THE ROLE OF THE LATERAL SPINAL NUCLEUS IN NOCICEPTION

by

Paul Michael Rea

A thesis presented for the degree of Doctor of Philosophy in Neuroscience and Molecular Pharmacology, Faculty of Biomedical and Life Sciences, University of Glasgow

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SUMMARY

The lateral spinal nucleus (LSN), located in the dorsolateral funiculus, is an area that has been poorly understood, but has been implicated in nociception. To investigate the function of this nucleus, three broad areas were investigated: responses to nociceptive stimuli, neurochemical relations to the NK-1 receptor, and projections from this nucleus to several brain centres, to try to gain a greater understanding of the functions of this nucleus. The following conclusions can be drawn from the studies undertaken here:

- A series of double-labelling experiments for confocal microscopy were carried out in the rat (Sprague-Dawley) to investigate the LSN responses to a variety of peripheral cutaneous noxious stimuli. It was found that the LSN responds to both thermal and chemical peripheral cutaneous noxious stimulation. However, unlike as previously thought, only a small number of neurons in the LSN are activated by a peripheral noxious stimulus, with hot water (55°C applied to the hind-paw) activating the most, as revealed by Fos immunoreactivity. Only 15% of LSN neurons showed response to this peripheral noxious stimulus. Interestingly, unlike the superficial dorsal horn (SDH), bilateral activation of LSN neurons after the application of a peripheral noxious stimulus was found in most of the experiments carried out.

- Triple and quadruple-labelling experiments for confocal microscopy were carried out in the rat to investigate neurochemical relations at this site. It was found that although the LSN is abundant in staining for substance P, the number of LSN neurons showing immunoreactivity for the target of substance P (the NK-1 receptor) represented only one-third of all neurons at this site. However, substance P and nitric oxide synthase were associated with NK-1 neurons, and specifically nitric oxide synthase terminals were preferentially associated with NK-1 neuronal cell bodies. However, unlike the
superficial dorsal horn, nitric oxide synthase terminals were not associated with inhibitory GABAergic neurons.

- Using retrograde injection techniques (in the rat) combined with multiple immunolabelling for confocal microscopy, the LSN was shown to project to areas traditionally associated with nociception (caudal ventrolateral medulla and mediodorsal thalamus) but also projected to the hypothalamus and also the lateral globus pallidus. Indeed, the regions found to have the most projections from the LSN were the lateral and medial hypothalamus, with most of those neurons (>80%) possessing the NK-1 receptor. Interestingly, although numbers of retrogradely labelled neurons were low, they represented 30% of all labelled neurons that projected from the LSN to the lateral globus pallidus.

In conclusion, the extent of involvement of the LSN in nociception is less than previously thought, but with projections to the hypothalamus, it could be postulated that the LSN functions as an integrative nucleus for autonomic and homeostatic functions, and related motivational and affective responses to autonomic function.
ACKNOWLEDGEMENTS

I would like to dedicate this thesis to my parents, Nancy and Paul, and to my dearest brother Jaimie, for all sharing the rollercoaster with me.

Many warmest heartfelt thanks to dear friends and colleagues who have been an immense support to me during this challenging period, thank-you Mr Richard Locke and Dr John Shaw-Dunn. A special thank-you to Ms Caroline Morris, who has always been there for me.

Last, and no means least, a supervisor who has been a strength of support, even through the toughest times, Professor David Maxwell, thank-you!

DECLARATION

I certify that the authorship of this thesis is entirely my own and that the work presented in it is substantially my own. Professor David J. Maxwell contributed to this work by performing transcardial perfusions and stereotaxic brain injections.
### LIST OF ABBREVIATIONS

(Terms in italics are not defined in the text)

<table>
<thead>
<tr>
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<th>Definition</th>
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<tbody>
<tr>
<td>AHA</td>
<td>Anterior hypothalamic area, anterior part</td>
</tr>
<tr>
<td>AHC</td>
<td>Anterior hypothalamic area, central part</td>
</tr>
<tr>
<td>AHP</td>
<td>Anterior hypothalamic area, posterior part</td>
</tr>
<tr>
<td>AM</td>
<td>Anteromedial thalamic nucleus</td>
</tr>
<tr>
<td>AMPA</td>
<td><em>alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid</em></td>
</tr>
<tr>
<td>AP</td>
<td>Anterior-posterior</td>
</tr>
<tr>
<td>AVDM</td>
<td>Anteroventral thalamic nucleus, dorsomedial part</td>
</tr>
<tr>
<td>AVVL</td>
<td>Anteroventral thalamic nucleus, ventrolateral part</td>
</tr>
<tr>
<td>CaM</td>
<td>Calmodulin</td>
</tr>
<tr>
<td>c-Fos</td>
<td>Cellular proto-oncogene from the immediate early gene transcription factors</td>
</tr>
<tr>
<td>cGMP</td>
<td>Cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>CGRP</td>
<td>Calcitonin gene related peptide</td>
</tr>
<tr>
<td>ChAT</td>
<td>Choline acetyltransferase</td>
</tr>
<tr>
<td>CM</td>
<td>Central medial thalamic nucleus</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>Cpu</td>
<td>Caudate putamen (striatum)</td>
</tr>
<tr>
<td>CTb</td>
<td>Cholera toxin B subunit</td>
</tr>
<tr>
<td>Cu</td>
<td>Cuneate nucleus</td>
</tr>
<tr>
<td>CVLM</td>
<td>Caudal ventrolateral medulla</td>
</tr>
<tr>
<td>DAB</td>
<td>Diaminobenzidine</td>
</tr>
<tr>
<td>DG</td>
<td>Dentate gyrus</td>
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<tr>
<td>DMD</td>
<td>Dorsomedial hypothalamic nucleus, dorsal part</td>
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<tr>
<td>DRt</td>
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<tr>
<td>DV</td>
<td>Dorsal-ventral</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular related kinase</td>
</tr>
<tr>
<td>FG</td>
<td>Fluoro-Gold</td>
</tr>
<tr>
<td>Fi</td>
<td>Flocculus</td>
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<td>FMRF</td>
<td><em>L-phenylalanyl-L-methionyl-L-arginyl-L-phenylalaninamide</em></td>
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<tr>
<td>Fos</td>
<td>Nuclear phosphorylated protein</td>
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<td>GABA</td>
<td><em>Gamma-aminobutyric acid</em></td>
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<tr>
<td>GAD</td>
<td>Glutamic acid decarboxylase</td>
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<tr>
<td>Gly-T2</td>
<td><em>Glycine transporter 2</em></td>
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<tr>
<td>GP</td>
<td>Globus pallidus</td>
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<tr>
<td>Gr</td>
<td>Gracile nucleus</td>
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<td>Gracile fasciculus</td>
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<tr>
<td>Hi</td>
<td>Hippocampus</td>
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<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
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<tr>
<td>IAM</td>
<td>Interanteromedial thalamic nucleus</td>
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<tr>
<td>ic</td>
<td>Internal capsule</td>
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<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
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<tr>
<td>ip</td>
<td>Intraperitoneal</td>
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<tr>
<td>LCN</td>
<td>Lateral cervical nucleus</td>
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<td>LGP</td>
<td>Lateral globus pallidus</td>
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<td>LH</td>
<td>Lateral hypothalamus</td>
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<td>Abbreviation</td>
<td>Term</td>
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<tr>
<td>LHbL</td>
<td>Lateral habenular nucleus, lateral part</td>
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<tr>
<td>LHbM</td>
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<tr>
<td>L-NAME</td>
<td>N\textsuperscript{ω}-nitro-L-arginine methyl ester</td>
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<td>LPMR</td>
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<td>Lateral reticular nucleus</td>
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<tr>
<td>LSN</td>
<td>Lateral spinal nucleus</td>
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<tr>
<td>LTP</td>
<td>Long term potentiation</td>
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<td>MdD</td>
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<td>MDL</td>
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<td>MdV</td>
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<td>Mfb</td>
<td>Medial forebrain bundle</td>
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<td>MH</td>
<td>Medial hypothalamus</td>
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<td>Medial lemniscus</td>
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<td>ML</td>
<td>Medial-lateral</td>
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<td>mlf</td>
<td>Medial longitudinal fasciculus</td>
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<td>MPC</td>
<td>Medial prefrontal cortex</td>
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<td>MRF</td>
<td>Medullary reticular formation</td>
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<td>NADPH-d</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
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<tr>
<td>NeuN</td>
<td>Neuronal marker</td>
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<td>NK-1</td>
<td>Neurokinin-1</td>
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<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartic acid</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>NPY</td>
<td>Neuropeptide Y</td>
</tr>
<tr>
<td>NTS</td>
<td>Nucleus tractus solitarius</td>
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<tr>
<td>Opt</td>
<td>Optic tract</td>
</tr>
<tr>
<td>ox</td>
<td>Optic chiasm</td>
</tr>
<tr>
<td>PAG</td>
<td>Periaqueudal grey</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PH</td>
<td>Posterior hypothalamic area</td>
</tr>
<tr>
<td>PHA-L</td>
<td>Phaseolus vulgaris leucoagglutinin</td>
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<tr>
<td>PKC-γ</td>
<td>Protein kinase C\textgreek{\gamma}</td>
</tr>
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<td>Po</td>
<td>Posterior thalamic nuclear group</td>
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<tr>
<td>PRV</td>
<td>Pseudorabies virus</td>
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<td>PVP</td>
<td>Paraventricular thalamic nucleus, posterior part</td>
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<tr>
<td>Py</td>
<td>Pyramidal tract</td>
</tr>
<tr>
<td>Pyx</td>
<td>Pyramidal decussation</td>
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<td>RAmb</td>
<td>Retroambiguus nucleus</td>
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<tr>
<td>RHO-D</td>
<td>Tetramethylrhodamine dextran</td>
</tr>
<tr>
<td>Rt</td>
<td>Reticular thalamic nucleus</td>
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<tr>
<td>RVM</td>
<td>Rostroventromedial thalamus</td>
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<tr>
<td>SDH</td>
<td>Superficial dorsal horn</td>
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<tr>
<td>SI</td>
<td>Substantia innominata</td>
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<td>SM</td>
<td>Nucleus of the stria medullaris</td>
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<td>S\text{\textacute{m}}</td>
<td>Stria medullaris of the thalamus</td>
</tr>
<tr>
<td>SP</td>
<td>Substance P</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
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<tr>
<td>Sp5I</td>
<td>Spinal trigeminal nucleus, interpolar part</td>
</tr>
<tr>
<td>SpV</td>
<td>Spinal trigeminal nucleus</td>
</tr>
<tr>
<td>Sub</td>
<td>Submedius thalamic nucleus</td>
</tr>
<tr>
<td>TC</td>
<td>Tuber cinereum area</td>
</tr>
<tr>
<td>Th</td>
<td>Thalamus</td>
</tr>
<tr>
<td>VA</td>
<td>Ventral anterior thalamic nucleus</td>
</tr>
<tr>
<td>VDB</td>
<td>Nucleus of the vertical limb of the diagonal band</td>
</tr>
<tr>
<td>VGLUT</td>
<td>Vesicular glutamate transporter</td>
</tr>
<tr>
<td>VH</td>
<td>Ventral hypothalamus</td>
</tr>
<tr>
<td>VIP</td>
<td>Vasoactive intestinal polypeptide</td>
</tr>
<tr>
<td>VL</td>
<td>Ventrolateral thalamic nucleus</td>
</tr>
<tr>
<td>VM</td>
<td>Ventromedial thalamic nucleus</td>
</tr>
<tr>
<td>VMH</td>
<td>Ventromedial hypothalamic nucleus</td>
</tr>
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<td>VMHA</td>
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</tr>
<tr>
<td>VMHC</td>
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</tr>
<tr>
<td>VMHVL</td>
<td>Ventromedial hypothalamic nucleus, ventrolateral part</td>
</tr>
<tr>
<td>VP</td>
<td>Ventral pallidum</td>
</tr>
<tr>
<td>VPL</td>
<td>Ventroposterolateral nucleus of the thalamus</td>
</tr>
<tr>
<td>VPM</td>
<td>Ventral posteromedial thalamic nucleus</td>
</tr>
<tr>
<td>VRt</td>
<td>Ventral reticular nucleus</td>
</tr>
<tr>
<td>WGA</td>
<td>Wheat germ agglutinin</td>
</tr>
<tr>
<td>XII</td>
<td>Hypoglossal nucleus</td>
</tr>
<tr>
<td>ZI</td>
<td>Zona incerta</td>
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Chapter 1

General Introduction
Pain is such a versatile sensation, and differs from other somatosensory modalities in that emotions such as fear, anxiety and feelings of unpleasantness are experienced along with it. However, over the centuries, pain has not been as clearly defined as it is today. The Romans and the Greeks were the first to put forward an idea of sensation, with the thought that the brain and nervous system have a role in the perception of pain. However, it was not until the Middle Ages and into the Renaissance (1400’s-1600’s) that the evidence developed to support these earlier theories. Leonardo Da Vinci and his contemporaries came to believe that the brain was the central organ for sensation, with Da Vinci himself later developing the idea that the spinal cord was the route that sensations were transmitted to the brain.

Into the 16th and 17th centuries, the study of the body and the senses continued to be a mystery and wonder for the world’s philosophers. Moving into the 19th century, pain came to dwell under a new domain – science, with an increasing knowledge base. Indeed today, pain research is an enormous, ever-growing field, with much interest in trying to unravel the complex neural circuitry of the brain and spinal cord. With a greater understanding of these pathways, therapeutic options for acute and chronic pain conditions were developed, which is something that could only have been dreamt of several centuries ago.

Nowadays, the spinal cord is established as essential for the transmission of sensory information to the brain, and for the regulation of motor and autonomic functions. It receives sensory information from somatic and visceral receptors passing through dorsal roots, transmitting to higher regions of the brain through ascending tracts and sends information to somatic and visceral targets via the ventral roots.
1. General layout of the spinal cord

When the spinal cord is examined in transverse section, it is composed of a central grey matter (butterfly-shaped) comprising cell columns oriented along the rostro-caudal axis (containing neuronal cell bodies, dendrites and axons that are both myelinated and unmyelinated), surrounded by the white matter comprising the ascending and descending myelinated and unmyelinated fasciculi (tracts). The general layout of the spinal cord is shown in Figure 1.1.

In each half of the spinal cord there are three funiculi: the dorsal funiculus (between the dorsal horn and the dorsal median septum), the lateral funiculus (located where the dorsal roots enter and the ventral roots exit) and the ventral funiculus (found between the ventral median fissure and the exit point of the ventral roots).

Based on detailed studies of neuronal soma size (revealed using the Nissl stain), Rexed (1952) proposed that the spinal grey matter is arranged in the dorso-ventral axis into laminae and designated them into ten groupings of neurons identified as I – X.

Lamina I contains the terminals of fine myelinated and unmyelinated dorsal root fibres that pass first through the zone of Lissauer (dorsolateral funiculus) and then enter lamina I mediating pain and temperature sensation (Christensen and Perl, 1970; Menétrey et al., 1977; Craig and Kniffki, 1985; Bester et al., 2000). The neurons here have been divided into small neurons and large marginal cells characterised by wide-ranging horizontal dendrites (Willis and Coggeshall, 1991). They then synapse on the posteromarginal nucleus. From here the axons of these cells pass to the opposite side and ascend as the lateral spinothalamic tract.

Lamina II is immediately below lamina I, referred to as the substantia gelatinosa. Neurons here modulate the activity of pain and temperature afferent fibres, though intrinsic neurons here do not contain the target for substance P, the NK-1 receptor (Bleazard et al.,
Lamina II has been sub-divided into an outer (dorsal) lamina II (II₀) and an inner (ventral) lamina II (IIᵢ) based on the morphology of these layers with stalked cells found in larger numbers in lamina II₀ but stalked and islet cells were found throughout lamina II (Todd and Lewis, 1986). Indeed, lamina II, was also found to be different in its neurochemical profile with a greater predominance of protein kinase C-γ (PKC-γ), an important enzyme in signal transduction, which will be discussed in greater detail in Chapter 4. Lamina II is the region which receives an extensive unmyelinated primary afferent input, with very little from large myelinated primary afferents (except for distal parts of hair follicle afferents in some animals; Willis and Coggeshall, 1991). The axonal projections from here are wide and varied with some neurons projecting from the spinal cord (projection neurons), some passing to different laminae and some with axons confined to a lamina in the region of the dendritic tree of that cell e.g. intralaminar interneurons, local interneurons and Golgi Type II cells (Todd, 1996).

Lamina III is distinguished from lamina II in that it has slightly larger cells, but with a neuropil similar to that of lamina II. The classical input to this lamina comes from hair follicles and other types of coarse primary afferent fibres which includes Pacinian corpuscles and rapidly and slowly adapted fibres.

Lamina IV is a relatively thick layer that extends across the dorsal horn. Its medial border is the white matter of the dorsal column, and its lateral border is the ventral bend of laminae I – III. The neurons in this layer are of various sizes ranging from small to large and the afferent input here is from collaterals and from large primary afferent fibres (Willis and Coggeshall, 1991). Input also arises from the substantia gelatinosa (lamina II) and contributes to pain, temperature and crude touch via the spinothalamic tract (Siegel and Sapru, 2006).
Lamina V extends as a thick band across the narrowest part of the dorsal horn. It occupies the zone often called the neck of the dorsal horn. It has a well demarcated edge against the dorsal funiculus, but an indistinct lateral boundary against the white matter due to the many longitudinally oriented myelinated fibres coursing through this area. The cell types are very homogeneous in this area, with some being slightly larger than in lamina IV (Willis and Coggeshall, 1991). Again, like lamina IV, primary afferent input into this region is from large primary afferent collaterals as well as receiving descending fibres from the corticospinal and rubrospinal tracts with axons also contributing to the spinothalamic tracts (Siegel and Sapru, 2006). In addition, in the thoracolumbar segments (T1 – L2/3) the reticulated division of lamina V contains projections to sympathetic preganglionic neurons (Cabot et al., 1994).

Lamina VI is present only in the cervical and lumbar segments. Its medial segment receives joint and muscle spindle afferents, with the lateral segment receiving the rubrospinal and corticospinal pathways. The neurons here are involved in the integration of somatic motor processes.

Lamina VII present in the intermediate region of the spinal grey matter contains Clarke’s nucleus extending from C8 – L2. This nucleus receives tendon and muscle afferents with the axons of Clarke’s nucleus forming the dorsal spinocerebellar tract relaying information to the ipsilateral cerebellum (Snyder et al., 1978). Also within lamina VII are the sympathetic preganglionic neurons constituting the intermediolateral cell column in the thoracolumbar (T1 – L2/3) and the parasympathetic neurons located in the lateral aspect of the sacral cord (S2 – 4). In addition Renshaw cells are located in lamina VII and are inhibitory interneurons which synapse on the alpha motor neurons and receive excitatory collaterals from the same neurons (Renshaw, 1946; Siegel and Sapru, 2006).
Lamina VIII and IX are found in the ventral grey matter of the spinal cord. Neurons here receive descending motor tracts from the cerebral cortex and the brainstem and has both alpha and gamma motor neurons here which innervate skeletal muscles (Afifi and Bergman, 2005). Somatotopic organisation is present where those neurons innervating the extensor muscles are ventral to those innervating the flexors, and neurons innervating the axial musculature are medial to those innervating muscles in the distal extremities (Siegel and Sapru, 2006).

Lamina X is the grey matter surrounding the central canal and represents an important region for the convergence of somatic and visceral primary afferent input conveying nociceptive and mechanoreceptive information (Nahin et al., 1983; Honda, 1985; Honda and Lee, 1985; Honda and Perl, 1985). In addition lamina X in the lumbar region also contains preganglionic autonomic neurons as well as an important spinothalamic pathway (Ju et al., 1987a,b; Nicholas et al., 1999).

Extensive literature exists in this complex circuitry, with a great deal of research on the spinal cord focussing on the grey matter. Although much progress in understanding has developed since Rexed’s (1952) first classification of the grey matter into laminae from his classical works using the cat, an area that has received little attention, and is poorly understood, is the lateral spinal nucleus (LSN) located in the dorsolateral funiculus.

From this point on the aim of this introductory chapter is to review the limited literature available regarding the LSN. The review has been subdivided into sections examining the existing evidence which ascribes potential roles to this unusual nucleus. The framework is set out as follows:

- Definition of the LSN
- Morphology of cells in the LSN
- Neurochemical profile of the LSN
• Mechanisms of activation, and projections of, the LSN
2. The definition of the LSN

In 1951, Rexed and Brodal described a separate nucleus forming a longitudinal cell column situated ventrolateral to the dorsal horn in the lateral funiculus of the first and second cervical segments of the cat’s spinal cord. It was believed that this nucleus projected to the cerebellum and that it received afferent fibres ascending in the spinal cord. This nucleus was referred to as the nucleus cervicalis lateralis, or the lateral cervical nucleus (LCN). However, in 1951, Rexed also found that in the rat, guinea pig, mouse, rabbit and man, the lateral cervical nucleus was either non-existent, or existed in a form different from that described in the cat (though no details of these findings were presented). Lund and Webster (1967), however, reported the presence of cells in the dorsolateral funiculus of the upper two cervical segments of the spinal cord of the rat and they considered this group of cells to represent the LCN as described in other species. Their findings therefore contradicted those of Rexed (1951).

However, through observing the distribution of acetylcholinesterase activity in the spinal cord of the rat, Gwyn and Waldron (1968) demonstrated a group of cells present in the dorsolateral funiculus extending in a continuous column from the spinomedullary junction to sacral levels of the spinal cord. They occupied a position ventral to the dorsal horn, similar to that described for the LCN in the cat by Rexed and Brodal (1951) and by Brodal and Rexed (1953), but, whereas in the cat, the nucleus is restricted to the upper two cervical segments of the spinal cord, in the rat, the column extends throughout the length of the spinal cord, defined then as the lateral spinal nucleus (LSN). Figure 1.1 demonstrates the position of the LSN in the spinal cord. Later evidence revealed however, that the enzyme acetylcholinesterase was not a specific marker for cholinergic cells (Albanese and Butcher, 1980; Greenfield, 1991), but it was noted that the LSN differed from the superficial dorsal horn in the nature of the neuropil.
3. The morphology of the LSN and its neurons

The neurons in the LSN have been shown to have a multipolar appearance through Golgi studies (Réthelyi, 2003), which have confirmed earlier descriptions by Alvarez et al. (2000) who used metabotropic glutamate receptor immunostaining. Jiang et al. (1999), through single cell staining following intracellular recordings, also demonstrated multipolar cells. Fusiform cell bodies have been noted in the LSN (Giesler et al., 1979; Giesler and Elde, 1985). Spindle-shaped cells have also been identified at all segmental levels (Gwyn and Waldron, 1968), though in Réthelyi’s (2003) Golgi study, they occurred only rarely.

The cells in the LSN are small (compared with anterior horn cells), ranging from 8 to 36µm by 4 to 12µm (Gwyn and Waldron, 1968). Réthelyi (2003) noted similar sized perikarya with his study showing cells in the range of 20 to 35µm. This compares to the medium to large cells of the cat LCN, where they were shown to be 20 to 50µm. (Rexed, 1951; Morin and Catalano, 1955).

The LSN neurons form a continuous column underneath the pial surface of the dorsolateral funiculus. Some of the dendrites remain within the column of the perikarya, while others have been identified passing either laterally or medially (Réthelyi, 2003). Of those medially oriented LSN neurons, Menétrey et al. (1982) demonstrated that they passed into lamina I, whereas Bresnahan et al. (1984) found occasional dendrites almost apposing the pial surface. However, Réthelyi’s (2003) electron micrographs revealed that the dendrites also pass to the pial surface. This brings into question the possibility that the LSN neurons may be under the influence of components of the cerebrospinal fluid surrounding the spinal cord (Vigh et al., 2004).
4. Neurochemical profile of the LSN and its involvement in nociception

As previously mentioned, Gwyn and Waldron (1968, 1969) were the first to investigate the profile of the LSN using acetylcholinesterase, but this was subsequently shown not to be a specific marker for cholinergic cells (Albanese and Butcher, 1980; Greenfield, 1991). Leah and co-workers (1988) were the first group to investigate, in detail, the neurochemical profile of the LSN from examining ascending tract cells in the rat lumbosacral region containing neuropeptides. They revealed that 90% of peptidergic ascending tract cells were congregated in two distinct areas – the LSN and the region surrounding the central canal. The LSN had the highest percentage of neuropeptides containing ascending tract cells which included vasoactive intestinal polypeptide (VIP), bombesin, dynorphin and substance P (SP), with their axons projecting in a variety of tracts including the spinomesencephalic, spinoreticular and spinosolitary tracts.

4.1 Excitatory Interneurons in the LSN

4.1.1 Substance P in the LSN

Ljungdahl et al. (1978) first reported that large numbers of fibres in the LSN were richly stained for SP with others confirming this (Gibson et al., 1981; Dalsgaard et al. 1982; Senba, 1982). However, in the first examination of the origins of SP containing fibres within the LSN, Barber et al. (1979) reported that transection of lumbar dorsal roots markedly reduced the number of labelled fibres in the nucleus ipsilaterally. They also noted that, since combined hemisection of the cord and dorsal rhizotomy failed to eliminate SP labelling in the LSN, it was likely that some of the labelled fibres within the LSN originated segmentally. In further studies, however, the possibility of an input from SP containing dorsal root fibres to the LSN has been questioned. Larabi et al. (1983) re-examined the effects of dorsal rhizotomy on fibres in the LSN and concluded that, although
such operations markedly reduced the number of labelled fibres in the adjacent dorsal horn, little if any reduction was produced in the LSN unless the radicular arteries were obstructed or cut during rhizotomies. Bresnahan et al. (1984) and Cliffer et al. (1988) also showed that rhizotomies have little, if any, effect on SP immunostained fibres in the LSN. In addition, Bresnahan et al. (1984) found that application of horseradish peroxidase (HRP) to the proximal stumps of cut dorsal roots labelled very few primary afferent terminals in the LSN (the adjacent dorsal horn contained a large number of labelled fibres). The failure to label more than a few fibres in the LSN following application of HRP to dorsal roots strongly suggests that the majority of SP stained fibres within the LSN are not central processes of primary afferent fibres. This finding also indicated that very few of the fibres within the LSN (whether they contain a peptide or not) originate in dorsal root ganglia. Bresnahan et al. (1984) did find HRP labelling of terminals in the LSN when HRP was injected into the dorsal horn, a finding that suggests the existence of a projection from neurons in the adjacent dorsal horn to the LSN. In other studies where colchicine was injected intrathecally to increase the peptidergic labelling of cell bodies (Seybold and Elde, 1980; Sasek et al., 1984), many dorsal horn neurons (Ljungdahl et al., 1978; Barber et al., 1979; Bresnahan et al., 1984) and the majority of LSN cell bodies were found to be immunoreactive for SP. In addition, unlike the superficial dorsal horn where all SP containing primary afferents are thought to contain calcitonin gene-related peptide (CGRP), the marker associated with large myelinated primary afferents (Chung et al., 1988; Naim et al., 1997; Todd et al., 2003), the LSN does not contain any CGRP (Olave and Maxwell, 2004). It therefore appears that the primary sources of SP stained fibres in the LSN is the adjacent dorsal horn, and perhaps, the LSN itself.

As SP exerts its biological actions by a high affinity interaction with the SP, or neurokinin–1 (NK-1) receptor, it could be postulated that the LSN may be involved in
nociception, as the majority of lamina I neurons with the NK-1 receptor are thought to be activated by nociceptors, as most show internalisation of the receptor (Mantyh et al., 1995) or express c-Fos (Doyle and Hunt, 1999; Todd et al., 2005).

### 4.1.2 Glutamate in the LSN

Glutamate is the major excitatory neurotransmitter in the central nervous system (CNS) with synaptic terminals of spinal sensory afferents strongly immunoreactive for glutamate, indicating a glutamatergic function (De Biasi and Rustioni, 1988, 1990; Broman et al., 1993; Valschanoff et al., 1994). This conclusion is confirmed by extensive physiological evidence of AMPA- and NMDA-mediated synaptic responses in spinal neurons evoked by dorsal root afferent stimulation (Salt and Hill, 1983; Jessel et al., 1986; Schneider and Perl, 1994; Li et al., 1998) and the presence of AMPA and NMDA receptor subunits postsynaptic to spinal sensory synapses (Alvarez et al., 1994; Popratiloff et al., 1996, 1998a,b).

Recently, three glutamate vesicular transporters (VGLUTs) have been characterised (reviewed by Fremeau et al., 2004) and have been shown to be present in axons belonging to largely non-overlapping populations of glutamatergic neurons throughout the CNS (Ni et al., 1995; Bellocchio et al., 1998, 2000; Aihara et al., 2000; Takamori et al., 2000; Fremeau et al., 2001; Sakata-Haga et al., 2001; Kaneko et al., 2002). Varoqui et al. (2002) reported initially that VGLUT1 and VGLUT2 were present in the spinal cord, but in differing locations: VGLUT2 throughout the grey matter, with VGLUT1 found in laminae III – VI, the intermediate grey matter and the ventral horn. In addition, Todd et al. (2003) showed that myelinated primary afferents in lamina I were VGLTU2 immunoreactive whereas all those in deeper lamina were VGLTU1 immunoreactive, with some in laminae III – VI appearing to contain both transporters. VGLUT3 on the other hand has been demonstrated
in the cortex and hippocampus (reviewed by Fremeau et al., 2004) where scattered cells showed VGLUT3 mRNA and protein were identified (Schafer et al., 2002; Fremeau et al., 2002), however these also co-localised with glutamic acid decarboxylase (GAD), the enzyme responsible for synthesising GABA (Fremeau et al., 2002) where symmetric synapses onto the cell bodies and proximal dendritic shafts of pyramidal cells by immunoelectron microscopy, raised the possibility of glutamate co-release with GABA at inhibitory synapses. In addition, Gras et al. (2002) also demonstrated that the VGLUT3 was also found in all cholinergic interneurons of the striatum, as well as in serotoninergic neurons from the raphe magnus.

Alvarez et al. (2004) demonstrated only minimal labeling of VGLUT1 within the LSN, with others unable to detect VGLUT1 at all (Li et al., 2003; Todd et al., 2003; Olave and Maxwell, 2004). However, VGLUT2 has been shown in abundance in the LSN as demonstrated by Li et al., 2003; Todd et al., 2003 and Alvarez et al., 2004. In addition, Olave and Maxwell (2004) also found VGLUT2 labeling to be abundant and co-localised with the adrenergic receptor α2c. As VGLUT2 has been found in the superficial dorsal horn, especially within lamina I (an area established in its role in nociception), is found in peptidergic axons likely to be derived from intrinsic neurons containing neurotensin, enkephalin, somatostatin and SP (suggesting that these are also glutamatergic (Todd et al., 2003)) and has been established in its role in nociception (Moechars et al., 2006), the VGLUT2 within the LSN may serve a role in nociceptive processing. However, unlike the superficial dorsal horn which receives direct primary afferent input, the LSN does not, and it may be that the VGLUT2 serves a very different role in nociception at this site.
4.2 Inhibitory interneurons in the LSN

4.2.1 GABA and glycine in the LSN

GABA is the major inhibitory neurotransmitter in the dorsal horn of the spinal cord and also plays a role in the ventral horn (Todd and Maxwell, 2000). GABA is synthesised by glutamic acid decarboxylase (GAD) which exists in two isoforms with slightly different molecular weights referred to as GAD65 and GAD67 (Martin and Tobin, 2000), coded by different genes (Erlander and Tobin, 1991).

GABA can produce presynaptic inhibition of primary afferents through axoaxonic synapses, and inhibition postsynaptically of spinal neurons, mediated through axosomatic and axodendritic synapses. Indeed, the inhibitory role of GABA can be seen when the GABA\(_A\) receptor antagonist bicuculline (or the glycine receptor antagonist strychnine) is locally applied to the spinal cord in the rat where it produces behavioural signs of tactile allodynia (Yaksh, 1989), and can cause low threshold mechanical stimuli to produce a flexion withdrawal reflex (Sivilotti and Woolf, 1994).

Specifically, in the superficial dorsal horn (SDH), where nociceptive information is first processed, approximately 30% of neurons in lamina I and II, and 45% of lamina III neurons are GABA immunoreactive (Todd and Sullivan, 1990), and these cells are assumed to be GABAergic inhibitory neurons. Glycine like immunoreactivity is also present in neuronal cell bodies in these laminae, but is limited to those that are GABA immunoreactive. Of those neurons in laminae I and II, 33% and 43% respectively contain GABA and glycine. However, as much as 64% of neurons contain both GABA and glycine in lamina III (Todd and Sullivan, 1990).

GABA is produced through decarboxylation of L-glutamate by the enzyme GAD. Two isoforms of this GAD exist, coded by different genes (Erlander and Tobin, 1991) and have been classified as GAD65 and GAD67 based on their molecular weights. Within the LSN,
although not formally discussed, it can be seen from the photomicrographs, that the LSN shows mild to moderate staining with GAD (McLaughlin et al., 1975). In addition, Mackie et al. (2003) also have shown both GAD65 and GAD67 exists within the LSN, with moderate staining. Olave and Maxwell (2004), through studies with the $\alpha_{2c}$ adrenergic receptor, also demonstrated moderate to abundant GAD staining, and of those approximately 10% co-localised with the $\alpha_{2c}$ adrenergic receptor, however most of the adrenergic receptors co-localised with VGLUT2.

Within the SDH, GABA immunoreactive axon terminals and cell bodies frequently contain relatively high levels of glycine (Todd and Sullivan, 1990; Todd et al., 1996), and there is evidence that GABA and glycine can act as co-transmitters at synapses in the spinal cord (Jonas et al., 1998; Keller et al., 2001). However, in the LSN there is only minimal staining with Gly-T2 (Olave and Maxwell, 2004), a marker for glycine (Zafra et al., 1995), and it may be that with such low levels, the GABA in the LSN may not be related to glycine at all, unlike the situation in the SDH.

4.2.2 GABA and NOS in the LSN

Previously it had been reported that neuronal nitric oxide synthase (nNOS) and the enzyme responsible for its synthesis — nicotinamide adenine dinucleotide phosphate (NADPH-d) — are located in the superficial dorsal horn, the area around the central canal (Dun et al., 1992; Valschanoff et al., 1992a), the intermediolateral nucleus and the LSN (Valschanoff et al., 1992a; Nazli and Morris, 2000). Nadelhaft and Booth (1984) demonstrated that most nNOS immunoreactive fibres terminating in the dorsal horn arose from dorsal root ganglion (DRG) neurons. Later, Aimi et al. (1991) revealed that the number of NOS positive immunoreactive DRG cells over all spinal segments may not be as large as first anticipated, suggesting an intrinsic origin of the NOS would be more likely.
Laing et al. (1994) completed an extensive investigation characterising the NOS immunoreactive neurons showing them to be most numerous in lamina II – III. Valtschanoff et al. (1992b) demonstrated that of those NOS immunoreactive axonal boutons in lamina II, many possessed GABA immunoreactivity, suggesting an inhibitory role. This was confirmed by Laing et al. (1994) who showed glycinergic immunoreactivity in NOS neurons in lamina I – II. However, NOS immunoreactive neurons in lamina III were not found to co-localise with glycine. In addition, Spike et al. (1993) revealed that choline acetyltransferase (ChAT) immunoreactive neurons in lamina III were found to be NADPH-d positive, suggesting that those neurons in this lamina which contain GABA and acetylcholine, are also capable of synthesising NO. Blottner and Baumgarten (1992) also revealed that of all NOS immunostained neurons in the intermediolateral column, nearly half co-localised with ChAT. However, unlike the large NO interneurons of the monkey cerebral cortex that express neuropeptide Y (NPY) immunoreactivity, none of the lamina I – III NOS immunoreactive neurons expressed NPY.

The exact function of NOS is still under great debate. Behavioural (Malmberg and Yaksh, 1993) and pharmacological studies (Kawabata et al., 1994; Semos and Headley, 1994; Lin et al., 1999) have indicated that NO is a modulator of nociceptive processes, but there is no agreement on its precise role and it may have both hyperalgesic and analgesic effects (Hoheisel et al., 2005). As NOS is found in the LSN (Valtschanoff et al., 1992a; Nazli and Morris, 2000), it may well be associated with GABA as it is in the SDH, but may not be related to glycine, due to the low levels of Gly-T2 (Olave and Maxwell, 2004). In addition, NOS in the LSN has also been found to co-localise with interferon-γ and synaptophysin (Vikman et al., 1998) and using Nitro-L-arginine-ester (L-NAME), a blocker of NOS, can at least partially inhibit the nociceptive response to interferon-γ in rats.
(Xu et al., 1994). Therefore, the role of NOS in the LSN may serve a role in nociception, although may be very different to what is understood regarding the SDH.

5. Mechanisms of activation, and projections of, the lateral spinal nucleus

Ling et al. (2003) suggested that the LSN could be directly innervated by primary afferents. Using electrophysiological techniques, they identified the C afferent fibres from the gastrocnemius muscle and performed iontophoretic injections of Phaseolus vulgaris leucoagglutinin (PHA-L). This showed that the unmyelinated muscle primary afferents projected rostrocaudally in the dorsolateral funiculus with projections both in a medial and lateral direction (from observation of their diagrammatic representations) and also passed into lamina I and II, with smaller numbers projecting to lamina III. However, most of the research into the activation of the LSN suggests that in fact it is not directly influenced by cutaneous stimulation, but actually from collateral activation.

Through electrophysiological studies, Grudt and Perl (2002) demonstrated that some lamina I neurons were relatively large with extensive dendritic arborisation in the horizontal dimension and possessed a prominent thick axon. These axons were noted to pass ventrally and course to the contralateral side to project in the ventral lateral funiculus. This raises the distinct possibility that this type of lamina I neuron is activated directly by a primary afferent responding to peripheral noxious stimulation, and, from there, activates the LSN neuron through an axon collateral (Han et al., 1998; Craig et al., 2001; Grudt and Perl, 2002; Todd et al., 2002; Olave and Maxwell, 2004). This strengthens previous electrophysiological studies demonstrating collateral activation of the LSN, rather than direct primary afferent input (Giesler et al., 1979; Menétrey et al., 1980). Indeed, as previously mentioned, the source of SP in the LSN is thought to be either from the adjacent SDH, or the LSN itself (Barber et al., 1979; Larabi et al., 1983; Bresnahan et al., 1984;
Cliffer et al., 1988). As the LSN is stimulated by spinal interneurons with a rich content of SP, VGLUT2, enkephalin, dynorphin, somatostatin, vasoactive intestinal polypeptide, bombesin and FMRF (Jessel et al., 1978; Seybold and Elde, 1980; Giesler and Elde, 1985; Cliffer et al., 1988; Olave and Maxwell, 2004), this adds further weight to the suggestion that the LSN is not directly activated by primary afferent fibres.

However, Neuhuber (1982) and Neuhuber et al. (1986) demonstrated that some afferent fibres from the greater splanchnic nerve, the inferior mesenteric plexus, and the hypogastric nerve also terminate in the LSN. This would suggest that the LSN could receive a visceral input, which will be discussed later. Therefore, there may be more primary afferents terminating in the white matter either directly, or close to, the LSN than was first appreciated.

Additionally, neurons in the LSN are not directly activated by cutaneous stimulation, have no spontaneous activity and possess axons with slow conduction velocities (i.e. the unmyelinated range) and can project contralaterally (46%), and bilaterally (40%) (Menétrey and Besson, 1981).

Petkó and Antal (2000) used anterograde and retrograde labelling techniques and showed that neurons in the lateral part of one superficial dorsal horn could project to the contralateral superficial part of the dorsal horn, with the possibility of involvement of the LSN. This could suggest that one LSN, could be activated by both the ipsilateral and contralateral lateral dorsal horn (with commissural fibres going between one LSN and the opposite). Also, from their diagrams, the LSN neurons themselves may project contralaterally and could be activated reciprocally. This could also explain the work by Olave and Maxwell (2004) that showed bilateral activation of c-Fos in the LSN. The knowledge at the moment of the mechanisms underlying bilateral activation of LSN neurons is limited.
LSN neurons may also be activated by descending fibres that originate from supraspinal nuclei, which in turn are activated by ascending fibres of spinal projection neurons located in the superficial dorsal horn. Suzuki et al. (2002) showed that superficial dorsal horn neurons which express the NK-1 receptor activate descending pathways which control spinal excitability. This could also explain the c-Fos activation bilaterally in Olave and Maxwell’s study (2004) as there could be ascending activation of descending systems which project bilaterally to the LSN.

Pechura and Liu (1986) used retrograde fluorescent double labelling of the periaqueductal grey (PAG) and the medullary reticular formation (MRF) which showed that double labelled neurons in the LSN were present. As the PAG and the MRF are strongly implicated in descending modulation of spinal neuronal activity, especially that resulting from noxious stimulation (Basbaum and Fields, 1984), this could imply that the LSN has a role in nociceptive pathways.

Initially, retrograde labelling studies showed that LSN neurons do not project to the thalamus (Giesler et al., 1979; Kevetter and Willis, 1982). However, Gauriau and Bernard (2004) used anterograde labelling (in the rat) with the sensitive markers PHA-L and/or tetramethylrhodamine-dextran (RHO-D) injected microiontophoretically. They found that when the LSN was labelled, it was very specific in its bilateral projection to the lateral and medial portions of the caudal region of the mediodorsal thalamic nuclei, as well as to the posterior thalamic group (triangular part). There was some spread into the most lateral portion of the laminae I-III but retrograde studies that have been performed prior to Gauriau and Bernard (2004) indicate that, in the cervical enlargement of the rat, the lateral portion of laminae I-III projects scarcely, or not at all to the thalamus, whereas numerous LSN neurons project to the thalamus (Granum, 1986; Burstein et al., 1990b). This pathway from the LSN, to the thalamus, and from there to the forebrain, forms part of the
somatosensory relays. This could form a direct and specific role in the emotional affective component of pain (Gauriau and Bernard, 2004). This is significant in that the mediodorsal thalamic nuclei project to the medial and orbital regions of the prefrontal cortex and the LSN-mediodorsal pathway could be implicated in the emotional and cognitive aspects of pain (Gauriau and Bernard, 2004).

Using the retrograde tracer Fluoro-Gold (FG), Burstein et al. (1990a) demonstrated extensive projections of the LSN to the hypothalamus. It was shown that up to 25% of the input to the hypothalamus came from the LSN, throughout the full length of the rat spinal cord. Work by Li et al. (1997) also confirmed projections of the LSN to the hypothalamus.

Retrograde labelling studies and SP immunoreactivity also showed that the LSN has extensive projections to the hypothalamus, as well as to the septal region (Li et al., 1997). The hypothalamic projection sites included, not only the lateral hypothalamus but also to the paraventricular hypothalamic nucleus and the posterior hypothalamic area. The paraventricular nucleus is composed of magnocellular neurosecretory neurons constituting the paraventriculohypothalamic system and non-endocrine neurons, some of which send projection fibres to the pain-related brainstem structures including the PAG, raphe nuclei, and parabrachial nuclei, with the posterior hypothalamic area being involved in the generation of emotional stress and the regulation of body temperature (Armstrong, 1995; Saper, 1995). In addition two neuroendocrine systems are activated in the animal when coping with stressors: the hypothalamicpituitary-adrenocortical system, in which the paraventricular hypothalamic nucleus is involved, and the hypothalamosympathico-adrenomedullary system in which the posterior hypothalamic area seems to be involved. (Agnati et al., 1991; Armstrong, 1995) Thus, from the study by Li et al. (1997), the projection fibres from the spinal cord that have SP immunoreactivity that project to the paraventricular hypothalamic nucleus and/or the hypothalamic area are likely to be
implicated in the elicitation of stress response. In particular, projection fibres from the LSN of the lower lumbar and sacral cord segments may mediate nociceptive information of visceral origin to the paraventricular hypothalamic nucleus and exert regulatory influences on the endocrine and autonomic systems through the paraventriculohypophyseal system. However affective motivational responses to peripheral stimulation is served by the septal area. The limbic system including the septal region is necessarily involved in nociception because it is strongly coupled with emotional and affective states. Thus the direct projections from the LSN, and deep part of the dorsal horn to the septal region, are presumed to be involved in motivational-affective aspects of nociception (Li et al., 1997).

Also, transneuronal studies have been done using pseudorabies virus (PRV) injected into the kidney (Schramm et al., 1993) and stellate ganglion (Jansen et al., 1995) have shown that the LSN innervates different types of sympathetic preganglionic neurons. Jansen and Loewy (1997) have also shown that neurons in the LSN become transneuronally labelled after PRV injections into the superior cervical ganglion, stellate ganglion, celiac ganglion or adrenal gland. Also, they showed direct projection of the LSN to sympathetic preganglionic neurons using PHA-L. These results suggest that the LSN has a descending sympathetic projection system, and may trigger a sympathetic response (eg. increase in blood pressure, release of catecholamines) during intense, acute visceral pain. So, as well as being influenced by visceral nociceptive information, the LSN may have a feedback to the sympathetic system in response to this.
Figure 1.1. **Position of the LSN in the spinal cord.** A single transverse section of L5 with immunofluorescence for the neuronal marker NeuN (red) to highlight the neuronal population predominantly in the grey matter on the left hand side of this Figure, with a diagrammatic representation of Rexed’s laminae (1952) being highlighted to the right hand side (as indicated by Roman numerals for each of the laminae). The position of the LSN can be seen in the dorsolateral funiculus, close to the superficial dorsal horn. Scale bar = 500µm.
Chapter 2

Aims and General Experimental Procedures
1. Aims

The purpose of this first section is to give a general account of the principal aims pursued, hypotheses to be tested and for each of the investigations, an outline of the experimental approaches. The specific details of each of these procedures will be detailed, as appropriate, in the experimental sub-division in each chapter.

There are three main areas of focus of this study that will give a greater understanding of the nucleus of the dorsolateral funiculus, the LSN:

• Nociception and the LSN

• NK-1 and excitatory and inhibitory terminals in the LSN

• NK-1 projection targets of LSN neurons to brain centres, including the brainstem

Investigation 1

Hypotheses:

a) LSN neurons receive cutaneous information

b) LSN neurons are activated by noxious cutaneous stimulation

c) There is a variable degree of expression of Fos depending on the stimulus, as in the superficial dorsal horn (SDH)

Aims:

a) Identify if LSN neurons receive information from the skin

b) Identify the types of stimuli that activate LSN neurons

c) Quantify the extent of LSN neurons that respond to different types of peripheral noxious stimuli
Expression of the immediate early gene c-Fos was induced by the application of a noxious thermal or chemical stimulus to the left hind-paw. This method was used to identify LSN neurons that could be activated by different types of nociceptive stimuli, and allowed direct comparison with the SDH. Combining this approach with double labelling immunocytochemistry (with the neuronal marker NeuN) allowed quantification of the neuronal populations in the LSN, both ipsilateral and contralateral to the stimulus. It also allowed direct comparison between different stimuli that may have influenced the LSN, and proportionally how many neurons were activated by different stimuli.

Investigation 2

Hypothesis 1:

a) As SP is present in abundance in the LSN, the majority of LSN neurons are NK-1 immunoreactive

b) If a majority of LSN neurons are immunoreactive for the NK-1 receptor, only a minority be immunoreactive for protein kinase C-γ (PKC-γ), which like the NK-1 receptor, has also been associated with nociceptive processing (Malmberg et al., 1997)

Aims:

a) To identify the relationship between SP and the NK-1 receptor in the LSN, and quantify the total neuronal population that is immunoreactive for the NK-1 receptor

b) To quantify the total neuronal population in the LSN that are immunoreactive for PKC-γ

c) To identify if any relationship exists between NK-1 and PKC-γ in the LSN

The NK-1 receptor, the target of SP, is found within the LSN and the role of NK-1 is well established as playing a role in nociception (especially within the SDH). As the LSN
has been implicated in nociception (as previously discussed), triple labelling immunocytochemistry (for examination with the confocal microscope) was used to quantify the total neuronal population in the LSN (using NeuN) that possessed this receptor, and that of protein kinase C-\(\gamma\) (PKC-\(\gamma\)) which also serves a role in nociceptive processing (Malmberg et al., 1997)).

Hypothesis 2:

a) As nitric oxide synthase (NOS; the enzyme responsible for synthesising nitric oxide (NO)), has been found to enhance the release of SP in the SDH (Garry et al., 1994; Aimar et al., 1998; Kamasaki et al., 1995), SP and NOS will be intimately related immunocytochemically in the LSN

b) If a close relationship exists between SP and NOS, as in the SDH, then the same will hold for the relationship of NOS and the target of SP, the NK-1 receptor in the LSN

c) As in the SDH, the NOS terminals in the LSN will be associated with inhibitory GABAergic neurons

Aims:

a) To identify the relationship that exists between SP and NOS in the LSN

b) To identify the contact nature of NOS terminals to NK-1 neurons, and then quantify this relationship

c) To identify, and then quantify, the relationship and proportion of NOS terminals to excitatory (glutamatergic) and inhibitory (GABAergic) terminals in the LSN

With both NOS and the NK-1 receptor involved in nociception in differing ways as previously discussed, triple labelling immunocytochemistry (for examination with the confocal microscope) was used to identify if a relationship between SP, NK-1 and NOS in
the LSN existed. In addition, quadruple labelling immunocytochemistry was used to identify NK-1 neurons and NOS terminals in the LSN, and identify the co-localisation patterns of NOS terminals and their contact relationship with NK-1 neurons.

Within the SDH, many NOS containing neurons are associated with inhibitory GABAergic neurons, as well as glycineogenic and cholinergic neurons (Valtschanof et al., 1992b; Laing et al., 1994). However, within the LSN there are few cholinergic terminals (Olave and Maxwell, 2004) and no GlyT-2 – a marker associated with glycineogenic terminals (Zafra et al., 1995). Therefore, within the LSN they may be associated with only GABAergic (glutamic acid decarboxylase (GAD) expressing neurons). To ensure they do not arise from excitatory terminals, immunocytochemistry was also combined with VGLUT2, known to exist in glutamatergic neurons throughout the CNS, as discussed in Chapter 1, page 8.

Investigation 3

Hypothesis:

a) If NK-1 neurons are abundant in the LSN, then many will be projection neurons and target brain areas known to be involved in nociception

Aim:

a) to quantify the proportion of NK-1 projection neurons that project to several brain and brainstem regions from the LSN known to be involved in nociception, and related aspects of pain

Retrograde labelling of projection neurons was combined with either triple or quadruple labelling immunocytochemistry (for examination with the confocal microscope). The retrograde tracer cholera toxin B subunit (CTb) was combined with NeuN and NK-1 to
quantify LSN neurons that contained each of these markers that projected to several brain regions that have been implicated in nociception. For the quadruple labelling experiments, two brain regions were injected – one with CTb and one with Fluoro-Gold (FG), and this was combined with NeuN and the NK-1 receptor, again to quantify the LSN projections possessing these markers.

2. General Experimental Procedures

The purpose of this section is to give a broader understanding of some of the techniques that have been used throughout this study, and to provide a basis for the more specific detailed experimental sub-division that will be detailed later in chapters 3, 4 and 5.

2.1 Multiple immunolabelling for confocal microscopy

This is a technique which allows the identification of several antigens in the same section, say for example (as in this work) the spinal cord. The antigens can be peptides, neurotransmitters, enzymes or receptors. The basis of immunolabelling for confocal microscopy is generally two-fold:

- Incubation of sections of the spinal cord with a cocktail of primary antibodies (up to four), which have been raised in different species. This technique allows the combination of various different antigens to be detected in a single section.

- Incubation of the sections with a cocktail of species-specific secondary antibodies, each one of which is coupled to a different fluorophore. The secondary antibody is an immunoglobulin (Ig) raised in donkey, which is directed against a specific species e.g. rabbit, rat, goat etc. The resulting secondary antibody will bind to any antigen of the species, and therefore readily identified by the fluorophore which it is also attached to it.
After the appropriate incubations and rinses, the sections are mounted and subsequently ready to be scanned (or stored in a freezer at -20°C until ready to be examined). Immunoreactivity for each of these antigens can be visualised separately by the corresponding fluorophore coupled secondary antibody. Images from the same optical section can also be merged to allow the relative spatial distribution of the antigens to be studied.

Exceptionally two primary antibodies from the same species can be used provided that the antigens identify different cellular compartments. For instance, the NK-1 receptor is localised to the cell membrane and VGLUT2 is localised to terminals. For this reason both antigens can be labelled with a primary antibody of the same species and the same secondary antibody and it is still possible to differentiate the two types of immunoreactivity. Figures 4.4 and 4.5 illustrate immunoreactivity for the NK-1 receptor and VGLUT2 that has been obtained using primary antibodies raised in the same species (i.e. guinea-pig anti-NK-1 and guinea-pig anti-VGLUT2), which were revealed with the same secondary antibody (i.e. donkey anti-guinea-pig IgG coupled to the fluorophore rhodamine-red).

2.2 Confocal microscopy

The Radiance 2100 (Hemel-Hempstead, UK) confocal laser scanning microscope was used in all investigations. It is equipped with four lasers: argon, green helium neon, red diode and blue diode which allowed the scanning of sections that had been labelled with four secondary antibodies, each one being coupled to a different fluorophores (Table 2.1). This method allowed the same region of the cell to be identified with different fluorophores. With the Radiance 2100 microscope, scanning can be performed simultaneously.
2.3 Control experiments used in immunocytochemical methods

Positive and negative controls

Although immunocytochemistry is a powerful investigative tool, it depends on the specificity of the antibody binding e.g. to the epitope of the protein used as an immunogen, and could be affected by fixation or detergents (Josephsen et al., 1999). The antibody specificity requires that the antibody binds only to the protein that contained the immunogen peptide. Today, many antibodies are generated to synthetic peptides and are purified with the immunising peptide based on affinity thus resulting in a reduction in possibility that the antibody binds to epitopes not found on the original peptide (Burry, 2000).

Therefore, controls are crucial in ensuring that the detection of the appropriate antigen is what results in the labelling seen using immunocytochemistry. Initially, to demonstrate that the labelling found is due to the primary antibody specifically, the primary antibody can be either omitted (Schuster and Powers, 2005) or replaced with similarly diluted normal serum from the same species, keeping all other experimental procedures the same i.e. a negative control (Petrusz et al., 1976). In addition to negative controls, positive controls can be done using tissue selected to contain (or not contain) the protein to confirm the specificity of the antibody. This should demonstrate immunoreactivity for this protein at the appropriate sites, but a lack of immunoreactivity in those components that are known not to contain that protein. Josephsen et al. (1999) also showed that sections could be used with several different antibodies that are directed against the same structure for labelling.
Absorption controls

The specificity of an antibody as demonstrated by appropriate controls is crucial in understanding the localisation of compounds in cells/tissues. One valuable control in the detection of a specific molecule is the absorption or pre-absorption technique where the antigen being examined needs to be present in its pure states in great amounts. The primary antibody is incubated with the pure, exogenous antigen, and then applied to the tissue being investigated. If the antibody is specific for the exogenous antigen, the “pre-absorption” should prevent the antibody from recognising the tissue antigen (Hockfield et al., 1993). However, this technique demonstrates only the specificity of the antibody for the incubating peptide/protein but does not prove the specificity of the antibody for the protein in whatever tissue is being examined (Swaab et al., 1977; Willingham, 1999).

Controls for double labelling immunocytochemistry

Double (multiple) labelling in immunocytochemistry allows two (or greater) different antigens to be examined in a single preparation (Wessendorf and Elde, 1985). Therefore it allows the determination of whether or not two different cell types can express unique antigens.

Two types of double labelling can be undertaken – direct and indirect. Direct double labelling uses two primary antibodies, where at least one is conjugated directly to a marker, and can be from the same species or of the same isotype (Hockfield et al., 1993). However, performing indirect double labelling can be undertaken with unlabelled primary antibodies from different species or unlabelled primary antibodies from the same species but a different immunoglobulin (isotype). Then, isotype- or species-specific labelled secondary antibodies can then be utilised in identifying the antigens recognised by the primary antibodies. However, when the antibodies are from identical species or isotype, indirect
double labelling can also be undertaken by the addition of blocking steps and sequential incubations with a primary antibody, the relevant secondary antibody, an alternative primary antibody, and the next relevant secondary antibody. However, as well as the control experiments which have to be undertaken for verification of the specificity of the antibodies used, additional controls have to be used.

If double labelling experiments are undertaken using primary antibodies from differing isotypes or species, the final concentration of each antibody should be identical to that established for single labelling. Mixtures of two primary antibodies, if produced in different species, or if they have unique isotypes, can be used if say, for example, fluorescent secondary antibodies are available for detection of these primary antibodies. However, an essential negative control in indirect double labelling is to make sure that each individual secondary antibody is specific for the appropriate primary antibody. This is identified by incubation of a separate tissue sample with each primary antibody and then with an inappropriate secondary antibody (Hockfield et al., 1993). No labelling should be found in this case i.e. no cross-reactivity should be present within the tissue examined.

If, on the other hand, double label immunofluorescence is performed using primary antibodies from the same isotype or species, control experiments have to be undertaken in addition to the ones detailed above. As a positive control, the order should be reversed in primary antibody incubation and the labelling should be identical to that undertaken initially. For a negative control, the tissue should be incubated only with the primary antibody, but no secondary antibody conjugated to a fluorochrome. This is then followed by incubation with the first primary antibody, with incubation in an excess unlabelled secondary antibody solution, and then with an incubation in which the secondary antibody is conjugated to the secondary fluorochrome (Hockfield et al., 1993). If primary antibody sites are saturated, this should result in tissue that is unlabelled. However, if labelling is
present, the experiments are repeated using a greater concentration of the unlabelled secondary antibody, or a reduced secondary antibody concentration conjugated with the secondary fluorochrome. If, after this, cross-reactivity is still found, the antibodies are not used for double labelling experiments.

*Antibodies used within the subsequent studies*

The studies undertaken in subsequent chapters involve a total of ten different primary antibodies and three fluorescent secondary antibodies, all of which have been extensively documented in the literature, and gave staining patterns consistent with those seen previously.

The mouse monoclonal antibody used was generated against cell nuclei extracted from mouse brain and was found to react with the protein specific to neurons (Mullen et al., 1992). This antibody labels all neurons (and no glial cells) within the rat spinal cord (Todd et al., 1998). The rabbit c-Fos antiserum was raised against a synthetic peptide sequence common to all c-Fos proteins (Hunt et al., 1987) and has been used in many immunocytochemical studies of the spinal cord (Hunt et al., 1987; Williams et al., 1989, 1990a,b; Olave and Maxwell, 2004; Todd et al., 1994, 2005).

The sheep antiserum against neuronal nitric oxide synthase used later (nNOS), or the K205, has been assessed using Western blotting (Herbison et al., 1996). They showed that the antibody recognised one main protein with a molecular mass of 155Kd in lanes with the recombinant nNOS and rat hypothalamus. Other smaller protein bands were present in both lanes and were assumed to be fragments of the nNOS. Herbison et al. (1996) also performed liquid phase absorption experiments and demonstrated that all immunoreactivity was abolished by absorption of the K205 antiserum with the recombinant nNOS protein.
The guinea pig antiserum against the vesicular glutamate transporter 2 (VGLUT2) has been examined by Mathur and Deutch (2008). They examined the specificity of the VGLUT2 antibody, and also the vesicular glutamate transporter 3 (VGLUT3) antibody by performing controls eliminating the primary antibodies and found no staining. In addition, they also incubated sections in a solution containing the VGLUT3 primary antibody, but not the VGLUT2 antibody, having both secondary antibodies present. From that, they demonstrated only VGLUT3, and no VGLUT2 immunoreactivity and no non-specific immunofluorescence with either the VGLUT2 or VGLUT3 primary antibodies. As well as that study, numerous other authors have shown the terminal staining that is found using this antibody, as discussed in detail in chapter 4.

The guinea-pig antiserum against the NK-1 receptor used in subsequent studies showed staining identical to that from the well characterised rabbit anti-NK1 receptor antibody (Vigna et al., 1994; Polgár et al., 1999) and the rat NK-1 receptor (Spike et al., 2003).

The substance P monoclonal antibody recognises the C-terminal of the peptide and therefore does not distinguish between substance P and the related tachykinins neurokinin A and B (Cuello et al., 1979). Substance P and neurokinin A originate from the same gene (preprotachykinin I), but neurokinin B arises from preprotachykinin II. Although neurokinin B is present in the dorsal horn, it is not detectable in primary afferents and is thought to originate from neurons within the spinal cord (Ogawa et al., 1985; Warden and Young, 1988; Too and Maggio, 1991). The rat substance P has been well characterised previously by Naim et al. (1997) and Todd et al. (2000).

The protein kinase C-γ (PKC-γ; C-19) used in chapter 4, investigation 2 is an affinity purified rabbit polyclonal antibody raised against a peptide mapping at the C-terminus of PKC-γ of mouse origin and has been shown to be highly specific by numerous authors (Takai et al., 1979; Nishizuka, 1984a; Osada et al., 1992; Polgár et al., 1999).
The goat polyclonal antibody was raised against CTb, and rabbit was raised against Fluorogold. Specificity of each of these tracer antibodies was demonstrated by a lack of staining in areas of the central nervous system that did not contain neurons that had taken up and transported the tracer and by immunostaining found in populations of neurons that are known to project to the injection sites, as discussed in detail in chapter 5. In addition, the specificity of the Fluorogold antibody was directly confirmed by comparing the fluorescence (seen through a UV filter set) with that for the anti-Fluorogold in individual neurons. All experiments examined had a perfect match between the two types of fluorescence.

Therefore, to minimise suffering to animals, as requested under the UK Animals (Scientific Procedures) Act 1986, and the fact that both the primary and secondary antibodies have been extensively characterised in the literature as quoted throughout the work presented, control experiments were not deemed appropriate.
### Table 2.1

<table>
<thead>
<tr>
<th>Fluorophore</th>
<th>Excitation $\lambda$</th>
<th>Emission $\lambda$</th>
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<tbody>
<tr>
<td>Alexa 488</td>
<td>494</td>
<td>518</td>
</tr>
<tr>
<td>Rhodamine-Red</td>
<td>570</td>
<td>590</td>
</tr>
<tr>
<td>Cyanine 5.18</td>
<td>650</td>
<td>670</td>
</tr>
</tbody>
</table>

*Table 2.1* Excitation-emission wavelengths corresponding to the fluorophores used.
Chapter 3

Investigation 1:

Nociceptive stimuli that activate LSN neurons
1. Introduction

C-Fos is an immediate early gene which is rapidly and transiently expressed in neurons in response to stimulation (Harris, 1998). Transcriptional activation of the gene occurs within minutes of stimulation, with the accumulation of mRNA reaching its peak approximately 30 to 40 minutes later. The gene encodes for the nuclear protein Fos, and levels peak about two hours after induction of gene transcription. Many stimuli have been found to activate neurons in the superficial laminae including noxious mechanical stimuli and heat and cold stimuli (Price et al., 1978; Ferrington et al., 1987; Han et al., 1998; Craig et al., 2001).

After noxious peripheral stimulation, Fos immunoreactive neurons are found throughout the spinal grey matter, but there is general agreement that they are concentrated in the superficial dorsal horn (laminae I and II) and in a band across the deep part of the dorsal horn and intermediate grey matter, extending from the lateral reticulated part of lamina V, towards the area around the central canal (lamina X). There are differences in the distribution in Fos immunoreactive neurons within the superficial dorsal horn following different types of acute noxious stimulus: cutaneous and subcutaneous stimulation giving rise to immunoreactive neurons throughout lamina I and II, whereas after noxious stimulation of muscles, joints or viscera, immunoreactive neurons are common in lamina I, but rare in lamina II (Hunt et al., 1987; Menétrey et al., 1989). There are also reports that repeated innocuous mechanical stimulation results in Fos immunoreactivity in neurons in laminae III and IV (Hunt et al., 1987; Jasmin et al., 1994; Menétrey et al., 1989).

Some cells which develop Fos immunoreactivity after noxious stimulation are known to be projection neurons, with axons that terminate in the brainstem or thalamus (Menétrey et al., 1989; Tavares et al., 1993), however it is likely from the very high density of Fos immunoreactive cells that most are interneurons. Neurons in laminae I to III of the dorsal
horn are highly diverse in terms of their neurochemical profiles (Willis and Coggeshall, 1991; Todd and Spike, 1993). Many of these neurons are not inhibitory, but it has been shown that approximately one-third are GABAergic in laminae I – III, and also use glycine or acetylcholine as a transmitter, (Todd and Sullivan, 1990; Todd, 1991). Interestingly, Todd et al. (1994) showed that approximately one-fifth of c-Fos immunoreactive neurons in the SDH (laminae I – II) showed GABAergic immunoreactivity.

More recently, Olave and Maxwell (2004) retrogradely labelled LSN neurons that projected to an area established in nociceptive processing, the caudal ventrolateral medulla (CVLM; Morton et al., 1983; Janss and Gebhart, 1988; Liu and Zhao, 1992) and combined this with the induction of c-Fos with peripheral noxious stimuli and NK-1 receptor immunoreactivity. They observed that a proportion of LSN neurons had been activated bilaterally by noxious stimulation, though NK-1 projection neurons that expressed Fos were not very numerous. As the SDH expresses Fos only unilateral to the side of noxious peripheral stimulation (Hunt et al., 1987; Harris, 1998; Todd et al., 1994, 2002), this suggests that the LSN receives nociceptive information in a very different way to the adjacent SDH.

In the past Fos has been useful in demonstrating noxious mechanical, hot and cold stimuli activating neurons in the SDH (Hunt et al., 1987; Ferrington et al., 1987; Han et al., 1998; Craig et al., 2001). Therefore, the purpose of this study was to a) identify if LSN neurons received cutaneous information, b) to determine if the LSN neurons responded specifically to noxious cutaneous stimulation and finally, c) to use four different noxious peripheral stimuli to identify if there was a variable degree of expression of Fos dependent on the stimulus used.
2. Experimental Procedures

Induction of c-Fos

Twelve adult male Sprague Dawley rats (Harlan, Loughborough, UK; 190-250g) were used in this study where they were housed under conditions of a 12 hour light-dark cycle with food and water provided ad libitum. All experiments were performed in accordance with the UK Animals (Scientific Procedures) Act 1986 and the European Communities Council Directive (86/609/EEC). They were anaesthetised initially with halothane in an enclosed chamber followed by injecting a ketamine and xylazine mixture (7.33 and 0.73mg/100g i.p., respectively). They were divided into four groups receiving one of the following noxious stimuli to the left hind-paw: 1) immersion of the hind-paw in hot water at 55°C for 20 seconds (n = 3); 2) immersion of the hind-paw in cold water maintained at 4°C for 30 seconds every 2 minutes over a 2 hour period (n = 3); 3) topical application of 100% mustard oil to the hind-paw (n = 3); 4) a subcutaneous injection of 50ml of 2% formaldehyde to the hind-paw (n = 3). The application of a peripheral noxious stimulus to one limb has been shown to induce the expression of Fos mainly in neurons in the ipsilateral SDH, especially in the medial portion (Hunt et al., 1987; Todd et al., 1994, 2002). The animals were maintained under anaesthetic for 2 hours after the application of the noxious stimulus, and then perfused through the left ventricle with 4% freshly depolymerised formaldehyde. All efforts were made to minimise the numbers of animals used and any unnecessary discomfort.

Immunocytochemical labelling for confocal microscopy

Mid-lumbar spinal cord segments (L3 – 5) were removed and post-fixed overnight. Transverse 50µm sections were cut with a Vibratome, and alternate sections selected with 10 collected from each of the spinal segments (resulting in a total of 30 per animal).
Sections were then immersed in 50% ethanol for 30 minutes to enhance antibody penetration (Llewellyn-Smith and Minson, 1992). For each of the above categories of Fos induced animals, immunocytochemical labelling was undertaken with mouse anti-NeuN antiserum (diluted 1:1000; Millipore, Watford, UK) and rabbit anti-c-Fos antiserum (diluted 1:2500, Santa Cruz, USA). After a 48 hour incubation period, sections were rinsed in phosphate buffered saline (PBS) and incubated for 3 hours in solutions containing species-specific secondary antibodies raised in donkey, conjugated to rhodamine-red-anti-mouse immunoglobulin (IgG; diluted 1:500, Jackson Immunoresearch) to detect NeuN, and Alexa-488-anti-rabbit IgG (diluted 1:100, Molecular Probes) to detect Fos. Following rinses in PBS, sections were mounted in anti-fade medium (Vectashield; Vector Laboratories, Peterborough, UK) and stored until required for analysis in a freezer at -20ºC.

Quantitative analysis

In all experiments, the left and right LSNs were scanned systematically by using a Radiance 2100 confocal laser microscope (Hemel-Hempstead, UK). Tissue was scanned with a 40X and 60X oil-immersion lens and each image was captured at 1µm intervals in the z-axis with a zoom factor of 1. In each animal, ten alternate sections were used for analysis from spinal segments L3 – 5 (i.e. a total of 30 per animal) and 2 fields were taken from each section (one from the left LSN and one from the right LSN). Therefore, for each animal, a total of 60 fields were taken covering all spinal segments. Image analysis was undertaken for all experiments by using Neurolucida for Confocal (MicroBrightField, Inc., Colchester, VT). The total number of neurons in the LSN was recorded (from NeuN staining) and then the green frequency was used to identify Fos immunoreactive neurons in each of the four categories of stimuli used.
3. Results

Fos immunoreactive neurons in the LSN after the application of a noxious stimulus

The average number of sampled neurons over each spinal segment (including ± SD), as detected by the neuronal marker NeuN, was very consistent between experiments and also between the left (ipsilateral to stimulation) and right (contralateral to stimulation) sides of the spinal cord (left LSN, average ± S.D. = 78 ± 4.65; right LSN, 82 ± 6.21). Following counting for each of the defined categories with one of four stimuli (hot water at 55°C, cold water at 4°C, 100% mustard oil or 50ml of 2% formaldehyde; n = 3 in each category), average percentages (± S.D.) of the LSN neuronal population identified through NeuN were calculated.

There was a mean number of Fos immunoreactive neurons for each of the categories as follows: hot water at 55°C, 8.58 ± 2.97% ipsilateral and 6.94 ± 2.06% contralateral to the side of stimulus (typical examples shown in Figs 3.1 and 3.2); cold water at 4°C, 4.86 ± 1.77% ipsilateral and 3.98 ± 1.80% contralateral to the side of stimulus; mustard oil (100%), 3.47 ± 1.46% ipsilateral and 2.45 ± 0.56% contralateral to the side of the stimulus; formaldehyde (2%, 50ml), 1.37 ± 1.06% ipsilateral and 0% contralateral to the side of the stimulus. These results are summarised in the histogram in Figure 3.3

The average percentage of Fos positive neuronal cells (as identified by NeuN) in the left LSN (ipsilateral to the stimulus applied) and the right (contralateral to the stimulus applied) was very similar in each of the categories of stimulus applied. In addition, using a one-way ANOVA, none of the differences between the left and right LSNs for each of the experimental categories were found to be significant statistically. However, what is notable in this study is the bilateral activation of the LSN neurons with a peripheral noxious stimulus (apart from the use of formaldehyde, but here overall number of c-Fos immunoreactive neurons in the ipsilateral LSN was very low). This is in contrast to the
SDH where it is known that a peripheral noxious stimulus applied to the right hind limb induces the expression of Fos mainly in neurons in the ipsilateral dorsal horn, i.e. right, especially in the medial part of the SDH (Hunt et al., 1997; Todd et al., 1994; Doyle and Hunt, 1999; Todd et al., 2002). The relations of Fos immunoreactivity between the LSN and the SDH ipsilateral and contralateral to the sides of stimulus are demonstrated in Figure 3.1.

However, there was a notable difference in the numbers of Fos immunoreactive neurons between the different categories of stimulus applied, with the hot water at 55°C showing the greatest numbers of neurons activated, with the formaldehyde stimulus demonstrating only very small numbers of Fos immunoreactivity suggesting that the LSN neurons are activated to a greater degree with a hot water stimulus compared to the other stimuli applied.
Figure 3.1. Fos labelling in the SDH and the LSN. A shows Fos labelling in the ipsilateral dorsal horn (as indicated by the arrows) as well as the LSN (as indicated by the *), whereas B shows Fos labelling in the contralateral LSN (as indicated by the *) but no labelling in the contralateral dorsal horn following application of the peripheral noxious stimulus (hot water at 55°C). Scale bar = 100µm, valid for both images.
Figure 3.2.  Fos neuron in the LSN. Image of a transverse section of the LSN, superficial dorsal horn (SDH) and lateral funiculus shown in A – C (red, NeuN; green, Fos). In A – C, 1 indicates a NeuN (red) labelled neuron in the LSN which shows Fos (green) immunoreactivity contralateral to the side of stimulation. 2 indicates a neuron in the lateral funiculus also immunoreactive for Fos. Numerous neurons are present in the LSN that do not demonstrate Fos immunoreactivity after hot water stimulation (55ºC). Scale bar = 20µm.
Figure 3.3. Histogram summarising the percentage of NeuN cells in the LSN that showed Fos immunoreactivity for each of the four stimuli used. Three animals were used in each of the four categories. Ipsilateral is the side of the stimulus i.e. left side, error bars = ± S.D.
% of Fos immunoreactive neurons in the LSN

% of NeuN neurons

- Ipsilateral
- Contralateral

<table>
<thead>
<tr>
<th>Condition</th>
<th>Ipsilateral</th>
<th>Contralateral</th>
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<tbody>
<tr>
<td>55°C</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>4°C</td>
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<td>2</td>
</tr>
<tr>
<td>Mustard oil</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>1</td>
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</tr>
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</table>
4. Discussion

Technical considerations

The mouse monoclonal antibody used in this study (and subsequent studies in the following chapters) was generated against cell nuclei extracted from mouse brain and was found to react with the protein specific to neurons (Mullen et al., 1992). This antibody labels all neurons (and no glial cells) within the rat spinal cord (Todd et al., 1998). The c-Fos antiserum was raised against a synthetic peptide sequence common to all c-Fos proteins (Hunt et al., 1987) and has been used in many immunocytochemical studies of the spinal cord (Hunt et al., 1987; Williams et al., 1989, 1990a,b; Olave and Maxwell, 2004; Todd et al., 1994, 2005).

The distribution of c-Fos immunoreactivity after noxious peripheral stimulation was found throughout the spinal grey matter on the side ipsilateral to the stimulus applied (only very sparse contralateral labelling) but, in agreement with others, was concentrated in laminae I and II (especially the outer (dorsal) part of lamina II) and in a band across the deep part of the dorsal horn and intermediate grey matter, extending from the lateral reticulated area of lamina V, towards the area around the central canal (Lantéri-Minet et al., 1993; Menétry et al., 1989; Lima et al., 1993; Tavares et al., 1993; Todd et al., 1994, 2005). These areas are known to correspond to the terminal fields of primary nociceptive afferent fibres and to the distribution of nocireactive neurons identified by electrophysiological recordings (Bullitt, 1991; Hunt et al., 1987; Presley et al., 1990). In addition to this, there was greater c-Fos immunoreactivity in the more medial parts of laminae I and II, but a less tightly organised arrangement of immunoreactivity in lamina V, in agreement with Bullitt (1991).

Previous investigators (using the cat) have shown that there is indeed a somatotopic arrangement of the dorsal horn arranged mediadorsally, with the foot and toes represented
in the medial two thirds of the dorsal horn, and with the proximal leg and hip represented more laterally (Wall, 1953; Brown and Fuchs, 1975; Pubols and Golberger, 1980; Brown and Culberson, 1981; Light and Durkovic, 1984). The rat also has a similar pattern of primary afferent termination (Ygge and Grant, 1983; Swett and Woolf, 1985; Molander and Grant, 1985, 1986). As the stimulus was applied to the hindpaw, this would explain the arrangement of the Fos immunoreactivity in laminae I and II (greater proportion of Fos immunoreactivity in the medial portion of these laminae) and also acts as a “positive” control for the LSN results which will be dealt with later. In addition, the experiments undertaken within this study also show “positive” control staining for Fos in the lateral aspect of laminae I and II (Figures 3.1). However, where a stimulus to induce Fos was not applied, there was little or no immunolabelling for Fos in the spinal cord, as shown by Olave and Maxwell (2004).

Other types of stimulation have been shown to cause Fos immunoreactivity within the spinal cord; however there is a different distribution in response to noxious stimulation and non-noxious (tactile) stimulation. For example, in contrast to the Fos expression in response to chemical and thermal stimulation, non-noxious stimulation (brushing of hairs, gentle manipulation of joints and walking) induces Fos immunoreactivity in the deeper laminae, namely III – VI (Hunt et al., 1987; Jasmin et al., 1994). However, in numerous control studies where no stimulus was applied, only very sparse Fos immunoreactivity was identified in deeper laminae, including laminae III and IV (Menétry et al., 1989; Presley et al., 1990; Jasmin et al., 1994; Hagihari et al., 1997). Therefore, as it has been proven that only very little Fos immunoreactivity is present in “negative” controls, further sacrifice of animals was not undertaken in this present study to minimise further pain and unnecessary suffering under the UK Animals (Scientific Procedures) Act 1986.
Two anaesthetic combinations were used in this study – inhalational halothane and a mixture of ketamine and xylazine. It has been shown that halothane as an inhalational anaesthetic can result in the suppression of Fos immunoreactivity, however only in the deeper layers of the grey matter (laminae III and IV) and not within neuronal populations in laminae I and II; suppression is minor compared to inhalational nitrous oxide, which was avoided in this, and subsequent studies (Hagihara et al., 1997). As there was abundant Fos staining with the four stimuli used in laminae I and II, especially in the medial aspect (but also some staining found in the lateral aspect – see Figure 3.1) and the lateral reticulated area of lamina V, extending to the area around the central canal, the influence of this anaesthetic could be assumed as minimal on Fos expression to the stimuli used.

Ketamine (a non-competitive NMDA receptor antagonist acting at an allosteric site, Harrison and Simmonds, (1985)) and xylazine (an α2 adrenergic receptor agonist (McCurnin and Bassert (2002)) have not been shown to influence Fos expression upon noxious stimulation, and it has been suggested that these act supraspinally rather than at the dorsal horn level (Yi and Barr, 1996). Again, the effects of this combination on Fos expression throughout the spinal cord (including the LSN) can be seen as minimal and will not significantly alter Fos expression with the stimuli used here.

Fos immunoreactivity in the LSN

The main finding of this study is that although Fos immunoreactivity has been detected in the LSN, particularly in response to hot water, overall Fos immunoreactivity is actually lower than what was anticipated.

Olave and Maxwell (2004) quantified the number of LSN neurons projecting to the caudal ventrolateral medulla (CVLM; an area established in nociceptive processing) that also possessed the NK-1 receptor. They had shown that more than one-fifth of all neurons
examined (including those projecting to the CVLM and/or containing the NK-1 receptor) showed Fos immunoreactivity upon using a thermal noxious stimulus peripherally (hot water). Compared to the Olave and Maxwell (2004) study, this work shows a smaller number of LSN neurons that demonstrate Fos immunoreactivity, even when using the same noxious stimulus peripherally.

Unlike the SDH, where the highest concentration of Fos immunoreactive neurons are located ipsilateral to the peripheral stimulus (Hunt et al., 1987; Todd et al., 1994, 2002), as well as immunoreactivity found in the lateral reticulated area of lamina V, VII, VIII and X (Menétrey et al., 1989), LSN neurons showing Fos immunoreactivity were found bilaterally, and of approximately equal proportions.

LSN neurons not only project to numerous supraspinal sites, but also receive descending projection fibres from supraspinal nuclei including the raphe nuclei, brainstem reticular formation nuclei, dorsal column nuclei and the periaqueductal grey (PAG) (Carlton et al., 1985; Masson et al., 1991). These in turn could be activated by ascending fibres of spinal projection neurons located in the SDH. Suzuki et al. (2002) showed that SDH neurons possessing NK-1 immunoreactivity activated descending pathways that influenced spinal excitability. Therefore, the bilateral expression of Fos in the LSNs could be the result of ascending activation of descending systems projecting bilaterally to this nucleus. In addition to this, Grudt and Perl (2002) demonstrated that some axon collaterals from lamina I neurons entered the area of the LSN, and also had their axons which passed ventrally, then contralaterally to project in the ventral white funiculus. From this, these neurons in lamina I could be activated by primary afferent input to the SDH and then activate LSN neurons through collaterals. Also, Petkó and Antal (2000) demonstrated that neurons in the lateral area of the SDH had commissural axons passing to the SDH on the contralateral side. They also showed that labelling occurred in the LSNs after lateral dorsal
horn injections. Taken together, this evidence could account for the bilateral Fos expression in the LSN after the application of noxious stimuli.

The other significant finding of this study also demonstrates is that, although the immediate early gene c-Fos is rapidly and transiently expressed in neurons in response to stimuli (Morgan and Curran, 1989, 1991), there has been a notable difference dependent on the stimulus involved in the LSN, though not in the superficial dorsal horn. Many types of stimuli have been documented to produce c-Fos expression and it has been assumed that neuronal activity in relation to nociception and the presence of Fos is related (Morgan et al., 1987; Hunt et al., 1987; Sagar et al., 1988).

In addition, stimulation has to be strong and prolonged before quantifiable levels of Fos expression are achieved (Bullitt et al., 1992; Lima and Avelino, 1994) and not all neurons express the gene when activated (Dragunow and Faull, 1989). Even the ventral posterolateral nucleus of the thalamus (VPL), an area having an established role in nociception, did not result in Fos expression after noxious stimulation (Willis, 1985, 1989; Bullit, 1990).

However, what the present study does add is that although the LSN appears to be activated bilaterally by cutaneous stimulation (specifically noxious stimuli) to varying degrees depending on the stimulus used, and confirms and extends other studies that have implicated the LSN in nociception (Harmann et al., 1988; Battaglia and Rustioni, 1992; Schafer et al., 1994; Feil and Herbert, 1995; Jiang et al., 1999; Olave and Maxwell, 2004), the proportion of LSN neurons responding to a peripheral noxious stimulus is much lower than may have been expected. Indeed, with the stimulus showing the greatest Fos immunoreactivity, it only revealed a maximum of approximately 15% of all NeuN labelled neurons in the LSNs.
In summary, all stimuli that were used in the experiments detailed previously have shown high levels of Fos immunoreactivity in the superficial dorsal horn (especially the medial parts of laminae I and II). However, even with the stimulus that demonstrated greatest Fos immunoreactivity in the LSN (hot water at 55°C), the overall numbers were low, representing at most 15% of all neurons. It could be postulated that as there was a notable difference between the LSN and the superficial dorsal horn, it may well be that this nucleus could receive nociceptive input from areas other than that stimulated in these experiments i.e. nociceptive information from sites other than the hind-paw and may have a very different somatotopic organisation to the superficial dorsal horn.
Chapter 4

Investigation 2:

Relationship of axon terminals possessing NOS with NK-1 neurons in the LSN
1. Introduction

Nitric oxide synthase (NOS), a nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d; Garthwaite, 1991), is the enzyme responsible for producing nitric oxide (NO) and its neuronal form (nNOS) is found in a variety of CNS neurons (Schmidt et al., 1991; Ruscheweyh et al., 2006). NO is an unconventional diffusible intercellular factor (Garthwaite and Boulton, 1995) and its principal target is soluble guanylyl cyclase which induces cyclic 3',5'-guanosine monophosphate (cGMP). In the spinal cord, many cell bodies containing NOS and NADPH-d are located in the SDH (Dun et al., 1992; Valtschanoff et al., 1992a) and a dense plexus of NOS-immunoreactive axon terminals is present in laminae I – III (Valtschanoff et al., 1992b; Laing et al., 1994; Bernardi et al., 1995) which also contain co-localised GABA.

Behavioural (Malmberg and Yaksh, 1993) and pharmacological studies (Kawabata et al., 1994; Semos and Headley, 1994; Lin et al., 1999) have indicated that NO is a modulator of nociceptive processes but there is no agreement on its precise role and it may have hyperalgesic and analgesic effects (Hoheisel et al., 2005). NO expression in the SDH is known to increase following peripheral application of noxious stimuli (Soyguder et al., 1994; Lam et al., 1996). This is often accompanied by induction of c-Fos which is abolished following administration of the NOS inhibitor, N\textsuperscript{\omega}-nitro-L-arginine methyl ester (L-NAME; Lee et al., 1992; Roche et al. 1996; Wu et al., 2000). Laminae I and IIo of the SDH are the principal termination sites of SP and glutamate-containing unmyelinated nociceptive cutaneous primary afferent fibres. It has been suggested that NO has an important role in the development of hyperalgesia (Meller and Gebhart, 1993). This involves activation of N-methyl D-aspartate (NMDA) receptors which in turn activate NOS to produce a NO/cGMP cascade which enhances the release of CGRP and SP from primary afferents fibres (Garry et al., 1994; Kamasaki et al., 1995; Aimar et al., 1998). Enhanced
release of glutamate and substance P is associated with central sensitisation (Wu et al., 2000) which may be an underlying mechanism of hyperalgesia.

Protein kinase C (PKC) on the other hand, is an enzyme that is important as a common mechanism for signal transduction of various extra-cellular signals into the cell to control many physiologic processes (Nishizuka, 1984a,b, 1986). In addition, it is believed to be involved in the process of sensitisation of dorsal horn neurons (Coderre, 1992; Mao et al., 1992; Palečk et al., 1994). PKC is activated by a rise in intracellular Ca\(^{2+}\) (for example through NMDA receptors) and through phospholipase C, which stimulates the formation of inositol triphosphate (IP3) and diacylglycerol which is mediated by certain G-protein coupled receptors (Nishizuka, 1984a; Berridge and Irvine, 1989; Bockaert et al., 1993; Schoepp and Conn, 1993). Candidate receptors in the dorsal horn include type I metabotropic glutamate receptors (mGluR1 and 5) and NK-1 receptors (Coderre, 1992; Palečk et al., 1994; Schoepp and Conn, 1993; Sluka and Willis, 1995). Interestingly, Polgár et al. (1999a) have shown that some neurons with protein kinase C-γ (PKC-γ) in lamina I and in lamina III also possessed the NK-1 receptor. It may well be postulated that substance P (which targets the NK-1 receptor) may also activate PKC-γ in those cells. Also, the involvement of excitatory amino acids and NK-1 receptors in both injury induced neuronal plasticity and in the stimulation of intracellular cascades leads to the translocation and activation of PKC, suggesting that PKC may be involved in neuronal changes produced by peripheral tissue injury that contribute to persistent nociception (Yashpal et al., 1995). Malmberg et al. (1997) also showed, using knock-out PKC-γ mice, that PKC-γ is linked to the development of neuropathic pain as mice that lacked PKC-γ displayed normal responses to acute pain, but they almost completely failed to develop the neuropathic pain syndrome after partial sciatic nerve sectioning.
With the relations of PKC-\(\gamma\) to the NK-1 receptor as previously discussed in the superficial dorsal horn (Polgár et al., 199), and the fact that it requires an increase in intracellular \(\text{Ca}^{2+}\) like NOS (via calmodulin (Bredt and Snyder, 1990)), there may well be a close interactive relation between PKC-\(\gamma\) and the NK-1 receptor in the LSN, like the superficial dorsal horn.

Therefore, the purpose of this study was to: a) identify the relationship between SP and the NK-1 receptor (as SP is abundant (Chapter 1, Pages 6 – 8)) in the LSN b) to quantify the proportion of neurons containing NK-1 in the LSN (and also protein kinase C-\(\gamma\) (PKC-\(\gamma\)) which, like the NK-1 receptor, is also associated with nociceptive processing (Malmberg et al., 1997)) c) identify if a close relationship (as present in the SDH) exists between the abundant SP staining (Chapter 1, Pages 6 – 8) and abundant NOS staining (Valtschanoff et al., 1992a; Nazli and Morris, 2000) in the LSN d) identify and quantify the relationship between NOS and the NK-1 receptor and PKC-\(\gamma\) e) identify the co-localisation pattern of NOS terminals in the LSN, i.e. are they associated with GABAergic terminals, as in the SDH

2. Experimental Procedures

*Immunocytochemical labelling for confocal microscopy*

A total of ten male Sprague-Dawley rats (150-250g) were used in this study where they were housed under conditions of a 12 hour light-dark cycle with food and water provided *ad libitum*. All experiments were performed in accordance with the UK Animals (Scientific Procedures) Act 1986 and the European Communities Council Directive (86/609/EEC). Each animal was anaesthetised with halothane in an anaesthetic chamber followed by i.p. administration of sodium pentobarbitone (1ml; 200mg/ml). They were
perfused through the left ventricle initially with saline followed by a fixative containing 4% freshly depolymerised formaldehyde in phosphate buffer pH 7.6.

The L3 – 5 lumbar segments were removed and post-fixed in the same solution for eight hours. Transverse sections (50µm) were cut by using a Vibratome and placed in 50% ethanol for 30 minutes to enhance antibody penetration (Llewellyn-Smith and Minson, 1992) and washed in phosphate buffer. Sections from the 10 animals were then placed into one of four groups for immunocytochemical reactions. Sections from the first group were processed for triple labelling immunofluorescence with the following primary antibodies: rabbit anti-PKC-γ (1:1000; Santa Cruz Biotechnology), guinea pig anti-NK-1 (1:1000; Sigma) and mouse anti-NeuN antiserum (1:1000; Millipore, Watford, UK) (n = 3). The second group was processed for triple immunofluorescence with rabbit anti-PKC-γ (1:1000; Santa Cruz Biotechnology), guinea-pig anti-NK-1 (1:1000; Sigma) and sheep anti-NOS antiserum (1:2000; gifted by Dr P. Emson, Cambridge) (n = 3). The third group was processed for quadruple labelling with rabbit anti-GAD (1:1000; Sigma), guinea-pig anti-VGLUT2 (1:5000; Chemicon), guinea pig anti-NK-1 (1:1000; Sigma) and sheep anti-NOS antiserum (1:2000; gifted by Dr P. Emson, Cambridge) (n = 3). Sections from the fourth group were processed for SP (1:100; Oxford Biotechnology), sheep anti-NOS antiserum (1:2000; gifted by Dr P. Emson, Cambridge) and guinea pig anti-NK-1 (1:1000; Sigma) (n = 3). After a 48 hour incubation period at 4°C, sections were rinsed in PBS and incubated for 2 hours in solutions containing species-specific secondary antibodies (all raised in donkey) coupled to fluorophores: Alexa 488 immunoglobulin (diluted 1:500; Molecular Probes) to identify either PKC-γ (experiments 1 and 2), GAD (experiment 3) or SP (experiment 4), rhodamine-red immunoglobulin (1:100; Jackson Immunoresearch) to identify NK-1 (all experiments) and VGLUT2 (experiment 3) and cyanine 5.18 immunoglobulin (1:100; Jackson Immunoresearch) raised against the corresponding animal
in the primary antibody to identify either NeuN (experiment 1) or NOS (experiments 2 and 4). Note that in the third group of experiments, both VGLUT2 and the NK-1 receptor were labelled with the same secondary antibody. This was possible because the NK-1 receptor is located principally on cell membranes whereas the VGLUT2 is found within axonal boutons. For this reason both antigens can be labelled with a primary antibody of the same species (in this case guinea pig) coupled with the same secondary antibody thus allowing differentiation between the two types of immunoreactivity. All antibodies were diluted in PBS with 0.3% Triton X-100. Following the incubations in secondary antibodies, sections were rinsed thoroughly in PBS, mounted in anti-fade medium (Vectashield; Vector Laboratories, Peterborough, UK) and stored until required for analysis in a freezer at -20°C.

Quantitative analysis

In all experiments, the left and right LSNs were scanned systematically by using a Radiance 2100 confocal laser microscope (Hemel-Hempstead, UK). Tissue was scanned with a 40X oil-immersion lens for experiments 1 (PKC-γ, the NK-1 receptor and NeuN) and 4 (SP, NOS and the NK-1 receptor). The 60X oil-immersion lens was used for experiments 2 (PKC-γ, NOS and the NK-1 receptor) and 3 (GAD, VGLUT2, the NK-1 receptor and NOS). Each image was captured at 1µm intervals (all 4 experiments) in the z-axis with a zoom factor between 1 and 5. In each animal, ten alternate sections were used for analysis from segments L3 – 5 (i.e. a total of 30 per animal) and two fields were taken from each section (one from the left LSN and one from the right LSN). For each animal therefore, a total of 60 fields were taken covering all spinal segments. Image analysis was undertaken for all experiments by using Neurolucida for Confocal (MicroBrightField, Inc., Colchester, VT).
Quantitative analysis involved the following steps:

*Experiment 1*: PKC-\(\gamma\), NK-1 and NeuN (\(n=3\)): 1) LSN neurons were identified (by NeuN) and counted after image capture. This enabled an estimation of the mean number of cells for segments L3 – 5. 2) The neurons were then placed into one of three categories: (A) those immunoreactive for NK-1; B) those immunoreactive for PKC-\(\gamma\); (C) those double labelled for both NK-1 and PKC-\(\gamma\).

*Experiment 2*: PKC-\(\gamma\), NOS and NK-1 (\(n=3\)): 30 immunoreactive NK-1, PKC-\(\gamma\) and NK-1/PKC-\(\gamma\) double-labelled neurons were selected in total. Ten NK-1, ten PKC-\(\gamma\) and ten double-labelled neurons were selected from each animal (3 from L3, 3 from L4 and 4 from L5 in each animal). The mean total contact of NOS terminals per 100\(\mu\)m\(^2\) on labelled neuron was determined by using Neurolucida Explorer on cells digitised with the Neurolucida programme.

*Experiment 3*: GAD, VGLUT2, NK-1 and NOS (\(n=3\)): The LSN was identified, and using the blue channel (for NOS terminals), five NOS terminals were selected at random from a single optical section from the left and right hand side of each 50\(\mu\)m section (i.e. 10 NOS terminals per 50\(\mu\)m section) and 10 sections were examined for segments L3 – 5 i.e. 100 terminals per segment. As three segments were analysed for each of the three animals, this resulted in 300 terminals per animal i.e. 900 terminals analysed in total. Initially, NOS terminals were visualised in the blue channel alone and marked. The other channels were selected in turn (i.e. red, VGLUT2 and NK-1; green, GAD) and co-localisation with either VGLUT2 and/or GAD was recorded for each of the NOS terminals selected.

*Experiment 4*: SP, NOS and NK-1 (\(n=3\)): This category was used to determine the relationship of SP terminals and NK-1 immunoreactive neurons in the LSN. 10 sections for each spinal segment were examined (i.e. 90 in total).
3. Results

*SP, NK-1 receptor and PKC-γ neurons within the LSN*

It was found that SP was abundant within the LSN, and found surrounding the neuronal cell body and dendritic tree of NK-1 neurons (Figure 4.1). In addition, NOS was found surrounding primarily the NK-1 cell body, and closely related to the SP staining around the NK-1 labelled neuron in the LSN.

The average number of sampled neurons (including ± S.D.) determined by the neuronal marker NeuN for the L3 – 5 segments combined in the three animals was 196 ± 47.6. Of those LSN neurons, 30.99±5.2% were immunoreactive for the NK-1 receptor, 9.02±1.2% were immunoreactive for PKC-γ and 2.78±0.76% were immunoreactive for both markers (Figure 4.2). Therefore the majority (57.2 ± 6.8%) of LSN neurons (shown by NeuN labelling) were not immunoreactive for either NK-1 or PKC-γ (Figure 4.3)

*Relationship of NOS terminals to NK-1 and PKC-γ neuronal cell bodies and dendrites*

Three patterns of distribution of NOS terminals in the LSN were identified: 1) those scattered throughout the LSN (Figures 4.4 – 4.5), 2) those clustered around NK-1 labelled neurons (Figure 4.4), or 3) clustering of NOS terminals around unidentified neurons (Figure 4.5). Of those NK-1 immunoreactive neurons in the LSN, 72.4% had clusters of NOS terminals around the cell body and dendritic tree. However, only 6.4% of non-NK-1 immunoreactive neurons (i.e. labelled only with NeuN) had clusters of NOS terminals surrounding the cell.

Figure 4.6 shows the frequency of NOS terminal contacts on NK-1, PKC-γ and NK-1/ PKC-γ cells. From these data it can be seen that the greatest density of NOS terminal contacts is associated with cell bodies of NK-1 immunoreactive neurons in the LSN (mean total contact / 100μm² ± S.D. of 6.68 ± 4.29). This compares with 1.24 ± 0.73 for PKC-γ
immunoreactive neurons and 1.28 ± 0.39 mean total contacts / 100µm² of NOS terminals on double-labelled NK-1 and PKC-γ cells. Very small number of NOS terminals contacted proximal dendrites of NK-1 and dual labelled neurons (0.4 ± 0.32, 0 and 0.59 ± 0.36 mean total contacts / 100µm² of NOS terminals respectively). Dendritic trees of PKC-γ immunoreactive neurons were insufficiently labelled to permit this type of analysis. Contact densities of NOS terminals on the cell bodies of NK-1 immunoreactive neurons were significantly different from those associated with NK-1 dendrites, PKC-γ cell bodies and double-labelled cells (one-way ANOVA p<0.0005). This suggests that NOS immunoreactive terminals preferentially target the cell bodies of NK-1 immunoreactive neurons.

**Distribution of NOS-immunoreactive fibres within the LSN and patterns of co-localisation with GAD and VGLUT2**

Subsequently, random NOS terminals were analysed to determine their relationship with the markers VGLUT2 and GAD (markers for excitatory or inhibitory amino acid transmitters respectively). It has been shown previously that GABA is co-localised within most NOS immunoreactive boutons in inner lamina II (Valtschanoff et al., 1992b). However, in the LSN, only 22.1% of NOS terminals contained co-localised GAD and no co-localisation was found between NOS and VGLUT2 (Figure 4.7).
Figure 4.1. The relationship of SP to the NK-1 receptor in the LSN. A – C represent a confocal image built from 9 projected images. A represents NK-1 in red, B represents SP in green, C represents NOS and D is the merged image of A – C. Note that # represents NK-1 neurons in the LSN. SDH = superficial dorsal horn. Scale bar = 20µm.
Figure 4.2. Neurochemical properties of LSN cells. A - C, represents a series of confocal images of a transverse section of the LSN built from ten projected images (A, NeuN; B, PKC-γ; C, NK-1; D = merged image of A, B & C). A PKC-γ immunoreactive cell (1) is adjacent to an NK-1 labelled neuron (2) and NeuN cells that are not labelled for either marker (3). The superficial dorsal horn is present at the top left of each image (SDH). Scale bar = 50µm.
Figure 4.3. Histogram summarising the frequency of NK-1, PKC-γ and double-labelled NK-1 and PKC-γ cells as a proportion of the total numbers of neurons in the LSN, identified with the neuronal marker NeuN. The majority (57.2%) of neurons in the LSN are not labelled for either NK-1 and/or PKC-γ; n=3; error bars = ± S.D.
Frequency of NK-1 and PKC-γ cells in the LSN

![Graph showing the frequency of NK-1 and PKC-γ cells in the LSN](image)
Figure 4.4. Clustering of NOS terminals around an NK-1 cell in the LSN. A - C, Single optical section in the transverse plane (A, NK1 and VGLUT2; B, GAD; C, NOS; D, merged image of A, B & C). An NK-1 immunoreactive neuron (*) is associated with a dense plexus of NOS terminals which surround its cell body and also partly extend to its dendritic tree. Some GAD and VGLUT2 terminals are also associated with the NK-1 labelled cell. The superficial dorsal horn (SDH) can be seen immediately to the left of the NK-1 neuron. Scale bar = 20µm.
Figure 4.5. Clustering of NOS, GAD and VGLUT2 around an NK-1 negative cell. A – C represents 9 confocal images projected showing NK1 and VGLUT2 (A), GAD (B) and NOS (C). A merged image of A, B & C is shown in D. An NK-1 immunoreactive neuron can be seen to the left of this image (*) which is not associated with a cluster of NOS terminals. An unlabelled neuron is present at the right of the field (1) which is surrounded by NOS and VGLUT2 terminals with some GAD. Note the lack of clustering of VGLUT2, NOS and GAD around an NK-1 cell. Scale bar = 20μm.
Figure 4.6. A histogram showing the packing density of NOS terminal contacts on neuronal cell bodies (cb) and dendrites (d) of NK-1, PKC-γ and double-labelled cells in the LSN. n = 3; error bars = ± S.D.
Packing density of NOS terminals on cell bodies and dendrites in the LSN

Mean total contacts / 100um²

- NK-1 (cb)
- NK-1 (d)
- NK-1 + PKCγ (cb)
- NK-1 + PKCγ (d)
- PKCγ (cb)
- PKCγ (d)
Figure 4.7. Co-localisation of GAD in some NOS terminals. A - C represents a single optical section (X60 oil immersion lens, zoom factor of 5) showing immunoreactivity for VGLUT2 (A), GAD (B) and NOS (C). D is a merged image of A, B & C. NOS terminals that co-localised with GAD are indicated by the arrows. Note that VGLUT2 immunoreactivity is not co-localised within any of the NOS terminals and that the majority of NOS terminals do not show immunoreactivity for GAD. Scale bar = 50µm.
4. Discussion

The main findings of this study are threefold: firstly, although the LSN displays strong immunoreactivity for SP, and is related to NK-1 neurons (e.g. see Barber et al., 1979 and Figure 4.1), the proportion of LSN neurons labelled with the NK-1 receptor is only approximately one-third of the total neuronal population. Secondly, a sub-population of NK-1 immunoreactive cell bodies (almost three-quarters of NK-1 neurons in the LSN) are densely innervated by NOS terminals (as well as SP), but this arrangement was not found for cells that possess immunoreactivity for PKC-γ or those possessing both types of immunoreactivity. Thirdly, while NOS-immunoreactive terminals are abundant in the LSN, less than a quarter of them contain GAD. Therefore, unlike the SDH, NOS terminals in the LSN are not derived principally from GABAergic inhibitory neurons.

Origins of Substance P in the Lateral Spinal Nucleus

Leah and co-workers (1988) performed a detailed investigation of neuropeptide-containing ascending tract cells in the rat lumbosacral spinal cord. They found that 90% of peptidergic ascending tract cells were congregated into two distinct areas; the LSN and the region surrounding the central canal. The neuropeptide-containing tract cells in the LSN were found principally to contain SP, VIP, bombesin and dynorphin (Barber et al., 1979; Bresnahan et al., 1984; Cliffer et al., 1988). In addition, axon terminals containing CGRP are not present within the LSN (Olave and Maxwell, 2004). As SP is co-localised with CGRP in peptidergic primary afferents, this is further evidence against a primary afferent source of SP axons in the LSN. It also seems unlikely that the SP axons in the LSN originate from a descending source as Cliffer et al. (1988) have shown that spinal transections do not reduce the numbers of SP-immunoreactive fibres. It therefore appears
that the source of SP fibres in the LSN is principally segmental and it is likely that they originate from the adjacent dorsal horn, and perhaps also within the LSN itself.

The NK-1 receptor and the LSN

This study has shown that one-third of all neurons in the LSN possess the target of SP, the NK-1 receptor, which is well established as serving a role in nociceptive transmission in the superficial dorsal horn (Kuraishi et al., 1985; McCarson and Goldstein, 1991). This in itself highlights an additional contrast to the SDH. Indeed, although more than three-quarters of spinothalamic tract neurons in lamina I possess the NK-1 receptor (Marshall et al., 1996), NK-1 neurons constitute only 10% of the total at this site that project to established areas for nociceptive information processing e.g. the thalamus and the parabrachial nucleus (Ding et al., 1995; Marshall et al., 1996). Therefore, it may well be that the LSN serves a role in nociception. Olave and Maxwell (2004) further developed the idea that the LSN may be involved in nociceptive processing when they demonstrated that a small proportion (approximately 10%) of these NK-1 neurons also expressed the nuclear protein Fos, in response to noxious thermal stimulation (see also Chapter 3). Most of the NK-1 projection neurons that responded to noxious stimulation projected to the caudal ventrolateral medulla (CVLM), an area of the brainstem that may have a role as an inhibitory modulator of nociceptive transmission (Tavares and Lima, 2002).

The Lateral Spinal Nucleus and NOS

In the SDH, NOS arises principally from GABAergic and glycinergeric neurons and is also found in a sub-population of cholinergic neurons (Valtschanof et al., 1992b; Laing et al., 1994) but the pattern of co-localisation in the LSN appears to be different as GAD is found within only a minority of NOS terminals in the LSN.
However, in common with the SDH, NOS terminals in the LSN do not contain VGLUT2. As VGLUT1 terminals are not present in the LSN (Olave and Maxwell, 2004) it is probable that the majority of NOS terminals in the LSN do not contain classical excitatory or inhibitory neurotransmitters. This raises important questions about the origin of the NOS terminals in the LSN. No NOS containing neuronal cell bodies were identified in the LSN from the sections examined in this study suggesting that an intrinsic origin is unlikely. In addition, it seems unlikely that many of them arise from the SDH, but one possibility is that they originate from the population of NOS cells found adjacent to the central canal which do not contain GABA or glycine (Laing et al., 1994). Nevertheless the precise origin of NOS fibres in the LSN remains to be determined.

*NOS and cells possessing the NK-1 receptor*

In the present study, a close relationship between NOS terminals in the LSN and the majority of NK-1 receptor-expressing neuronal cell bodies has been shown. This relationship was not observed for PKC-\(\gamma\)-containing neurons (which have an established role in nociceptive processing in the SDH (Malmberg et al., 1997)) or cells possessing immunoreactivity for both NK-1 and PKC-\(\gamma\). Therefore, despite the close relationship that exists between the NK-1 receptor and PKC-\(\gamma\) in laminae I and II of the superficial dorsal horn (Polgár et al., 1999), the same does not hold true within the LSN. Also, with so few neurons possessing PKC-\(\gamma\) immunoreactivity (and dual NK-1 and PKC-\(\gamma\) immunoreactivity), the LSN may not be involved in processing neuropathic pain, as occurs in the dorsal horn (Malmberg et al., 1997) via PKC-\(\gamma\) dependant means.

This study has not shown that the relationship between NOS axons and NK-1 cells is synaptic, but as NO is a diffusable neuromodulator, and given the intense concentration of NOS axons around these cells (mainly their cell bodies) it is reasonable to suggest that
when NOS axons are active, the levels of NO around these cells will be high. However, not all NK-1 cells were associated with NOS clusters (approximately one-quarter) and some cells which did not possess NK-1 immunoreactivity were associated with these clusters, although their numbers were low, accounting for only 6% of the total neuronal population (Figure 4.5). Therefore the original hypothesis that NO is preferentially associated with (and may even influence the activity of) NK-1 cells in the LSN selectively may only be partially correct and some other property of these cells may be the key factor which governs NOS clustering. Axons of LSN cells form components of a number of ascending tracts, including the spinomesencephalic (Menétrey et al., 1982), spinosolitary (Pechura and Liu, 1986), spinothalamic (Granum, 1986; Burstein et al., 1990a; Gauriau and Bernard, 2004) and spinohypothalamic tracts (Burstein et al., 1990b; Li et al., 1997) and it may be that the combination of projection target and neurochemical signature is the key determinant.
Chapter 5

Investigation 3:

Brain projections of LSN neurons with NK-1 immunoreactivity
a) **Spinohypothalamic projections from the LSN**

1. **Introduction**

   It is widely believed different regions of the hypothalamus play differing roles in a variety of autonomic and neuroendocrine functions (reviewed by Siegel and Sapru, 2006). Stimulation of the lateral hypothalamus causes the parasympathetic outflow to predominate (Milam et al., 1980; Yoshimatsu et al., 1984), and as it has one of the largest descending inputs to the periaqueductal grey (PAG) of the rat, is implicated in descending modulation of spinal neuronal activity, especially that resulting from noxious stimulation, and without affecting reactions to other stimuli (Beitz, 1982; Basbaum and Fields, 1984; Jensen and Yaksh, 1984; Aimone and Gebhart, 1987; Tasker et al., 1987; Aimone et al., 1988). Stimulation of the medial hypothalamus and specifically the ventromedial hypothalamic nucleus, results in domination of the sympathetic outflow (Inoue et al., 1977; Niijima et al., 1984; Yoshimatsu et al., 1984; Saito et al., 1989; Uyama et al., 2004). In addition, the medial hypothalamus (especially the ventromedial area) has been suggested to have an additional role in the motivational reaction to a noxious stimulus (Bester et al., 1995; Braz et al., 2005). Specifically, it has been suggested that it is involved in processing information that may threaten the animal, and organises the execution of innate defensive behaviours (Siegel, 2005; Borszcz, 2006). The paraventricular area on the other hand consists of several nuclei and controls the autonomic nervous system, regulation of visceral organs (Kannan et al., 1987; Uyama et al., 2004) and coordinates neurosecretions influencing the pituitary gland (Freund-Mercier et al., 1981).

   Previously, a direct spinohypothalamic pathway was not identified (Bowsher, 1957; Mehler et al., 1960; Boivie, 1979; Craig and Burton, 1985) and it was believed that the afferent pathway for somatosensory information to the hypothalamus was transmitted exclusively via indirect, multi-synaptic projections. The earliest suggestion of a direct
projection from the spinal cord to the hypothalamus was based on anatomical studies in the monkey. Chang and Ruch (1949) demonstrated that sectioning the monkey spinal cord resulted in degeneration at the supraoptic decussation at several levels of the hypothalamus bilaterally. Since then numerous anatomical and electrophysiological studies have revealed that somatosensory and visceral information can reach the hypothalamus through monosynaptic pathways that originate in medullary dorsal horn neurons and from all levels of the spinal cord (Burstein et al., 1987; Katter et al., 1996a,b; Kostarczyk et al., 1997; Zhang et al., 1999; Malick et al., 2000). The physiological studies in the cervical (Dado et al., 1994a), thoracic (Zhang et al., 2002) and lumbosacral segments (Burstein et al., 1987, 1991) have shown that the majority of spinal cord neurons projecting to the hypothalamus are strongly activated by noxious thermal and mechanical stimuli with sacral segments activated by noxious stimulation of both visceral and cutaneous structures (Katter et al., 1996a,b).

In addition to physiological studies, Burstein et al. (1987) performed several retrograde tracing techniques by injecting Fluoro-Gold (FG) into the hypothalamus and demonstrated a large number of labelled neurons bilaterally throughout the length of the spinal cord, with approximately half located in the lateral reticulated area and a lesser proportion around the central canal and marginal zone. From their retrograde tracing studies, they also showed a relatively large number of cells in the contralateral superficial dorsal horn, though mainly in the lower cervical cord of the rat. Kayalioglu et al. (1999) injected FG into the rat hypothalamus and from careful observations of their diagrams, demonstrated not only labelled neurons in the deeper laminae and the area around the central canal, but also in lamina I. However, the numbers in lamina I were considerably less than in the area around the central canal. This contrasts with anterograde studies done by Gauriau and Bernard (2004), who found that most of the projecting neurons to the
hypothalamus were located in the deeper laminae, with most of these being in the lateral reticulated area of lamina V. However, their study was limited to the cervical segments of the rat.

One feature common to these studies is the bilateral projections to the hypothalamus from the LSN. This nucleus has been shown to project through a variety of tracts including the spinohypothalamic (Burstein et al., 1996), spinomesencephalic (Pechura and Liu, 1986), spinosolitary (Leah et al., 1988) and spinothalamic tracts (Gauriau and Bernard, 2004) but there is still uncertainty regarding the function of this nucleus. Olave and Maxwell (2004) suggested a nociceptive function of this nucleus and this has been supported to a degree in chapters 3 and 4.

A possible role in visceroreception and visceronociception could be postulated as a role for the LSN as Neuhuber (1982) and Neuhuber et al. (1986) have shown that afferents from the greater splanchnic nerve, the inferior mesenteric plexus, and the hypogastric nerve terminate in the LSN. In addition, transneuronal studies using pseudorabies virus (PRV) injected into the kidney (Schramm et al., 1993) and stellate ganglion (Jansen et al., 1995) have shown that the LSN innervates different types of sympathetic pre-ganglionic neurons. Jansen and Loewy (1997) have also shown that neurons in the LSN become transneuronally labelled after PRV injections into the superior cervical ganglion, stellate ganglion, celiac ganglion or adrenal gland. It may well be that, along with lamina I cells (which are also activated by acute visceral information, like LSN cells (Menétrey and de Pommery, 1991), the LSN may trigger sympathetic responses during intense and acute visceral pain (Jansen and Loewy, 1997). So, as well as being influenced by visceral nociceptive information, a loop system may exist between the LSN, the aforementioned ganglia and the hypothalamus.
The aim of the present investigation was to quantify the laminar distribution (including the LSN) of NK-1 projection neurons to both the lateral and medial hypothalamus. In the first instance, retrograde labelling of spinohypothalamic projection neurons with CTb was combined with triple-immunofluorescence to examine the relationship of projection neurons that possess the NK-1 receptor, and for accurate quantification of the LSN neurons projecting to either the lateral or medial hypothalamus, the neuronal marker NeuN was also used. Neurons of this type are likely to be involved in the transmission of nociceptive information (Naim et al., 1997; Todd et al., 2002).

2. Experimental Procedures

Confocal microscopy and quantitative analysis

Eight adult male Sprague-Dawley rats (150-250 g; Harlan, UK) were used in this study. They were housed under conditions of a 12 hour light-dark cycle with food and water provided ad libitum. All experiments were performed in accordance with the UK Animals (Scientific Procedures) Act 1986. Each animal was deeply anaesthetised (ketamine/xylazine mixture, 7.33 and 0.73mg/100 g i.p.), placed in a stereotaxic frame and a craniotomy performed. An aqueous solution of CTb (200nl of 1% CTb; Sigma, Poole, Dorset, UK) was injected into either the left lateral hypothalamus (co-ordinates anterior-posterior [AP] + 7.2; dorsal-ventral [DV] +1.6; medial-lateral [ML] +1.8; Paxinos and Watson, 1997; n=4) or the left medial hypothalamus (AP + 7.2; DV +1.5; ML +0.8; Paxinos and Watson, 1997; n=4) through a glass micropipette which was connected to an air-pressure microinjection system with injections which were performed vertically into the appropriate site. After injection of the CTb, the pipette was left in-situ for 5 minutes backtracking of the tracer. The wound was sutured after the procedure and the rats were allowed to recover. Following 3 days survival, the animals were placed in a chamber
containing halothane and once anaesthetised, received a lethal dose of sodium pentobarbitone (1 ml i.p; 200mg/ml) and then perfused through the left ventricle with saline followed by a fixative containing 4% freshly depolymerised formaldehyde in phosphate buffer pH 7.6. The spinal segments C1 – 2, C5 and L3 – 5 were removed from each animal, notched on the left side to indicate the ipsilateral side to the injection, and post-fixed for 8 hours in the same solution. Following fixation, transverse spinal cord sections (50µm thick) were cut using a Vibratome and alternate sections retrieved for examination from each spinal segment.

Initially, sections were treated with 50% ethanol to enhance antibody penetration (Llewellyn-Smith and Minson, 1992) before undergoing triple-labelling immunofluorescence with a goat anti-CTb antiserum (diluted 1:5000; Sigma, Poole), mouse anti-NeuN antiserum (diluted 1:1000; Millipore, Watford, UK) and guinea pig anti-NK-1 antiserum (diluted 1:1000; Sigma). After 48 hrs incubation, sections were rinsed and incubated overnight in solutions containing three different species-specific secondary antibodies that were raised in donkey and coupled to fluorophores: rhodamine-red-anti-goat immunoglobulin G (IgG; diluted 1:500; Jackson Immunoresearch) to identify CTb immunoreactivity; cyanine 5.18-anti-mouse IgG (diluted 1:500; Jackson Immunoresearch) to identify the neuronal marker NeuN and Alexa-488-anti-guinea-pig IgG (diluted 1:100; Molecular Probes) to identify the NK-1 receptor. All antibodies were diluted in PBS containing 0.3% Triton X-100. After extensive rinsing of the sections, they were mounted using an anti-fade medium (Vectashield; Vector Laboratories) and stored in a freezer maintained at -20°C until analysis was undertaken.

The injection sites were confirmed using histological means. Following perfusion, the brains were stored overnight in the 4% freshly depolymerised formaldehyde with 30% sucrose in PBS at pH 7.6. The next day, the brains were cut on the freezing microtome into
100µm thick sections and incubated with goat anti-CTb antiserum (diluted 1:50,000; Sigma) for 48 hrs followed by the avidin-biotin-horseradish peroxidase (HRP) complex (Vector Elite) for 1 h. Standard DAB reactions were performed followed by dehydration and mounting on glass slides. The sections were then examined using a transmitted light microscope to identify the extent of the spread of the tracer. Reconstruction of the injection sites for each of the experiments was undertaken using the Paxinos and Watson atlas (1997) and composed graphically using the 2-D vector graphics editor software program Xara Xtreme (Xara Group Ltd., Hemel-Hempstead, UK).

Ten alternate sections were examined from C1 - C2, C5, and each of the lumbar spinal segments (L3 - 5), resulting in a total of 50 sections (each 50µm thick) from each rat. The retrogradely labelled neurons (as revealed using the red channel to visualise CTb) were identified and then scanned with a confocal microscope (Bio-Rad MRC 1024; Bio-Rad, Hemel-Hempstead, UK) through dry (4X, 10X, 20X) and oil-immersion (40X, 60X) lenses. To prevent over-counting which may arise if transected cells are at the section surface, cells were only included if the nucleus (seen as a filling defect) was entirely contained within the Vibratome section, or if part of the nucleus was present in the first optical section from the z-series. However, they were excluded if part of the nucleus was present in the last optical section in the z-series (Spike et al., 2003). The low magnification images were used to plot the position of the CTb labelled cells on an outline of the spinal cord. Dark-field microscopy was used to distinguish laminar boundaries, with retrogradely labelled neurons counted as lamina I if they were close to the dorsal border of the dorsal horn or lay dorsal to the dark band identified as lamina II with dark-field microscopy. Other laminar boundaries were created individually for each section based on standard reconstructions seen in Paxinos and Watson (1997). After the laminar boundaries were determined, the precise location of each retrogradely labelled neuron was established. Using the image
analysis programme Neurolucida for Confocal software (MicroBrightField, Inc., Colchester, VT), the green channel was examined to see if the retrogradely labelled neurons possessed the NK-1 receptor. Finally, the LSN neurons were examined to identify the proportion of NeuN labelled neurons (observed through the blue channel) that were retrogradely labelled, and of them, what proportion possessed the NK-1 receptor.

3. Results

To examine the total population of neurons in the spinal cord (including the LSN) that projected to the hypothalamus, as well as identifying if there are differences in projections to the medial and lateral hypothalamus, the retrograde tracer CTb was injected into eight rats. Four of these rats received a CTb injection to the lateral hypothalamus (experiment numbers 1 - 4) and four received it to the medial hypothalamus (experiment numbers 5 - 8).

Injection sites

For the lateral hypothalamic injection experiments, reconstructions of the anterior and posterior extents including the centre of the injections are indicated in Figure 5.1. The most representative photomicrograph is indicated above each of the reconstructions. In experiment 1 (Figure 5.1a) slight spread of tracer was found in the ventrolateral part of the anteroventral thalamic nucleus and the ventral anterior thalamic nucleus. In experiment 2 (Figure 5.1b,) there was leakage of the tracer into the mediodorsal thalamic nuclei, ventromedial thalamic nucleus, interanteromedial thalamic nucleus and the tuber cinereum area. In experiment 3 (Figure 5.1c), there was the most extensive spread of the CTb tracer into the anteroventral thalamic nuclei (both dorsomedial and ventrolateral parts), ventromedial and lateral thalamic nuclei, some of the mediodorsal thalamic nucleus and, due to its proximity to the hypothalamus, a small part of the internal capsule. In
experiment 4 (Figure 5.1d), there was least leakage of tracer with only a small amount identified in the reticular thalamic nucleus. No retrograde tracer passed to the contralateral side in any of the four experiments.

For the medial hypothalamic injection experiments, the reconstructions of the most anterior and posterior extents of the spread of retrograde tracer after medial hypothalamic injections in the four experiments (experiment numbers 5 – 8) can be seen in Figure 5.2. Again, the most representative image of the injection site is shown above the reconstructions for each of the experiments. Experiment number 5 was the most focal (Figure 5.2a), with least spread of tracer into surrounding structures. There was passage of tracer into the anterior hypothalamic area, zona incerta, central medial hypothalamic area, the ventrolateral and anterior parts of the ventromedial hypothalamic nucleus, dorsomedial hypothalamic nucleus (dorsal part) and the submedius thalamic nucleus. No leakage to the contralateral side was noted. The sixth experiment in this series (Figure 5.2b) had the greatest spread of the tracer to other nearby structures. Tracer was found in the anterior hypothalamic area, central and ventrolateral parts of the ventromedial hypothalamic nucleus and the dorsal part of the dorsomedial hypothalamic nucleus. In addition, there was considerable leakage of tracer into the central medial thalamic, centrolateral and mediodorsal thalamic nuclei. There was also leakage of the tracer into the contralateral submedius thalamic nucleus. The seventh experiment in this series (Figure 5.2c) had tracer located in the ventral anterior and ventromedial thalamic nuclei with only some in the medial part of the mediodorsal thalamic nucleus. The eighth experiment (Figure 5.2d) had some leakage of tracer into the anteromedial thalamic, the interanteromedial thalamic nucleus and the ventral anterior thalamic nucleus. No leakage to the contralateral side was identified.
The distributions of retrogradely labelled neurons were generally similar across all eight experiments where CTb was injected into either the lateral or medial hypothalamus (Figures 5.3 and 5.4 respectively). However, with experiment 6, where greatest leakage into surrounding structures occurred (with almost twelve times the number of retrogradely labelled neurons compared to the next largest in the medial hypothalamic injection series), this was not included to allow for fair statistical comparison between the other more focal hypothalamic injections. Retrogradely labelled neurons were found bilaterally in the spinal cord segments i.e. both ipsilateral and contralateral to the injection sites. In addition, retrogradely labelled neurons were predominantly found in two laminae – V and VII. They constituted 55.6 ± 11.6% and 53.4 ± 10.1% of all retrogradely labelled neurons for the lateral and medial hypothalamic injections respectively. A typical lamina V neuron is shown in Figure 5.5. Figure 5.6 summarises the laminar distribution of retrogradely labelled neurons after lateral and medial hypothalamic injections of the retrograde tracer CTb. For both the lateral and medial hypothalamic injections, the greatest number of retrogradely labelled neurons was located in the spinal segment C1 – 2 with almost a third located there for each of the hypothalamic regions injected with CTb (31.5 ± 13.4%, lateral hypothalamus; 31.9 ± 3.9%, medial hypothalamus). The least numbers of retrogradely labelled neurons after either lateral hypothalamic or medial hypothalamic injections occurred in the C5 spinal segment (9.72 ± 5.6% and 8.9 ± 2.5% respectively). Figure 5.7 shows the distribution of retrogradely labelled neurons over the spinal segments examined.

Interestingly, however, where there was leakage into surrounding thalamic structures, retrogradely labelled neurons were identified in laminae I – II in both the lateral and medial hypothalamic injections with CTb, whereas if the injection was focussed on primarily the lateral or medial hypothalamic target, no laminae I – II retrogradely labelled neurons were
noted. In experiment 6 (medial hypothalamic injection) where there was the greatest leakage of CTb tracer (of all experiments) into surrounding thalamic structures, a high number of retrogradely labelled neurons were identified in laminae I – II (11.4% of all retrogradely labelled neurons) and the white matter extending from the lateral reticulated area of lamina V towards the LSN (9.9%) but primarily the area around the central canal (26.8% of all retrogradely labelled neurons).

Retrogradely labelled neurons in the LSN

Common to both lateral and medial hypothalamic injections of the retrograde tracer CTb, the LSN contained a large proportion of all the retrogradely labelled neurons (25.7 ± 5.6% and 21.3 ± 7.3% respectively). A typical retrogradely labelled LSN neuron is shown in Figure 5.8. Of all the retrogradely labelled neurons found throughout the vertebral segments that were immunoreactive for the NK-1 receptor, almost half were located in the LSN (45 ± 8.2%, lateral hypothalamus; 47.9 ± 4.5%, medial hypothalamus). However, the retrogradely labelled neurons from hypothalamic injections constituted only a small proportion of the total neuronal population (revealed by NeuN) of the LSN (Figure 5.9), yet greater than 80% of those retrogradely labelled LSN neurons possessed the NK-1 receptor.
Figure 5.1. Reconstruction of the injection sites to the lateral hypothalamus (n=4). a - d represents each of the experiments 1 – 4 respectively. A representative photomicrograph is shown above each of the reconstructions for each animal. The spread of the tracer is represented by the dark grey area and in c, the paler area represents the leakage of CTb. Note that there is variable leakage into the thalamus, with most occurring in experiment c, and least in d. The numbers below each tracing indicates the interaural co-ordinate (Paxinos and Watson, 1997).
Figure 5.2. Reconstruction of injection sites to the medial hypothalamus (n=4). a - d represents experiments 5 - 8 respectively. The spread of tracer is represented by the dark grey area, and in b, the paler area represents the caudal spread of CTb. Note the extensive leakage of the tracer into the thalamus in experiment 6 (and contralateral spread). The numbers below each tracing indicates the interaural co-ordinate (Paxinos and Watson, 1997).
Figure 5.3. Location of retrogradely labelled neurons after lateral hypothalamic injections. a – d represents experiments 1 - 4 where CTb was injected into the lateral hypothalamus (each corresponding to the reconstructions of the injection sites in Figures 5.1 a – d respectively). Each dark filled circle represents a single CTb labelled neuron in each of the named spinal segments. The numbers of labelled neurons found in each side of the indicated segment are indicated in the ventral funiculus from each of the alternately selected segments. Ten alternate sections were examined for each spinal segment, each 50µm thick. The figure in parentheses shows the total number of CTb labelled neurons in the LSN. The left side of each segment diagram is contralateral to the injection site.
Figure 5.4. Location of retrogradely labelled neurons after medial hypothalamic injections. **a - d** represents experiments 5 - 8 with each corresponding the reconstructions shown in Figure 5.2 **a - d** respectively in each of the stated spinal segments. Each dark filled circle represents a single CTb labelled neuron in each of the named spinal segments. The numbers in the ventral funiculus represents the total labelled neurons over ten alternately selected 50µm transverse spinal segments. The figure in parentheses shows the total number of CTb labelled neurons in the LSN. The right side of each spinal segment diagram is ipsilateral to the injection.
Figure 5.5. Example of a lamina V labelled neuron after CTb injection to the medial hypothalamus. A – C, merged images of a transverse section of the contralateral lamina V (red, CTb; green, NK-1; blue, NeuN) built from 10 projected images. A represents a single CTb labelled neuron in the centre of the field that is not immunoreactive for NK-1 (B), and shows NeuN immunoreactivity (C). D is the merged image of A - C. Scale bar = 50µm
Figure 5.6. Histogram summarising laminar distributions of ALL retrogradely labelled spinohypothalamic neurons. A represents the lateral hypothalamus, and B represents the medial hypothalamus. Red = right (contralateral) to the injection, blue = left (ipsilateral) to the injection; error bars = ± S.D.
A. LATERAL HYPOTHALAMUS

![Graph showing mean % of SHT neurons in different areas of the lateral hypothalamus.]

B. MEDIAL HYPOTHALAMUS

![Graph showing mean % of SHT neurons in different areas of the medial hypothalamus.]

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Figure 5.7. Histograms summarising the spinal segment distributions. A represents the spinal segment distribution of ALL retrogradely labelled neurons after lateral hypothalamic injections, and B represents the spinal segment distribution of ALL retrogradely labelled neurons after medial hypothalamic experiments; error bars = ± S.D.
A.

Lateral Hypothalamus

![Bar graph showing mean % of neurons in different regions of the lateral hypothalamus.]

B.

Medial Hypothalamus

![Bar graph showing mean % of neurons in different regions of the medial hypothalamus.]
Figure 5.8. Triple labelling of CTb, NeuN and NK-1 in the LSN. A – C, merged images of a transverse section of the LSN built from 10 projected confocal images (A, CTb; B, NK-1; C, NeuN; D, merged image of A - C). A contralateral retrogradely (from lateral hypothalamic injection of tracer) labelled (CTb) neuron is in the LSN. The SDH is present at the top right of each image. Scale bar = 50µm.
Figure 5.9. Histograms summarising the proportion of only LSN neurons (as labelled by NeuN) that were retrogradely labelled from either the lateral or medial hypothalamus. Histogram 1 (shown in A) shows the average percentages of NeuN labelled LSN neurons that were labelled either from the lateral or medial hypothalamic injections of CTb (green: right side, contralateral to injection; red: left side, ipsilateral to injection). Histogram 2 (shown in B) shows the average percentages of retrogradely labelled LSN neurons that possessed the NK-1 receptor either from the lateral or medial hypothalamic injections. Error bars = ± S.D.
A.

Histogram 1. Percentage of LSN neurons retrogradely labelled

B.

Histogram 2. Percentages of LSN neurons retrogradely labelled that possessed the NK-1 receptor
4. Discussion

There are three main conclusions based on this study: firstly, there are approximately the same numbers of projections to both the lateral and medial hypothalamus from the rat spinal cord, with the majority of retrogradely labelled neurons in laminae V and VII. Secondly, it was more common for NK-1 retrogradely labelled neurons to be located in laminae V, VII and the LSN. Finally, a similar amount of retrogradely labelled neurons projected to both the lateral (25.7 ± 5.6% of the total labelled) and medial (21.3 ± 7.3% of the total labelled) hypothalamus from the LSN, with the vast majority of those possessing the NK-1 receptor (>80%).

The Spinohypothalamic Tract in the Rat

This present study confirms and extends previous anatomical and physiological observations made on spinal cord neurons that project bilaterally to both the medial and lateral hypothalamus. On examination of the experiments where the injection of the retrograde tracer was found only, or primarily in the hypothalamic sites, there were almost identical distributions within the spinal cord. Lamina V and lamina VII were consistently found with the greatest numbers of retrogradely labelled neurons when injection sites were located to the hypothalamic territories, as also shown by Burstein et al., 1990a and Katter et al., 1991. This could be relevant regarding pain pathways as these two regions serve a role in nociceptive and proprioceptive processing (Hillman and Wall, 1969; Menétréy et al., 1977; Light and Perl, 1979; Kevetter and Willis, 1982; Menétrey et al., 1984; Granum, 1986; Sugiura et al., 1986; De Koninck et al., 1992; Mouton and Holstege, 1994; Littlewood et al., 1995). It may then be that the hypothalamus serves a role in the autonomic responses to somatosensory stimulation, including painful stimuli. Regardless of the area of the hypothalamus that was injected approximately half of all retrogradely
labelled neurons were located contralaterally, which is in agreement with Burstein et al. (1990a).

However, in the study by Burstein et al. (1990a), they also found a proportion of retrogradely labelled neurons (using FG) were located in lamina I, and the area around the central canal, something echoed by others (Carstens et al., 1990; Katter et al., 1991; Menétrey and de Pommery, 1991; Kostarczyk et al., 1997; Kayalioglu et al., 1999). With the experiments conducted in this study very few lamina I neurons were identified where only or mainly the hypothalamus was injected. However, where there was leakage into many surrounding structures in experiment 6 (medial hypothalamic injection), there were large numbers of retrogradely labelled neurons in lamina I, and the area around the central canal.

Projections from lamina I to the thalamus have been demonstrated in numerous species including cats (Trevino and Carstens, 1975; Craig et al., 1989; Zhang et al., 1996; Klop et al., 2004), primates (Apkarian and Hodge, 1989; Zhang and Craig, 1997) and also rats (Lima and Coimbra, 1988; Marshall et al., 1996; Yu et al., 2005). This could explain why there are a large number of retrogradely labelled neurons identified in lamina I in experiment 6, as there was extensive leakage of the tracer into the mediodorsal and ventral posteromedial thalamic nuclei (areas established as receiving lamina I projections).

Recently, Gauriau and Bernard (2004) presented a very substantial projection from the SDH of the rat cervical spinal cord to the triangular part of the posterior thalamic nuclear group (PoT). In addition, Al-Khater et al. (2008) demonstrated that there is a very specific projection from lamina I neurons possessing the NK-1 receptor to the PoT. On careful examination of the reconstructions for experiment 6 (Figure 5.2b), it can be seen that there is some leakage of the retrograde tracer into the posterior thalamic nuclear group, though
not into the PoT as that lies at the extreme caudal end of the thalamus, providing a possible route that the tracer may have passed to result in the lamina I labelled neurons.

**Spinohypothalamic Tract Neurons and the NK-1 receptor**

The NK-1 receptor is the target of the tachykinin neuropeptide substance P (SP), which is secreted by small diameter, primary afferent fibres, many of which respond to noxious stimuli (Duggan and Hendry, 1986; Lawson et al., 1997). The NK-1 receptor is concentrated in lamina I but also scattered throughout the remaining of the dorsal horn and the area around the central canal (Bleazard et al., 1994; Nakaya et al., 1994; Brown et al., 1995; Todd et al., 1998). Interestingly, however, very few neurons in lamina II appear to have the receptor, despite this lamina having a rich innervation from SP containing axons (Marshall et al., 1996). However, they have dorsally oriented dendrites that enter the superficial laminae and receive substantial SP input monosynaptically from primary afferents (Naim et al., 1997). In addition, lamina V and the area round the central canal are established as receiving nociceptive afferent fibres (Bessou and Perl, 1969; Cuello and Kanazawa, 1978; Light and Perl, 1979; Mense and Praghakar, 1986; Sugiura et al., 1986; Willis and Coggeshall, 1991) with lamina VII also showing SP immunoreactivity, suggesting the presence locally of the NK-1 receptor and second order nociceptive processing here (De Lanerolle and LaMotte, 1982). We have shown that many of the neurons in lamina V and VII, and also the LSN projecting to either the medial or lateral hypothalamus, possess the NK-1 receptor suggesting a role of these projection neurons in nociception.
Almost one fourth of retrogradely labelled neurons was located in the LSN with very similar numbers projecting to either the lateral or medial hypothalamus (25.7 ± 5.6% and 21.3 ± 7.3% respectively). Regardless of the area of the hypothalamus that was injected, greater than 80% of all the retrogradely labelled neurons possessed the NK-1 receptor.

LSN neurons have been shown to contain peptides like vasoactive intestinal polypeptide, bombesin, SP and dynorphin (Leah et al., 1988) and project through many diverse tracts including the spinothalamic tract (Granum, 1986; Burstein et al., 1990b; Gauriau and Bernard, 2004), spinoreticular and spinomesencephalic tracts (Menétrey, 1982; Pechura and Liu, 1986). Our findings support and extend studies done by other authors who have demonstrated a spinohypothalamic tract from the LSN (Burstein et al., 1990a; Li et al., 1997), though some have only considered it from either the cervical (Dado et al., 1994a,b; Gauriau and Bernard, 2004), lumbar (Zhang et al., 1999) or sacral enlargements (Katter et al., 1996b) in isolation.

An additional feature of the LSN is that within this nucleus there are a large numbers of peptidergic varicosities. Experimental work involving interrupting either descending pathways or primary afferent input to the LSN did not affect plexi of SP, enkephalin, dynorphin, FMRF amide (a neuropeptide Y-like substance) or somatostatin at this site (Jessel et al., 1978; Larabi et al., 1983; Seybold and Elde, 1980; Giesler and Elde, 1985; Cliffer et al., 1988). This shows that the peptidergic input to the LSN arises either at the same level, or segmental levels nearby, of the spinal cord. Our findings support other authors’ studies that have shown an LSN-hypothalamic pathway, reinforcing work done by Li et al. (1997) who demonstrated that the LSN neurons projecting to the hypothalamus also possessed the substance P receptor. We have also shown that of those that do, greater than 80% contain the NK-1 receptor, one of the G-protein coupled receptors, suggesting an
involvement of the LSN as an integrative nucleus involved in visceroception and/or
ticereonociception and therefore, of autonomic and neuroendocrine regulation (Jansen and
Loewy, 1997; Workman and Lumb, 1997; Hudson et al., 2000; Vergnano et al., 2008).
b) Caudal ventrolateral medulla and mediadorsal thalamic projections from the LSN

1. Introduction

The mediadorsal thalamus (MDT) is a major component of the thalamus of all mammals, and is especially developed in humans (Le Gros Clark, 1932a,b). Indeed the functions of the MDT are wide and varied with it being well established that it plays a major role in emotional changes, anterograde amnesia where post-operative learning is severely affected especially in visual memory tasks (Schulman, 1957; Zola-Morgan and Squire, 1985; Parker et al., 1997; Gaffan and Watkins, 1991; Gaffan and Parker, 2000; Mitchell et al., 2007) and memory acquisition (Mitchell and Gaffan, 2008).

Major reciprocal connections exist between the MDT and the prefrontal cortex (Parker and Gaffan, 1998; McFarland and Haber, 2002; Erickson and Lewis, 2004) and (along with the orbitofrontal cortex) has descending pathways linked to the amygdala and the hypothalamic nuclei (Barbas et al., 2003) in the primate. As the MDT has been shown to serve a role in nociceptive processing (Casey, 1966; Palestini et al., 1987; Dostrovsky and Guilbaud, 1990), it could be said that a loop exists forming the “basolateral limbic system” composed of the MDT, amygdala, orbitofrontal cortex including that also of the rat (Krettek and Price, 1977; Sarter and Markowitsch, 1983, 1984; Bachevalier and Mishkin, 1986; Cassell and Wright, 1986; Gaffan et al., 1993) and the prefrontal cortex (Fuster, 1997). Therefore, the MDT could serve a role in the motivational and affective components of pain, including that of visceroreception and visceronociception. As well as the thalamus playing a major role in nociceptive processing, in recent years an increasing number of studies have been undertaken in examining the role of the medulla oblongata in its role in nociception.

The medulla oblongata has the highest density of pain modulation areas in the brain. Several areas of the medulla are involved in endogenous antinociceptive processes including the rostroventromedial medulla (RVM), the nucleus tractus solitarius (NTS), ventral reticular
nucleus (VRt), dorsal reticular nucleus (DRt) and the caudal ventrolateral medulla (CVLM). In recent times, the CVLM has been shown to have a significant role in modulation of nociception. Indeed, the CVLM has a very important role in the inhibitory modulation of pain, and has been shown to inhibit nociceptive dorsal horn neurons (through monitoring electrical thresholds for inhibition and the magnitude of duration of suppression of nociceptive reflexes (Gebhart and Ossipov, 1986; Janss and Gebhart, 1987, 1988)) more than areas including the locus coeruleus (Jones and Gebhart, 1986a,b), the periaqueductal grey (Carstens and Watkins, 1986; Jensen and Yaksh, 1984) and the RVM (Satoh et al., 1983; Ness and Gebhart, 1987).

Stimulation of the CVLM has a potent antinociceptive effect, as demonstrated by the resulting marked inhibition of nociceptive spinal dorsal horn neurons (Morton et al., 1983; Janss and Gebhart, 1988; Liu and Zhao, 1992), which the CVLM forms a loop system with (reviewed by Tavares and Lima, 2002), and specifically lamina I neurons, but communications also exist between lamina IV – V and X (Fields et al., 1990). The CVLM depresses nociceptive reflexes (Gebhart and Ossipov, 1986; Janss and Gebhart, 1987) through apposition to spinally projecting neurons only in the pontine A5 noradrenergic cell group and the RVM (Tavares et al., 1996). Specifically, it is the lateral part of the CVLM where the $\alpha_2$-adrenoceptor anti-nociception produced in the CVLM is triggered in its lateral part, and mediated by the A5 noradrenergic cell group then passing to the spinal cord (and subsequent superficial dorsal horn) via the dorsolateral funiculus (Janss and Gebhart, 1988).

It has been suggested that the LSN plays a role in nociception (as discussed in previous chapters), and projects to both the CVLM (Olave and Maxwell, 2004) and MDT (Gauriau and Bernard, 2004). Therefore, this study was undertaken to identify the laminar distribution (including the LSN) of NK-1 projection neurons that were labelled from either/both the CVLM and the MDT. Firstly, the retrograde tracers CTb and Fluoro-Gold (FG) were
injected into the CVLM and the MDT respectively. This was combined with quadruple labelling immunofluorescence for the NK-1 receptor and the neuronal marker NeuN.

2. Experimental Procedures

Experimental materials and methodology used in this study were the same as for the spinohypothalamic studies. However, each rat used here (n = 3), received a single injection of 50nl 4% FG (Fluorochrome Inc., Englewood, CO) through a glass micropipette into the mediodorsal thalamus [coordinates = AP + 6.2; DV + 4.0; ML + 0.7] and 200nl of 1% cholera toxin B subunit (CTb; Sigma, Poole, UK) into the caudal ventrolateral medulla (CVLM) [coordinates = AP – 4.8; DV – 0.6; ML + 2.1], specifically the region between the spinal trigeminal nucleus and the lateral reticular nucleus.

In addition to the same primary and secondary antibodies used for the spinohypothalamic studies, rabbit anti-FG (diluted 1:1000; Chemicon) was used as the primary antibody, and this was coupled to Alexa-488-anti-guinea-pig (diluted 1:500; Molecular Probes) to reveal FG immunoreactivity (as well as the NK-1 receptor). Data collection and analysis were undertaken as previously described in the spinohypothalamic study.

3. Results

Injection Sites

To examine the total population of neurons in the spinal cord (including the LSN) that projected to the mediodorsal thalamus and/or the caudal ventrolateral medulla, the retrograde tracers FG and CTb (respectively) were injected into three rats.
**Injection site – mediodorsal thalamus**

Reconstructions of the anterior and posterior extents of the spread of tracers, including the centre of the injections, are indicated in Figures 5.10 (A - C). The light micrograph image, epifluorescent image, merged image (both light and epifluorescent images combined) and reconstructions are shown for each of the experiments used. The first of the three experiments studied had the least amount of FG tracer in the mediodorsal thalamus, whereas the other two cases filled the mediodorsal thalamus more extensively with greater leakage of tracer into nearby structures. In the first experiment in this series (A), there was filling of the mediodorsal thalamic nuclei (central, lateral and medial), with some leakage into the dentate gyrus, paraventricular thalamic nucleus, medial and lateral habenular nucleus, hippocampus and some leakage into the posterior thalamic nuclear group. In addition, there was some leakage into the contralateral central and lateral mediodorsal thalamus. In the second experiment (B), more of the mediodorsal thalamic nucleus was filled ipsilaterally but also more leakage into the central medial thalamic nucleus, centrolateral thalamic nucleus, ventrolateral and ventromedial thalamic nucleus, submedius thalamic nucleus, ventral and dorsal parts of the subcoeruleus nucleus, medial and lateral habenular nucleus, posterior thalamic nuclear group and the hippocampus. No contralateral spread was present. In the third experiment (C), there was extensive filling of the mediodorsal thalamic nuclei, with filling of the central medial thalamic nucleus, ventral anterior thalamic nucleus, ventromedial thalamic nucleus, submedius thalamic nucleus, medial and lateral habenular nucleus, and the posterior thalamic nuclear group. No tracer spread to the contralateral side. In all experiments, there were small foci of necrosis identified with the retrograde tracer FG, generally centrally located in the injection site.
**Injection site – caudal ventrolateral medulla**

Injections into the dorsal part of the caudal medulla (Figure 5.10, D) were centred on the reticular nucleus reaching the lateral reticular nucleus, dorsal and ventral reticular nucleus, with spread into the spinal trigeminal nucleus laterally. No contralateral spread was identified.

**Retrogradely labelled neurons**

The greatest numbers of retrogradely labelled neurons were double labelled with both FG and CTb, with the least number of retrogradely labelled neurons showing labelling with only CTb immunoreactivity (Figure 5.11). Retrogradely labelled neurons were found bilaterally (i.e. ipsilateral and contralateral to the injection sites). In general, the greatest numbers of retrogradely labelled neurons occurred in the C1-2 spinal segment (38.7%, CVLM and MDT; 27.9% MDT) though there were slightly more labelled neurons from only the CVLM in the L3 segment (25.9%; Figure 5.11). The spinal segmental distribution of retrogradely labelled neurons is shown in Figure 5.12. Indeed, over all experimental groups that were retrogradely labelled from both the CVLM and the MDT, only the MDT or only to the CVLM, there was a similar laminar distribution of neurons (Figure 5.13). Retrogradely labelled neurons were found in one of four laminar territories: I–II, V and VII, and to a lesser degree the LSN. Figures 5.14 and 5.15 show typical examples of lamina I and lamina VII retrogradely labelled neurons respectively.

For each of the categories of retrogradely labelled neurons, the greatest frequency of labelled neurons occurred in the following laminae: laminae I–II that were retrogradely labelled from both the CVLM and MDT (38.7 ± 8.9%); laminae V–VII (59.5 ± 4.8%) that were retrogradely labelled from only the CVLM and laminae I–II (26.4 ± 4.4%) that were retrogradely labelled from the MDT.
NK-1 receptor labelling over all experimental groups was in greatest numbers found in
the SDH in laminae I – II. Of the total NK-1 receptor labelling for those neurons that were
retrogradely labelled from both the CVLM and the MDT, 65.4 ± 4.7% were found in laminae
I – II. 47.5 ± 4.7% of NK-1 receptor immunoreactivity where retrograde labelling occurred
only from the MDT were found in laminae I – II. Of the total NK-1 receptor labelling in the
category of neurons that were retrogradely labelled only from the CVLM was 50 ± 10% in
laminae I – II.

Retrogradely labelled neurons in the LSN

Unlike the retrogradely labelled neurons identified after hypothalamic injections
discussed in Investigation 3a), the total retrogradely labelled neurons in the after CVLM and
MDT injections was lower proportionally, of all retrograde neurons identified in each
grouping.

As a total of all retrogradely labelled neurons that were double labelled from both the
CVLM and the MDT, the proportion found in the LSN was 11.5 ±2.5%. Of all retrogradely
labelled neurons from the CVLM, the LSN constituted 14.7 ± 5.3% of the total. For
retrogradely labelled neurons only from the MDT, the LSN constituted 6.9 ± 4.3% of the
total labelled neurons. As a proportion of all LSN NeuN labelled neurons, they constituted a
small proportion of the total (revealed by NeuN) as shown in Figure 5.16.

A similar proportion of LSN neurons possessed the NK-1 receptor that were double
labelled from both the CVLM and the MDT (Figures 5.17, 5.18) and only labelled from the
CVLM (52.5 ± 5.9% and 52.2 ± 8.7% respectively), but a smaller proportion only labelled
from the MDT (25.3 ± 3.2%).
Figure 5.10. Photomicrographs and diagrams showing the spread of the tracer for the CVLM and MDT injections. A, B and C represent light micrograph images of the position of the retrograde tracer FG which was injected into the mediodorsal thalamus. Ai, Bi and Ci represent epifluorescent images of the same site. Aii, Bii and Cii are the merged photomicrograph and the epifluorescent images for each experiment. Aiii, Biii and Ciii are reconstructions of the anterior and posterior extents of the leakage of FG. D represents diagrams showing the spread of the retrograde tracer CTb after injection into the caudal ventrolateral medulla where i – iii represent experiments 1 – 3 as seen in A – C respectively. Numbers related to each of the reconstruction diagrams represents the approximate position of the section anterior (+) or posterior (-) to the ear bar. Drawings based on those of Paxinos and Watson (1997). Scale bar = 1µm.
Figure 5.11. Location of ALL retrogradely labelled neurons after injections to the CVLM and the MDT, including those with the NK-1 receptor. A represents those retrogradely labelled neurons from both the CVLM and the MDT, B represents those retrogradely labelled neurons from only the CVLM and C represents those retrogradely labelled neurons from only the MDT. The left hand side of each diagram is contralateral to the injection sites. The number in the ventral white matter indicates the total in each vertebral segment on each side. The number in parenthesis indicates the total number in the LSN.
Figure 5.12. Mean percentages (including standard deviation) of ALL retrogradely labelled neurons. Projections to both the CVLM and the MDT (A), only to the CVLM (B) or only to the MDT (C) according to ipsilateral or contralateral to the injection site across the spinal segments C1 – 2, C5 and L3 – 5.
A. CVLM and MDT

B. CVLM

C. MDT
Figure 5.13. Histograms summarising the laminar distributions of ALL retrogradely labelled neurons. The laminar distributions of retrogradely labelled neurons from the CVLM and the MDT, only the CVLM and only the MDT (including those possessing the NK-1 receptor). The contralateral and ipsilateral sides are shown respectively. Error bars = ± S.D.
Figure 5.14. Quadruple labelling of CTb, FG, NK-1 and FG in the SDH. A – C shows a single optical transverse section of the SDH (A, red, CTb; B, green, FG and NK-1; C, blue, NeuN; D, merged image of A, B and C). 1 represents an NK-1 labelled neuron (as shown by NeuN, blue) in lamina I which has not been retrogradely labelled from either the CVLM or MDT. 2 represents a lamina I neuron (labelled with NeuN, blue) that has been labelled from both the CVLM (CTB, red) and the MDT (FG, green), but not showing immunoreactivity for the NK-1 receptor. 3 represents a lamina I neuron (labelled with NeuN, blue) which shows immunoreactivity for the NK-1 receptor and has been retrogradely labelled from both the CVLM (CTB, red) and the MDT (FG, green). Scale bar = 50µm.
Figure 5.15. Quadruple labelling of CTb, FG, NK-1 and FG in lamina VII. A – C, represents optical transverse image of lamina VII (A, CTb, red; B, FG and NK-1, green; C, NeuN, blue; D, merged image of A - C). A contralateral retrogradely labelled neuron (shown by immunoreactivity for NeuN, blue) in lamina VII that is labelled from both the CVLM (CTb, red) and the MDT (FG, green), though does not display immunoreactivity for the NK-1 receptor. This neuron is found at the centre of each of the images A – D. Scale bar = 20µm
Figure 5.16. **Histograms summarising the percentages of only retrogradely labelled LSN neurons (as revealed by NeuN) from both the CVLM and MDT, only the CVLM and only the MDT.** Histogram 1 (shown in A) shows the average percentages (within the LSN) of only NeuN labelled LSN neurons that were labelled from each of the sites. Histogram 2 (shown in B) shows the average percentages (of only NeuN labelled) LSN retrogradely labelled neurons that possessed the NK-1 receptor.
A. 

Histogram 1. Percentage of LSN neurons retrogradely labelled

B. 

Histogram 2. Percentages of LSN neurons retrogradely labelled that possessed the NK-1 receptor
Figure 5.17. Quadruple labelling of CTb, FG, NK-1 and FG in the LSN. A – C represents 9 projected confocal images of a transverse section of the region of the LSN (A, CTb, red; B, FG and NK-1, green; C, NeuN, blue; D, merged image of A, B and C). A contralateral retrogradely labelled neuron in the LSN that is labelled from both the CVLM (CTb, red) and the MDT (FG, green). This neuron (as indicated by *) is also immunoreactive for the NK-1 receptor. The superficial dorsal horn (SDH) is to the right of each of the images A – D. Scale bar = 50μm.
Figure 5.18. Quadruple labelling of CTb, FG, NK-1 and FG in the LSN. A – C represents the neuron indicated by * in Fig. 5.18 at a higher magnification (A, CTb, red; B, FG and NK-1, green; C, NeuN, blue; D, merged image of A, B and C). Scale bar = 10μm.
4. Discussion

The main finding of this study was that the majority of neurons in the spinal cord, whose cell bodies are primarily located in laminae I – II, V, VII and the LSN, project to both the CVLM and the MDT, rather than just to either the CVLM or the MDT, which are areas responsible for very different components of pain (Casey, 1966; Feltz et al., 1967; Gebhart and Ossipov, 1986; Sotgiu, 1986; Janss and Gebhart, 1987; Palestini et al., 1987; Price, 1995). In addition, more of the projection neurons to both of these sites were located in the cervical segments (especially the C1 – 2 spinal segments) compared to the lumbar segments. Those neurons projecting to both the CVLM and the MDT also possessed the NK-1 receptor, though were more numerous where this receptor has been established to be present i.e. laminae I – IV, X and in the LSN (Bleazard et al., 1994; Liu et al., 1994; Nakaya et al., 1994; Brown et al., 1995; Littlewood et al., 1995).

It is well established that a closed reciprocal loop exists with laminae I – II and the lateral part of the CVLM with terminal boutons labelled from these laminae having round vesicles and making asymmetrical synapses with this brain region projecting to those spinal laminae (Tavares and Lima, 2004). Also, in lamina I there are flattened boutons which are labelled from the lateral caudal ventrolateral medulla. As it has been shown that terminal boutons with round vesicles are excitatory, and those with flattened boutons are inhibitory (Uchizono, 1965; Gray, 1969; Todd, 1991), the lamina I neurons delivering nociceptive information to the medulla at that point will result in descending modulation of pain through both excitatory and inhibitory means.

This study has also identified many projection neurons to the medullary and thalamic sites in lamina V and VII. Tavares and Lima (2004), in their diagrammatic representation of the circuitry between the spinal cord and the medulla, show that lamina V (and lamina IV) receives projections from the lateral ventrolateral medulla, thus explaining why many of the
neurons in our study have been labelled at this site. However, lamina VII contains mainly pre-motor interneurons, and is targeted exclusively by the lateral reticular nucleus (Tavares and Lima, 2002). On careful examination of the injection sites carried out in this study where CTb was injected into the CVLM, it can be seen that there is indeed filling of the lateral reticulated nucleus in all three experiments, explaining the lamina VII labelled neurons from filling of this site. As the lateral reticular nucleus can result in inhibition of nociceptive spinal neurons, as shown by electrical or glutamate stimulation (Morton et al., 1983; Gebhart and Ossipov, 1986) of the lateral reticulated nucleus (Janss and Gebhart, 1988), it also responds to noxious visceral and cutaneous stimulation (Ness et al., 1998) with the electrical stimulation required to produce analgesia from this site lower than in other caudal ventrolateral medullary sites (Gebhart and Ossipov, 1986), with involvement of the LSN also in these roles through circuitry between laminae I, V and VII.

In addition to the projections to the CVLM, many of these retrogradely labelled neurons were also labelled from FG injected into the MDT. However, although it has been established that the neurons projecting to the medial thalamus have been found to be in the intermediate zone, laminae V and VII and the ventral horn, as this study also proves, many neurons were also retrogradely labelled that are present in lamina I, traditionally labelled from the lateral, and not the medial thalamus (Carstens and Trevino, 1978; Giesler et al., 1979; Willis et al., 1979).

As the diaminobenzidine (DAB) horseradish peroxides (HRP) procedure had been used in some of these previous studies, it may not have been as sensitive as the tetramethylbenzidine reactions used by Craig and Burton (1981). However, Craig and Burton (1981) used the anterograde tracer HRP and autoradiographic techniques and were the first to establish a unique projection of lamina I neurons to the nucleus submedius in the medial thalamus. Examination of the injection sites in the second and third experiments used
in this study shows extensive leakage into this nucleus, perhaps explaining the numbers in this study in the superficial laminae. Indeed, Craig and Burton (1981) also reported the existence of a direct projection from the spinal cord and the caudal part of the spinal trigeminal nucleus (Sp5C) to the submedius nucleus of the thalamus. Of note was that the cells of origin of both the spinothalamic and trigeminothalamic pathways (in the cat) were almost exclusively located in the marginal layer of both the spinal dorsal horn and the Sp5C (Craig and Burton, 1981), the area where there was also leakage of the tracer in the dorsal medullary injections in this study, but established as regions known to contain primarily nociceptive and thermoreceptive neurons (Dostrovsky and Hellon, 1978; Hu et al., 1981; Dubner and Bennett, 1983; Craig and Kniffki, 1985; Besson and Chaouch, 1987). Their original study concentrated on the cat, but in the rat, the cells of origin of the spinal projection to the submedius nucleus are located in the deeper layers (Menétrey et al., 1984; Dado and Giesler, 1990) in contrast to their mainly exclusive location in the marginal layer in the cat. In the rat trigeminal sensory nucleus less than 20% of the neurons are located in the marginal layer of the Sp5C and 60% are actually located in the interpolar part of the spinal trigeminal nucleus (Sp5I) rather than in Sp5C (Yoshida et al., 1991). However, in our studies, leakage also occurred into the Sp5I. Initially, Craig and Burton (1981) reported that lamina I neurons, after injections of HRP and titrated amino acids, projected to the nucleus submedius in the medial thalamus in the cat, monkey and rat. They, however, used considerably more cats (14) compared to monkeys (2) and rats (3). However, since that study Peschanski (1984) and then Iwata et al. (1992) also confirmed the lamina I spinal neurons projecting to the nucleus submedius but Dado and Giesler (1990), Cliffer et al. (1991) and Yoshida et al. (1991) were all unable to find this projection from both spinal and medullary superficial laminae. These three investigators only used rats in their experimental work suggesting perhaps a species difference. Therefore, the lamina I projections to the
nucleus submedius may not be as significant as first thought. Indeed, in the rat, the projection arising from the cervical enlargement is very sparse (Dado and Giesler, 1990), but from the trigeminal projection appears to originate from two regions as shown by Dado and Giesler (1990) and Yoshida et al. (1991): the interpolaris region and the ventral portion of the caudalis division. Therefore, the lamina I labelled neurons could be explained by the leakage into the posterior thalamic group, as shown by Gauriau and Bernard (2004) and discussed earlier.

Specifically, the ventral posterolateral thalamic nucleus (VPL), ventral posteromedial thalamic nucleus (VPM) and the posterior thalamic group (Po) regions contain numerous nociceptive neurons in the rat (Guilbaud et al., 1980; Peschanski et al., 1980). In the rat, these nociceptive properties were from both superficial and deep spinal/trigeminal laminae (Lund and Webster, 1967; Peschanski et al., 1980; Granum, 1986; Burstein et al., 1990b; Cliffer et al., 1991; Iwata et al., 1992). However, Gauriau and Bernard (2004) showed that most of the lamina I neurons projected to the VPL/VPM/Po regions. We, however, have shown that many lamina I neurons project to both the mediodorsal thalamus and the caudal ventrolateral medulla, not the lateral areas of the thalamus mentioned in other studies.

However, the injection sites in the three experiments used in this part of the study had leakage into the Po in all cases, explaining the greater number of lamina I neurons in our study, both for neurons retrogradely labelled from both the caudal ventrolateral medulla and the mediodorsal thalamus, and also for the retrogradely labelled neurons only from the mediodorsal thalamus as according to Gauriau and Bernard (2004). More recently, Al-Khater et al. (2008) showed, using fluorescent latex microspheres, a very discrete projection from lamina I to the PoT with the NK-1 receptor, and it may be that the lamina I neurons in this study also specifically project to this site via the leakage into the Po.
Interestingly, a significant number of retrogradely labelled neurons exist in the LSN that project to both the CVLM and the MDT, with approximately half possessing the NK-1 receptor. The LSN has also been shown to have a role in the processing of visceral information (Menétrey et al., 1980; Leah et al., 1988; Schramm et al., 1993; Jansen et al., 1995; Jansen et al., 1995), and potentially involved in nociception as previously discussed, with this nucleus projecting also through the spinothalamic (Gauriau and Bernard, 2004), spinoreticular, spinomesencephalic (Menétrey and Basbaum, 1987; Leah et al., 1988) and the spinohypothalamic tract (Burstein et al., 1996). It is also known that LSN neurons project to laminae I, II, V and VII (Jansen and Loewy, 1997) and electrophysiological studies demonstrated that these neurons exhibit a variety of intrinsic properties, which could significantly contribute to sensory processing, including nociceptive processing (Jiang et al., 1999). Therefore, the LSN may serve an integrative role between these laminae and be involved in processing a variety of nociceptive pathways, including descending modulation (Carlton et al., 1985; Masson et al., 1991), and as the mediodorsal thalamus projects to the medial and orbital regions of the prefrontal cortex (areas strategically involved in autonomic visceromotor and cognitive functions (Neafsey et al., 1986, 1993; Loewy, 1991; Verberne and Owens, 1998; Gabbot et al., 2005)), it could be suggested that the LSN could have an integrative role in visceral function and visceralnociception providing the emotional and motivational aspects of pain.
c) **Lateral globus pallidus projections from the LSN**

1. **Introduction**

The corpus striatum comprises the striatum (caudate nucleus and putamen) and the pallidum (globus pallidus), and forms the anatomical basis of the basal ganglia. It is well established that the corpus striatum collectively is the site in which instructions for parts of learned movements are “remembered” and from which they transmit to the motor cortex for integration by corticospinal and reticulospinal pathways to the motor neurons. However, in addition to the areas traditionally associated with nociceptive processing, as previously discussed, and the spinothalamic and spinoreticular pathways thought to constitute the major tracts transmitting nociceptive information, the globus pallidus has been suggested to also play a role in somatosensory transmission. Studies have shown that the basal ganglia are important for processing sensory information, where electrical and mechanical stimulation of trigeminal receptive fields are important in activating neurons both in the caudate nucleus and the globus pallidus (Carelli and West, 1991; Levine et al., 1987; Lidsky et al., 1978; Manetto and Lidsky, 1989; Schneider et al., 1982, 1985; Schneider and Lidsky, 1981). This is further enhanced by high concentrations of endogenous opiate receptors in both the neostriatum and the globus pallidus from immunocytochemistry and radioimmunoassay activity (Atweh and Kuhar, 1977; Hong et al., 1977a,b; Gros et al., 1978; Sar et al., 1978; Gramsch et al., 1979; Pickel et al., 1980; Herkenham and Pert, 1981; Jones et al., 1991; La Motte et al., 1978). Further to this, the globus pallidus also has been implicated in nociception as injecting morphine into the neostriatum or globus pallidus resulted in a naloxone reversible analgesia (Anagnostakis et al., 1992). In addition, the caudate nucleus and putamen receive afferent fibres from the intralaminar nuclei of the thalamus (Jones and Leavitt, 1974; Van der Kooy, 1979; Veening
et al., 1980; Herkenham and Pert, 1981; Kaufman and Rosenquist, 1985; Kincaid et al., 1991; Sadikot et al., 1992a,b) and the primary somatosensory cortex (Webster et al., 1961; Carman et al., 1965; Mercier et al., 1990). This could offer an explanation to related motor activities that can be seen in response to pain. Indeed, some patients with nerve injury induced persistent pain conditions have profound abnormalities of posture and motor control. In addition, the positions of injured limbs are in resembles that occurring in patients with extra-pyramidal lesions (Schwartzman and Kerrigan, 1990).

Spinopallidal connections, however, were studied using traditional anterograde tracing studies in the rat (Cliffer et al., 1991; Gauriau and Bernard, 2004) and the primate (Newman et al., 1996) that showed occasional spinal cord axons entering the globus pallidus. These studies showed that the spinopallidal projection was, in fact, a minor one. However, more recently, Braz et al. (2005) showed that there was a significant projection from the spinal cord to the globus pallidus.

Using transgenic mice that expressed the transneuronal tracer WGA, Braz et al. (2005) induced this tracer in dorsal root ganglion neurons that expressed the voltage gated tetrodotoxin resistant Na\(^+\) channel (Na\(_{\text{v}}\)1.8) which, as they state, through mosaic express of the transgene, resulted in labelling for the Na\(_{\text{v}}\)1.8 positive neurons that corresponded to the non-peptide class of primary afferent nociceptors. They had shown that lamina II interneurons were part of the major ascending pathways targeted by that class of nociceptors. From lamina II, Braz et al. (2005) stated that those interneurons contacted lamina V projection neurons, which projected to amongst other areas, the globus pallidus and constituted a greater pathway to this site than previously thought. They expanded by showing that there was no transneuronal labelling in subcortical regions which link the spinal cord and the globus pallidus e.g., the pedunculopontine nucleus (Nakano, 2000), midline thalamic nuclei, parafascicularis and submedius (Groenewegen et al., 1990) or the
parabrachial nuclei (Bernard and Besson, 1990). Interestingly though, in the study undertaken by Braz et al. (2005) using transneuronal transport of the genetically expressed lectin tracer, no labelling occurred in areas traditionally associated with the spinal cord projections for nociception like the ventroposterolateral nucleus of the thalamus.

As there appears to be a discrepancy between studies regarding input to the globus pallidus, especially direct spinopallidal projections, a series of retrograde injections were undertaken to identify those projection neurons to the globus pallidus from the spinal cord, including the LSN. Differences exist in input to different regions of the globus pallidus (Bernard et al., 1991) so this study used the retrograde tracer CTb injected into the lateral globus pallidus combined with immunocytochemistry for the tracer and the NK-1 receptor to quantify both the laminar distribution of spinopallidal projection neurons, and to identify those LSN neurons which may sub-serve a role for the motor activity, potentially related to pain.

2. Experimental Procedures

Experimental procedures and methodologies used were similar to previous retrograde experiments. Three adult male Sprague-Dawley rats (150-250g) were used where CTb was injected into the lateral GP (coordinates = anterior-posterior [AP] +6.9; dorsal-ventral [DV] + 3.0; medial-lateral [ML] + 3.6), and the spinal segments (C1 – 2, C5, L3 – 5) were processed and analysed for CTb, NeuN and the NK-1 receptor as previously described using the same primary and secondary antibodies and processing techniques.
3. Results

The retrograde tracer CTb was injected into three rats, and immunocytochemistry for this was combined with the NK-1 receptor to establish if this pathway may be involved in nociceptive processing, and if so, to what extent.

Injection sites

Reconstructions of the anterior and posterior extent of the spread of the tracer CTb, is demonstrated in Figure 5.19. Above each reconstruction (based on the atlas by Paxinos and Watson (1997)) is the most representative photomicrograph. In the first experiment in this series (Figure 5.19a), CTb was found mainly in the lateral globus pallidus, with some spread into internal capsule, posterior part of the anterior commissure and the ventral pallidum. The second of the experiments (Figure 5.19b) was the most focal of the three undertaken for the lateral globus pallidus where only a very small amount of tracer was found in the internal capsule, posterior part of the anterior commissure and the caudate putamen. The final experiment (Figure 5.19c) had greatest leakage outside the lateral globus pallidus with more extensive leakage into the caudate putamen and also into the anterodorsal part of the medial amygdaloid nucleus, central amygdaloid nucleus (medial, lateral and capsular parts), interstitial nucleus of the posterior limb of the anterior commissure, basal nucleus, reticular thalamic nucleus and the stria terminalis. No retrograde tracer was found on the contralateral side.

Distribution of retrogradely labelled neurons

The distribution of retrogradely labelled neurons after CTb injection to the lateral globus pallidus can be seen in Figure 5.20. Overall, the numbers of retrogradely labelled neurons was low, with only 43 neurons identified with CTb and were found bilaterally, but with predominantly contralateral to the injection site (67.4 ± 2.7%). As with previous
retrograde labelling experiments, retrogradely labelled neurons were found in laminae V – VII with $53.4 \pm 3.2\%$ located in these locations. Interestingly, although total numbers were very low for retrograde labelling, the LSN comprised $29.9\%$ of all CTb labelled neurons, were found bilaterally but preferentially on the contralateral side to the injection ($20.67 \pm 9.2\%$, contralateral; $9.4 \pm 8.3\%$ ipsilateral). This is summarised in Figure 5.21.

Similar to previous retrograde labelling experiments, the greatest number proportionally of CTb labelling occurred in the cervical segments, specifically C1 – 2 ($34.7 \pm 3.5\%$). The least number of retrogradely labelled neurons were found in L5 ($7.3 \pm 7.2\%$). This is summarised in Figure 5.21.

This study also used immunocytochemistry for the NK-1 receptor, and interestingly none of the retrogradely labelled neurons from the lateral globus pallidus contained this receptor. Representative images of a retrogradely labelled neuron in lamina V and the LSN are shown in Figure 5.22 and 5.23 respectively.
Figure 5.19. Reconstruction of injections to the lateral globus pallidus (n = 3). a - c represents experiments 1 - 3 respectively in this series. The spread of CTb tracer is represented by the dark grey area. The numbers below each tracing indicates the interaural co-ordinate (Paxinos and Watson, 1997).
Figure 5.20. Location of retrogradely labelled neurons after lateral globus pallidus injections. a - c represents experiments 1 - 3 with each corresponding with the reconstructions shown in Figure 5.19a – c respectively, in each of the stated spinal segments. Each dark filled circle represents a single CTb labelled neuron in each of the named spinal segments. The numbers in the ventral funiculus represents the total labelled neurons over ten alternately selected 50µm transverse spinal segments. The figure in parentheses shows the total number of CTb labelled neurons in the LSN. The right side of each spinal level diagram is ipsilateral to the injection.
Figure 5.21. Distribution of ALL retrogradely labelled neurons after lateral globus pallidus injections. A shows the mean percentage of retrogradely labelled neurons as distributed over the spinal segments C1 – 2, C5 and L3 – 5. B shows the laminar distribution of retrogradely labelled neurons, including the LSN.
A. Globus Pallidus

- Mean % of neurons
  - C1 – 2
  - C5
  - L3
  - L4
  - L5

B. Globus Pallidus

- Mean % of neurons
  - I – IV
  - V – VII
  - VIII – X
  - LSN

Contralateral
Ipsilateral
Figure 5.22. Example of a lamina VII neuron labelled after CTb injection to the lateral globus pallidus. A transverse section of lamina VII built from 9 projected confocal images (red, CTb; green, NK-1; blue, NeuN). A represents the single lamina VII CTb labelled neuron in the middle of the field, that is not immunoreactive for NK-1 (B), and C represents the neuronal population. D is the merged image of A, B and C. Scale bar = 20µm
Figure 5.23. Example of a LSN neuron labelled after CTb injection to the lateral globus pallidus. Projected image of a transverse section of the ipsilateral LSN (red, CTb; green, NK-1; blue, NeuN) built from 9 confocal images. A represents a single CTb labelled neuron identified by 1, that is not immunoreactive for NK-1 (B), and shows NeuN immunoreactivity (C). 2 represents an NK-1 immunoreactive neuron in the LSN that is not retrogradely labelled by CTb injected to the lateral globus pallidus. D is the merged image of A - C. Scale bar = 50μm
4. Discussion

The main findings of this study are fourfold. Firstly, the overall number of retrogradely labelled neurons identified was relatively small. Secondly, of those retrogradely labelled neurons identified with CTb immunocytochemistry, the most common laminar distribution was in laminae V and VII. Thirdly, almost one-third of all retrogradely labelled neurons were found in the LSN. Fourthly, of those retrogradely labelled neurons found, none possessed the NK-1 receptor.

The Spinopallidal Tract in the Rat

Recently, Braz et al. (2005) used transneuronal transport of a genetically expressed lectin tracer WGA in sensory neurons of Na\textsubscript{V}1.8-expressing mice. They showed the densest accumulation of transganglionic transportation of WGA was found in terminals and cell bodies within neurons in lamina II, and corresponded to primarily to the IB4 non-peptide class. Indeed, they also stated that there was extensive transportation of WGA to the lateral aspect of the globus pallidus, and did not project to the subthalamic nucleus and substantia nigra pars reticulata due to a lack of co-localisation with parvalbumin (Ruskin and Marshall, 1997). They claimed because no labelling was found in subcortical regions that link the spinal cord and the globus pallidus e.g. submedius, parafascicularis and the midline thalamic nucleus (Groenewegen et al., 1990), pedunculopontine nucleus (Nakano, 2000) or parabrachial nucleus (Bernard and Besson, 1990), that it must be a direct pathway.

However, the results presented here show that the spinopallidal projection is not as significant as Braz et al. (2005) may have suggested. Newan et al. (1996) had used the anterograde tracer Phaesolus vulgaris-leucoagglutinin (PHA-L) and biotinylated dextranamine injected it into the upper cervical spinal cord (C1 – 2) and the cervical enlargement at C5, and had showed a large number of labelled terminals in the globus
pallidus, many of which were from C1 – 2, in agreement with this study. However, the limitation of that study was that the anterograde transport was only examined in the upper cervical spinal cord and the cervical enlargement in the rat. Although the material presented here also demonstrates predominance in the upper cervical segments (C1 – 2), the overall numbers were indeed very low.

This study is also in agreement with anterograde tracing studies in the rat (Cliffer et al., 1991; Gauriau and Bernard, 2004) that showed that a direct spinopallidal tract is in fact minor. Interestingly, although Braz et al. (2005) demonstrated large numbers of transneuronal labelling in the globus pallidus, very little labelling was found in traditional targets of the spinal cord like the ventroposterolateral nucleus of the thalamus. As this study shows that it is laminae V and VII neurons that were labelled more frequently (despite overall numbers being low), Braz et al. (2005) propose that these neurons arising from this site could be part of the nociceptive circuit engaged especially by lamina V neurons that receive an input from the non-peptide Nav1.8 expressing population of primary afferent nociceptors. Gauriau and Bernard (2004) have demonstrated that it is the deeper laminae that target the globus pallidus, though in contrast to Braz et al. (2005), they have shown that the numbers (from the cervical segments only) are indeed low, in agreement with the results presented here.

Although the globus pallidus is a target of deeper spinal laminae, with wide dynamic range neurons projecting there (Bernard et al., 1992; Chudler et al., 1993), and the globus pallidus established (amongst many other functions) in nociception (Richards and Taylor, 1992; Lin et al., 1985; Bernard et al., 1992; Chudler et al., 1993; Chudler and Dong, 1995), the actual numbers of retrogradely labelled neurons found in this study are relatively small compared to other traditional nociceptive pathways. As this study has shown there to be relatively few retrogradely labelled neurons (with only some in the LSN), and none
possessing the NK-1 receptor, a direct nociceptive pathway to the globus pallidus from the spinal cord may not exist to the extent as some authors have claimed previously. Therefore, although the globus pallidus is considered to be an output of the basal ganglia system and concerned in regulation of movement (Delong, 1990; Chesselet and Delfs, 1996) and has been demonstrated to be involved in sensory and cognitive processing (Brown et al., 1997), the spinopallidal pathway is indeed very complex in the relations of pain and movement.
Chapter 6

General Discussion
The aim of this chapter is to discuss further the functional significance of the results discussed in previous chapters. The main conclusions of each investigation are summarised briefly followed by hypothetical functional models.

1. Conclusions and models

**Investigation 1:**

The hypotheses initially proposed (p25) suggested the following:

a) LSN neurons receive cutaneous information

b) LSN neurons are activated by noxious cutaneous stimulation

c) There is a variable degree of expression of Fos depending on the stimulus, as in the SDH

From this it can be concluded that the LSN does indeed receive cutaneous information, and in this case from peripheral cutaneous noxious stimuli (thermal and chemical). However, only a small number of LSN neurons are activated by these various stimuli (in this case four stimuli were used). A hot thermal stimulus activated the most of those neurons as demonstrated by Fos immunoreactivity, but approximately 15% of the total neuronal population at the site of the LSN were activated. Interestingly, unlike the SDH, LSN neurons were activated on both the ipsilateral and contralateral sides to the application of the noxious stimulus applied (apart from formaldehyde application to the hind-paw, which showed only Fos immunoreactivity in the LSN ipsilateral to the side of the stimulus).

The LSN could play a role in nociception based on its neurochemical profile (Ljungdahl et al., 1978; Barber et al., 1979; Seybold and Elde, 1980; Bresnahan et al., 1984; Sasek et al., 1984; Vikman et al., 1998; Aarnisalo and Panula, 1998; Olave and
Maxwell, 2004) and target projection sites (Pechura and Liu, 1986; Leah et al., 1988; Burstein et al., 1996; Jansen and Loewy, 1997; Gauriau and Bernard, 2004), though perhaps not to the extent as previously suggested.

The work presented here has shown varying degrees of Fos immunoreactivity dependent on the stimulus used, with hot water activating most LSN neurons, with approximately 15% of all LSN neurons showing Fos immunoreactivity. However, despite the advantages of using c-Fos immunoreactivity to demonstrate nociceptive neurons, it also comes with its drawbacks. Unlike electrophysiological studies, it is not a dynamic way of recording nociceptive responses of the neurons. Indeed, stimulus intensity and duration, play key factors in showing Fos immunoreactivity (Bullitt et al., 1992; Lima and Avelino, 1994). In addition, not all neurons express the gene when activated (Dragunow and Faull, 1989). Even the ventroposterolateral nucleus of the thalamus, an area clearly established in nociception, failed to elicit Fos immunoreactivity after the application of noxious stimulation (Bullitt, 1990). More recently, activity dependent phosphorylation of extracellular related kinases 1 and 2 (p-ERK1/2) has been shown to highlight nociceptive neurons within 5 minutes of noxious mechanical, thermal or chemical stimulation (Polgár et al., 2007), and this may well be better at demonstrating nociceptive neurons, though further studies will be needed to determine that.

The LSN neurons project to spinal laminae I, II, V and VII (Jansen and Loewy, 1997) and a loop system may well exist for the processing of nociceptive information at this site, before projecting to higher brain centres that process nociceptive stimuli. In addition, the LSN neurons form a continuous column underneath the pial surface and some of the dendrites pass laterally (Réthelyi, 2003) and some almost appose the pial surface Bresnahan et al. (1984). Indeed, on close inspection of Réthelyi’s (2003) electron micrographs, the dendrites actually do pass to the pial surface. That brings into question the
possibility that the LSN neurons may also be under the influence of components of the cerebrospinal fluid surrounding the spinal cord (Vigh et al., 2004) as well having a small role in nociception.

In addition, SDH neurons that express the NK-1 receptor (more than 80% (Ding et al., 1995; Marshall et al., 1996; Li et al., 1998; Todd et al., 2000; Spike et al., 2003)) activate descending pathways that control spinal excitability (Suzuki et al., 2002). It may be that the descending pathways are activated by the ascending NK-1 neurons from the SDH and activates those descending paths (that project bilaterally), and could explain the bilateral expression of Fos in the LSN.

Another potential source of LSN activation could be from lamina I neurons. Grudt and Perl (2002) showed that some lamina I neurons had axon collaterals which entered the dorsolateral funiculus, in and around the region of the LSN. It may be that, those lamina I neurons (also with thick axons that passed to the contralateral ventrolateral funiculus) are activated by primary afferent input and through the axon collateral, activate the LSN neurons. Indeed, neurons in the lateral dorsal horn have also been shown to have commissural axons which project to the lateral region of the dorsal horn on the contralateral side (Petkó and Antal, 2000), and could also activate the LSN on the contralateral side thus again explaining the bilateral activation of the LSN. Figure 6.1 summarises a potential circuit for the LSN, its role in nociceptive processing, and other influences on it.
Investigation 2:

The hypotheses initially proposed (p26-27) suggested the following:

a) As SP is present in abundance in the LSN, the majority of LSN neurons are NK-1 immunoreactive

b) If a majority of LSN neurons are immunoreactive for the NK-1 receptor, only a minority be immunoreactive for PKC-γ, which like the NK-1 receptor, has also been associated with nociceptive processing (Malmberg et al., 1997)

c) As NOS has been found to enhance the release of SP in the SDH (Garry et al., 1994; Aimar et al., 1998; Kamasaki et al., 1995), SP and NOS will be intimately related immunocytochemically in the LSN

d) If a close relationship exists between SP and NOS, as in the SDH, then the same will hold for the relationship of NOS and the target of SP, the NK-1 receptor in the LSN

e) As in the SDH, the NOS terminals in the LSN will be associated with inhibitory GABAergic neurons

From this it can be concluded that although the LSN is abundant in SP, the total number of NK-1 immunoreactive neurons there represented only approximately one-third of the entire population, with PKC-γ representing even less (at approximately 10%). Also, SP and NOS immunoreactivity in the LSN were closely related, and the NOS preferentially targeted the cell bodies of NK-1 immunoreactive neurons, whereas SP was related to both the cell bodies and the dendritic tree of those neurons. However, unlike in the SDH, the work presented previously shows that NOS in the LSN (as revealed by GAD) is not derived principally from GABAergic inhibitory neurons (nor that from excitatory glutamatergic neurons). Therefore, the exact source of NOS in the LSN is yet to be determined. A possibility that could be used in the future is combining NOS immunoreactivity with the use of the vesicular GABA transporter (VGAT), which has been shown to localise in
synaptic vesicles in both glycinergic and GABAergic neurons (Chaudry et al., 1998). Indeed, they found that although the vast majority of nerve terminals that contained GABA or glycine co-localised with VGAT, there are subpopulations of terminals that were rich in GABA or glycine that were not immunoreactive for VGAT. On the contrary, it may be that there are more GABAergic terminals in the LSN that have not been revealed with the antibodies used in this study, and may be revealed with VGAT.

Whatever the source of NOS is in the LSN, the precise role of NO in the superficial dorsal horn has also been the subject of debate. There is abundant evidence to support the idea that NO has a role in pain but its precise role remains unclear. There are a variety of reasons for this, for example NO modulates nociception at spinal and supraspinal levels and NOS inhibitors have different effects depending upon whether they are administered systemically, intrathecally or spinal (Kitto et al., 1992; Meller et al. 1992; Yonehara et al., 1997; Osborne and Coderre, 1999; Hoheisel et al., 2005).

Furthermore, in addition to nNOS, two other variants of NOS have been identified (the endothelial and inducible isoforms) and these may also have a role in nociceptive modulation (Ruscheweyh et al., 2006). We have shown that NOS terminals in the LSN are preferentially associated with NK-1 neuronal cell bodies. As discussed in the introduction, NO may enhance release of SP from spinal axon terminals (Garry et al., 1994; Aimar et al., 1998) which has a well established role in transmission of nociceptive information. NO was shown to regulate release of SP from rat spinal cord synaptosomes, but in contrast to the studies cited above, it was reported to attenuate release (Kamisaki et al., 1995). An alternative possibility is that NO may generate long term potentiation (LTP) at nociceptive synapses, (Sandkühler, 2000) a phenomenon that occurs in lamina I projection cells but not in interneurons (Ruscheweyh et al., 2006). These findings provide a morphological basis for both of these possible modes of action of NO in the LSN. A possible functional model

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based on work within the CNS is demonstrated in Figure 6.2 and suggests how SP, NOS and NK-1 may be related to each other in the LSN.

**Investigation 3:**

The hypothesis initially proposed (p28) suggested the following:

a) If NK-1 neurons are abundant in the LSN, then many will be projection neurons and target brain areas known to be involved in nociception

Using retrograde injection techniques, this Investigation focussed on the projections of LSN neurons to areas of the brain traditionally associated with nociceptive processing, but also to the globus pallidus and to the hypothalamus. It shows that of all retrogradely labelled neurons in each of the three sets of experiments, many LSN neurons project to these sites, with a bilateral projection system existing to the CVLM, MDT, lateral and medial hypothalamus and also the lateral globus pallidus.

Of all retrogradely labelled neurons in each of the three sets of experiments, LSN neurons constituted proportionally: 25% projecting to the lateral hypothalamus, 21% projecting to the medial hypothalamus, 12% projecting to both the CVLM and MDT, 15% projecting only to the CVLM, 7% projecting only to the MDT and 30% projecting to the lateral globus pallidus (although numbers were very low that were retrogradely labelled).

Although the NK-1 receptor represent only approximately 30% of all neurons within the LSN (as discussed in Investigation 2), of all those LSN retrogradely labelled neurons, NK-1 receptor immunoreactivity represents: 80% for either the lateral or medial hypothalamic projections, more than 50% for either the CVLM and the MDT or just the CVLM projections, 25% for MDT projections, but none of the LSN projection neurons to the lateral globus pallidus. Figure 6.3 presents a summary of the findings of these studies.
Although the LSN and the SDH share similar characteristics, they both possess very unique properties, suggesting that they sub-serve divergent functions that are probably complimentary. There is a great deal of research into the roles and functions within the grey matter, including that of the SDH, though the precise role of the LSN has remained uncertain.

This Investigation along with the previous work presented, shows that although the LSN has been implicated in nociception in the literature, the extent of its involvement in this process is less than previously thought. Interestingly, approximately one-quarter of LSN neurons project to either the lateral or medial hypothalamus, areas traditionally associated with autonomic and homeostatic processing, with many of them possessing the NK-1 receptor associated with nociceptive transmission especially in the SDH. Interestingly, LSN neurons have also been shown to project to the MDT (and also the CVLM) which projects to the prefrontal cortex. It could be postulated that the LSN functions as an integrative nucleus for autonomic and homeostatic functions, and with the projections to the MDT (and then to the prefrontal cortex), could be involved in the motivational and affective components of autonomic function.
Figure 6.1. A model of a possible mechanism of activation of the LSN. LSN neurons could be activated by a number of means (see p159). They could be activated by NK-1 neurons in the SDH that activate descending fibres that originate from supraspinal nuclei (1); lamina I neurons could pass to the dorsolateral funiculus via their axon collaterals, thus activating LSN neurons (2), or those neurons in the lateral part of the dorsal horn could project (via commissural axons) to the lateral region of the contralateral dorsal horn, and from there activate the contralateral LSN (3).
Figure 6.2. **Possible interactions and relations of NOS in the LSN.** The close relationship of NOS terminals to NK-1 neuronal cell bodies, and the relation of SP terminals to the NK-1 neuron in the LSN could be activated as shown in the diagram. Glutamate that is released from SP containing presynaptic terminals, perhaps from lamina I neurons, will act on NMDA and AMPA receptors. When the postsynaptic site (e.g. LSN NOS terminal) is depolarised, Ca$^{2+}$ enters, and via calmodulin (CaM), activation of NOS occurs (Bredt and Snyder, 1990). The NO may then have some type of regulatory effect on the SP release that is targeting the NK-1 neuronal cell body (Investigation 2) in the LSN, including activation of guanylate cyclase in the SP/glutamate containing cell, and perhaps local astrocyte processes.
Figure 6.3. Summary diagram representing the projection targets of NK-1 immunoreactive LSN neurons. There is a bilateral projection from the LSN to each of the areas studied previously. The thickness of the lines indicates how many NK-1 neurons in the LSN project to each of the brain regions, with projections to the lateral and medial hypothalamus (LH and MH respectively) being greater than projections to the CVLM and MDT and the lateral globus pallidus (GP).
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