPA receptor proteins, resulting in a reduced presynaptic expression following peripheral nerve damage, which may account for the changes seen following spinal nerve ligation. Indeed nerve injury is thought to result in a large degree of neuronal plasticity (Basbaum 1999) and synaptic plasticity resulting from nerve injury has been shown to affect the expression of AMPA receptors on motorneurones within the CNS (Alvarez et al. 2000).
The effect of CNQX was rather different from that of NBQX on normal lamina I dorsal horn neuronal responses. These differences could be attributed to a greater affinity for kainate receptors, as well as AMPA receptors exerted by CNQX compared to NBQX. CNQX may potentially have better access to the receptors after spinal application. Overall, CNQX resulted in larger reductions in input and A-β fibre response values in lamina I neurones (5μg/50μl & 50μg/50μl; 21-69% inhibition). Interestingly, CNQX did not cause large reductions in the post-discharge and A-δ fibre response as seen by NBQX (greatest effect seen at top dose, 50μg/50μl; 27% reduction and 50μg/50μl; 26% reduction, respectively). However, similar effects were seen on the C-fibre and XS spike responses (largest reduction at top dose, 50μg/50μl; 59% and 80% inhibition, respectively). Smaller reductions in the receptive field area of lamina I neurones were observed, confirming the role that both AMPA and kainate receptors play within lamina I, in processing sensory information from the periphery.

These results suggest that kainate receptors may play a larger role in A-β fibre, and therefore innocuous sensory transmission. It is also evident that kainate receptors may be expressed to a larger extent on pre-synaptic primary afferents, unlike AMPA receptors. Indeed the small effect exerted by NBQX on the input response may be due to its small affinity for kainate receptors. There is also immunocytochemical evidence supporting the finding that kainate receptors are pre-synaptic in origin and modulate sensory transmission in the superficial dorsal horn (Hwang et al. 2001) (Kerchner et al. 2001b; Kerchner et al. 2002; Kidd et al. 2002). The smaller effects exerted by CNQX on post-discharge and A-δ fibre responses suggest a smaller role for kainate receptors in lamina I neuronal hyperexcitability and possibly the majority of innocuous and noxious mechanically evoked sensory transmission as well. However, reductions in C-fibre and XS spike responses suggest a role for both AMPA and kainate receptors in noxious sensory transmission, largely supported by a variety of previous studies (Procter et al. 1998). Studies demonstrating the presence of kainate and AMPA receptors on unmyelinated C-fibre afferents in the superficial dorsal horn also confirm these effects (Popratiloff et al. 1996b; Lee et al. 2001). Furthermore, block of such excitatory amino acid receptors has been shown to exert distinct antinociceptive results (Raiigorodsky and Urca 1990). Interestingly, the effect of spinally applied LY293558 differed from the quinoxaline derivatives in normal animals. Large reductions in the input, C-fibre and A-δ fibre responses were
observed across the dose range in normal animals (largest effects seen at the top
dose 50µg/50µl; between 83-100% reduction).

These results largely support a distinct role for kainate receptors in noxious sensory
transmission and confirm a possible presynaptic location for kainate receptors (as
opposed to AMPA receptors) on primary afferent nerve terminals in lamina I.
Reductions in the A-β fibre responses were observed following application of
LY293558 (50µg/50µl; 38.5% inhibition), again supporting a larger role for kainate
receptors in innocuous sensory transmission in superficial lamina. However although
small reductions in post-discharge and XS-spike were seen, these were not
statistically significant at any dose of LY293558 and suggest that kainate receptors
do not play a major role in contributing to neuronal hyperexcitability, unlike AMPA
receptors. Interestingly, unpaired t-tests found no significant difference between
sham and SNL animals and similar reductions in post-discharge and XS-spike
responses (5µg/50µl & 50µg/50µl; between 55-5% reduction) were observed
following application of LY293558 in SNL and sham operated animals. Furthermore,
the inhibitory effect of LY293558 on the A-δ fibre response was largely diminished in
SNL groups, compared to sham operated control groups.

These results suggest that kainate receptors alone may have an enhanced role in
nociceptive transmission and neuronal hyperexcitability following nerve injury, as
these results were not observed after application of NBQX. It is also clear that
kainate receptors exert a reduced function in A-δ fibre responses following nerve
injury, which may suggest a down regulation. However, most studies suggest that
block of kainate receptors may attenuate pain related symptoms such as thermal and
mechanical allodynia which are frequently associated with nerve injury (Sutton et al.
1999; Ta et al. 2000). This would largely implicate an up-regulation in kainate
receptor expression overall. However, these results may just as easily suggest
degeneration of A-δ fibres following nerve injury.

Overall, when comparing the effects of LY293558 to NBQX in SNL and sham
operated animals, LY293558 appeared to cause greater reductions in the A-β fibre,
C-fibre, input and post-discharge responses in SNL groups (50µg/50µl; 33%, 92%,
97% and 95% reductions, respectively). This may be attributed to the combined block
of both AMPA and kainate receptors and possible upregulation of kainate receptors
following nerve injury. However, less inhibition was noted in XS-spike and A-δ fibre
responses. In sham operated animals, LY293558 caused greater reductions in the input, C-fibre, post-discharge and XS-spike responses (most notably 50μg/50μl; 92%, 82%, 84% & 82% reductions, respectively) compared to SNL groups.

It appears that both AMPA and kainate receptors play a large role in noxious sensory transmission, as seen by the effect on lamina I neuronal response properties. It is clear that although slight, and not statistically significant, small changes to arise following nerve injury that may account for the development of some, if not all, abnormal pain related behaviours. The functions of kainate and AMPA receptor are different, and it appears that this may reflect the location of these receptors on spinal neuronal circuits. Overall, both receptors appear to be vital for the transmission of nociception to the higher centres of the CNS, whereby such sensations may be perceived and emotionally reacted upon. The next step in this direction would be to analyse the role of specific AMPA/kainate receptor subunits at this level of synaptic sensory transmission. As with many other receptor systems, selective drugs would aid this aim.
### 4.5 Tables

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Table 4.2.1: Showing Bonferroni corrections for P values at 95% and 99% confidence intervals.

Table 4.3.1.a: Lamina I responses to electrical stimuli in normal, sham and SNL operated animal models and the effect of NBQX on these lamina I response properties in these different animal models. Results are means expressed as % control. Paired t-test analysis for dose related effects. Unpaired t-test analysis for comparative effects between each animal group. Bonferroni corrections for posthoc analyses utilised, therefore statistical significance values set accordingly. *P < 0.05, **P < 0.01, paired t-test analysis for dose related effects. Bonferroni corrected values, whereby α/n (n=no. of tests) sets the significance of post-drug values.
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Table 4.3.1.b. Lamina I responses to electrical stimuli in normal animals and the effect of CNQX on these lamina I response. *P < 0.05, **P < 0.01, paired t-test analysis for dose related effects. Bonferroni corrections for posthoc analyses utilised, therefore statistical significance values set accordingly. Bonferroni corrected values, whereby o/n (n=no. of tests) sets the significance of post-drug values.
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Table 4.3.2.a. Lamina I responses to electrical stimuli in normal, sham and SNL operated animal models and the effect of LY 293558 on these lamina I response properties in these different animal models. Results mean % control. Paired t-test analysis for dose related effects. Unpaired t-test analysis for comparative effects between each animal group. Bonferroni corrections for posthoc analyses utilised, therefore statistical significance values set accordingly. *P < 0.05, **P < 0.01, paired t-test analysis for dose related effects. Bonferroni corrected values, whereby o/n (n=no. of tests) sets the significance of post-drug values.
CHAPTER 5

THE ROLE OF THE NMDA RECEPTOR IN
LAMINA I NEURONES OF THE RAT
SUPERFICIAL DORSAL HORN
5.0 The Role of the NMDA Receptor in Lamina I Neurones of the Rat
Superficial Dorsal Horn

5.1 Introduction

The excitatory amino acids glutamate and aspartate act on a variety of different ionotropic and metabotropic receptors. In this chapter we will be focussing on the role of the ionotropic NMDA receptor subtype, which is located throughout the central nervous system. As mentioned in section 1.9.1 the NMDA receptor is quite unique in its physiological properties and function. These distinct properties have largely implicated NMDA receptors in sensory and nociceptive transmission and this will be greatly expanded upon in this chapter. Furthermore, the majority of electrophysiological in vivo work, has so far been focussed on the pharmacology of deep convergent dorsal horn neurones. Less is known regarding the pharmacology of lamina I dorsal horn neurones responsible for the majority of ascending projections to higher supraspinal levels (Craig 1996). Early studies investigated the role of the excitatory neurotransmitter glutamate and found evidence that glutamate had a predominant role in nociceptive transmission. Not only was glutamate largely distributed throughout the dorsal roots, as opposed to the ventral roots of the spinal cord, it was also abundantly localised throughout the DRG of sensory neurones. In addition, application of glutamate to the spinal cord resulted in long-lasting depolarisation of affected dorsal horn neurones.

The NMDA receptor is widely distributed throughout the central nervous system (Yung 1998). It is thought to be involved in a variety of physiological phenomena throughout the central nervous system, including LTP associated with synaptic plasticity and learning, plasticity throughout the developmental nervous system and neurotoxicity, resulting in cell death (Mori and Mishina 1995). Indeed much work has been focussed on the development of NMDA receptor antagonists in brain ischaemia and stroke (Kemp and McKernan 2002). However, one of the most well recognised phenomena associated with NMDA receptors and nociceptive transmission is wind-up (Mori and Mishina 1995).
From very early on it was recognised that application of a fixed intensity repetitive stimulus to most neurones, resulted in a large frequency-dependent incremental facilitation in their responses (Mendell 1966). The described stimulus necessary was mostly electrical and the progressive increases observed were thus termed "wind-up" (Mendell 1966; Herrero et al. 2000). It was originally proposed that wind-up was a product of unmyelinated C-fibre afferents terminating on spinal interneurones and as such these interneurones evoked cumulatively larger magnitude discharges as each stimuli, transmitted via C-fibres, arrived in the spinal cord (Mendell 1966; Herrero et al. 2000). These early studies were remarkably accurate in their findings. However the mechanisms leading to the development of wind-up are still yet completely unravelled (Herrero et al. 2000).

Wind-up is a topic of much interest particularly in the study of sensory processing and nociception. Following the initial description of wind-up by Lorne Mendell (Mendell 1966), the pharmacological mechanisms underlying wind-up, its modulation and the physiological need for such a mechanism were important questions to be answered (Herrero et al. 2000). It was realised that application of two widely used NMDA receptor antagonists (Ketamine & APV) resulted in substantial reductions in the development of wind-up in electrophysiological in vivo studies (Davies and Lodge 1987; Dickenson and Sullivan 1987; Dickenson 1990; Dickenson and Sullivan 1990; Herrero et al. 2000). Interestingly, this can be extended to human studies whereby application of noxious thermal stimuli to the hands of male volunteers results in wind-up of 'second pain', which is sensitive to intravenous administration of the NMDA receptor antagonist, ketamine (Hughes et al. 2002). Furthermore, it was evident that following the development of wind-up in spinal neurones, long-lasting facilitations in neuronal excitability ensued (Cervero et al. 1984; Cook et al. 1987; Herrero et al. 2000). Indeed NMDA receptor block is thought to have analgesic activity in the majority of persistent pain states described (Aanonsen et al. 1990; Coderre and Melzack 1992; McMahon et al. 1993; Neugebauer et al. 1993; Ren 1994; Dickenson et al. 1997; Carpenter and Dickenson 1999; Ren and Dubner 1999; Sorkin and Wallace 1999; Eide 2000; Carpenter and Dickenson 2001; Kemp and McKernan 2002; Berrino et al. 2003). It is also clear that NMDA receptors play a role in the development of wind-up and consequential hyperexcitability, which may also be involved in the development of such persistent pain states (Herrero et al. 2000).
Indeed, studies have suggested that peripheral nerve damage, underlying neuropathic pain syndromes as well as inflammation, may lead to wind-up and neuronal hyper-excitability in the dorsal horn, both of which contribute to ‘central sensitisation’ and thus abnormal pain behaviours such as hyperalgesia (Li et al. 1999a).

It is important to realise that wind-up is not entirely dependent on the activity of glutamate at NMDA receptors, and that other neurotransmitter systems also contribute to the development of this phenomena (Herrero et al. 2000). It is also crucial to be aware that wind-up is not solely responsible for the development of central sensitisation implicated in many persistent pains conditions (Herrero et al. 2000). However, the role of NMDA receptors in the generation of wind-up and in turn central sensitisation is constantly implicated in a variety of related studies and thus serves as one of the main targets, for the reduction of central hyperexcitability, often associated with chronic nociceptive states.

It was evident very early on that there was a substantial difference in both the pharmacological and physiological nociceptive response properties of dorsal horn neurones within different laminae. In vivo electrophysiological studies have compared the effects of two excitatory amino acid receptor antagonists (APV, a selective NMDA receptor antagonist and DGG, a combined NMDA and non-NMDA receptor antagonist) in lamina I, lamina II and lamina V & VI dorsal horn neurones in normal rats (Dickenson and Sullivan 1990). Interestingly, whereas DGG results in a universal reduction in A- and C-fibre response properties, APV has small effects on lamina I dorsal horn neurones yet, exhibits a reduction in post-discharge and C-fibre evoked responses in deep dorsal horn neurones (Dickenson and Sullivan 1990). From these studies it is clear that NMDA receptors do not mediate C-fibre evoked activity in lamina I dorsal horn neurones as compared to deep dorsal horn neurones (Dickenson and Sullivan 1990). Such findings are interesting, based on the fact that a huge number of studies have localised NMDA receptors within the lamina I dorsal horn of the spinal cord, yet their role in this superficial layer appears to be quite different to their role in deep convergent dorsal horn neurones (Yung 1998). Furthermore, both antagonists have predominantly facilitatory effects on lamina II neuronal responses, suggesting this layer is composed primarily of inhibitory interneurones and disinhibition is suggested as the cause of this opposite effect (Dickenson and Sullivan 1990).
NR2B subunit containing NMDA Receptors

NMDA receptors are heteromeric and consist of many subunit proteins, which may form a wide variety of NMDA receptor subtypes encoded by multiple subunit genes (Loftis and Janowsky 2003). The NR1 subunit is derived from one such gene and is essential for NMDA receptor function as well as glycine binding (Laube et al. 1997; Loftis and Janowsky 2003). Numerous functional splice forms exist, due to alternative splicing of this gene (Loftis and Janowsky 2003). The NR2B subunit, which when combined with NR1 subunits comprises a functional NMDA receptor, is thought to be important in most NMDA mediated physiological and pharmacological functions (Laube et al. 1997; Loftis and Janowsky 2003). NR2B subunits in NMDA receptor systems form both a site for the glycine antagonist CGP61594 and the glutamate-binding area (Honore et al. 1988; Laube et al. 1997; Loftis and Janowsky 2003). There are a variety of genes encoding numerous NR2 subunits and these are often expressed in varying amounts throughout development and in different areas of the central nervous system (Loftis and Janowsky 2003). NR2B containing NMDA receptors are implicated in learning and memory, as well as other vital physiological functions (Loftis and Janowsky 2003). The NR2B subunit is located in the superficial dorsal horn of the spinal cord, as well as other supraspinal locations within the central nervous system (Yung 1998; Loftis and Janowsky 2003). It is also evident that NR2B subunits are located on small diameter nerve fibres which correspond to C-fibre primary afferents, and may play a regulatory role on C-fibre terminals in the release of neurotransmitters (Loftis and Janowsky 2003).

Ifenprodil is a successful non-competitive NR2B selective NMDA receptor antagonist (Gallagher et al. 1996; Loftis and Janowsky 2003) and since its development many other similar compounds have been produced e.g. eliprodil, CP-101, 606 and Ro 25-6981 (Loftis and Janowsky 2003). Voltage clamp recordings have demonstrated the inhibitory effect of ifenprodil at both NR1A/ NR2B and NR1A/ NR2A in xenopus oocytes and have suggested a distinct mechanism of antagonism at the NR1A/ NR2B recombinant receptor (Williams 1993). This may involve non-competitive block of glycine effects at the NMDA receptor (Williams 1993). However, it has been shown that Ifenprodil has a 140-fold selectivity for NR2B, rather than NR2A subunits and acts predominantly by inhibiting NMDA receptor channel opening (Gallagher et al. 1996; Chenard and Menniti 1999; Loftis and Janowsky 2003).
Interestingly, it has been shown that this inhibition of NMDA receptor channel opening is due to ifenprodil blocking the polyamine site of the NMDA receptor in a voltage-independent activity-dependent manner (Carter et al. 1989; Chizh et al. 2001a). As polyamines exhibit a preference for the NR2B subunit, this may explain the selectivity of ifenprodil for NR2B containing NMDA receptors (Carter et al. 1989; Gallagher et al. 1996; Gallagher et al. 1997). Interestingly, [H\textsuperscript{3}]ifenprodil binding experiments have shown that ifenprodil is also an effective blocker at human NR1/NR2B recombinant receptors as well (Grimwood et al. 2000). It is also thought that ifenprodil may also show some selectivity towards the 5-HT\textsubscript{3} receptors and \(\alpha\text{_{1}}\)-adrenoceptors (McCool and Lovinger 1995; Chenard and Menniti 1999; Chizh et al. 2001a). Eliprodil, CP-101, 606, Ro 25-6981 and other such agents all act by binding to the same area as ifenprodil and are therefore also useful pharmacological agents (Taniguchi et al. 1997; Mutel et al. 1998; Chenard and Menniti 1999; Loftis and Janowsky 2003). NR2B selective antagonists, as a whole, have been implicated as useful tools for unravelling the localisation, as well as the function, of NR2B containing receptors (Chenard and Menniti 1999; Loftis and Janowsky 2003).

NR2B containing NMDA receptors may play a role in a variety of pathological and physiological processes within the central nervous system. LTP, often associated with NMDA receptor action, is partly mediated by NR2B containing NMDA receptors (Loftis and Janowsky 2003). In knock-down studies, animals exhibit substantially less LTP and diminished spatial learning after such NR2B antisense treatment (Clayton et al. 2002; Loftis and Janowsky 2003). Furthermore, NMDAR2B receptors are upregulated in certain brain regions in some pathological conditions such as schizophrenia, and in contrast, expression is decreased in particular CNS regions in Parkinsons disease (Grimwood et al. 1999; Dunah et al. 2000; Loftis and Janowsky 2003). Furthermore, NR2B containing NMDA receptors are also thought to play a role in Huntingdons and Alzheimers diseases, seizure disorders, ischemia and diabetes (Loftis and Janowsky 2003).

As a consequence, NR2B selective antagonists have a variety of useful effects in the clinic, one such useful property is their neuroprotective role, as seen following some neurodegenerative diseases (Chenard and Menniti 1999; Chazot et al. 2002; Loftis and Janowsky 2003). Interestingly, it has been shown that ifenprodil and CP-101,606 treatment may be beneficial in the restoration of locomotor activity and the reduction in catalepsy in Parkinsons disease models (Nash et al. 1999; Steece-Collier et al. 2000; Loftis and Janowsky 2003). NR2B selective compounds may be useful as
analgesics (Taniguchi et al. 1997), or more particularly anti-hyperalgesia agents (Loftis and Janowsky 2003). Indeed, studies have analysed the usefulness of NR2B selective agents in reducing the side-effect profiles of general NMDA receptor antagonists used as antinociceptive drugs (Boyce et al. 1999). NMDA receptor blockers have some beneficial analgesic properties, however these effects are limited by the large side effect profile resulting from universal block of NMDA receptors throughout the CNS (Dickenson and Sullivan 1987; Dickenson 1990; Dickenson and Sullivan 1990; Dickenson and Sullivan 1991; Gordh et al. 1995; Dickenson 1997; Christensen et al. 1999; Garry et al. 2000; Chizh et al. 2001a; Garry et al. 2003). In contrast, NR2B selective compounds may retain the useful analgesic properties in neuropathic and other such pain conditions, with less psychotomimetic and unpleasant effects attributed to general NMDA receptor antagonists (Boyce et al. 1999). Furthermore, NR2B overexpression in forebrain areas appears to facilitate the response to a variety of peripherally applied inflammatory stimuli suggesting that NR2B receptors within the forebrain may play a role in the development of inflammatory pain (Wei et al. 2001).

Contradictory to these findings, some studies have been unable to find an effective role for NR2B receptors (Momiyama 2000; Chizh et al. 2001b). This is despite the fact that their presence within the superficial dorsal horn and on small primary afferent neurones, makes them highly probable targets sites in the search for newer and more effective analgesics (Chizh et al. 2001a). In fact both spinal and supraspinal sites contain NR2B containing NMDA receptors and have been implicated in nociceptive modulation (Chizh et al. 2001a). Some electrophysiological studies have even suggested a predominantly supraspinal antinociceptive role of NR2B selective antagonists, particularly compared to general NMDA receptor channel blockers (Chizh et al. 2001a) but this is at variance with many spinal studies. However, regardless of the site of action, the majority of related studies have demonstrated significant roles for NR2B receptors in spinal nociception. It is thought that during different pain conditions, concentrations of endogenous polyamines such as spermine and spermidine may rise, thus facilitating the function of NR2B NMDA receptors in nociception (Chizh et al. 2001a). Thus the effect of NR2B block in this instance will be far greater and potentially more analgesic (Chizh et al. 2001a). It is also evident that more efficient block of NR2B receptors is achieved following greater agonist induced activation of these receptors (Chizh et al. 2001a).
Therefore, in nociceptive conditions, whereby activation of NMDA receptors is increased, the analgesic role of NR2B blockers is heightened (Chizh et al. 2001a). Interestingly, it is believed that NR2B selective compounds may be beneficial in neuropathic pain related behaviours as well (Chizh et al. 2001a). The aim of the study was to elucidate the role NMDA receptors and NR2B containing NMDA receptors play in lamina I of the superficial dorsal horn.

5.2 Methods

Tracheal cannulation and a laminectomy allowed the exposure of L3-L4 regions of the spinal cord, so that electrophysiological recordings of anaesthetised rats could be performed. Isolation of one neurone within the superficial dorsal horn (0-250 μm), enabled single recordings to be made of lamina I dorsal horn neurones in response to peripheral electrical stimulation at 3 x the C-fibre threshold value. Bending of the electrode at a 45° angle from the spinal horizontal plane, allowed deeper penetration and a larger cross-sectional area for lamina I neurones to be found. One lamina I neurone was used per experiment and the effect of spinally applied drugs were examined following suitable and stable controls. Each dose of drug was followed for 40-60 minutes to achieve the maximal drug effect. All drugs were tested in normal animal models, however two drugs were tested in animals that had undergone spinal nerve ligation of L5/L6 peripheral nerves as described by (Kim and Chung 1992). Those tested in SNL rats were also tested in a sham operated group, whereby rats underwent similar surgery without ligation of L5/L6 nerves.

5.2.1 NMDA Receptor Antagonists

APV Lithium Salt (DL-2-Amino-5-Phosphonovaleric Acid) was obtained from Sigma-Aldrich Company Ltd., Poole, Dorset, U.K. The drug was dissolved in 0.9% saline to give dilutions of 50, 100 and 500μg/50μl and stored in glass vials at 4°C. All three doses were tested in every experiment performed. APV was tested in normal animals and compared to animals that had undergone spinal nerve ligation (L5/ L6 spinal nerves) two weeks prior to electrophysiological recordings (Kim and Chung 1992). Sham operated animals were used as controls for SNL surgery.
5.2.2 NR2B-selective NMDA receptor Antagonists

Ifenprodil Tartrate Salt, (α-[4-Hydroxyphenyl]-β-methyl-4-benzyl-1-piperidineethanol) was obtained from Sigma-Aldrich Company Ltd., Poole, Dorset, UK. The drug was dissolved in 0.9% saline to give dilutions of 1, 40, 400µg/50µl and stored in glass vials at 4°C. Ifenprodil was tested in normal animals and compared to animals that had undergone spinal nerve ligation (L5/ L6 spinal nerves) two weeks prior to electrophysiological recordings (Kim and Chung 1992). Sham operated animals were used as controls for SNL surgery. The effects of Ifenprodil on lamina I neurones in normal animal models were compared to data extracted from Kate Carpenter, Thesis, 2001 in which the effect of Ifenprodil was tested on deep convergent dorsal horn neurones (lamina V/ VI) in normal animal models. Ifenprodil was diluted in the exactly the same way and the doses used were also the same, allowing accurate comparisons between these two studies. K. Carpenter originally used Kruskal Wallis non parametric ANOVA statistical tests to evaluate the statistical significance of increasing doses, as well as Dunnett’s multiple comparison tests to assess the significance of each dose compared to control values. Although the significant effects found by K. Carpenter are marked in tables 5.3.2.a-c. Paired t-tests were performed on these results. It was thought paired t-tests would be more appropriate for the results obtained in this study as has been consistently used throughout this thesis, thus for comparisons sake K. Carpenter’s results were analysed using paired t-test analysis. Unpaired t-tests were performed between lamina I and lamina V/VI neuronal effects.

ACEA-1244 IUPAC name 1-[2-(4-Hydroxy-phenoxy)-ethyl]-4-(4-methyl-benzyl)-piperidin-4-ol, was kindly provided by Parke-Davis. The drug, which is not soluble in water, was dissolved in ethanol, after which cremaphor was added and finally saline after the solution had been sonicated for 30 minutes. Proportions were 33% Cremaphor, 7% Ethanol and 60% Saline. Dilutions were made to give 50, 100, 250 and 500µg/50µl concentrations. ACEA-1244 50, 100, 250 and 500µg/50µl were considerably more viscous than the other drugs dissolved in saline, which made spinal administration problematical. Application of ACEA-1244 was thus performed using a Gilson pipette as opposed to the 50µl Hamilton syringe. All four doses were used per experiment. ACEA-1244 was tested in naïve rats alone due to time constrictions. ACEA-1244 data in this study was compared to that extracted from K. Carpenter’s thesis, 2001 in which the effect of ACEA-1244 was tested on deep

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convergent dorsal horn neurones (lamina V/VI). Unpaired t-tests were performed between lamina I and lamina V/VI neuronal effects. ACEA-1244 was diluted in the exact same conditions and the doses used were the same, allowing accurate comparisons between these two studies. Again paired t-tests were used on K. Carpenters results as explained above. In K. Carpenters study, vehicle alone was also tested on lamina V/VI dorsal horn neurones and compared to ACEA-1244 effects, however time restrictions meant that this was not possible in this study. Vehicle was comprised of the exact same proportions of cremaphor, ethanol and saline used to dilute ACEA-1244 in 500µg/50µl (Carpenter 2001). From this saline was added to give the same proportions of cremaphor, ethanol and saline in 50 and 100µg/50µl dilutions as well as 500µg/50µl, giving 3 vehicle doses (K. Carpenter, Thesis, 2001). Below are the proportions of cremaphor, ethanol and saline used in each ACEA-1244 dilution as given by K. Carpenter, Thesis, 2001 (Table. 5.2.2.a)

Paired t-tests were used to test the statistical significance of the dose dependent effects of all three drugs used and unpaired t-tests were used to compare statistically significant differences between the drug effect on different animal groups. Bonferroni corrections for post-hoc statistical analysis were used when performing paired t-tests to account for multiple comparisons made to the pre-drug control values.
Often there was some differences in the n numbers for different response types (low control values were discarded). The effects of each drug was more than often clear and easily interpreted.

5.3 Results

To demonstrate the effect of each spinally applied NMDA receptor antagonist, maximal effects were taken within each dose range, for each response type. These maximal effects were calculated as a % of the control value and the mean was calculated. It is important to note that not all responses of each lamina I neurone recorded were used to calculate the pharmacological effect of each drug used as lamina I neurones typically exerted extremely small response ranges. Neurones were selected if they exerted a good C-fibre response (>100 action potentials), but due to the nature of lamina I neurones it was often very difficult to obtain neurones with a good all round response profile. Therefore if the control response was not large enough to calculate a clear effect it was not used to measure the drugs effect. On rare occasion an experiment may have been terminated before all doses were applied, due to loss of the neurone or premature death of the animal.

5.3.1 NMDA Receptor Antagonists

The NMDA receptor antagonist, APV, was studied in this experiment. Cumulative doses of APV (50, 100, 500 µg/50µl) were administered spinally, and the effects following stabilised controls were recorded as % of mean control values. One lamina I neurone was used per experiment in each animal. The effects of APV were tested on 16 lamina I neurones in naive animals, 8 lamina I neurones in sham operated animals and 9 lamina I neurones in SNL operated animals. The mean depths for lamina I neurones in normal, sham and SNL operated groups were 223±47µm, 353±64µm and 449±47µm respectively using a bent electrode (45°).

Using simple trigonometry described in section 2.2 this meant lamina I neurones with depths of approximately 158±33µm, 250±45µm and 317±33µm were recorded from in these different animal models, respectively. The mean thresholds for lamina I neurones in naive, sham and SNL operated rats were 3.3mA, 1.25±0.95mA and 2.3±0.3mA respectively. APV had a tendency to reduce the input in normal animals (See Graph 5.3.1.1 & refer to Table 5.3.1.a). A-δ fibre responses were also inhibited
by 31-42% at the top doses of APV (100μg /50μl, p=0.0067, 500μg /50μl, p=0.0092) (See Graph 5.3.1.iii). There was a small tendency for APV to facilitate post-discharge and XS-spike response values yet only a slight tendency to reduce the C-fibre response in naïve animals; however overall these effects proved to be statistically insignificant (See Graphs 5.3.1.iv, v & vi). Significant reductions in the input (50μg/50μl, p=0.0173) and A-β fibre responses (50μg, 100μg and 500μg /50μl, p=0.0016, p=0.0076 & p=0.0043) were evident in sham operated animal groups (See Graphs 5.3.1.i & ii and refer to Table 5.3.1.a). There was also a tendency for reduction of C-fibre and XS-spike responses in sham operated rats, at the top dose of APV (Graphs 5.3.1.iv & vi). XS-spike was reduced by 78% at the top dose and surprisingly, the A-β fibre response was reduced maximally at 500μg /50μl by 64% in these sham operated animals. APV reduced the input (50μg, 100μg and 500μg /50μl, p=0.0043, p=0.0016 & p=0.0083) and C-fibre responses (50μg, 100μg and 500μg /50μl, p=0.0130, p=0.0035 & p=0.0016) in the neuropathic animal group (Graphs 5.3.1.i & iv). There was also a tendency for reduction of the post-discharge response in SNL operated animals (See Graphs 5.3.1.v). In SNL animals, the C-fibre response was reduced by 55% at the top dose, compared to 19% in normal animals and 39% in sham operated animals.

When assessing the changes in NMDA receptor function following peripheral nerve damage it is interesting to note that there was in fact a distinct difference in the drug effects on the sham control and the SNL animal models. A distinct reduction in the input response, in sham operated and SNL animals, was evident across all dose ranges. There was a large reduction in A-β fibre responses in sham operated animals compared to SNL rats, following APV application at all three doses. Sham operated animals exhibited a substantial reduction in A-δ fibre responses (51% reduction at the top dose) following APV application, which was not seen in SNL animal groups (23% reduction at low dose and 28% facilitation at the top dose).

SNL animal groups exhibited a significant and slightly larger reduction in C-fibre responses compared to sham animal models across all dose ranges as mentioned above. Lastly, sham operated animals showed a distinct reduction in XS-spike responses (500μg /50μl; 78% reduction), not seen in SNL groups. However, there was no statistical significance between animal groups in this study and these differences only represent a tendency for difference between these groups. APV had no effect on the ‘reduced wind-up’ response in naïve animals, across the dose range.
(See Graphs 5.3.1.vii). Interestingly, when looking at the 'reduced' wind-up response, APV had a small effect on the sham operated reduced wind-up effect and a small inhibitory effect on the 'reduced' wind-up observed in a neuropathic animal (See Graphs 5.3.1.viii & ix).
Graph 5.3.1.1. The effect of APV on the input response in lamina I neurones in the naive, sham-operated and SNL-operated rat superficial dorsal horn. Results mean % control. Paired t-test analysis for dose related effects.

*P < 0.05, **P < 0.01, paired t-test analysis for dose related effects.

Bonferroni corrected values, whereby a/n (n=no. of tests) sets the significance of post-drug values.
Graph 5.3.1.ii. The effect of APV on the A-beta fibre response in lamina I neurones in the naive, sham-operated and SNL-operated rat superficial dorsal horn.

Results mean % control. Paired t-test analysis for dose related effects.

*P < 0.05, **P < 0.01, paired t-test analysis for dose related effects.

Bonferroni corrected values, whereby a/n (n=no. of tests) sets the significance of post-drug values.
Graph 5.3.1.iii. The effect of APV on the A-delta fibre response in lamina I neurones in the naive, sham-operated and SNL-operated rat superficial dorsal horn.
Results mean % control. Paired t-test analysis for dose related effects.
*P < 0.05, **P < 0.01, paired t-test analysis for dose related effects.
Bonferroni corrected values, whereby a/n (n=no. of tests) sets the significance of post-drug values.
Graph 5.3.1.iv. The effect of APV on the C-fibre response in lamina I neurones in
the naive, sham-operated and SNL-operated rat superficial dorsal horn.
Results mean % control. Paired t-test analysis for dose related effects.
*P < 0.05, **P < 0.01, paired t-test analysis for dose related effects.
Bonferroni corrected values, whereby a/n (n=no. of tests) sets the significance
of post-drug values.
Graph 5.3.1.v. The effect of APV on the post discharge response in lamina I neurones in the naive, sham-operated and SNL-operated rat superficial dorsal horn.
Graph 5.3.1.vi. The effect of APV on the XS-spike response in lamina I neurones in the naive, sham-operated and SNL-operated rat superficial dorsal horn.
Graph 5.3.1.vii. The effect of APV on the 'reduced wind-up' response of one lamina I neurone recorded in the naive rat superficial dorsal horn
Graph 5.3.1.viii & ix. The effect of APV on the 'reduced wind-up' response in one lamina I neurone recorded from a sham (viii) and SNL (ix) operated rat superficial dorsal horn
5.3.2 NR2B-selective NMDA receptor Antagonists

Two NR2B selective antagonists were used in this study, ifenprodil (1, 40, 400μg/50 μl) and ACEA-1244 (50, 100, 250 & 500μg/50 μl). Both drugs were administered spinally and their effects on lamina I dorsal horn neuronal response properties were studied. Ifenprodil was investigated in naive, sham and SNL animal models. ACEA-1244 was tested in a naive rats alone. The effect of both Ifenprodil and ACEA-1244, on lamina I neuronal response properties in naïve rats was compared to data obtained from (Carpenter 2001), whereby the effects of these drugs were tested in the exact same manner in lamina V/VI deep convergent dorsal horn neuronal responses.

In K. Carpenter’s study, 5 neurones were used to study the effect of spinal ifenprodil (1, 40, 400μg/50 μl). The mean threshold for these lamina V/VI cells was 1.66±0.47mA in the ifenprodil study and the mean depth was 900±51μm, (Carpenter 2001) used a straight electrode. (Carpenter 2001) describes the exact same procedure in her thesis. The mean C-fibre threshold for lamina I neurones used in the ifenprodil study in naïve rats was 1.6±0.24mA, in sham and SNL animal models the threshold value was 2.2±0.38mA and 2.24±0.24mA respectively. The mean depth for neurones used in the ifenprodil study was 278±88μm, 491.7±75.7μm and 315±108.8μm in naïve, sham and SNL operated animal groups respectively using a bent electrode (45°). Using simple trigonometry described in section 2.2 this meant lamina I neurones with depths of approximately 197±62μm, 348±54μm and 223±77μm were recorded from in these different animal models, respectively.

Ifenprodil tended to reduce the input response of lamina I neurones in normal animals. Ifenprodil (400μg/50μl, p=0.0119) had a small statistically significant inhibitory effect on the input response in sham operated animals, not seen in SNL operated animals suggesting nerve injury evoked plasticity (See Graph 5.3.2.i & refer Table 5.3.2.a). A-β fibre responses were significantly reduced by ifenprodil in normal, sham and SNL operated animals (normals; 40μg/50μl; p=0.0067 & 400μg/50μl; p=0.0034, shams; 1μg/50μl; p=0.0003 and SNL groups; 40μg/50μl; p=0.003 & 400μg/50μl; p=0.0001 respectively) (See Graph 5.3.2.ii). The greatest reduction was seen in SNL operated animals (400μg/50μl; 45% inhibition), however only marginally larger than that seen in sham models at the lower dose. A-δ fibres were significantly reduced in normal and sham animals (1μg/50μl; p=0.001 & 40μg/50μl;
p=0.0134 and 1μg/50μl; p=0.0226 & 40μg/50μl; p=0.0092 respectively) (See Graph 5.3.2.iii). The greatest reduction in A-δ fibre responses was seen following 1μg/50μl ifenprodil in normal animals (51% inhibition). Ifenprodil had a tendency to reduce the A-δ fibre responses in SNL operated animals as well (400μg/50μl; 37% inhibition), however this was not a statistically significant effect and was far less than that seen in sham controls. Interestingly, ifenprodil reduced C-fibre responses in naive and sham operated animals (1μg/50μl; p=0.0156 & 40μg/50μl; p=0.0012 and 1μg/50μl; p=0.0057, 40μg/50μl; p=0.0129 & 400μg/50μl; p=0.0013 respectively) (See Graph 5.3.2.iv). The greatest reductions in C-fibre response values were seen in sham operated animals (400μg/50μl; 57% inhibition). There was a smaller tendency to reduce the C-fibre response in SNL operated animals (1μg/50μl; 28% reduction), however this was not statistically significant and far less than that seen in shams. Naive and sham operated animals exhibited the greatest reductions in post-discharge (1μg/50μl; p=0.0007 & 400μg/50μl; p=0.0019 in normal animals) (See Graph 5.3.2.v). Similar reductions were seen in these two animal groups (400μg/50μl; 63% & 70% inhibition, normal and sham groups respectively). There was a small reduction in SNL operated animals (1μg/50μl; 30% inhibition), however these effects were not statistically significant and again less than that seen in the sham groups.

Lastly, statistically significant reductions were noted in naive and sham operated XS-spike response values (40μg/50μl; p=0.001 & 400μg/50μl; p=0.0024 & 1μg/50μl; p=0.0222, 40μg/50μl; p=0.0011 & 400μg/50μl; p=0.0044 respectively) (See Graph 5.3.2.vi). The reduction observed in normal animals (40μg/50μl; 82% inhibition), was very similar to the reduction seen in sham operated animals at the same dose (40μg/50μl; 81% inhibition). Large reductions were not seen in SNL operated animal groups, however there was still a small tendency for ifenprodil to reduce the XS spike response (1μg/50μl; 27% reduction) in the lower dose range, however this effect was not as pronounced as in the sham groups.

Overall, it is evident that the greatest inhibitions were seen in naive and sham operated animals, particularly in input, A-δ, C-fibre, post-discharge and XS-spike response values compared to SNL operated animals. However, there was a small tendency for these responses to be reduced in SNL operated animals, particularly at the lower dose ranges. As the sham operated model acts as a control for those SNL animals, differences in the effect of ifenprodil could be compared in these two groups.
and it was evident that block of the NR2B site had a tendency to produce far less of an effect following peripheral nerve damage. However, no statistically significant differences were found. The 'reduced wind-up' response graphs show large reductions in an example normal and sham lamina I neurone indicative of the XS-spike response, yet only a comparatively small reduction in the SNL lamina I neurone represented here (See Graphs 5.3.2.vii, viii & ix). Unpaired statistical analysis revealed no statistical differences between ifenprodil's effect on the electrical responses in each animal group.

When comparing the effect of ifenprodil on lamina I and lamina V neurones, there was a substantial difference seen on the A-β fibre, A-δ fibre, C-fibre, post-discharge and XS-spike responses. Greater reductions were seen on input, A-β fibre and XS-spike responses in lamina I neurones (see Graphs 5.3.2.x, xi &xv and refer Table 5.3.2.b), compared to lamina V neurones (Input; 1μg/ 50μl; 83%, A-β fibre; 1μg/ 50μl; 20%, 40μg/ 50μl; 33% & 400μg/ 50μl; 34% and XS-spike; 40μg/ 50μl; 82% inhibition in lamina I neurones respectively). A statistical difference in the effect of ifenprodil on lamina I and lamina V XS-spike response was seen at 40μg/ 50μl; p=0.0308 using unpaired t-test analysis, whereby a far greater reduction in XS-spike response was evident in lamina I neurones. However, there was a greater reduction in lamina V neuronal responses compared to lamina I neuronal responses at 400μg/ 50μl, in A-δ (82% reduction), C-fibre (59% reduction, p=0.0018) and post-discharge response properties (84% reduction, p=0.0116) (See Graphs 5.3.2.xii, xiii & xiv). The greater reductions following ifenprodil application in lamina I neuronal responses were generally seen in the lower dose range. Overall ifenprodil clearly reduced the 'wind-up' response in lamina V neurones, as well as the lamina I 'reduced wind-up' effect (See Graphs 5.3.2.vii & xvi).

In the study looking at the NR2B receptor antagonist ACEA-1244, the mean thresholds for lamina I neurones recorded in normal rats used in this study were 2±0.66mA. The mean depths were 250±40μm using a bent electrode (45°), which once corrected using simple trigonometry, meant mean depths of 176±28μm were used in this study. In Carpenter's study (2001), 18 deep dorsal horn neurones were used to study the effect of spinally administered ACEA-1244 (50, 100, 250 & 500μg/ 50 μl). The mean threshold for these lamina V/ VI cells was 1.96 ±mA. The mean depth was 891±47μm, (Carpenter 2001) used a straight electrode. K. Carpenter describes the exact same procedure in her thesis, although she describes difficulty in
recording the effects of all four doses of ACEA-1244 on lamina V/VI neurones, due to the highly viscous property of the ACEA-1244 solutions. Therefore, one neurone was not always used to record the effect of all four ACEA-1244 doses.

K. Carpenter also describes considerable background activity evoked by application of these ACEA-1244 dilutions, also seen following application of vehicle alone. These findings were not evident in the lamina I neuronal recordings following application of ifenprodil. Interestingly, in Carpenter's study (2001), the effect of the highest dose of ACEA-1244 was not significantly different from that of the highest vehicle 'dose' on any electrically evoked neuronal response lending doubt to the overall effects seen in lamina V/VI neurones following ACEA-1244 application (Carpenter 2001).

Overall, when comparing the effect of these two NMDA NR2B selective receptor antagonists, ACEA-1244 appeared to result in smaller reductions in input, A-β, C-fibre, post-discharge and XS-spike response properties in lamina I neurones compared to ifenprodil's effects on lamina I neurones (See Graphs 5.3.2.xvii, xviii, xx, xxi & xxii). However, similar reductions were seen in the lamina I A-δ fibre response properties (See Graphs 5.3.2.iii & 5.3.2.xix). Interestingly, ACEA-1244 resulted in smaller reductions in the input, A-δ and post-discharge responses in lamina V neurones compared to ifenprodil, (see Graphs 5.3.2.xvii, xix & xxi and refer Table 5.3.2.c) yet similar effects were seen in A-β and XS-spike response properties (See Graphs 5.3.2.xviii & xxii) compared to ifenprodil in lamina V neurones. Comparatively, ACEA-1244 appeared to have a greater inhibitory effect on the C-fibre responses in lamina V neurones, although these effects were very similar to that of ifenprodil (See Graphs 5.3.2.xc & xiii).

When comparing the effect of ACEA-1244 on lamina I and lamina V neuronal response properties, similar effects were observed in the A-β fibre responses in lamina I and lamina V neurones (See Graphs 5.3.2.xviii and refer to Table 5.3.2.c). However, distinct differences were evident in the other electrically evoked responses recorded in these two neuronal populations. The greatest inhibitions in A-δ (250μg/50μl; 68% inhibition, p=0.0008), C-fibre, input and post-discharge response values were observed in lamina V neuronal responses (See Graphs 5.3.2.xix, xx, xxiv & xxi). Interestingly, lamina I neurones exhibited greater reductions in the XS-spike responses (100μg/50μl; 56% inhibition; p=0.0103) following ACEA-1244 application.
(See Graph 5.3.2.xxii). The lamina V XS-spike response was also reduced by ACEA-1244 administration (250µg/50µl, 52%, p=0.0033) (See Graph 5.3.2.xxii).

When comparing the wind-up response in a lamina V single neurone as represented by a 'wind-up' graph, ACEA-1244 had a large inhibitory effect with increasing doses (See Graph 5.3.2.xxiv). However, the effect of ACEA-1244 on a single example lamina I neurone, as represented by the 'reduced wind-up graph' illustrates only a small reduction with increasing doses (See Graph 5.3.2.xxiii). Interestingly unpaired t-test analysis demonstrated a significant difference between the effects of ACEA-1244 on C-fibre in this neuronal population, whereby the smaller doses exerted greater inhibitory effects on lamina I neuronal response properties as compared to lamina V (50µg/50µl; p=0.0045). K. Carpenter investigated the possibility that the vehicle solution could exert some effect on the neuronal response, whereby effects were found (Carpenter 2001).
Graph 5.3.2.i. The effect of Ifenprodil on the input response in lamina I neurones in the naive, sham-operated and SNL-operated rat superficial dorsal horn.

Results mean % control. Paired t-test analysis for dose related effects.

*P < 0.05, **P < 0.01, paired t-test analysis for dose related effects.

Bonferroni corrected values, whereby a/n (n=no. of tests) sets the significance of post-drug values.
Graph 5.3.2.ii. The effect of Ifenprodil on the A-beta fibre response in lamina I neurones in the naive, sham-operated and SNL-operated rat superficial dorsal horn. Results mean % control. Paired t-test analysis for dose related effects.

*P < 0.05, **P < 0.01, paired t-test analysis for dose related effects.

Bonferroni corrected values, whereby a/n (n=no. of tests) sets the significance of post-drug values.
Graph 5.3.2.iii. The effect of Ifenprodil on the A-delta fibre response in lamina I neurones in the naive, sham-operated and SNL-operated rat superficial dorsal horn.

Results mean % control. Paired t-test analysis for dose related effects.

*P < 0.05, **P < 0.01, paired t-test analysis for dose related effects.

Bonferroni corrected values, whereby a/n (n=no. of tests) sets the significance of post-drug values.
Graph 5.3.2.iv. The effect of ifenprodil on the C-fibre response in lamina I neurones in the normal, sham-operated and SNL-operated rat superficial dorsal horn. Results mean % control. Paired t-test analysis for dose related effects. *P < 0.05, **P < 0.01, paired t-test analysis for dose related effects. Bonferroni corrected values, whereby a/n (n=no. of tests) sets the significance of post-drug values.
Graph 5.3.2.v. The effect of Ifenprodil on the post-discharge response in lamina I neurones in the naive, sham-operated and SNL-operated rat superficial dorsal horn. Results mean % control. Paired t-test analysis for dose related effects.

*P < 0.05, **P < 0.01, paired t-test analysis for dose related effects.

Bonferroni corrected values, whereby a/n (n=no. of tests) sets the significance of post-drug values.
Graph 5.3.2.vi. The effect of Ifenprodil on the XS-spike response in lamina I neurones in the naive, sham-operated and SNL-operated rat superficial dorsal horn. Results mean % control. Paired t-test analysis for dose related effects.

*P < 0.05, **P < 0.01, paired t-test analysis for dose related effects.

Bonferroni corrected values, whereby a/n (n=no. of tests) sets the significance of post-drug values.
Graph 5.3.2.vii. The effect of Ifenprodil on the 'reduced wind-up' response in one lamina I neurone of the superficial dorsal horn in a normal rat.
Graph 5.3.2.viii. The effect of Ifenprodil on the 'reduced wind-up' response in one lamina I neurone of the superficial dorsal horn in a sham-operated rat.
Graph 5.3.2.ix. The effect of Ifenprodil on the 'reduced wind-up' response in one lamina I neurone of the superficial dorsal horn in an SNL-operated rat.
Graph 5.3.2.x. The effect of Ifenprodil on the input response in lamina I and lamina V neurones in the normal rat superficial dorsal horn. Lamina V data extracted from Carpenter (2001).
Graph 5.3.2.xi. The effect of Ifenprodil on the A-beta fibre response in lamina I and lamina V neurones in the naive rat superficial dorsal horn. Lamina V data extracted from Carpenter (2001). Results mean % control. Paired t-test analysis for dose related effects. *P < 0.05, **P < 0.01, paired t-test analysis for dose related effects. Bonferroni corrected values, whereby a/n (n=no. of tests) sets the significance of post-drug values.
Graph 5.3.2.xii. The effect of Ifenprodil on the A-delta fibre response in lamina I and lamina V neurones in the naive rat superficial dorsal horn. Lamina V data extracted from Carpenter (2001). Results mean % control. Paired t-test analysis for dose related effects. *P < 0.05, **P < 0.01, paired t-test analysis for dose related effects. Bonferroni corrected values, whereby a/n (n=no. of tests) sets the significance of post-drug values.
Graph 5.3.2.xiii. The effect of Ifenprodil on the C-fibre response in lamina I and lamina V neurones in the naive rat superficial dorsal horn. Lamina V data extracted from Carpenter (2001). Results mean % control. Paired t-test analysis for dose related effects. *P < 0.05, **P < 0.01, paired t-test analysis for dose related effects. Bonferroni corrected values, whereby a/n (n=no. of tests) sets the significance of post-drug values.
Graph 5.3.2.xiv. The effect of Ifenprodil on the post-discharge response in lamina I and lamina V neurones in the naive rat superficial dorsal horn. Lamina V data extracted from Carpenter (2001). Results mean % control. Paired t-test analysis for dose related effects. *P < 0.05, **P < 0.01, paired t-test analysis for dose related effects. Bonferroni corrected values, whereby a/n (n=no. of tests) sets the significance of post-drug values.
Graph 5.3.2.v. The effect of Ifenprodil on the XS-spike response in lamina I and lamina V neurones in the naive rat superficial dorsal horn. Lamina V data extracted from Carpenter (2001). Results mean % control. Paired t-test analysis for dose related effects. *P < 0.05, **P < 0.01, paired t-test analysis for dose related effects. Bonferroni corrected values, whereby a/n (n=no. of tests) sets the significance of post-drug values.
Graph 5.3.2.xvi. Effect of ifenprodil on the wind-up response of one lamina V neurone in the naive rat dorsal horn. Data extracted from Carpenter (2001).
Graph 5.3.2.xix. The effect of ACEA-1244 on the A-delta fibre response in lamina I and lamina V neurones in the naive rat superficial dorsal horn. Lamina V data extracted from Carpenter (2001). Results mean % control. Paired t-test analysis for dose related effects. *P < 0.05, **P < 0.01, paired t-test analysis for dose related effects. Bonferroni corrected values, whereby a/n (n=no. of tests) sets the significance of post-drug values.
Graph 5.3.2.xxii. The effect of ACEA-1244 on the XS-spike response in lamina I and lamina V neurones in the naive rat superficial dorsal horn. Lamina V data extracted from Carpenter (2001). Results mean % control. Paired t-test analysis for dose related effects. *P < 0.05, **P < 0.01, paired t-test analysis for dose related effects. Bonferroni corrected values, whereby a/n (n=no. of tests) sets the significance of post-drug values.
Graph 5.3.2.xxiii. The effect of ACEA-1244 on the 'reduced wind-up' response in one lamina I neurone of the superficial dorsal horn in a naive rat.
Graph 5.3.2.xxv. The effect of ACEA-1244 on the 'wind-up' response in one lamina V neurone in the dorsal horn of a naive rat. Lamina V data extracted from Carpenter (2001).
5.4 Discussion

The effects of a general NMDA receptor antagonist (APV) and two NR2B selective antagonists (Ifenprodil & ACEA-1244) were observed on the electrically evoked responses of dorsal horn lamina I neurones, before and after peripheral nerve ligation, in the rat. APV had little effect on lamina I neuronal response properties in normal animals as seen by A-ô fibre, C-fibre, XS-spike and post-discharge response values, which were all relatively unchanged following APV application. This suggested only a small role for NMDA receptors in lamina I neuronal response properties in normal models of nociception. However a statistically significant reduction in the A-ô fibre response in normal animal models by APV was evident, suggesting the presence of functional NMDA receptors controlling A-ô fibre primary afferents terminals within lamina I. A tendency for a reduction in the input response suggested some involvement of NMDA receptors in the non-potentiated response of lamina I neurones and therefore a possible pre-synaptic location. Sham operated rats exhibited a similar reduction in the A-ô fibre responses, and greater reductions in the input response following APV. However statistically significant reductions in A-ô fibre neuronal responses in sham groups were also clear, suggesting an increased involvement of NMDA receptors in the transmission of innocuous stimuli following spinal nerve exposure, which may be related to the possible development of inflammation and hypersensitivity in the operated region. There was also a clear reduction exhibited by APV in the lamina I XS-spike response in sham groups, illustrating a large role exerted by NMDA receptors in the reduced wind up response, which seems to be an important characteristic of lamina I neurones in intact rats.

Interestingly, following peripheral nerve ligation (L5/L6), a greater inhibition of both the C-fibre and post-discharge responses by APV, compared to sham operated controls. Such results suggest that the role of NMDA receptors in nociception increases following peripheral nerve injury, and thus may contribute towards neuropathic pain related behaviours. Indeed, the increased effectiveness of the NMDA receptor antagonist, in rat models of peripheral nerve injury is supported by studies demonstrating an upregulation of glutamate receptors in neuropathic pain models (Harris et al. 1996; Popratiloff et al. 1996a; Croul et al. 1998). Large reductions in input were observed, similar to that seen in sham groups, demonstrating predominantly functional presynaptic NMDA receptors within lamina I in neuropathic animals. One interesting finding was that although NMDA receptors
appeared to be involved in A-δ fibre sensory transmission within lamina I neurones, following nerve injury this was not evident. The proposed location of NMDA receptors on primary afferent A-δ fibre terminals within lamina I of normal and sham operated rats is not true of nerve injured animals, suggesting a down regulation of NMDA receptors at these sites and a possible upregulation on unmyelinated C-fibre afferents.

Interestingly the effects of a general NMDA receptor blocker APV can be compared to a similar study using the same experimental technique, which demonstrated the effect of APV on both superficial (lamina I) and deep neurones in intact animal models. (Dickenson and Sullivan 1990) showed that APV had virtually no effect on C-fibre response values in lamina I neurones. However when they looked at the effect of APV on deep neurones, corresponding to lamina V/VI, it was found that APV produced small reductions in the C-fibre response and profound reductions in the post-discharge response (Dickenson 1995b). Interestingly, no change in the A-fibre responses were noted, however based on the latency of these A-fibre responses, they appear to correspond to the A-β fibre band recorded in our experiments (Dickenson 1995b). Thus, the effects of APV on the A-δ fibre responses have not been accounted for. These results suggest that in general NMDA receptors play a much larger role in noxious transmission in the deep dorsal horn compared to the superficial dorsal horn. (Dickenson and Sullivan 1990) concluded that the lack of effect exerted by APV on lamina I neuronal responses, was due to a small number of C-fibre inputs in lamina I, as unmyelinated C-fibres terminate predominantly in lamina II. This is supported by the comparatively small C-fibre response values compared to lamina V neurones. A smaller number of C fibre inputs, would feasibly result in less glutamate and peptide release, and thus insufficient depolarisation to remove NMDA receptor Mg ²⁺ block in lamina I (Dickenson and Sullivan 1990). However, the observed reductions in A-δ fibre responses seen in this study, suggest that glutamate release in the superficial dorsal horn is enough to remove NMDA Mg ²⁺ on responses from these thinly myelinated fibres.
Another electrophysiological study performed in rats following spinal nerve ligation compared the effect of different uncompetitive NMDA receptor antagonists on deep dorsal horn neurones. Ketamine was able to block electrically evoked C-fibre and post-discharge responses in deep dorsal horn neurones in sham and nerve injured groups, similar to the those findings in lamina V neurones following APV application (Suzuki et al. 2001). However, ketamine inhibited the post-discharge response, as well as naturally evoked mechanical and thermal stimuli more efficiently in nerve injured groups than in control sham groups (Suzuki et al. 2001). Interestingly, memantine also blocked the wind-up response in neuropathic animals as well (Suzuki et al. 2001). In general, this electrophysiological study suggests that not only do NMDA receptors play a larger role in the noxious evoked response properties of deep dorsal horn neurones, but that similar to lamina I, their contribution in nociceptive transmission is increased following peripheral nerve injury. In lamina I neurones, NMDA receptors appear to play a role in A-δ fibre responses, however in general their involvement is far less than that seen in deep dorsal horn neurones. Following peripheral nerve ligation, the effect of APV on lamina I neuronal responses appears to be similar to that of deep dorsal horn neurones. Overall, it is evident that NMDA receptors contribute towards dorsal horn neuronal sensitisation and thus neuropathic and other related chronic pain conditions (Dickenson 1995a).

It is clear that NMDA receptor antagonists are useful agents for reducing nociceptive transmission in models of nerve injury as the analgesic effect of NMDA blockers seem to be more potent in neuropathic pain models. However, the use of NMDA receptor blockers is restricted due to the distinct psychomimetic side effect profile associated with their use (Chizh et al. 2001a). The search for drugs specific for the NMDA receptor subunits directly associated with nerve injury evoked plasticity has meant that the NR2B subunit has been intensely researched in recent years due to its restricted distribution in the forebrain and the superficial dorsal horn (Chizh et al. 2001a).
Ifenprodil, an NR2B selective NMDA receptor antagonist, was tested on both normal, sham and SNL operated animal models, however ACEA-1244 was tested only on normal animals due to time restrictions. Reductions in A-β fibre responses were seen in all three animal models following application of Ifenprodil. This suggested a larger role for NR2B containing NMDA receptors in innocuous sensory transmission in normal and neuropathic animals, as well as the sham operated controls, despite the fact it is thought NR2B subunits are localised predominantly on small diameter C-fibre afferents (Ma and Hargreaves 2000). This role was enhanced following nerve injury, suggesting a small up-regulation of NR2B NMDA receptors on myelinated non-nociceptive nerve fibres after nerve injury, which may contribute towards neuropathic pain related behaviours. Based on previous studies, it is thought that following nerve injury NMDA receptors are responsible for Fos-like immunoreactivity within lamina I/II, following application of an innocuous stimuli (Kosai et al. 2001).

Myelinated A-β fibres are thought to undergo plastic changes following peripheral nerve injury, which mean that they express Fos proteins, despite the fact that these are normally expressed in nociceptive nerve fibres following a noxious stimulus (Kosai et al. 2001). Interestingly, the expression of these proteins can be modulated via application of an NMDA receptor antagonist (Kosai et al. 2001). As it is thought that the NR2B subunit is the most commonly expressed subunit in the dorsal horn of the rat spinal cord, my results may be in agreement with this study (Karlsson et al. 2002). However, no significant alterations in the expression of NR2B subunits following axotomy using retrograde tracing techniques and glutamate induced currents have been identified.

These contradictory observations therefore suggest that NR2B subunits may not be responsible for increased noxious activity, thought to be exerted by myelinated A-β fibres (Karlsson et al. 2002; Nabekura et al. 2002). However, changes in NR2A subunit expression have been observed (Karlsson et al. 2002; Nabekura et al. 2002). Statistically significant reductions in input, following ifenprodil application, were seen in sham operated control groups. However there was a tendency for input to be reduced in normal animals, similar to that seen following APV application. It may be that these NR2B containing NMDA receptors are responsible for the majority of NMDA receptors located on primary afferent neurones terminating in lamina I particularly as NR2B subunits are densely distributed throughout the superficial dorsal horn (Loftis and Janowsky 2003). A-δ fibre responses were reduced in normal
as well as sham operated groups with a tendency for reduction in SNL groups, which not only suggests that the NMDA receptors located on A-\(\delta\) fibres in lamina I may be primarily NR2B subtypes, but that they have a pre-synaptic origin. As A-\(\delta\) fibres are thought to terminate principally in the lamina I layer, it is highly probable that these results reflect NMDA receptor distribution in the dorsal horn (Woolf 1987). Interestingly Ifenprodil had remarkably different effects on the C-fibre, post-discharge and XS-spike response values compared to APV. C-fibre, post-discharge and XS-spike responses were largely reduced in the normal and sham control groups, yet less so in nerve injured groups whereby no statistically significant reductions were seen. It is evident that NR2B subtypes play a larger role in nociceptive transmission in normal and sham control groups, as APV does not exert similar inhibitory effects in normal animals. However, it may be that whilst NR2B subtypes are down regulated following nerve injury, other previously poorly expressed NMDA receptor subtypes are upregulated and play a much larger role. Such results may be useful in allowing the clinician to differentiate and treat normal pain conditions differently from neuropathic pain conditions by the use of subtype selective NMDA receptor antagonists.

ACEA-1244, had much smaller effects on the input, A-\(\beta\) fibre, C-fibre, post-discharge and XS-spike responses in normal animals compared to ifenprodil suggesting a lower affinity for the NR2B subtype. However ACEA-1244 did have similar effects on the A-\(\delta\) fibre response (21-53\% reduction), compared to ifenprodil (33-51\% reduction), confirming that NR2B selective antagonists play a distinct role in A-\(\delta\) fibre responses in normal animals.

In the latter part of this study, the effects of the two NR2B selective antagonists on lamina I neuronal responses in the normal rat, were compared to similar electrophysiological experiments recording from deep convergent dorsal horn neurones (as performed by (Carpenter 2001)). Ifenprodil tended to have greater inhibitory effects on lamina V neurones as compared to lamina I neurones, however there were two interesting differences. The largest effects of ifenprodil were generally seen in the lower dose range on lamina I neurones, whereas ifenprodil tended to have greater effects at increasing doses in lamina V neurones. Greater reductions in the input and XS-spike responses were observed in lamina I neurones following application of ifenprodil, yet in all other responses recorded the largest reductions evoked by ifenprodil were seen in lamina V. Comparatively,
ACEA-1244 generally exerted smaller effects than ifenprodil, yet greater inhibitions were generally seen in lamina V neuronal response values following ACEA-1244 application, compared to lamina I. However the XS-spike response in lamina I neurones was inhibited to a larger degree. The greater reductions in lamina V neuronal responses following application of these two NR2B selective blockers suggest that NR2B subunit receptors play a larger role in lamina V neuronal responses to predominantly noxious stimuli, as seen with the general NMDA receptor antagonist APV. It is important to note however, that the initial control values are much smaller in lamina I neurones than lamina V neurones, a characteristic of lamina I neurones in general, particularly in C-fibre, post-discharge and XS-spike values. Percentage reductions in lamina I neurones may therefore correspond to much smaller decreases in action potential firing, compared to lamina V.

This may explain some large differences in the effects of different drugs on lamina I and lamina V neurones, particularly evident in the XS-spike response following both ACEA-1244 and ifenprodil application. A recent study using SP-SAP to ablate NK1 expressing neurones in lamina I (Suzuki et al. 2002) showed reduced wind-up in deep dorsal horn neurones, independently of changes in descending controls exerted on other neuronal responses. This is in keeping with the idea that wind-up is an intrinsic spinal mechanism that can be seen in slices of dorsal horn and in spinal animals (Mendell 1966; Herrero et al. 2000). Thus the reductions in the input and some other responses of lamina I cells may in turn reduce the excitability of the deep cells and attenuate wind-up. Since the feeling is that peptides are needed to release the magnesium block of the NMDA receptor then a role for NK1 expressing neurones in lamina I seems feasible. Alternatively, NMDA receptors located on interneurones, the dendrites and soma of deep dorsal horn cells may also make contributions to wind-up.

Overall it was clear that there were changes in the role of both NMDA receptors and NR2B subtypes between normal, sham and SNL animal groups. These results implicate a considerable amount of plasticity evoked by peripheral nerve injury and interestingly it is known that considerable amounts of structural reorganisation arise following peripheral damage which results in distinct behavioural changes (Goff et al. 1998). In addition it is widely believed that NMDA receptors in particular, are involved in the development of these neuropathic pain related behaviours. It is evident that tactile hypersensitivity on the ipsilateral hindpaw arises following loose ligation of the rat inferior alveolar nerves and intraperitoneal administration of the
general NMDA antagonist MK-801 has been shown to reduce the appearance of this allodynia (Yonehara et al. 2003). Indeed, there are a great many studies which show the effectiveness of NMDA receptor antagonists in blocking neuropathic pain related behaviours like allodynia and hyperalgesia (Suzuki et al. 2001). MK-801, memantine, ketamine and other related NMDA receptor antagonists have demonstrated reductions in spontaneously evoked pain, hyperalgesia and allodynia in neuropathic models in a whole host of studies (Davar et al. 1991; Seltzer et al. 1991; Mao et al. 1992; Yamamoto and Yaksh 1992b; Yamamoto and Yaksh 1992a; Kawamata and Omote 1996; Kim et al. 1997b; Nichols et al. 1997; Bian et al. 1999; Munglani et al. 1999; Quartaroli et al. 2001; Suzuki et al. 2001; Karadag et al. 2003; Villetti et al. 2003). One particular study demonstrated the development of NMDA induced allodynia in mice, which was effectively blocked by application of the NR2B antagonist CP-101,606 (Minami et al. 2001).

Another study showed that spinally administered NMDA receptor antagonists were anti-allodynic, but not when supraspinally applied (Chaplan et al. 1997). It appears as though NR2B receptors may partly mediate neuropathic pain related symptoms and do so via spinal sites of action, however paradoxically, some studies have implicated NR2B subunits within the forebrain for the development of persistent pain states (Zhuo 2002). Furthermore, following spinal cord injury, the NMDA receptor antagonist and NOS inhibitor agmatine is thought to greatly improve both locomotor function, as well as reduce tissue damage induced by such trauma (Yu et al. 2000). However, in humans memantine has been shown to have a very low therapeutic profile in the treatment of amputation and surgically induced neuropathic pain (Nikolajsen et al. 2000). These findings show NR2B selective blockers may be useful for neuropathic pain treatment in animals, however extrapolation into the clinic has as yet proved successful.

Overall this study has demonstrated the small role NMDA receptors play in lamina I of the superficial dorsal horn. Although under normal conditions block of NMDA receptors produce reductions in A-δ fibre responses as well as the XS-spike response, following nerve injury it was evident that there was no longer any block of A-δ fibre and XS-spike responses yet a larger block of C-fibre and post-discharge, particularly compared to the sham controls. Block of the NR2B receptor with ifenprodil produced reductions in all electrically evoked lamina I responses, although reductions in A-β fibre, A-δ fibre and C-fibre responses were small. Following nerve
injury, no reduction in the input response was evident and smaller reductions in the other electrically evoked response properties were seen, as compared to sham control models. Overall block of NR2B receptors caused larger reductions in the lamina V electrically evoked response properties as compared to lamina I neurones, particularly in A-\(\delta\) fibre, input, C-fibre and post-discharge responses however slightly greater reductions were seen in XS-spike and A-\(\beta\) fibre responses in lamina I in the ifenprodil study. The ACEA-1244 study produced similar if less pronounced results and reductions tended to be greater in the lamina V population of neurones recorded by (Carpenter 2001).
5.5 Tables

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<td>33%</td>
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<td>16.50%</td>
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<td>80%</td>
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<td>6.60%</td>
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<td>92%</td>
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<td>0.70%</td>
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Table 5.2.2.a. Proportions of cremaphor, ethanol and saline used in ACEA-1244 dilutions

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Table 5.2.2b showing Bonferroni corrections for P values at 95% and 99% confidence intervals.
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Table 5.3.1.a. Lamina I responses to electrical stimuli in normal, sham and SNL operated animal models and the effect of APV on these lamina I response properties in these different animal models. Results mean % control. Paired t-test analysis for drug effects. Unpaired t-test analysis for comparative effects between each animal group. Bonferroni corrections for posthoc analyses utilised, therefore statistical significance values set accordingly. *P < 0.05, **P < 0.01, paired t-test analysis for dose dependant effects. Bonferroni corrected values, whereby o/n (n=no. of tests) sets the significance of post-drug values.
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Table 5.3.2.a. Lamina I responses to electrical stimuli in normal, sham and SNL operated animal models and the effect of Ifenprodil on these lamina I response properties in these different animal models. Results mean % control. Paired t-test analysis for drug effects. Unpaired t-test analysis for comparative effects between each animal group. Bonferroni corrections for posthoc analyses utilised, therefore statistical significance values set accordingly. *P < 0.05, **P < 0.01, paired t-test analysis for dose dependant effects. Bonferroni corrected values, whereby α/n (n=no. of tests) sets the significance of post-drug values.
Table 5.3.2.b. Lamina I and lamina V responses to electrical stimuli in normal animal models and the effect of ifenprodil on these neuronal response properties. Results as % mean controls. *P < 0.05, **P < 0.01, paired t-test analysis for dose dependant effects. Bonferroni corrections for posthoc analyses utilised, therefore statistical significance values set accordingly. *P < 0.05, **P < 0.01, paired t-test analysis for dose drug effects. Bonferroni corrected values, whereby c/n (n=no. of tests) sets the significance of post-drug values. *P < 0.05 and **P < 0.01, unpaired t-test analysis for comparative effects between each cell type and * or ** used as above to denote significance at related confidence levels. tP<0.05, fP<0.01, ftfP<0.001 repeated measure ANOVA, significant effect with larger doses, ♠P<0.05, ♣P<0.01, Dunnett’s multiple comparison test, significant effect of each dose in comparison to control values.

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<td>47±12</td>
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<td>n=11</td>
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<tr>
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Table 5.3.2.c. Lamina I and lamina V responses to electrical stimuli in normal animal models and the effect of ACEA-1244 on these neuronal response properties. Results as % mean controls. *P < 0.05, **P < 0.01, paired t-test analysis for dose dependant effects. Bonferroni corrections for posthoc analyses utilised, therefore statistical significance values set accordingly. *P < 0.05, **P < 0.01, paired t-test analysis for drug effects. Bonferroni corrected values, whereby a/n (n=no. of tests) sets the significance of post-drug values. P < 0.05 and P < 0.01, unpaired t-test analysis for comparative effects between each cell type. tP<0.05, tPP<0.01, tttP<0.001 repeated measure ANOVA, significant effect with larger doses, *P<0.05, **P<0.01, Dunnett’s multiple comparison test, significant effect of each dose in comparison to control values.
CHAPTER 6

THE ROLE OF $\text{GABA}_A$ AND GLYCINE RECEPTORS IN LAMINA I OF THE SUPERFICIAL DORSAL HORN AND ALTERATIONS IN GABA FUNCTION FOLLOWING PERIPHERAL NERVE INJURY
6.0 The Role of $\text{GABA}_A$ and Glycine Receptors in Lamina I of the superficial dorsal horn and alterations in GABA function following peripheral nerve injury

6.1 Introduction

GABA (γ-amino-butyric acid) and glycine are the two main inhibitory neurotransmitters in the central nervous system. GABA is suggested to be involved in around 40% of inhibitory neurotransmission within the CNS (Malcangio and Bowery 1993; Malcangio and Bowery 1996). Immunohistochemical staining has shown that GABA is found throughout the dorsal horn and is densely distributed throughout lamina I-III (Todd and McKenzie 1989; Malcangio and Bowery 1996). GABA is predominantly located in spinal interneurones or islet cells in the superficial dorsal horn, where it can contact cell bodies, dendrites and axons of local neurones mediating sensory transmission (Todd and McKenzie 1989; Lin et al. 1994; Malcangio and Bowery 1996; Bonanno et al. 1997; Curtis and Lacey 1998). Light microscopic post-embedding immunohistochemistry has revealed that GABAergic neurones within the superficial dorsal horn contact central terminals of type 2 glomeruli (C2) which are derived from myelinated afferents, suggesting that the inhibitory gating of sensory inputs within the superficial dorsal horn is highly selective (Bernardi et al. 1995; Todd 1996). GABA is also found throughout the ventral horn whereby it controls motorneurone activity (Malcangio and Bowery 1996). $\text{GABA}_A$ and $\text{GABA}_B$ receptors are distributed throughout the superficial dorsal horn of the spinal cord, although $\text{GABA}_A$ receptors can be found in deeper dorsal horn laminae as well (Malcangio and Bowery 1996). It is thought that these two receptor populations are expressed both on A-δ and C-fibre primary afferent fibres (Desarmenien et al. 1984), as well as interneurones and motoneurones in the case of $\text{GABA}_A$ receptors (Malcangio and Bowery 1996). Interestingly $\text{GABA}_B$ receptors are localised predominantly at pre-synaptic sites, whereas $\text{GABA}_A$ receptors are primarily post-synaptic in location. However conflicting reports clearly demonstrate pre-synaptic $\text{GABA}_A$ receptors in vertebrate synapses (Xi and Akasu 1996; Chery and De Koninck 2000). In vitro studies have shown that $\text{GABA}_B$ receptors are often activated when the release of GABA at inhibitory synapses is incapable of activating $\text{GABA}_A$ receptors on the post-synaptic target demonstrating synergistic roles played by these two receptor subtypes in modulating inhibitory spinal processes (Chery and De Koninck 2000).
There is much literature concerning GABA's involvement in the transmission of nociceptive information from primary afferent neurones within the spinal cord (Malcangio and Bowery 1996). Indeed it has been shown in rats, that agonists at both GABA_A and GABA_B receptors reduce pain-related behaviours (Malcangio and Bowery 1996). Some studies suggest the antinociceptive properties of GABA_A receptors are mediated primarily from their location at higher centres in the brain (Malcangio and Bowery 1996) however, many studies demonstrate spinal actions. Some reports suggest GABA_A receptors do play a distinct role in mediating nociception at the spinal level, particularly in chronic pain conditions (Desarnenien et al. 1984; Kontinen et al. 2001; Malan et al. 2002). Spinal Bicuculline (GABA_A Receptor Antagonist) has been shown to increase activity evoked by mechanical stimuli in the peripheral receptive field area of nociceptive dorsal horn neurones in a variety of studies (Sorkin et al. 1998; Kontinen et al. 2001). GABA_B receptors are thought to play a major role in both supraspinal and spinal antinociception as well (Sawynok 1987; Sharma et al. 1993; Lacey 1996; Malcangio and Bowery 1996; Teoh et al. 1996; Chery and De Koninck 2000; Iyadomi et al. 2000; Yang et al. 2001).

Interestingly, GABA and glycine are both found in the same spinal synaptic vesicles (Malcangio and Bowery 1996; Chery and De Koninck 2000). Consequently they are likely to be released from the same neurones within the superficial dorsal horn and it is possible that these two inhibitory neurotransmitters act as co-transmitters at these sites (Todd 1990; Todd and Sullivan 1990; Todd et al. 1996; Chery et al. 2000). Indeed it is believed that glycine is found in 30%, 45% and 65% of GABA containing neurones within lamina I, II and III respectively (Todd 1990; Todd and Sullivan 1990; Todd 1996). However, post-embedding immunogold studies have suggested that there are distinct differences in GABA and glycine immunoreactivity of C1 and C2 glomeruli within the superficial dorsal (Todd 1996). Glycine acts at glycine receptors to produce inhibitory effects within the dorsal horn of the spinal cord (Curtis and Johnston 1970; Game and Lodge 1975; Schneider and Fyffe 1992; Yoshimura and Nishi 1995; Todd 1996). It has been shown that block of glycine receptors within the spinal cord of the rat can reproduce a distinct allodynic-like condition. Similarly, following administration of intrathecal strychnine (a glycine receptor antagonist) in awake rats, application of an innocuous stimuli can evoke noticeable pain-like behaviours previously undetected (Beyer et al. 1985; Yaksh 1989; Sherman and Loomis 1995; Sherman and Loomis 1996).
Such studies suggest that glycine receptors play a highly influential role in the modulation of innocuous, rather than noxious, sensory processing at the spinal level (Beyer et al. 1985; Sherman and Loomis 1996).

Electrophysiological studies have demonstrated that the GABA\textsubscript{A} receptor antagonist bicuculline causes a facilitation of electrically evoked A-\textgreek{d} fibre activity in deep dorsal horn neurones, recorded from normal rat models (Reeve et al. 1998; Kontinen et al. 2001). Following peripheral nerve damage, bicuculline now also produces increases in electrically evoked C-fibre activity (Kontinen et al. 2001). Interestingly, upon application of strychnine no significant effect on the responses to innocuous or noxious stimuli was demonstrated on these deep dorsal horn neurones, suggesting that glycine receptors play a limited role in modulating sensory transmission within the spinal cord (Kontinen et al. 2001). It is thought that following nerve injury, which results in neuropathic pain conditions, there is a loss of GABAergic activity within the spinal cord which leads to the development of allodynia and hyperalgesia (Yamamoto and Yaksh 1993; Malan et al. 2002). Indeed block of GABA\textsubscript{A} receptors in the spinal cord by bicuculline simulates allodynic-like states similar to that seen following nerve injury (Yaksh 1989). Both glycine and GABA have been strongly localised within the superficial dorsal horn. Much work is focussed on the GABA\textsubscript{B} receptor in lamina I-III, and most in vivo electrophysiological studies looking at the role of GABA\textsubscript{A} and glycine receptors have recorded from deeper dorsal horn neurones. It is the aim of this chapter to elucidate the role of GABA\textsubscript{A} and glycine receptors in lamina I of the superficial dorsal horn, and to look at the changes in the role GABA\textsubscript{A} receptors play following a peripheral nerve injury.

6.2 Methods

Electrophysiological recordings in anaesthetised rats were conducted. Isolation of one neurone within lamina I enabled single recordings to be made at 3 x the C-fibre threshold value. Bending of the electrode at a 45° angle from the spinal horizontal plane, allowed deeper penetration and a larger cross-sectional area for lamina I neurones to be found as described in previous chapters. Both the glycine receptor antagonist strychnine and the GABA\textsubscript{A} receptor antagonist bicuculline were used in this study, to assess the role of glycine and GABA\textsubscript{A} receptors in the superficial dorsal horn (refer to section 2.3 for details on bicuculline and strychnine).
All drugs were tested in normal animal models, however bicuculline was also tested in animals following spinal nerve ligation of L5 and L6 nerves as described by Kim and Chung (Kim and Chung 1992) as well as sham operated control models. Due to time restrictions, experiments using strychnine on neuropathic and sham operated animals were not possible. All drugs were applied spinally once neuronal responses were stable. Responses to electrical and natural stimuli were followed for 40-60 minutes. Results were taken as the maximal effect seen within this time frame. Three doses of bicuculline were used in this study (0.5, 5 and 50 μg), and both the electrically evoked responses and mechanical and heat evoked responses of lamina I neurones were examined. The neurones used in this set of experiments had similar response properties to those recorded in previous characterisation studies. In the bicuculline study, the full dose range was tested in a total of 8 different lamina I neurones selected for the purpose of this study in normal animal models. In normal animals, lamina I neuronal responses to electrically evoked, mechanical (von Frey 9 & 30g) and thermal (32 & 45°C) stimuli were measured. These were presented as mean % control values. The response to 32°C was deemed a neutral temperature, and was thus used as a representative of the mechanical response of lamina I neurones to the water jet. Responses to 32°C were subtracted from the response to 45°C to give an accurate representation of the response of lamina I neurones to a noxious thermal stimuli. In sham and SNL operated animals, the responses of lamina I neurones to electrical, mechanical and thermal stimuli were tested, although a much wider range of stimuli were applied (4, 35, 40, 45, 48, 50 °C, 1,5,9,12,15,30,75,125g and brush). As bicuculline elicited pronounced effects in the normal animal model group, it was decided that the effects of bicuculline in the sham and SNL operated groups, would be better examined over a larger range of stimuli. The time taken to test this fuller stimuli range meant tests were performed every 20 minutes in SNL and sham models, as opposed to 10 minutes in the normal group. Maximal responses to both thermal and mechanical stimuli were presented as raw data values, rather than mean % control values. This range of stimuli was chosen and presented in line with recent experiments performed in this lab and the responses to 32°C were not subtracted from the more noxious thermal stimuli (Suzuki et al. 2002). The mechanical component that this response elicited was taken into account when analysing these results.

In sham and SNL operated groups, a total of 9 lamina I neurones were examined (5 in SNL animal groups and 4 in sham groups) for their electrical, mechanical and
thermal responses. Lamina I receptive field areas were mapped onto the hindpaw pre-drug application and the receptive field area was also mapped at 20 minutes post-drug application.

Paired t-tests were used to test the statistical significance of the dose related effects of all three drugs used and unpaired t-tests were used to compare statistically significant differences between drug effect on different animal groups. Bonferroni corrections for post-hoc statistical analysis were used when performing paired t-tests to account for multiple comparisons made to the pre-drug control values.

6.3 Results

6.3.1 GABA<sub>A</sub> receptor antagonists

Mean depths of lamina I neurones in normal animals were 466±66μm using a bent electrode (45°). Using simple trigonometry described in section 2.2 this meant lamina I neurones with depths of approximately 329±47μm were found. Mean C-fibre thresholds for neurones recorded were 2.09±0.3mA. Mean depths of lamina I neurones in sham animals were 450±76μm using a bent electrode (45°). Using simple trigonometry described in section 2.2 this meant lamina I neurones with depths of approximately 318±54μm were recorded from. Mean C-fibre thresholds for neurones recorded were 2.5±0.3mA. Likewise mean depths of lamina I neurones in SNL operated animals were 275±25μm using a bent electrode (45°). Using simple trigonometry this meant lamina I neurones with depths of approximately 194±18μm, were recorded. Mean C-fibre thresholds for neurones recorded were 2±0.4mA. The full dose range of Bicuculline was then tested on these responses in all neurones tested. Unfortunately time restrictions meant that a larger, and thus more predictive population of animals (and thus neurones) were not tested in sham and SNL models. However, it was felt that the results presented are largely indicative of the actions Bicuculline elicits in rat lamina I neurones in sham and SNL animal models.

Overall, in normal animals, C-fibres control values averaged 190 ±10 spikes and Aδ fibre values averaged 27±4 spikes (See Graphs 6.3.1.i & ii and refer Table 6.3.1.a). A restricted range of natural stimuli was initially applied to lamina I neurones, in order to avoid sensitisation. Despite this, lamina I neurones recorded in these normal animals were loosely categorised as nociceptive-specific (60%) and WDR (40%)
based on their response to von Frey 9 (innocuous) and von Frey 30 (noxious) as well as thermal stimuli. The lamina I neuronal responses to noxious thermal stimuli (45°C-32°C) were 121 ± 32 spikes and the response to von Frey 9 was 59 ± 18 spikes and 143 ± 42 spikes for von Frey 30 (see table 6.3.1.b). Spinal bicuculline had a small effect on A-β, input and reduced wind-up responses in lamina I neurones (See Graphs 6.3.1.i & iv). However, there was a minor reduction at the lowest dose of C-fibre and post-discharge responses to between 65 – 81 % of pre-drug control values (See Graphs 6.3.1.ii). Interestingly, there was also a large significant facilitation of the A-δ-evoked electrical responses (to 169±39% of pre-drug control values, 5μg and 279±69% of pre-drug control values, 50 μg, p=0.0125) (See Graph 6.3.1.i). Reduced wind up graphs in normal animals demonstrate very little effect of bicuculline in these lamina I neurones (See Graph 6.3.1.iv).

Bicuculline (0.5, 5 and 50 ug) also resulted in a facilitation of lamina I neurones to von Frey 30 (5 μg and 50 μg, 173 – 277% of pre-drug control values, with statistical significance shown P=0.0005 at 5 μg) (See Graph 6.3.1.v). Bicuculline also resulted in a facilitation of von Frey 9 responses in lamina I neurones (between 135 – 308 % of pre-drug control values, 0.5-50 μg, P=0.0065 at 0.5 μg only) (See Graph 6.3.1.v). Bicuculline had no obvious or significant effects on the receptive field areas of lamina I neurones (See Graph 6.3.1.iii). However, application of bicuculline appeared to reduce the responses of lamina I neurones to heat (Graph 6.3.1.vi). The two top doses (5 and 50μg) resulted in an inhibition compared to pre-drug control values (5 and 50 μg; 9-33% of pre-drug controls for 45°C-32°C, with statistical significance P=0.0047 at 50μg). However, without accommodating for the mechanical component of the heat evoked response, one can see there is less of a reduction in the noxious heat response (see Table 6.3.1.b). Thus from these studies, there is a clear GABA_α control of A-δ inputs. This is also manifest as an increase in both 9 and 30g von Frey stimuli after block of this receptor, strongly suggesting that A-δ fibres mediate these low to moderate mechanical inputs and an active GABA_α control of these modalities.

Bicuculline (0.5, 5 and 50 ug) had pronounced effects on sham and SNL operated lamina I neuronal response properties (Refer to Table 6.3.1.c). Lamina I input (50 μg; 216±62% & 170±108% in sham & SNL respectively) and A-δ fibre (50 μg; 175±15% & 283±75% in sham & SNL respectively) responses were both facilitated by bicuculline in both animal groups (Graphs 6.3.1.vii & ix). Facilitation of the input response was less pronounced in the SNL animal group compared to sham lamina I neuronal responses, yet there was a larger facilitatory effect in SNL lamina I A-δ fibre
response values than in sham lamina I neurones. Small and insignificant effects on C-fibre and A-β responses were noted in the lamina I response values of both animal groups (See Graph 6.3.1.viii & x).

Interestingly, bicuculline had confusing effects on the post-discharge and XS-spike responses, exerting both facilitation and inhibition (See Graph 6.3.1.xi & xii). However, a large facilitatory effect was noted on the post-discharge response in sham operated lamina I neuronal responses (5µg; 251±148%) and in SNL operated lamina I neuronal responses (50 µg; 232±116%). The large error bars suggest that these responses were highly variable between the different lamina I neurones recorded, thus suggesting a larger population would be required to correctly decipher the effect of bicuculline on post-discharge in lamina I neurones. The lamina I XS-spike response appeared to be reduced in sham operated animals (50 µg; 14±6%). However, a large facilitation of the lamina I XS-spike response was noted in SNL operated animals (0.5µg; 160±58%), despite the fact that the top dose resulted in a reduction of this lamina I XS-spike response (50 µg; 42% reduction). Interestingly the reduced wind up graphs demonstrate a small facilitation in the reduced wind-up of a lamina I neurone in sham operated animals and a slight decrease in SNL animal groups (See Graphs 6.3.1.xiv & xv). Receptive field areas were largely unaffected in lamina I neurones, following application of bicuculline (See Graph 6.3.1.xiii).

The lamina I neuronal control response to noxious mechanical stimuli was far greater in SNL operated animals than sham operated animals (135-278% greater at von Frey ≥12g in SNL lamina I neurones) (See Graphs 6.3.1.xvi & xvii and refer to Table 6.3.1.d). Overall, bicuculline exerted no obvious effect on the mechanical response of lamina I neurones recorded from SNL operated rats.

In sham operated animals the lamina I response to von Frey 5g and above (which corresponds to both the innocuous and noxious range), appeared to be facilitated in all doses. A large facilitation of the von Frey 30g lamina I neuronal response was particularly evident (126-291%, in 0.5, 5 &50µg/ 50µl and statistical significance P=0.0011 at 50µg/ 50µl). There was a small reduction in lamina I responses to von Frey 1-12g in SNL animals following bicuculline administration, and a slight facilitation in responses to 15-75g in SNL animals however these changes were too small to suggest any genuine role of GABA_A receptor systems. Small facilitations in the brush response were noticeable in both sham and SNL animal groups. See
Graph 6.3.1.xviii for comparison between sham and SNL effects. No statistical significance was seen between the effect of bicuculline on these two animal groups.

When observing the control responses in these two animal groups the response to noxious thermal responses were far greater in sham models compared to SNL models (138-171% greater) (See Graphs 6.3.1.xix & xx and refer to Table 6.3.1.e). This together with the enhanced responses to mechanical stimuli seen in this group may provide a substrate for the allodynia/hyperalgesia seen in behavioural tests in these animals. Overall, bicuculline exhibited very small effects on the thermal response in lamina I neurones recorded in the rat in both sham and SNL operated animal groups.

Lamina I neuronal responses to noxious thermal (48-50°C) were facilitated by bicuculline across the dose range, in both animal groups (48°C; 73-136%, 50°C; 80-156% in sham animals and 48°C; 94-182%, 50°C; 86-130% in SNL animals). The lamina I neuronal response to noxious cold stimuli (4°C) was also facilitated in both sham and SNL animal groups (158-269%, 115-279% in sham & SNL animals respectively). No statistical significance was seen between the effect of bicuculline on these two animal groups.
Graph 6.3.1.i. The effect of bicuculline on the electrical responses of lamina I neurones in the rat superficial dorsal horn.

Results mean % control. Paired t-test analysis for dose related effects.

*P < 0.05, **P < 0.01, paired t-test analysis for dose related effects.

Bonferroni corrected values, whereby a/n (n=no. of tests) sets the significance of post-drug values.
Graph 6.3.1.ii. The effect of bicuculline on the electrical responses of lamina I neurones in the rat superficial dorsal horn.
Graph 6.3.1.iii. The effect of bicuculline on the peripheral receptive field area of lamina I neurones in the rat superficial dorsal horn in normal rats.
Graph 6.3.1.iv. The effect of bicuculline on the 'reduced wind-up' response in one lamina I neurone of the superficial dorsal horn in a normal rat.
6.3.1.v. The effect of bicuculline on the response of lamina I neurones to punctate mechanical stimuli in the normal rat superficial dorsal horn. Results mean % control. Paired t-test analysis for dose related effects. *P < 0.05, **P < 0.01, paired t-test analysis for dose related effects. Bonferroni corrected values, whereby a/n (n=no. of tests) sets the significance of post-drug values.
6.3.1.vi. The effect of bicuculline on the lamina I neuronal response to thermal stimuli (45-32°C) in the naive rat. Results mean % control. Paired t-test analysis for dose related effects. *P < 0.05, **P < 0.01, paired t-test analysis for dose related effects. Bonferroni corrected values, whereby a/n (n=no. of tests) sets the significance of post-drug values.
Graph 6.3.1.vii. The effect of bicuculline on the input response in lamina I neurones in the superficial dorsal horn of sham-operated and SNL rats.
Graph 6.3.1.ix. The effect of bicuculline on the A-delta fibre response in lamina I neurones in the superficial dorsal horn of sham-operated and SNL rats.
Graph 6.3.1.x. The effect of bicuculline on the C-fibre response in lamina I neurones in the superficial dorsal horn of sham-operated and SNL rats.
Graph 6.3.1.xi. The effect of bicuculline on the post discharge response in lamina I neurones in the superficial dorsal horn of sham-operated and SNL rats.
Graph 6.3.1.xii. The effect of bicuculline on the XS-spike response in lamina I neurones in the superficial dorsal horn of sham-operated and SNL rats.
Graph 6.3.1.xiii. The effect of bicuculline on the peripheral receptive field area of lamina I neurones in the rat superficial dorsal horn in sham and SNL-operated rats.
Graph 6.3.1.xiv. The effect of bicuculline on the 'reduced wind-up' response in one lamina I neurone of the superficial dorsal horn in a sham operated rat.
Graph 6.3.1.xv. The effect of bicuculline on the 'reduced wind-up' response in one lamina I neurone of the superficial dorsal horn in an SNL rat.
Graph 6.3.1.xvi. The effect of bicuculline on lamina I neuronal responses to peripherally applied punctate mechanical stimuli in sham operated rats.

Results mean % control. Paired t-test analysis for dose related effects.

*P < 0.05, **P < 0.01, paired t-test analysis for dose related effects.

Bonferroni corrected values, whereby a/n (n=no. of tests) sets the significance of post-drug values.
Graph 6.3.1.xvii. The effect of bicuculline on lamina I neuronal responses to peripherally applied punctate mechanical stimuli in SNL rats.
Graph 6.3.1.xviii. Lamina I neuronal responses to peripherally applied punctate mechanical stimuli in sham operated and SNL rats and the effect of 50ug bicuculline
Graph 6.3.1.xix. The effect of bicuculline on lamina I neuronal responses to peripherally applied thermal stimuli in sham operated rats.
Graph 6.3.1.xx. The effect of bicuculline on lamina I neuronal responses to peripherally applied thermal stimuli in SNL rats.
6.3.2. Glycine receptor antagonists

The effect of strychnine (5 & 50 µg) was tested in a total of 12 neurones in normal animals alone. Mean depths of lamina I neurones were 463±36µm using a bent electrode (45°). Using simple trigonometry described in section 2.2 this meant lamina I neurones with depths of approximately 327±25µm were recorded from. Mean C-fibre thresholds for neurones recorded were 1.36±0.2mA. Time restrictions meant that strychnine was not tested in sham and SNL operated animals, as bicuculline was. The response to both thermal and von Frey filaments were presented as mean % control and like with the bicuculline study in normal animals, thermal stimuli was presented as 45 - 32°C. There was a small reduction in lamina I neuronal receptive field area following strychnine administration (77% of control receptive field area).

Overall strychnine caused little or no effect in A-β fibre and A-δ fibre-evoked responses (See Graph 6.3.2.i). Small inhibitions were observed in post-discharge (5 & 50 µg; 72±19 & 77±27%) and XS-spike-evoked (5µg; 53±21 & 50µg; 37±15) responses (See Graph 6.3.2.ii). Interestingly a facilitatory effect was evident in C-fibre (between 130±16 & 132±20% of pre-drug controls), input (5µg; 138±26% & 50µg; 184±25%, p=0.0081) evoked responses in lamina I neurones following peripheral administered electrical stimuli (See Graphs 6.3.2.i & ii). Intriguingly lamina I neuronal response to thermal stimuli, particularly in the noxious range (50µg; 161±42%) was facilitated (See Graphs 6.3.2.v &vi). The lamina I neuronal response to von Freys 9 and 30 were largely increased as well, especially in the innocuous range (50µg; 455±41%) (See Graphs 6.3.2.iv). There was also a small facilitation in the thermal response in lamina I neurones following strychnine administration, particularly evident in the noxious thermal range (50µg; 149±36% control) (See Graphs 6.3.2.vi).
Graph 6.3.2.i. The effect of strychnine on the electrical responses of lamina I neurones in the rat superficial dorsal horn. Results mean % control. Paired t-test analysis for dose related effects. *P < 0.05, **P < 0.01, paired t-test analysis for dose related effects. Bonferroni corrected values, whereby a/n (n=no. of tests) sets the significance of post-drug values.
Graph 6.3.2.ii. The effect of Strychnine on the electrical responses of lamina I neurones in the rat superficial dorsal horn.
Graph 6.3.2.iii. The effect of Strychnine on the peripheral receptive field area of lamina I neurones in the rat superficial dorsal horn.
Graph 6.3.2.iv. The effect of Strychnine on the mechanically evoked responses of lamina I neurones in the rat superficial dorsal horn.
Graph 6.3.2.v. The effect of Strychnine on the thermally evoked responses of lamina I neurones in the rat superficial dorsal horn.
Graph 6.3.2.vi. The effect of Strychnine on the thermally evoked responses of lamina I neurones in the rat superficial dorsal horn (45-32°C). The Graph illustrates the effect of Strychnine on the heat response alone, once the mechanical component of the water jet has been discounted using.
6.4 Discussion

6.4.1. GABA<sub>α</sub> Receptor Antagonists

Studies have shown that a large proportion of Waldeyer cells within lamina I contain the glycine-receptor associated protein gephyrin and are thought to project to the parabrachial area in the brain (Puskar et al. 2001). Furthermore the majority of these gephyrin rich neurones are thought to be highly innervated by GABAergic neurones and do not contain the NK1 receptor (Puskar et al. 2001). They do, however, contain the glycine receptor subunit α1, although only a small proportion contain the glycine transporter GLYT2 (glycine receptor marker) (Puskar et al. 2001). Overall one can presume that the high threshold and small evoked responses of lamina I neurones recorded in normal animals could be due, partly, to the tonic or evoked inhibitions acting centrally to suppress the responses of lamina I neurones and curtail their receptive fields. Spinal block of GABA<sub>α</sub> receptors in normal animals results in a facilitatory effect of lamina I A-δ fibre responses, as well as innocuous and noxious von Frey response properties, which suggests a common substrate for these changes. Confusingly small reductions in lamina I post-discharge and C-fibre responses were evident in the lower dose range, as well as a substantial and significant dose related reduction in the lamina I thermal response. In addition, to potential dis-inhibitory effects of GABA, complicating analysis, the reduction in thermal stimuli may have arisen from a depolarizing block of the neurone as activity increased although no reduction in spike amplitude could be observed. Overall these findings may implicate an entirely different mechanism for the control of thermal as opposed to mechanical stimuli within the superficial dorsal horn of the spinal cord, which is worth further investigation. However, the water jet technique used to evoke thermal stimulation of lamina I neurones in this study, meant that facilitation of mechanical responses could interfere with the responses of lamina I neurones to thermal stimuli since the thermal response includes a mechanical component. If bicuculline is causing a facilitation of the innocuous mechanical response, then one may expect the response to the water jet to increase regardless of the temperature. In table 6.3.1.b it is clear that the response to 32°C has increased as the 45-32°C values are far smaller than the action potentials evoked by 45°C alone.
GABA$_A$ receptors appear to control the mechanical responsivity of lamina I neurones and this appears to be primarily mediated by A-δ fibres. Studies in the dorsal horn of the cat observed increases in background and evoked activity, particularly to low and high threshold mechanical stimulation following bicuculline application (Sorkin et al. 1998). Under the same experimental conditions, similar increases in A-δ and mechanical responses were seen in deep dorsal horn neurones (Kontinen et al. 2001). GABA therefore plays a distinct role in mediating tonic inhibitory control systems throughout the dorsal horn and this suggests that the responses of lamina I cells are suppressed by GABA$_A$ receptors. In particular, the enhanced von Frey and A-δ fibre responses confirm the frequently reported location of GABAergic synapses on A-δ fibre terminals (Bernardi et al. 1995). Thus some of the high threshold nature of lamina I cells are due to active inhibition.

Interestingly, ablation of the majority of NK1 containing lamina I neurones, using the cytotoxin saporin conjugated to substance P, reduces noxious responses to acute nociceptive stimuli (Marshall et al. 1996; Todd et al. 2000). Thus powerful reductions in the responses of deep dorsal horn neurones to both mechanical and thermal coding, wind-up, receptive field size and in behavioural studies, responses to formalin and various forms of hyperalgesia are seen (Mantyh et al. 1997; Wiley and Lappi 1999; Suzuki et al. 2002). It is likely that these behavioural changes are attributed to loss of these NK1 expressing neurones. Gephyrin rich neurones, which lack the NK1 receptor (Puskar et al. 2001), have a large density of GABAergic inhibitory inputs. Antagonism of the GABA$_A$ receptor, which results in facilitation of both the A-δ fibre and mechanical responses is likely to be via gephyrin-rich neurones (Suzuki et al. 2002). These results suggest that the two populations of neurones may play different roles in pain processing. What is not known is that there may be reciprocal connections between these two neuronal populations within lamina I and in chapter 7.0.1 I have attempted to address this.

In sham and SNL operated animals a slightly different experimental protocol was utilised for this study, in line with more recent studies performed using the same experimental procedure (Suzuki et al. 2002).
In both sham and SNL operated animals input and A-δ fibre responses were largely augmented, however in SNL operated animals the facilitation of the A-δ fibre response was greater, yet the facilitation of input less than sham operated animals. The lamina I post-discharge response was facilitated in both sham and SNL operated animals, yet this effect was quite variable as inhibitions were seen with different doses, suggesting a larger n value would be required to reach a firm conclusion. A facilitation of the XS-spike response was also evident in SNL operated animals, however this effect was also highly variable suggesting a larger population would be required to clearly decipher the role GABA<sub>A</sub> receptors play in the lamina I XS-spike response. Interestingly, block of GABA<sub>A</sub> receptors did not appear to cause any clear effect on the response to innocuous and noxious von Frey filaments, applied to the receptive field area of lamina I neurones in SNL animals. This was in complete contrast to the results seen in normal animals. Consistent with this, in sham operated animals, lamina I neuronal responses to von Frey 5g and above, were facilitated in a dose related fashion. This is consistent with the facilitatory effects exerted by bicuculline in normal animals.

In both sham and SNL operated animals the response to thermally evoked stimuli appeared to be facilitated in the noxious range (4, 48 and 50°C), although the mechanical component of the water jet has to be considered. In line with more recent studies performed using the same experimental protocol temperatures of 4, 35, 40, 45, 48 and 50°C were tested (Suzuki et al. 2002). 35°C was seen as a neutral stimulus and considered to reflect the mechanical aspect of spraying a water jet on the lamina I receptive field area. 40°C was seen as an innocuous thermal stimulus, whereas 45, 48 and 50°C were seen as noxious thermal stimuli. Interestingly, lamina I neuronal responses to 35°C were somewhat confusing to interpret in SNL animals. The neuronal response to 5μg/50μl bicuculline was a 21% reduction in the control response, yet at 50μg/50μl bicuculline the response was 123% of the control neuronal response. However, in sham operated animals the lower dose resulted in a 43% reduction in the response of lamina I neurones to 35°C, yet the top dose of bicuculline resulted in a response 199% that of the lamina I control neuronal response to 35°C.

This suggests that much of the facilitation observed in the noxious thermal response may be attributed to the mechanical aspect of the water jet stimulus, particularly in sham operated animals. In sham operated animals, lamina I responses to 40 - 50°C resulted in some facilitatory effects, but if the response to 35°C were subtracted, one
may see an inhibitory effect similar to that observed in normal animals which was largely ascribed to depolarisation block. However, in SNL operated animals, a small facilitatory effect would still be evident. Interestingly though, the facilitation of the noxious cold response was far greater than facilitation evoked by 35°C in both sham and SNL animal groups suggesting GABAergic control of noxious cold stimuli.

Overall it is obvious that the lamina I responses of sham and normal animals, to electrically and naturally evoked peripheral stimuli are very similar. However, the larger stimulus range analysed in sham operated animals provide a greater insight as to the role GABA_A receptors play in lamina I neuronal responses. In SNL operated animals, stark differences were seen in the GABAergic control of lamina I neuronal responses. Lamina I A-δ fibre responses were facilitated in SNL animals to a greater extent than sham operated lamina I neurones. However when compared to the A-δ fibre response of lamina I neurones in normal animals the effect is very similar.

It may be that given a greater population this difference would not be so large. Small inhibitions of post-discharge responses in lamina I neurones recorded in normal animals were evident, however bicuculline resulted in a large facilitation of the post-discharge response in sham and SNL operated animals. This was far more consistent in lamina I neurones following peripheral nerve injury, suggesting a greater role for GABA_A receptors on neuronal hyperexcitability in SNL animal groups. These findings confirm suggestions from previous studies using similar experimental protocols, which observe a greater GABAergic role in deep dorsal horn neuronal responses following nerve injury (Kontinen et al. 2001). However unlike in deep dorsal horn neurones, there was no clear increase in C-fibre evoked activity in lamina I neurones following nerve injury suggesting that compensatory increases in GABAergic tone following increased neuronal excitability in deep dorsal horn neurones do not arise in lamina I (Kontinen et al. 2001).

Interestingly, characterisation studies (chapter 3.0) analysing the responses of lamina I neurones in both normal and SNL animal groups suggest no such changes in neuronal excitability occurs between these two animal groups, therefore it would be unlikely that there would be any compensatory increases in GABAergic tone in lamina I neurones. However, SNL lamina I neurones generally exert larger responses than sham lamina I neurones at 30 and 75g von Frey stimuli as seen in chapter 3.0 (see Graph 3.3.vi), however this is not seen at any other von Frey
stimulus. However a loss of GABAergic neurones following nerve injury, particularly evident in the superficial dorsal horn, would mean that block of these receptors would not have such a large effect compared to the naive animals (Moore et al. 2002). This idea is supported by my findings that a block of GABA\textsubscript{A} receptors exerted no effect on the von Frey responses following peripheral nerve injury. Furthermore, control responses were far greater in the SNL animal group in this small population of lamina I neurones, than in the sham operated group suggesting a loss of inhibition or increase in excitability in superficial spinal cord. The characterisation studies (chapter 3.0) demonstrated small and insignificant differences between the mechanical responses of lamina I neurones before and after nerve injury. 4/5 (80%) neurones used in SNL animals for this bicuculline study were NS compared to 63% in all lamina I neurones characterised in SNL groups. Due to the small group of lamina I neurones tested in this study it was hard to decipher a clear difference between the effect of bicuculline on WDR as compared to NS neurones. Many previous studies have suggested a selective loss of GABA immunoreactivity in the spinal cord following peripheral nerve injury, partly attributed to a down regulation in GABAergic synthesis (Castro-Lopes et al. 1993; Eaton et al. 1999a; Eaton et al. 1999b; Polgar et al. 2003). This has been shown to occur in the superficial dorsal horn of isolated spinal cords in various models of nerve injury (Moore et al. 2002). Interestingly, quantitative stereological analysis studies show no correlation between thermal hyperalgesia and the selective loss of GABAergic and glycinergic neurones following chronic nerve constriction, consistent with the findings in this study which show no effect of lamina I thermal responses following block of GABA\textsubscript{A} in neuropathic rats (Polgar et al. 2003). However, most studies do report significant correlations between the inhibitory amino acids GABA and glycine and the manifestation of alldynia following nerve injury (Sivilotti and Woolf 1994). Overall these findings suggest that there is a loss of GABAergic inhibitory control in lamina I neurones following peripheral nerve injury, which may manifest itself as alldynia and other neuropathic pain related behaviours due to a remaining increase in excitability within the spinal cord and CNS. Increased excitability is though to contribute towards central sensitisation and as such abnormal processing of normal sensory information.
6.4.2. Glycine Receptor Antagonists

Strychnine exerted slight facilitation of C-fibre responses in normal animals, yet a small reduction in post discharge responses. None of which proved to be statistically significant. The top dose of strychnine facilitated the lamina I input response yet reduced the XS-spike response, however strychnine had no effect on the A-β and A-δ fibre responses recorded in lamina I neurones. Overall there was a small facilitation of the noxious thermal response observed at the top dose of strychnine and a tendency for facilitation of both von Frey 9 and 30g mechanically induced lamina I neuronal responses. This was particularly evident at von Frey 9, which was largely facilitated at 50μg/50μl strychnine. Overall these results suggest a tendency for glycine-mediated control of lamina I mechanical response properties, particularly in the innocuous range and a small glycine mediated inhibitory control of C-fibre, input and thermally evoked responses. The small reduction in post-discharge and XS-spike was not statistically significant, yet could suggest a distinct regulatory control different from the other response properties. Interestingly allodynia is evident in behavioural studies upon glycine block, which suggests a glycine-mediated control of mechanical response properties, following nerve injury (Yaksh 1989; Polgar et al. 2003) although it may be that this results from interactions with motor systems as well as sensory systems. Interestingly, immunohistochemical studies have revealed that glycine immunoreactive neurones are found throughout the superficial dorsal horn, predominantly as presynaptic axons in lamina III (Todd 1990). My study demonstrates that block of spinal glycine receptors does have an effect on lamina I neuronal response properties, largely validating the observation that a population of lamina I neurones contain this receptor and that these may be via pre-synaptic mechanisms as indicated by the input response. Interestingly, the majority of glycicnergic neurones have been shown to receive contact from low threshold myelinated cutaneous primary afferents (Todd 1990).

Whilst no effect on A-β, and indeed A-δ fibres was evident, a large facilitation of innocuous von Frey responses suggests a large glycine mediated control of low threshold mechanical responses in line with these immunohistochemical observations (Todd 1990). A small facilitation in the noxious thermal responses of lamina I neurones following application of strychnine suggests a glycicnergic control of the noxious thermal coding of lamina I neurones. Overall these results suggest that both GABA and glycine play a substantial role in mediating nociceptive transmission in the superficial dorsal horn. Lamina I A-δ fibre and mechanical response properties
appear to be largely mediated by GABA\textsubscript{A} receptors, and following nerve injury it appears that the role GABA\textsubscript{A} plays in mediating A-\textdelta fibre responses is increased, although loss of inhibitory control on mechanical response properties induced by peripheral nerve injury, mean that block of GABAergic receptors no longer has a facilitatory effect. Therefore, following nerve injury certain changes in the neurochemistry of the superficial dorsal horn mean that GABA plays an enhanced role in mediating the transfer of sensory information from the periphery to the spinal cord and thus higher centres of the central nervous system. Overall lamina I neuronal responses appear to be partly mediated by glycinergic controls. This is demonstrated by the tendency for facilitatory effects exerted by Strychnine on C-fibre, input, thermal and mechanical (especially innocuous mechanical) response modalities. This suggests that modulation by pre-synaptic intrinsic inhibitory controls may contribute towards the high-threshold nociceptive specific nature of lamina I neurones.
### 6.5 Tables

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<th>Confidence Interval</th>
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Table 6.2.1 showing Bonferroni corrections for $P$ values at 95% and 99% confidence intervals.

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<th>50 ug</th>
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<td>12±4 (n=8)</td>
<td>110±14</td>
<td>123±20</td>
<td>116±25</td>
</tr>
<tr>
<td>A-beta</td>
<td>113±16 (n=8)</td>
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<td>86±12</td>
<td>87±14</td>
</tr>
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<td>A-delta</td>
<td>27±4 (n=8)</td>
<td>78±26</td>
<td>169±39</td>
<td>279±69*</td>
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<tr>
<td>C-fibre</td>
<td>190±10 (n=8)</td>
<td>81±10</td>
<td>99±8</td>
<td>104±25</td>
</tr>
<tr>
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<td>101±29</td>
<td>83±41</td>
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Table 6.3.1.a. Lamina I responses to electrical stimuli in normal animal models and the effect of bicuculline on these lamina I response properties. Results are means expressed as % control. Results are mean % control in normal animals with the raw values (spikes) given in the first column (control). Paired t-test analysis for drug effects. Bonferroni corrections for posthoc analyses utilised, therefore statistical significance values set accordingly. *$P<0.05$, **$P<0.01$, paired t-test analysis for drug effects. Bonferroni corrected values, whereby $\alpha/n$ (n=no. of tests) sets the significance of post-drug values.

<table>
<thead>
<tr>
<th>Response</th>
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<th>5 ug</th>
<th>50 ug</th>
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<td>277±96 (n=8)</td>
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Table 6.3.1.b. Lamina I responses to naturally evoked mechanical and thermal stimuli in normal animal models and the effect of bicuculline on these lamina I response properties. Results are means expressed as % control. Results mean % control in normal animals. Paired t-test analysis for drug effects. Bonferroni corrections for posthoc analyses utilised, therefore statistical significance values set accordingly. *$P<0.05$, **$P<0.01$, paired t-test analysis for drug effects. Bonferroni corrected values, whereby $\alpha/n$ (n=no. of tests) sets the significance of post-drug values.
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Table 6.3.1.c. Lamina I responses to electrical stimuli in sham and SNL operated animal models and the effect of bicuculline on the lamina I response properties in these different animal models. Results are means expressed as % control. Results mean % control. Paired t-test analysis for dose related effects. Unpaired t-test analysis for comparative effects between each animal group. Bonferroni corrections for posthoc analyses utilised, therefore statistical significance values set accordingly. *P < 0.05, **P < 0.01, paired t-test analysis for dose dependant effects. Bonferroni corrected values, whereby Δ/n (n=no. of tests) sets the significance of post-drug values.
<table>
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<td>185±59</td>
</tr>
<tr>
<td></td>
<td>SNL</td>
<td>186±37</td>
<td>196±25</td>
<td>197±31</td>
<td>204±41</td>
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Table 6.3.1.d. Lamina I responses to naturally evoked mechanical stimuli in sham and SNL operated animal models and the effect of bicuculline on the lamina I response properties in these different animal models. Results shown as raw data values. Paired t-test analysis for drug effects. Unpaired t-test analysis for comparative effects between each animal group. Bonferroni corrections for posthoc analyses utilized, therefore statistical significance values set accordingly. *P < 0.05, **P < 0.01, paired t-test analysis for drug effects. Bonferroni corrected values, whereby α/n (n=no. of tests) sets the significance of post-drug values.
<table>
<thead>
<tr>
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<tbody>
<tr>
<td>4</td>
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Table 6.3.1.e. Lamina I responses to naturally evoked thermal stimuli in sham and SNL operated animal models and the effect of bicuculline on the lamina I response properties in these different animal models. Results are means expressed as raw data values. Paired t-test analysis for drug effects. Unpaired t-test analysis for comparative effects between each animal group. Bonferroni corrections for posthoc analyses utilised, therefore statistical significance values set accordingly. *P < 0.05, **P < 0.01, paired t-test analysis for drug effects. Bonferroni corrected values, whereby a/n (n=no. of tests) sets the significance of post-drug values.
### Response Control

<table>
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<tr>
<td>A-delta</td>
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</tr>
<tr>
<td>C-fibre</td>
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<td>72±19 (n=9)</td>
<td>77±27 (n=9)</td>
</tr>
<tr>
<td>Post Discharge</td>
<td>120±26</td>
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<td>37±15 (n=9)</td>
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<td>173±52 (n=12)</td>
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6.3.2.a. Lamina I responses to electrical and naturally evoked stimuli in normal animal models and the effect of strychnine on these lamina I response properties in these different animal models. Results are means expressed as % control. Results mean % control in normal animals. Paired t-test analysis for dose related effects. Unpaired t-test analysis for comparative effects between each animal group. Bonferroni corrections for posthoc analyses utilised, therefore statistical significance values set accordingly. *P < 0.05, **P < 0.01, paired t-test analysis for dose related effects. Bonferroni corrected values, whereby α/n (α=no. tests) sets the significance of post-drug values. * or ** used as above to denote significance at related confidence levels.
CHAPTER 7

THE ROLE NK1 AND CGRP RECEPTOR EXPRESSING NEURONES PLAY IN LAMINA I OF THE RAT SUPERFICIAL DORSAL HORN AND DESCENDING CONTROL EXERTED BY THE 5HT₃ RECEPTOR
7.0 The Role NK1 and CGRP Receptor Expressing Neurones Play in Lamina I of the Rat Superficial Dorsal Horn and Descending Control Exerted by the $5\text{HT}_3$ Receptor.

7.1 Introduction

There are multiple ascending and descending pathways from the spinal cord to the brain, which enable sensory information to be both perceived, acted upon and regulated. The superficial dorsal horn of the spinal cord comprises the main output centre for ascending neurones emerging from the spinal cord to the brain, projecting to areas such as the parabrachial nucleus, thalamus, medulla and periaqueductal grey (Craig and Burton 1985; Craig 1991; Bernard et al. 1995; Craig 1995; Bester et al. 2000; Craig 2002; Andrew et al. 2003). The spinal cord however, also receives inputs from supraspinal centres via descending pathways of various origins. These descending pathways can be either facilitatory (DF’s) or inhibitory (DI’s), therefore modulating sensory processing in different ways (Craig 2002; Millan 2002). Nociceptive information from the periphery is transmitted primarily by nociceptive specific neurones, terminating predominantly in the superficial dorsal horn onto other dorsal horn neurones (Millan 1999; Millan 2002). These dorsal horn neurones are subject to descending regulation and this is important in understanding the modulation of pain processing (Hunt and Mantyh 2001; Craig 2002; Millan 2002).

Descending controls have a variety of other modulatory functions, among which, regulation of motor and autonomic activity are examples (Craig 2002; Millan 2002).

Many structures within the brain send descending projection neurones to the spinal cord for the purpose of regulating spinal neuronal activity transmitted from the periphery. Various hypothalamic regions, including the paraventricular nucleus and arcuate nucleus project directly to the dorsal horn (Millan 2002). The parabrachial nucleus and the periaqueductal grey which are located lower down in the midbrain, have direct projections to the superficial dorsal horn whereby noxious information transmitted from the periphery can be intensely modulated (Hunt and Mantyh 2001; Millan 2002). The rostroventromedial medulla (RVM), nucleus tractus solitarius (NTS), locus coeruleus (LC) and dorsal reticular nucleus, located in the medulla, send descending projections to modulate dorsal horn neuronal activity (Mason 1999; Millan 2002).
It is thought the dorsal reticular nucleus is involved in the emergence of 'Diffuse Noxious Inhibitory Controls', and it is also believed that the NTS predominantly regulates the transmission of visceral information (Millan 2002). The RVM, particularly the nucleus raphe magnus (NRM) located within the surrounding RVM of the medulla, has direct descending projections to both the superficial and deep dorsal horn (Mason 1999; Millan 2002). See Diagram 7.1a. It is now hypothesised that 'ON' or 'OFF' cells in the NRM may mediate the transmission of noxious information by either descending excitatory or inhibitory mechanisms (Aimone et al. 1987; Chiang et al. 1994; Millan 2002). Interestingly, lesioning the rostral medial medulla attenuates the development of thermal hyperalgesia in models of secondary hyperalgesia but not primary hyperalgesia illustrating the extremely specific functions of descending modulation that stem from different areas of the brain (Urban et al. 1999). It is thought that the NRM transmits neuronal information predominantly via 5HT, which acts on 5HT_{1A,1B,2,3} receptor subtypes to regulate dorsal horn neuronal activity (Roberts 1984; Chiang et al. 1994; Ali et al. 1996; Millan 2002).

Noradrenergic and serotonergic systems are widely implicated in the majority of descending transmission from the brain to the spinal cord. The dorsal raphe nucleus in the brainstem sends serotonergic projections to the spinal cord, as does the NRM which is thought to be the main origin of serotonergic inputs to the spinal cord (Chiang et al. 1994; Millan 2002). Importantly, it appears that the superficial dorsal horn contains the largest amount of serotonergic fibres, with 5HT receptors located on primary afferent terminals and primary neurones (Roberts 1984; Millan 2002). Overall, it is proposed that serotonergic descending pathways inhibit the transmission of nociceptive information in the spinal cord (Roberts 1984; Chiang et al. 1994; Millan 2002). Ionotropic 5-HT_3 receptors, densely expressed on C-fibre terminals within the superficial dorsal horn, can be separated into two subunit populations, 5-HT_{3A} and 5-HT_{3B} receptors. Both receptors have facilitatory pronociceptive effects on the neurones that express them, along with 5HT_{2A} and 5-HT_{4} receptors which are often located on the same neuronal populations (Millan 2002). These receptors are located predominantly at primary afferent fibre terminals within the spinal cord, whereby they facilitate the release of substance P (SP) in the dorsal horn (Ali et al. 1996; Oyama et al. 1996; Millan 2002) and likely, the co-existing glutamate.
Substance P is a peptide located throughout the central nervous system. It is found predominantly in A-δ and C-fibres, which convey high threshold noxious stimuli (Otsuka and Yanagisawa 1990; Hunt and Mantyh 2001). Interestingly, there are two main C-fibre populations which have been categorised based on their substance P content (Hunt and Mantyh 2001). One group express P2X3 (purine receptor) and GDNF receptors as well as the IB4-lectin-binding site, the other group both synthesise and express the peptides substance P and CGRP, as well as TrkA (NGF receptor) (Hunt and Mantyh 2001). Interestingly, it is thought that these peptide-containing neurones terminate more superficially within the spinal cord, whereas the IB4 positive group of C-fibres project predominantly to the substantia gelatinosa in lamina II (Hunt and Mantyh 2001). It is thought only a small proportion of lamina I neurones contain the NK1 receptor for substance P, however a large proportion of spinoparabrachial neurones (80%) express NK1 receptors (Puskar et al. 2001; Ikeda et al. 2003). Studies performed in monkey spinothalamic lamina I neurones have shown that NK1 receptors for substance P are located predominantly in fusiform and multipolar cell types. This suggests that a large proportion of nociceptive neurones within lamina I are NK1 positive (Yu et al. 1999). Retrograde labelling studies have shown that the majority of NK1 expressing neurones in the superficial and deep dorsal horn, project to the contralateral lateral reticular nucleus (>90%) (Todd et al. 2000). Many also have axonal projections within the lateral parabrachial area (>60%) (Todd et al. 2000).

The majority of studies investigating the role of substance P responsive neurones in the development of various pain conditions, have utilised a model using the selective cytotoxin saporin which is conjugated to substance P. This selectively ablates NK1 expressing neurones in the superficial dorsal horn, through internalization of the ligand-receptor complex therefore allowing the neurotoxin saporin access into the neurone (Mantyh et al. 1997; Nichols et al. 1999). NK1 receptors are internalised in the majority of dorsal horn neurones following intense peripheral stimulation, by exploiting this effect NK1 expressing neurones can be killed by internalisation of this cell toxin (Todd et al. 2000). Substance P is involved in the development of central hyper-excitability and hypersensitivity to noxious stimuli following repeated high threshold peripheral stimulation (De Felipe et al. 1998). Mutant mice lacking the NK1 receptor do not exhibit a wind-up response following noxious stimulation and furthermore, they cannot code the intensity of nociceptive reflexes (De Felipe et al. 1998).
Indeed, it has been shown that application of increasing thermal stimulus intensities evokes a graded release of substance P within the spinal cord (Allen et al. 1997). Furthermore, Fos expression studies show that lamina I NK-1 positive neurones are able to encode cutaneously administered noxious cooling intensities efficiently (Doyle and Hunt 1999a). It is also evident from studies performed in gene knock-out mice that NK1 receptors are important in the manifestation of responses elicited by noxious stimuli (King et al. 2000; Bester et al. 2001). The majority of studies utilising the substance P-saporin model have been extremely informative and have allowed a great insight into the role substance P plays in the processing and development of pain behaviours. Loss of NK1 expressing neurones in lamina I/III of the spinal cord produces a reduction of thermal hyperalgesia and mechanical allodynia in models of neuropathic and inflammatory pain (King et al. 1997; Nichols et al. 1999). Similar studies also demonstrated a selective loss of hyperalgesia in NK1 deficient animal models (Mantyh et al. 1997). It is also evident that animals lacking NK1 expressing neurones in the superficial dorsal horn exhibit lower responses to intraplantar capsaicin (Khasabov et al. 2002). Furthermore, nociceptive neurones in these animal models do not exhibit a wind-up response consistent with findings from the genetically modified animals mentioned above (Khasabov et al. 2002). Immunostaining studies have revealed that peripheral stimulation of different intensities is coded very efficiently by lamina I NK1-positive neurones, quite unlike deeper dorsal horn neurones (Doyle and Hunt 1999b). However, inflammation is sufficient to evoke large increases in Fos expression in NK1 positive neurones in the deep dorsal horn (Doyle and Hunt 1999b). These findings support the fact that NK1 positive neurones within the superficial dorsal horn are involved in intensity coding and perception, yet deeper dorsal horn neurones distinguish the stimulus modality. Interestingly it was found that deeper NK1 positive dorsal horn neurones can identify the origin of noxious stimuli as well (Doyle and Hunt 1999b).

CGRP is another peptide found throughout the spinal cord, including the dorsal horn, of man and many other species (Gibson et al. 1984). CGRP is thought to be confined to small and intermediate sized cells within the DRG (Gibson et al. 1984). CGRP is also often co-localised with substance P in small DRG cells (Gibson et al. 1984). In the primate, CGRP immunostaining studies have demonstrated fibres within a dense plexus in lamina I, IIo, V and the central canal (Carlton et al. 1987; Carlton et al. 1988).
These studies suggested that the intervaricose fibres containing CGRP were mostly small unmyelinated or thinly myelinated fibres, consistent with C- and A-δ fibre populations in the lumbar spinal cord (Carlton et al. 1987; Carlton et al. 1988). It is also of interest that CGRP immunoreactive terminals were consistently presynaptic to other primary afferent terminals in such studies, often those also containing CGRP (Carlton et al. 1987; Carlton et al. 1988). Interestingly in the substantia gelatinosa of the guinea pig lumbar spinal cord, post-embedding double immunogold labelling as well as pre-embedding PAP studies have found that nociceptive primary afferent nerve endings can transmit painful stimuli to intrinsic inhibitory interneurones and can therefore function to regulate high threshold nociceptive information by a postsynaptic neuronal circuit (Hiura et al. 1998). In the rat lumbar dorsal horn, application of CGRP has been shown to potentiate the excitatory role that substance P plays on noxious evoked neuronal activity. This effect illustrates the excitatory role CGRP plays in the processing of high threshold and nociceptive information in the dorsal horn of the rat (Biella et al. 1991). Furthermore, in vitro studies have shown that CGRP can potentiate the release of substance P from primary afferent terminals following infusion of capsaicin in slices taken from the dorsal half of the spinal cord, as well as induce nociceptive transmission evoked by noxious mechanical stimuli (Oku et al. 1987). Interestingly, following nerve injury reductions in CGRP and substance P have been demonstrated in the rat spinal cord (Na et al. 2001).

Descending influences in lamina I have a large impact on the transmission of nociceptive information within the dorsal horn of the spinal cord. It is clear that 5HT is one of the prominent neurotransmitters within descending neurones and evidence suggests that 5HT₃ receptors are abundant within lamina I. The majority of ascending pathways stem from NK1-positive neurones within the superficial dorsal horn and these receptors play a prominent role in the development of nociceptive behaviours. It was thus thought vital that the effect of 5HT₃ dependent descending pathways following selective ablation of NK1 positive neurones be tested. In a similar study performed in SP-SAP animal models changes in mechanical and thermal stimulus coding, peripheral receptive field size and central sensitization of lamina V dorsal horn neurones evoked by ablation of NK1 expressing neurones were reversed following block of 5HT₃ receptors in the spinal cord (Suzuki et al. 2002).
It is thus the aim of this chapter to observe the effect of selective ablation of lamina I NK1 receptors on the neuronal response of lamina I neurones following application of the 5HT3 receptor antagonist Ondansetron. Furthermore, I used a rat calcitonin gene related peptide (CGRP) receptor antagonist CGRP8-37 to gauge the role of the peptide in the responses of these lamina I neurones. Interestingly ondansetron is utilised in the clinic as an anti-emetic in both chemotherapy and opioid treated cancer and post-surgical patients (Deegan 1992; Roila et al. 1997; Chung et al. 1999).

7.2 Methods

In this set of experiments spinal application of the 5HT3 receptor antagonist ondansetron (Zofran) and the rat calcitonin gene related peptide (CGRP) receptor antagonist CGRP8-37 were tested on spinal lamina I neurones. Ondansetron was obtained from Glaxowellcome, U.K and was dissolved in 0.9% saline and kept in glass vials stored at 4°C. Ondansetron was tested in two animal groups. One group (SP-SAP's) had been treated with intrathecally administered ribosome-inactivating protein saporin conjugated to substance P, 28 days prior to electrophysiology. This procedure was performed in order to selectively ablate substance P receptor (NK1) expressing lamina I spinal neurones as described by (Mantyh et al. 1997). The second group of animals (SAP's) had been treated 28 days earlier with intrathecally administered ribosome-inactivating protein saporin, without conjugated substance P. This second group acted as a control. Ondansetron was not tested on a normal group of animals – this has previously been studied (Suzuki et al. 2002) and due to the procedure, the SAP control group is the true control for the intrathecal injection and size of animal. These procedures were kindly performed by Dr Rie Suzuki, Dr Wahida Rahman & Dr. Idil Maie. CGRP8-37 was obtained from Tocris Cookson Ltd. The drug was dissolved in saline 0.9% and stored in separate aliquots at -20°C. CGRP8-37 was tested in normal animals as time restrictions meant that further analysis in other animal models was not possible. However, these results are a good indication of the role of CGRP and its receptor in spinal lamina I neuronal processing of sensory and noxious information.

All rats were anaesthetised prior to exposure of the lumbar spinal cord and electrophysiological testing. One lamina I neurone was used per experiment and the effect of spinally administered drugs were examined, following suitable stable controls. The effect of ondansetron (10, 50 & 100 μg/ 50 μl) was tested in SP-SAP
and SAP animal models. In these experiments, the response to peripheral electrical stimulation, von Frey filaments (1,5,9,12,15,30,75,125g) and thermal stimuli (4,35,40,45,48,50°C) applied to the peripheral receptive field area was tested every 20 minutes. The receptive field area to a pinch stimulus was mapped on a standard paper template and recorded prior to drug and also at each 20 minute time point thereafter. Each dose of drug was followed for 60 minutes in total, in order to achieve the maximal drug effect. CGRP was tested in normal animals alone. The response of lamina I neurones to peripheral electrical stimuli was tested before and after CGRP8-37 application. CGRP8-37 was applied directly to the spinal cord in cumulative doses of 0.5, 5, 50 µg/ 50 µl, and each dose was followed for 40-60 minutes post application. The short acting nature of this peptide antagonist meant that it was more appropriate to test the responses of spinal lamina I neurones every 10 minutes. Therefore in this set of experiments, the effect of the CGRP receptor antagonist on naturally evoked responses was not examined.

To demonstrate the effect of each drug tested, maximal effects were taken within each dose range, following electrical stimuli. These maximal effects were calculated as a % of the control value and averaged. For naturally evoked stimuli, responses were measured as raw data values and graphs were presented accordingly. However, maximal effects were always taken at the 20 minute time as this time point tended to consistently be when the peak effect was seen. Receptive fields were calculated via weight analysis, as described in the methods section 2.0. Paired t-tests were used to compare the drug effect to the pre-drug control values. Unpaired t-tests were used to compare the drug effect between animal populations. Bonferroni corrections were made for post hoc analysis of data. A non-parametric test (Mann Whitney) was also performed to test the drug effect between animal populations.
7.3 Results

7.3.1. 5HT3 receptor antagonist in the substance P saporin NK1 receptor ablated model

The mean C-fibre threshold for SP-SAP lamina I neurones recorded in this study was 1.95±0.3 mA. Mean C-fibre threshold in SAP lamina I neurones was 1.83±0.3 mA. Mean depth of SP-SAP lamina I neurones was 225±64μm, and that of SAP lamina I neurones recorded was 217±60mA using a bent electrode (45°). Using simple trigonometry described in section 2.2 this meant lamina I neurones with depths of approximately 159±45μm, and 153±42μm were recorded from these different animal models, respectively. 'N' numbers are not listed in the tables, however, n=11 were achieved for the SP-SAP study and due to restrictions in time only an n=5 SAP animals were used for the ondansetron study, however this served as a good control for the SP-SAP group. Occasionally a neurone did not exhibit sufficient control values in all response properties tested, for a pharmacological effect to be analysed and these responses were thus removed from further analysis. However all doses of ondansetron were tested on each neurone used.

The majority of lamina I electrical responses were similar in SAP and SP-SAP animal groups. XS-spike responses appeared to be smaller in lamina I neurones of SP-SAP animals compared to SAP animal groups (35% smaller) (Refer to Table 7.3.1.a), yet the A-δ fibre response was far smaller in lamina I neurones recorded in SAP animal models (65% smaller). Following application of ondansetron, similar reductions in evoked lamina I post-discharge responses (SP-SAP; 10 μg/ 50μl; 66% reduction; P=0.006 & 100 μg/ 50μl; 49% reduction; P=0.0039 and SAP; 10 μg/ 50μl; 56%; P=0.0151 reduction) were evident (See Graph 7.3.1.v), in both SAP and SP-SAP models. There was a tendency for reduction of C-fibre evoked responses in SAP models (50 μg/ 50μl; 46% reduction), whereas in SP-SAP models these responses had the tendency to be facilitated, if unaffected (100 μg/ 50μl; response 110% that of control value) (See Graph 7.3.1.iv). XS-spike evoked responses were significantly reduced by the 5HT3 receptor antagonist in SAP animal models in the lower dose range (10 μg/ 50μl; 43% reduction, P=0.0247 & 50 μg/ 50μl; 65% reduction, P=0.0096) (See Graph 7.3.1.vi). XS-spike responses in SP-SAP had the tendency for slight facilitation following ondansetron application. The reduced wind-up graphs showing the effect of ondansetron demonstrated a larger XS-spike response in SAP
animal groups and a general reduction in these responses following ondansetron application (See Graph 7.3.1.viii). Ondansetron had similar inhibitory effects on the input response in both animal models, yet a larger effect was evident in SAP groups compared to SP-SAP groups, although no significant difference was recorded between these two groups (See Graph 7.3.1.i). A significant reduction in the SP-SAP input response was evident at the lower dose range (10 μg/50μl; 46% reduction; P=0.0012). Similarly, ondansetron evoked reductions in the Aβ fibre response in both animal groups (50 μg/50μl; SAP; 44% and SP-SAP; 16% reduction), yet this was far more pronounced in SAP groups than in SP-SAP groups (See Graph 7.3.1.ii). This was particularly evident in the lower dose range, whereby a statistically significant reduction was evident (10 μg/50μl; 33% reduction; P=0.018). A statistically significant difference was seen overall between these two groups using unpaired t-test analyses (P=0.0004). However no statistical difference in individual neuronal response properties was not evident.

Ondansetron had small effects on the receptive field area of lamina I neurones (See Graph 7.3.1.vii and refer to Table 7.3.1.b). There was a small reduction in SAP receptive field area (50μg/50μl; 44% reduction) yet a small facilitation at the top dose. A small dose related reduction in receptive field area was also evident in SP-SAP animals.

Smaller lamina I neuronal responses to mechanical stimuli were evident in SAP animal groups compared to SP-SAP animal groups (Refer to Table 7.3.1.c and see Graphs 7.3.1.x & xi). This was true for the entire range and would therefore suggest that the putative NK1 expressing neurones, sampled in the SAP group, have smaller responses than the non-NK1 population. Overall reductions in the response to innocuous von Frey stimuli (>12g) were evident in both animal groups after ondansetron, with a much larger effect evident in the SAP animal groups (See Graphs 7.3.1.xii & xiii). These results vary from the section 3, which may be due to there being a smaller population of neurones used in this pharmacological study.

At all doses and mechanical forces the effect of ondansetron was diminished in the SP-SAP group. Large drug induced reductions were evident in the more noxious range (>12g), with particular reference to SAP groups in which the greatest effect
was evident (von Frey 125g; 10μg/ 50μl; 22% reduction; p=0.0211 & von Frey 125g; 100μg/ 50μl; 54% reduction; p=0.0089). Although unpaired t-tests found no significant difference between the drug effect on these two neuronal populations, Mann Whitney non-parametric tests revealed significance between the effect of Ondansetron on von Frey 1, 5 and 30g (Not marked on Graphs; 1g: 50μg/ 50 μl & 100μg/ 50 μl; P=0.0079 & P=0.0159, 5g; 50μg/ 50 μl; p=0.03 and 30g; 100μg/ 50 μl; P=0.048). Reductions in the brush response in SAP animals were also evident, but this effect was far less pronounced in SP-SAP animal groups. Thus, the NK1 population of neurones appear to determine the effectiveness of ondansetron, in keeping with the idea that these neurones are at the origins of the ascending arm of the facilitatory 5HT3 receptor mediated loop.

Overall, lamina I neuronal responses to thermal stimuli were smaller in SAP animals groups than SP-SAP animal groups (Refer to Table 7.3.1.d and see Graphs 7.3.1.xiv & xx). Similar reductions in the cold response of lamina I neurones was evident, following the top dose of ondansetron, in both SAP and SP-SAP animal groups (SP-SAP; 40% & SAP; 39% reductions). At all doses and applied temperatures the effect of ondansetron was considerably less in the SP-SAP group, compared to the SAP controls although reductions were evident in both groups. Larger relative reductions in innocuous thermal stimuli were evident in the SAP animal groups, as compared to the SP-SAP animal groups. This was also evident in response to noxious thermal stimuli in SAP animal groups. Reductions in SAP groups were evident at the lower dose range of ondansetron (50°C; 10μg/ 50μl; 22% reduction).
Graph 7.3.1.1. The effect of ondansetron on the input response in lamina I neurones in the superficial dorsal horn of saporin and substance P-saporin treated rats. Results mean % control. Paired t-test analysis for dose related effects.

*P < 0.05, **P < 0.01, paired t-test analysis for dose related effects.
Bonferroni corrected values, whereby a/n (n=no. of tests) sets the significance of post-drug values.
Graph 7.3.1.ii. The effect of ondansetron on the A-beta fibre response in lamina I neurones in the superficial dorsal horn of saporin and substance P-saporin treated rats.
Graph 7.3.1.iii. The effect of ondansetron on the A-delta fibre response in lamina I neurones in the superficial dorsal horn of saporin and substance P-saporin treated rats.
Graph 7.3.1.iv. The effect of ondansetron on the C-fibre response in lamina I neurones in the superficial dorsal horn of saporin and substance P-saporin treated rats.
Graph 7.3.1.v. The effect of ondansetron on the post discharge response in lamina I neurones in the superficial dorsal horn of saporin and substance P-saporin treated rats. Results mean % control. Paired t-test analysis for dose related effects.

*P < 0.05, **P < 0.01, paired t-test analysis for dose related effects.

Bonferroni corrected values, whereby a/n (n=no. of tests) sets the significance of post-drug values.
Graph 7.3.1.vi. The effect of ondansetron on the XS-spike response in lamina I neurones in the superficial dorsal horn of saporin and substance P-saporin treated rats. Results mean % control. Paired t-test analysis for dose related effects. *P < 0.05, **P < 0.01, paired t-test analysis for dose related effects. Bonferroni corrected values, whereby a/n (n=no. of tests) sets the significance of post-drug values.
Graph 7.3.1.vii. The effect of ondansetron on the peripheral receptive field area of lamina I neurones in the rat superficial dorsal horn in saporin and substance P saporin-treated rats.
Graph 7.3.1.viii. The effect of ondansetron on the 'reduced wind-up' response in one lamina I neurone of the superficial dorsal horn in a saporin treated rat.
Graph 7.3.1.ix. The effect of ondansetron on the 'reduced wind-up' response in one lamina I neurone of the superficial dorsal horn in a substance P-saporin treated rat.
Graph 7.3.1.x. The effect of ondansetron on lamina I neuronal responses to peripherally applied punctate mechanical stimuli in saporin treated rats.

P≤0.05* P≤0.01** Paired T-Test showing statistical significance for dose effects compared to control values.
Graph 7.3.1.xi. The effect of ondansetron on lamina I neuronal responses to peripherally applied punctate mechanical stimuli in substance P-saporin treated rats.
Graph 7.3.1.xii. The effect of ondansetron on lamina I neuronal responses to peripherally applied thermal stimuli in saporin treated rats.
Graph 7.3.1.xiii. The effect of ondansetron on lamina I neuronal responses to peripherally applied thermal stimuli in substance P-saporin treated rats.
7.3.2. CGRP receptor antagonists in naive animal models

The mean threshold of lamina I neurones in normal animals, used for studying the effect of CGRP_{8-37}, was 1.96\pm0.4 mA. Depths of lamina I neurones was 505\pm33\mu m using a bent electrode (45°). Using simple trigonometry described in section 2.2 this meant lamina I neurones with depths of approximately 357\pm23\mu m were recorded from. CGRP_{8-37} had a small overall inhibitory effect on lamina I neuronal responses to peripheral electrical stimulation. Overall no statistically significant effects were evident in these neuronal responses following application of rat CGRP_{8-37}. Small inhibitory effects were noticeable in the A-\beta fibre, A-\delta fibre and input responses, particularly in the lower dose range (A-\beta fibre; 50\mu g/ 50\mu l; 20\%, A-\delta fibre; 0.5\mu g/ 50\mu l; 21\% & input; 5\mu g/ 50\mu l; 31\% reductions respectively) (See Graph 7.3.2.i). Interestingly, C-fibre, post-discharge and XS-spike responses had a tendency for facilitation following the top dose of CGRP_{8-37} administration (50\mu g/ 50\mu l; 112\%, 148\% & 158\% of control responses respectively) (See Graph 7.3.2.ii). However, when looking at the reduced wind-up response in one example lamina I neurone, the top two doses appeared to reduce the reduced wind-up effect, with the top dose (50\mu g/ 50\mu l) causing a complete reduction. (See Graph 7.3.2.iii)
Graph 7.3.2.i. The effect of the CGRP Antagonist on the electrical responses of lamina I neurones in the rat superficial dorsal horn.
Graph 7.3.2.ii. The effect of the CGRP Antagonist on the electrical responses of lamina I neurones in the rat superficial dorsal horn.
Graph 7.3.2.iii. The effect of CGRP antagonist on the reduced wind-up response of one lamina I neurones in the rat superficial dorsal horn.
7.4 Discussion

Overall, lamina I neuronal responses in SAP and SP-SAP animal groups were similar in terms of their response to peripheral electrical stimuli. The control XS-spike response was noticeably larger in SAP animals, yet the A-δ fibre response was greater in SP-SAP animals. Marked elevations in both lamina I von Frey and thermally induced control responses were evident in SP-SAP animal groups compared to SAP groups. Overall, these results suggest that the putative NK1 expressing neurones, part of the population sampled in the SAP group, have smaller responses than the population of neurones in the SAP-SP group, now being predominantly the non-NK1 population. Following application of ondansetron on lamina I neuronal responses, more pronounced reductions were evident in SAP animal groups, as compared to SP-SAP animal groups in response to electrical, mechanical and thermal stimuli. The rat CGRP antagonist CGRP_{8-37} had very small effects on lamina I neuronal response properties, none of which proved to be statistically significant.

Overall the marked increase in responses of lamina I neurones following von Frey and thermal stimuli in SP-SAP animals suggest either response differences between the NK1 expressing and other neurones or compensatory increases in neuronal activity in remaining non-NK1 expressing neurones, following ablation of the NK1 expressing neurones. This finding may seem to contradict an NK1/- knock-out mice study, which demonstrated distinct deficits in mechanical and thermal coding properties in deep dorsal horn neurones (Suzuki et al. 2003). However, although it is shown that the NK1 population of cells can influence dorsal horn deep neurones, either through intrinsic or descending pathways, the non-NK1 cells may not share this role. Electrical activity was mostly very similar in lamina I neurones recorded from the two animal groups, yet the decrease in XS-spike responses largely mimics that seen in deep dorsal horn neurones following ablation of NK1 containing neurones (Suzuki et al. 2002). In fact NK1/- knock-out studies also demonstrate a distinct loss of wind-up in deep dorsal horn neurones (Suzuki et al. 2003). It is thought that disruption of local spinal circuits, following ablation of NK1 containing neurones, is responsible for this reduction in wind-up (Suzuki et al. 2002).

Indeed it is known that neurones in the superficial dorsal horn contribute to wind-up in the deep dorsal horn neurones explaining the similar effects seen in both
superficial and deep dorsal horn neurones in SP-SAP models (Suzuki et al. 2002). Following ondansetron application, pronounced reductions in C-fibre and particularly XS-spike responses were noticeable in lamina I neurones recorded from SAP groups. Drug induced reductions in post-discharge, input and A-β fibre responses were evident in both animal groups, yet the reductions observed in A-β fibres were far more pronounced in SAP animal groups. Such results confirmed the idea of a pronociceptive role of 5HT₃ receptors in lamina I neuronal response properties (Millan 2002) as well as in deep WDR neurones. Interestingly low dose ondansetron significantly reduced the input response in SP-SAP groups, suggesting that ablation of NK1 containing lamina I neurones increases the pronociceptive role 5HT₃ receptors play in non-potentiated input response properties. Inhibition of brush and noxious von Frey (125g) responses were far more pronounced in SAP animal groups, however reductions in lamina I responses to mechanical stimuli were evident in both the innocuous and noxious range in both animal groups. A reduction in the lamina I cold response, following ondansetron administration, was clear in SAP and SP-SAP groups. Yet the thermal response appeared to be more inhibited in SAP groups as compared with SP-SAP groups.

Interestingly, the results obtained here, where ondansetron inhibited both neuronal groups are somewhat different from that obtained in a similar study, whereby the effect of the 5HT₃ receptor was tested in deep dorsal horn neurones (Suzuki et al. 2002). Here the responses of deep dorsal horn neurones were recorded in SAP and SP-SAP animals thereby demonstrating distinct deficits in thermal and mechanical coding in SP-SAP animal models (Suzuki et al. 2002). Such deficits, which were not present in SAP animals following punctate mechanical and thermal stimuli, were mimicked by application of the 5HT₃ receptor antagonist ondansetron (Suzuki et al. 2002). However, ondansetron exerted no effects on the mechanical and thermal lamina V response properties in the SP-SAP groups. Reductions in the pre-drug wind-up and input responses were evident in SP-SAP treated rats compared to SAP controls, yet ondansetron exerted no effect on electrically evoked neuronal response properties in SAP and SP-SAP deep dorsal horn neurones (Suzuki et al. 2002). I found, however, that although the effect of ondansetron was inhibition in both groups, SP-SAP treatment resulted in neurones with larger pre-drug control responses, particularly to mechanical and thermal stimuli, however this was not so evident in section 3.3 with a larger population of neurones.
Larger reductions in mechanical and thermally induced responses, following ondansetron administration were evident in SAP rats. Overall it appears that block of 5HT₃ receptors results in reductions in electrical activity in lamina I neurones in both SAP and SP-SAP animals, unlike that seen in deep dorsal horn neurones where 5HT₃ receptor blockade only alters natural evoked responses. Interestingly the drug induced reduction in XS-spike responses in lamina I neurones largely mimicked the smaller XS-spike responses in SP-SAP models. This suggests that descending pathways regulate nociceptive activity in the superficial dorsal horn. In this case, even with this 5HT₃ excitatory control, lamina I neurones are high threshold with small responses and have a 'reduced wind-up effect' as compared to deeper dorsal horn neurones. Removal of lamina I NK1 expressing projection neurones, evidently removes the activation of descending pathways and attenuates the pronociceptive dorsal horn 5HT₃ influences on C-fibre afferents in lamina I (See Diagram 7.4.a). As C-fibres are thought to be responsible for the 'wind-up; due to their ability to induce NMDA receptor activation, it is deemed possible that this is also the mechanism for production of the XS-spike response as well (Dickenson 1990; Dickenson and Sullivan 1991; Dickenson 1995a; Dickenson et al. 1997; Suzuki et al. 2002). However, it appears that the reduction in neuronal hyperexcitability, the non-potentiated input response and the A-β fibre response in lamina I neurones of both SAP and SP-SAP animal groups by 5HT₃ block, are dependent on descending neurones but differ in extent. As such these descending serotonergic influences are mostly NK1 neurone mediated (less in the SP-SAP group) but are also mediated by non-NK1 expressing projection neurones or by local spinal circuits contacted by 5HT and 5HT₃ receptors, independent of descending modulation altogether (Suzuki et al. 2002). As it is proposed that only a small proportion of lamina I neurones express NK1 receptors this may be the case (Todd et al. 1998; Nichols et al. 1999; Todd et al. 2002).

The reductions in mechanical responses in SAP animal groups following ondansetron application are far less pronounced than that seen in deep dorsal horn neurones, suggesting that descending 5HT₃ pathways exert greater controls on systems deeper in the spinal cord. Since the 5HT₃ receptor is largely presynaptic, this may arise from a differential control of afferent terminals into the deep and superficial cord or differential control of intrinsic pathways. Furthermore, enhanced pre-drug responses of lamina I neurones to thermal and mechanical stimuli in SP-SAP animal groups suggest that the NK1 containing neurones have lower responses
Diagram 7.4a  Effect on Descending Pathways by the removal of NK1 expressing Neurones
than non-NK1 containing dorsal horn neurones. The increased neuronal responses to mechanical and thermal stimuli may either be due to compensatory increases in activity or the removal of descending inhibitory influences on these lamina I neurones. Indeed studies performed in NK1 -/- mice have demonstrated reductions in descending inhibitory controls terminating in the dorsal horn, projecting from the brainstem (Bester et al. 2001). The fact that such increases are not mimicked by 5HT3 receptor block, suggest that lamina I neurones are controlled via other descending pathways as well. (Bester et al. 2001) suggested that these descending inhibitory controls originated from the brainstem RVM, similar to the pronociceptive serotonergic pathways implicated in the regulation of deep dorsal horn neurones (Suzuki et al. 2002). However, inhibitory influences may stem from pontomedullary raphe magnus (RM) ‘OFF’ cells whereas facilitatory influences stem from RM ‘ON’ cells, furthermore differences in the 5HT receptor targets in the dorsal horn may also underlie the differences in these effects (Mason 1999). Other studies suggest that antinociceptive pathways originate from the PAG located in the midbrain (Mason 1999). Indeed, only recently have diffuse inhibitory controls (DNIC's) been implicated in the control of NS neurones located within lamina I parabrachial neurones (Bester et al. 2000; Bester et al. 2001).

Interestingly the small reductions in lamina I responses in both SAP and SP-SAP animal groups to brush, cold and innocuous as well as noxious von Frey stimuli, suggest that serotonergic pathways have only influences on spinal lamina I neuronal coding of these stimuli. However this, once again, appears to be independent of sensory information projected via NK1 expressing neurones in lamina I. The tendency for reduction of thermal responses seen in SAP groups alone, largely mimic that seen in deep dorsal horn neurones, and suggest similar modulatory functions, although it is thought that this may not be entirely due to activation by NK1 projecting neurones.

Interestingly, a recent immunohistochemistry study demonstrated the high levels of gephyrin (the glycine receptor–associated protein) on the cell bodies and dendrites of giant waldeyer cells found within lamina I of the spinal cord (Puskar et al. 2001). It was evident that these gephyrin-rich neurones primarily projected to the parabrachial area (as illustrated from retrograde labelling following cholera toxin B subunit injection) and as a result of noxious stimulation, expressed the immediate early gene c-fos, protein product, Fos (Puskar et al. 2001).
Furthermore, these gephyrin rich cells appeared to mostly lack the NK1 receptor for substance P and those that did express it only containing very low levels, quite unlike the majority of lamina I projection neurones (Puskar et al. 2001). Interestingly, it was found that these gephyrin rich cells predominantly neighboured GABAergic neurones (identified by the presence of glutamate decarboxylase) and were presumably modulated by inhibitory neurones (Puskar et al. 2001). These studies would therefore suggest that following the removal of NK1 expressing neurones in the superficial dorsal horn, gephyrin rich neurones under inhibitory control would predominate within the superficial dorsal horn. One would also presume that as a result of this, smaller responses in lamina I neurones of SP-SAP animal models would prevail. However, as a number of studies have demonstrated NK1 neurones comprise only 5 -45% of lamina I neurones, thus removal of NK1 expressing neurones may not affect the neuronal responses of the remaining neurones quite as dramatically as this (Todd et al. 1998; Todd et al. 2002).

The CGRP receptor antagonist (CGRP$_{8-37}$) exerted very little effect overall on the response of lamina I neurones to peripheral electrical stimuli in normal animals. There was a tendency toward slight inhibition of A-beta, A-delta fibre and input responses in these animals. Paradoxically there was a tendency toward facilitation of post-discharge and XS-spike responses following administration of CGRP$_{8-37}$. As no statistical significance was found attached to these responses, one can more or less assume that CGRP$_{8-37}$ exerted no pronounced effect on lamina I neuronal responses in general. Very little is known regarding the function of CGRP in the dorsal horn. It is thought that CGRP is contained predominantly in small diameter primary afferent neurones and therefore contributes to nociceptive activity (Bennett et al. 2000). It has been shown that CGRP can promote distinct nociceptive behaviours when applied directly to the spinal cord of rats implicating a large contribution towards nociceptive transmission (Bennett et al. 2000). It thus feasible that within the superficial dorsal horn, CGRP plays a small role, but should recordings be made from deeper dorsal horn neurones CGRP may play a far more pronounced role in the transmission of nociceptive information. Interestingly, CGRP is thought to play a distinct role in nociceptive activity in chronic central neuropathic pain syndromes (Bennett et al. 2000). However, due to time limitations the effect of CGRP$_{8-37}$ in lamina I neurones and deep dorsal horn neurones could not be tested in a model of peripheral nerve injury used in this PhD study.
Overall it is clear that NK1 expressing neurones play a key role in conducting nociceptive and sensory information to the supraspinal nervous system, which in turn stimulates serotonergic descending controls that play a pronociceptive function in lamina I dorsal horn neurones. The lamina I NK1 expressing neurones stimulate a descending serotonergic pathway. This appears to regulate unmyelinated C-fibres and XS-spike responses in remaining lamina I neurones, as well as noxious mechanical and thermal response properties. Non-NK1 expressing neurones appear to regulate a descending serotonergic pathway which modulates innocuous mechanical and other electrical responses recorded in lamina I neurones. Overall, CGRP plays a small role in lamina I neuronal responses, and it is thought that it may play a more pronounced role following nerve injury and central nerve damage, as well as in deep dorsal horn neuronal responses.
7.5 Tables

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Table 7.2.a. Bonferroni corrections of P values, calculated for statistical significance.

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Table 7.3.1.a. The effect of SP-SAP on lamina I neuronal responses to electrical stimuli and the effects of ondansetron on these responses. Results mean % control. Paired t-test analysis for dose related effects. Unpaired t-test analysis for comparative effects between each animal group. Bonferroni corrections for posthoc analyses utilised, therefore statistical significance values set accordingly. *P < 0.05, **P < 0.01, paired t-test analysis for dose related effects. Bonferroni corrected values, whereby α/n (n=no. of tests) sets the significance of post-drug values. ϕ P< 0.05 and ϕ ϕ P< 0.01, unpaired t-test analysis for comparative effects between each animal group and * or ** used as above to denote significance at related confidence levels.

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Table 7.3.1.b. The effect of ondansetron on the peripheral receptive field area of lamina I neurones. Units are % Control Value.
Table 7.3.1.c. The effect of SP-SAP on lamina I neuronal responses to punctate mechanical stimuli and the effect of ondansetron administration on these response properties. Results shown as raw data values. Paired t-test analysis for dose related effects. Unpaired t-test analysis for comparative effects between each animal group. Non-parametric Mann Whitney test for comparison between animal groups ∆P < 0.05 & ∆∆P < 0.01. Bonferroni corrections for posthoc analyses utilised, therefore statistical significance values set accordingly. *P < 0.05, **P < 0.01, paired t-test analysis for dose related effects. Bonferroni corrected values, whereby a/n (n=no. of tests) sets the significance of post-drug values. ∗ or ∗∗ used as above to denote significance at related confidence levels.

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Table 7.3.1.d. The effect of SP-SAP on lamina I neuronal responses to thermal stimuli and the effect of ondansetron administration on these response properties. Results shown as raw data values. Paired t-test analysis for dose related effects. Unpaired t-test analysis for comparative effects between each animal group. Bonferroni corrections for posthoc analyses utilised, therefore statistical significance values set accordingly. *P < 0.05, **P < 0.01, paired t-test analysis for dose related effects. Bonferroni corrected values, whereby a/n (n=no. of tests) sets the significance of post-drug values. * or ** used as above to denote significance at related confidence levels.

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</tr>
<tr>
<td>45</td>
<td>362±118 (n=10)</td>
<td>222±21</td>
<td>257±89</td>
<td>178±109</td>
<td>414±191</td>
<td>128±57</td>
<td>343±101</td>
<td>107±82</td>
</tr>
<tr>
<td>48</td>
<td>536±166 (n=7)</td>
<td>392±182</td>
<td>379±107</td>
<td>317±147</td>
<td>316±80</td>
<td>153±69</td>
<td>395±108</td>
<td>163±71</td>
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<tr>
<td>50</td>
<td>568±151 (n=7)</td>
<td>965±220</td>
<td>468±144</td>
<td>439±213</td>
<td>550±202</td>
<td>264±135</td>
<td>514±188</td>
<td>355±136</td>
</tr>
</tbody>
</table>
Table 7.3.2.a. The effect of the CGRP receptor antagonist CGRP8-37 on the electrically evoked responses of normal animals. Results shown as mean % control. Paired t-test analysis for dose related effects. Unpaired t-test analysis for comparative effects between each animal group. Bonferroni corrections for posthoc analyses utilised, therefore statistical significance values set accordingly. *P < 0.05, **P < 0.01, paired t-test analysis for dose related effects. Bonferroni corrected values, whereby α/n (n=no. of tests) sets the significance level of post-drug values and *or ** used as above to denote significance at related confidence levels.

<table>
<thead>
<tr>
<th>Response</th>
<th>Control (No of AP's)</th>
<th>CGRP 0.5 ug (% Control)</th>
<th>CGRP 5 ug (% Control)</th>
<th>CGRP 50 ug (% Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Electrical</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A-beta</td>
<td>15±1.8 (n=10)</td>
<td>95±8 (n=10)</td>
<td>89±10 (n=10)</td>
<td>80±10 (n=10)</td>
</tr>
<tr>
<td>A-delta</td>
<td>44±1.3 (n=8)</td>
<td>79±20 (n=8)</td>
<td>84±27 (n=8)</td>
<td>102±39 (n=8)</td>
</tr>
<tr>
<td>C-fibre</td>
<td>146±15 (n=10)</td>
<td>106±13 (n=10)</td>
<td>103±10 (n=10)</td>
<td>112±25 (n=10)</td>
</tr>
<tr>
<td>Post Discharge</td>
<td>58±1.4 (n=8)</td>
<td>133±44 (n=8)</td>
<td>118±34 (n=8)</td>
<td>148±40 (n=8)</td>
</tr>
<tr>
<td>XS-Spikes</td>
<td>111±2.4 (n=6)</td>
<td>69±31 (n=6)</td>
<td>119±58 (n=6)</td>
<td>158±63 (n=6)</td>
</tr>
<tr>
<td>Input</td>
<td>11±1 (n=6)</td>
<td>90±16 (n=6)</td>
<td>70±10 (n=6)</td>
<td>92±21 (n=6)</td>
</tr>
</tbody>
</table>
CHAPTER 8

DISCUSSION
8.0 Discussion

The responses of lamina I neurones have been fully analysed in naive, neuropathic (SNL) and sham operated animal models, as well as in animals where lamina I/III NK1 receptor expressing neurones have been ablated (SP-SAP) and the associated saporin (SAP) controls. Lamina I neurones recorded in naïve rats were compared to previously characterised deep convergent dorsal horn neurones. Overall lamina I neurones had different biophysical and physiological response characteristics to deeper dorsal horn neurones. Lamina I neurones had a characteristic double spike, and the majority were nociceptive specific (NS) in their response to peripherally applied electrical and natural stimuli. Paradoxically, the majority of lamina I neurones exhibited a characteristic brush response although lacking a response to von Frey stimuli below 12g.

Overall lamina I neurones exhibited much lower responses to peripherally applied electrical, mechanical and thermal stimuli compared to that seen in deep dorsal horn neurones. This suggests that either a smaller proportion of sensory fibres terminate within lamina I, compared to deeper dorsal horn neurones, which could be associated with their smaller active receptive fields and different membrane properties or indeed less excitatory or more inhibitory receptor systems act on these superficial neurones. Interestingly, lamina I neurones also exerted a 'reduced' wind-up response compared to the characteristic wind-up effect widely documented in deep neurones (Dickenson 1990; Dickenson and Sullivan 1991; Dickenson 1997). Repetitive electrical stimuli was enough to evoke a small incremental potentiation of the majority of lamina I neuronal responses, yet a characteristic large NMDA mediated potentiation was not evident in these neurones. These findings suggested less excitatory influences or in turn more inhibitory influences within lamina I of the spinal dorsal horn. These ideas led to the use of different pharmacological tools to assess the excitatory and inhibitory influences within lamina I, as compared to previously documented findings in deep dorsal horn neurones, which have been widely studied in this laboratory. Numbers of nociceptive neurones containing NMDA receptor populations underlying the development of wind-up and related central sensitisation, may therefore be low in the superficial dorsal horn as compared to deep dorsal horn neurones (Dickenson and Sullivan 1990; Dickenson and Sullivan 1991; Dickenson 1995b).
Alternatively, GABAergic or even glycinergic neuronal populations may strongly innervate lamina I of the superficial dorsal horn compared to the deeper dorsal horn and thus dampen the neuronal responses of these neurones to a variety of sensory stimuli (Kontinen et al. 2001). Indeed as shall be discussed, distinct differences in the pharmacology of lamina I and lamina V neurones are evident in the naïve rat (See Table 8.a).
<table>
<thead>
<tr>
<th>Drug</th>
<th>Lamina I</th>
<th>Lamina V</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACEA-1244</td>
<td>↓ Aδ-fibre &amp; XS-spikes at lower dose range</td>
<td>↓ Aβ-fibre, Aδ-fibre, C-fibre, Post Discharge &amp; XS-spike at all dose ranges</td>
</tr>
<tr>
<td>AP</td>
<td>↓ Aδ-fibre (58±21% control value, 500μg) Small ↑ Post-Discharge &amp; XS-Spikes</td>
<td>↓ Aβ fibre, ↓ C-fibre, ↓ Post-Discharge (51±6% inhibition; 500μg) Abolishes 'wind-up' in 73% neurones</td>
</tr>
<tr>
<td>Bicuculline</td>
<td>↑ Aδ-fibre (279±6% facilitation, 50μg) ↑ Innocuous and Noxious Mechanical Responses ↓ Noxious Thermal Responses</td>
<td>↑ Aδ-fibre (approximately 450% facilitation of control response, 50μg) Small ↑ Post-Discharge Mechanical and Thermal Data Unavailable</td>
</tr>
<tr>
<td>CGRP Antagonist</td>
<td>No Effect on Electrically evoked Responses</td>
<td>Data Unavailable</td>
</tr>
<tr>
<td>CNQX</td>
<td>↓ C-fibre, Input &amp; XS-spikes (41±10%, 31±12%, 20±8% control value, 50μg)</td>
<td>Data Unavailable</td>
</tr>
<tr>
<td>Ifenprodil</td>
<td>↓ Aβ fibre, Post Discharge, XS-spikes (66±13%, 37±9%, 49±27% control value, 400μg)</td>
<td>↓ Input, Aδ-fibre, C-fibre, Post Discharge, XS-spikes (28±9%, 18±8%, 41±8%, 16±7%, 32±12% control value, 400μg)</td>
</tr>
<tr>
<td>LY293558</td>
<td>↓ Input, Aδ-fibre, C-fibre (0±0%, 17±12%, 13±8% control value, 50μg) Smaller reductions in Aβ fibre, Post Discharge and XS-spike response</td>
<td>Data Unavailable</td>
</tr>
<tr>
<td>NBQX</td>
<td>↓ Aδ-fibre &amp; C-fibre (31±13% &amp; 38±17% control value, 50μg) ↓ Post Discharge and XS-Spike response</td>
<td>↓ Input, C-fibre &amp; Post Discharge (99.1±0.9%, 73.6±3.0%, 41.5±18.6% reduction, 50μg) ↓ Wind-up, at top dose (50μg) only</td>
</tr>
<tr>
<td>Ondansetron</td>
<td>↓ Electrically and Mechanical evoked responses in SAP Models Small ↓ Aδ-fibre, Input and Post-Discharge</td>
<td>↓ Innocuous &amp; Noxious Mechanical and Thermal in SAP Model</td>
</tr>
<tr>
<td>Strychnine</td>
<td>↑ Input (184±25% facilitation, 50μg) Small facilitation of C-fibre response Small reduction in Post Discharge and XS-spike response Tendency for Facilitation of Thermal and Mechanical responses</td>
<td>No effect on Aβ-, Aδ-, C-fibre and Post-Discharge in Sham (SNL) Control Models</td>
</tr>
</tbody>
</table>

Table 8.0.a. Summary of the pharmacological actions of the different drugs tested in lamina I neurones in this thesis, as compared to previously described effects in lamina V deep dorsal horn neurones. (Lamina V data obtained from Kontinen et al, 2001; Dickenson & Sullivan, 1990; Carpenter, 2001; Stanfa, 1999).
Following nerve injury certain evoked responses of lamina I neurones were largely unchanged. A tendency for larger A-\(\delta\) fibre responses following nerve injury suggested anatomical changes and receptor upregulations, specific to these nerve fibres, which are evidently evoked by peripheral nerve damage. The electrically evoked responses of lamina I neurones were similar in SAP and SP-SAP animal models. However SP-SAP lamina I neurones tended to exhibit slightly larger responses following naturally evoked stimuli than lamina I neurones in SAP control groups. This suggests differences in response characteristics between the NK1 expressing and non-NK1 expressing neurones. Alternatively there may be compensatory increases in neuronal activity in remaining non-NK1 expressing neurones, following ablation of NK1 expressing neurones.

Based on the response properties of lamina I neurones to punctate mechanical and thermal stimuli, the dorsal horn lamina I neurones were classified as primarily NS with a small population of polymodal nociceptive (HPC) and wide dynamic range neurones (WDR). However, brush is clearly an innocuous stimulus and thus there is some discrepancy regarding our classification system compared to previously documented NS lamina I neurones (although many previous studies have been in cat and monkey) (Andrew and Craig 2001a; Craig et al. 2001; Andrew and Craig 2002b). The responses of these individual neuronal populations could be compared to each other and to that of the corresponding neuronal population in different animal models. Overall it was found that WDR neurones consistently had larger responses to that of the NS and HPC neuronal populations in all animal models tested. HPC neurones, as one would expect, had large cold responses. In sham animals HPC neurones had larger responses to thermal stimuli compared to NS neurones and in SNL animals they exhibited larger responses to mechanical stimuli. However in normal animals the responses were similar.

Following nerve injury it was apparent that the responses of NS neurones to mechanical stimuli were smaller, yet the HPC responses to mechanical stimuli were larger in SNL animals suggesting some plasticity in the responses of these individual neuronal populations induced by peripheral nerve damage. In SP-SAP animals NS and HPC neuronal populations exerted similar responses, yet in SAP animals NS neurones exerted larger responses than HPC neurones. Interestingly, removal of NK1 receptors in lamina I-III implicated that HPC neurones no longer have the ability to code noxious thermal stimuli as they do in SAP animals. Thus changes in HPC
neurones may contribute to the allodynia to mechanical and cold stimuli that follow nerve injury.

It appears that both AMPA and kainate receptors play a large role in noxious sensory transmission, as seen by the effect of antagonists on lamina I neuronal response properties. The functions of kainate and AMPA receptors are different, and the location of both these receptors may also differ. Block of AMPA receptors reduces both the receptive field area of lamina I neurones and the electrically evoked responses in normal, sham and SNL animal groups. Reductions in A-δ, C-fibre, post discharge and XS spike values suggest a role for AMPA receptors in noxious sensory transmission, as opposed to innocuous sensory transmission. Block of kainate receptors suggest that these receptors play a larger role in the transmission of innocuous transmission in lamina I as seen by reductions in A-β fibre responses. It was also suggestive that kainate receptors, unlike AMPA receptors, are predominantly pre-synaptic in location within lamina I. Following nerve injury, smaller reductions in A-δ fibre response properties following block of kainate receptors implicate changes in these thinly myelinated sensory fibres. However, interestingly larger reductions in both post discharge and XS spike values within lamina I suggest an enhanced role played by kainate receptors in nociceptive transmission and neuronal hyperexcitability following peripheral nerve injury. It is therefore clear that although small, there is a tendency for minor changes following nerve injury, which may account for the development of some, if not all, abnormal pain related behaviours.

The role of the excitatory NMDA receptor was also examined in lamina I of the superficial dorsal horn. Block of the NMDA receptor by APV had minimal effects on the electrical response properties of lamina I neurones overall. However reductions in A-δ and input responses suggested a small population of NMDA receptors on these thinly myelinated sensory neurones and a possible presynaptic location, as implicated by the reduction in the non-potentiated input response. Interestingly in sham operated animals, some NMDA receptor plasticity was evident. Larger reductions in A-β and XS spike values were clear, indicating a role in innocuous sensory responses as well as 'reduced' wind up in lamina I neurones. It was presumed that this was induced by the invasive surgical procedure used to ligate L5 and L6 peripheral nerves. However, following peripheral nerve ligation itself, greater reductions in C-fibre and post discharge suggested an increased role for NMDA
receptors in neuronal hyperexcitability and noxious sensory transmission, driven by peripheral nerve damage.

Interestingly, following block of NR2B NMDA receptors by ifenprodil, reductions in A-β fibre responses in normal, sham and neuropathic animal groups suggested the presence of NR2B NMDA receptors on myelinated sensory neurones within lamina I. Reductions in the non-potentiated input response in normal animals indicated a presynaptic location for NR2B containing receptors in lamina I neurones, yet this was less evident following nerve injury and in sham controls, which proposed that NR2B receptors are also located on postsynaptic neurones within lamina I. Reductions in A-δ fibre responses in normal, as well as sham control animals, produced by NMDA antagonism supported the idea that NMDA receptor populations play a distinct role in the responses of A-δ fibres. Furthermore, it suggested that the majority of these NMDA receptors are NR2B containing receptors. Similar to APV, ifenprodil did not exert a reduction in the A-δ fibre response in neuropathic animal groups. This suggested that there is either a down regulation of NMDA receptors (particularly NR2B containing NMDA receptors) on A-δ fibre neurones or a degeneration of A-δ fibres within lamina I of the spinal cord. Interestingly, reductions in C-fibre, post discharge and XS spike responses, which were clear in sham controls, following block of NR2B NMDA receptors, were not evident following nerve injury. This suggests that NR2B receptors do not play any role in increased noxious sensory transmission, which may underlie certain pain behaviours in neuropathic animals, such as hyperalgesia. The tendency for larger reductions in these electrical response properties in lamina I neurones following APV application in neuropathic animals, suggest that down regulations of NR2B receptors allow for an upregulation in other NMDA receptor subunits.

This may therefore help to facilitate the heightened noxious sensory transmission that may produce such abnormal sensory behaviours. Greater reductions in deep dorsal horn neurones exerted by block of the NR2B NMDA receptor populations in the spinal cord, demonstrates the larger role that this receptor population plays in sensory transmission in deep convergent dorsal horn neurones as compared to lamina I. Interestingly greater reductions in XS spike responses and input responses were evident in lamina I, however as explained in the characterisation studies the XS spike values are considerably smaller in lamina I and this may influence the % changes seen following drug application. It is also possible that a larger proportion of
lamina I neurones are contacted by presynaptic NR2B NMDA receptors than deep dorsal horn neurones. Overall, ACEA-1244 (an experimental NR2B receptor antagonist) produced less pronounced inhibitory effects than ifenprodil, largely suggesting reduced specificity. However, this compound also produced greater reductions in lamina V neuronal response as compared to lamina I. Similar to that seen with ifenprodil, greater reductions in the XS spike response were clear. Overall these results suggest that NR2B play a larger role in deep dorsal horn neuronal responses than lamina I neuronal responses. It is also clear that NMDA receptors, on the whole, do not play a large role in lamina I neuronal responses compared to deep dorsal horn neurones. This may go some way to explain the reduced responses observed in lamina I as compared to deep dorsal horn neurones. Following nerve injury small differences are evident in the role NMDA receptors exert in lamina I neuronal response properties, again, quite unlike that seen in deep dorsal horn neurones (Suzuki et al. 2001) where NMDA blockade produces enhanced inhibitions in the nerve injured animals.

A marked functional role of the inhibitory amino acid receptors GABA_A and glycine were investigated in lamina I to assess whether larger inhibitory influences could account for the high threshold and small responses exerted by lamina I neurones in the superficial dorsal horn. Strychnine resulted in minor reductions in post discharge responses, however more importantly increases in both C-fibre, XS spike and input responses suggested that glycine receptors play a small role in dampening the responses of lamina I neurones under normal conditions. Studies performed in deep dorsal horn neurones did not observe such effects (Kontinen et al. 2001). Furthermore increases in both thermal and mechanical responses after the antagonist was applied also suggested that glycinergic control may explain why both lamina I thermal and mechanical responses are much lower than deep dorsal horn neuronal responses properties.

The role of GABA_A receptors in lamina I neuronal responses has been investigated in deep dorsal horn neurones, and in lamina I neurones, block of GABA_A receptors has similar effects. Both the A-ô and mechanical responses of lamina I neurones were facilitated, which suggests a GABAergic influence on these lamina I neuronal response properties. Following nerve injury a larger facilitation of A-ô fibres following block of GABA_A receptors, than that seen in sham control groups, demonstrated a possible upregulation and a larger influence exerted by GABA_A receptors on the responses of A-ô fibre sensory neurones. Most interestingly, block of GABA_A
receptors did not have an effect on the mechanical response properties of lamina I neurones in neuropathic animal groups, quite unlike that seen in normal and sham control groups. This demonstrated a possible down-regulation of GABA$_A$ receptors in lamina I neurones following peripheral nerve damage, which may lead to increased neuronal excitability. Furthermore control values for mechanical responses were much larger in SNL rats than in sham controls, however given a larger population as in the characterisation chapter, only minor differences were noted between these two populations. It could be that although GABA$_A$ receptors are down regulated following nerve injury, compensatory increases in other receptor systems prevent there being any distinct changed in the mechanical responses of lamina I neurones overall. As the majority of neurones in the bicuculline study were nociceptive specific (NS), the fact that other physiological lamina I neuronal types make up the population of neurones recorded (as in the characterisation study), may suggest the down regulation of GABA$_A$ receptors are confined to nociceptive specific neurones. Interestingly, the majority of neurones projecting to the parabrachial nucleus, which is thought to be involved in the affective emotional aspects of pain are nociceptive specific (Bester et al. 2000).

The role of the peptide CGRP as well as substance P NK1 containing lamina I neurones was examined on the neuronal responses of lamina I neurones in the superficial dorsal horn. Overall, block of the CGRP receptor resulted in only minor effects on the neuronal response properties of lamina I neurones, suggesting that CGRP and the CGRP receptor play only a small role in these neurones. Removal of NK1 receptor containing neurones within lamina I/II of the dorsal horn was also examined on the lamina I neuronal responses as well as the effect of blocking the 5HT$_3$ receptor in these NK1 deficient lamina I neurones.

Originally studies performed in deep dorsal horn neurones in SP-SAP and SAP animal models demonstrated reductions in both input and wind-up responses, following ablation of NK1 containing neurones in the superficial dorsal horn, as well as deficits in both thermal and mechanical coding (Suzuki et al. 2002). Using the 5HT$_3$ receptor antagonist ondansetron, the deficits in both mechanical and thermal coding could be mimicked in the control saporin treated animal groups suggesting a NK1 neuronal projection pathway which influences descending 5HT$_3$ receptor mediated pathways projecting to the dorsal horn (Suzuki et al. 2002). However, no such deficits were evident in lamina I neuronal mechanical and thermal response properties in NK1 ablated animal groups. Furthermore, block of 5HT$_3$ receptors
resulted in reductions in the electrical responses of lamina I neurones in both animal groups (particularly post discharge and input responses) suggesting 5HT₃ receptor mediated serotonergic descending pathways which influence both NK1 and non-NK1 containing receptors in the superficial dorsal horn. However larger reductions in both C-fibre and XS -spike responses were seen in saporin treated control animals, suggesting greater influences of pronociceptive sertonergic pathways in the noxious response properties of NK1 expressing neurones. Reductions in the thermal, noxious mechanical and brush responses in saporin treated lamina I neurones were similar to that seen in deep dorsal horn neurones. However, reductions in mechanical and thermal responses were also evident in the lamina I neuronal responses of NK1 ablated groups, which demonstrated that such 5HT₃ receptor mediated descending serotonergic controls are still present despite the absence of NK1 projection neurones. This suggests that pro-nociceptive serotonergic controls, independent of NK1 projection neurones, may exist in lamina I.

Overall both the characterisation studies and pharmacological studies incorporated in this PhD have lent support to the individual and distinct neuronal response properties and receptor composition of lamina I neurones in the superficial dorsal horn, as opposed to the well characterised deep dorsal horn neurones. Interestingly it is known that A-δ fibres terminate within lamina I, whereas A-β and C-fibres tend to project to deeper dorsal horn laminae. The most pronounced effects following both block of GABAₐ and NMDA receptors were often seen on A-δ fibre neuronal responses. Furthermore, A-δ fibre responses were very similar to that recorded from deep dorsal horn neurones, suggesting that the reduced pharmacological effects and high threshold nature of lamina I neurones could be due to the limited sensory fibres inputs emerging from the periphery within lamina I.

Interestingly small changes in the lamina I neuronal responses take place following nerve injury, however the plasticity that emerges may go some way to account for the abnormal pain related behaviours that evolve following peripheral nerve damage. Interestingly, the descending controls regulating lamina I neuronal responses are evidently different to that documented in lamina V neurones. This study thus proposes for the first time dissimilar pharmacological mechanisms within lamina I, compared to deep dorsal horn neurones, and supports a distinct role for lamina I neurones in the transmission of nociceptive information and sensory processing.
AMPA and kainate receptors play a large role in the nociceptive processing of noxious sensory information within lamina I neurones, however it was evident that the NMDA receptor played a far smaller role in lamina I responses, which may underlie the high threshold nature of lamina I neurones. However, it appears that glycine and GABAergic influences contribute towards the dampening down of lamina I neuronal responses. The small contribution made by NMDA receptors and the considerable contribution from inhibitory amino acid receptors, may be the key to the smaller response properties and nociceptive specific nature of lamina I neurones. Furthermore, following nerve injury the reduced role exerted by GABAergic receptors and the heightened role exerted by NMDA receptors provide an interesting theory behind the emergence of abnormal pain behaviours and the perception of heightened sensory inputs.

It is known that a large proportion of lamina I neurones project to the parabrachial area, thought to be involved in the emotional aspects of pain perception (Hylden et al. 1985; Hylden et al. 1989; Bester et al. 1997; Gauriau and Bernard 2002). The majority of lamina I neurones appear to be nociceptive specific, high threshold and small responding to electrical and naturally evoked stimuli, as demonstrated in this thesis as well as other such studies (Bester et al. 2000). It is clear that these lamina I neurones may play a large role in the perception of unpleasant sensations emanating from the periphery. However, even more intriguingly is that these lamina I neurones also appear to exert a distinct brush response, even the so-called 'nociceptive specific' neurones. Thus neurones projecting to an area thought to be involved in the emotional aspects of sensory perception (Gauriau and Bernard 2002), display response properties often perceived as both 'pleasant' and 'unpleasant'. However, innocuous punctate mechanical and innocuous thermal stimuli are not classically described as pleasant or unpleasant, but merely innocuous, although this may often depend on who and what is administering it!

Overall this thesis aims to provide novel theories in an in vivo rat electrophysiological study, regarding the pharmacological properties, response characteristics and ensuing plasticity following peripheral nerve injury and ablation of NK1 receptors, of lamina I neurones in the superficial dorsal horn. It has successfully shown a population of dorsal horn neurones that are primarily devoted to the transmission of nociceptive information to higher supraspinal centres, that have responses dampened by a fine balance between reduced excitatory and heightened inhibitory controls and which also appear to contribute to abnormal pain behaviours following peripheral nerve injury. More intriguingly, unlike deep dorsal horn neurones these
neurones also receive descending modulation from supraspinal centres independent of NK1 projections and appear to compensate for the removal of NK1 receptor expressing populations.
CHAPTER 9

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9.0 References


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CHAPTER 10

PUBLICATIONS
Electrophysiological characterisations of rat lamina I dorsal horn neurones and the involvement of excitatory amino acid receptors

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Abstract

Lamina I of the spinal cord plays a key role in sensory transmission between afferent activity and the CNS. Studies have shown lamina I neurones to have distinct response properties compared to deep dorsal horn neurones, but little is known regarding excitatory amino acid mechanisms in their responses. Spinal electrophysiological recordings of lamina I neurones confirmed that the majority of these neurones (74%) are nociceptive specific (NS) in their responses, of which 18% can be termed polymodal nociceptive (HPC) (13% of the total population). The remainder (26%) were wide dynamic range. Lamina I neurones had smaller mechanical and heat-evoked responses compared to deeper dorsal horn neurones. The electrically evoked responses were also smaller, with a distinct lack of an NMDA-mediated 'wind-up' effect. NBQX (AMPA receptor antagonist, 0.5, 5, 50 μg/50 μl) produced dose-dependent inhibitions of the electrically evoked neuronal responses, but APV (NMDA receptor antagonist, 50, 100, 500 μg/50 μl) had minimal effects on their responses. These results implicate mainly AMPA receptors in the responses of lamina I neurones. Bicuculline (GABA_A receptor antagonist, 0.5, 5, 50 μg/50 μl) demonstrated a role exerted by GABA_A receptors in the control of A-δ fibre-mediated mechanical responses in lamina I. Overall, this study describes a high threshold, AMPA receptor possessing population of lamina I neurones, which seem to lack functional NMDA receptors, and are partially controlled by GABA_A receptor activity.

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Keywords: Lamina I neurones; Spinothalamic tract; Electrophysiological recordings

1. Introduction

The first synapses between the periphery to the brain occur in the superficial dorsal horn of the spinal cord. This marginal layer is an intrinsic constituent of the central representation of both pain and temperature responsiveness with major ascending output to higher centres unlike lamina II, which is mainly small neurones terminating locally (Bowsher and Abdel-Maguid, 1984; Woolf and Fitzgerald, 1983). Lamina I comprises modality-selective nociceptive and thermoreceptive neurones (Craig et al., 2001). Three major classes predominate, in accordance with their responses to natural cutaneous stimuli, namely nociceptive specific (NS), polymodal and thermoceptive cells (Craig et al., 2001).

Superficial dorsal horn neurones receive monosynaptic input from A-δ and C-fibre primary afferent nociceptors, and respond to A-δ and C-fibre-mediated innocuous thermal and mechanical stimuli (Rethelyi et al., 1982; Sugiura et al., 1986, 1993). Most studies suggest that lamina I receives input primarily from high threshold mechanoreceptors. However, input from polymodal high threshold nociceptors is also evident, and responses to mechanical, thermal, histamine and cold stimuli have been reported (Andrew and Craig, 2001a,b, 2002a,b; Bester et al., 2000a,b; Craig and Andrew, 2002; Craig et al., 2001; Woolf and Fitzgerald, 1983). Morphological studies in the cat and monkey reveal that lamina I spinothalamic tract (STT) neurones can be categorised on the basis of these physiological responses (Han et al., 1998; Zhang et al., 1996).

The primary ascending output from the superficial dorsal horn is via lamina I. Lamina I axons in cats and primates project contralaterally in the lateral STT. Crucial projections to spinal autonomic and brainstem homeostatic integration sites are also present (Craig and Dostrovsky, 2001) including lateral periaqueductal gray and the parabrachial area, PBA. The PBA, a main projection site in
the rat, not only impinges upon some descending controls, but also plays a role in affective reactions to pain (Bester et al., 2000a,b; Blomqvist et al., 1989; Wall et al., 1988).

Ablation of neurokinin-1 (NK-1) expressing lamina I neurones, using a substance P and sapolin conjugate (SP-SAP), results in behavioural deficits to noxious stimuli (Nichols et al., 1999) and reduced coding of both thermal and mechanical stimuli in deep dorsal horn neurones but via descending controls (Suzuki et al., 2002).

Other than immunohistochemical studies less is known regarding the pharmacology of lamina I neuronal responses (Furuyama et al., 1993; Munoz et al., 1999; Tachibana et al., 1994) other than in vitro or in neonates (Cheunsuang et al., 2002; Engelman et al., 1999). In vivo studies show excitatory amino acid receptors play a role in the neuronal responses of lamina I (Dickenson and Sullivan, 1990; Green and Gibb, 2001; Kontinen et al., 2001; Schoenburg et al., 1986). We determined the roles of excitatory and inhibitory amino acids in the physiologically characterised responses of lamina I neurones and compared to deep dorsal horn neuronal response properties where the contributions of amino acid receptors have been extensively studied (Dickenson and Sullivan, 1990; Dickenson et al., 1997).

2. Materials and methods

Intact anaesthetised male Sprague-Dawley rats (200–250 g) were used to perform these experiments, in concurrence with the UK Animals (Scientific Procedures) Act, 1986 and IASP guidelines. The rats were anaesthetised with halothane (3%) in 33% O₂/66% N₂O, and reduced to 2% halothane for the ensuing surgery. The rats’ body temperature was regulated using a thermostatically controlled heating blanket, maintaining the core temperature of 37 °C. Tracheal cannulation was used to maintain anaesthesia throughout the experiment, and the rats were secured in a stereotaxic frame by metal clamps attached to the vertebral column. The spinal cord was exposed via a laminectomy at vertebrae L1–L3. The level of anaesthesia was then reduced to approximately 1.6%, which was deemed sufficient to retain a state of complete areflexia. Following this, electrophysiological recording began.

2.1. Electrophysiological recording

Lamina I neurones were located in the superficial marginal layer of the spinal cord using a parylene-coated tungsten electrode, which then enabled single-unit extracellular recordings to be made. The electrode was positioned to enter the spinal cord at an angle of 45° horizontal to the spinal cord, enabling it to penetrate the spinal cord at an angle that allowed a large cross-sectional area of the superficial layer to be searched for neuronal activity. A separate population of lamina V cells had been previously characterised in the same manner, in order to compare electrical and naturally evoked neuronal response characteristics, the majority of these neurones (77%) were wide dynamic range (WDR) in characteristic. In each experiment, 3–4 neurones were found and fully characterised (approximately 30 min was left between locating and characterising each lamina I neurone). The last neurone per experiment (randomly selected following each characterisation study) was used to perform one pharmacological study, whereby a drug antagonist (as described later) was applied intrathecally following a number of control tests. Only one neurone, per animal, was used to test the effect of each drug and there was no correlation between the physiological response of the neurone and the order in which it was found in any given experiment. Lamina I neurones were identified according to their depth (0–250 μm) from the surface of the spinal cord where no neuronal activity could be detected. A calculation was used to correct the depth, as the electrode used to record neuronal activity was bent at 45°, using simple trigonometry. On the basis that the electrode trajectory was 45°, a simple right angled triangle could be used to calculate the correction using the cosine equation (cos45°* electrode depth), which meant depths of up to 450 μm could be considered to be within the lamina I layer. The mean depths of the lamina I neurones used in this experiment were 293 ± 18 μm (using bent and straight electrodes), compared to the mean depths of lamina V neurones (n = 69) which were 791 ± 24 μm (using straight electrodes). To ensure correct identity of lamina I, as well as lamina V neurones, measures other than depth examination were used. It was important to ensure lamina I neurones were recorded as opposed to neurones in lamina II and deeper lamina, particularly as location of lamina I neurones are restricted to the thin layer in which they comprise in the dorsal horn of the spinal cord. Indeed, prior studies have illustrated that both lamina II and deep dorsal horn neurones exhibit entirely different response profiles to that of lamina I neurones (Dickenson and Sullivan, 1990). Therefore, the characteristics of lamina I neurones along with depth were intensely analysed. Lamina I neurones were consistently double spiked and appeared and sounded very different to lamina V neurones, which were typically single spiked (see Fig. 1). Lamina I neurones had larger threshold values, and much lower magnitudes of responses, particularly C-fibre, post-discharge and XS spike responses, compared to lamina V neurones. However, it is important to note that lack of histological confirmation means it is difficult to absolutely classify these neurones as definite lamina I and lamina V neurones and they are therefore deemed ‘probable’ lamina I and V neurones. A variety of innocuous and noxious stimuli were used to characterise the neuronal responses in lamina I using an assortment of hot, warm and cold water jets, brush and von Frey hairs. Temperatures of −4, 32, 35, 40 42, 45, 48, 50, 52 °C were applied for 10 s, via a water jet in strict ascending order. Temperatures of 32 °C were taken as neutral and represented...
any mechanical activity elicited by the force of the water jet. Von Frey filaments (Scientific Marketing Associates, 189/191 High Street, Barnet, Herts, EN5 5SU. Cat No. 18011) were also applied in ascending order 1, 5, 9, 12, 15, 30, 75 and 125 g for 10 s. Lastly, a fine brush was applied in gentle stroking patterns to the receptive field area for 10 s. A time interval of 2 min was left between each thermal and mechanical stimulation to prevent desensitisation of the neurone, and 20 min was left between each set of tests. All spontaneous activity of the recorded neurone (always low, approx 0.1–1 Hz), was subtracted from the neuronal response to each stimulus. The response of these neurones to electrical stimuli was also measured by delivering electrical stimuli through two needles inserted into the neuronal peripheral receptive field area, located on the ipsilateral hindpaw. The C-fibre threshold of the neurone was detected and a train of 16 electrical stimuli (0.5 Hz, 2 ms pulse) was applied at three times this level. The threshold was taken as the necessary current to elicit action potential firing of C-fibre latency (90–300 ms post-stimulus, incorporating the average latency firing of C-fibre inputs) in the lamina I neurone. Following each ‘test’ the responses of A-fibres and C-fibres were captured and plotted as a post-stimulus histogram (spike2 software CED Cambridge, UK). The responses were quantified by separating the action potential firing on a latency basis. This was 0–20 and 20–90 ms, respectively, for A-β and A-δ fibres, based on their known conduction velocities, and the distance between the neuronal receptive field and the spinal cord (approximately 12 cm; Gasser and Erlanger, 1927). As well as the responses attributed to different fibre types, post-discharge could be measured.

Post-discharge is the result of repeated C-fibre activity which induces ‘wind-up’, as seen in deep dorsal horn neurones, and is measured as the neuronal activity beyond the C-fibre latency band (number of action potentials fired) between 300 and 800 ms post-stimulus (Dickenson, 1990; Dickenson and Sullivan, 1987, 1990). However, many lamina I neurones did not exhibit a wind-up response as classically described by Mendell (1966) and seen in previous electrophysiological studies in deeper dorsal horn neurones (Dickenson, 1990; Herrero et al., 2000); the response observed was merely a small increase in action potential firing as the stimulus was repeated. This response imitated a slow incremental potentiation and seemed to be a characteristic of these lamina I cells. Therefore the lamina I wind-up effect was different from wind-up observed in lamina V neurones, and therefore deemed ‘reduced wind-up’. Reduced wind-up was presented in the same way as wind-up in that the response to each stimulus was plotted against the stimulus number.

The input response, and excess spikes were also measured. The input was taken as being the number of action potentials, at C-fibre latencies, following the first electrical stimuli in the train of 16. Excess spikes (XS) were calculated by multiplying the input response by 16 (to account for the 16 stimuli applied to the peripheral receptive field) and subtracting this value from the cumulative total number of action potentials fired following

Fig. 1. Post-stimulus histograms showing two typical neuronal responses: (a) probable lamina I, (b) probable lamina V. The figure shows the number of action potentials plotted against latency over a period of 800 ms. The responses of those generated by 16 peripherally applied electrical stimuli at 3 × C-fibre threshold. An example of the double spike characteristic of lamina I neurones is shown in (a).
2.3. Statistical analysis

The maximal effects were shown as means ± SEM. Statistical analyses of these values were performed using a paired t-test on the maximal drug effects compared to pre-drug controls, using the raw data. A 95% CI was used for significance.

3. Results

3.1. Lamina I cell characterisation

A total of 204 probable lamina I neurones were characterised for their electrical response properties, 54 of which were used to fully characterise the naturally evoked response properties. From this population, 18 probable lamina I cells were used in pharmacological studies. The mean depths of the lamina I neurones used in this experiment were 293 ± 18 µm. We are convinced that this population of neurones were lamina I neurones in the most part, however, we did not histologically stain the neurones recorded and therefore absolute confirmation is unavailable. The mean depths of lamina V neurones (n = 69) were 791 ± 24 µm (using straight electrodes). Lamina I neurones were consistently double spiked (see Fig. 1), whereas lamina V neurones typically exhibited single spikes. All lamina I neurones used in the experiment had characterised receptive field areas in the toe/plantar region of the ipsilateral rat hindpaw. Receptive field areas of lamina I neurones to pinch covered 35% (± 0.005) of the rat hindpaw region. C-fibre thresholds for lamina I neurones were 2.9 mA (± 0.2), compared to 1.2–1.5 mA for lamina V neurones. The difference in electrically evoked responses at 3 × C-fibre thresholds, of lamina I and V A-β fibre values, was only marginal. Vast differences in the overall response properties of lamina I and lamina V neurones, of which the majority (77%) were WDR, were evident. In particular, the differences in electrical response values of A-β fibres, C-fibre, post-discharge and excess spikes was large, so that the lamina I responses ranged from 43 to 59% of those recorded in the deep neurones (Table 1). Fig. 1 shows an example of lamina I and lamina V neuronal responses to peripheral electrical stimulation and demonstrates the double spikes elicited by lamina I neurones.

Interestingly, when comparing the superficial and deep cells, lamina I neurones did not exhibit a typical wind-up

<table>
<thead>
<tr>
<th>Neuronal response</th>
<th>Lamina I</th>
<th>Lamina V</th>
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<tbody>
<tr>
<td>A-β fibres</td>
<td>132 ± 0.4</td>
<td>100 ± 4</td>
</tr>
<tr>
<td>A-δ fibres</td>
<td>34 ± 2.5</td>
<td>58 ± 4</td>
</tr>
<tr>
<td>C-fibres</td>
<td>140 ± 7.5</td>
<td>315 ± 14</td>
</tr>
<tr>
<td>Post-discharge</td>
<td>105 ± 7.5</td>
<td>183 ± 19</td>
</tr>
<tr>
<td>XS-spikes</td>
<td>122 ± 11.1</td>
<td>281 ± 30</td>
</tr>
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Table 1
Mean electrical response values of dorsal horn neurones
effect as seen in lamina V cells. Repeated stimulation evoked a much smaller response in these superficial dorsal horn neurones. Wind-up, seen in the large majority of deep dorsal horn neurones, is a sudden increase in evoked responses to constant repeated peripheral stimulation at C-fibre strength, which then leads to neuronal activity beyond the C-fibre latency band (300–800 ms). However, these probable lamina I neurones did not exhibit this typical wind-up response, merely a small increase in action potential firing as the stimulus was repeated. This was termed reduced wind-up, typically seen as a slow incremental potentiation to the repeated stimuli, which enhanced responses by 2–3 fold, rather than the 10–20 fold increases produced by wind-up in deeper dorsal horn neurones. Reduced wind-up was a characteristic of the lamina I cells recorded and the differences can be seen in the lower XS-Spike values exhibited by lamina I neurones compared to lamina V neurones. Lamina I neuronal XS-Spike responses are 57% smaller than lamina V XS-Spike response values. Interestingly, 50% of lamina I neurones had an XS-Spike value < 50 action potentials (Fig. 2).

Of the 54 lamina I neurones characterised for their natural responses (responses greater than 50 action potentials being termed a response, within a 10 s time frame), 74% responded to noxious mechanical (12 g or above), noxious heat (42 °C or above), and noxious cold stimuli alone (−4 °C). These were termed nociceptive specific (NS) (Andrew and Craig, 2002a,b; Craig, 2001). However, the 18% of these NS neurones that responded to noxious cold, were therefore sub-categorised as being polymodal nociceptive (HPC), making up 13% of the whole population and leaving 61% as NS (Craig et al., 2001; Craig and Andrew, 2002). The remainder (26%) was termed WDR, as they responded to both innocuous and noxious mechanical and thermal stimuli. These WDR neurones coded the responses to the stimuli administrated, so that noxious heat and mechanical stimuli elicited larger responses than less noxious or innocuous stimuli. A total of 86% of the entire neuronal population (54 cells) responded to brush stimuli, this could be sub-divided, illustrating that 90% of NS, 71% of HPC and all WDR responded to brush stimuli even though the NS cells were high threshold to punctate mechanical stimulation. It is important to mention the paradox that responses of probable lamina I neurones to brush stimuli could be found for neurones with only high thresholds to von Frey stimuli. This makes it difficult to ascertain whether these neurones are entirely NS in characteristic. However, lack of responses to innocuous punctate stimuli, and in addition, an inability to code stimuli other than that in the noxious range, makes it difficult to classify these neurones as WDR. It is unlikely that sensitisation of the neurones, following peripheral application of both electrical and mechanical stimuli is responsible for the brush responses since neurones with this property could be found in the first penetration into the spinal cord.

WDR neuronal responses could be sub-categorised. Of the WDR neurones 31% were WDR to only heat stimuli and their mechanical response mimicked an NS neurone. A further 31% of WDR neurones were WDR to mechanical stimuli alone and appeared NS to heat stimuli. The remaining 38% were WDR to both stimulus modalities. This meant that from the total population 9% were WDR to both stimulus modalities and 7% to either mechanical or thermal stimuli. From the population of WDR neurones, 54% had a response to noxious cold. Out of the NS neuronal population, 2% had no responses to mechanical or heat responses, 9% had no response to the thermal stimuli and 6% had no response to mechanical stimuli. It is important to note that no correlation was found between the type of neurone characterised and the order and timing in which it was found throughout each experiment. When comparing lamina I to lamina V response values, von Frey forces of 9 g and over produced larger responses in lamina V neurones with the difference consistently between 42 and 64%, with increasing force. At von Frey forces of 75 g in the very noxious range, lamina I responses are 42% of lamina V.

![Graph](image)

**Fig. 2.** Number of action potentials produced in response to each of the 16 electrical stimuli, applied to the peripheral receptive field at 3 x C-fibre threshold. In this example the lamina I neurone did not show a typical wind-up response, whereas the lamina V neurone clearly shows wind-up. We termed the responses of these lamina I cells reduced wind-up.

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**Table 2.** Mean mechanical response values of dorsal horn neurones

<table>
<thead>
<tr>
<th>von Frey (g)</th>
<th>Lamina I</th>
<th>Lamina V</th>
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<tbody>
<tr>
<td>5</td>
<td>56 ± 9.6</td>
<td>52 ± 10</td>
</tr>
<tr>
<td>9</td>
<td>60 ± 10.9</td>
<td>115 ± 22</td>
</tr>
<tr>
<td>12</td>
<td>85 ± 16.1</td>
<td>133 ± 21</td>
</tr>
<tr>
<td>15</td>
<td>124 ± 20.2</td>
<td>239 ± 35</td>
</tr>
<tr>
<td>30</td>
<td>187 ± 26.8</td>
<td>385 ± 52</td>
</tr>
<tr>
<td>75</td>
<td>273 ± 34</td>
<td>655 ± 74</td>
</tr>
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</table>
neuronal responses (Table 2). Comparison of the heat response in both lamina I and V neurones, demonstrates large differences in response values in the very noxious range (>48 °C). At 48 °C, the response of lamina I neurones is 78% of the lamina V response. At 50 °C the response is just 54% of lamina V responses and at 52 °C it was reduced further to 40% (Table 3).

Interestingly, the majority of lamina I neurones had a heat threshold of 45 °C, which is clearly in the noxious range. Furthermore, 18% of lamina I neurones responded only to the highest von Frey force of 125 g, 10% to 75 g, 24% to 30 g, 18% to 15 g and 14% to 12 g. The remainder of the population (16%) responded to the innocuous von Frey forces (1, 5 and 9 g).

A random selection of lamina I neurones were selected for pharmacological studies, so that no discrimination was made between NS, HPC and WDR lamina I neurones. Therefore neurones used for the effects of each receptor antagonist was based on a population of lamina I cells with the same proportions of each physiologically categorised type mentioned above.

3.2. Effect of spinal NBQX on the electrical evoked responses of lamina I neurones

The results of three doses of NBQX (0.5, 5, 50 µg), administered intrathecally, were tested on the electrically evoked response and the peripheral receptive field size of a group of eight lamina I neurones. Spinal NBQX did not alter the A-β fibre responses of lamina I neurones, however, it did produce a reduction in the input response (57% of pre-drug control value, at 50 µg NBQX). The A-δ, C-fibre, post-discharge and excess spike responses were significantly inhibited with reductions to 18–66% of pre-drug control values, seen after 5 and 50 µg (Fig. 3). Receptive field areas were also significantly reduced by spinal NBQX to between 34 and 44% pre-drug control values (5 and 50 µg) (Fig. 4). The reduced wind-up response, exhibited by lamina I neurones, was reduced by all three doses of NBQX and completely eliminated by 50 µg NBQX (Fig. 5).

3.3. Effect of spinal APV on the electrical evoked responses of lamina I neurones

The results of three doses of APV (50, 100, 500 µg), administered intrathecally, were tested on the electrically evoked response of eight lamina I neurones. Spinal APV had little effect on A-β, C-fibre, post-discharge and excess spike-evoked responses. However, there were significant reductions in the A-δ-evoked responses and input by APV to between 60 and 65% of the pre-drug control value (Fig. 6). APV had no effect on the reduced wind-up response of lamina I neurones (Fig. 7).

3.4. Effect of spinal bicuculline on electrical and natural evoked responses of lamina I neurones

Bicuculline was applied intrathecally in three doses (0.5, 5 and 50 µg), and both the electrically evoked responses and mechanical and heat-evoked responses of eight lamina I neurones were examined. The neurones used in this set of

<table>
<thead>
<tr>
<th>Heat stimuli (°C)</th>
<th>Lamina I</th>
<th>Lamina V</th>
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</thead>
<tbody>
<tr>
<td>32</td>
<td>149 ± 24</td>
<td>158 ± 39</td>
</tr>
<tr>
<td>35</td>
<td>154 ± 25</td>
<td>212 ± 50</td>
</tr>
<tr>
<td>40</td>
<td>183 ± 27.5</td>
<td>206 ± 41</td>
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<tr>
<td>42</td>
<td>183 ± 26.7</td>
<td>221 ± 43</td>
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<tr>
<td>45</td>
<td>258 ± 32</td>
<td>289 ± 44</td>
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<tr>
<td>48</td>
<td>341 ± 38</td>
<td>439 ± 59</td>
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<tr>
<td>50</td>
<td>447 ± 53.1</td>
<td>833 ± 92</td>
</tr>
<tr>
<td>52</td>
<td>427 ± 24</td>
<td>1077 ± 97</td>
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</table>
experiments had similar response properties to those recorded overall, C-fibre values averaged 190 ± 10 spikes and A-β fibre values averaged 27.3 ± 4 spikes. As a restricted amount of natural stimuli was applied to these neurones to avoid sensitisation, we loosely categorised them as NS (60%) and WDR (40%) based on their response to von Frey 9 (innocuous) and von Frey 30 (noxious) as well as thermal stimuli.

Their response to noxious thermal stimuli (45 °C) was 121 ± 32.2 spikes and the response to von Frey 9 was 59 ± 18 spikes and 146 ± 43 spikes for von Frey 30, all small compared to those of deeper neurones. Spinal bicuculline had virtually no effect on A-β, input and the reduced wind-up responses observed. However, there was a significant reduction at low doses of C-fibre and post-discharge-evoked responses to between 64.9 and 81% of pre-drug control values, C-fibre, 0.5 μg, post-discharge, 0.5 μg. Interestingly, there was also a large and significant facilitation of A-β-evoked electrical responses (to 279% of pre-drug control values, 50 μg) (Fig. 8).

Bicuculline (0.5, 5 and 50 μg) also resulted in a facilitation of lamina 1 neurones to von Frey 30 (173–277% of pre-drug control values, 5 and 50 μg). Bicuculline also resulted in a facilitation of von Frey 9 responses in lamina 1 neurones (between 135 and 308% of pre-drug control values, 0.5 μg) (Fig. 9). Bicuculline had

Fig. 4. Effect of spinal NBQX on the receptive field area of lamina I neurones. Changes in lamina I neuronal receptive field area following NBQX administration are expressed as a percentage of the control response. Mean ± SEM of the maximal effect in a 60 min time interval following NBQX administration is shown with n = 8. *P < 0.05, **P < 0.001 illustrate that the maximal effect is significantly different from the pre-drug control values.

Fig. 5. Effect of NBQX on the reduced wind-up response in probable lamina I neurones. Graph shows the effect of NBQX on a single lamina I neuronal response. As in Fig. 2, the number of action potentials produced per stimulus is plotted.

Fig. 6. Effect of spinal APV (50, 100, 500 μg) on the electrically evoked neuronal responses. (a) Input, A-β, A-δ fibres; (b) C-fibre, post-discharge, excess spikes. Graph shows mean ± SEM of the maximal effect of APV over 60 min, n = 8. *P < 0.05, **P < 0.001, illustrates that the maximal effect is significantly different to pre-drug control values.
Fig. 7. Lack of effect of APV on the reduced wind-up response in lamina I neurones. Graph shows the effect of APV on a single lamina I neuronal response. Number of action potentials produced per stimulus is plotted.

Fig. 8. Effect of spinal bicuculline (0.5, 5 and 50 μg) on the naturally evoked neuronal response to von Frey forces 9 and 30 g. Graph shows mean ± SEM of the maximal effect of bicuculline over 60 min, n = 8. *P < 0.05, **P < 0.001 illustrate that the maximal effect is significantly different to pre-drug control values.

Fig. 9. Effect of spinal bicuculline (0.5, 5 and 50 μg) on the naturally evoked neuronal response to von Frey forces 9 and 30 g. Graph shows mean ± SEM of the maximal effect of bicuculline over 60 min, n = 8. *P < 0.05, **P < 0.001 illustrate that the maximal effect is significantly different to pre-drug control values.

no significant effects on the receptive field areas of these neurones (Fig. 10). Application of bicuculline appeared to significantly reduce the responses of lamina I neurones to heat. The two top doses (5 and 50 μg) resulted in an inhibition compared to pre-drug control values (33–9% of pre-drug controls, 45–32 °C) (Fig. 11).

4. Discussion

We have characterised a population of high threshold lamina I neurones in the rat lumbar spinal cord with a response profile, location and reduced wind-up that distinguishes them from deep dorsal horn neurones. Andrew and Craig (2002a,b), studying STT lamina I neurones, divided them into NS, polymodal nociceptive (HPC), cold or warm. NS neurones respond to only pinch and/or heat (Craig and Hunsley, 1991; Craig and Serrano, 1994; Craig et al., 1994, 1996) and HPC neurones also to noxious cold. We conclude that 13% of our neuronal population was HPC neurones, and 61% NS. However, unlike Andrew and Craig, in the cat, we also observed 26% WDR neurones.
We sub-categorised these into those responding to mechanical stimuli alone (31% of the WDR population) and WDR neurones to heat stimuli alone (31%). The remaining neurones could not be termed merely WDR since they were NS to the other modality. These results are complicated by a frequent innocuous brush response that may be species dependent.

Our lamina I neurones are similar to rat lamina I spinoparabrachial neurones (Bester et al., 2000a,b) where 75% were NS, although 35% were likely to be HPC (Bester et al., 2000a,b). If we group our HPC with NS populations, 74% of neurones are NS, of which 18% are HPC, almost identical to Bester et al. Out of the total population, 28% had a cold response in addition to their mechanical and heat responses. These cold units had small receptive fields, as did the majority of neurones encountered. Of the lamina I cells, 75% had receptive field areas less than 50% of the total rat hindpaw area. The large majority of the lamina I cells only responded to von Frey forces of 12 g and above (the majority with thresholds of 30 g) which corresponds to the noxious range, and temperatures of >45 °C, also in the noxious range. C-fibre responses evoked by transcutaneous electrical stimuli were small and few of the neurones exhibited a typical wind-up response as described in deep dorsal horn neurones. Thus with a train of 16 stimuli there was no sudden increase in response after the first few stimuli, followed by 5–10 fold increases in activity, a key characteristic of NMDA-mediated wind-up seen in the majority of lamina V neurones (Dickenson, 1990; Dickenson and Sullivan, 1987). By contrast, all lamina I neurones demonstrated a slow progressive increase in activity with each successive stimulus—we have termed this reduced wind-up. This was true for all cells whether NS or WDR. Correspondingly, only 50% of lamina I neurones showed any excess activity and this was much less than that produced by wind-up in lamina V neurones. These characteristics agree well with those on spinoparabrachial neurones in the rat (Bester et al., 2000a,b). Our pharmacological data further supports the idea that lamina I neurones lack functional NMDA components to their responses. The slow sequential increases in activity (reduced wind-up) seen here, may well derive from summation of slow peptide-mediated depolarisations.

Comparing these lamina I to deep dorsal horn neurones, lamina I neurones had smaller responses to electrical stimulation than lamina V neurones. The largest differences were seen with C-fibres, post-discharge and excess spikes, where the lamina I neuronal response was approximately half. Reduced responses were also evident when comparing the von Frey and thermal responses. Lamina I had smaller responses at von Frey forces of 9 g and above and to heat at 42 °C and above compared to deep neurones. Although lamina I cells had smaller receptive field areas than deeper dorsal horn neurones they had a similar somatotopic organisation (Woolf and Fitzgerald, 1983).

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**Fig. 10.** Effect of spinal bicuculline (0.5, 5 and 50 μg) on the receptive field area of lamina I neurones. Graph shows mean ± SEM receptive field area (weight representation) at 40 min, n = 8.

**Fig. 11.** Effect of spinal bicuculline (0.5, 5 and 50 μg) on the naturally evoked neuronal response to a thermal stimulus at 32 and 45 °C. Graph shows mean ± SEM of the maximal effect of bicuculline over 60 min, n = 8. *p < 0.05, **p < 0.001 illustrate that the maximal effect is significantly different to pre-drug control values.
Glutamate-like immunoreactivity is seen in neurones, fibres and terminals throughout the superficial dorsal horn (Miller et al., 1988) and in vitro studies show NMDA receptor expression and function at synapses (Bardoni et al., 2000; Li et al., 1999a,b). Other studies describe high levels of both glutamate and receptor-binding sites within the outer dorsal laminae (Engelman et al., 1999; Hwang et al., 2001; Kerchner et al., 2001; Swett et al., 1985). We examined the pharmacological mechanisms involved in the responses of lamina I neurones. NBQX (AMPA receptor antagonist) had marked dose-dependent inhibitory effects on the neuronal responses and also reduced the receptive field areas of lamina I neurones. Although there was only a minor reduction in the A-beta-evoked responses, the A-beta fibre, C-fibre, post-discharge and excess spikes were all reduced by approximately 60–80% with the highest dose. The input was only reduced by 40%. The characteristic reduced wind-up response in such neurones was also clearly inhibited as reflected by the excess spike measure. In marked contrast, APV (NMDA receptor antagonist) inhibited the evoked lamina I neuronal responses to a much lesser degree. However, there was a significant reduction in the A-beta fibre electrical responses (40% inhibition). Input, a measure of pre-synaptic transmission, was also reduced by 40%. APV had no effect on the reduced wind-up response in lamina I neurones confirming the marked difference in this parameter in the two areas of the dorsal horn.

These results indicate that the majority of synaptic transmission from primary afferents onto lamina I neurones is AMPA receptor mediated with minimal NMDA receptor contributions. However, the reduction in the input responses seen with both NBQX and APV indicates that there may be pre-synaptic AMPA/Kainate and NMDA receptors. Nevertheless, as the effects of these antagonists on input were considerably less than the effect of NBQX on other measures, we would conclude that post-synaptic AMPA receptors dominate the responses of these cells to noxious and innocuous stimuli. The lack of any functional NMDA component to the responses may be altered in pathological states. In this regard, it is interesting to note that very high frequency stimulation can elicit an NMDA-mediated LTP of these neurones (Sandkühler et al., 1989, 1997).

The high threshold and small evoked responses of these NS neurones could partly be due to the reduced wind-up response, but also tonic or evoked inhibitions could suppress the responses of these cells. We therefore blocked the GABA_A receptor with bicuculline. A-beta responses were markedly augmented, yet C-fibre and post-discharge were slightly decreased. A-beta input responses and receptive field were unaltered. The increase in A-beta-evoked activity was paralleled by an increase in the von Frey responses. However, a significant inhibition of the noxious heat-evoked responses was also observed. This may have arisen from a depolarising block or may implicate a different mechanism for the control of thermal as opposed to mechanical stimuli within lamina I. However, bicuculline-induced alterations in mechanical response properties could influence thermal response properties using this contact thermal stimulation. Thus GABA_A receptors appear to mainly control the mechanical responsivity of these lamina I neurones and this appears to be primarily mediated by A-beta fibres. Studies in the cat observed increases in activity, particularly mechanical stimulation after bicuculline (Sorkin et al., 1998) and we reported similar increases in A-beta and mechanical responses in deep dorsal horn neurones (Kontinen et al., 2001). These studies confirm GABA_A’s distinct role in mediating inhibitory controls and suggest that some of the responses of lamina I cells are suppressed by GABA_A receptors. In particular, the enhanced von Frey and A-beta responses fit well with the preferential location of GABAergic synapses on A-beta terminals (Bernardi et al., 1995). Thus some of the high threshold nature of lamina I cells is due to active inhibition.

Ablation of the majority of NK1-containing lamina I neurones using SP-SAP reduces responses to acute nociceptive stimuli (Marshall et al., 1996; Todd et al., 2000). There are also marked reductions in the mechanical and thermal coding of deep dorsal horn neurones, wind-up, receptive field size and in behavioural studies, responses to formalin and various forms of hyperalgesia (Mantyh et al., 1997; Suzuki et al., 2002; Wiley and Lappi, 1999). Gephyrin-rich neurones, which lack the NK1 receptor (Puskár et al., 2001), have a large density of GABAergic inhibitory inputs. This suggests that antagonism of the GABA_A receptor, resulting in facilitation of both the A-beta fibre and mechanical responses, is via gephyrin-rich neurones and not the NK1-containing neurones in lamina I.

In conclusion, these studies describe a distinct population of neurones located in the superficial dorsal horn that are mainly high threshold nociceptive—which may be partly due to their lack of the typical NMDA-mediated wind-up response seen in deeper dorsal horn neurones. Pharmacological studies suggest a vital role of the AMPA receptor in lamina I neuronal responses, particularly in their A-beta and C-fibre responses and a GABAergic control of A-beta fibre and mechanical responses. This suggests that information relayed to areas, such as the parabrachial area in the rat, will be dominated by noxious inputs and remains constant throughout repeated stimuli. This may allow the emotional and autonomic areas of the brain to be sent different information from that ascending in the spinthalamic tract, mostly arising from deep WDR neurones that possess quite different physiological and pharmacological properties.

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Sandkuhler J, Chen JG, Cheng G, Randic M. Low-frequency stimulation of afferent Adelta-fibers induces long-term depression at primary afferent
Aim of Investigation: To study the synaptic activity of excitatory circuitry in the dorsal horn, synaptic responses were recorded from spinal dorsal horn neurons following the activation of central terminal VR1 receptors and P2X receptors. Methods: Using an upright microscope equipped with IR-DIC system, patch clamp recordings were made from lamina II and V neurons. Results: The activation of central terminal VR1 receptors with capsaicin increased frequency of mEPSCs in superficial dorsal horn neurons. The capsaicin effects were due to the enhancement of glutamate release from the central terminals of capsaicin-sensitive afferent fibers. The capsaicin-induced activity spreads polysynaptically to deep dorsal horn neurons, resulting in a large increase in the frequency of mEPSCs. In contrast to the polysynaptic properties of capsaicin-induced increases of sEPSC frequency in lamina V, we found that lamina V neurons received monosynaptic inputs from abmATP-sensitive/capsaicin-insensitive terminals. Activation of P2X receptors on these terminals with abmATP resulted in a large increase in the frequency of mEPSCs due to the synaptic release of glutamate onto lamina V neurons. The convergence of these inputs generates high frequency action potential firing due to a temporal summation. Conclusions: These observations provide a mechanism of sensory integration for different sensory inputs and may have implications in sensory hypersensitivity.

1190-P106 DESCENDING MODULATION OF THORACIC VISCEROCEPTIVE TRANSMISSION BY UPPER CERVICAL SPINAL NEURONS IN RATS

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Aim of Investigation: Our previous studies show that upper cervical spinal neurons modulate activity of lumbosacral spinal cells receiving colorectal inputs. The purpose of this study was to examine effects of exciting upper cervical spinal neurons on spontaneous and evoked activity of thoracic spinal neurons responsive to noxious cardiac and esophageal stimuli.

Methods: Extracellular potentials of single T3 neurons were recorded in pentobarbital anesthetized male rats. A catheter was placed in the pericardial sac to administer bradykinin (10^5 M, 0.2 ml, 1 min) as a noxious cardiac stimulus and saline as control. Thoracic esophageal distension (0.1-0.5 ml, 20 s) was produced by water inflation of a latex balloon. A glutamate pledget (1 M, 1-3 min) was placed on the surface of C1-C2 segments to chemically activate upper cervical spinal neurons.

Results: Glutamate at C1-C2 inhibited spontaneous activity and/or excitatory responses of 34/44 (77%) neurons to noxious esophageal distension. After spinal transection at the rostral C1 segment, glutamate at C1-C2 still significantly reduced spontaneous activity and/or excitatory responses of 5/6 neurons to intrapericardial bradykinin.

Conclusions: Chemical activation of C1-C2 spinal neurons primarily produced descending inhibition of background activity and excitatory responses of thoracic spinal neurons to noxious cardiac and/or esophageal stimuli. Modulation from upper cervical spinal neurons on activity of distant thoracic neurons did not require supraspinal structures.

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1191-P107 INTRATHECAL PERTUSSIS TOXIN INDUCED-THERMAL HYPERALGESIA: INVOLVEMENT OF NMDA-PKC γ SYSTEM

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Aim of Investigation: The present study examined the effect of intrathecal (i.t.) pertussis toxin (PTX) on rat nociceptive threshold and protein kinase C (PKC) expression in the spinal cord. The role of NMDA receptor on these changes was also examined.

Methods: Male Wistar rats were implanted with two i.t. catheters. One catheter was connected to a mini-osmotic pump for saline or D-AP5 (2µg/hr) infusion. Two days later, saline or PTX (2µg) was administered via the other catheter and flushed with 10µl saline. On day 4 PTX or saline injection and hyperalgesia were examined. Then rats were sacrificed by decapitation and the dorsal part of the lumbosacral spinal segments were removed for western blot.

Results: In PTX-treated rats, not only was thermal hyperalgesia exhibited, but the content of PKC γ in synaptosomal and cytosolic fraction was significantly increased. Neither α, β1 nor β2 isoforms were affected by the PTX treatment in synaptosomal and cytosolic fraction. Infusion of NMDA antagonist D-AP5 for 6 days apparently prevented the thermal hyperalgesia and the increase of PKC γ isoform expression in PTX-treated rats.

Conclusions: These data show that i.t. PTX injection induced a thermal hyperalgesia accompanied by an increase of PKC γ in synaptosomal membrane and cytosolic expression of the dorsal part of lamina spinal cord. These effects were inhibited by the NMDA receptor antagonist.
A antagonist) were applied spinaly.

Results: A population of superficial neurones was defined with much smaller responses to peripheral stimuli than deeper neurones. Many of these neurones had very high thresholds to natural stimuli. The majority of cells showed a slow incremental potentiation (SIP) to repeated C-fibre stimuli that was clearly different from wind-up. NBQX markedly inhibited C-fibre evoked responses and reduced receptive field size but lacked any selective effect on SIP. A-beta responses were likewise unaltered. The A- and C-fibre responses and SIP were not altered by AP-5. Bicuculline selectively enhanced the A-beta-evoked and von Frey 30 g responses.

Conclusions: It would therefore appear that much of the synaptic responses of these lamina 1 neurones is through AMPA mediated transmission and that these cells do not possess functional NMDA receptors under these conditions. GABA-A receptors control A-beta responses of these cells and SIP is likely to involve peptide rather than glutamate and NMDA wind-up mechanisms.

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1193-P109 THE ROLE OF NEURONAL NITRIC OXIDE SYNTHASE (NOS) IN DEVELOPING RAT SPINAL NOCICEPTIVE RESPONSES
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Aim of Investigation: To investigate the role of nNOS in the development and modulation of spinal nociceptive responses in the rat.

Methods: In vivo extracellular recordings were made of wide dynamic range neurones in lamina I of the dorsal horn of anaesthetized rats at postnatal ages (P) 14, 21, 28 and adult as described by Urch et al (Dev. Brain Res. 2001, 126; 81-89). NOS blockers were applied directly onto the spinal cord in a volume of 20 µl at the following doses 7NI: 10 µg, 100 µg, 1000 µg and L-NAME: 100 µg, 500 µg and 2000 µg. The response to each dose was followed for one hour. There were at least 6 animals per group.

Results: A dose dependent inhibition of the C fibre and post-discharge evoked responses and the excess counts (windup) was seen with both 7NI and L-NAME. There was no difference between age groups. However the primary evoked response at 1000 µg 7NI and 500 µg L-NAME was significantly different between the P14 age group and the P21, 28 and adult groups (p<0.035).

Conclusions: The data supports the role of nNOS/N0 in the modulation of the nociceptive responses and synaptic plasticity rather than a role in postnatal development and maturation. The expression of nNOS appears to parallel C fibre function rather than to direct maturation in contrast to the NMDA receptor. Of note is the variation in primary evoked response of the immature P14 pups to 7NI and L-NAME. This may be due to the lack of mature descending inhibitory pathway. This combined with NO inhibition revealed a relative lack of presynaptic control in the P14 age group. In conclusion the nNOS/NO pathway modulates nociceptive transmission in a highly complex manner and appears to be fully mature and functional from P14 onwards.

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1194-P110 ROLE OF METABOTROPIC GLUTAMATE RECEPTORS-5 IN THE INITIATION BUT NOT THE MAINTENANCE OF PAIN
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Aim of Investigation: Group I metabotropic glutamate receptors (mGluR) include the mGluR1 and mGluR5 subtypes and are found in the spinal dorsal horn. Although the group I mGluR are believed to have a role in injury-induced pain, the role of each subtype, particularly the mGluR5, has not been well characterized. The effect of a subtype selective and potent mGluR5 antagonist, 2-methyl-6- (phenylethynyl) pyridine (MPEP) on acute activation of spinal group I mGluR and nerve injury-evoked pain was examined.

Methods: Rats with intrathecal (i.t.) catheters were prepared. Rats were i.t. injected with the group I mGluR-selective agonist RS-3,5-dihydroxyphenylglycine (DHPG) and MPEP. The rats were placed on a cold surface and their responses (total number of lifts, cumulative duration of lifting) were observed. Additional rats with a chronic constriction injury (CCI) with i.t. catheters were prepared. These rats were i.t. injected with MPEP and evaluated on the cold plate test.

Results: Intrathecal pretreatment with MPEP blocked the DHPG-evoked behaviors on the cold plate test. In contrast, i.t. injection of MPEP after DHPG did not suppress DHPG-evoked behaviors. Following a CCI, rats displayed increased lifting and duration of lifting from the cold surface. Intrathecal injection of MPEP did not affect CCI-induced cold sensitivity.

Conclusions: Blockade of the mGluR5 prior to activation of spinal mGluR5 with DHPG prevents the onset of enhanced cold sensitivity. This suggests that the mGluR5 is involved in the initiation of abnormal nociception. However, ongoing cold sensitivity, associated with either nerve injury or prior i.t. injection of DHPG, may not be dependent on spinal mGluR5. Thus, spinal mGluR5 may be involved in the initiation but not maintenance of chronic pain.

1195-P111 PLASTICITY OF WIDE DYNAMIC RANGE NEURONES FOLLOWING SITE-SELECTIVE ABLATION OF NK1 RECEPTOR EXPRESSING LAMINA I NEURONES IN RAT SPINAL CORD
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Aim of Investigation: To investigate the electrophysiological consequences of ablating lamina I NK1-receptor expressing neurones on the responses of deep dorsal horn neurones.

Methods: Substance P (SP) is implicated in spinal nociceptive transmission. A conjugate of SP and saporin (SAP) was given intrathecally to selectively ablate NK1-receptor expressing neurones in lamina I of the lumbar spinal cord. Separate groups of animals were infused with SAP alone or saline. In vivo electrophysiology was conducted under halothane anaesthesia 28 days later, and the responses of deep lamina V wide dynamic range (WDR) neurones were characterised. In a separate study, the responses to peripheral formalin injection were quantified.

Results: WDR neurones in SP-SAP treated rats had a significant deficit in mechanical and thermal coding, prominent in the noxious range of von Frey filaments (30-75 grams), and in the warm to noxious temperature range (42-48 degrees). SP-SAP resulted in a significant reduction of the receptive field size and in the magnitude of wind-up of spinal neurones. Similarly, there was a marked attenuation of the second phase of the formalin response, as compared to SAP or saline controls.

Conclusions: Ablation of lamina I NK1-Rs significantly reduces the central excitability of WDR neurones, as manifested by the reduction in wind-up and receptive field sizes. Our results suggest that lamina I cells are critical links between peripheral afferent traffic and the full accurate coding of sensory information in deep dorsal horn neurones.