Primary Afferent Input to Neurons in Laminae III and IV of the Rat Spinal Cord Which Possess the Neurokinin-1 (NK-1) Receptor

BY

Magda Mohamed Naim B.Sc. Msc. (Med. Sci)

A Thesis Presented for the Degree of Doctor of Philosophy in the Institute of Biomedical and Life Sciences, University of Glasgow

December 1998

Laboratory of Human Anatomy
Institute of Biomedical and Life Sciences
University of Glasgow

Summary

Neurokinin-1 receptor-immunoreactivity is present on many neurons in the spinal cord. A population of cells which possess the receptor is located in laminae III and IV and these cells have dorsal dendrites that penetrate the superficial laminae (I and II) of the dorsal horn. Since the dendrites of these neurons traverse several laminae, it has been suggested that they might receive input from different classes of primary afferents that terminate in these laminae. The present study investigated the input which these cells receive from two types of primary afferents: those which contain substance P (and are thought to function as nociceptors) and those with myelinated axons that terminate in laminae III-V (most of which are low-threshold mechanoreceptors).

With the immunofluorescence technique and confocal microscopy, all neurons of this type were found to receive contacts from substance P-immunoreactive varicosities and in most cases the contacts onto dorsal dendrites were very numerous. The great majority of substance P-immunoreactive varicosities which formed contacts were also calcitonin gene-related peptide-immunoreactive, indicating that they were of primary afferent origin. Combined confocal and electron microscopy revealed that synapses were present at sites of contacts between substance P-immunoreactive primary afferent boutons and the dendrites of the NK-1 receptor-immunoreactive cells. Some neurons of this type belong to the spinothalamic tract and cells retrogradely-labelled after thalamic injection of cholera toxin B subunit, were therefore examined for the presence of contacts from substance P-immunoreactive axon terminals. The results indicated that the spinothalamic tract neurons which possess the NK-1 receptor and have dorsally directed dendrites also receive contacts from substance P-immunoreactive axonal varicosities onto their dorsal dendrites.

By using the transganglionic transport technique, input from myelinated primary afferents which were labelled by injection of cholera toxin B subunit into the sciatic nerve were also examined. Dual-immunofluorescence and confocal microscopy revealed the presence of contacts between labelled primary afferent terminals and all of the NK-1 receptor-immunoreactive cells examined, however these contacts were much less numerous than those which the cells received from substance P-containing primary afferents. Electron microscopy revealed that synapses were present at some of the contacts between the myelinated afferents and the NK-1 receptor-immunoreactive neurons.

Since cells of this type were found to receive monosynaptic input from two different classes of primary afferents, (a major input from substance P-containing afferents and a less dense input from myelinated primary afferents) it can be concluded that they are likely to have wide-dynamic-range receptive fields but with a relatively strong nociceptive component.

Acknowledgements

I would like to thank my supervisor Dr. A.J. Todd very much for his continuous help, support and encouragement throughout the whole period of my study. I would also like to thank all of my supervisor's team for their help and support especially Dr. R.C. Spike. I wish to thank Professor A.P. Payne very much for giving me this chance to study in his Department. I am grateful to all of the staff members and technicians who helped me in the Department. I am especially very grateful to people who kindly looked after my children during my work in this study.

CONTENTS

Chapter 1

Introduction

The dorsal horn of the spinal cord has a complex organization. It contains a wide variety of neurons including projection neurons as well as excitatory and inhibitory interneurons. It receives an input from primary afferents and from axons which descend form neurons in the brainstem. The input from primary afferents to dorsal horn neurons forms the first synaptic relay in important ascending somatosensory pathways and this input can be modified by activity in spinal interneurons and descending axons.

In the present study, the relationship between a group of morphologically characterized dorsal horn neurons and two types of primary afferents was examined. There is evidence that these neurons are involved in processing information from nociceptors, and the present study was designed to help in understanding their wiring which would throw some light on the function of these neurons.

In order to understand the mechanisms by which the the somatosensory information is processed the following introduction will deal with some different aspects in the organization of the dorsal horn of the spinal cord. This will include a general description of the cytoarchitecture of the dorsal horn and the anatomy and physiology of some of its neurons with special reference to neurons studied in the present work. Classification of primary afferent neurons, in addition to the course and termination of their fibres and their receptive field properties is also included.

1. Dorsal Horn of the Spinal Cord

On studying thick sections of Nissl stained cat spinal cord, Rexed (1952, 1954) showed that the grey matter of the spinal dorsal horn can be divided into 6 parallel laminae. In Rexed's scheme lamina I corresponds to the marginal zone and lamina II to the substantia gelatinosa while laminae III and IV are equal to the nucleus proprius described by previous authors. This cytoarchitectonic scheme is now generally used and it has been shown that similar arrangements can be observed in other species including rat (Molander et al., 1984), monkey (Beal and Cooper, 1978; Ralston, 1979) and human (Schoenen, 1982). As described by Rexed (1952), lamina I is a thin layer which contains small neurons as well as large marginal neurons. Lamina II is characterized by its gelatinous appearance due to the concentration of small neurons (including stalked and islet cells; Gobel, 1978) and their processes, and the absence of myelinated axons (Willis and Coggeshall, 1991). Subdivision of lamina II has recently been reported (Ribeiro-da-Silva and Coimbra, 1982) and it is commonly divided into an outer part (IIo) and an inner part (IIi). Lamina III contains small neurons (similar to those of lamina II but less closely packed) and also some large neurons. Laminae IV, V and VI are characterized by the presence of large cells (Rexed, 1952).

The dorsal horn of the spinal cord contains a wide variety of neurons including cells which project to the brain as well as numerous interneurons. In addition to the major input from primary afferents which enter through the dorsal roots, the dorsal horn receives input from axons descending from various parts of the brain. Since it contains the first synapse of somatosensory pathways which convey sensations that are perceived as pain, it has attracted a great deal of interest and there have been numerous studies which have attempted to define the neuronal circuitry within it.

2. Primary Afferents

Somatosensory primary afferent neurons consist of a cell body located in a dorsal root ganglion (DRG) or the trigeminal ganglion and an axon which is divided into a peripheral process travelling in a peripheral nerve and a central process which travels centrally to the spinal cord through a dorsal root, or to the brain stem via a cranial nerve. Early knowledge on primary afferent fibres was obtained from electrophysiological studies of cutaneous receptors in mammals. These formed the basis for classification of different functional types of DRG cells.

2.1. Functional Classification of Axons in Peripheral Nerves

The early division of mammalian nerve fibres into A, B and C groups suggested by Erlanger and Gasser (1937) has been widely accepted. Group A which consists of myelinated fibres was further subdivided into α, β, γ and δ fibres on the basis of conduction velocity and function. Of the large myelinated axons, Aα fibres are concerned with proprioception and somatic motor function, while Aβ fibres are concerned with innocuous tactile sensations. Aγ fibres are motor to intrafusal fibres of muscle spindles and the thinly myelinated Aδ fibres are concerned with nociception, cold detection and touch sensation. B fibres belong to preganglionic autonomic neurons while the unmyelinated C-fibres include postganglionic autonomic efferents as well as sensory fibres carrying information about pain and temperature, some low threshold mechanoreceptors and visceral afferents (Ganong,1995; Guyton and Hall, 1996).

2.2. Classification of Primary Afferents Based on Cell Body Appearance

Many studies have subdivided DRG neurons according to different parameters such as the cell size, development, morphological markers or physiological characteristics. The first widely accepted subdivision was by cell size. Most reports used the terms

large light neurons (L neurons) which have uneven lightly stained cytoplasm and small dark neurons (SD neurons) which have even darkly stained cytoplasm (Lawson, 1979; Rambourg et al., 1983). Although by statistical analysis of cell size measurements, there was a general agreement that there were two population of cells in several species including mouse, rat, cat, and human (Lawson,1979; Lawson and Biscoe,1979; Lawson et al., 1984,1985; Lee et al., 1986; Ohnishi and Ogawa,1986) the histograms showed considerable overlap between the two populations. Other studies have described either one population (in C2 and T11 ganglia in cats; Warrington and Griffiths, 1904) or three populations of cells, in certain human ganglia (Kawamura and Dyck, 1978) or in the rat (Kitao et al., 1996). Willis and Coggeshall (1991) stated that the dark cells are always small but the light cells are of different sizes.

By the use of *in vitro* intracellular recording in mouse,Yoshida and Matsuda (1979) were able to find a roughly linear relationship between axon diameters and cell body diameter of DRG neurons, such that large cells gave rise to large axons and small cells to small axons. A linear relationship between DRG cell body size and the conduction velocity of their processes was also described (Yoshida and Matsuda, 1979; Cameron et al., 1986). Thus conduction velocity is more frequently used to estimate the axonal size rather than the morphological measurements of axon diameters. The correlation which was described between diameters of both the cell body and the axon of DRG neurons is consistent with previous observation of loss of small DRG cells and unmyelinated axons, after neonatal capsiacin (Fitzgerald, 1983) which is a neurotoxin that affects mainly the unmyelinated primary afferent fibres (Nagy et al., 1981; Nagy and Hunt, 1983), and in certain diseases (Ohnishi and Ogawa, 1986). Lee et al. (1986) were able to find this correlation in cat L5-S3 DRG cells whose processes had conduction velocity more than 2.5 m/s but no correlation was found for cells whose processes conducted at less than 2.5 m/s.

By the use of intracellular recording and injection technique, cells with C-fibre coduction velocity were all found to have somata in the size range of the SD neurons and the size distribution of fast conducting A-fibre somata was similar to that of the L cell population in both the rat (Harper and Lawson,1985) and the cat (Lee et al.,1986).

The use of neurofilament (NF) immuoreactivity can distinguish better between the two types of cells. It was reported that the L neurons are NF rich and the SD neurons are NF poor (Sharp et al., 1982; Lawson et al., 1984). Lawson et al. (1984) have shown that RT97 (a monoclonal antibody against the phosphorylated form of the 200-KDa NF subunit) can label the L but not the SD neuron population in the rat DRG. By using intracellular recording in rat DRG (where neurons were characterized by the conduction velocity of their processes) combined with immunocytochemistry, Lawson and Waddell (1991) have shown that L neurons (RT97-positive) conduct in the A-fibre-range and thus are presumably myelinated, whereas SD neurons (RT97-negative) have C-fibre range of conduction velocity, and thus are presumably unmyelinated. The subdivision into L and SD neurons was also emphasized developmentally by the reported difference in their birth dates (Lawson et al., 1974; Kitao et al., 1996).

2.3. Primary Afferents Classified by Axonal Conduction Velocity

2.3.1. C-Fibres

Because of their small size, there have been very few studies which have achieved successful intracellular labelling of C-fibres, however there have been numerous studies which have examined their chemistry.

2.3.1.1. Chemistry

By the use of immunocytochemistry, it has been established that there is a large number of biochemically distinct subpopulations of DRG cells which can be

characterised by their peptide or enzyme content, or by the presence of cell surface markers (Nagy and Hunt, 1981; Hunt and Rossi, 1985; McCarthy and Lawson, 1990; Hunt et al., 1992). It has been reported that peptides are found in DRG neurons which have C or Aδ fibers but rarely in those with Aβ fibres (Hunt et al., 1992). In addition, the enzyme fluoride resistant acid phosphatase (FRAP) is present in a discrete population of unmyelinated primary afferents. Therefore immunocytochemistry, enzyme histochemistry and the identification of cell surface markers have allowed the study of the cell bodies of C-fibres in DRG and their terminals in the spinal cord. C-fibres have been classified into two main subgroups: those which contain neuropeptides and those which do not (Hunt and Rossi, 1985; Hunt et al., 1992; Averill et al., 1995).

2.3.1.1.1. Peptide-Containing Afferents

Several neuropeptides have been localized in primary afferent neurons. Although early studies looked at individual peptides, combinations of three or more peptides may be found in some cells (Ju et al., 1987). There is general agreement that there are variations in peptide distribution in different species and the present work will concentrate on findings in the rat. Various problems are encountered in interpreting studies of biochemically defined DRG cell populations, the first was the use of colchicine in earlier experiments to block axoplasmic transport and raise the level of peptides in the soma. The results of this technique must be interpreted carefully because there is evidence that colchicine might alter peptide synthesis (Kiyama and Emson, 1991) and this may affect cell counts. Another problem which may affect the cell counts is the sampling method and quantitative techniques used. As discussed by Lawson (1995), DRG cell measurements carried out without stereological methods may be biased towards the large neurons due to greater probability of multiple nuclear sections through large than small neuronal nuclei. This problem of overestimation of

large cells was also reported by Robertson and Grant (1989) and Woolf et al. (1995). In addition, data obtained from electrophysiological recording may also be biased towards large neurons since these are easier to penetrate, while it is difficult to make stable penetration of cell bodies of the small C-fibres (Lawson, 1995). The problem of antibody cross-reactivity was encountered in early studies which demonstrated cholecystokinin (CCK)-LI in DRG neurons. However it is believed now that it was a cross-reaction with CGRP (Ju et al., 1987). Also the problem of sensitivity can lead to false negative results when the amount of peptides in the cell bodies are small and need more sensitive detection method to be demonstrated. Peptides localized in DRG neurons included calcitonin-gene related peptide (CGRP), substance P, somatostatin, galanin, CCK, vasoactive intestinal peptide (VIP), bombesin and opioid peptides.

2.3.1.1.1.1. Calcitonin-Gene Related Peptide (CGRP)

It is generally accepted that the most widely distributed peptide in DRG cells is CGRP (Hunt et al., 1992). Most studies have found CGRP-like immunoreactivity (LI) in small DRG neurons (Ju et al., 1987; McCarthy and Lawson, 1990). McCarthy and Lawson (1990) carried out double-labelling immunocytochemistry of the rat L4 DRG with an antibody to CGRP and with the monoclonal antibody RT97. Without the use of colchicine, they found CGRP-immunoreactivity in an average of 46.5% of all neurons (a percentage which is similar to that reported by Ju et al., 1987 after colchicine treatment). Sixty-two percent of the RT97 negative cells and 30% of the RT97 positive cells showed CGRP-LI. According to their report, approximately one third (32%) of the cells with peptide immunoreactivity were RT97 positive whereas about two-thirds (68%) were RT97 negative cells. CGRP-LI therefore appears to be present in neurons with A fibres as well as in those with C-fibres (McCarthy and Lawson, 1990) and this is compatible with previous studies in which CGRP-LI has been localized in DRG neurons

with a wide range of cell sizes (Gibson et al., 1984b) as well as in myelinated and unmyelinated nerve fibres (Harmann et al., 1988). In addition, intracellular electrophysiological recordings followed by dye injection and immunocytochemistry have shown that conduction velocities (CV) of neurons with CGRP-LI ranged from 0.5 to 28.6 m/s which means that CGRP-LI occurred in C-fibre neurons (CV < 1.3 m/s), Aδ fibre neurons (CV 2-12 m/s) as well as Aα/β fibre neurons (CV >12 m/s) (McCarthy and Lawson, 1990). It was noted that the group of RT97-positive CGRP-LI neurons spanned the small, medium and large size ranges within the ganglion although CGRP-LI was not seen in the very largest neurons of the ganglion.

Many authors have reported colocalization between CGRP and other peptides, for example substance P, somatostatin, VIP and galanin (Hokfelt et al., 1976, 1987; Nagy and Hunt, 1982; Molander et al., 1987; Gibbins et al., 1985, 1987; Leah et al., 1985a).

Numerous immunohistochemical studies have indicated that CGRP-immunoreactivity is present in the spinal dorsal horn. CGRP-immunoreactivity was reported in laminae I and II with less immunoreactivity elswhere in the dorsal horn (Gibson et al., 1984b; Skofitsch and Jacobowitz, 1985; Carlton et al., 1988; Chung et al., 1988; Harmann et al., 1988; Leah et al., 1988; McNeill et al., 1988; Traub et al., 1989; Todd and Spike, 1993) and is generally believed to be of primary afferent origin since the great majority of CGRP-LI in the dorsal horn disappears after dorsal root section (Chung et al., 1988; Traub et al., 1989), and *in situ* hybridization histochemistry has failed to find cells containing CGRP mRNA in the dorsal horn (Gibson et al., 1988; Rethelyi et al., 1989).

Several investigators have studied the ultrastructure of CGRP-immunoreactive axons in different species (Carlton et al., 1988; Harmann et al., 1988; McNeill et al., 1988; Merighi et al., 1991; Zhang et al., 1993; Ribeiro-da-Silva, 1995). There was general agreement that CGRP immunoreactivity in primary afferent terminals was

associated with large dense core vesicles. In the rat, McNeill et al. (1988) reported that labelled terminals almost exclusively formed the presynaptic elements at simple axodendritic synapses, however a small proportion of immunoreactive axon terminals formed the central parts of type I synaptic glomeruli (described by Ribeiro-da-Silva and Coimbra, 1982). In contrast, in the monkey a much higher proportion of CGRP-immunoreactive boutons formed the central axons of glomeruli (Carlton et al., 1988). Ribeiro-Da-Silva (1995) found that although most of the CGRP-immunoreactive boutons were nonglomerular, in lamina II nearly 30% of them were the central elements of type I glomeruli and these constituted approximately 20% of the central elements of type I golmeruli (Ribeiro-Da-Silva and Cuello, 1990). In the study by Ribeiro-da-Silva (1995), the pattern of colocalization of CGRP-LI with both substance P- and somatostatin-immunoreactivity has been investigated with electron microscopy. The results confirmed the non-glomerular nature of most of the double-labelled terminals in agreement with both Merighi et al. (1991) and Zhang et al. (1993). Ribeiro-da-Silva (1995) emphasized that boutons (either glomerular or non-glomerular) in which somatostatin- and CGRP-immunoreactivity were colocalized appeared identical to those in which substance P- and CGRP-immunoreactivity were colocalized.

2.3.1.1.1.2. Substance P

The tachykinin substance P has been the most widely studied peptide because of its involvement in pain. Substance P was found in 18-20% of rat lumbar DRG neurons when no colchicine treatment was used (McCarthy and Lawson, 1989), however the percentage reported by Ju et al. (1987) after colchicine treatment was 30%. Similarly Boehmer et al., (1989) found that 28% of DRG neurons express the mRNA for substance P. Substance P-LI has consistently been found in small DRG neurons with few medium-sized-immunoreactive neurons (Hokfelt et al., 1976; Tuchscherer and

Seybold,1985; McCarthy and Lawson,1989). Lawson and Waddell (1991) reported that 20-30% of substance P-LI neurons were part of the RT97-positive population which would presumably give rise to myelinated axons. A study of conduction velocity of substance P-LI neurons in the rat lumbar DRG has shown that approximately 50% of C-fibre cells, 20% of Aδ-fibre cells and no fast conducting Aβ-fibre cells have substance P-LI (McCarthy and Lawson,1989). Although similar figures to the previous ones were also recently published by McCarthy and Lawson (1997), in a more recent study by Lawson et al. (1997) in guinea pig, the authors reported in their sample that 10 of 66 A fibre neurons exhibited substance P-LI, and these included 2 fibres which conducted in the Aαβ range.

Substance P has been found to colocalize with a number of other peptides including neurokinin A, CGRP and galanin. All substance P-immunoreactive cells in the rat DRG were found to contain CGRP-immunoreactivity (Ju et al., 1987). Since both substance P and neurokinin A are derived from the same gene (preprotachykinin I; Helke et al., 1990), all DRG cells in the rat that have neurokinin A-LI would be expected to contain substance P-LI and this was found to be the case (Dalsgaard et al., 1985). Ju et al., (1987) demonstrated that many of the galanin-immunoreactive DRG cells also contained substance P-immunoreactivity. The extent of colocalization of substance P- with somatostatin- and VIP-immunoreactivity is species dependent. There appears to be no coexistence of substance P with either of these peptides in the rat although there is substantial coexistence in other species such as the cat (Lawson, 1995). There is little or no overlap betweeen substance P-immunoreactivity and FRAP in rat DRG cells (Lawson, 1992).

In the spinal dorsal horn, substance P-immunoreactivity is dense in lamina I and the dorsal part of lamina II, moderate in the ventral part of lamina II and light in lamina III (Hokfelt et al., 1975; Ljungdahl et al., 1978; Gibson et al., 1981; DeLanerolle and

LaMotte, 1982, 1983; Charnay et al., 1983; Ruda et al., 1986; Chung et al., 1989; Todd and Spike, 1993). Substance P in the dorsal horn is thought to be mainly located in primary afferents (Ruda et al., 1986) as shown by marked decrease of its level in the dorsal horn after dorsal rhizotomy as well as its accumulation distal to a ligature of a dorsal root (Takahashi and Otsuka, 1975). However there is evidence that it is also derived from other sources since it has been found in some cell bodies in lamina I-III (Hokfelt et al., 1977; Ljungdahl et al., 1978; Tessler et al., 1980, 1981; Hunt et al., 1981; Yoshida et al., 1990; Ribeiro-da-Silva et al., 1991; Battaglia and Rustioni,1992) and also in pathways descending from brainstem to spinal cord (Hokfelt et al., 1978; Gilbert et al., 1982; Helke et al., 1982; Menetrey and Basbaum, 1987; Maxwell et al., 1996) where it may be colocalized with serotonin (Chan-Palay et al., 1978; Hokfelt et al., 1978; Johansson et al., 1981; Maxwell et al., 1996). This substance P orginating from descending fibres has been found to project to the intermediolateral cell column and ventral horn but not to the dorsal horn (Helke et al., 1982; Menetrey and Basbaum, 1987; Maxwell et al., 1996).

Ribeiro-Da-Silva et al. (1989) examined the ultrastructure of substance P-immunoreactive axon terminals. In lamina I of the spinal cord, almost all substance P-immunoreactive profiles were non-glomerular however, in the ventral part of lamina II, substance P-LI occurred in a significant proportion of type I synaptic glomeruli (15%; Ribeiro-Da-Silva and Cuello, 1990) which are characterized by a dark small central terminal, of indented contour with closely packed spherical vesicles of variable diameter and few mitochondria (Ribeiro-Da-Silva and Coimbra, 1982). At the ultrastructural level, substance P was found in dense-core synaptic vesicles (Cuello et al., 1977) although immunoreactivity was also found in association with other structures in the synaptic terminals (DeLanerolle and LaMotte, 1983).

2.3.1.1.1.3. Somatostatin

Somatostatin immunoreactivity was found in about 5-15% of rat DRG neurons with the highest percentage in the lumbar region (Lawson 1992). Somatostatin immunoreactive DRG cells in the rat were described as being exclusively (Molander et al., 1987) or mostly (Ju et al., 1987) small. Unlike substance P and CGRP, somatostatin-immunoreactive cells never show RT97-immunoreactivity, and therefore they presumably all have unmyelinated axons (Lawson et al., 1993). Lawson (1995) has tested 38 intracellularly injected rat DRG neurons for somatostatin-LI. She was able to identify 8 cells with C-fibre conduction velocity of which only one showed somatostatin-LI while none of 30 A-fibre cells was immunoreactive for somatostatin. In the rat, there is little or no coexistence between somatostatin-LI and substance P-LI (Garry et al., 1989). Coexistence with CGRP varied between species, but in rat all somatostatin-LI cells had CGRP-LI (Ju et al., 1987). As for substance P-LI, very few somatostatin-LI neurons were FRAP positive (Lawson, 1992).

The highest density of somatostatin-immunoreactive fibres demonstrated in the rat dorsal horn was in the dorsal half of lamina II with a lower concetration of fibres in lamina I and the ventral half of lamina II (Hunt et al., 1981; Alvarez and Priestley, 1990a; Ribeiro-da-Silva and Cuello, 1990; Todd and Spike, 1993). Although it was originally thought that somatostatin-containing fibres within the dorsal horn were exclusively primary afferents, there is evidence that many are derived from local interneurons (Schroder, 1984; Ribeiro-da-Silva and Cuello, 1990) and there may be also a contribution from neurons in the brain with descending axons (Millhorn et al., 1987).

Ribeiro-Da-Silva and Cuello (1990) studied the ultrastructural distribution of somatostatin immunoreactivity in laminae I-III of the rat cervical spinal cord. Somatostatin-immunoreactive axonal varicosities contained round agranular synaptic vesicles and some large granular vesicles. Two types of varicosities could be identified:

light type and dense type. The light type varicosities had relatively low density of synaptic vesicles, and they were mainly dome-shaped and established mostly symmetric synapses with dendrites or rarely with cell bodies. Varcosities of the dense type had closely packed synaptic vesicles, and they were either scalloped-or dome-shaped and established asymmetric synapses. A small number of varicositics (5-7%) in the middle of lamina II were the central elements of type I synaptic glomeruli. Ribeiro-da-Silva and Cuello (1990) reported that substance P-, CGRP- and somatostatin-immunoreactive glomerular central varicosities share the same morphological characteristics. All have more than three large granular vesicle per profile per ultrathin section and relatively simple synaptic architecture. Few studies have attempted to distinguish between primary afferent and local somatostatin-immunoreactive axons (Alvarez and Priestley, 1990b; Ribeiro-da-Silva and Cuello, 1990). Ribeiro-da-Silva (1995) was able to distinguish between them by colocalization with CGRP as a marker for primary afferents. Most varicosities immunoreactive for somatostatin only were dome-shaped and some of them established symmetric synapses in contrast to those with colocalized CGRP-immunoreactivity which usually had a scalloped outline and never established symmetric synapses.

2.3.1.1.1.4. Galanin

After colchicine treatment, the number of galanin-immunoreactive DRG neurons reported by Ju et al. (1987) was less than 15% of the total population. Galanin-immunoreactive cells were small and were all found to contain CGRP-LI. Using immunofluorescence staining, Zhang et al. (1993) found many galanin-LI nerve terminals and a few immunoreactive cell bodies in laminae I and II of the dorsal horn of the spinal cord as well as a few nerve fibres in deeper laminae. They also demonstrated that galanin-LI colocalized with substance P- and CGRP-LI in many nerve fibres and

terminals in laminae I and II. These are galanin-containing primary afferent nerve terminals as shown by the disappearance of many of them after dorsal root section (Ch'ng et al., 1985) as well as by the presence of CGRP-LI within them. Galanin-immunoreactive axons are presumably also derived from local neurons (Ch'ng et al., 1985; Melander et al., 1986) which are GABAergic (Simmons et al., 1995), and possibly from descending fibres (Holets et al., 1988).

At the ultrastructural level, Zhang et al. (1993) used pre-embedding immunocytochemistry and found galanin-LI in central terminals of type I synaptic glomeruli. These terminals contained many densely packed synaptic vesicles and several large dense-core vesicles. Zhang et al. (1993) also reported that a few type II glomeruli were weakly galanin-immunoreactive. In lamina III, galanin-immunoreactive terminals formed only simple axo-dendritic synapses.

2.3.1.1.1.5. Cholecystokinin (CCK)

There has been controversy concerning the presence of CCK in primary afferents because although several immunocytochemical studies have apparently shown CCK-LI in DRG neurons, *in situ* hybridization histochemistry has failed to demonstrate CCK mRNA in the rat DRG (Seroogy et al., 1990; Cortes et al., 1990; Schiffmann et al., 1991). Ju et al., (1986, 1987) reported a complete identity between CGRP- and CCK-immunoreactive DRG cell bodies and suggested that CCK antibodies were cross-reacting with CGRP. However, *in situ* hybridization histochemistry has shown that CCK mRNA is present in guinea pig DRG and this appears to reflect species variation (Seroogy et al., 1990).

2.3.1.1.1.6. Vasoactive Intestinal Polypeptide

Very few VIP-immunoreactive cells were seen in DRG of normal rats either with or without colchicine treatment (Ju et al., 1987; however see below).

2.3.1.1.2. Fluoride-Resistant Acid Phosphatase (FRAP)

Many small DRG neurons do not appear to contain neuropeptides but are characterized by the presence of FRAP (Nagy and Hunt, 1982; Hunt and Rossi, 1985). This is an extralysosomal enzyme belonging to the acid phosphatase family (Ribeiro-da-Silva et al., 1986). Originally, FRAP was described as occurring only in rodents but more recently, DRG neurons with FRAP have been described in rabbit, cat, dog, monkey, cow and human (Knyihar-Csillik and Csillik, 1981; Silverman and Kruger, 1988). FRAP is present in small DRG neurons and also in axon terminals in a narrow band near the centre of lamina II of the spinal cord (Hunt and Rossi, 1985; Ribeiro-da-Silva et al., 1986). It is believed that FRAP fibres in the dorsal horn are exclusively primary afferents because there is evidence of its complete loss after dorsal root section (Knyihar et al., 1974) as well as after treatment with capsaicin in adult (Jessell et al., 1978) and neonatal rats (Nagy et al., 1981).

In rat DRG neurons, little or no overlap was found between FRAP and either substance P-LI or somatostatin-LI. Based on the almost complete lack of coexistence of substance P and FRAP, Nagy and Hunt (1982) and Hunt and Rossi (1985) suggested that separate populations of peptide-containing and non-peptide-containing C-fibres could be distinguished. However Carr and co-workers (1990) found colocalization of CGRP in 50% of FRAP-containing DRG neurons in the rat. In agreement with this, several other recent studies reported colocalization between FRAP and CGRP although the exact percentage of coexistence is disputed (Silverman and Kruger, 1990; Alvarez et al., 1991; Wang et al., 1994). Averill et al., (1995) divided DRG cells into three

minimally overlapping subgroups: the group of large light cells identified by being RT97-immunoreactive, a second group identified with markers such as the monoclonal antibody LA4 (Alvarez et al., 1991) or the capacity to bind the lectin *Bandeiraea simplicifolia* IB4 (BSI-B4), which appears to correspond more or less to the cell group expressing FRAP (Silverman and Kruger, 1990) and a third group of peptide-expressing cells.

Another enzyme which has been studied with histochemistry in the dorsal horn is thiamine monophosphatase (TMP; Knyihar-Csillik et al., 1986). TMP and FRAP are both fluoride resistant and have a similar distribution in DRG and dorsal horn and it has therefore been suggested that TMP and FRAP are the same enzyme (Knyihar-Csillik et al., 1986).

At the electron microscopic level, FRAP occurs only in the central terminals of type I synaptic glomeruli in lamina II and is totally absent from type II synaptic glomeruli (Ribeiro-da-Silva et al., 1986). In the zone corresponding to a band of FRAP seen with the light microscopy, nearly 89% of central axons of type I glomeruli were FRAP positive and this percentage fell to 29% of those in lamina IIo and to 7.4% in the ventral-most 20μm thick stripe of lamina IIi.

2.3.1.1.3. Plasticity of Neuropeptides and FRAP in Primary Afferents

The distribution of peptides and FRAP in primary afferents described above is that seen in normal rats. However there is a great deal of evidence that in certain pathological states, there are dramatic changes in the peptide and enzyme content of primary afferents. Transection of a peripheral nerve has been shown to induce dramatic changes in expression of peptides in DRG cells and their terminals in the dorsal horn of the spinal cord. Thus it was demonstrated that sciatic nerve section causes marked depletion of substance P (Jessel et al., 1979), CGRP (Noguchi et al., 1990a), and

somatostatin (Shehab and Atkinson, 1984) as well as the non-peptide marker FRAP (Devor and Claman, 1980). On the other hand, several other peptides have been shown to be upregulated in primary afferents, including VIP (McGregor et al., 1984; Shehab and Atkinson, 1984, 1986a), galanin (Hokfelt et al., 1987) and neuropeptide Y (NPY; Wakisaka et al., 1991, 1992; Munglani et al., 1995). After sciatic nerve section the increased staining of VIP was restricted to areas of the third, fourth and fifth lumbar segments from which the other peptides (substance P and somatostatin) were depleted and FRAP was absent (Shehab and Atkinson, 1986a). Shehab and Atkinson (1986b) showed that an intact dorsal root was necessary for increases in VIP in the spinal cord and that VIP was increased in DRG neurons, indicating the primary afferent origin of the up-regulated peptide. The increases in VIP and galanin include small fibre populations in laminae I and II (McGregor et al., 1984; Shehab and Atkinson, 1986a; Hokfelt et al., 1987).

NPY-LI is absent in the normal DRG cells and is present in all laminae of the lumbar spinal cord with an especially dense concentration in laminae I and II of the dorsal horn, originating from local neurons (Gibson et al., 1984a). Wakisaka et al. (1991) have shown that sciatic nerve injury produced an increase in NPY-LI in laminae III-V of the affected segments and induction of NPY-immunoreactivity in many DRG cells. Interestingly, they found that the nerve injury-evoked increase in NPY-immunoreactivity occurred mostly in myelinated primary afferent neurons with very few if any C-fibre neurons as evidenced by analysis of cell sizes and observation of the laminar distribution of the increased immunoreactive-terminals.

2.3.1.2. Intracellular Injection Studies

Unmyelinated C-fibres are the most numerous primary sensory fibres of vertebrates (Nagy et al., 1983; Sugiura et al., 1986). Although the recent technique of intra-axonal

recording and injection of tracer substances such as horseradish peroxidase (HRP) has been used successfully to study the course and termination of both Aβ fibres (Brown, 1981) and Aδ fibres (Light and Perl, 1979) it has proven very difficult in the case of C-fibres because of their extremely small diameter (less than 1 μm). However, Alvarez et al. (1993) have been able to carry out intraxonal recording and injection of the tracer IIRP in two unmyelinated C-fibres of the monkey. In this technique, the receptive field properties of the fibre were first investigated followed by injection of HRP which was allowed to be actively transported out towards the terminals, and then the course and termination of the axon and its collaterals could be demonstrated by a histochemical method. One of the most important advantages of this technique over the anatomical techniques such as Golgi staining (Szentagothai, 1964; Scheible and Schieble, 1968) and degeneration techniques (Szentagothai,1964; Sterling and Kuypners, 1967) was the ability to provide identification of collaterals and terminals in terms of their receptive field (functional) types. However, even the method of intraaxonal staining does not allow the total number of collaterals arising from a single axon to be determined because there is likely to be incomplete filling. Sugiura et al., (1986) have developed an alternative approach which involved injection of tracer into the cell bodies of C-fibres in the DRG rather than into the axons themselves. This method has been developed in guinea pig. They were able to overcome the problem of tracer transport through these very fine fibres to the spinal cord by two modifications of the standard procedure, firstly the use of a plant lectin *Phaseolus vulgaris* leukoagglutinin (PHA-L) which can fill distant processes by orthograde transport and can be iontophoresed into cell bodies from micropipette electrodes and secondly by using prolonged survival time (2 to 7 days). Sugiura et al., (1986) have carried out an intracellular recording and tracer injection at the level of C2 and L6 DRG of guinea pigs and have successfully stained 7 afferents of which 1 was high-threshold mechanoreceptor, 2 were polymodal nociceptors, 2 were

mechanical cold nociceptors and 2 were low-threshold mechanoreceptors. Subsequent immunocytochemical processing of the injected ganglia, their dorsal roots and adjacent spinal cord to reveal PHA-L showed that all but one of the C-fibres passed from the dorsal root and ran for some distance either in Lissauer's tract or rostrocaudally in the superficial dorsal horn itself. The parent fibre gave off branches at different loci before ending in terminal arborizations. All of their fibre sample ended mainly in the superficial dorsal horn (laminae I and II) except the lumbar polymodal nociceptors which also had terminals deep into laminae III and IV. In another study aimed at comparing the central projections of somatic and visceral C-afferents, Sugiura et al. (1989) found that the visceral afferents terminated in the superficial dorsal horn (laminae I and II) as well as in laminae IV,V and X, occasionally in the dorsal and lateral funiculi and few collaterals were traced to the contralateral laminae V and X. They also reported that visceral afferent C-fibre projection showed an extensive rostrocaudal distribution of more than 5 spinal segments which provides an anatomic basis for the poor localization of visceral sensation and possibly for referred sensation (Cervero, 1983).

In the study by Alvarez et al. (1993), the electron microscopic appearance of C-fibre terminals within the spinal cord has been demonstrated. They studied two C-fibres in the monkey, one of which was identified as a C-nociceptor. Postembedding immunocytochemical technique for CGRP, substance P and GABA was used to study the synaptic interaction and neuropeptide content of the terminals. Alvarez et al. (1993) found that the terminals of these two fibres commonly (65%) formed simple axodendritic connections and rarely formed typical synaptic glomeruli (fewer than 10%). They reported that the terminals sampled in their study differed from the terminals of Aδ nociceptors which they had previously examined (Alvarez et al., 1992) as these C-fibre terminals rarely had glomerular endings, never received presynaptic

input from GABA-immunoreactive terminals or any other profile and they contained CGRP-LI. Alvarez et al. (1992) suggested that this difference between the two different types of primary afferent may represent a different processing mechanism.

2.3.1.3. Receptive Field Properties

The receptive field properties of C-afferent fibres have been examined in several studies of rat peripheral nerves by using extracellular recording (Lynn and Carpenter, 1982; Fleischer et al., 1983; Leem et al., 1993). In a survey study of the physiological properties of cutaneous units recorded extracellularly in the saphenous nerve, Lynn and Carpenter (1982) found that of 149 C-fibre units studied, a large proportion (73%) were polymodal nociceptors with small receptive fields that responded to noxious pressure and heating. Sensitive mechanoreceptors represented 12% of the sample while cold thermoreceptors accounted for 4% and the remaining units (11%) were either very insensitive or completely inexcitable. Fleischer et al. (1983) studied responses of unmyelinated fibres in the saphenous and coccygeal nerves and identified mechano- and heat-sensitive units, which correspond to polymodal nociceptors (56% in saphenous and 74% in coccygeal), mechanoreceptive units (30% in saphenous and 5% in coccygeal) as well as cold-sensitive which were more frequent in the coccygeal nerve. Leem et al. (1993) studied 574 cutaneous afferent units in the sural and plantar nerves supplying the skin of the rat foot, of which 120 were C-fibres. In the sural nerve, they found that 44% of the units were nociceptors, 33% were mechanoreceptors and 21% were cold receptors. The plantar C-fibre sample included nociceptors (77%) and cold receptors (23%). Their results indicated that all known types of cutaneous receptors except warm receptors exist in the skin of the rat foot and that nociceptor units were the dominant type of C-fibre in both nerves.

2.3.1.4. Chemistry and Peripheral Targets

It has been suggested that one way to study the function of primary afferent neurons is to correlate peptide immunolocalization with patterns of peripheral innervation (Willis and Coggeshall, 1991). Recently, many investigators have tried to find a correlation between the biochemistry of primary afferents and their peripheral targets by the use of combined retrograde labelling from a particular part of the body and immunocytochemistry (McMahon et al., 1984; Gibbins et al., 1987; Molander et al., 1987; Ositelu et al., 1987; O'Brein et al., 1989; Perry and Lawson, 1998). McMahon et al. (1984) reported that there are substantial biochemical differences in the population of C-fibres supplying muscle compared with those supplying skin. Molander et al. (1987) found that somatostatin-immunoreactive DRG cells project into somatic nerves but not into visceral nerves, however other peptide immunoreactivity (CGRP and substance P) and also FRAP were found in both types of nerve. In trigeminal ganglion, Ositelu et al. (1987) demonstrated that somatostatin-immunoreactive cells innervate facial skin but not masticatory muscle or tongue. In a study in the guinea-pig, Gibbins et al. (1987) were able to find a relatively precise correlations of the part of the body innervated with colocalization of different peptides. Neurons that were immunoreactive to a combination of substance P, CGRP, CCK and dynorphin were distributed mainly to the skin. Neurons that contained immunoreactivity to substance P, CGRP and CCK but not dynorphin were distributed mainly to small blood vessels of skletal muscles while those which contained immunoreactivity to substance P, CGRP and dynorphin but not CCK were distibuted mainly to pelvic viscera and airways. Neurons containing immunoreactivity to both substance P and CGRP but not CCK or dynorphin were distributed mainly to the heart, systemic blood vessels, blood vessels of the abdominal viscera, airways and sympathetic ganglia. O'Brien et al. (1989) injected the fluorescent dye Fast Blue into either skin, muscle or knee joint of the hind limb of the rat to

differentiate between the chemical expression of primary afferent neurons (including C-fibres) innervating different target tissue. Among the small dark cells, they found that TMP (which is identical to FRAP) and somatostatin-LI predominate in DRG cells innervating skin while CGRP- and substance P-immunoreactivity were more common in muscle and joint afferents. Perry and Lawson (1998) found that skin afferents contained the highest proportion of neurons expressing somatostatin while the visceral afferents had the highest proportion of neuronal profiles labelled for CGRP and substance P. These previous studies concluded that the chemical expression of primary afferents is characteristic of the peripheral target they innervate.

On the other hand because of technical difficulties, few authors have managed to study the relationship between physiological and biochemical properties of DRG neurons with unmyelinated axons. Lynn and Hunt (1984) reported that there is limited evidence on which histochemical profiles are matched with particular functional types such that VIP is assosiated with some visceral C-fibres, substance P is found in some C polymodal nociceptors and FRAP may be associated in part with C mechanoreceptor fibres. In cats, Leah et al. (1985b) combined intracellular dye injection of single sensory neurons of known fibre type and sensory modality with peptide immunohistochemistry. Twenty-four hours prior to electrophysiological recording, they used colchicine to enhance peptide immunostaining. They found no clear correlation between the content of neuropeptides (substance P, somatostatin, colecystokinin and VIP) and receptive field properties of cells. Surprisingly they were not able to detect substance P-immunoreactivity in many nociceptive sensory neurons even though it could be demonstrated with the same technique in many neurons which did not have cutaneous receptive fields. A recent study in guinea-pig by Lawson et al. (1997) disagreed with that of Leah et al. (1985b), although the same technique was used. Interestingly, Lawson et al. (1997) found that all substance P-LI positive units (29, of which 19 were

C-fibre neurons and 10 were A-fibre neurons) with identified receptive properties were nociceptive but not all categories of nociceptors were substance P-immunoreactive. There was a tendency for nociceptive neurons with slower conduction velocities and/or smaller cell bodies to show substance P-LI. On the basis of conduction velocity and location of receptive field, nociceptive units were further subdivided into superficial cutaneous, deep cutaneous or subcutaneous. The most intensely labelled units for substance P were the deep cutaneous and some of the polymodal nociceptors in glabrous skin. It was concluded that substance P content is related to afferent receptive properties, the tissue in which the peripheral receptive terminals are located, the conduction velocity and the soma size.

2.3.2. Aδ Fibres

2.3.2.1. Receptive Field Properties

The receptive field properties of Aδ fibres have been reported in numerous studies which used extracellular recording and the results of these studies are summarised in Willis and Coggeshall (1991). Two main classes of Aδ afferents have been identified: mechanical nociceptors and down-hair follicle afferents.

2.3.2.2. Intracellular Injection Studies

By the use of intraaxonal recording, HRP iontophoresis and subsequent histochemical reactions, Light and Perl (1979) were able to study the spinal termination of functionally identified Aδ fibres from cutaneous receptors of the cat and monkey at the light microscope level. They noted that the pattern of termination was identical in the two species. Light and Perl (1979) reported that mechanical nociceptor fibres terminated mainly in lamina I and to a lesser extent in lamina V while the down-hair primary afferent axons displayed an entirely different pattern and terminated in the inner

part of lamina II as well as laminae III and IV.

A further study (Rethelyi et al., 1982) in the same laboratory was carried out with both the light and electron microscope to examine the nature and organization of the synaptic contacts of these fibres. Rethelyi et al. (1982) noticed that each collateral which arose from the primary afferent had a large number of *en passant* enlargements before terminating as a terminal enlargement. Neither the part of the collateral proximal to the enlargements nor the portion connecting enlargments was seen to participate in a synaptic specialization. They also found that synaptic glomeruli were common to both mechanical nociceptors and the down-hair axons. In lamina I of the cat, they found that myelinated mechanical nociceptor enlargements either had simple axodendritic contacts or were the central terminals in glomeruli. These glomerular central terminals were presynaptic to several dendritic profiles and postsynaptic to either an axon terminal or a vesicle containing dendrite. In lamina V the synaptic complexes were simpler and the afferents received presynaptic contacts from only axonal profiles. In the monkey, Aδ nociceptor terminals were associated with glomeruli in both laminae I and V. In these glomeruli, the labelled afferents were presynaptic to dendritic profiles and postynaptic to axon terminals. Down-hair axons which terminated in laminae IIi, III and IV formed the central terminals in glomeruli and were presynaptic to both ordinary and vesicle-containing dendrites, and also postsynaptic to axonal profiles. These glomeruli resembled type II glomeruli seen in the rat (Ribeiro-da-Silva and Coimbra, 1982).

As an extension of these studies, Alvarez et al. (1992) have analyzed the synaptic interaction between the central terminals of physiologically identified Aδ high-threshold mechanoreceptors and GABA immunoreactive profiles in both monkey and cat. By the use of postembedding technique and GABA immunoreactivity, they found that all peripheral axon terminals which were presynaptic to the Aδ high-threshold mechanoreceptors terminals showed GABA immunoreactivity and 28% of the

postsynaptic dendritic profiles displayed weak GABA immunoreactivity. They emphasized that there is similar synaptology between both monkey and cat.

2.3.3. Aβ Fibres

2.3.3.1. Receptive Field Properties

The receptive field properties of Aβ fibres have been characterized in many studies into hair follicle afferents, Pacinian corpuscle afferents and various types of cutaneous mechanoreceptors (reviewed in Brown, 1981; Willis and Coggeshall, 1991). Cutaneous mechanoreceptors which are activated by mechanical forces in the skin are classified on the basis of their adaptation rate to a maintained stimulus into rapidly adapting and two types of slowly adapting (type I and type II). Slowly adapting type I mechanoreceptors are associated with Merkel cell complexes in the epidermis, whereas type II are associated with Ruffini endings in the dermis of the skin. In the absence of a stimulus, slowly adapting type I endings are normally silent while type II usually have a background discharge. Slowly adapting type I afferents discharge following indentation of the skin directly over the ending but are relatively insensitive to displacement of the skin immediately adjacent to the ending or to skin stretch. In contrast, slowly adapting type II respond to dispacement of the skin either directly over the receptor or as a result of skin stretch from a distance. Rapidly adapting mechanoreceptors have been identified with encapsulated endings of the Krause corpuscles in the dermis of the skin in cat's foot and toe pads, and in Meissner's corpuscle in rat and primate including humans. Hair follicle receptors which respond to movement of hairs are associated with 3 types of hairs, tylotrichs, guard and down hairs. Pacinian corpuscles which are rapidly adapting are located in the subcutaneous tissue and respond to vibration.

2.3.3.2. Intracellular Injection Studies

An extensive series of studies of Aβ fibres in the cat has been carried out by Brown and his colleagues (Brown et al., 1977, 1978, 1980a, 1981; Brown, 1981) using the technique of intraaxonal recording and injection of the tracer HRP. These studies demonstrated the course and termination of axons innervating hair follicles, Pacinian corpuscles, as well as rapidly adapting mechanoreceptors and slowly adapting type I and type II mechanoreceptors. On entering the cord from dorsal roots, axons of these large myelinated afferents followed a general pattern. Most of them bifurcated into rostral and caudal branches within the dorsal column. Each branch gave off numerous collaterals at nearly regular intervals then each collateral ended by terminal arborizations. All arborizations from a single axon were approximately in the same general medio-lateral position. Termials of all Aβ fibres were located in deep laminae of the dorsal horn (III-VI) however each type of afferent had a characteristic branching pattern and laminar distribution of its boutons.

Reconstructions of hair follicle afferent fibre collaterals made from serial transverse sections showed the characteristic morphology of "flame shaped arbors" first described by Scheibel and Scheibel (1968). According to the description of Brown et al. (1977), the collaterals enter the dorsal horn at its dorsal border and descend through the first four or five laminae of the cord, and then in lamina IV or V they make a "U"-shaped turn and ascend back into laminae IV and III where they undergo repeated subdivion and break up into terminal arborizations centred on lamina III with only slight extension into the most dorsal part of lamina IV and occasionally, minimal intrusion into lamina II. Brown et al. (1977) emphasized that only a few collaterals had terminals that entered even the most ventral parts of lamina II and attributed the difference berween their findings and the results shown with the Golgi method (Szentagothai, 1964; Scheibel and Scheibel, 1968; Rethelyi and Szentagothai 1973) to changes which occur during post-

natal development since most Golgi studies used materials from new-born kittens. This suggestion was supported by Rethelyi (1977) who presented results consistent with those of Brown et al. (1977) after carrying out a Golgi study in adult cats. In their description of the synaptic boutons, Brown et al. (1977) stated that the terminal arborization of hair follicle afferent collaterals contain a very high density of synaptic boutons greater than that for any other type of collateral from myelinated afferent fibres and that the boutons are more clearly confined to lamina III than are the flame shaped arbors. They considered that hair follicle afferent fibres are unique among Aβ afferent fibres in that they have recurving collaterals that give rise to the flame-shaped arbors described by the Scheibels (1968), their input to the cord is restricted to lamina III, they form a continuous sheet of terminals without interruptions and in addition most of them do not bifurcate upon entering the cord but turn rostrally and ascend.

Following the usual pattern of large primary afferent fibres, the axons of Pacinian corpuscle units were found to bifurcate into rostral and caudal branches shortly after entering the cord, and each branch gave off numerous collaterals (Brown et al., 1980a). Reconstructions of axons from Pacinian corpuscles of the foot and toe pads showed that the distribution of the collaterals was in the medial third or less of the dorsal horn. One of the characteristic features was that each collateral had terminal arborizations in two main areas of the dorsal horn: a main termination dorsally in laminae III and IV where the axons tended to run in the longitudinal direction and a lesser area of termination ventrally in laminae V and VI where axons ran in a dorso-ventral direction. Another characteristic feature was that Pacinian corpuscle afferent collaterals had the largest dorso-ventral extension of any Aβ afferent in cutaneous nerves. As regards the synaptic boutons, Pacinian corpuscles were described as having larger boutons than those of any other primary afferent fibres in cutaneous or muscle nerves.

Brown et al. (1980a) studied a sample of three axons innervating rapidly adapting mechanoreceptors in glabrous skin of the foot and toe pads. Collaterals were distributed to the most medial parts of the dorsal horn, sometimes to the medial quarter or less. Brown et al. (1980a) found some morphological similarity between the rapidly adapting mechanoreceptors of the glabrous skin and hair follicle afferents. The rapidly adapting mechanoreceptors showed the general morphology of "flame-shaped arbors" and their terminal arborization were distributed mainly to lamina III although some collaterals reached lamina IV. However, while hair follicle afferent collaterals formed a continuous sheet of arborizations running up and down the cord for many millimetres, with arborization from adjacent collaterals overlapping, those of the rapidly adapting mechanoreceptors were discontinuous in the longitudinal axis. The synaptic boutons were found mainly in lamina III but some collaterals had boutons in lamina IV. Many of the boutons were of the *en passant* variety although some clusters were also observed.

Brown et al. (1978) described the physiological characteristics, the central projection and the morphology of axons of type I slowly adapting mechanoreceptors both in hairy and glabrous skin. Studying 13 axons of type I units, nine from hairy skin and four from glabrous skin revealed that they were similar to the great majority of cutaneous and muscle afferents in that they nearly all bifurcated upon entering the cord. The collaterals descended through the dorsal four or five laminae sometimes dividing on the way and then at the level of lamina IV or V, they showed a broad "C"-shaped curve with convexity of the "C" nearly always towards the lateral surface of the cord. Collaterals from axons innervating glabrous skin were distributed to the medial third of the dorsal horn and were often having an "L"-shape. The terminal arborizations were discontinuous and occupied laminae III, IV and V (dorsal part of lamina V for axons from hairy skin and most of lamina V for axons from glabrous skin). Compared to

collaterals of hair follicle and Pacinian corpuscle afferents, the density of synaptic boutons of slowly adapting type I afferent collaterals was considerably less.

Receptive fields of axons innervating slowly adapting type II receptors in the cat were found either in hairy skin of the limbs or in the skin around the base of the claw however, the sample of type II axons that had been injected in the study of Brown et al. (1981) were all of the claw variety because these were much more common. On entering the cord, type II axons followed the usual pattern however in some cases a collateral was given off before the parent axon bifurcated, a feature which seemed to be more common in slowly adapting type II axons than in others. The axon collaterals formed a series of plates or sheets 100-300 μm thick in the rostrocaudal axis of the spinal cord across nearly a third of the dorsal horn in laminae III-V or III-IV. The density of synaptic boutons was greater in laminae III and IV than in laminae V and VI and the arrangement of the boutons varied according to the target lamina.

Woolf (1987) studied the morphology and the central termination of three different types of low-threshold mechanoreceptors in the rat spinal cord, by using the same technique of intraaxonal injection of HRP into physiologically characterized afferent fibres. His sample included 5 rapidly adapting glabrous skin mechanoreceptors, 6 hair follicle afferents and 4 slowly adapting type I afferent fibres (2 from glabrous and 2 from hairy skin). Although there were similarities between the morphology of the central terminals of cutaneous low-threshold mechanoreceptors in the rat and those previously described in the cat (Brown et al., 1977, 1978, 1980a), some significant differences have been found. Woolf (1987) reported that the dimension of the arborizations in the rat are smaller than those in the cat, but the total number of collaterals per axon is higher in the rat. In addition, variability of collaterals morphology was a characteristic feature in the rat. For example, the lateral convexity of collaterals of slowly adapting type I fibres was not a consistent feature. Some

differences in the laminar location between the two species were also observed. Forty-four percent of the arborizations of hair follicle afferents in the rat had boutons in inner lamina II, while this arrangement was only occasionally encountered in the cat (Brown et al., 1977). While the synaptic boutons of the rapidly adapting afferents innervating glabrous skin were present mainly in lamina III in the cat (Brown et al., 1980a), they tended to be concentrated in laminae IV and V in rat. In addition, those of slowly adapting type I afferents were distributed in laminae III-V in the cat (Brown et al., 1978) but did not reach lamina III in the rat.

Shortland et al. (1989) studied the morphology of the central collateral arborizations of 24 Aβ hair follicle afferents innervating differents regions of the skin of the hind limb in the rat. They observed that the morphology of afferents may be related to their peripheral innervation since the collaterals of axons innervating the lateral and medial leg and dorsum of the foot had "flame-shaped arbors" while afferents with receptive fields on the glabrous-hairy skin border consistently had extra terminal branches running mediolaterally into laminae IV or V.

Maxwell et al. (1982a, 1984a) and Bannatyne et al. (1984) studied the ultrastructure of different types of Aβ afferent fibres. Their results indicated that there was considerable overlap in the ultrastructural characteristics of all types of boutons belonging to large myelinated cutaneous afferents. Synaptic boutons of hair follicle afferent collaterals were usually of the *en passant* type and made asymmetrical contacts with dendrites (Maxwell et al., 1982a). Boutons from Pacinian corpuscle afferents and rapidly adapting afferents had a similar ultrastructural appearance and formed between 1 and 3 synaptic junctions with dendritic shafts and spines (Maxwell et al., 1984a). Terminals from slowly adapting type I and II both formed contacts with 1 to 5 post-synaptic profiles including dendritic shafts and spine heads (Bannatyne et al., 1984). All types of myelinated Aβ afferents received axo-axonic synapses which are thought to be

the anatomical substrate for presynaptic inhibition (Gray, 1962). A few of the boutons from Pacinian corpuscle afferents and rapidly adapting afferents were observed to form structures resembling the central elements of glomeruli but this finding was not observed with the other types studied.

2.4. Primary Afferents Studied by the Use of Transganglionic Tracers

The central terminations of primary afferents have also been studied by means of axonal transport methods with a variety of tracer substances such as the enzyme HRP, lectins (e.g. wheat germ agglutinin, WGA; BSI-B4) or the B subunit of the cholera toxin (CTb, choleragenoid). These compounds have been applied to peripheral nerves or in some cases injected into peripheral tissues after which they are transported through the dorsal root ganglia to central terminals of primary afferents, a process termed, transganglionic labelling (Swett and Woolf, 1985; Molander and Grant, 1985, 1986; Woolf and Fitzgerald, 1986; LaMotte et al., 1991; Kobayashi and Matsumura, 1996). This 'bulk-labelling' method provides an alternative approach to the intraaxonal injection of markers into physiologically characterized afferents. It has been shown that different classes of primary afferents are selective in terms of the tracers which they transport, and this has been attributed to the expression of distinct cell surface carbohydrate residues to which the tracer can bind (Dodd and Jessell, 1985; LaMotte et al., 1991; Robertson et al., 1991). It has been reported that the precise pattern of labelling may vary somewhat depending on the survival time and mode of application (LaMotte et al., 1991).

2.4.1. Unconjugated HRP

Since being introduced nearly 25 years ago as a method of studying neuronal connectivity, HRP histochemistry has become one of the most widely used techniques

for such studies. However it is now used much less in central nervous system because more sensitive methods are available. It has been found that ligand-HRP conjugates (CTb-HRP and WGA-HRP) are far superior to the unconjugated HRP, as orthogradely and retrogradely transported neuroanatomical markers (Wan et al., 1982). These differences are thought to be due to differences in effectiveness of different uptake mechanisms, unconjugated HRP being taken up by fluid phase endocytosis (Silverstein et al., 1977) while the conjugated forms are internalized by adsorptive endocytosis (Gonatas et al., 1979; Robertson and Grant, 1985). There is also evidence of delayed clearance of both conjugates from the injection site compared to free HRP (Wan et al., 1982).

When unconjugated HRP is applied to peripheral nerves it is transported to laminae I, III and IV, the sites of termination of Aβ and Aδ afferents (Molander and Grant, 1986; Woolf and Fitzgerald, 1986; LaMotte et al., 1991).

2.4.2. WGA

This lectin has been used extensively as a neuroanatomical tracer both in the central nervous system and peripheral nerves. It has been used as a conjugate with HRP which can be detected by enzyme histochemistry or unconjugated, in which case it is detected with immunocytochemistry. Transganglionically transported WGA-HRP almost exclusively labels afferents projecting to laminae I and II of the dorsal horn, with little label elsewhere and is therefore likely to be transported mainly by C-afferent fibres (Molander and Grant, 1985; Robertson and Arvidsson, 1985; Swett and Woolf, 1985; LaMotte et al., 1991). LaMotte et al. (1991) injected WGA-HRP into a peripheral nerve and found selective labelling of small ganglion cells. They also reported that after sciatic nerve injection, counts of WGA-HRP-labelled axons made from montages of an L4 dorsal rootlet showed that 16% of labelled afferents were myelinated and 84% were

unmyelinated. A binding study of WGA carried out by Robertson (1990) suggested a non-uniform affinity of WGA for different subpopulations of DRG neurons. Robertson (1990) reported that WGA appears to bind to both small and large types of DRG cells and therefore cannot be used as a specific marker for any of these subpopulations. Transneuronal transport (reaction product observed in neuronal cell bodies in the dorsal horn) has been reported both for WGA-HRP (Gerfen et al., 1982; Trojanowski and Schmidt, 1984; Robertson and Arvidsson, 1985) and also for WGA (Ruda and Coulter, 1982). Molander and Grant (1985) reported the occurrence of transneuronal transport of WGA-HRP in some cases after 3 days survival and regularly in cases after 4 and 5 days survival time.

2.4.3. CTb

Cholera toxin consists of two subunits: the A subunit which is responsible for the activation of the adenylate cyclase and the B subunit which is not toxic but is responsible for the binding of the toxic A subunit. Recently the B subunit has been widely used as a neuronal tracer (Wan et al., 1982; Robertson and Arvidsson, 1985; Robertson and Grant, 1985; Rivero-Melian and Grant, 1990; 1991; LaMotte et al., 1991). When CTb-HRP is applied to a peripheral nerve, labelled afferents are present throughout the dorsal horn except for lamina II where there is labelling only in the ventral part. No difference in distribution of labelling could be detected after changing the dosage of the tracer however it was reported that the density was lower when a smaller volume was injected (Robertson and Grant, 1985). LaMotte et al. (1991) reported that injection of CTb-HRP into a peripheral nerve resulted in selective labelling of medium sized and large ganglion cells, with labelling of only a very few small neurons. They found that after injection into the sciatic nerve, the great majority of labelled afferents in an L4 dorsal rootlet were myelinated. Similarly, a binding study

carried out by Robertson et al. (1991) revealed that of all the neurons that were immunoreactive to RT97 (a marker of myelinated afferents) 82% showed CTb-LI, and of those that showed CTb-LI, 94% were immunoreactive to RT97. LaMotte et al. (1991) also reported that both WGA-HRP and CTb-HRP can label small myelinated fibres since both labels can stain profiles in lamina I which is one of the targets of Aδ afferents. Their ultrastrucural studies have addressed the identity of these profiles in lamina I, since other studies had suggested that these were axons of passage directed toward the deep laminae (Woolf and Fitzgerald, 1986; Molander and Grant, 1986). LaMotte et al. (1991) found that the majority of these profiles (52%) were terminals and only (18%) were non-varicose axons. No transneuronal labelling was seen with CTb (Robertson and Grant, 1985).

2.4.4. BSI-B4

Several lectins including the I-B4 isolectin from *Bandeiraea simplicifolia* and soybean agglutinin which recognize α-D-galactose carbohydrate residues have been found to bind to a subset of small dorsal root and trigeminal ganglion cells, and to presumed unmyelinated primary afferent fibres and terminals in the dorsal horn laminae I and II (Tajti et al., 1988; Plenderleith et al., 1989; Silverman and Kruger, 1990; Ambalavanar and Morris, 1992, 1993; Kitchener et al., 1993; Wang et al., 1994). It has been reported that BSI-B4 either free or conjugated to HRP can be used as a transganglionic tracer (Kitchener et al., 1993; Wang et al., 1994) . BSI-B4 has been also used on sections of either spinal cord or medulla to examine labelled afferents, however the advantage of its transganglionic transport over the binding histochemistry is the ability to demonstrate the terminal fields of a known subpopulation of afferents within chosen peripheral nerves (Kitchener et al., 1993). After injection of BSI-B4 or BSI-B4-HRP conjugate into a rat peripheral nerve, label is found only in presumed unmyelinated

afferents in lamina II with the most intense labelling in lamina IIi and with no transneuronal labelling (Kitchener et al., 1993; Wang et al., 1994). It has also been used to demonstrate the central projections from the rat trigeminal nerve after injection into the facial dermis and oral mucosa (Kobayashi and Matsumura, 1996). Although Kitchener et al. (1993) observed no labelling outside lamina II, Wang et al. (1994) detected additional labelling in laminae I, III as well as lamina X and considered that this finding was due to the use of larger amount of the injected tracer, since they found that the higher the concentration they used the more intense and extensive was the labelling. Kitchener et al. (1993) emphasized that BSI-B4 is selective for cutaneous afferents, as they had reported that BSI-B4 binding within the DRG was almost completely restricted to neurons which projected to cutaneous targets (Plenderleith and Snow, 1993). Wang et al. (1994) concluded that the labelling in lamina X could be derived from visceral afferents, which form a longitudinal bundle ventral to the central canal. Wang et al. (1994) found that almost all DRG cells that transported BSI-B4-HRP also bound BSI-B4. Double-labelling studies carried out in trigeminal ganglion neurons by Ambalavanar and Morris (1992) revealed that BSI-B4 had the ability to bind to all somatostatin-containing neurons whereas it bound to only 23% of those that had CGRP-immunoreactivity and 15% of neurons that showed substance P-immunoreactivity. Similar results have also been presented by Wang et al. (1994) who found that all somatostatin-immunoreactive cells and almost all (95%) of the FRAP-positive cells were contained within the BSI-B4 positive population.

The transganglionic transport technique has led to the possibility of mapping the central terminal projections of individual peripheral nerves. Studies of this type demonstrated that there is a highly detailed somatotopic relationship between afferent fibres innervating each portion of the skin surface and the location and size of their central terminals in the superficial dorsal horn (Molander and Grant, 1985,1986; Woolf

and Fitzgerald, 1986; LaMotte et al., 1991). Other techniques such as the anterograde transport of either WGA-HRP or CTb-HRP after injection into the DRGs has led to the conclusion that visceral sensory ganglion cells and the large light subpopulation of somatic DRG cells, both bind CTb whereas the small dark somatic cells show affinity for WGA but rarely for CTb, which supports the observations of Robertson and Grant (1985) and Robertson et al. (1992) concerning selectivity of transport.

3. Neurons in Laminae III and IV of the Dorsal Horn of the Spinal Cord

In Nissl-stained sections of the cat spinal cord, Rexed (1952) described the cells of lamina III as spindle shaped and having relatively little cytoplasm, whereas lamina IV cells were of heterogenous size, ranging from small to large, and although the largest cells were relatively infrequent they were so prominent that he regarded it the layer with large cells. In the rat, Molander and Grant (1995) stated that lamina IV cells were more loosely arranged than those in lamina III and some of them were multipolar and considerably larger. There have been many studies describing the morphology of cells in laminae III and IV with either the Golgi technique or intracellular injection of HRP. It is to be noted that in some of the early literature, neurons in lamina III were grouped together with those in II because both laminae contain neurons with similar shapes. This region was referred to as substantia gelatinosa (Szentagothai, 1964) or "gelatinosal complex" (Scheibel and Scheibel, 1968; Beal and Cooper, 1978). However, Maxwell et al. (1983) stated that it is appropriate to consider lamina III as a morphologically and functionally separate entity from lamina II which receives different afferent input and contains neurons whose dendrites are mainly confined to the lamina, which is not the case for lamina III cells. Additional evidence was also reported by the same authors who considered the heterogeneous morphology of terminals associated with lamina III neurons a major difference between them and neurons of lamina II.

Studies have examined both unidentified neurons (in which neither the physiological receptive field properties nor the axonal termination were known) as well as neurons identified as projection cells belonging to spinocervical tract (Brown et al., 1980a,b), postsynaptic dorsal column pathway (PSDC; Brown and Fyffe, 1981b), spinothalamic tract (Kevetter and Willis, 1982, 1983), spinoreticular tract (Lima, 1990; Lima and Coimbra, 1990; Lima et al., 1991) and intracellularly stained neurons whose physiological properties have been characterized. It has been also reported that the majority of the spinal cord neurons are local interneurons (Willis and Coggeshall, 1991).

3.1. Unidentified neurons

3.1.1. Morphological Features of Unidentified Neurons

The main feature of the dendritic trees of lamina III cells seen in Golgi studies of the cat spinal cord was that they were flattened mediolaterally and extended rostrocaudally (Szentagothai, 1964; Scheibel and Scheibel, 1968). Mannen and Sugiura (1976) reconstructed 3 cells in serial sections of Golgi material and found that these cells were slightly flattened in the mediolateral axis and that their dendritic fields were more complicated than what previously reported and spanned more laminae (laminae I-V).

Szentagothai (1964) was the first to draw the attention to the large lamina IV neurons of the cat which have characteristically dorsally directed dendrites that reach up to the superficial laminae. He stated that although the relation of these cells to the substantia gelatinosa had been clearly described and illustrated by Ramon Y Cajal (1909), its significance had not been realized before. He suggested that these neurons have ample opportunity to establish synapses with several different types of primary afferent including those which terminate in lamina II (which he regarded as a closed system with no direct forward conduction). Brown (1981) described similar

intracellularly labelled pyramidal neurons in lamina III of the cat whose dendrites extend dorsally through laminae I-II and ventrally through laminae IV-V. Since their dendrites span most laminae of the dorsal horn, Brown (1981) also suggested that these cells could receive direct synaptic input from several types of primary afferent. In the rat, similar cells have been also observed by Todd (1989). Todd (1989) was able to confirm the suggestion proposed earlier by Szentagothai that these cells might form a target for primary afferents in lamina II since he found that Golgi-stained cells of this type received synapses from degenerated axons in lamina II after dorsal rhizotomy.

Rethelyi and Szentagothai (1973) subdivided cells in laminae III, IV and the dorsal part of V according to dendritic orientation into three types: antenna-type neurons which are smaller in lamina III and larger in lamina IV with dendrites oriented mainly towards lamina II (as described earlier by Szentagothai, 1964), central cells with longitudinally oriented dendrites and a third group mainly in lamina V but also in lamina IV with transversely oriented dendrites. However, they mentioned that this subdivision was not clearcut because a considerable number of cells in laminae IV and V showed different shapes of dendritic orientations in combination so that the classification could be only applied unambiguously to a limited number of neurons in these laminae. Proshansky and Egger (1977) described lamina IV neurons in kittens and adult cats as a heterogenous population of cells displaying a variety of sizes and dendritic forms. Dendrites of these neurons were highly branched with a bushy appearance. They were frequently travelling in the dorsal direction penetrating into laminae III and II however the ventral spread of dendritic trees was less extensive. Beal and Cooper (1978), in their description of the dendritic arbor of the cells in monkey gelatinosal complex (which they considered to be lamina II and III) mentioned that dendrites of lamina III cells usually extended dorsally. Some neurons also gave rise to dendrites which spread longitudinally with little mediolateral spread. In a study of the dendritic organization of

the human spinal cord, Schoenen (1982) found that lamina III contained a mixed population of antenna-like neurons with a vertical cone shaped dendritic domain and radial cells characterized by a small spherical dendritic territory. He also reported that all lamina IV neurons were medium or large sized antenna-like neurons with dorsally oriented, conical dendritic domains. Similar results have been reported by Bowsher and Abdel-Maguid (1984) who found neurons that resembled the radial cells of Schoenen, and another type with dorsal dendrites that resembled the antenna-like cells described by Schoenen (1982).

Maxwell et al. (1983) studied the morphological properties of physiologically characterized lamina III neurons in the cat spinal cord. They found that smaller lamina III neurons were heterogeneous in their morphological configurations but could be divided into two broad classes: those with rostrocaudally orientated dendritic arbors that are limited to lamina III and those with dendritic arbors orientated in the dorsoventral plane that traverse several laminae including lamina II. They reported that neurons of the first class are capable of receiving a monosynaptic input from larger afferent fibres only (Aβ and Aδ) whereas those of the second class could receive monosynaptic inputs from both large and small primary afferent fibres (Aβ, Aδ and C-fibres). The examined neurons showed also variance in their response properties, while one neuron responded to light brushing of its receptive field, four cells were excited by strong pressure. There was no correlation between the physiological characteristics of these neurons and their morphology. Maxwell et al. (1983) and Maxwell (1985) examined the synaptic arrangements associated with intracellularly labelled lamina III neurons. Electron microscopic analysis of the neurons revealed that they were associated with many different types of boutons thus suggesting that they receive synaptic input from various sources and again there was no correlation between the ultrastructure of the neurons and their morphological or physiological properties.

3.1.2. Physiological Properties of Unidentified Neurons

Many electrophysiological experiments have been carried out in order to try and classify dorsal horn neurons in terms of their responses to peripheral stimulation (for example, Eccles et al., 1960; Mendell, 1966; Wall, 1967; Price and Browe, 1973; Price and Mayer, 1974; Handwerker et al., 1975; Radhakrishnan and Henry, 1993, 1995).

Several ways of classifying response properties of dorsal horn neurons have been suggested. Mendell (1966) described cells in lamina IV that only responded to innocuous mechanical stimuli (narrow-dynamic-range cells or low threshold cells) as well as cells that responded to both innocuous and noxious stimuli, which he called wide-dynamic-range cells. This classification has been confirmed and extended by Radhakrishnan and Henry (1993, 1995) who classified the dorsal horn neurons into non-nociceptive, nociceptive specific and wide-dynamic-range cells. Five classes of cells have been described by other investigators: touch units, touch-pressure units, touch-pressure-pinch units, pressure-pinch units and pinch units (Price and Browe, 1973; Price and Mayer, 1974). Handwerker et al. (1975) suggested the presence of 2 classes, class 1 are cells excited only by large myelinated afferent fibres while class 2 are cells excited by large myelinated and unmyelinated fibres.

One of the most useful schemes was that of Wall (1967) who used the laminar terminology of Rexed (1952; 1954) and noted that the receptive fields of dorsal horn neurons changed in a predictable way from lamina to lamina. However Wall (1967) was careful to point out that the laminae described by him should be regarded as zones of concentration of particular cell types rather than separate laminae of distinct specialization. In the study by Wall (1967), responses of neurons in the deep dorsal horn to natural stimuli have been described. Neurons recorded in lamina IV could often be excited by hair movement, touch, and stimulation of tactile domes, however some cells could also be excited by pressure and pinch, thus these cells were either low-

threshold or wide-dynamic-range cells. Wall's findings have been confirmed and extended by other investigators (Gregor and Zimmermann, 1972; Menetrey et al., 1977) who found that some neurons in laminae III and IV only responded to weak cutaneous mechanical stimuli, while others in lamina IV either responded only to intense stimuli or were wide-dynamic-range cells . Although Wall (1967) did not find input from deep receptors onto neurons recorded in lamina IV, other investigators reported a proprioceptive input (Fukushima and Kato, 1975) as well as a visceral input (Alarcon and Cervero, 1990) to cells of this type.

Mendell and Wall (1965) and Mendell (1966) estimated the percentage of neurons in lamina IV of the dorsal horn which respond to activation of both A and C-fibres as 60% whereas the other 40% respond only to stimulation of A fibres. It was reported that repetitive stimulation of C-fibres resulted in increasing response of many dorsal horn neurons, a phenomenon referred to "wind-up" (Mendell and Wall, 1965; Mendell, 1966; Fitzgerald and Wall, 1980; Woolf and King, 1987). Other neurons show a decreasing response to repeated C-fibre stimulation, which was termed "wind-down" or habituation (Fitzgerald and Wall, 1980; Woolf and King, 1987; Alarcon and Cervero, 1990). These latter cells are probably similar to a population recorded in laminae V and VI which has been reported earlier and called "novelty detection" cells by Wall (1967).

Early studies (Wall, 1960; Hillman and Wall, 1969) showed that dorsal horn neurons have beside their excitatory receptive fields, inhibitory receptive fields. Two types of inhibition can affect dorsal horn neurons, local inhibition and descending inhibition. The local inhibition depends upon a segmental mechanism involving activation of inhibitory interneurons by primary afferents which is presumably responsible for the inhibitory receptive fields of these dorsal horn neurons. Many dorsal horn neurons are also under control of descending inhibition from various regions of the brain including the periaqueductal gray, nucleus raphe magnus, locus coeruleus,

hypothalamus and others (reviewed in Willis and Coggeshall, 1991).

3.2. Projection Neurons

3.2.1. Spinocervical Tract Neurons

The spinocervical tract is a somatosensory pathway which carries information from the trunk, limbs and tail and projects through the lateral cervical nucleus (LCN) and via the medial lemniscus to the thalamus (Brown, 1981). The lateral cervical nucleus consists of a group of neurons within the white matter just ventrolateral to the dorsal horn in the uppermost cervical region (C1-C3). Although the LCN has been described in different species, its relative size varies and its presence in human is controversial (Willis and Coggeshall, 1991). Nearly all of the spinocervical tract neurons are excited by movement of hair (Brown, 1981).

The distribution of the cells of origin of the spinocervical tract in different species such as rat, cat and dog has been mapped following retrograde labelling with HRP injected into the LCN (Craig, 1976, 1978; Baker and Giesler, 1984) and also with electrophysiological methods (both intracellular and extracellular recording; Brown et al., 1980b) from antidromically activated neurons. Brown et al. (1980b) combined the retrograde transport with intracellular and extracellular recording and found that the intracellular recording technique was biased towards the larger neurons and those located in the more superficial laminae of the dorsal horn. The electrophysiological criteria for identification of spinocervical tract neurons included antidromic firing (demonstrated by collision between an antidromic and an orthodromic impulse) from below the LCN with a stimulating electrode at the level of C3 segment associated with absence of antidromic excitation at the level of C1 or higher (Brown et al., 1980b). To ensure identification of spinocervical tract neurons, transection of the dorsal column caudal to the C3 stimulating electrode has been carried out. Craig (1978) mapped cells

of origin of spinocervical tract in spinal cord of cats and dogs using the retrograde HRP technique. Labelled cells were found at all levels of the ipsilateral side of the cord predominantly within lamina IV with some in medial lamina V and few in cervical laminae I, VI and VII. There were also a few labelled cells on the contralateral side. Brown et al. (1980b) reported that most of the labelled cells in the cat were in laminae III-V with some in lamina I in the ipsilateral dorsal horn, however there were a few in lamina I, III-V, and VII-VIII contralaterally. In the rat, Baker and Giesler (1984) were able to localize spinocervical tract neurons by making small injections of HRP which were restricted to the LCN. Labelled cells were found in ipsilateral laminae III and IV at all levels of the spinal cord however few cells have also been found in lamina II.

Morphology of individual spinocervical tract cells has been studied using either intracellular injections or retrograde labelling (Brown et al., 1980b, c; Sedivec et al., 1986). Although the neurons do not constitute a homogeneous morphological population, they characteristically have well developed dorsal dendrites. Some of these neurons have their dendritic trees limited almost exclusively to dendrites that ascend in the dorsal direction. The dorsal dendrites rarely extend dorsally into lamina II, but instead run rostrocaudally along the border between laminae II and III, and therefore they are unlikely to receive a major monosynaptic input from unmyelinated axons. Consequently their responses to noxious stimuli are thought to be mediated via interneurons (Brown, 1981). According to the description of Brown et al. (1980b) many spinocervical tract neurons have dendrites ramifying at the level of the cell body and also travelling ventrally in addition to their dorsal dendrites. They suggested that the deep dendrites in laminae IV or V or even deeper may receive input from sources other than hair follicle afferent fibres, such as excitatory or inhibitory interneurons. Brown et al. (1980c) investigated the relationship between dendritic trees and receptive fields of adjacent neurons. They showed that when the dendritic trees of adjacent spinocervical

tract neurons interdigitated, their receptive field overlaped completely or in part. Conversely, when the receptive fields of spinocervical tract neurons did not overlap, the dendritic trees of the cells occupied separate volumes of the dorsal horn.

Maxwell et al. (1982b, 1984b) examined the ultrastructure of intracellularly injected spinocervical tract neurons. The cells were found to have dendritic spines in confirmation of the reported light microscopic observation. Maxwell et al. (1982b, 1984b) identified two types of asymmetrical synapse on these neurons. The most common type contained round vesicles and a few dense core vesicles and were thought to originate form hair follicle afferent fibres since they degenerated after dorsal rhizotomy while the other type contained flattened vesicles and were thought to be derived from inhibitory interneurons because they survived dorsal rhizotomy. Occasional boutons with highly flattened vesicles were also seen. Synapses from boutons with flattened vesicles were more common on proximal than on distal dendrites. No axoaxonic synapses or glomeruli were found in relation to the spinocervical tract cells.

Although the spinocervical tract is concerned primarily with hair movement, neurons excited only by noxious stimuli were found however they were the minority (less than 5%; Bryan et al., 1973; Brown,1971). Bryan et al. (1973, 1974) examined the receptive fields of spinocervical tract cells in cats and monkeys. The cells were identified by antidromic activation from the dorsolateral funiculus. Most of them responded to hair movement or to tactile stimulation of the skin while some also responded to pressure and a few responded only to pressure or pinch. These authors found that the receptive fields of spinocervical tract cells in the monkey were often located on glabrous skin (Bryan et al., 1974) although comparable receptive fields were not found on the glabrous skin of cats (Brown and Franz, 1969; Bryan et al., 1973). However Kunze et al. (1987) were able to demonstrate that some spinocervical tract

cells located in the medial part of the dorsal horn in cats have receptive fields on the glabrous skin of the footpads. Results similar to those of Bryan et al. (1974) have been reported by Downie et al. (1988) using the same approach in the spinal cord of monkey where the cells were classified as low-threshold, wide-dynamic-range, and high-threshold.

Further studies have been carried out to reveal receptive field organization of the spinocervical tract neurons. Brown and Noble (1982) demonstrated the presence of contacts between intracellularly labelled hair follicle afferent fibres and spinocervical tract cells when both units share the same receptive field. A somatotopic relationship between the locations of the spinocervical tract cells in the dorsal horn of cats and monkeys and the position of the receptive fields on the limb have also been described (Bryan et al., 1973, 1974; Brown, 1981).

3.2.2. Postsynaptic Dorsal Column Neurons

The postsynaptic dorsal column pathway is a projection from second order neurons in the dorsal horn to the dorsal column (gracile and cuneate) nuclei. Its cells of origin have been labelled retrogradely in different species by injection of tracer into the dorsal column nuclei (Rustioni and Kaufman, 1977; Rustioni et al., 1979; Giesler et al., 1984) or by injection of HRP into electrophysiologically identified PSDC neurons (Brown and Fyffe, 1981b; Bennett et al., 1984). PSDC neurons were identified by antidromic excitation with a stimulating electrode at C2 below a dorsal column lesion and above a dorsolateral funiculus lesion. Antidromic excitation was confirmed by collision between orthodromic and antidromic impulses, in addition to frequency following and fixed latency of response (Brown and Fyffe, 1981b). In the rat, neurons retrogradely labelled from dorsal column nuclei were located in laminae III and IV and the projection was somatotopic since the nucleus gracilis received an input from cells in the

lumbosacral enlargement while the nucleus cuneatus received an input from cells in the cervical enlargement (Giesler et al., 1984). In cats, although all investigators agree that PSDC neurons are concentrated in lamina IV, different reports included other laminae (III, V, VI, VII) as well (Rustioni and Kaufman, 1977; Brown and Fyffe, 1981b; Bennett et al., 1984). Cells of origin in the monkey were found in laminae IV-VI (Rustioni et al., 1979). The number of cells of both PSDC and spinocervical tract tracts in different laminae were about the same, the main difference being the aggregation of somata in medial lamina V in the former (Brown, 1981). It was also assumed that both tracts have the same density of neurons.

Brown and Fyffe (1981b) described three morphological types of PSDC neurons in the cat in which the form of dendritic trees varied with the position of the soma in the dorsal horn. The first type had somata in lamina III or III-IV border and dendritic trees that were developed mainly in the dorsal direction but restricted in the mediolateral and rostrocaudal directions. All of these cells had dendrites that reached into lamina II, often extending throughout its thickness and into lamina I. The second type consisted of neurons in the medial parts of lamina V and deep IV which had dendritic trees that were very extensive in the transverse plane but restricted in the parasagittal plane and with limited rostrocaudal spread. The third type had cell bodies in lamina IV and dendrites that were mainly directed dorsally with less transverse or longitudinal spread. These neurons had ten or more primary dendrites and the dorsal ones reached into lamina II and could extend throughout its thickness. Differences in the morphological characteristics between PSDC and spinocervical tract neurons were observed (Brown, 1981). While dendrites of spinocervical tract neurons showed well developed spread in the rostrocaudal direction, those of PSDC neurons had limitied rostrocaudal spread. Only a minority of spinocervical tract neurons had dendrites that extended into lamina II and only its inner portion, however PSDC neurons commonly had dendrites travelling

dorsally into the superficial dorsal horn. A different morphological description has been reported by Bennett et al. (1984) although they used the same species and even used Brown and Fyffe's method of identification for some cells in their sample. All PSDC neurons they identified had dendritic arbors that were elongated rostrocaudally but narrow mediolaterally. Only one neuron of their sample had a significant amount of dendritic spread into laminae I and II. Surprisingly they emphasized that all of the neurons identified with Brown and Fyffe's method resembled the other neurons in the sample with respect to the shapes of their dendritic arbors, location of somata, axonal conduction velocities and responses to natural and electical stimulation. They suggested two possible explanations for the difference between their results and those of Brown and Fyffe (1981b). The first was that they might have sampled a different subset of the PSDC population and the second was that differences may have been due to incomplete reconstruction by Brown and Fyffe who used transverse sections. An additional discrepancy between the two studies concerned the extension of dendrites into lamina II. Brown and Fyffe (1981b) found that the dorsal dendrites of PSDC neurons usually penetrated into lamina II and often throughout its complete thickness, whereas Bennett et al. (1984) emphasized that most PSDC neurons either had no dendrites in lamina II or had only the distalmost tips of a few of their terminal dendrites penetrating the ventral part of lamina II. Bennett et al. (1984) reported that the spinocervical tract and PSDC neurons were very similar in dendritic organization and suggested that many neurons might belong to both tracts however the work of the Brown group indicated that the spinocervical tract and PSDC cells are quite distictive (Brown and Fyffe, 1981b; Brown, 1981; Maxwell et al., 1982b).

Bannatyne et al. (1987) examined the ultrastructure of synaptic input to PSDC neurons. The synaptic arrangements included contacts by small boutons which contained either spherical or elongated vesicles. Unlike the spinocervical tract, some

central elements of glomeruli and axoaxonal complexes were also seen.

The response properties of PSDC neurons to natural stimuli were found to be different in rats and in cats (Angaut-Petit, 1975a, b; Brown and Fyffe, 1981b; Giesler and Cliffer, 1985; Kamogawa and Bennett, 1986). Giesler and Cliffer (1985) examined the physiological properties of PSDC neurons in rats. They found that 64% of the cells responded exclusively to innocuous stimuli while the remainder responded to strong mechanical stimuli in addition. Ninety three percent of the examined cells showed no response to repeated noxious heating of the skin. It was concluded that PSDC pathway is propably not important for nociception in rat. Angaut-Petit (1975a, b) found that 16% of PSDC neurons identified in the cat were activated by innocuous stimuli only, whereas 77% responded to both innocuous and noxious stimuli. A few of the neurons (6%) were exicted only by noxious mechanical (but not thermal stimuli). Similar results were also presented by Brown and Fyffe (1981b). Kamogawa and Bennett (1986) tested the effects of repeated noxious heat stimulation on the responses of PSDC neurons in cats. Unlike cells of this pathway in rats (Giesler and Cliffer 1985), sensitization of the skin in cats led to a response to noxious heat stimuli which led Kamogawa and Bennett (1986) to coclude that many of these neurons in the cat receive an input from Aδ nociceptors. Thus the PSDC pathway in cat may differ from that in rat and is more likely to contribute to nociception in the cat (Willis and Coggeshall, 1991). The receptive field properties of PSDC neurons in the cat are different from those of spinocervical tract cells since spinocervical tract cells are not activated either by slowly adapting type I receptors or Pacinian corpuscles (which excite PSDC cells) and also seldom have receptive fields on glabrous skin (Brown, 1981).

To determine whether any of the PSDC neurons of the rat which have cell bodies in laminae III-IV and dorsally-directed dendrites belong to the population expressing NK-1 receptor immunoreactivity, Polgar, E., Shehab, S.A.S., Watt, C. and Todd, A.J.

(unpublished observations) combined retrograde labelling from the gracile nucleus with immunocytochemistry and found that PSDC cells were not NK-1 receptor-immunoreactive.

3.2.3. Spinothalamic Tract Neurons

The spinothalamic tract is thought to be one of the most important pathways responsible for mediating pain sensation in humans (White and Sweet, 1969). It has been found in all orders of mammals that have been examined, with some variation between different species such that it is moderate sized in cats and of great size and complexity in primates. It arises largely from neurons in the dorsal horn of the spinal cord although some cells are located in the intermediate region and ventral horn (Willis and Coggeshall, 1991). The location of cells of origin of spinothalamic tract has been mapped in various species including rat, cat and monkey by using retrograde tracing methods. In rats, investigators used different tracers such as HRP (Giesler et al., 1979; Kevetter and Willis, 1982, 1983; Granum, 1986; Kemplay and Webster, 1986), WGA-HRP (Lima and Coimbra, 1988), fluorescent dyes (Kevetter and Willis, 1982, 1983; Burstein et al., 1990) or CTb (Lima and Coimbra, 1988; Marshall et al., 1996) however the most effective labelling has been obtained with Fluorogold (Burstein et al., 1990) or CTb (Lima and Coimbra, 1988; Marshall et al., 1996). In addition to the retrograde labelling technique, the distribution of spinothalamic tract cells has also been demonstrated by using antidromic activation of their axons in the thalamus (Dilly et al., 1968; Giesler et al., 1976). In general, the locations of antidromically activated spinothalamic tract cells agreed well with maps produced by using retrograde tracers although the antidromic technique undoubtedly underestimated the number of small neurons (Willis and Coggeshall, 1991). Retrograde labelling studies in the rat have shown that the largest concentration of labelled cells is in the upper cervical segments

(C1 and C2). In segments below the upper cervical cord, spinothalamic tract cells were found chiefly in the cervical and lumbosacral enlargements, with the greatest concentration in lamina I, deep dorsal horn (laminae IV and V) and medial intermediate grey (internal basilar column) with scattered cells in lamina III, around the central canal and in the ventral horn. Most of the labelled cells at these levels were contralateral to the thalamic injection site (Giesler et al., 1979; Kevetter and Willis, 1982, 1983; Lima and Coimbra, 1988; Burstein et al., 1990; Marshall et al., 1996). In the upper cervical segments of the cat, retrogradely labelled neurons were found in laminae V-VII contralateral and in laminae VII and VIII ipsilateral to the thalamic injection (Carstens and Trevino, 1978b). Below the upper cervical level, labelled neurons were concentrated in laminae I, IV-VI and in laminae VII and VIII. Unlike the arrangement reported in the rat, the spinothalamic tract neurons in deeper laminae of the dorsal horn of the cat were not concentrated medially but were distributed across the width of the dorsal horn (Carstens and Trevino, 1978a; Craig et al., 1989). Following injection of WGA-HRP into the thalamus of monkey, labelled neurons in the upper cervical regions were found in laminae IV-VIII contralateral and lamina VIII ipsilateral to the injection. Below the upper cervical segments, labelled cells were concentrated in lamina I and lateral part of lamina V with some labelled neurons in laminae VII-VIII and a few in laminae II and III (Apkarian and Hodge, 1989).

Projection targets of the spinothalamic tract cells in the rat include three main regions, the ventrobasal complex, the intralaminar nuclei (primarily the central lateral nucleus) and the posterior complex (Lund and Webster, 1967). Albe-Fessard et al. (1985) suggested that nociceptive neurons in the ventrobasal complex are responsible for sensory and discriminative aspects of painful stimulus whereas neurons in the more medial intralaminar nuclei are responsible for the motivational and affective aspects of pain.

The morphology of individual spinothalamic tract cells has been described after the use of either retrograde labelling studies or intracellular injection technique, however the retrograde labelling was unable to show the whole dendritic tree of the cells (Willis and Coggeshall, 1991). In the rat, Lima and Coimbra (1988), studied the morpholgoy of retrogradely labelled neurons following injection of CTb into the thalamus and found that CTb gave Golgi-like staining and good dendritic labelling. They reported that labelled cells in lamina I were either pyramial or flattened. In the cat, Zhang et al. (1996) identified three distinct retrogradely labelled spinothalamic tract cell types in lamina I: fusiform, pyramidal and multipolar after the use of either CTb or Fast Blue. Following intracellular injection of HRP, Surmeier et al. (1988) reconstructed spinothalamic tract neurons in laminae IV-VI of the monkey spinal cord. They found that labelled neurons had long branched dendrites that could extend dorsally as far as lamina I, laterally into the lateral funiculus and ventrally into the ventral horn. Some cells showed extension of their dendrites dorsally into the superficial dorsal horn while others had dendrites that arborized mainly ventrally.

Ultrastructural analysis of functionally identified primate spinothalamic tract neurons in lamina V revealed that the synaptic terminals associated with these neurons were variable in their content of vesicles (round clear, flattened clear and small or large dense core vesicles; Carlton et al., 1989). With electron microscopy, several substances have been located in synaptic endings apposed to the spinothalamic tract neurons, including enkephalin (Ruda et al., 1984), substance P (Carlton et al., 1985), serotonin (LaMotte et al., 1988) CGRP (Carlton et al., 1990), dopamine-β-hydroxylase (Westlund et al., 1990), GABA and glutamate (Westlund et al., 1992). Immunocytochemical studies revealed the presence of neuropeptides in some spinothalamic tract neurons in rat. Most of these peptidergic neurons were found in lamina X (Leah et al., 1988). Neurons in laminae VI, VII and X were found to contain enkephalin or dynorphin

(Coffield and Miletic, 1987; Nahin, 1988) and those in lamina X showed immunoreactivity to cholecystokinin, bombesin or galanin (Leah et al., 1988). In the lateral spinal nucleus, some spinothalamic tract cells have been observed to contain VIP (Nahin, 1988) or bombesin (Leah et al., 1988).

A recent study was carried out by Marshall et al. (1996) to determine whether spinothalamic tract neurons in the lumbar spinal cord of the rat possess the NK-1 receptor immunoreactivity. Dual-labelling immunocytochemistry revealed the retrogradely labelled cells after CTb injection into the thalamus as well as the NK-1 receptor immunoreactivity. On the side contralateral to the injection, 77% of spinothalamic tract neurons in lamina I showed receptor immunoreactivity while 33% of cells in laminae III-IV and 14% of the ventromedial group were immunoreactive to the receptor. The authors found that several of the double labelled neurons had cell bodies in lamina III or IV and dendrites extending to the superficial dorsal horn however these are only likely to account for a small minority of neurons of this morphological type.

There have been relatively few reports of the physiological properties of spinothalamic tract neurons in the rat spinal cord (Willis and Coggeshall, 1991). However Palecek et al. (1992) studied the activity of spinothalamic tract neurons in the rat dorsal horn and found that most neurons in the superficial laminae responded most vigorously to noxious cutaneous stimuli while some in the deep laminae were mechanoreceptive. Spinothalamic tract cells in the intermediate region and ventral horn respond best to activation of receptors in the subcutaneous tissue (Willis et al., 1995). Studies of the response properties of primate spinothalamic tract neurons showed that the cells usually respond to noxious mechanical stimulation (high-threshold) and often to innocuous stimulation as well (wide-dynamic-range), although some low-threshold neurons have also been found (Chung et al., 1979; Surmeier et al., 1988). Lamina I neurons were reported to have smaller receptive fields than those in deeper laminae

which suggests provision of information useful for stimulus localization. A somatotopic relationship between the distribution of the receptive fields of spinothalamic tract cells and their locations in the superficial laminae of the dorsal horn of the monkey has been described: in the lateral part of laminae I-IV, spinothalamic tract neurons have receptive fields on the extensor surface of the hindlimb, whereas cells in the medial part of the dorsal horn have fields on the flexor surface (Willis et al., 1974).

3.2.4. Spinoreticular Tract Neurons

The spinoreticular tract is a projection of spinal cord neurons to various nuclei in the brain stem reticular formation (Willis and Coggeshall, 1991). Most axons of spinoreticular tract cells ascend to the brainstem in the ventrolateral white matter of the spinal cord, however Lima (1990) suggested that the ascending pathway from the dorsal horn is mainly in the dorsal column. Since section of the ventrolateral funiculus of the spinal cord through which spinothalamic tract and spinoreticular tract travel can alleviate pain, both tracts were implicated in the conduction of nociceptive information (Kevetter et al., 1982). Three major components of the spinoreticular tract have been identified, one projects to the lateral reticular nucleus and one to the medial pontomedullary reticular formation (Willis and Coggeshall, 1991), in addition to a recently identified separate projection to the dorsal reticular nucleus of the medulla (Lima, 1990; Lima and Coimbra, 1990).

The cells of origin of spinoreticular tract have been mapped in several species including rat, cat and monkey with two different approaches: the retrograde tracer technique and the antidromic mapping in physiological experiments. Following large injections of HRP into the medial reticular formation in the rat, labelled cells were located in laminae V, VII, VIII, and X with most cells contralateral to the injections (Kevetter and Willis, 1982; 1983). Injection of CTb in the dorsal reticular nucleus of

the medulla chiefly labelled neurons in laminae I, X and also some neurons in laminae II-IV and in deeper laminae (Lima, 1990; Lima and Coimbra, 1990). Projection from dorsal horn was mostly ipsilateral whereas that from laminae VII and X and was bilateral. Large injections of CTb into the lateral reticular nucleus resulted in labelling of spinal neurons in lateral spinal nucleus, laminae I-III and V, VI, VII with scatterd cells in lamina IV and X (Lima et al., 1991). Labelled cells occurred bilaterally but ipsilateral labelling usually predominated. Generally similar distributions of labelled spinoreticular tract neurons were also reported for the cat (Abols and Basbaum, 1981) and monkey (Kevetter et al., 1982), following injections of HRP into the medial reticular formation.

Following injections of the dorsal reticular nucleus of the rat medulla, Lima (1990) and Lima and Coimbra (1990) described the morphological features of individual retrogradely labelled spinoreticular tract neurons. In lamina I, most of labelled cells were multipolar (70%) with a minority either pyramidal or flattened (Lima and Coimbra, 1990). Lamina II neurons were generally similar to those of lamina I whereas lamina III cells were mainly fusiform with dendrites oriented dorsoventrally. Labelled neurons in lamina IV had either ovoid or triangular somata while those in laminae V and VI were fusiform (Lima, 1990). After injection of the lateral reticular nucleus, retrogradely labelled neurons in lamina I were mostly fusiform with a minority pyramidal or flattened (Lima et al., 1991). Morphology of retrogradely labelled spinoreticular tract neurons (from the medial reticular formation) in the monkey were studied by Kevetter et al. (1982). Most labelled cells in lamina I were small, fusiform or round however occasional large cells were observed. In lamina V labelled cells were commonly multipolar and less commonly fusiform or round. The dendritic trees of many of the labelled cells were extensive.

Immunocytochemical studies have shown that some spinoreticular tract neurons in laminae VII and X contain enkephalin immunoreactivity (Nahin and Micevych, 1986). Many spinoreticular tract neurons in laminae I, III and IV have been found to possess the NK-1 receptor immunoreactivity (Naim, M.M., Shehab, S.A.S. and Todd, A.J., unpublished observations).

In a study of the physiological properties of spinoreticular tract neurons in the monkey, Haber et al. (1982) found that a few of the identified neurons in the lumbar enlargement could also be activated antidromically from the contralateral thalamus. They suggested that some spinothalamic tract axons give off collaterals into the reticular formation of the lower brainstem.

Fields et al. (1977) studied the physiological properties of spinoreticular tract neurons in the cat. They found that neurons located in the dorsal horn had cutaneous receptive fields, and were either wide-dynamic-range or high-threshold. Other widely distributed neurons were excited by stimulation of deep structures such as joint capsule, muscle or tendon. Stimulation of visceral structures was also reported to excite spinoreticular tract neurons in the cat (Blair et al., 1984). In monkey, Haber et al. (1982) found that approximately half of their sample of spinoreticular tract neurons were not activated by any peripheral natural stimuli tested. However the other half of the sample was responsive, of which most neurons were high-threshold and a smaller number were wide-dynamic-range or low-threshold.

4. Neurokinin-1 Receptor in the Dorsal Horn of the Spinal Cord

It was as early as 1931 when von Euler and Gaddum isolated a hypotensive agent from equine brain which they named substance P due its powdered form. About 50 years later, two similar peptides were independently isolated in mammals (Nawa et al., 1983) and named neurokinin A (NKA) and neurokinin B (NKB). This family of

peptides has been named tachykinins because they have an immediate stimulant action on extravascular smooth muscles, as compared with the delayed and slow stimulation produced by bradykinins (Bertaccini, 1976). In 1971, Tregear et al. were able to synthesize the peptide. The tachykinins are found extensively in the periphery where they function as vasodilators and potent constrictors of many smooth muscles, as well as in the central nervous system where they are thought to be involved in neurotransmission (Otsuka and Yoshioka, 1993; Regoli et al., 1994).

The early observation that the dorsal roots contain more substance P than the ventral roots led to the hypothesis that substance P may be the mediator of signals from primary sensory fibres (Lembeck, 1953). This hypothesis was subsequently supported by Otsuka et al. (1972) who showed that substance P exerts a powerful excitatory action on spinal motor neurons of the frog and is nearly 200 times more potent than L-glutamate. Otsuka and Konishi (1976) were also able to show that immunoreactive substance P increases in the perfusate of isolated spinal cord of newborn rats after electrical stimulation of the dorsal roots. Moreover, Hokfelt et al. (1975) demonstrated the presence of substance P in the cat primary sensory neurons by immunofluorescence, and it was later found in the dorsal horn of the rat spinal cord (Hokfelt et al., 1977).

4.1. Neurokinin Peptides

Five naturally occurring related peptides have been reported (Regoli et al., 1994): eledoisin, substance P, physalaemin, NKA and NKB. They have in common the COOH-terminal pentapeptide sequence Phe-X-Gly-Leu-Met-NH2.

4.2. Neurokinin Receptors

Three receptor types, NK-1, NK-2, NK-3 were identified when Erspamer (1981) noted different potencies for substance P and its analogues in different assays. The

existence of these receptor subtypes has been confirmed by molecular biology studies and their genes have been isolated and cloned (Nakanishi, 1991). In the rat, NK-1 receptor contains 407 amino acid residues, NK-2 receptor contains 390, and NK-3 receptor contains 452 (Masu et al., 1987). They share significant sequence similarity with other receptors in the G-protein coupled receptor family, and like all G-protein coupled receptors, have seven α helical transmembrane (hydrophobic) domains, three extracellular loops, three cytoplasmic loops and a cytoplasmic C-terminal region (Nakanishi, 1991). The three receptors share 54-66% homology in their transmembrane and cytoplasmic regions. This considerable homology is consistent with the fact that all three neurokinins are capable of binding to each receptor subtype (Routh and Helke, 1995). NK-1 receptor shows high affinity for substance P, NK-2 receptor shows high affinity for NKA and NK-3 receptor shows high affinity for NKB although it must be remembered that the naturally occurring neurokinins have a relatively high affinity for, and act as full agonists on all the three receptors, which therefore show poor selectivity. Despite this, the order of potency of the neurokinins is still used as a criterion for receptor classification (Regoli et al., 1994). Selectivity for each receptor has been markedly improved by agonists obtained by structural modification of the neurokinin COOH terminal sequence (Regoli et al., 1988) because the majority of evidence indicates that the C-terminal portions of the neurokinin peptides is the site which interacts with the receptor (Fong et al., 1992). These selective agonists have been very useful for the study of neurokinin physiology and pharmacology. Studies in which residues in the different receptors were substituted (point mutation) have indicated that both the extracellular and transmembrane domains are crucial for affinity and specificity (Fong et al., 1992).

4.3. Neurokinin Antagonists

Peptide and non-peptide antagonists have been discovered primarily through random screening followed in some cases by chemical optimization of the first identified lead (Regoli et al., 1994). The non-peptide antagonists have been found to be potent and selective for their specific receptors. Different types of antagonists have been described for example CP 96,345, CP 99,994 and RP 67,580 which are selective NK-1 receptor antagonist, SR 48,968 which is a NK-2 receptor antagonist and R 486 which is a NK-3 receptor antagonist. The NK-1 receptor was reported to have high sensitivity to naturally occurring agents in the following order of potency: substance P > NKA > NKB as well as high affinity for CP 96,345 and much less affinity for NK-2 and NK-3 receptor antagonists. The NK-2 receptor can be described as sensitive to NKA and displaying the following order of potency: NKA > NKB > substance P in addition to having high affinity for SR 48,968 and being insensitive to the other antagonists. The NK-3 receptor is characterized by a high sensitivity to NKB (order of potency: NKB >NKA > substance P) as well as high affinity to R 486 and low afffinity for the other receptor antagonists. In spite of the importance of the antagonists, CP 96,345 did not appear to be appropriate to evaluate the role of NK-1 receptor in nociception because it is too weak in the most commonly used animal models that are performed in the mouse and the rat, two species relatively insensitive to CP 96,345 (Regoli et al., 1994).

4.4. Invovement of Substance P in Pain Transmission

Substance P has been implicated in a variety of physiological processes including cardiovascular, respiratory, gastrointestinal functions and inflammatory responses (Otsuka and Yoshioka, 1993), in addition to functions in the nervous system, such as involvement in axon guidance during embryonic development (De Felipe et al., 1995), regulation of ganglionic, spinal, supraspinal reflexes, release of neurochemicals and

hormones from the pituitary (Regoli et al., 1994), emotional behaviour as well as a nociceptive function (Cao et al., 1998; De Felipe et al., 1998).

The neurotransmitter function of substance P which was originally proposed by Lembeck in 1953, based on the finding that the content of substance P is higher in the dorsal than in the ventral roots of spinal cord, remained unclear until recently. There have been many pieces of evidence supporting the suggestion that substance P has a role in pain transmission. Release of substance P from C-afferent terminals in lamina I of the spinal cord has been reported after noxious stimulation (Duggan et al., 1987) and the peptide has been found to evoke slow excitatory postsynapic potentials (EPSP) in second-order sensory neurons in the dorsal horn and to facilitate their activation, an effect which was blocked with specific antagonists (De Koninck and Henry, 1991; Radhakrishnan and Henry, 1995). Hylden and Wilcox (1981) have shown that intrathecal application of substance P in the mouse induces scratching, biting, and licking, the typical reactive syndrome that reflects pain sensation. Furthermore, Yashpal and Henry (1983) observed that the intrathecal application of substance P in rats facilitates a spinal nociceptive reflex, a finding that was confirmed by Otsuka and Yanagisawa (1988) and also supported by the observation that C-afferent stimulation has the same effect as substance P (Wiesenfeld-Hallin, 1986). Further evidence has been presented by Lawson et al. (1997) who reported that all substance P containing primary afferent fibres were nociceptors. Experiments showing NK-1 receptor internalization following noxious (mechanical or chemical) stimulation of the rat hindpaw *in vivo* added a further support for the nociceptive role of substance P in the spinal dorsal horn (Mantyh et al., 1995). Ma and Woolf (1995) investigated the role of NK-1 receptor in nociception and found that the induction but not the maintenance of hyperalgesia (enhanced pain and pain related behaviour in response to noxious stimuli) and allodynia (pain and pain related behaviour in response to normally innocuous

stimuli) is NK-1 receptor dependent, unlike NMDA receptor which is involved in both the induction and maintenance.

In spite of the evidence supporting a role of substance P in nociception, results obtained with NK-1 receptor antagonists (e.g. Rubniak et al., 1996) did not entirely support a definite role of substance P in pain transmission. These results were also less easy to interpret due to the species differences in antagonist potency and the effect of antagonists on motor behaviour which could interfere with the behaviour tests (Regoli et al., 1994; Routh and Helke, 1995; Iversen, 1998; Cao et al., 1998; De Felipe et al., 1998; Zimmer et al., 1998).

Very recently, the nociceptive function of substance P has been confirmed by 3 studies (Zimmer et al., 1998; Cao et al., 1998; De Felipe et al., 1998) in mice, which showed that when the function of substance P is genetically disrupted, the animals show reduced responses to painful stimuli. In both of the studies by Cao et al. (1998) and Zimmer et al. (1998), mice with a targeted deletion of preprotachykinin I gene were used. Because this gene encodes the precursor from which both substance P and NKA are made, the mice lacked any detectable substance P or NKA, however their development, ability to reproduce and general behaviour were unaffected. Zimmer et al. (1998) reported that these mutant mice displayed no significant behavioural responses following subcutaneous formalin injection into the hind paw and have an increased withdrawal threshold in the hot plate test. On the other hand, the mice reacted normally in the tail flick and acetic acid induced writhing tests, which demonstrate that substance P has an essential function in specific types of pain responses. Zimmer et al. (1998) stated that they could not rule out the possibility that this deficiency in nociception could have resulted from functional alterations in substance P-containing neurons in supraspinal sites involved in the processing of painful stimuli, such as thalamic nuclei, the periaqueductal grey and the locus coeruleus. The other two studies by Cao et al.

(1998) and De Felipe et al. (1998) also supported a role for substance P and NK-1 receptor in pain mechanisms. In the study by Cao et al. (1998), mice with disrupted preprotachykinin I gene showed normal thresholds for reaction to various painful stimuli such as heat, mechanical pressure or chemical irritants, but when the intensities of the painful stimuli were increased, the knockout mice showed blunted responses evidenced by increased latencies in the response to the stimuli. Thus substance P and/or NKA appear to be required only for a certain window of pain intensities, and when the intensity of the painful stimulus is further increased, the responses of the knockout animals did not differ significantly from those of the wild-type mice. Cao et al. (1998) reported that the contribution of substance P/NKA is neither modality- nor tissue-specific since pain behaviours were evoked by thermal, mechanical and chemical stimulation of somatic and visceral tissue. They also found that the neurogenic inflammation (a local inflammatory response to certain types of injury or infection) was impaired in the mutant mice. Moreover, the local response to injection of capsaicin in the skin of the ear was absent or considerably reduced. By contrast the inflammation produced by complete Freund's adjuvant (CFA), which is non-neurogenic (and therefore does not depend on the integrity of primary afferent fibres), was the same in the wild type and mutant mice.

De Felipe et al. (1998) investigated the effect of disrupting the gene encoding NK-1 receptor in mice. The animals resembled those studied by Cao et al. (1998) in that they did not show any changes from the wild type in acute pain thresholds in mechanical, electrical or noxious heat tests but their responses were blunted in tests that involved more intense noxious stimuli. In addition, they also had impairment of neurogenic inflammation but not non-neurogenic inflammation. Electrophysiological investigation showed that wind-up (in which repetitive activation of C-fibres but not A-fibres increases the response of dorsal horn neurons to subsequent C-fibre input) was

completely absent in the NK-1 receptor knockout mice. De Felipe et al. (1998) also reported that, in these knockout mice, the analgesic response to cold-water swim stress was considerably impaired indicating that substance P activates stress-induced analgesia. The mutant mice were considerably less aggressive and the authors therefore concluded that substance P is important for orchestrating the response of the animal to major stressors such as pain, injury or invasion of territory. The results of these studies with the knockout mice, suggested that substance P/NK-1 receptor is not the only mechanism involved in pain perception (Iversen, 1998). Mild and severe pain presumably involve other neurochemical mechanisms, such as glutamate and its receptors (Dubner and Basbaum, 1994).

4.5. Anatomy of Neurokinin Receptor in the Spinal Cord

Numerous studies have been carried out to localize NK-1 receptor in the spinal cord. Early studies used radioligand binding (Charlton and Helke, 1985; Helke et al., 1986; Mantyh and Hunt, 1985; Yashpal et al., 1990, 1991) and these have recently been confirmed and extended by studies using *in situ* hybridization histochemistry (Elde et al., 1990; Maeno et al., 1993) for NK-1 receptor mRNA, and immunocytochemistry with antisera raised against synthetic peptide sequences corresponding to parts of the receptor (Moussaoui et al., 1992a; Bleazard et al., 1994; Liu et al., 1994; Brown et al., 1995; Littlewood et al., 1995). In the immunocytochemical approach, three different antisera raised against parts of the NK-1 receptor sequence have been used on spinal cord sections, two of these antisera were directed at the C-terminal part of the receptor protein (residues 349-407; Shigemoto et al., 1993 or 393-407; Vigna et al., 1994) and the other at the N-terminal end (residues 19-32; Moussaoui et al., 1992a).

In immunocytochemical studies, NK-1 receptor immunoreacitvity is densest in lamina I, light in lamina II and moderate in laminae III-IV and the area around the

central canal, as well as the autonomic areas and certain motor nuclei (Bleazard et al., 1994; Liu et al., 1994; Nakaya et al., 1994; Brown et al., 1995; Littewood et al., 1995). High magnification showed that the immunoreactivity was associated mainly with the surface membranes of dendrites and cell bodies of certain neurons, a picture which could be described as Golgi-like staining (Bleazard et al., 1994; Liu et al., 1994). Liu et al. (1994) used both light and electron microscopic immunocytochemistry and reported a significant mismach between the location of the NK-1 receptor- and substance P-immunoreactivity in the grey matter in particular in lamina II where there is a high concentration of substance P-immunoreactive axons but there is little NK-1 receptor-labelling. This mismach was confirmed at the electron microscopic level since the authors showed that although substance P-containing axon terminals had synaptic contacts with NK-1 receptor bearing neurons in the superficial dorsal horn, many NK-1 receptor-immunoreactive processes were not associated with substance P- containing terminals. This finding at the ultrastructural level suggested that the insertion of the receptor was not directed to a particular region on the neuronal surface. Liu et al. (1994) suggested that almost the entire surface of neurons that express NK-1 receptor may be acted upon by substance P, assuming that substance P can diffuse a considerable distance from its site of release, a process termed volume transmission. The suggestion that substance P can diffuse is supported by the observation that high intensity stimulation evokes release of substance P (presumably from the superficial laminae) into the spinal CSF (Go and Yaksh, 1987) and conversely, intrathecal injection of substance P evokes a profound caudally-directed biting and scratching behaviour (Hylden and Wilcox, 1981) which is thought to result from diffusion of the peptide from the CSF into the spinal cord tissue. Further evidence came from studies with antibody-coated microelectrode (Duggan et al., 1990, 1992) which characterized the spread of a variety of peptides in the dorsal horn. Nakaya et al. (1994) also reported a significant mismatch

between substance P- and NK-1 receptor-immunoreactivity in other regions of the central nervous system including the entopeduncular nucleus, substantia nigra pars reticulata and lateral part of the interpeduncular nucleus. The low level of NK-1 receptor immunoreactivity reported in lamina II is consistent with a recent report (Bleazard et al., 1994) demonstrating that very few lamina II neurons respond to the selective NK-1 receptor agonist [$Sar^{9}Met(O_2)^{11}$]-substance P in a neonatal rat slice preparation, which indicates that substance P presumably does not have a functional role on lamina II neurons. Bleazard et al. (1994) suggested that it is likely that lamina II neurons have a monosynaptic primary afferent input mainly from non-peptidergic afferents (Hunt and Rossi, 1985). Although other investigators hypothesized that NKA rather than substance P mediates the primary afferent evoked responses in lamina II interneurons, the study of Bleazard et al. (1994) did not confirm this since they found that lamina II neurons were equally insensitive to selective NK-2 and NK-3 agonists.

Brown et al. (1995) studied the morphological characterization of NK-1 receptor immunoreactive neurons throughout the rostro-caudal extent of the rat spinal cord. Their results indicated that relatively few, but morphologically distinct subclasses of spinal cord neurons express NK-1 receptor and the majority but not all of these neurons are located in regions that contain neurons responding to noxious stimulation. In lamina I, they observed a dense population of fusiform immunoreactive neurons as well as marginal neurons which resembled the large Waldeyer type, and estimated that 5% of lamina I neurons possessed the NK-1 receptor. Very few NK-1 receptor-labelled neurons were found in lamina II and none of them resembled the stalked or islet cells which are characteristic of this lamina (Gobel, 1978). A distinct population of NK-1 receptor-immunoreactive neurons were located in laminae III-V. Many of these neurons had the characteristic feature of large dorsally directed dendritic arbor that traversed lamina II to reach lamina I. Brown et al. (1995) also found an extensive labelling of

neurons in the intermediolateral cell column and in the ventral horn. The labelling in the ventral horn was associated with clusters of motoneurons. The results of Brown et al. (1995) were consistent with those obtained by Littlewood et al. (1995), who carried out a study of the types of dorsal horn neurons which possess the NK-1 receptor in the rat. In order to provide more information about these neurons the authors carried out pre-embedding immunocytochemistry with NK-1 receptor antibody on sections of rat lumbar spinal cord and combined this with post-embedding reaction to detect GABA and glycine. Interestingly, the great majority of spinal neurons which possessed the NK-1 receptor immunoreactivity were not GABA or glycine immunoreactive, however a few cells in the deep part of the dorsal horn and the lateral spinal nucleus and several cells near the central canal were GABA-immunoreactive, and some of these were also glycine immunoreactive. This suggests that substance P acts through neurokinin-1 receptors mainly on excitatory neurons within the spinal cord. Todd et al. (1998) estimated the number of spinal cord neurons which express NK-1 receptor immunoreactivity. They found that the approximate percentages of neurons which possessed the receptor were 45% of neurons in lamina I, 6% of neurons in lamina II, 11% of those in lamina III, 23-29% of neurons in laminae IV-VI and 18% of those in lamina X.

Most of the studies concerned with the distribution of NK-1 receptor immunoreactivity in the rat spinal cord (Bleazard et al., 1994; Liu et al., 1994; Brown et al., 1995; Littlewood et al., 1995; Mantyh et al., 1995) have confirmed the presence of a morphologically distinct population of cells in laminae III and IV expressing the receptor. These cells have the characteristic features of the antenna like neurons described by Rethelyi and Szentagothai (1973) in the cat. They are of interest because their dendritic tree frequently extends from lamina I to V and so they could potentially receive input from various classes of primary afferents (Szentagothai, 1964; Brown,

1981). In addition, some of them have been shown to belong to the spinothalamic tract which is thought to be important in pain sensation (Marshall et al., 1995). There is evidence that these cells are activated by noxious stimulation. Mantyh et al. (1995) reported that following acute noxious stimulation, these cells showed internalization of the NK-1 receptor on their distal dendrites, presumably due to activation of the receptor by substance P released from nociceptive primary afferents. Furthermore, in rats with persistent hindpaw inflammation, internalization of the receptor occurred on the cell body and proximal dendrites of these cells in response to both noxious and innocuous stimulation (Abbadie et al., 1997). Additional recent evidence came from the observation of Doyle et al. (1997) that some of these neurons express *c-fos* in response to noxious chemical stimulation (application of mustard oil to the skin).

Therefore the aims of the present study were to examine two types of primary afferents to laminae III/IV NK-1 receptor-immunoreactive neurons which have dorsally directed dendrites: substance P-containing afferents (labelled with immunocytochemistry) and myelinated afferents (labelled by transganglionic transport). In each case, the aims were to look for contacts, with the confocal microscope and to confirm that synapses are present at sites of contacts by the electron microscope.

Chapter 2

Materials and Methods

1. Substance P Primary Afferent Input

1.1. Immunofluorescence

Four adult Albino Swiss rats (either sex, 220-310 g) were deeply anaethetized with an intraperitoneal injection of sodium pentobarbital (26 mg/kg) and perfused intracardially with 4% formaldehyde (freshly prepared from paraformaldehyde) in 0.1 M phosphate buffer (PB), pH 7.3. Lumbar spinal cord segments were dissected out, postfixed for (2-4) hours in the same fixative, rinsed in PB, and cut into transverse or parasagittal sections (60 μm thick) in fresh PB with a Vibratome. The spinal cord sections were treated with 50% ethanol for 30 mins to enhance antibody penetration (Llewellyn-smith and Minson, 1992), they were then given three 5 minute rinses in phosphate buffered saline (PBS). Sections were incubated in blocking serum (10% normal donkey serum (NDS) in 0.3% Triton-X 100 PBS) for 1 hour and for 1-3 days in the primary antibodies. The primary antibodies used were, rabbit antiserum to NK-1 receptor which was raised against a synthetic peptide corresponding to the C-terminal 15 amino acids of the rat NK-1 receptor, coupled to bovine thyroglobulin (Vigna et al., 1994; diluted 1:10,000), rat monoclonal antibody to substance P (obtained from the NC1/34 hybridoma cell line; Cuello et al., 1979) directed against the C terminal part of the substance P molecule, which thus recognizes all the 3 tachykinins (Biogenesis, Poole, U.K.; diluted 1:200). For some sections, sheep antiserum to CGRP (Affiniti, Exeter, U.K.; diluted 1:5,000) was also included . The diluent for both primary and secondary antibodies was PBS containing 0.3%Triton and 5% NDS. After PBS rinses, sections were incubated in mixed species-specific secondary antibodies overnight. The mixure consisted of biotinylated anti-rabbit (diluted 1:500), anti-rat IgG conjugated to cyanine 5-18 (Cy5; diluted 1:100) and, when antibody to CGRP was used, anti-goat IgG conjugated to lissamine rhodamine (LRSC; diluted 1:100) was used. The secondary antibodies were all raised in donkey and were obtained from Jackson Immunoresearch

(West Grove, PA, U.S.A.). Further PBS rinses were followed by incubation in fluorescein (FITC) conjugated to avidin (Vector Laboratories, Peterborough, U.K.; diluted 1:10,000) in PBS Triton for 4-24 hours. The sections were rinsed in PBS and then in bicarbonate buffer (pH 8.2). Finally they were mounted between glass coverslips in antifade medium (Vectashield, Vector Laboratories), sealed with nail varnish and stored in a freezer until used.

Control sections were treated in the same way, except that the peptide (SP or CGRP, Sigma, Poole, U.K.; each 10^{-6} M) was added to the antibody solution for 1 hour before use. For other sections, the NK-1 receptor antibody was omitted.

Spinal cord sections were initially viewed with epifluorescence and NK-1 receptor immunoreactive cells with soma either in lamina III or IV and dendrites passing up to the superficial dorsal horn were identified. The selected cells were then viewed with a Bio-Rad MRC 1024 confocal laser scanning microscope (Bio Rad Hemel Hempstead; U.K.) equipped with a Krypton-Argon laser. The cells all had their cell bodies at a depth between 150-300 μm below the dorsal white matter to ensure that they were located in lamina III or IV, since the laminar boundaries are difficult to identify particularly in parasagittal sections. The 20× objective lens and the 488 nm line of the Krypton-Argon laser (which excites FITC) were initially used to collect z-series through the whole of the cell bodies and dendritic tree of the selected NK-1 receptor-immunoreactive neurons. The cell bodies and dendritic trees of the neurons were then scanned with two or three lines of laser and the 60× oil immersion lens to reveal NK-1 receptor, substance P and CGRP. A thorough search was carried out to examine any contacts between substance P-immunoreactive axonal varicosities and the NK-1 receptor-immunoreactive dendrites or cell bodies. Approximately 90 cells were examined in this part of the study. Half of them were used to examine the association

between NK-1 receptor and substance P, while the other half were scanned to reveal NK-1 receptor, substance P and CGRP.

1.2. Electron Microscopy

Electron microscopy was used to determine whether synapses were present at contacts from substance P-immunoreactive afferents. For that purpose a modified technique combining confocal (triple-labelling immunocytochemical technique) and electron microscopy was used (Todd, 1997). Two adult rats (male, 270, 350 g) were deeply anaesthetised and perfused with a fixative consisting of 0.2% glutaraldehyde and 4% formaldehyde in 0.1 M PB. Lumbar spinal cord segments were dissected out, postfixed in the same fixative for 4 hours and cut into parasagittal sections (60 μm thick). The sections were treated with 50% ethanol for 30 mins, followed by PBS rinses and 1% sodium borohydride in PBS for 30 mins to reverse the effect of glutaraldehyde. This was followed by extensive PBS rinsing for 90 mins and then incubation with blocking serum (10 % NDS in PBS without Triton). The sections were incubated in a mixture of primary antibodies for 1-3 days. The mixture consisted of antibodies to NK-1 receptor, substance P and CGRP at the same concentrations used in the previous immunofluorescence technique. After rinsing, sections were incubated in mixed secondary antibodies overnight. These were anti-rabbit IgG conjugated to FITC, anti-rat IgG conjugated to Cy5 and anti-goat IgG conjugated to LRSC (Jackson Immunoresearch; all raised in donkey; diluted 1:100). The diluent for primary and secondary antibodies was 5% NDS in PBS without Triton. The sections were further rinsed in PBS then in bicarbonate buffer and mounted between glass coverslips with antifade medium.

The immunoreactive sections were viewed initially with epifluorescence and then with the confocal microscope to select suitable cells (one from each animal) which had

cell bodies in lamina III or IV and dorsal dendrites which received numerous substance P-immunoreactive contacts. Since penetration of the peroxidase reaction product is limited in this technique, cells which had numerous contacts close to the surface of the Vibratome section were chosen. In addition, cells which had dendrites that ran parallel to the surface (rather than obliquely) were preferred to reduce the number of ultrathin sections needed. For each of these cells, z-series were obtained with the 488 nm line and 20× objective lens to reveal the dendritic tree and then sequential series with the three lines of laser and the 60× lens were obtained from parts of the dendrites to record SP and CGRP contacts.

The next step was to reveal both the NK-1 receptor and SP staining with diaminobenzedine (DAB). Sections containing these neurons were removed from coverslips, rinsed in PBS to wash out the mounting medium and then incubated with biotinylated secondary antibodies for 3 days. Both biotinylated anti-rabbit and biotinylated anti-rat (Jackson Immunoresearch raised in donkey) were used at a concentration 1:200 in 5% NDS in PBS with no Triton. After further rinsing, the sections were incubated in a solution of Extravidin-peroxidase (Sigma; diluted 1:1000 in PBS) for another 3 days. Before DAB reaction the sections were rinsed in PBS then PB. They were then reacted with filtered freshly prepared DAB solution (PB containing 0.05% DAB and 0.01% hydrogen peroxide) for 5 mins. Four rinses in PB were carried out before the sections were postfixed in 1% osmium tetroxide in 0.1 PB for 30 mins and then rinsed in water. The sections were dehydrated in acetone, flat embedded between glass coverslips in Durcupan and cured at 60 °C for 24-48 hours.

The DAB reacted sections were examined with light microscopy and the selected parts of the NK-1 receptor-immunoreactive neurons were identified and photographed. One of the coverslips was removed and the sections were mounted onto cured resin blocks Ultrathin sections were cut through the region of interest using a diamond knife

and collected on single-slot copper grids (coated with Formvar), stained with lead citrate and viewed with the transmission electron microscope (Philips CM100).

Identification of the immunoreactive structures seen with the electron microscope was made possible by comparing the electron micrographs with the corresponding confocal images and light microscope photographs.

1.3. Labelling of Spinothalamic Tract Neurons

1.3.1. Operative Procedure

Three adult rats (male, 280-320 g) were deeply anaesthetised using halothane inhalation and placed in a stereotaxic frame. CTb was then injected into the left thalamus through a glass micropipette. The injections were at three anteroposterior planes: 4.8, 5.9 and 6.9 mm anterior to the ear bar (Paxinos and Watson, 1982) and at each plane three vertical tracks: 0.5 or 1.0, 2.0 and 3.0 mm lateral to the midline were made. In each track, 450 nl of 1% CTb (List Biological Laboratories, Campbell, CA, U.S.A.) was injected over a period of approximately 5 mins giving a total volume of 4.05µl.

After 3-4 days survival period, the rats were re-anaesthetized and perfused with 4% formaldehyde. The brain and the whole lumbar spinal cord were removed and placed in the same fixative for a further 2-4 hours.

1.3.2. Immunocytochemical Procedure

1.3.2.1. Injection Sites

One hundred micrometer thick coronal sections of the brain were cut through the injection site with a Vibratome and reacted with an immunoperoxidase method to reveal the injection site and spread of tracer. They were first rinsed in PBS then incubated with the primary antiserum (goat anti-CTb, List Biological Laboratories,

diluted 1:80,000 in Triton PBS) for a period of 1-3 days. Following PBS rinses, biotinylated anti-goat raised in donkey was used (Jackson Immunoresearch diluted 1:500) for 1 hour. This was followed by PBS rinses, Extravidin-peroxidase (diluted 1:1,000 in PBS) for 1 hour, and further PBS and PB rinses. DAB reaction was carried out (PB containing 0.05% DAB, 0.07% nickel chloride and 0.005% hydrogen peroxide) for 5 mins. The sections were extensively rinsed in PB, mounted on gelatinized slides and air dried. The dried sections were further rinsed in water to dissolve any salt, dehydrated in ascending grades of alcohol, cleared in xylene then mounted with DPX using glass coverslips.

1.3.2.2. Spinal Cord Sections

Both contralateral and ipsilateral halves of the lumbar spinal cord were cut into 60 μm thick sagittal sections with a Vibratome. The immunofluorescent triple labelling method described above was used except that goat anti-CTb (diluted 1: 5000) was substituted for anti-CGRP and donkey serum was omitted

All the lumbar spinal cord sections were examined with epifluorescence and retrogradely labelled spinothalamic tract cells were identified. Neurons in lamina III or IV which were both CTb-labelled and NK-1 receptor-immunoreactive, and with dendrites which could be followed into superficial laminae of the dorsal horn were selected for further study. With the use of the confocal microscope, the selected cells were examined for contacts from substance P-immunoreactive varicosities made onto their cell bodies or dendrites.

2. Contacts from Myelinated Sciatic Afferents

2.1. Operative Technique and Immunocytochemistry

Three adults rats (male, 290-340 g) were used in this part of the study. They were anaesthetised either with halothane or with an intraperitoneal injection of ketamine (70mg/kg) and xylazine (7mg/kg). The left sciatic nerve was exposed by an incision in the middle of the thigh and an injection of 4 or 5 µl of 1% CTb was made into the exposed nerve. After three or four days survival, the animals were re-anaesthetised with pentobarbitone and perfused through the left ventricle with 4% formaldehyde in 0.1M PB. Laminectomy was performed and the segments L3-5 of the spinal cord were taken out and postfixed in the same fixative overnight. The segments were rinsed in PB and the left side halves (ipsilateral sides) were cut in PB into sections 60 µm thick in the parasagittal plane. The sections were treated with 50% ethanol for 30 minutes and rinsed with PBS before application of primary antibodies. A mixture of the primary antibodies consisting of rabbit polyclonal antiserum to NK-1 receptor (diluted 1:10000) and goat polyclonal antiserum to CTb (diluted 1:5000) was used overnight. The sections were rinsed in PBS, then incubated overnight in secondary antibodies: biotinylated anti-rabbit (diluted 1:500), and donkey anti-goat IgG conjugated to LRSC (Jackson Immunoresearch, West Grove, PA, U.S.A.; diluted 1:100). The diluent for both primary and secondary antibodies was PBS Triton. After 3 PBS rises, sections were incubated in FITC conjugated to avidin (Vector Laboratories, Peterborough, U.K.; diluted 1:10,000 in PBS Triton) overnight. They were then rinsed in PBS and in bicarbonate buffer and finally mounted between glass coverslips in antifade medium, sealed with nail varnish and stored in a freezer until use.

The sections were examined initially with the epifluorescence microscope, and a filter set was used to reveal LRSC (CTb-immunoreactivity) and select sections from the sciatic nerve territory, in which a dense plexus of CTb-immunoreactive axons was

present in the deep dorsal horn (laminae III-VI). The same sections were examined using a filter set for FITC to show the NK-1 receptor-immunoreactive cells. Twenty neurons were selected for further analysis; 6 or 7 from each rat. They all had somata at a depth of between 150-300 μm below the dorsal white matter (in laminae III or IV) and dendrites that passed up in the dorsal direction to lamina II or I. The selection was made on the basis of presence of CTb labelling in the sciatic nerve territory and the morphological criteria of the cells, before examination of the contacts with a confocal microscope. The cells were scanned with confocal microscope. With a 20× objective lens and the 488 nm line of the laser to reveal fluorescein (NK-1 receptor), a complete series of scans was made through the cell body and dendritic tree of each cell to reveal the dendritic arborization. Projections of these z series were used to reconstruct the shape of the cell body and dendritic tree of each cell with the aid of a computer drawing program. The boundary between the grey and overlying white matter and the dorsal limit of the dense plexus of CTb-immunoreactive axons were indicated in the drawings.

A search was made for any contacts formed onto the cell bodies or dendrites of the neurons by varicosities that were CTb-immunoreactive. For each of the 20 neurons, the cell body and as much of the dendritic tree as was present on the section was scanned with the 488 and 568 nm lines through a 40× oil immersion lens to reveal both FITC and LRSC. All contacts between CTb-immunoreactive axonal varicosities and NK-1 receptor immunoreactive cells were plotted onto the cell drawing and a detailed analysis of the densitity of contacts was performed by measuring the lengths of the dendrites from the z series using a computer program (Neurolucida for Confocal, MicroBrightField Inc.). Because there was a dramatic difference between the density of contacts on dendrites which lay within the plexus of CTb-immunoreactive axons and the density of contacts on dendrites which lay dorsal to the plexus, the dendrites in each of these regions were analysed separately for each cell.

2.2. Combined Confocal and Electron Microscopy

Two adult rats (male 270, 300g) were anaesthetised either with halothane or with an intraperitoneal injection of ketamine (70mg/kg) and xylazine (7mg/kg) and received an injection of CTb into the left sciatic nerve as described above. After 3 or 4 days survival period the animals were re-anaethetised with pentobarbitone and perfused through the left ventricle with a fixative consisting of 0.2% glutaraldehyde and 4% formaldehyde in 0.1M PB. Segments L3-5 of the spinal cord were dissected out, post-fixed overnight in the same fixative, rinsed in PB and cut into transverse sections 60 μm thick with the use of a Vibratome. Transverse sections were used to ensure better penetration of the immunoperoxidase reaction product. All spinal cord sections were treated with 50% ethanol for 30 minutes, rinsed with PBS and then 1% sodium borohydride was used for 30 minutes. Extensive rinses with PBS for 90 minutes were carried out and the sections were then incubated overnight in the primary antisera (goat polyclonal antiserum to CTb and rabbit polyclonal antiserum to NK-1 receptor at the same concentrations as above). After rinsing, the sections were incubated overnight in species-specific secondary antibodies: donkey anti-rabbit IgG conjugated to FITC and donkey anti-goat conjugated to LRSC (both from Jackson Immunoresearch, West Grove, PA, U.S.A.; diluted 1:100). Both primary and secondary antibodies were made up in PBS without Triton. The spinal cord sections were rinsed in PBS, then in bicarbonate buffer and then they were coverslipped with antifade medium, sealed with nail varnish and stored in a freezer.

Using epifluorescence initially and then the confocal microscope (as described above) 3 cells were selected from the two animals and processed for the combined technique. The chosen cells had somata in lamina III or IV and long dorsal dendrites. They were all located in the area of sciatic nerve termination and had a significant number of contacts.

For each of these 3 cells, z series were obtained with the 488 nm line and 20× objective to reveal the dendritic tree. They were then re-examined with a 40× oil-immersion lens, and z series through parts of the cells which received contacts were obtained by scanning sequentially with 488 and 568 nm lines to reveal the contacts from CTb-labelled axons for subsequent comparison with electron microscope images of the corresponding regions (Todd, 1997).

As described above, the sections containing these cells were processed to reveal NK-1 receptor- and CTb-immunoreactivities with the chromogen DAB. The sections were rinsed in PBS to wash out the mounting medium and incubated for 72 hours in a cocktail of biotinylated antibodies against rabbit and goat IgG (both raised in donkey; Jackson Immunoresearch; diluted 1:200). After rinsing, incubation in Extravidin-peroxidase conjugate for 72 hours and further rinses, they were reacted with DAB and hydrogen peroxide to reveal the peroxidase. The sections were osmicated for 30 minutes in 1% osmium tetroxide in 0.1 PB then stained with uranyl acetate, dehydrated in ascending grades of acetone and flat-embedded in Durcupan resin between acetate sheets.

The light microscope was used to examine the cured sections, and the selected regions of the NK-1 receptor-immunoreactive neurons which had been shown to receive contacts with confocal microscopy were identified and photographed. One of the acetate sheets was removed and the sections were mounted onto blocks of cured resin. Ultrathin sections through the region of interest of each cell were cut with a diamond knife, collected on single-slot Formvar-coated copper grids, stained with lead citrate, and viewed with a Phillips CM100 transmission electron microscope. Comparing the electron micrographs with the confocal images allowed the identification of the immunoreactive structures.

Chapter 3

Results

1. Substance P Primary Afferent Input

1.1. Confocal Microscopy

1.1.1. NK-1 Receptor Immunoreactivity

The appearance of NK-1 receptor-immnoreactivity in the lumbar spinal cord was very similar to that reported previously using the same antiserum (Bleazard et al., 1994; Liu et al., 1994; Brown et al., 1995; Littlewood et al., 1995; Marshall et al., 1996). The labelling was dense in lamina I, less dense in the lateral spinal nucleus and the area around the central canal, and moderate in both laminae III and IV. The immunoreactivity was mostly associated with dendrites but many labelled cell bodies could be also seen in different areas such as lamina I which contained heavily labelled neurons, laminae III, IV (figs. 1, 2), the lateral spinal nucleus and the area around the central canal. In contrast to the dense labelling in lamina I, there was much lower level of immunoreactivity in lamina II and this was mostly associated with dendrites that belonged to neurons with cell bodies located in lamina I, III or IV. Occasionally large weakly immunoreactive cells were also observed in lamina II. With confocal microscopy, NK-1 receptor immunoreactivity appeared to decorate the entire cell body and dendritic tree of the neurons, which gave them a Golgi-like appearance (figs. 1, 2), as has been described earlier by Liu et al. (1994).

In laminae III and IV, a characteristic population of large immunostained-cells was present. Different shapes of the cell bodies could be identified as reported previously by Brown et al. (1995), for example, pyramidal (fig. 2a) and multipolar (figs. 1, 2b) cell bodies were commonly seen however very few of the neurons had a fusiform shape. The dendritic morphology was somewhat variable, however the proximal dendrites were often directed dorsally where they commonly gave off a few secondary branches and then passed up into laminae II and I, or even reached the border between the grey and the white matter. In the superficial laminae, the dorsal dendrites often showed extensive

Figure 1. A confocal picture showing a low-magnification view of a NK-1 receptor-immunoreactive neuron with cell body in lamina III. The image was built from a series of 15 optical sections 1 μm apart. The series was obtained from a transverse section of the dorsal horn of the spinal cord. Laminae of the dorsal horn are indicated (I, II and III). The boxes e, f, g show regions of the cell illustrated in fig. 3. Scale bar, 50 μm.

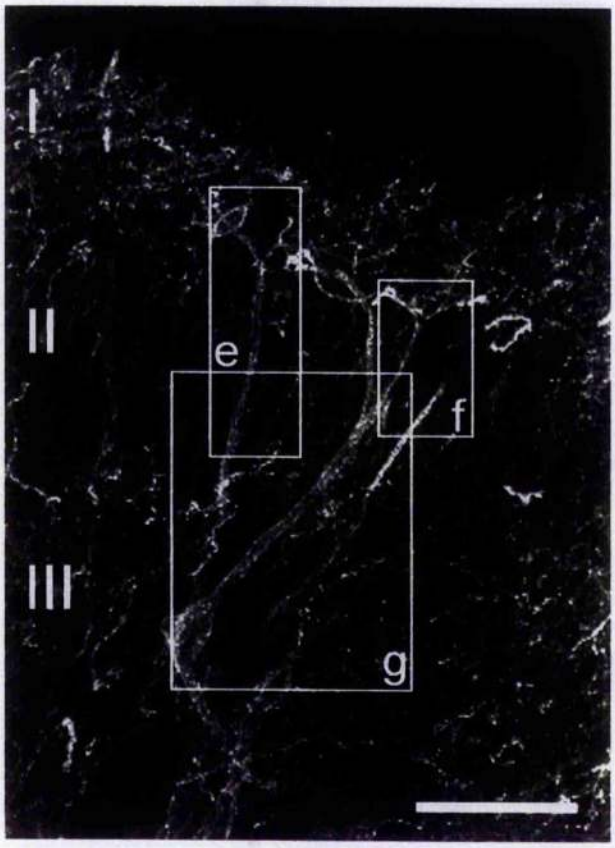
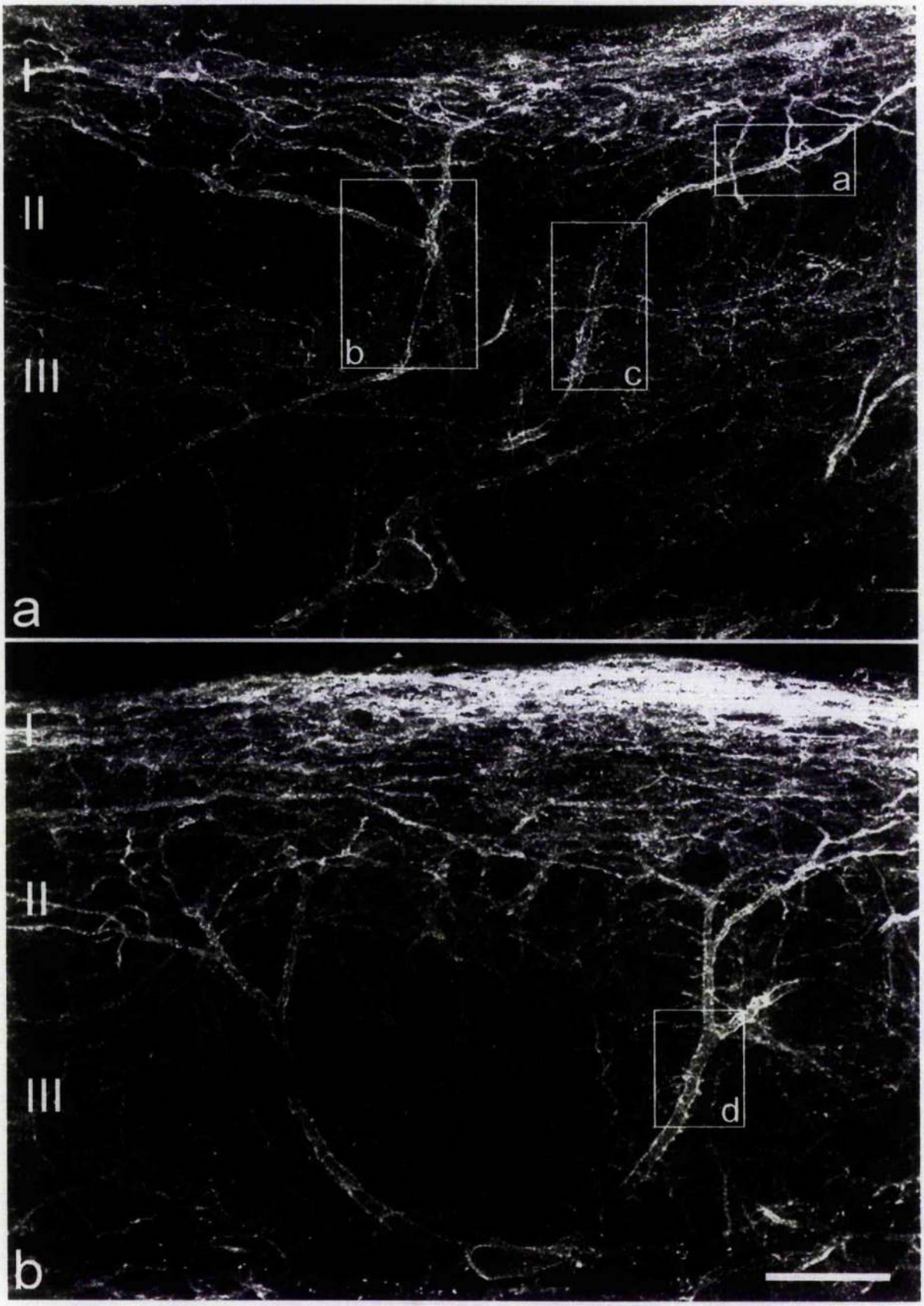

I
II
III
e
f
g

Figure 2. a, A confocal picture showing a low-magnification view of a NK-1 receptor-immunoreactive neuron with a pyramidal cell body in lamina IV and dorsal dendrites that reach lamina I. The image was built from 11 optical sections 2 μm apart, obtained from a sagittal section of the spinal cord. Laminae of the dorsal horn are indicated (I, II, III). Parts of the dendritic tree which are included in the boxes a, b and c are shown at higher magnification in fig. 3. b, Low-magnification confocal image showing a NK-1 receptor-immunoreactive neuron in a sagittal section of the spinal cord with a cell body in the deep part of lamina III. The picture was built from a series of 11 optical sections at 2 μm interval. The part of the dorsal dendrite in box d is illustrated at higher magnification in fig. 3. Scale bar, 50 μm.


I
II
III
a
b
c
a
I
II
III
d
b

branching, however these branches could be followed in the dense plexus of NK-1 receptor-immunoreactive dendrites in lamina I with the confocal microscope. In addition to the dorsal dendrites, rostrocaudally-orientated dendrites and ventral dendrites were also common and often extended for long distances but the dendritic trees were compressed in the medio-lateral axis, and therefore the cells were best seen in the parasagittal sections where the dendritic tree was often almost complete. Dendritic spines were occasionally seen on these cells. Very rarely, beaded structures like axons were seen, which when followed with a high power lens were found to join a dendrite.

1.1.2. Substance P Immunoreactivity

Immunostaining with the substance P antibody was similar to previous reports (Hokfelt et al., 1975; Ljungdahl et al., 1978; Gibson et al., 1981; DeLanerolle and LaMotte, 1982, 1983; Charnay et al., 1983; Ruda et al., 1986; Chung et al., 1989; Todd and Spike, 1993). There was a dense plexus of immunoreactive-fibres and varicosities in lamina I and the outer part of lamina II, while the inner part of lamina II had moderate staining. A few immunoreactive fibres were also seen in the rest of the dorsal horn (fig. 3). The varicosities seen in lamina I and outer part of II were mostly running in the rostro-caudal axis while those in the remaining part of the dorsal horn ran in the dorso-ventral direction. Immunoreactive cell bodies were not seen.

1.1.3. CGRP Immunoreactivity

CGRP-immunoreactive boutons were also concentrated in laminae I and II, with a lower density of boutons occurring in the deeper laminae (fig. 3e-g), as has been reported previously (Gibson et al., 1984b; Skofitsch and Jacobowitz, 1985; Carlton et al., 1988; Chung et al., 1988; Harmann et al., 1988; Leah et al., 1988; McNeill et al., 1988; Traub et al., 1989; Todd and Spike, 1993). The immunoreactive boutons were of

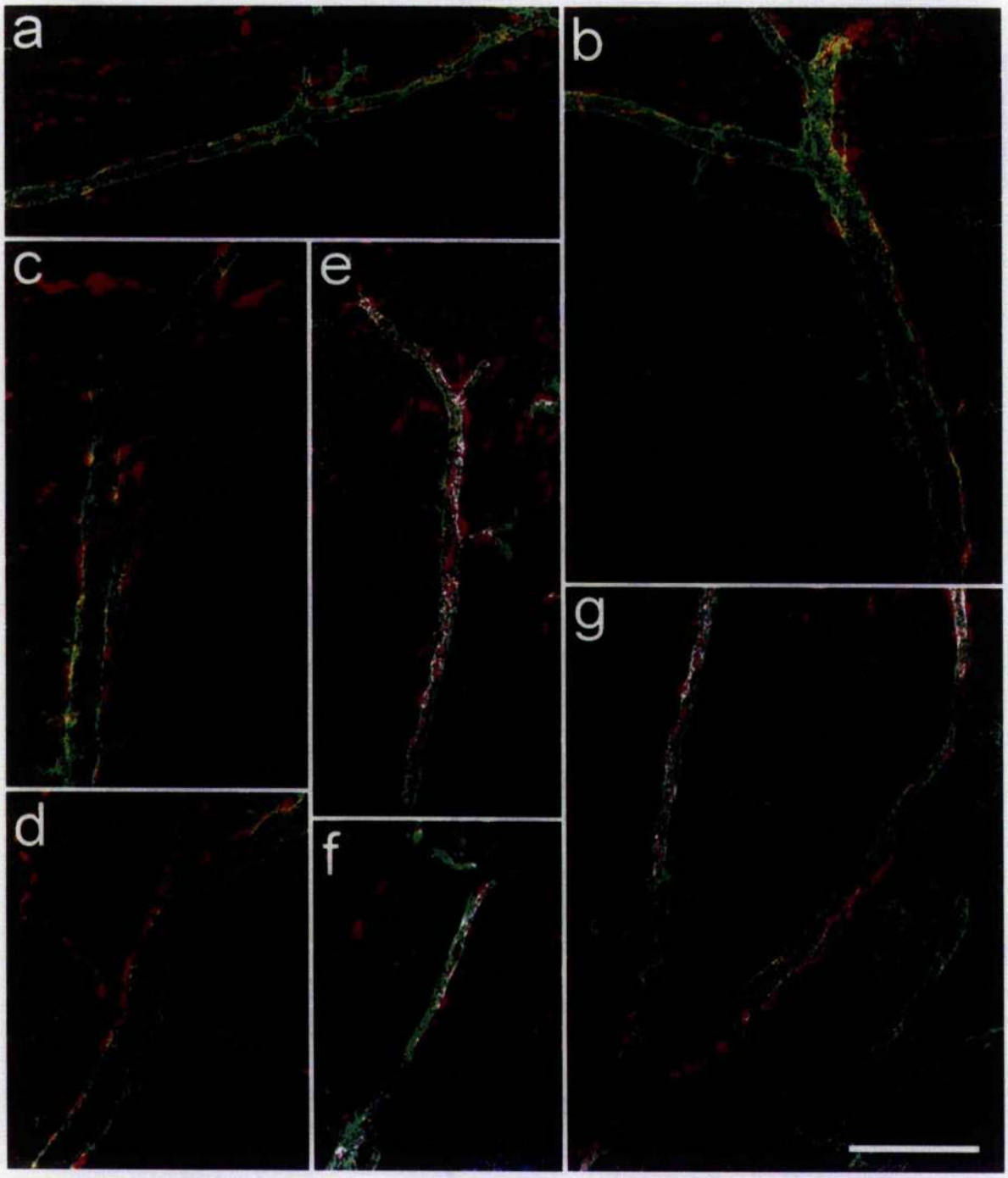
different sizes ranging from small to large size. No immunoreactive perikarya were seen in the dorsal horn.

The great majority of substance P-immunoreactive axon terminals in laminae I and II contained CGRP immunoreactivity as well, however axons showing only substance P or only CGRP immunoreactivity were also present. It was noticable that penetration of the immunonstaining with the substance P and CGRP antibodies was much better in transverse sections than in the parasagittal ones which sometimes showed a clear reduction in the density of immunoreactive profiles away from the cut surface of the section. However in some cases, penetration of peptide-immunostaining was uniform, even in parasagittal sections.

1.1.4. Contacts Between Substance P-Containing Axons and the NK-1 Receptor-Immunoreactive Neurons

All the cells examined in this part of the study received contacts from substance P-immunoreactive varicosities on their dorsal dendrites. These contacts were often extremely numerous especially on the large primary and secondary dendrites as they ran dorsally through laminae III and II (fig. 3). It was common to follow a single substance P-immunoreactive axon apposing the length of a dorsal dendrite with its varicosities and making many contacts. The fine distal dendritic branches which were running in the rostrocaudal direction in the superficial dorsal horn had fewer contacts than the dorsally directed ones, even though they lay within the substance P-immunoreactive plexus. Substance P-immunoreactive varicosities contacted both the cell bodies and ventral dendrites, however these contacts were much less numerous than those on the dorsal dendrites, confirming the observation made earlier by Liu et al. (1994) that not all of the plasma membrane of the immunoreactive neurons had contacts from substance P-containing axons. In sections reacted with antibodies to both peptides, the great

Figure 3. The boxes (a-g) show different regions of the dendritic trees from the 3 neurons illustrated in fig. 1 (e-g), fig. 2a (a-c) and 2b (d) at higher magnification. NK-1 receptor-immunoreactivity is shown in green and in boxes a-d, substance P-immunoreactivity is shown in red. In boxes e-g substance P-immunoreactivity is shown in blue, CGRP-immunoreactivity in red and when both types of peptide-immunoreacitivity colocalize, the colour appears purple. All of the NK-1 receptor-immunoreactive dendrites received numerous contacts from substance P-immunoreactive axonal varicosities. In boxes e-g which show triple-labelling (NK-1 receptor, substance P and CGRP) most of the contacts from substance P-immunoreactive varicosities also contain CGRP-immunoreactivity and when these double-labelled axons overlap with the NK-1 receptor-immunoreactivity, the colour appears white. a and c were obtained from 5 optical sections 0.5 μm apart, b and e was obtained from 8 optical sections 0.5 μm (b) and 1 μm apart (e) while d, f and g were obtained from 7, 4 and 6 optical sections respectively each at 1 μm separation. Scale bar, 20 μm.


a
b
c
e
d
f
g

majority of substance P-immunoreactive axons also contained CGRP-immunoreactivity (fig. 3e-g). However the cells also received occasional contacts from varicosities which had either substance P immunoreactivity alone or CGRP immunoreactivity alone.

1.2. Electron Microscopy

In series of ultrathin sections cut from parts of 2 different cells, (one from each rat), 13 substance P-immunoreactive varicosities contacting the dendritic trees of the NK-1 receptor-immunoreactive cells were identified (7 from one animal and 6 from the other). This was carried out by comparing the positions of DAB-labelled profiles in the ultrathin section series with the substance P-immunoreactive profiles which were Cy5-labelled in the confocal images (fig. 4). For each confocal image, an approximately corresponding ultrathin section could be identified. Since the electron microscope sections were thinner than the optical sections obtained with the confocal microscope, not all of the profiles seen on a confocal image were present on a single utrathin section. Of the 13 substance P-immunoreactive varicosities identified, 11 were also CGRP-immunoreactive. All of these 13 immunoreactive contacts examined with the electron microscope showed asymmetrical synapses, where the substance P-immunoreactive varicosity was presynaptic to the NK-1 receptor-immunoreactive dendrite (fig. 5).

1.3. Spinothalamic Tract Neurons

Light microscopic examination of the brain sections of the 3 thalamic injected rats which were reacted with the immunoperoxidase reaction, revealed that the spread of tracer was similar in all of the 3 experiments. The injections resulted in a variable amount of damage at the site of the pipette tracks and almost complete filling of the left thalmus with reaction product (fig. 6). Although there was spread of tracer in the region

Figure 4. Combined confocal and electron microscopy of a part of the dorsal dendrite of a NK-1 receptor-immunoreactive neuron. a, NK-1 receptor-immunoreactive (green colour) dendrite (D) with a small branch (arrow) given off from the main dendritic shaft. a, was obtained from 11 optical sections taken at 0.5 μm intervals. b, A confocal image obtained from a single optical section (taken from the series which was used to build the image in a), NK-1 receptor-immunoreactivity appears in green, substance P-immunoreactivity appears in blue and CGRP-immunoreactivity appears in red. Many varicosities show both types of peptide immunoreactivity and appear purple. Several substance P-immunoreactive varicosities are in contact with the NK-1 receptor-immunoreactive dendrite (D) or its small branch (large arrow) and 4 of these are indicated with the numbered small arrows. Number 2 varicosity shows only substance P-immunoreactivity while those numbered 1, 3 and 4 show both substance P and CGRP immunnoreactivity. c, The corresponding region of the immunoreactive dendrite shown approximately at the same focal plane (as in b) with light microscopy after the immunoperoxidase reaction. The immunoreactive dendrite (D) and part of its small branch (large arrow) are clearly visible while most of the right hand branch is out of focus. Many substance P-immunoreactive varicosities are also clearly seen including those indicated in b (numbered arrows). d, A low-magnification electron micrograph of the corresponding region at a depth nearly equivalent to the confocal picture in b. The main dendritic shaft (D) and its small branch (large arrow) are clearly seen as well as the substance P-immunoreactive varicosities which were indicated in b. Scale bar, 10 μm.

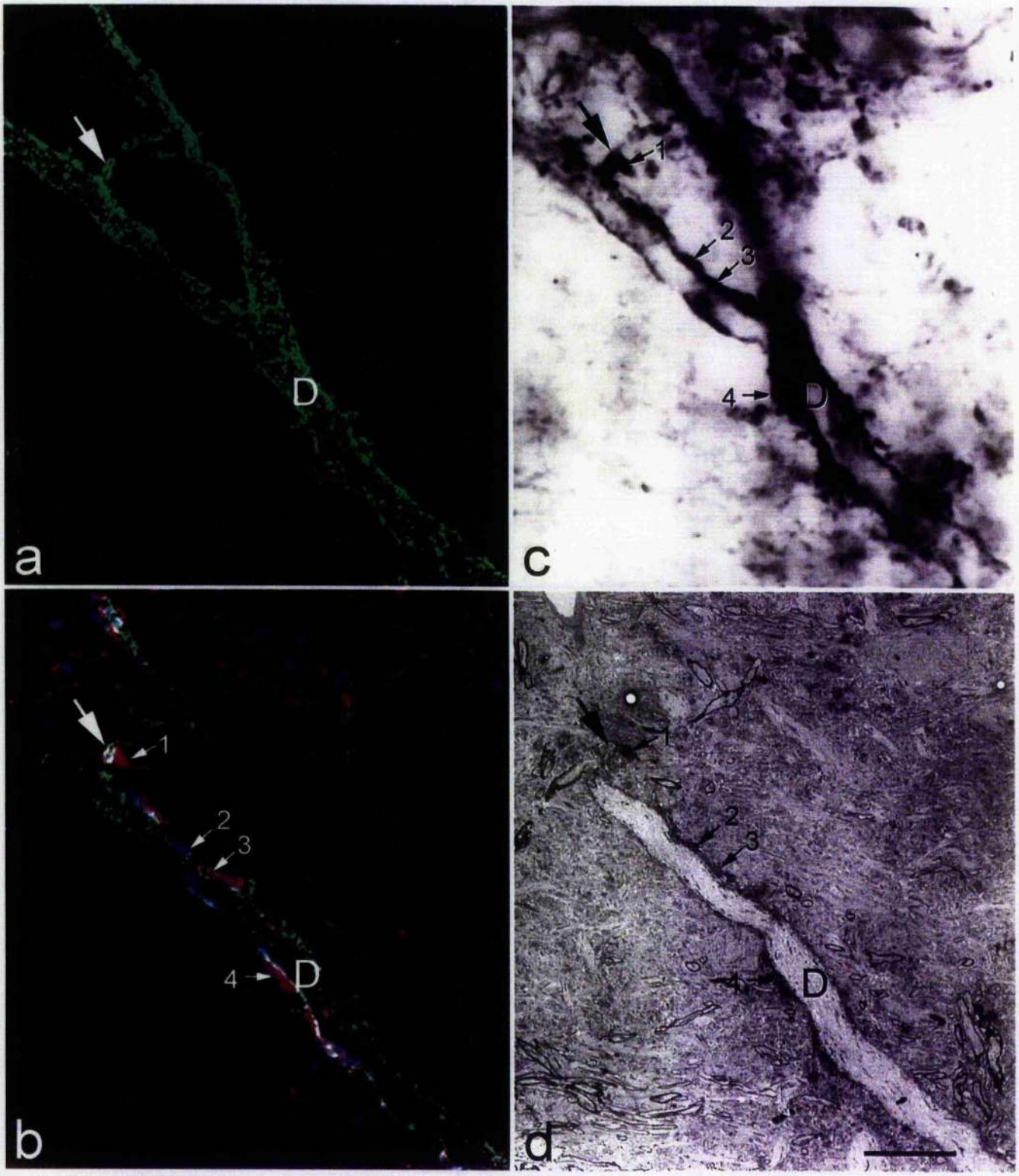
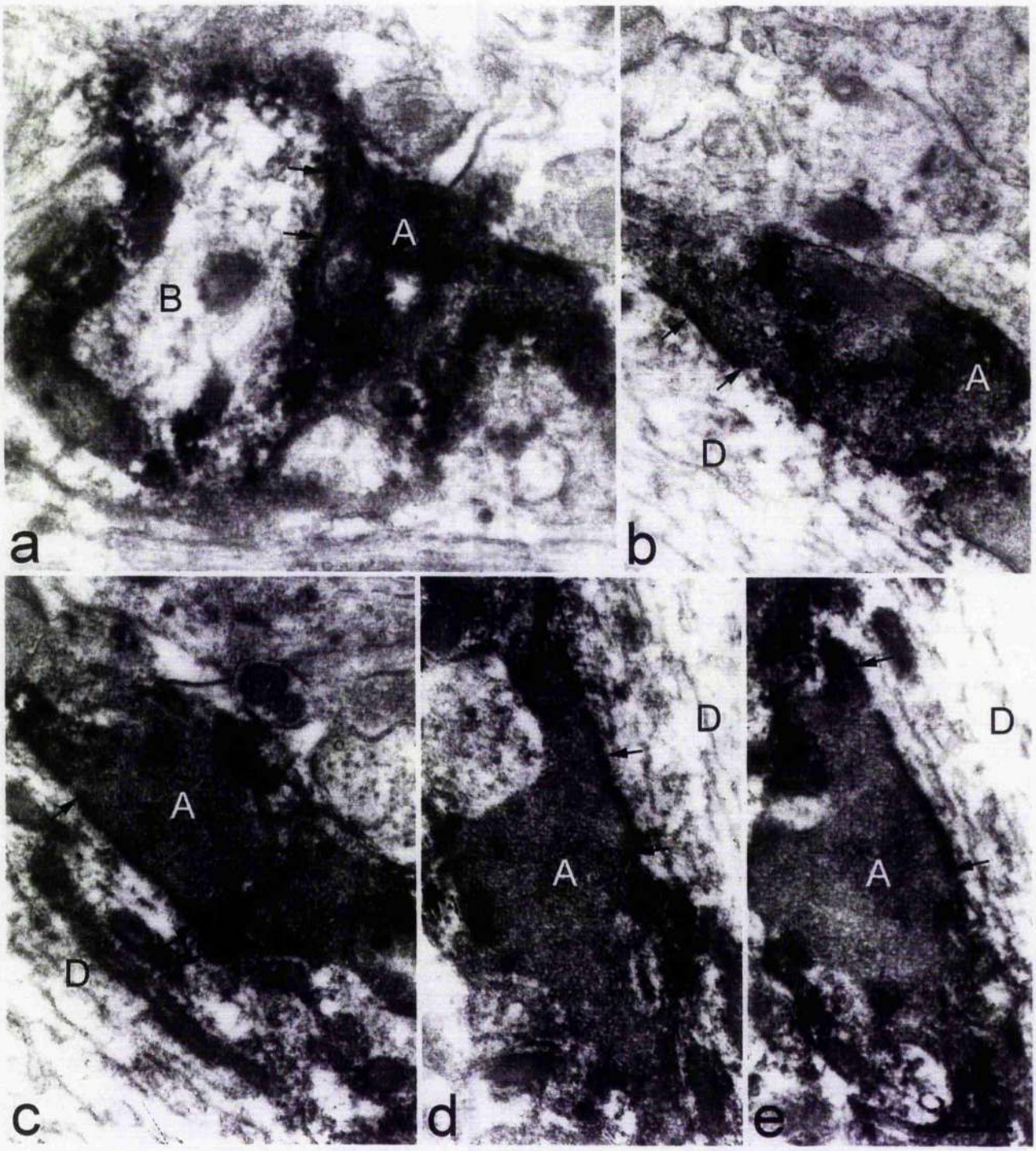

a
D
c
1
2
3
4
D
b
1
2
3
4
D
d
1
2
3
4
D

Figure 5. High magnification electron micrographs showing synspses between substance P-immunoreactive varicosities which were indicated in fig. 4b-d and the NK-1 receptor-immunoreactive dendrite (shown in fig. 4a). The micrographs b, d were taken from the ulrathin section illustrated in fig. 4d, whereas a, c and e were taken from nearby sections. a, The substance P-immunoreactive axon (A; indicated by the numbered small arrow 1 in fig. 4b-d) forms a synapse onto the small branch (B) which was given off the main dendritic shaft. b-d, The substance P-immunoreactive axonal boutons (A) (numbered small arrows 2-4 in fig. 4b-d, respectively) form synapses onto the dendrite of the immunoreactive neuron. e, The synapse illustrated in d is seen more clearly in a nearby ultrathin section. In each micrograph, an asymmetrical synaptic specialization is clearly seen (between arrows). Scale bar, 0.5 μm.

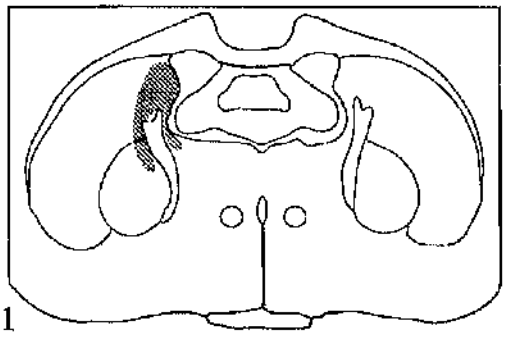

A
B
a
A
D
b
A
D
c
D
A
d
D
A
e

of cortex immediately surrounding the injection track, there was no spread into the hypothalamus or the midbrain.

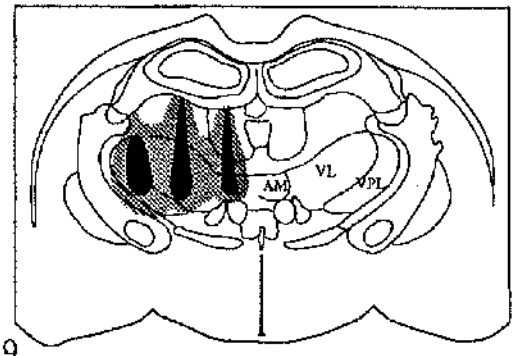
The retrogradely labelled spinothalamic tract neurons had a similar distribution in the lumbar spinal cord to that reported previously (Lima and Coimbra, 1988; Burstein et al., 1990; Marshall et al., 1996). Labelled cells were present in lamina I and the lateral spinal nucleus, and throughout a large area extending from the ventral part of lamina III to lamina VIII and also in lamina X. Although most labelled cells were present in the contralateral side of the injection, a significant number was seen in the same regions on the ipsilateral side. Higher power examination showed CTb immunoreactivity in retrogradely labelled cells as granular cytoplasmic fluorescence which generally filled the perikaryon and extended for variable distances into the dendritic tree (figs. 7b, 8b), however the staining was progressively paler with increasing distance from the cell body. Punctate staining was seen in the grey matter contralateral to the injection side. These presumably are due to the presence of axon terminals of anterogradely labelled corticospinal tract neurons whose cell bodies were in the vicinity of the injection track.

Ten NK-1 receptor-immunoreactive spinothalamic neurons with cell bodies in lamina III or IV and dendrites that entered the superficial dorsal horn were identified (figs. 7, 8) on the right side of the lumbar spinal cord (contralateral to the thalamic injection, between 2 and 5 from each rat). Although CTb immunoreactivity was restricted to the somata and proximal parts of the main dendrites, the rest of the dendritic trees could be followed easily as they were NK-1 receptor-immunoreactive. Thorough examination using the confocal microscope revealed that all of the ten labelled neurons received contacts from substance P-immunoreactive axons (fig. 8c-f). In five of these cases the contacts were very numerous however in the remaining 5 cases, fewer contacts were observed. This was attributed to the penetration of

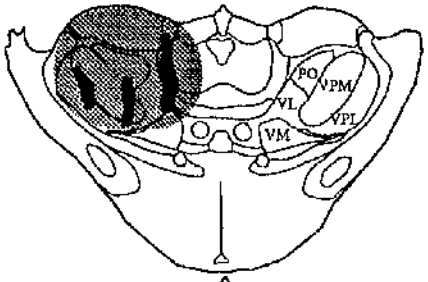
Figure 6. Drawings of the thalamic injection sites. Black areas indicate sites of damage due to the injection. The spread of tracer is indicated by shading. The numbers associated with each section refer to the distance in millimeters anterior to the ear bar. AM, Anteromedial thalamic nucleus; MG, medial geniculate nucleus; PF, parafascicular thalamic nucleus; PO, posterior thalamic nuclear group; VM, ventromedial thalamic nucleus; VL, ventrolateral thalamic nucleus; VPM, ventroposterior thalamic nucleus (medial); VPL, ventroposterior thalamic nucleus (lateral) (Paxinos and Watson, 1982).



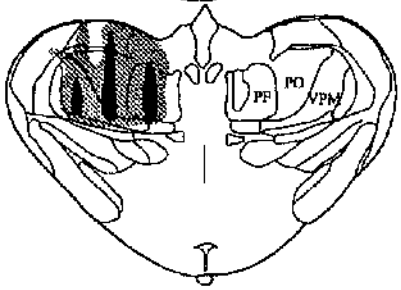
8.1



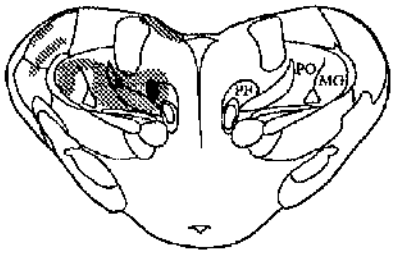
6.9



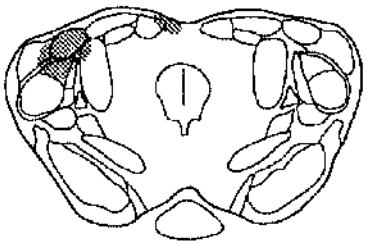
5.9



4.8



4.5



3.8

Figure 7. a, A low magnification confocal image showing a NK-1 receptor-immunoreactive spinothalamic tract neuron. NK-1 receptor-immunoractivity is shown in green. The cell body is located 260 μm below the dorsal white matter and the dendrites extend dorsally into lamina I. The picture was obtained from 20 optical sections 1.5 μm apart. Scale bar, 50 μm. b, NK-1 receptor- (green) and CTb-immunoreactivity (red) in a single optical section through the cell body of the neuron. Scale bar, 20 μm.

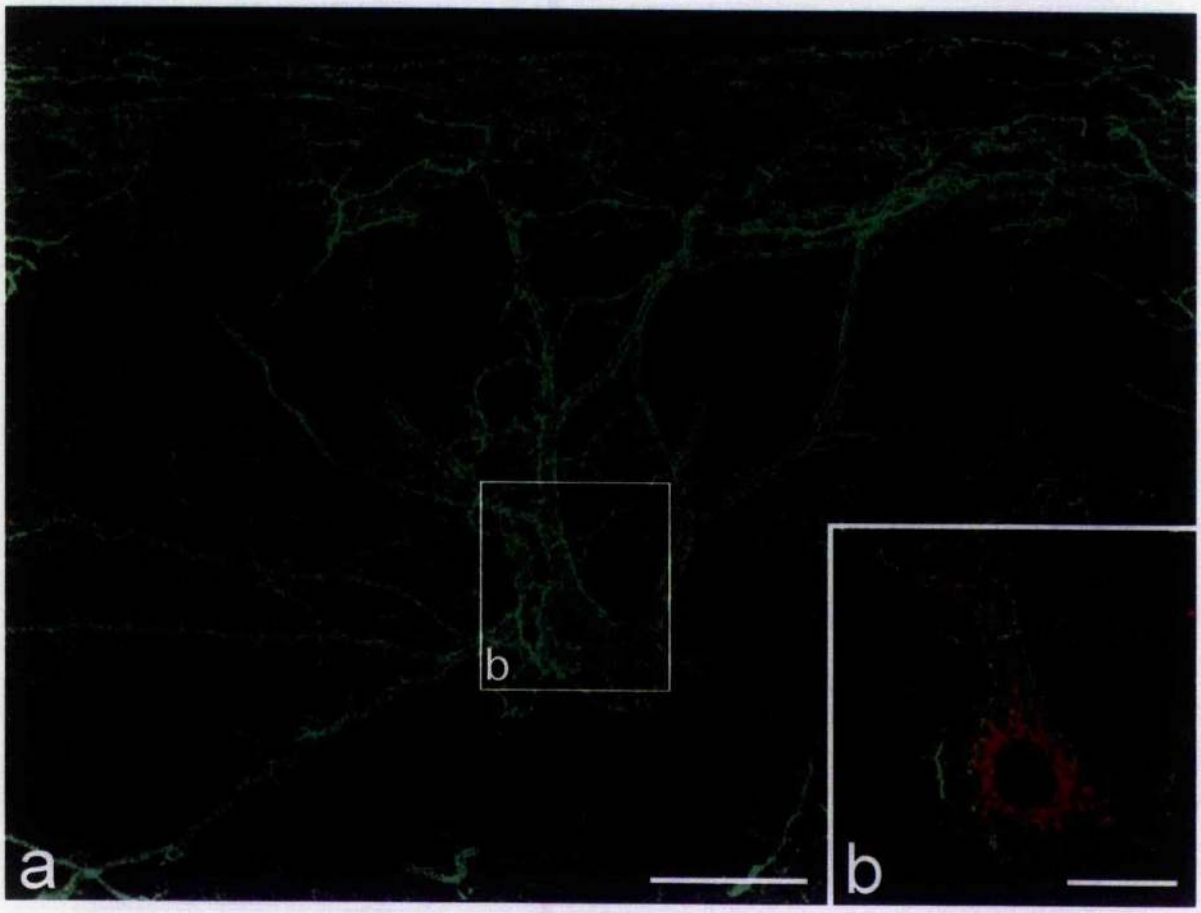
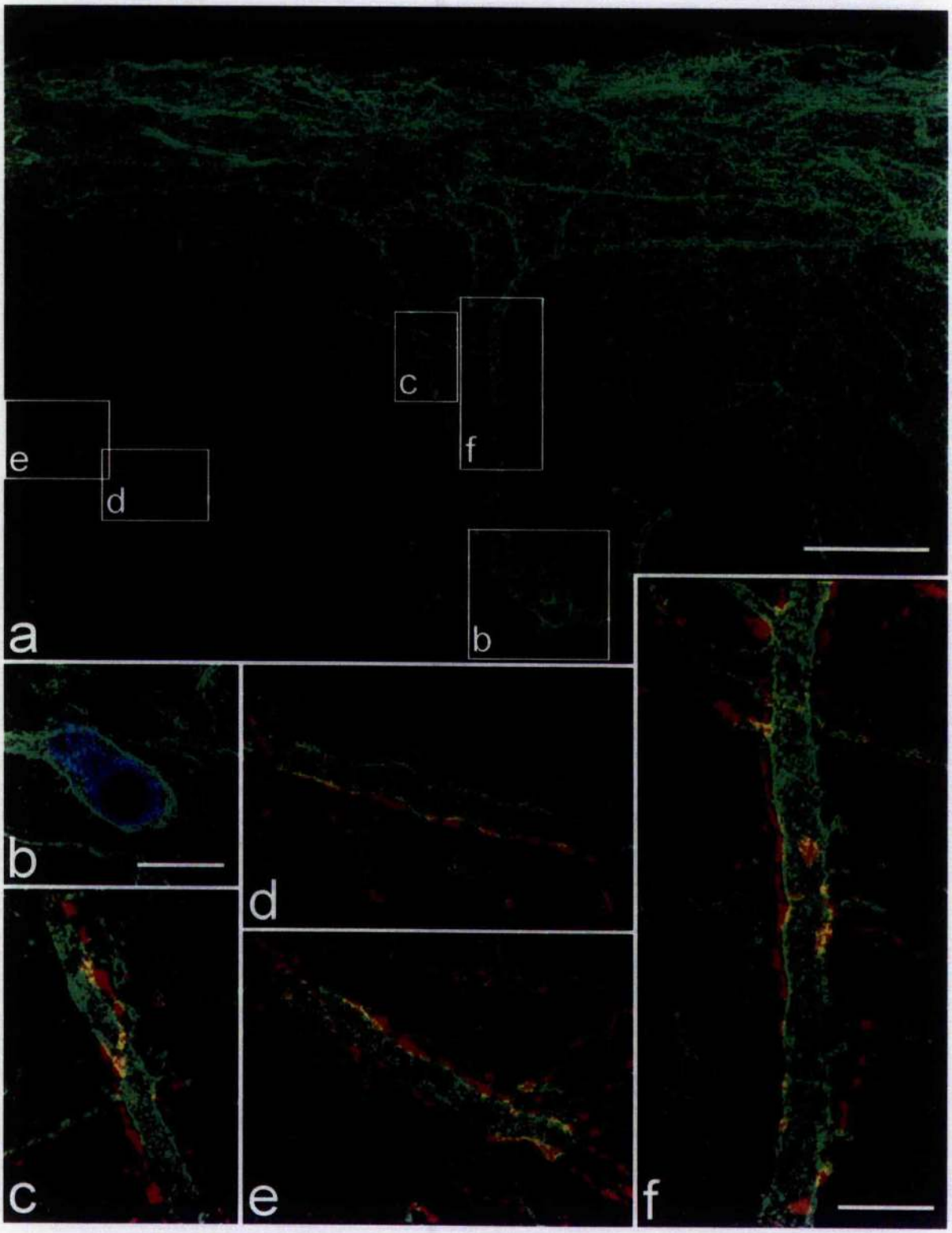

b
a
b

Figure 8. Confocal pictures showing substance P-immunoreactive contacts onto a NK-1 receptor-immunoreactive spinothalamic tract neuron. NK-1 receptor-immunoreactivity is shown in green (a-f), CTb-immunoreactivity in blue (b) and substance P-immunoreactivity in red (c-f). a, A confocal image showing NK-1 receptor-immunoreactivity in the retrogradely labelled spinothalamic tract cell. The cell body is located 235 μm below the dorsal white matter. Boxes indicate the regions illustrated in b-f. b, A higher magnification view of the cell body, showing NK-1 receptor- and CTb-immunoreactivity in a single optical section. c-f, Parts of the dendritic tree of the cell apposed by numerous contacts from substance P-immunoreactive varicosities. a, was built from a series of 9 optical sections 1.5 μm apart, c and e-f from 4 sections and d from 7 sections, each 0.5 μm apart. Scale bars, a, 50 μm; b, 20 μm; c-f, 10 μm.


a
b
c
d
e
f

substance P-immunostaining which was clearly incomplete in the sagittal sections which contained these five neurons.

2. Myelinated Primary Afferent Input

2.1. Confocal Microscopy

The distribution of CTb staining was similar to that which has been reported in previous literature following injections of either CTb or CTb-HRP into the sciatic nerve (Robertson and Grant, 1985; LaMotte et al.,1991; Rivero-Melian and Grant, 1991; Rivero-Melian et al., 1992; Woolf et al., 1995). By examining both the parasagittal and transverse sections, the CTb staining which appeared as large-sized granular profiles was clearly located in the territory of the sciatic nerve (occupying the medial two-thirds of dorsal horn at the level from mid-L4 to mid-L5). There was a dense plexus of CTb staining extending from lamina III to lamina VI (fig. 9a). Because it was difficult to be certain about the laminar boundaries in the immunostained material, both parasagittal and transverse sections were examined with dark field optics as well as LRSC fluorescence. Comparison between the views seen with both the dark field and the confocal microscope revealed the extent of the dorsal border of the CTb plexus which was found to be slightly dorsal to the border between laminae II and III. Therefore there were immunostained axonal varicosities in the ventralmost part of lamina II, as reported previously by other investigators (Robertson and Grant, 1985; Rivero-Melian and Grant, 1990; Broman et al., 1993) after using CTb-HRP. There were very few immunostained profiles in the remainder of lamina II, however there was faint to moderate labelling in lamina I (fig. 9a).

In the parasagittal sections, the CTb-labelled axons were not found to have any particular orientation, (fig. 9b) unlike the substance P-immunoreactive axons which generally ran along the rostrocaudal axis. At high magnification, although most CTb-

immunoreactivity was present in varicosities (axonal boutons; figs. 9b, 11a), a few labelled profiles resembling inter-varicose axons were seen. No labelling was detected in dendritic profiles or neuronal cell bodies in the dorsal horn however there was very intense staining in the motoneurons as well as occasional labelled axons in the ventral horn.

The appearance of NK-1 receptor immunoreactivity was the same as described above. In parasagittal sections, many NK-1 receptor immunoreactive neurons which had their cell bodies in either lamina III or IV and dorsal dendrites directed to the superficial laminae were found. Although there was an overlap between the CTb-labelled axons and the cell bodies, ventral dendrites and proximal parts of dorsal dendrites of the NK-1 receptor-immunoreactive neurons (fig. 9a), few contacts were found between them (fig. 9b). In laminae I and II the contacts onto the dorsal dendrites were extremely infrequent.

The total number of contacts on the cells varied from 24 to 99 per cell. As expected, there was a difference between the calculated densities of contacts made on dendrites contained within the plexus of CTb-immunoreactivity and dendrites that lay dorsal to the plexus (fig. 10). The density of contacts on dendrites within the CTb plexus was 2.62-10.26/100 μm (mean 5.92 ± 1.99 SD) while the density onto dendrites in lamina I and the dorsal part of lamina II was 0-0.7/100 μm (mean 0.24 ± 0.23 SD). The number of contacts on the cell body ranged from 0 to 12 (mean 3.75 ± 2.97 SD). Within the plexus of CTb-labelled axons, there was no detectable difference between the density of contacts on ventral dendrites and the density on the proximal parts of dorsal dendrites. In several cases labelled axons made contacts with dendritic spines of the NK-1 receptor-immunoreactive neurons.

Figure 9. Confocal pictures to show the relation between CTb-immunoreactive axon terminals (red) labelled by CTb injection into the sciatic nerve, and a NK-1 receptor-immunoreactive neuron (green) in a parasagittal section. Where the two types of immunoreactivity overlap the colour appears yellow. a, Low-magnification image obtained from 7 optical sections at 2 μm interval. Labelled CTb axons are seen in lamina I, the deep part only of lamina II (but they are absent from most of remaining part of lamina II), and there is a dense plexus of staining extending from the deep part of lamina II to lamina VI. The dorsal part of this plexus is seen in the lower half of the image. The NK-1-receptor immunoreactive neuron has a cell body located in lamina III and dorsal dendrites that extend into the superficial dorsal horn. The box shows the region of the cell illustrated in b. b, A higher magnification image obtained by a single confocal scan showing part of the cell body and a dorsal dendrite of the NK-1 receptor-immunoreactive neuron. Most of the CTb-immunoreactivity is in the form of varicosities (axonal boutons). Three contacts between CTb-immunoreactive boutons and the NK-1 receptor-immunoreactive dendrite are visible (arrows). Scale bars, a, 50 μm, b, 20 μm.

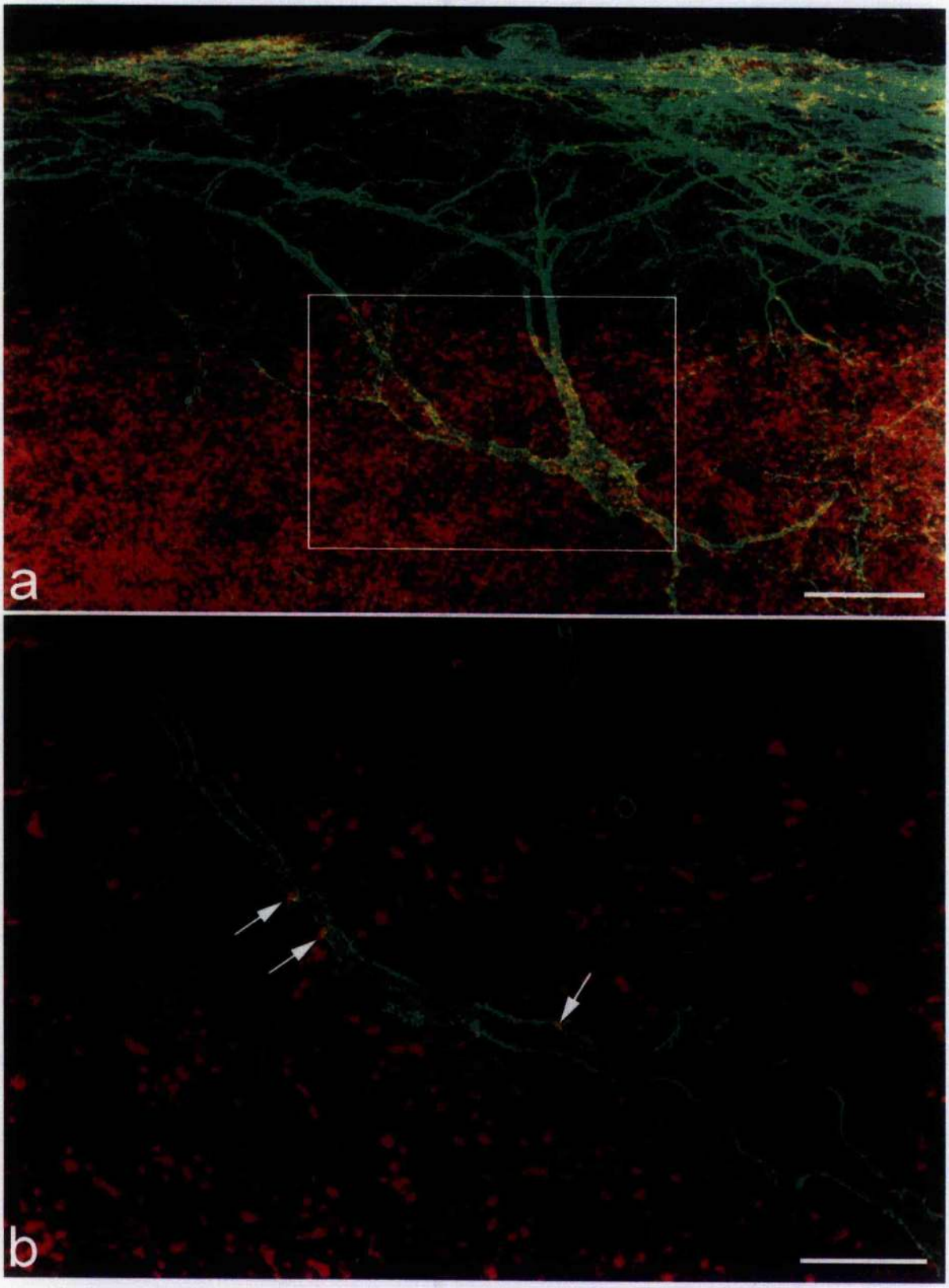
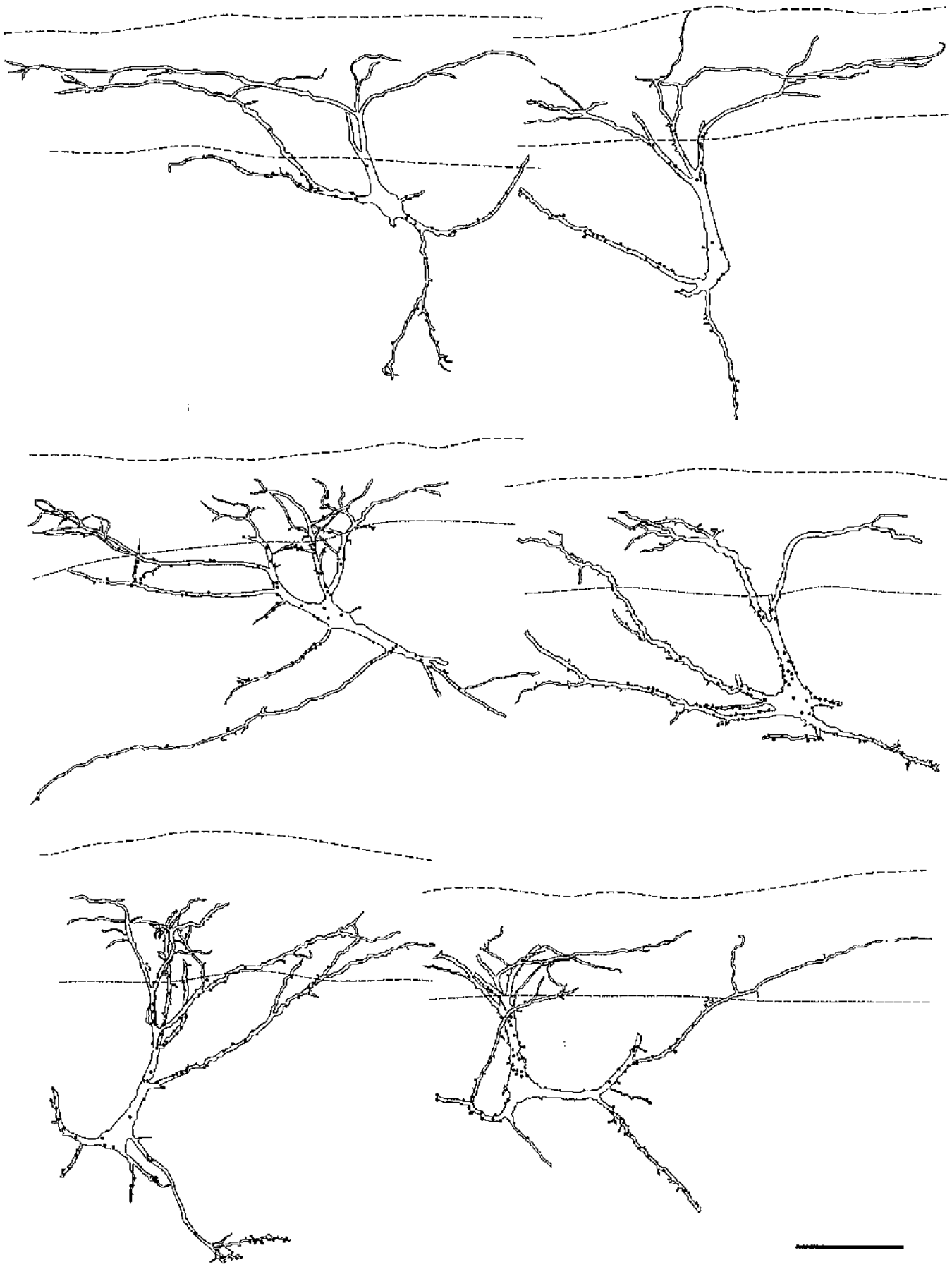

a
b

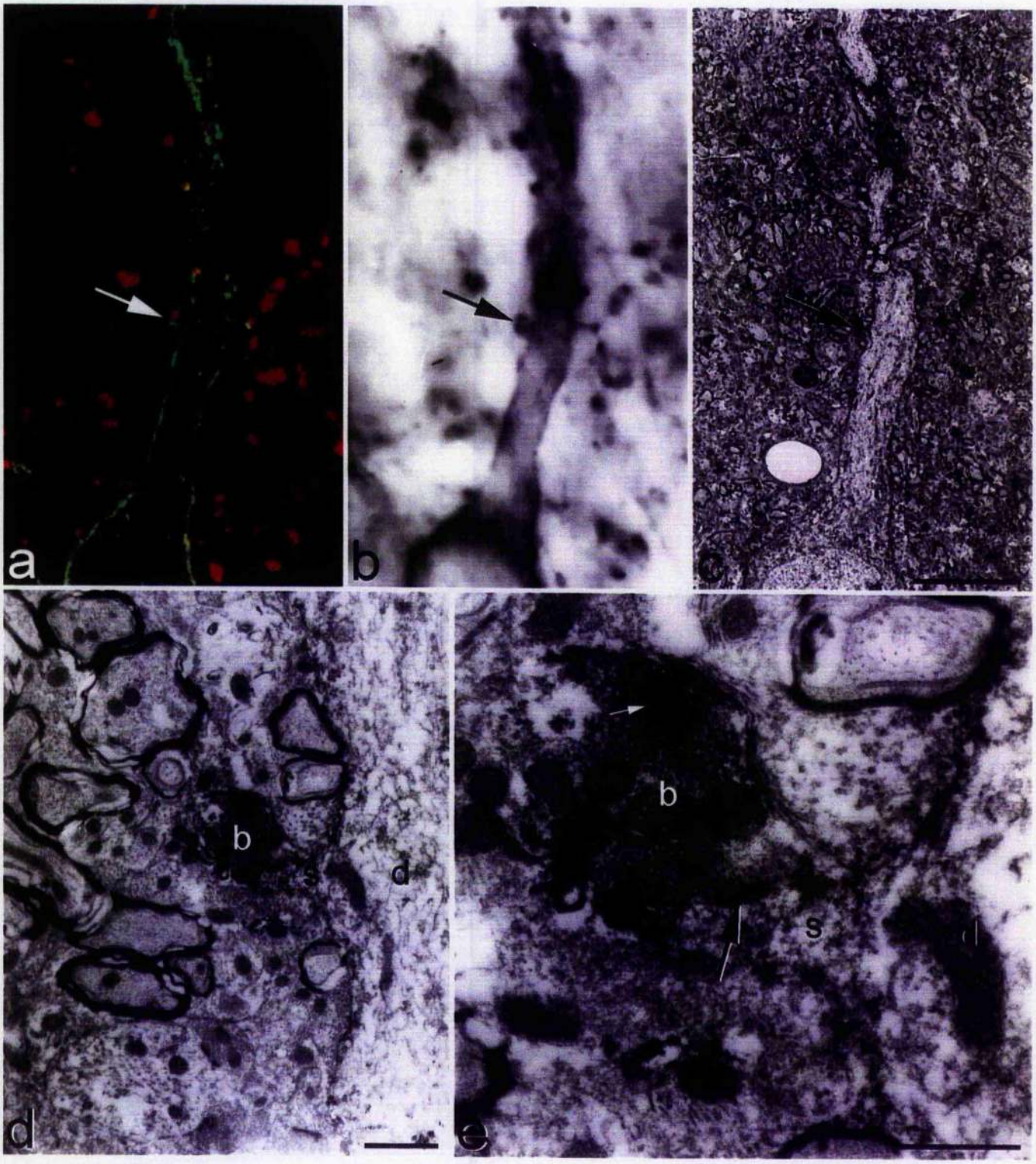
Figure 10. Drawings of six of the 20 NK-1 receptor-immunoreactive neurons which were analysed quantitatively (two from each of the 3 rats). These cells were from parasagittal sections. The upper dashed line represents the border between grey and white matter and the lower line indicates the dorsal limit of the dense CTb-immunoreactive plexus. Filled circles indicate the positions of contacts between CTb-immunoreactive varicosities and the NK-1 receptor-immunoreactive neurons. The top left cell is the one illustrated in fig. 9. Scale bar, 100 μm.



2.2. Combined Confocal and Electron Microscopy

Of forty-nine contacts found with the confocal microscope in the 3 cells processed by the combined method, twenty-six contacts were identified with the electron microscope. Eleven of these contacts were seen to form synapses which were always asymmetrical (see an example in fig. 11). One of the 3 neurons received 3 definite synapses (from 3 boutons), the second received 4 synapses (from 4 boutons) and the third received 5 synapses (from 4 boutons) because one of the boutons was associated with two synapses, one with the shaft of the dendrite and another with a dendritic spine. In 7 of the 26 boutons found, it was not possible to confirm whether a synapse was present or not because of the obliquity of the contacts. In 4 further cases, the bouton and the dendrite were seen with the electron microscope not to be in contact even though the confocal microscope had revealed an apparent contact. In the remaining 4 cases it was difficult to see the actual site of contact most probably because it was too oblique to the plane of the section. Of the 12 synapses identified, 7 involved dendritic spines, while 4 were associated with dendritic shafts and 1 was on a cell body. Two of the synapses identified were located in lamina II while the remaining 10 cases, synapses were located in lamina III or IV.

Figure 11. Combined confocal and electron microscopy to show the presence of a synapse between a CTb-labelled axon terminal and the dendrite of a lamina III NK-1 receptor-immunoreactive neuron in a transverse section. a, A confocal image obtained by a single confocal scan showing the cell body and dorsal dendrite of the NK-1 receptor-immunoreactive neuron (in green), surrounded by CTb-labelled axonal varicosities (red). Two dendritic spines emerge from opposite sites of the dendrite, and both receive contacts from CTb-immunoreactive axons. The left sided spine is indicated with an arrow in a-c. b, The corresponding region of the immunoreactive cell shown approximately at the same focal plane (as in a) with light microscopy after the immunoperoxidase reaction. c, A low-magnification electron micrograph of the same region at a level corresponding to the plane of section of the confocal image in a. d and e, are higher magnification electron micrographs showing the region of the same ultrathin sections as that illustrated in c. In each of the two views, the CTb-labelled axonal bouton (b) is seen in contact with the dendritic spine (s) which is emerging from the dendritic shaft (d). An asymmetrical synapse is seen at the site of contact (large arrow in e). Small arrow in e show a clump of reaction product representing CTb-immunoreactivity. It was not possible to determine whether there was a synapse between the other dendritic spine and the adjacent bouton because of the obliquity of the contact. Scale bars, a-c, 10 μm; d, 1 μm; e, 0.5 μm.


a
b
c
b
d
d
b
s
d

Chapter 4

Discussion

The main finding of the present study was that NK-1 receptor-immunoreactive neurons with cell bodies located in lamina III or IV and dorsal dendrites that reach the superficial dorsal horn, receive numerous contacts from substance P-immunoreactive primary afferents axonal varicosities and a much smaller number of contacts from myelinated primary afferents. Electron microscopy revealed that synapses were present at sites of contacts from substance P-immunoreactive varicosities and at some of those involving myelinated afferents.

1. Methodological Considerations

1.1. Specificity of the NK-1 Receptor Antibody

The specificity of the NK-1 receptor antibody for detecting the rat NK-1 receptor has been validated by Western blotting, radioimmunoassay, and immunocytochemistry (Vigna et al., 1994). The antibody was shown to recognize a protein band of 80-90 kDa on Western blots of membranes from cells transfected with the NK-1 receptor but not from cells transfected with the vector alone. It also recognized the receptor fragment (393-407 residue) as well as the intact receptor in radioimmunoassays. The antiserum localized immunoreactive NK-1 receptor to neurons in the dorsal horn of the spinal cord and to neurons and muscle cells in the periphery (in areas which are well defined sites for NK-1 receptor expression) and immunostaining was abolished by preabsorption of the antiserum with the peptide. An additional confirmation that the antibody only detects NK-1 receptor is the absence of any specific immunostaining with the antibody in mice in which the NK-1 receptor gene had been deleted, as reported recently by De Felipe et al. (1998). Omission of the antiserum in control sections of the present study also resulted in an absence of any fluorescence staining.

Recent pharmacological and biochemical studies have suggested that there are two molecular forms of the NK-1 receptor, a long and a short isoform differing in length of

their cytoplasmic carboxyl-terminal tail (Mantyh et al., 1996). The short form contains a very short carboxyl-terminal sequence extending only for 7 amino acids beyond the seventh transmembrane domain. To examine whether these different isoforms are expressed *in vivo*, Mantyh et al. (1996) compared the distribution of high affinity substance P binding sites (visualised by autoradiography with [^{125}I] substance P) with the distribution of the C-terminal epitope of the full length receptor (visualised with the NK-1 receptor antibody). They concluded that the former method labelled both long and short forms of the NK-1 receptor, while the latter labelled only the long form of the protein (since the epitope which it recognizes is not present in the short isoform). Mantyh et al. (1996) compared the situation in the central nervous system (striatum) with that in certain peripheral tissues (parotid and submaxillary glands). They found that the striatum exhibited a very similar pattern of binding and immunostaining which suggested that the long isoform was present in this tissue. Unlike the striatum, the parotid and submaxillary glands exhibited very high levels of binding but only very low levels of NK-1 receptor immunoreactivity suggesting that expression of the short isoform predominates in the periphery. It can be concluded that the long form of the receptor is present wherever NK-1 receptor immunoreactivity is found however this can not rule out the presence of the short form.

1.2. NK-1 Receptor Immunoreactivity in the Rat Lumbar Spinal Cord

The present results revealed dense NK-1 receptor immunoreactivity in lamina I and the area around the central canal, moderate immunoreactivity in laminae III-IV, and less immunoreactivity in lamina II. Most immunoreactive profiles exhibited the morphological appearance of dendrites, although many immunoreactive cell bodies were also seen in the grey matter (laminae I, III and IV) and lateral spinal nucleus. These results are in good agreement with several reports which have investigated the

distribution of NK-1 receptor immunoreactivity in the rat spinal cord (Bleazard et al., 1994; Liu et al., 1994; Nakaya et al., 1994; Brown et al., 1995; Ding et al., 1995; Littlewood et al., 1995). In each of these studies, one of two different antibodies directed against the C-terminal part of the NK-1 receptor was used (Shigemoto et al., 1993; Vigna et al., 1994). A third antibody was developed by Moussaoui et al. (1992a) against a peptide corresponding to 14 amino acid residues at the N-terminal end of the receptor, and this has also been used to examine the distribution of NK-1 receptor immunoreactivity in the thoracic region of the rat spinal cord, however it gave a pattern of staining which was significantly different than those seen with the C-terminal antibodies. Moussaoui et al. (1992a) reported that the NK-1 receptor immunolabelling was associated with membranes either of cell bodies or of neuronal processes, dendrites or axons. The highest density of NK-1 receptor like immunoreactivity was in the dorsal horn particularly in the lateral part of lamina II. They were able to recognize numerous cell bodies labelled in laminae IV, V but only a few cell bodies were observed in the superficial laminae where the most intensively labelled structures were dendrites. It is however not clear why this antibody gives such a different pattern, but the C-terminal antibodies have been more thoroughly characterised and the pattern they give matches substance P binding (see below).

In addition to the immunocytochemical approach which has recently been used to examine the distribution of NK-1 receptor, radioligand binding studies with substance P (Charlton and Helke, 1985; Mantyh and Hunt, 1985; Helke et al., 1986; Yashpal et al., 1990, 1991) as well as *in situ* hybridization histochemistry with probes directed against the mRNA for the receptor (Elde et al., 1990; Maeno et al., 1993) have also been used. The results from studies of each type within the spinal cord were reasonably consistent, however neither the techniques of *in situ* hybridization nor radioligand binding studies provided the resolution necessary to identify the populations of neurons that express the

receptor. *In situ* hybridization labels cell bodies which express the receptor (provided that the message is sufficient) but not dendrites, and this makes it impossible to identify the morphology of labelled neurons (Kiyama et al., 1993). Allthough the binding seen in radioligand studies presumably involves receptors on cell bodies and dendrites, the resolution is not adequate to identify individual neurons (Charlton and Helke, 1985; Helke et al., 1986; Yashpal et al., 1990).

A close correlation has been reported between NK-1 receptor immunoreactivity and substance P binding sites in most parts of the central nervous system including the autonomic areas, ventral horn and most of the dorsal horn of the spinal cord (Charlton and Helke, 1985; Liu et al., 1994; Nakaya et al., 1994), and this supports the suggestion that antisera raised against synthetic peptide sequences corresponding to the C-terminal part of the NK-1 receptor are reliable for detecting neurons which possess the receptor. The only significant mismatch reported between SP binding and NK-1 receptor immunocytochemistry in the spinal cord was the strong binding in lamina II where the receptor immunoreactivity was low. Littlewood et al. (1995) attributed this discrepancy to the relatively low spatial resolution of the autoradiographic technique in the thin lamina II which is less than 100 μm thick (Ribeiro-da-Silva and Coimbra, 1982). In the study of Charlton and Helke (1985) it was obvious that at least in certain spinal cord segments, the level of substance P binding in lamina II was significantly lower than that seen in lamina I (figs. 2A, 4A of Charlton and Helke, 1985). However an alternative explanation is that radioactive substance P also bound to another type of neurokinin receptor for example the NK-3 receptor. Seybold et al. (1997) examined the distribution of NK-3 receptor immunoreactivity in rat spinal cord (by using two antisera against sequences of amino acids contained in the C-terminal region of the NK-3 receptor) and found dense NK-3 receptor immunoreactivity in lamina II at all levels of the spinal cord with the majority of immunostained perikarya in the inner half of the lamina.

Although there have not been detailed *in situ* hybridization studies of NK-1 receptor mRNA in the spinal cord, Elde et al. (1990) reported strong signals over neurons in the dorsal horn, the area around the central canal and preganglionic autonomic neurons. Maeno et al. (1993) reported that generally the areas containing substance P immunoreactive terminals were associated with NK-1 receptor mRNA positive cells however they also observed a mismatch in lamina II, where they could only detect a few weakly labelled positive cells (despite the presence of numerous substance P-immunoreactive fibres) which is consistent with distribution of NK-1 receptor immunoreactivity.

1.3. CGRP Immunoreactivity and its Use as a Primary Afferent Marker

The CGRP antibody was raised in sheep against synthetic rat α-CGRP conjugated to bovine serum albumin, with glutaraldehyde. Two forms of CGRP have been identified: α-CGRP and β-CGRP, however α-CGRP is the predominant form in the sensory ganglia and all neurons which express mRNA for β-CGRP also express α-CGRP mRNA in the DRG (Noguchi et al., 1990b).

In the data presented here, CGRP immunoreactive fibres and varicosities were seen at high density in laminae I and II of the dorsal horn, and at a lower density in the deeper laminae of the dorsal horn. No immunoreactive cell bodies were observed. A similar distribution of CGRP-immunoreactivity has been reported in previous immunocytochemical studies in different species including rat, cat, monkey and human (Gibson et al., 1984b; Skofitsch and Jacobowitz, 1985; Carlton et al., 1988; Chung et al., 1988; Harmann et al., 1988; Leah et al., 1988; McNeill et al., 1988; Traub et al., 1989; Todd and Spike, 1993).

Several lines of evidence suggest that CGRP-LI in the dorsal horn of the spinal cord originates exclusively from primary afferent fibres and not dorsal horn neurons or

descending fibres. Numerous immunocytochemical studies have indicated the presence of CGRP immunoreactivity in the cell bodies of primary afferent neurons in DRG (Gibson et al., 1984b; Gibbins et al., 1985; Skofisch and Jacobwitz, 1985; Ju et al., 1987). Unilateral dorsal rhizotomies of consecutive segments in the lumbar enlargement have been shown to cause a substantial, although incomplete, loss of CGRP immunoreactivity in the dorsal horn of the corresponding segments of the spinal cord in both rat (Chung et al., 1988) and cat (Traub et al., 1989) ipsilateral to the lesion. Similar results have been observed in the medullary dorsal horn following retrogasserian rhizotomy (Henry et al., 1996). The most conclusive evidence of primary afferent source of CGRP was that bilateral dorsal rhizotomies and spinal cord transection that isolated a piece of the spinal cord, resulted in an elimination of all the dorsal spinal cord CGRP-LI in the rat (Chung et al., 1988). The great majority of immunocytochemical studies have failed to demonstrate CGRP immunoreactivity in the dorsal horn neurons in rat, cat, monkey and human (Gibson et al., 1984b; Skofitsch and Jacobwitz, 1985; Carlton et al., 1988; Chung et al., 1988; Harmann et al., 1988; Leah et al., 1988; McNeill et al., 1988). No CGRP-immunoreactive cell bodies were observed either following intrathecal adminstration of colchicine in cat or unilateral dorsal rhizotomy plus colchicine treatment in rat (Traub et al., 1989). The absence of a local neuronal source has been also confirmed by *in situ* hybridization histochemistry which has failed to demonstrate cells containing CGRP mRNA in the dorsal horn (Gibson et al., 1988; Rethelyi et al., 1989). Although GRRP-LI occurs in bulbospinal neurons, they do not seem to project to the spinal cord (Orazzo et al., 1993) and no evidence of a supraspinal source has been reported.

From the previous pieces of evidence, it could be concluded that CGRP-immunoreactive axons in the dorsal horn of the spinal cord are exclusively of primary afferent origin and therefore it seems reasonable to have used it as a primary afferent

marker in the present study. However an isolated study by Conrath et al. (1989) reported that in the cervical spinal cord of rats treated intrathecally with colchicine, weakly CGRP- immunoreactive cell bodies were present in the superficial three laminae and lateral spinal nucleus. With the electron microscope, Conrath et al. (1989) also reported the presence of a few immunoreactive-cell bodies even in animals which had not received colchicine in addition to relatively numerous immunoreactive dendrites in lamina II. The reaction in this study was thought to be specific since the antibody used did not cross-react with cholecystokinin which ruled out the possibility of detecting cholecystokinin-containing neurons. Conrath et al. (1989) suggested that the failure to detect CGRP-immunoreactive neurons in the superficial dorsal horn in previous studies may have been because of one of two reasons, the first was the masking of immunoreactive neurons by the dense plexus of immunoreactive axons, however this possibility is unlikely in the present study since the confocal microscope has high resolution and could show details of cells even through the dense plexus of immunoreactive substance P and CGRP. The second possibility suggested was that CGRP may be synthesized at a low rate by spinal neurons and this may have led to the failure to detect such cells in *in situ* hybridization studies. Merighi et al. (1990) also reported the presence of some CGRP-immunoreactive cell bodies in lamina I and II of the horse spinal cord. However since CGRP-immunoreactive axons are eliminated from the dorsal horn after extensive bilateral rhizotomies and spinal transection in the rat (Chung et al., 1988) it is likely that the reports of CGRP-immunoreactivity in the dorsal horn neurons are due either to cross-reactivity with some other unknown peptide or a species difference (Todd and Spike, 1993).

1.4. Substance P Immunoreactivity

The monoclonal antibody raised against substance P (Cuello et al., 1979) recognizes the C-terminal part of the peptide which is common to substance P, NKA and NKB and therefore the antibody does not distinguish between the three tachykinins. Both substance P and NKA are derived from the same gene (preprotachykinin I), and alternative splicing can result in the production of 3 different tachykinin-encoding mRNAs, each of which encode substance P (Helke et al., 1990). It would therefore be expected that all neurons which synthesize NKA would also produce substance P and this has been confirmed by Dalsgaard et al. (1985) who compared consecutive immunostained sections of the same cells in the DRG (after intrathecal injection of colchicine). They found that cells which contained NKA immunoreactivity also displayed substance P immunoreactivity but that not all cells with substance P immunoreactivity were immunoreactive to NKA. Dalsgaard et al. (1985) also observed a parallel decrease of NKA and substance P immunoreactivity in the dorsal horn following dorsal root section. By the use of radioimmunoassay combined with high performance liquid chromatography, Ogawa et al. (1985) found that the distribution of both substance P and NKA was identical in each of the spinal cord, dorsal roots and dorsal root ganglia. In confirmation of the observation by Dalsgaard et al. (1985), Ogawa et al. (1985), found that following unilateral dorsal rhizotomy, the magnitude of a decrease of NKA in the dorsal horn of the lesioned side was comparable to that of substance P. Also Helke et al. (1990) found that the distribution pattern of substance P and NKA were the same in both the spinal cord and sensory neurons. However substance P is present in higher concentration in both the spinal cord and the DRG neurons compared to NKA (Ogawa et al., 1985; Too et al., 1989; Moussaoui et al., 1992b). From these previous findings, it can be concluded that NKA does not occur in neurons without substance P.

Although NKB is present in the dorsal horn, it is derived from a different gene (preprotachykinin II) and neither NKB nor preprotachykinin II mRNA are detectable in primary afferents (Ogawa et al., 1985; Warden and Young, 1988; Moussaoui et al., 1992b). NKB is thought to originate from local spinal neurons since its level in the spinal cord was not affected by dorsal rhizotomy, spinal transection (Ogawa et al., 1985) or neonatal capsaicin treatment (Moussaoui et al., 1992b). Warden and Young (1988) found cells containing preprotachykinin II mRNA in the dorsal horn of the spinal cord and these cells are likely to be the source of NKB-containing axons in the dorsal horn. Although NKB would presumably have been detected with the substance P antibody used in this study, it will not have been present in those axons in which substance P- and CGRP-immunoreactivity coexisted, since these were of primary afferent origin.

Immunostaining obtained with substance P antibody in the present study was similar to that reported previously in the dorsal horn of the rat as well as other different species (Hokfelt et al., 1975; Ljungdahl et al., 1978; Gibson et al., 1981; DeLanerolle and LaMotte, 1982, 1983; Charnay et al., 1983; Ruda et al., 1986; Chung et al., 1989; Todd and Spike, 1993). An extensive plexus of immunoreactive fibres and varicosities was present in lamina I and dorsal part of lamina II with moderate immunoreactivity in the ventral part of lamina II and less immunoreactivity elsewhere in the dorsal horn. Many of the substance P-immunoreactive varicosities had a rostro-caudal orientation in lamina I and dorsal part of lamina II, however axons running in the dorso-ventral direction were also observed in the rest of the dorsal horn. This observation is consistent with the orientation of fibres reported by Ruda et al. (1986). In the present study, no substance P immunoreactive cell bodies were observed in the dorsal horn. Similarly in the human spinal cord, Chung et al. (1989) could not find any substance P-immunoreactive neurons at any level of the spinal cord. In the cat, Tessler et al. (1981) demonstrated the presence of substance P-immunoreactive neurons only in the ventral

horn when no colchicine was applied, however some neurons appeared in the dorsal horn after colchicine treatment. Many investigators have been able to observe immunoreactive neuronal cell bodies in laminae I-III of the dorsal horn after application of colchicine (Hokfelt et al., 1977; Seybold and Elde, 1980; Hunt et al., 1981; Nagy et al., 1981; Nahin, 1987; Leah et al., 1988; Yoshida et al., 1990; Ribeiro-da-Silva et al., 1991; Battaglia and Rustioni, 1992). Gibson et al. (1981) were able to observe occasional neurons in the dorsal horn without the use of colchicine, by using a different method of fixation (benzoquinone instead of paraformaldehyde) and a modified sensitive immunoperoxidase reaction. *In situ* hybridization histochemistry has confirmed the presence of preprotachykinin I mRNA (substance P precursor) in rat dorsal horn (Warden and Young, 1988). The failure of some immunocytochemical studies (including the present one) to detect these neurons is presumably due to very low levels of substance P in cell bodies of dorsal horn neurons.

1.5. CTb Labeling Following Injection into the Sciatic Nerve

The choice of CTb as a transganglionic tracer in the present study was based on evidence from several studies showing that CTb is a relatively specific marker for myelinated primary afferents in somatic nerves (Robertson and Grant, 1985, 1989; LaMotte et al., 1991; Robertson et al., 1991; Rivero-Melian et al., 1992). Robertson and Grant (1989) incubated sections from L5 rat DRG with CTb and then with an antibody against it, to reveal localization of the GM1 ganglioside which is a membrane receptor having a high affinity for CTb (Holmgren et al., 1973). CTb-immunoreactive cells were then examined for possible colocalization with different enzyme markers and neuropeptides. Robertson and Grant (1989) found that the majority of DRG neurons which exhibited CTb-LI were medium sized or large. These CTb-immunoreactive cells rarely contained any of the peptides examined (substance P-, somatostatin- or CGRP-LI)

or FRAP activity. Only 5% of the CTb-immunostained cells contained CGRP-LI and these were thought to give rise to small myelinated (Aδ) fibres, since the majority of cell bodies were medium sized. Similar observations have also been reported in guinea pig (Lindh et al., 1989). Absence of colocalization between CTb-immunoreactivity and either substance P- or CGRP-immunoreactivity was not only reported for DRG neurons but also for terminals labelled in the spinal cord by transganglionic transport of CTb from the sciatic nerve (Rivero-Melian et al., 1992). Conversely, almost all of the CTb-immunoreactive cells (approximately 97%) were found to show RT97 immunoreactivity suggesting that they had myelinated axons (Robertson and Grant,1989; Robertson et al., 1991). When CTb-HRP was injected into the L5 DRG, this resulted in anterograde labelling in lamina I and in the deep dorsal horn of the spinal cord (laminae III-VI) which are regions known to receive myelinated primary afferents (Aβ and Aδ). The central part of lamina II, which is thought to receive primary afferent input exclusively from unmyelinated fibres was devoid of staining (Robertson and Grant, 1985; Rivero-Melian and Grant, 1990). In addition LaMotte et al. (1991) reported that following application of CTb-HRP to the sciatic nerve, 98% of the immunoreactive-axons in the L4 dorsal root were myelinated. Although CTb is clearly transported by some Aδ afferents as shown by the presence of labelling in lamina I, it may be more effectively transported by Aβ fibres, because LaMotte et al. (1991) reported that a much higher proportion of myelinated fibres in the L4 dorsal root with diameters greater than 2μm were CTb-immunoreactive than was the case for small myelinated fibres after injection of CTb-HRP into the sciatic nerve. An additional advantage of CTb is that unlike certain other tracers such as WGA, transneuronal labelling of cells in the dorsal horn is not seen (Robertson and Grant, 1985; Robertson et al., 1992).

Although the most sensitive histochemical method for visualizing CTb-HRP or other HRP-conjugates appears to be the tetramethyl benzidine method, it is less suitable

for combined staining of the tracer and other intraneuronal substances (Lindh et al., 1989). On the other hand, unconjugated CTb has been reported to be a suitable marker for double labelling experiments, since it can be detected easily with immunofluorescence (Lindh et al., 1989; Robertson and Grant, 1989; Rivero-Melian et al., 1992) and that was the reason for its use in the present study. Injection of CTb or CTb-HRP into a peripheral nerve has been reported to be the best mode of application (LaMotte et al., 1991; Rivero-Melian and Grant, 1991). It was found to produce more consistent results and minimize spread to other tissue compared to application of the label to the end of a cut nerve or the use of a capsule containing the tracer.

Following injection of the tracer CTb into the sciatic nerve, the distribution of CTb-immunoreactivity in dorsal horn of the spinal cord was similar to results of previous reports which used either CTb or CTb-HRP injected into the nerve (Rivero-Melian and Grant, 1991; LaMotte et al., 1991; Rivero-Melian et al., 1992; Woolf et al., 1992, 1995). Examination of parasagittal sections from L3-L5 revealed CTb-immunoreactive axon terminals in the medial two-thirds of dorsal horn of the spinal cord. There was a dense plexus of staining extending from the deep part of lamina II to lamina VI. A faint to moderate staining was seen in lamina I. The extension of the anterogarde CTb labelling into the inner part of lamina II was also observed by other investigators (Robertson and Grant, 1985; Rivero-Melian and Grant, 1990, 1991; Broman et al., 1993). The finding in the present study that no labelling was detected in either dendritic profiles or neuronal cell bodies is consistent with other reports which used either CTb or its conjugated form as an anterogradely or transganglionically transported tracer (Robertson and Arvidsson, 1985; Robertson and Grant,1985; Rivero-Melian and Grant, 1990; Broman et al., 1993). Robertson and Grant (1985) reported that a small number of anterogradely labelled structures were in some cases seen on the contralateral side in the dorsal grey commissure and the deep dorsal horn in the most caudal part of L5 segment. As

discussed by Robertson and Grant (1985), this contralateral projection in the L5 segment following CTb-HRP injections into the L5 ganglion was restricted to few labelled structures, however they observed a larger number at the L6-S1 level contralaterally. Rivero-Melian and Grant (1990) reported that contralaterally labelled fibres were found consistently after injections into the L6 DRG but only occasionally after injections into L2-L5 DRGs. They stated that occurrence of contralateral projection of primary afferents depended upon the segmental level and also upon species. Contralateral projection was reported in most of the segments of the spinal cord including those innervating the tail (Light and Perl, 1979; Rethelyi et al., 1979).

1.6. Advantages of Immunofluorescence and Confocal Microscopy

The immunofluorescence technique and confocal microscopy proved extremely useful in the present study since they permitted the relationship between two different groups of primary afferents and an immunocytochemically defined population of neurons in the dorsal horn of the spinal cord to be examined. In the first part of the study, a triple-labelling immunofluorescence technique and the use of three wavelengths of light from the laser to reveal three different fluorochromes (FITC, LRSC, Cy5), made it possible not only to study contacts between the substance P containing varicosities and different parts of the NK-1 receptor-immunoreactive laminae III/IV cells but also to determine the origin of the substance P-containing axons, by demonstrating that in most of them, substance P was colocalized with CGRP. Triple-labelling also allowed the study of substance P input onto retrogradely labelled spinothalamic tract neurons which possessed the NK-1 receptor, in which the retrograde tracer CTb was also revealed with immunofluorescence. Because of the limitation of the laser to only 3 wavelengths, it was not possible to examine the origin of substance P axons which formed contacts onto these spinothalamic tract neurons, however since the

great majority of substance P axons which made contacts onto NK-1 receptor immunoreactive neurons of this type were CGRP-immunoreactive, and there was no obvious morphological difference between spinothalamic tract NK-1 receptor cells in laminae III and IV and the remainder of this population, it is likely that those forming contacts onto spinothalamic tract neurons were also primary afferents. It would be possible to test this by using Fluoro-Gold as a retrograde tracer. Fluoro-Gold is excited by ultraviolet light and can be revealed with epifluorescence but is not detected by the confocal microscope. It can therefore be used in conjunction with triple-labelling immunofluorescence (Pollock et al., 1997).

It is important to stress that double labelling of axons with substance P and CGRP antibodies did not occur as a consequence of either non specific reaction between secondary antibodies and inappropriate primary antibodies, or bleed-through fluorescence. Bleed-through, which results from detection of a fluorescent dye by an inappropriate photomultiplier has been shown by Brelje et al. (1993) to be virtually absent with the dyes, laser and filter combinations used in the present study. Pretreatment of the primary antibody mixture with either substance P or CGRP resulted in loss only of the corresponding type of fluorescence. In addition the presence of numerous strongly immunoreactive single-labelled axons as well as double-labelled ones and the complete lack of colocalization of either peptide with NK-1 receptor provided evidence for absence of either bleed-through or cross-reaction between inappropriate primary and secondary antibodies.

The use of the confocal microscope also pemitted detailed study of the morphological appearance of this population of neurons. With the confocal microscope, it was possible to follow the dorsal dendrites of these neurons even when they entered the dense plexus of immunoreactive dendrites in the superficial laminae.

1.7. Combined Confocal and Electron Microscopy

Minor modifications of the immunofluorescence technique (by including glutaraldehyde in the fixative and avoiding the use of Triton) allowed electron microscopy to be performed on tissues which had been examined with the confocal microscope (Todd, 1997). Although the primary antibodies were already labelled with fluorescent secondary antibodies it was still possible to reveal them with DAB by using biotinylated secondary antibodies and avidin-peroxidase which permitted electron microscopy to be used. Presumably the fluorescent secondary antibodies do not mask all of the sites on the primary antibodies, which means that they can still bind to biotinylated secondary antibodies. Both NK-1 receptor and substance P immunoreactivity were revealed with the same chromogen (DAB) but the two types of immunoreactivity could be easily distinguished by following serial ultrathin sections and comparing the appearance of immunoreactive structures in ultrathin sections with those in the confocal series.

2. NK-1 Receptor on Primary Afferents

In agreement with the previous reports, NK-1 receptor immunoreactivity in the present work was associated mainly with neuronal cell bodies and dendrites. However in addition, thin immunoreactive-profiles with varicosities resembling axons were also occasionally seen, and when followed these were found to originate from a dendrite. These structures might have been axon-like processes which were described by Beal and Cooper (1978) on dendrites of some neurons in the superficial dorsal horn. However the possibility that they were axons which arose from one of the main dendrites can not be ruled out until they are examined with the electron microscope. These immunoreactive beaded processes were also occasionally seen by Littlewood et al. (1995) in the dorsal horn and by Brown et al. (1995) in the ventral horn. In the dorsal

horn, these immunoreactive beaded processes could also be dendrites in which activation of NK-1 receptor (due to some sort of noxious stimulation) resulted in internalization of the receptor and reshaping of the dendrite into a beaded structure (Mantyh et al., 1995). Since these structures were rarely seen, it appears that NK-1 receptor is only infrequently (if at all) associated with axons in the spinal dorsal horn (Brown et al., 1995; Littlewood et al., 1995). This observation is consistent with the reported absence of NK-1 receptor on primary afferents (Brown et al., 1995). There has been controversy concerning the expression of NK-1 receptors on primary afferent neurons (Coggeshall and Carlton, 1997). Brown et al. (1995) did not find expression of NK-1 receptors in the dorsal root or trigeminal ganglia. They performed 3 manipulations (multiple dorsal rhizotomy, sciatic nerve ligation and neonatal capsaicin treatment) to exclude the possibility that the receptor protein was synthesized in DRG neurons but not stored in the cell bodies. No detectable decrease in amount of NK-1 receptor immunoreactivity in the superficial laminae of dorsal horn was observed after either dorsal rhizotomy or capsaicin treatment compared with normal rats. Also the receptor did not accumulate proximal to sciatic nerve ligation which would be expected if the receptor was transported by primary afferent fibres from the DRG neurons. By the use of quantitative autoradiography, Yashpal et al. (1991) found an increase in substance P binding in the superficial dorsal horn following dorsal rhizotomy (which is probably related to supersensitivity that occurs after these lesions). These previous observations, together with the very low level of substance P binding reported in the DRG neurons (Mantyh et al., 1984) suggested that primary afferent neurons do not possess the receptor. However there is some evidence from other studies that NK-1 receptor may be present on primary afferents. Carlton et al. (1996) were able to demonstrate NK-1 receptor immunoreactivity on more than 30% of the unmyelinated axons in the subepidermal plexus of the rat hindpaw skin however they used an immunoperoxidase

method with high concentrations (1: 1000-1:3000) of the same NK-1 receptor antibody used in the present study, and this may have resulted in cross-reactivity with other unkown substance. Their behavioral tests revealed that injection of substance P into the subcutaneous tissue of the third toe resulted in behavioral changes which were interpreted as resulting from pain and these were blocked by the NK-1 receptor antagonist CP 99,994-1 (given by subcutaneous injection) which also implied that these cutaneous fine sensory axons possess the receptor. Carlton et al. (1996) observed that high doses of substance P produced little or no behavioral effects, which they interpreted as desensitization of the receptors and attributed the relatively mild physiological effects of exogenous peripherally applied substance P reported previously (Fitzegerald and Lynn, 1979) to the use of inappropriate doses of substance P. In a further study, Carlton et al. (1998) confirmed the presence of both glutamate receptor- and NK-1 receptor-immunoreactivity in primary afferent sensory fibres in the skin of the rat tail. They reported that, with subcutaneous administration of both substance P and glutamate, substance P enhanced glutamate-induced nociception. Three possible explanations for the failure to detect NK-1 receptor-immunoreactivity on DRG cells were discussed by Carlton et al. (1996, 1998). The first was that NK-1 receptor immunoreactivity on axons in the periphery might reflect cross-reactivity with an unknown antigen, however they concluded that this was unlikely based on the specificity controls previously reported for the antibody (Vigna et al., 1994). A second explanation proposed by Carlton et al. (1996) was that NK-1 receptor may be synthesized by DRG neurons but rapidly transported only to the periphery, however this also appears to be unlikely since Brown et al. (1995) could not detect any accumulation of the receptor proximal to a sciatic nerve ligation. A more likely third possibility was that the level of the receptor on the cell bodies of primary afferents in the DRG might be below the detection level with immunocytochemistry.

Hu et al. (1997) presented pharmacological evidence for the existence of substance P autoreceptors in the membrane of rat DRG neurons. By the whole-cell patch-clamp technique, they identified neurons which responded to bath application of substance P. Seventeen of the identified neurons were chosen for immunocytochemical detection of substance P and 7 of these exhibited substance P immunoreactivity. These observations led them to conclude that some substance P-containing primary afferents express autoreceptors. Brechenmacher et al. (1998) demonstrated pharmacologically expression of all neurokinin receptor subtypes (NK-1, NK-2, NK-3) in cultured rat DRG neurons. Although there have been several electron microscopic studies of the NK-1 receptor in the spinal cord, none of them have provided evidence for axonal staining. Unfortunately there are no reported *in situ* hybridization studies for NK-1 receptor mRNA in DRG cells which would help to resolve this controversy.

3. NK-1 Receptor Neuronal Populations

In agreement with previous studies, the most intensely labelled NK-1 receptor-immunoreactive neurons in the dorsal horn had cell bodies in laminae I, III and IV. The population of large NK-1 receptor-immunoreactive neurons with cell bodies in lamina III/IV and dorsally directed dendrites travelling to the superficial dorsal horn were initially identified by Bleazard et al. (1994) and Liu et al. (1994). Subsequently cells of this type have been observed in several studies (Brown et al., 1995; Littlewood et al., 1995; Marshall et al., 1996). Nakaya et al. (1994) did not comment on these neurons however they are visible in their illustrations of the spinal cord (their fig. 15d). In another recent study by the same group (Ding et al., 1995), the presence of large neurons with well developed dendrites in lamina III and the lateral parts of laminae IV and V, (which presumably correspond to the same population examined in the present study) was also described.

On the basis of their dendritic arborsizations, Brown et al. (1995) recognized three different morphological types of these NK-1 receptor-immunoreactive neurons in laminae III and IV of the lumbar spinal cord, pyramidal, multipolar and fusiform, each type had dorsal dendrites which arborized in the superficial dorsal horn. Pyramidal neurons had large pyramidal cell bodies most often in lamina III and less commonly in lamina IV. These were characterized by large primary dendrite that often reached lamina I (having a length up to 300 μm). Brown et al. (1995) described these primary dendrites as having only few secondary branches however because of the extensive dendritic labelling of NK-1 receptor-immunoreactive neurons in lamina I, they could not rule out the possibility that the dendrites branched in lamina I. Multipolar neurons were distributed in laminae III-V, and had large cell bodies and thick dendrites that arborized in all directions. In sagittal sections, some of their dorsally directed dendrites which reached lamina I also had an extension up to 400 μm in the rostro-caudal direction. These multipolar neurons appeared to have two subtypes, neurons with cell bodies located in lamina III, often had dorsal dendrites that reached lamina I and others located more ventrally in lamina V with dorsal dendrites which rarely reached lamina II. Fusiform neurons were commonly found along the medial border of laminae IV-V. They had small fusiform cell bodies, and dendrites that arborized dorsally and ventrally but they did not show extension in the mediolateral plane. These dendrites were thin, aspiny, generally unbranched and in some cases thin dendritic processes that penetrated the medially adjacent dorsal column white matter were seen. Unlike NK-1 receptor-immunoreactive neurons in lamina I which had a relatively consistent pattern of immunoreactivity at all segments of the cord, Brown et al. (1995) reported that laminae III/IV neurons showed a significant segmental differences with respect to both the numbers and density of cell body labelling. Although these neurons showed intense labelling in the lumbar as well as the sacral spinal cord, they were rarely seen in both

cervical and thoracic regions. In the sacral spinal cord, these neurons were more commonly observed than in the lumbar cord. They were similar in morphology to those reported in the lumbar segments however in some cases medially directed dendritic branches were observed to cross the midline to the contralateral gray matter.

Neurons examined in the present work also had a range of cell shapes. Multipolar, pyramidal and very rarely fusiform types were also recognized, and this confirmed the observation made by Brown et al. (1995). Since the present study was mainly concentrated on cells with somata located between 150-300 μm below the dorsal white matter (because the laminar boundaries are difficult to recognize particularly in the parasagittal sections) and since the fusiform type of neurons are concentrated along the medial border of laminae IV and V (Brown et al., 1995), they were only rarely seen in the present study. These morphologically characterized cells which have well developed dorsally directed dendrites restricted in the medio-lateral direction but extensive in the rostro-caudal direction have also been described by previous investigators using different techniques such as Golgi studies (Scheibel and Scheibel, 1968; Rethelyi and Szentagothai, 1973; Mannen, 1975; Proshansky and Egger, 1977) and intracellular injection studies (Brown, 1981). They are similar to the antenna type neurons described by Rethelyi and Szentagothai (1973) and Maxwell et al. (1983) in the cat spinal cord and also similar to those reported in the rat by Todd (1989). Although cells of this morphological type possess NK-1 receptor immunoreactivity, it is not necessarily the case that all antenna type neurons possess the receptor.

3.1. Contacts between Substance P-Containing Axonal Varicosities and NK-1 Receptor-Immunoreactive Neurons

The NK-1 receptor-immunoreactive neurons studied in the present work were found to receive numerous contacts from substance P-immunoreactive varicosities not only on

superficial dendrites in laminae I and II (where the plexus of substance P-immunoreactivity was dense) but also on dendrites that lay in deeper laminae (in regions in which the density of substance P-containing axons is much lower). At the electron microscopic level, these contacts were shown to be synapses. Substance P in the spinal cord is known to be derived from 3 different sources (Ruda et al., 1986): primary afferents (in which it is colocalized with CGRP; Ribeiro-da-Silva, 1995), fibres descending from raphe nuclei of the brainstem (in which it is colocalized with serotonin; Hokfelt et al., 1978) and local spinal neurons (in which it may be colocalized with enkephalin; Senba et al., 1988; Ribeiro-da-Silva et al., 1991). Ribeiro-da-Silva et al. (1991) investigated substance P and enkephalin colocalization in laminae I-III of the rat cervical spinal cord using immunocytochemistry and electron microscopy. They found that substance P- and enkephalin-immunoreactivity colocalized in a considerable number of axonal varicosities in laminae I and II. These double-labelled varicosities were not found to be elements in synaptic glomeruli. Following colchicine treatment, both substance P- and enkephalin-immunoreactivity were also colocalized in small cell bodies of lamina II neurons and some of lamina I neurons (Senba et al., 1988; Ribeiro-da-Silva et al., 1991. Since enkephalin-immunoreactivity occurs in only a very small number of primary sensory neurons (Ruda et al., 1986) and since that substance P- and enkephalin-immunoreactivity have not been described to colocalize in axons descending from the brainstem and most of axons of such origin terminate in the ventral horn, Ribeiro-da-Silva et al. (1991) suggested that most of substance P and enkephalin double-labelled axons originate from interneurons. Because the great majority of substance P-immunoreactive boutons which contacted NK-1 receptor-immunoreactive laminae III/IV neurons showed immunoreactivity for CGRP, they were presumably of primary afferent origin. However a few of the substance P-immunoreactive boutons which contacted the dendrites did not show CGRP immunoreactivity which means that

they are likely to be derived from local spinal neurons, since there is evidence that substance P-containing fibres descending from the brainstem project to the intermediolateral cell column and ventral horn but not the dorsal horn (Helke et al., 1982; Menetrey and Basbaum, 1987; Maxwell et al., 1996). Maxwell et al. (1996) found that substance P and serotonin did not colocalize in the dorsal horn however they showed considerable colocalization in the ventral horn. Substance P input from local spinal neurons was also confirmed by the observation that some of the boutons which contacted the cells were double-labelled for substance P and enkephalin (Naim, M.M. and Todd, A.J., unpublished observation) however these were infrequent. Unlike the case for this local substance P input, the primary afferent input seemed to be very selective, and in many cases it was possible to see an individual substance P-immunoreactive axon running along the length of the dorsal dendrite of a NK-1 receptor-immunoreactive neuron and making numerous contacts with it. This observation suggests the presence of a very secure synaptic linkage between substance P-containing primary afferents and these neurons.

Physiological experiments have shown that fine primary afferents (which terminate in the superficial dorsal horn) can activate cells in deeper laminae of the dorsal horn of the spinal cord (Fitzegerald and Wall, 1980; Woolf and King, 1987). Todd (1989) suggested that one way in which input from fine primary afferents reaches the deeper laminae of the dorsal horn and excites some of its neurons is through monosynaptic connections with the dorsal dendrites of cells in laminae III and IV. Demonstration of dense input from substance P-immunoreactive varicosities to the dorsal dendrites of these cells in the present study confirmed this suggestion. Another route by which input from unmyelinated afferents could activate neurons in deeper laminae of the dorsal horn was proposed by Light and Kavookjian (1988) who used intracellular recording and injection in the cat and monkey and found that some lamina II neurons had axons that

projected ventrally and made synaptic contacts with deep dorsal horn neurons. The synapses had asymmetrical specialization which suggests that the injected cells may have been excitatory interneurons. The results of Light and Kavookjian (1988) could explain how the fine afferents activate deep neurons whose dendrites do not reach the superficial dorsal horn (Woof and King, 1987). Another possibility suggested by Todd (1989) is that the cells with long dorsal dendrites may have axon collaterals that relay the input from the fine afferents to neurons that do not possess long dorsal dendrites.

In a series of studies, De Koninck et al. (1992) and Ma et al. (1996, 1997) combined intracellular recording and injection in cat dorsal horn neurons *in vivo* with electron microscopic immunocytochemistry to examine the correlation between physiologically identified neurons and innervation by substance P immunoreactive fibres. On the basis of their response properties to different natural innocuous and noxious stimuli, the dorsal horn neurons were classified into nociceptive-specific, wide-dynamic-range and non-nociceptive. The nociceptive response emphasized in these studies was a slow EPSP following noxious mechanical stimulation of the skin. It is thought that this response is mediated by substance P since it has been shown to be blocked by a specific NK-1 receptor antagonist, CP 96, 345 (De Koninck and Henry, 1991; Radhakreshnan and Henry, 1995).

In these studies, 7 wide-dynamic-range neurons were successfully labelled with HRP, of which 1 was in lamina II (near the border between laminae II and III), 2 were in lamina III, 2 were in lamina IV and the remaining 2 had cell bodies in lamina V. The neuron located in lamina II had a stellate-shaped cell body. Most of its dendrites arborized in lamina II and III and some of these were spiny. This cell also had dorsal dendrites that penetrated into lamina I where they arborized into smaller branches. The 2 neurons with cell bodies in lamina III, were similar in morphology. They had stellate shaped somata and an extensive dendritic tree that arborized in lamina III however few

of their dendrites extended dorsally to the border between lamina I and II. Most of their dendrites were spiny except the dorsally directed ones. One of the neurons recorded in lamina IV had a large multipolar cell body. Although most of its dendrites branched within lamina IV, one dendrite extended dorsally as far as the middle third of lamina II and some dendrites reached lamina V. The dendrites of this cell were devoid of spines. The other lamina IV neuron had a triangular shaped cell body. Most of its dendrites passed dorsally to lamina III and inner part of lamina II, while a few branches extended ventrally to laminae V and VI. The two lamina V neurons had ovoid somata which gave rise to 3-5 main dendrites that were mostly oriented dorsally with a few extending to lamina VI. Some of the dorsal dendrites of these neurons reached to the border between laminae I and II. The sample of nociceptive-specific neurons in these three studies included 3 neurons in lamina I, while the non-nociceptive ones included 1 neuron in the inner part of lamina II, 1 neuron in lamina III and several neurons in lamina IV. The non-nociceptive lamina III neuron had dorsally directed dendrites and an axon which passed into the dorsal column suggesting that this cell may have belonged to the PSDC pathway (Brown, 1981). The neurons in lamina IV had multipolar cell bodies and dendritic trees that branched mostly within the limits of the lamina, however some of their dendrites extended dorsally and reached lamina II. Two of these lamina IV neurons had morphological characteristics similar to spinocervical tract neurons (Brown, 1981) with dendrites that extended rostrocaudally and axons that could be traced to the dorsolateral funiculus.

De Koninck et al. (1992) and Ma et al. (1996, 1997) found that all of the sample of nociceptive neurons (both nociceptive-specific and wide-dynamic-range) received a considerable number of substance P-immunoreactive varicosities which apposed the cell bodies and dendrites (with a greater density of contacts on dorsal dendrites compared to ventral dendrites and cell bodies). Their quantitative analysis indicated that nociceptive-

specific neurons had a significantly higher density of substance P-immunoreactive boutons contacting their cell bodies and proximal dendrites than the wide-dynamic-range neurons but, that this was not the case for their distal dendrites. Since they also found that wide-dynamic-range neurons with a strong nociceptive component to their receptive fields had a denser innervation from substance P-immunoreactive axons than other wide-dynamic-range cells, they suggested the presence of a correlation between the intensity of the nociceptive response and the degree of innervation by substance P-immunoreactive boutons. They emphasized that substance P-immunoreactive input was always associated with aspiny dendrites. In contrast to nociceptive neurons, the non-nociceptive neurons scarcely received contacts from substance P-immunoreactive varicosities. Furthermore, they found that a considerable number of substance P-immunoreactive boutons which contacted the nociceptive neurons contain enkephalin immunoreactivity (Ma et al., 1997).

From the detailed description of the intracellularly-labelled wide-dynamic-range neurons in these three studies, they appear to be different in their morphology from the population of NK-1 receptor-immunoreactive neurons examined in the present study. The dendritic arborizations of the neurons studied by De Koninck et al. (1992) and Ma et al. (1996, 1997) did not extend to lamina I, and some of them had different laminar locations (those with cell bodies in laminae II and V) and dendritic spines. Although the wide-dynamic-range neurons are likely to possess the NK-1 receptor since they responded to noxious stimulation with a slow prolonged EPSP which was thought to be mediated by substance P, they are apparently of a different type from the NK-1 receptor-immunoreactive neurons which were examined in the present study. In fact NK-1 receptor-immunoreactive neurons which were located either near the border between laminae II and III, or in lamina V were observed by Brown et al. (1995) and also in the present study, however the present study was concerned only with neurons that are

similar to antenna type cells.

At the utrastructural level, Ma et al. (1996) found synapses at 33.5% of the contacts made by substance P-immunoreactive profiles onto the dendrites of their recorded cells, whereas examination of serial sections in the present work revealed that synapses were present at all of the sample of substance P-immunoreactive contacts which were examined. As discussed by Ma et al. (1996), this apparent discrepancy was most probably because their quantification was based on studying isolated sections while the present data was based on studying serial sections, thus the percentage of boutons forming synapses would be much higher (possibly 100%) if they had used serial sections. The presence of numerous contacts from substance P-immunoreactive varicosities onto neurons examined in the present study allowed the selection of those for which the contact was at a right angle to the plane of section and this would have increased the chance of observing synapses. A more significant difference between the data presented here and that of Ma et al. (1996) is the high degree of coexistence between CGRP- and substance P-immunoreactivity in boutons that contacted the NK-1 receptor immunostained-profiles in the present work, whereas Ma et al. (1996) found that only 30% of the boutons were also CGRP-immunoreactive. As reported by Ma et al. (1996), this might have been because the postembedding immunocytochemistry they performed on ultrathin sections had a lower sensitivity for detecting CGRP than the immunofluorescent method used in the present study. Another likely explanation would be that the neuronal populations which they examined differed from the ones in this study. Ma et al. (1997) also reported that a considerable number of boutons which contained both substance P- and enkephalin-immunoreactivity (and were therefore probably derived from local interneurons; Senba et al., 1988; Ribeiro-da-Silva et al., 1991), contacted the nociceptive (nociceptive-specific and wide-dynamic-range) cells. However the enkephalin/substance P double-labelled varicosities which made contacts

on the NK-1 receptor-immunoreactive laminae III/IV cells were infrequent (Naim, M.M. and Todd, A.J., unpublished observations), which suggests that neurons of the type studied in the present work receive only a minor input from substance P-containing interneurons and a major input from primary afferents. Again this difference might have been due to the fact that different populations of neurons were examined.

Liu et al. (1994) studied the relationship between substance P-immunoreactive axon terminals and NK-1 receptor-immunoreactive neurons at both the light and electron microscopic level in different areas of the central nervous system of the rat, including the dorsal horn of the spinal cord. At the electron microscopic level, they found that although some substance P-immunoreactive terminals contacted the NK-1 receptor-immunoreactive membranes, no more than 15% of the membrane received contacts from these terminals. They described this as a mismatch between substance P and NK-1 receptor, since the receptor was present all over the somatic and dendritic membranes rather than being inserted only into regions of the neuronal surface where substance P-containing axons made contacts. Liu et al. (1994) concluded that almost the entire surface of neurons expressing the receptor (either synaptic or nonsynaptic locations) could be acted upon by substance P, assuming that the peptide could diffuse from its site of release (volume transmission). In agreement with this suggestion, they found that in 90% of substance P-immunoreactive synaptic profiles, the dense core vesicles (the storage site of peptides) were located away from the synaptic junction, which is also consistent with the evidence that dense core vesicles undergo exocytosis at sites distant from the synaptic zone (Zhu et al., 1986). The present results support the observation of Liu et al. (1994) that much of the plasma membrane of cells with NK-1 receptor is not in contact with substance P-containing boutons. However, the density of innervation of the neurons studied in the present work by substance P-containing afferents was much higher than that of choline acetyl transeferase (ChAT)-immunoreactive lamina III

neurons which do not possess NK-1 receptor (Naim et al., 1997). This suggests that the dorsal dendrites of the NK-1 receptor-immunoreactive neurons will be exposed to particularly high concentrations of the peptide after its release. The high concentration of substance P is likely to activate these neurons and this has been shown by Mantyh et al. (1995) who demonstrated internalization of NK-1 receptor in the distal parts of dorsal dendrites of this type of neurons in lamina III (following noxious stimulation of the skin).

Demonstration of asymmetrical synapses at sites of contacts between substance P-immunoreactive varicosites and NK-1 receptor-immunoreactive dendrites of laminae III/IV cells in the present study is in agreement with observations made in rat (Liu et al., 1994) and in cat (DeKoninck et al., 1992; Ma et al., 1996). These findings raise a question about the significance of these synapses if substance P is released from its storage sites away from the synaptic location and acts through volume transmission. The presence of the synapses is presumably significant for glutamatergic transmission because substance P-containing primary afferents are enriched with the fast transmitter glutamate which acts at these synapses (De Biasi and Rustioni, 1988). Several studies have suggested a combined role for both excitatory amino acid and substance P in transmission of sensory information including nociceptive information within the dorsal horn of the spinal cord (Salt and Eaton, 1989; Dougherty and Willis, 1991). It has been proposed that the non-NMDA glutamate receptors are associated with transmission of information from sensitive mechanoreceptors while NMDA receptors and receptors for peptides such as substance P participate in transmission from nociceptors to dorsal horn neurons (Salt and Hill, 1983; Morris, 1989). In the present study, it was demonstrated that the great majority of these presynaptic substance P-containing boutons also contained CGRP which is thought to be coreleased with substance P and inhibits its degradation through inhibition of an endopeptidase which degrades substance P (Le

Greves et al., 1985).

3.2. Substance P Input to Spinothalamic Tract Neurons

It has been previously reported that some of laminae III/IV NK-1 receptor-immunoreactive neurons of the type examined in the present work belong to the spinothalamic tract (Marshall et al., 1996). Marshall et al. (1996) suggested that the NK-1 receptor laminae III/IV cells which have dendrites that enter the superficial dorsal horn are one of two groups of spinothalamic tract neurons which are likely to be influenced directly through NK-1 receptors by substance P released from nociceptive primary afferents terminating in the superficial dorsal horn (the other group being those with cell bodies in lamina I). The present results supported this suggestion by demonstrating that these neurons received a dense innervation from substance P-immunoreactive axon terminals.

Demonstration of heavy input from substance P immunoreactive axons onto spinothalamic tract neurons presented here is in good agreement with results from electrophysiological and pharmacological studies carried out on primate spinothalamic tract neurons located in laminae I-VI of the dorsal horn (Dougherty and Willis, 1991; Dougherty et al., 1994; 1995). These experiments revealed potentiation of excitatory amino acid-induced discharges of spinothalamic tract neurons after co-adminstration of NMDA and substance P. In addition, Dougherty et al. (1994) demonstrated that NK-1 receptor antagonists prevent sensitization of monkey spinothalamic tract neurons after intradermal injection of capsaicin.

Since many neurons in the dorsal horn project to sites in the brain other than the thalamus (Willis and Coggeshall, 1991), NK-1 receptor-immunoreactive neurons in laminae III/IV might also belong to various other ascending tracts in addition to the spinothalamic tract. Ding et al. (1995) found that occasional NK-1 receptor-

immunoreactive neurons in lamina IV belong to the spinoparabrachial tract however they did not describe the morphology of these cells. Many neurons which project to the lateral reticular nucleus (Lima, 1990; Lima and Coimbra, 1990; Lima et al., 1991) were found to be NK-1 receptor-immunoreactive and these included cells in laminae III and IV with long dorsal dendrites (Naim M. M., Todd A. J. and Shehab S. A. S., unpublished observations). It is possible that at least some of the neurons examined in the present study also belong to this tract. However neurons of this type do not apparently give origin to the spinocervical tract or PSDC pathway, since the spinocervical tract neurons reported in the cat have a different morphology (with dorsal dendrites that rarely penetrate lamina II) while cells of the PSDC pathway were found not to possess the receptor (Polgar E., Shehab S. A. S., Watt C. and Todd A. J., unpublished observations)

3.3. Contacts from Myelinated Primary Afferents

There have been few reports which studied the density of input from myelinated primary afferents onto dorsal horn neurons. However Brown and Noble (1982) examined the relationship between the terminal arborizations of hair follicle afferent fibres and dendritic trees of spinocervical tract neurons using both the intraaxonal and intracellular injections of HRP in the cat. Their sample included 17 afferent-neuron pairs of which 10 had spinocervical tract neurons with a receptive field containing that of the hair follicle afferent, and 7 pairs had separate receptive fields. In the 10 cases where the receptive fields overlapped, numerous contacts (40-60) between the labelled afferent and the spinocervical tract neurons were observed, while in the remaining 7 cases no contacts were seen. Since this study was carried out by light microscopy, it was not possible to confirm that synapses were present at points of contacts.

Examination of double labelled sections in the present study revealed the presence

of a significant number of contacts from CTb-labelled primary afferent axon terminals onto NK-1 receptor-immunoreactive lamina III/IV neurons. Although most of these contacts were seen on cell bodies, proximal parts of dorsal dendrites and ventral dendrites (parts of these neurons which lay within the CTb-immunoreactive plexus), some of the cells also received contacts on their distal parts of dorsal dendrites in laminae I and II. In comparison to Aβ afferents input from hair follicles onto spinocervical tract neurons in laminae III/IV, which had been studied by Brown and Noble (1982), the density of myelinated input to NK-1 receptor-immunoreactive neurons examined in the present study appeared to be much lower. The number of contacts from a single hair follicle afferent onto spinocervical tract neurons reported by Brown and Noble (1982) ranged from 40-60 contacts, and each spinocervical tract cell presumably receive input from many hair follicle afferents. Most CTb-immunoreactive contacts on the NK-1 receptor-immunoreactive neurons were located on dendritic shafts however some contacts were on dendritic spines (unlike those from substance P-immunoreactive varicosities). This latter observation is consistent with the findings of Brown and Noble (1982) who reported that 5% of contacts from hair follicle afferents on spinocervical tract neurons involved dendritic spines. It is also consistent with findings obtained from other intracellular recording studies which suggested a direct association of dendritic spines with hair follicle afferent input (De Koninck et al., 1992).

From the present results, it is not possible to determine the type of myelinated primary afferents which contacted the NK-1 receptor-immunoreactive cells. However the CTb-labelled afferents seen in lamina I are presumably derived from Aδ high-threshold mechanoreceptors while those in the ventral part of lamina II are likely to have been Aδ down-hair afferents (Light and Perl, 1979). Labelled afferents in the dorsal part of lamina III are likely to be from either Aδ down hair or from Aβ mechanoreceptors including hair follicles, rapidly adapting, slowly adapting type I or

type II mechanoreceptors while those in the remaining part of the CTb plexus were presumably all from Aβ afferents (Brown, 1981).

The use of electron microscopy in the present study revealed that synapses were present at some of the CTb-immunoreactive contacts. Unlike the case for substance P-immunoreactive afferents, not all CTb-immunoreactive contacts seen with the confocal microscope were synapses. The failure to identify synapses was in some cases due to obliquity of the contact, however in some others no sign of synaptic specialization was observed even though the contact was not oblique. Moreover, the electron microscopic analysis revealed separation of some of the boutons from the NK-1 receptor-immunoreactive neuron, although the confocal microscope showed apparent contacts. This discrepancy could be attributed to the limitation of the confocal microscopic resolution in the Z axis. Therefore profiles which appear contacting with the confocal microscope can be found at different depth when viewed with the electron microscope. Some previous studies have assumed that contacts between primary afferents and spinal cord neurons are synapses (Brown and Fyffe, 1981a; Brown and Noble, 1982). Criteria used in these studies included the presence of a bouton on the terminal axonal arborizations and no evidence of a gap between the bouton and the neuron's dendrite or soma. Although these criteria were fulfilled at the light microscopic level in the present study, some of the contacts between the CTb-labelled primary afferents and NK-1 receptor-immunoreactive neurons were proved not to be in contact when examined with the electron microscope (as there were small profiles such as glial cells intervening). This highlights the need for electron microscopy to confirm that contacts are actually associated with synapses in studies of neuronal connections.

Quantitative analysis revealed that for the 20 neurons examined in detail, the number of contacts varied from 24 to 99 contacts, however this number was probably underestimated since the 60 μm thick sections did not always contain the whole of the

neuron. The estimated densities of contacts on these neurons are likely to have been underestimated since it is possible that the transganglionic transport did not reveal all of the myelinated afferents in the injected nerve. However LaMotte et al. (1991) reported that after sciatic nerve injection approximately 75% of L4 dorsal root fibres with diameters greater than 2 μm (Aβ fibres) were CTb-labelled. Since the L4 dorsal root contains fibres which do not travel in the sciatic nerve (for example those of the dorsal primary ramus), it seems likely that the great majority of Aβ fibres in the nerve had transported CTb. In addition, the high density of CTb-labelled varicosities seen with oil-immersion lens in a single confocal image (figs. 9b, 11a) suggests that a high proportion of the terminals of myelinated afferents were labelled.

Comparison between the densities of contacts from CTb-labelled axonal varicosities on NK-1 receptor-immunoreactive lamina III/IV neurons and those reported from substance P-immunoreactive axonal boutons (Naim et al., 1997) reveals that these cells receive a much higher input from substance P-containing afferents (dorsal dendrites within laminae I and II received 18.8 contacts per 100 μm, while dorsal dendrites below lamina II received 13.2 contacts per 100 μm and ventral dendrites received 5.5 contacts per 100 μm).

3.4. Other Inputs to Neurons of This Type

Primary afferent input to lamina III/IV neurons which have dendrites that enter the superficial dorsal horn was first examined by Todd (1989) who studied 5 Golgi-stained neurons, 3 of them were from animals in which 2 or 3 dorsal roots had been cut 26 or 30 hours peviously. All 3 neurons from the operated animals received numerous synapses in lamina II, and between 13 and 16% of these (24-31% of asymmetric synapses) were from degenerating axons. In lamina III, all the 3 cells were postsynaptic to degenerating axons as well. The 2 neurons selected from the unoperated animals also received many

synapses in lamina II. Very few of these synapses involved the central elements of glomeruli which suggested that they received a very minor input from non-peptidergic C-fibres, that terminate as the central axons of glumeruli. Only one example of a dendrodendritic synapse involving a stained dendrite was seen, which suggested that unlike the intrinsic neurons of lamina II which commonly receive dendrodendritic synapses (Todd, 1988), these neurons in deeper laminae were not a major target for presynaptic dendrites belonging to lamina II cells. Todd (1989) stated that the figures presented in his study for synapses involving degenerated axons in lamina II were likely to have been underestimated because only 2 or 3 dorsal roots were cut, therefore some primary afferent fibres may have survived the procedure. In addition the survival time may not have been enough for the complete degeneration of all primary afferents.

In addition to the primary afferent input, lamina III/IV neurons which have dorsally directed dendrites and possess NK-1 receptor have been shown to receive other inputs. Polgar and Todd (1997) demonstrated that these neurons receive a substantial input on their cell bodies and entire dendritic tree from synapses at which gephyrin (the glycine receptor associated protein) was present. Polgar, E., Shehab, S. A. S., Watt, C. and Todd, A. J. (unpublished observations) have recently found that neurons of this type also receive a dense innervation from NPY-immunoreactive axons, and synapses were found with electron microscopy. They demonstrated that the great majority of the NPY-immunoreactive boutons were GABA- immunoreactive which suggests that these axons are derived from local NPY-containing inhibitory interneurons which are known to be GABAergic (Rowan et al., 1993). This NPY input was specific for this type of dorsal horn neuron, since PSDC neurons which were also located in laminae III-V but did not possess the NK-1 receptors received only a small number of contacts from NPY-immunoreactive boutons. The NK-1 receptor-immunostained neurons were found to receive very few contacts from nitric oxide synthase (NOS)-immunoreactive axons,

which are derived from another population of local GABAergic neurons (Spike et al., 1993; Laing et al., 1994).

3.5. Functional Properties of Lamina III/IV NK-1 Receptor-Immunoreactive Neurons

Neurons examined in the present study were found to receive a substantial monosynaptic input from substance P-immunoreactive afferents as well as a less dense monosynaptic input from myelinated primary afferents. Since all substance P-containing primary afferents are thought to be nociceptors (Lawson et al., 1997), while the myelinated afferents observed in the present study are likely to have been low-threshold mechanoreceptors, these neurons are likely to have wide-dynamic-range receptive fields with a strong nociceptive component. There is some direct evidence that these neurons are activated by noxious stimulation. Doyle et al. (1997) demonstrated that some neurons of this type express *c-fos* after acute noxious chemical stimulation (topical application of mustard oil to the skin for 2 hours). *C-fos*-immunoreactivity has also been observed in neurons of this type following formalin injection into the rat foot (Todd, A.J. and Shehab, S.A.S., unpublished observation).

In response to either acute mechanical or chemical (subcutaneous injection of capsaicin into the rat hindpaw) noxious stimulation, the dorsal dendrites of these neurons were shown to internalize the NK-1 receptor, presumably due to activation by substance P that is released by noxious stimulation (Mantyh et al., 1995). In some cases, the thin distal dendrites showed evidence of structural reorganization in which the dendrite appeared as strings of swollen varicosities connected by thin fibres which is thought to be due to internalization of the plasma membrane in addition to the receptor. Mantyh et al. (1995) reported that internalization of the receptor occurred only in laminae I and II, the region of maximal substance P release (Duggan et al., 1987), and they could not detect internalization of the receptor on the proximal parts of dorsal

dendrites of these neurons (that lay in lamina III) or on the cell body. In contrast, in rats with persistent hindpaw inflammation, acute noxious stimulation caused internalization of the receptor in many of these neurons including the proximal parts of dendrites and the cell body (Abbadie et al., 1997). Furthermore innocuous stimulation (brush for 2 min) of the chronically inflamed hindpaw was found to induce internalization of NK-1 receptor on the cell body and proximal dendrites of these neurons in addition to the distal dendrites. This presumably indicates the development of peripheral sensitization in which substance P is released from primary afferents after innocuous stimulation, which may contribute to mechanical allodynia (Abbadie et al., 1997). This latter observation provides evidence that the altered properties of dorsal horn neurons which occur in the setting of injury or inflammation involve release of substance P and interaction with dorsal horn neurons that express the NK-1 receptor.

Failure to detect internalization of the receptor on dorsal dendrites that lay within lamina III following acute noxious stimultion in normal animal is an unexpected finding, since these dendrites often received numerous contacts from substance P-immunoreacitve varicosities in the present study. This anomaly could be related to various possibilities. It is possible that in the experiments carried out by Mantyh et al. (1995), substance P-containing axons that contacted the dendrites in lamina III did not release substance P in the normal animal even following acute noxious stimuli either because they were not activated by the type of the stimulus used, or because they act as "silent nociceptors" (Handwerker et al., 1991) that only become sensitized in the setting of inflammation. An alternative explanation is that these axons could release substance P but that the amount released in normal animal is not sufficient to internalize the receptor, compared to the large amounts released in laminae I and II by the numerous substance P afferents in these laminae. Different explanations of the changes in patterns of internalization that occur in the setting of chronic inflammation have been proposed.

Internalization of NK-1 receptor on the proximal parts of dendrites and cell bodies of the NK-1 receptor-immunoreactive neurons in the case of inflammation might result from a significant diffusion of enhanced substance P release (Duggan et al., 1988; Schaible et al., 1990) from the superficial to deep dorsal horn. Another possibility is that substance P input which induce internalization in the deep parts of these cells (in response to both noxious and innocuous stimulation) could derive from large diameter Aβ afferents which do not synthesize substance P in normal animals but synthesize it in cases of peripheral nerve injury (Noguchi et al., 1994) and inflammation (Neumann et al., 1996). Thus both small and large diameter afferents may be releasing substance P that induces internalization, the former to the distal parts of dorsally directed dendrites of these lamina III/IV neurons and the latter to the cell bodies and proximal dendrites in the deep dorsal horn (Abbadie et al., 1997).

References

Abbadie, C., Trafton, J., Liu, H., Mantyh, P.W. & Basbaum, A.I. (1997) Inflammation increases the distribution of dorsal horn neurons that internalize the neurokinin-1 receptor in response to noxious and non-noxious stimulation. Journal of Neuroscience, 17, 8049-8060.

Abols, I.A. & Basbaum, A.I. (1981) Afferent connections of the rostral medulla of the cat: a neural-substrate for midbrain-medullary interactions in the modulation of pain. Journal of Comparative Neurology, 201, 285-297.

Alarcon, G. & Cervero, F. (1990) The effects of electrical stimulation of A and C visceral afferent fibres on the excitability of viscerosomatic neurons in the thoracic spinal cord of the cat. Brain Research, 509, 24-30.

Albe-Fessard, D., Berkley, K.J., Kruger, L., Ralston, H.J. & Willis, W.D. (1985) Diencephalic mechanisms of pain sensation. Brain Research, 356, 217-296.

Alvarez, F.J. & Priestley, J.V. (1990a) Anatomy of somatostatin-immunoreactive fibres and cell bodies in the rat trigeminal subnucleus caudalis. Neuroscience, 38, 343-357.

Alvarez, F.J. & Priestley, J.V. (1990b) Ultrastructure of somatostatin-immunoreactive nerve terminals in lamina I and II of the rat trigeminal subnucleus caudalis. Neuroscience, 38, 359-371.

Alvarez, F.J., Kavookjian, A.M. & Light, A.R. (1992) Synaptic interactions between GABA-immunoreactive profiles and the terminals of functionally defined myelinated nociceptors in the monkey and cat spinal cord. Journal of Neuroscience, 12, 2901-2917.

Alvarez, F.J., Kavookjian, A.M. & Light, A.R. (1993) Ultrastructural morphology, sunaptic relationships and CGRP immunoreactivity of physiologically identified C-fibre terminals in the monkey spinal cord. Journal of Comparative Neurology, 329, 472-490.

Alvarez, F.J., Morris, H.R. & Priestly, J.V. (1991) Sub-populations of small diameter trigeminal primary afferent neurons defined by expression of calcitonin gene-related peptide and the cell surface oligosaccharide recognized by monoclonal antibody LA4. Journal of Neurocytology, 20, 716-731.

Ambalavanar, R. & Morris, R. (1992) The distribution of binding by isolectin I-B4 from Griffonia Simplicifolia in the trigeminal ganglion and brainstem trigeminal nuclei in the rat. Neuroscience, 47, 421-429.

Ambalavanar, R. & Morris, R. (1993) An ultrastructural study of the binding of an α-D-galactose specific lectin from Griffonia Simplicifolia to trigeminal ganglion neurons and the trigeminal nucleus caudalis in the rat. Neuroscience, 52, 699-709.

Angaut-Petit, D. (1975a) The dorsal column system: existence of long ascending postsynaptic fibres in the cat's fasciculus gracilis. Experimental Brain Research, 22, 457-470.

Angaut-Petit, D. (1975b) The dorsal column system: functional properties and bulbar relay of the postsynatic fibres of the cat' s fasciculus gracilis. Experimental Brain Research, 22, 471-493.

Apkarian, A.V. & Hodge, C.J. (1989) The primate spinothalamic pathways: I. a quantitative study of the cells of origin of the spinothalamic pathway. Journal of Comparative Neurology, 288, 447-473.

Averill, S., McMahon, S.B., Clary, D.O., Reichardt, L.F. & Priestley, J.V. (1995) Immunocytochemical localization of trkA receptors in chemically identified subgroups of adult rat sensory neurons. European Journal of Neuroscience, 7, 1484-1494.

Baker, M.L. & Giesler, G.J. (1984) Anatomical studies of the spinocervical tract of the rat. Somatosensory Research, 2, 1-18.

Bannatyne, B.A., Maxwell, D.J. & Brown, A.G. (1987) Fine structure of synapses associated with characterized postsynaptic dorsal column neurons in the cat. Neuroscience, 23, 597-612.

Bannatyne, B.A., Maxwell, D.J., Fyffe, R.E.W. & Brown, A.G. (1984) Fine structure of primary afferent axon terminals of slowly adapting cutaneous receptors in the cat. Quarterly Journal of Experimental Physiology, 69, 547-557.

Battaglia, G & Rustioni, A. (1992) Substance P innervation of the rat and cat thalamus. II. Cells of origin in the spinal cord. Journal of Comparative Neurology, 315, 473-486.

Beal, J.A. & Cooper, M.H. (1978) The neurons in the gelatinosal complex (laminae II and III) of the monkey (Macaca mulatta): a Golgi study. Journal of Comparative Neurology, 179, 89-122.

Bennett, G.J., Nishikawa, N, Lu, G. W., Hoffert, M.J. & Dubner, R. (1984) The morphology of dorsal column postsynaptic (DCPS) spinomedullary neurons in the cat. Journal of Comparative Neurology, 224, 568-578.

Bertaccini, G. (1976) Active polypeptides of non-mammalian origin. Pharmacological Reviews, 28, 127-177.

Blair, R.W., Ammons, W.S. & Foreman, R.D. (1984) Responses of thoracic spinothalamic and spinoreticular cells to coronary artery occlusion. Journal of Neurophysiology, 51, 636-648.

Bleazard, L., Hill, R.G. & Morris, R. (1994) The correlation between the distribution of the NK-1 receptor and the actions of tachykinin agonists in the dorsal horn of the rat indicates that substance P does not have a functional role on substantia gelatinosa (lamina II) neurons. Journal of Neuroscience, 14, 7655-7664.

Boehmer, C.G., Norman, J., Catton, M., Fine, L.G. & Mantyh, P.W. (1989) High levels of mRNA coding for substance P, somatostatin and alpha-tubulin are expressed by rat and rabbit dorsal root ganglion neurons. Peptides, 10, 1179-1194.

Bowsher, D. & Abdel-Maguid, T.E. (1984) Superficial dorsal horn of the adult human spinal cord. Neurosurgery, 15, 893-899.

Brechenmacher, C., Larmet, Y., Feltz, P. & Rodeau, J-L. (1998) Cultured rat sensory neurons express functional tachykinin receptor subtypes 1, 2 and 3. Neuroscience Letters, 241, 159-162.

Brelje, T.C., Wessendorf, M.W. & Sorenson, R.L. (1993) Multicolour laser scanning confocal immunofluorescence microscopy: practical applications and limitations. In Cell Biological Applications Of Confocal Microscopy, ed. Matsumoto, B., pp 97-181. San Diego: Academic Press.

Broman, J., Anderson, S. & Ottersen, O.P. (1993) Enrichment of glutamate-like immunoreactivity in primary afferent terminals throughout the spinal cord dorsal horn. European Journal of Neuroscience, 5, 1050-1061.

Brown, A.G. & Franz, D.N. (1969) Responses of spinocervical tract neurons to natural stimulation of identified cutaneous receptors. Experimental Brain Research, 7, 231-249.

Brown, A.G. & Fyffe, R.E.W. (1981a) Direct observations on the contacts made between Ia afferent fibres and α-motoneurons in the cat's lumbosacral spinal cord. Journal of Physiology, 313, 121-140.

Brown, A.G. & Fyffe, R.E.W. (1981b) Form and function of dorsal horn neurons with axons ascending the dorsal columns in cat. Journal of Physiology, 321, 31-47.

Brown, A.G. & Noble, R. (1982) Connexions between hair follicle afferent fibres and spinocervical tract neurons in the cat: the synthesis of receptive fields. Journal of Physiology, 232, 77-91.

Brown, A.G. (1971) Effects of descending impulses on transmission through the spinocervical tract. Journal of Physiology, 219, 103-125.

Brown, A.G. (1981) Organization in the Spinal Cord: the Anatomy and Physiology of Identified Neurons. Berlin: Springer-Verlag.

Brown, A.G., Fyffe, R.E.W, Rose, P.K. & Snow, P.J. (1981) Spinal cord collaterals from axons of type II slowly adapting units in the cat. Journal of Physiology, 316, 469-480.

Brown, A.G., Fyffe, R.E.W. & Noble, R. (1980a) Projections from Pacinian corpuscles and rapidly adapting mechanoreceptors of glabrous skin to the cat's spinal cord. Journal of Physiology, 307, 385-400.

Brown, A.G., Fyffe, R.E.W., Noble, R, Rose, P.K. & Snow, P.J. (1980b) The density, distribution and topographical organization of spinocervical tract neurons in the cat. Journal of Physiology, 300, 409-428.

Brown, A.G., Rose, P.K. & Snow, P.J. (1977) The morphology of hair follicle afferent fibre collaterals in the spinal cord of the cat. Journal of Physiology, 272, 779-797.

Brown, A.G., Rose, P.K. & Snow, P.J. (1978) Morpholgy and organization of axon collaterals from afferent fibres of slowly adapting type I units in cat spinal cord. Journal of Physiology, 277, 15-27.

Brown, A.G., Rose, P.K. & Snow, P.J. (1980c) Dendritic trees and cutaneous receptive fields of adjacent spinocervical tract neurons in the cat. Journal of Physiology, 300, 429-440.

Brown, J.L., Liu, H., Maggio, J.E., Vigna, S.R., Mantyh, P.W. & Basbaum, A.I. (1995) Morphological characterization of substance P receptor-immunoreactive neurons in the rat spinal cord and trigeminal nucleus caudalis. Journal of Comparative Neurology, 356, 327-344.

Bryan, R.N., Coulter, J.D. & Willis, W.D. (1974) Cells of origin of the spinocervical tract in the monkey. Experimental Neurology, 42, 574-586.

Bryan, R.N., Trevino, D.L., Coulter, J.D. & Willis, W.D. (1973) Location and somatotopic organization of the cells of origin of the spinocervical tract. Experimental Brain Research, 17, 177-189.

Burstein, R., Dado, R.J. & Giesler, G.J. (1990) The cells of origin of the spinothalamic tract of the rat: a quantitative reexamination. Brain Research, 511, 329-337.

Cameron, A.A., Leah, J.D. & Snow, P.J. (1986) The electrophysiological and morphological characteristics of feline dorsal root ganglion cells. Brain Research, 362, 1-6.

Cao, Y.Q., Mantyh, P.W., Carlson, E.J. Gillespie, A-M, Epstein, C.J. & Basbaum, A.I. (1998) Primay afferent tachykinins are requierd to experience moderate to intense pain. Nature, 392, 390-394.

Carlton, S.M., LaMotte, C.C., Honda, C.N., Surmeier, D.J., DeLanerolle, N. & Willis, W.D. (1989) Ultrastructural analysis of axosomatic contacts on functionally identified primate spinothalamic tract neurons. Journal of Comparative Neurology, 281, 555-566

Carlton, S.M., LaMotte, C.C., Honda, C.N., Surmeier, D.J., DeLanerolle, N. & Willis, W.D. (1985) Ultrastructural analysis of substance P and other synaptic profiles innervating an identified primate spinothalamic tract neuron. Society for Neuroscience Abstracts, 11, 578.

Carlton, S.M., McNeill, D.L., Chung, K. & Coggeshall. R.E. (1988) Organization of calcitonin gene-related peptide immunoreactive terminals in the primate dorsal horn. Journal of Comparative Neurology, 276, 527-536.

Carlton, S.M., Westlund, K.N., Zhang, D. Sorkin, L.S., & Willis, W.D. (1990) Calcitonin gene-related peptide containing primary afferent fibres synapse on

primate spinothalamic tract cells. Neuroscience Letters, 109, 76-81.

Carlton, S.M., Zhou, S. & Cogeshall, R.E. (1996) Localization and activation of substance P receptors in unmyelinated axons of rat glabrous skin. Brain Research, 734, 103-108.

Carlton, S.M., Zhou, S. & Cogeshall, R.E. (1998) Evidence for the interaction of glutamate and NK-1 receptors in the periphery. Brain Research, 790, 160-169.

Carr, P.A., Yamamoto, T. & Nagy, J.I. (1990) Calcitonin gene-related peptide in primary afferent neurons of rat: coexistence with fluoride-resistant acid phosphatase and depletion by neonatal capsaicin. Neuroscience, 36, 751-760.

Carstens, E. & Trevino, D.L. (1978a) Laminar origins of spinothalamic projections in the cat as determined by the retrograde transport of horseradish peroxidase. Journal of Comparative Neurology, 182, 151-166.

Carstens, E. & Trevino, D.L. (1978b) Anatomical and physiological properties of ipsilaterally projecting spinothalamic neurons in the second cervical segment of the cat's spinal cord. Journal of Comparative Neurology, 182, 167-184.

Cervero, F. (1983) Somatic and visceral inputs to the thoracic spinal cord of the cat: effects of noxious stimulation of the biliary system. Journal of Physiology, 337, 51-67.

Ch'ng, J.L.C., Christofides, N.D., Amand, P., Gison, S.J., Allan, Y.S., Su, H.C. & Tatemoto, K. (1985) Distribution of galanin-immunoreactivity in the central nervous system and the responses of galanin-containing neuronal pathways to injury. Neuroscience, 16, 343-354.

Chan-Palay, V., Jonsson, G. & Palay, S.L. (1978) Serotonin and substance P coexist in neurons of the rat's central nervous system. Proceedings of the National Academy of Sciences of the U.S.A., 75, 1582-1586.

Charlton, C.G. & Helke, C.J. (1985) Autoradiographic localization and characterization of spinal cord substance P binding sites: high densities in sensory, autonomic, phrenic and Onuf's motor nuclei. Journal of Neuroscience, 5, 1653-1661.

Charnay, Y., Paulin, C., Chayvialle, J-A. & Dubois, P.M. (1983) Distribution of substance P-like immunoreactivity in the spinal cord and dorsal root ganglia of the human foetus and infant. Neuroscience, 10, 41-55.

Chung, J.M., Kenshalo, D.R., Gerhart, K.D. & Willis, W.D. (1979) Excitaion of primate spinothalamic neurons by cutaneous C-fibre volleys. Journal of Neurophysiology, 42, 1354-1369.

Chung, K., Briner, R.P., Carlton, S.M. & Westlund, K.N. (1989) Immunohistochemical localization of seven different peptides in the human spinal cord. Journal of Comparative Neurology, 280, 158-170.

Chung, K., Lee, W.T. & Carlton, S.M. (1988) The effects of dorsal rhizotomy and spinal cord isolation on calcitonin gene-related peptide containing terminals in the rat lumbar dorsal horn. Neuroscience Letters, 90, 27-32.

Coffield, J.A. & Miletic, V. (1987) Immunoreactive enkephalin is contained within some trigeminal and spinal neurons projecting to the rat medial thalamus. Brain Research, 425, 380-383.

Coggeshall, R.E. & Carlton, S.M. (1997) Receptor localization in the mammalian dorsal horn and primary afferent neurons. Brain Research Reviews, 24, 28-66.

Conrath, M., Taquet, H., Pohl, M. & Carayon, A. (1989) Immunocytochemical evidence for calcitonin gene-related peptide-like neurons in the dorsal horn and lateral spinal nucleus of the rat cervical spinal cord. Journal of Chemical Neuroanatomy, 2, 335-347.

Cortes, R., Arvidsson, U., Schalling, M., Ceccatelli, S & Hokfelt, T. (1990) *In situ* hybridization studies on mRNAs for cholecystokinin, calcitonin gene-related

peptide and choline acetyltransferase in the lower brainstem, spinal cord and dorsal root ganglia of rat and guinea-pig with special reference to motoneurons. Journal of Chemical Neuroanatomy, 3, 467-485.

Craig, A.D. (1976) Spinocervical tract cells in cat and dog, labelled by the retrograde transport of horseradish peroxidase. Neuroscience Letters, 3, 173-177.

Craig, A.D. (1978) Spinal and medullary input to the lateral cervical nucleus. Journal of Comparative Neurology, 181, 729-744.

Craig, A.D., Linington, A.J. & Kniffki, K.D. (1989) Cells of origin of spinothalamic projections to medial and/or lateral thalamus in the cat. Journal of Comparative Neurology, 289, 568-585.

Cuello, A.C., Galfre, G. & Milstein, C. (1979) Detection of substance P in the central nervous system by a monoclonal antibody. Proceedings of the National Academy of Sciences of the U.S.A., 76, 3532-3536.

Cuello, A.C., Jessell, T.M., Kanazawa, I. & Iversen, L.L. (1977) Substance P: localization in synaptic vesicles in rat central nervous system. Journal of Neurochemisty, 29, 747-751.

Dalsgaard, C.J., Haegerstrand, A., Theodorrson-Norheim, E., Brodin, E. & Hokfelt, T. (1985) Neurokinin A-like immunoreactivity in rat primary sensory neurons: Coexistence with substance P. Histochemistry, 83, 37-39.

De Biasi, S. & Rustioni, A. (1988) Glutamate and substance P coexist in primary afferent terminals in the superficial laminae of spinal cord. Proceedings of the National Academy of Sciences of the U.S.A., 85, 7820-7824.

De Felipe, C., Herrero, J.F., O'Brien, J.A., Palmer, J.A., Doyle, C.A., Smith, A.J.H., Laird, J.M.A., Belmonte, C., Cervero, F. & Hunt, S.P. (1998). Altered nociception, analgesia and aggression in mice lacking the receptor for substance P. Nature, 392, 394-397.

De Felipe, C., Pinnock, R.D. & Hunt, S.P. (1995) Modulation of chemotropism in the developing spinal cord by substance P. Science, 267, 899-902.

De Koninck, Y. & Henry, J.L. (1991) SubstanceP-mediated slow excitatory postsynaptic potential elicited in dorsal horn neurons *in vivo* by noxious stimulation. Proceedings of the National Academy of Sciences of the U.S.A., 88, 11344-11348.

De Koninck, Y., Ribeiro-da-Silva, A., Henry, J.L. & Cuello, A.C. (1992) Spinal neurons exhibiting a specific nociceptive response receive abundant substance P-containing synaptic contacts. Proceedings of the National Academy of Sciences of the U.S.A., 89, 5073-5077.

DeLanerolle, N.C. & LaMotte, C.C. (1982) The human spinal cord: substance P and methionine-enkephalin immunoreactivity. Journal of Neuroscience, 2, 1369-1386.

DeLanerolle, N.C. & LaMotte, C.C. (1983) Ultrastructure of chemically defined neuron systems in the dorsal horn of the monkey. I. Substance P immunoreactivity. Brain Research, 274, 31-49.

Devor, L. & Claman, D. (1980) Mapping and plasticity of acid phosphatase afferents in the rat dorsal horn. Brain Research, 190, 17-28.

Dilly, P.N., Wall, P.D. & Webster, K.E. (1968) Cells of origin of the spinothalamic tract in the cat and rat. Experimental Neurology, 21, 550-562.

Ding, Y-Q., Takada, M., Shigemoto, R. & Mizuno, N. (1995) Spinoparabrachial tract neurons showing substance P receptor-like immunoreactivity in the lumbar spinal cord of the rat. Brain Research, 674, 336-340.

Dodd, J. & Jessel, T.M. (1985) Lactoseries carbohydrates specify subsets of dorsal root ganglion neurons projecting to the superficial dorsal horn of rat spinal cord. Journal of Neuroscience, 5, 3278-3294.

Dougherty, P.M. & Willis, W.D. (1991) Enhancement of spinothalamic neuron responses to chemical and mechanical stimuli following combined micro-

iontophoretic application of N-mehtyl-D-aspartic acid and substance P. Pain, 47, 85-93.

Dougherty, P.M., Palecek, J., Paleckova, V. & Willis, W.D. (1994) Neurokinin 1 and 2 antagonists attenuate the responses and NK-1 antagonists prevent the sensitization of primate spinothalamic tract neurons after intradermal capsaicin. Journal of Neurophysiology, 72, 1464-1475.

Dougherty, P.M., Palecek, J., Paleckova, V. & Willis, W.D. (1995) Infusion of substance P or neurokinin A by microdialysis alters responses of primate spinothalamic tract neurons to cutaneous stimuli and to iontophoretically released excitatory amino acids. Pain, 61, 411-425.

Downie, J.W., Ferrington, D.G., Sorkin, L.S. & Willis, W.D. (1988) The primate spinocervicothalamic pathway: responses of cells of the lateral cervical nucleus and spinocervical tract to innocuous and noxious stimuli. Journal of Neurophysiology, 59, 861-885.

Doyle, C.A., Palmer, J.A., Munglani, R. & Hunt, S.P. (1997) Molecular consequences of noxious stimulation. In Molecular Neurobiology of Pain, ed. Borsook, D., pp 145-169. Seattle: IASP Press.

Dubner, R. & Basbaum, A. (1994) In Textbook of Pain, ed. Wall, P.J. & Melzack, R., pp 225-241. London: Churchill Livingstone.

Duggan, A.W., Hendry, I.A., Morton, C.R., Hutchison, W.D. & Zhao, Z.Q. (1988) Cutaneous stimuli releasing immunoreactive substance P in the dorsal horn of the cat. Brain Research, 451, 261-273.

Duggan, A.W., Hope, P.J., Jarrott, B., Schaible, H.G. & Fleetwood-Walker, S. (1990) Release, spread and persistence of immunoreactive neurokinin A in the dorsal horn of the cat following noxious cutaneous stimulation. Studies with antibody microprobes. Neuroscience, 35, 195-202.

Duggan, A.W., Morton, C.R., Zhao, Z.Q. & Hendry, I.A. (1987) Noxious heating of the skin releases immunoreactive substance P in the substantia gelatinosa of the cat: a study with antibody microprobes. Brain Research, 403, 345-349.

Duggan, A.W., Schaible, H.G., Hope, P.J. & Lang, C.W. (1992) Effect of peptidase inhibition on the pattern of intraspinally released immunoreactive substance P detected with antibody microprobes. Brain Research, 579, 261-269.

Eccles, J.C., Eccles, R.M. & Lundberg, A.(1960) Types of neuron in and around the intremediate nucleus of the lumbosacral cord. Journal of Physiology, 154, 89-114.

Elde, R., Schalling, M., Ceccatelli, S., Nakanishi, S. & Hokfelt, T. (1990) Localization of neuropeptide receptor mRNA in rat brain: initial observations using probes for neurotensin and substance P receptors. Neuroscience Letters, 120, 134-138.

Erlanger, J. & Gasser, H.S. (1937) Electrical Signs of Nervous Activity. Philadelphia: University Pennsylvania Press.

Erspamer, V. (1981) The tachykinin peptide family. Trends in Neurosciences, 4, 267-269.

Fields, H.L., Clanton, C.H. & Anderson, S.D. (1977) Somatosensory properties of spinoreticular neurons in the cat. Brain Research, 120, 49-66.

Fitzegerald, M. & Lynn, B. (1979) The weak excitation of some cutaneous receptors in cats and rabbits by synthetic substance. Journal of Physiology (proceedings), 293, 66-67.

Fitzgerald, M. & Wall, P.D. (1980) The laminar organization of dorsal horn cells responding to peripheral C-fibres stimulation. Experimental Brain Research, 41, 36-44.

Fitzgerald, M. (1983) Capsaicin and sensory neurons-a review. Pain, 15, 109-130.

Fleischer, E., Handwerker, H.O. & Joukhadar, S. (1983) Unmyelinated nociceptive units in two skin areas of the rat. Brain Research, 267, 81-92.

Fong, T.M., Yu, H. & Strader, C.D. (1992) Molecular basis for the species selectivity of the neurokinin-1 receptor antagonists CP 96345 and RP 67580. Journal of Biological Chemistry, 267, 25668-25671.

Fukushima , K. & Kato, M. (1975) Spinal interneurons responding to group II muscle afferent fibres in the cat. Brain Research, 90, 307-312.

Ganong, W.F. (1995) Review of Medical Physiology. 17th ed. Englewood Cliffs, N.J., London: Prentice-Hall International.

Garry, M.G., Miller, K.E. & Seybold, V.S. (1989) Lumbar dorsal root ganglia of the cat: A quantitative study of peptide immunoreactivity and cell size. Journal of Comparative Neurology, 284, 36-47.

Gerfen, C.R., O'Leary, D.D.M. & Cowan, W.M. (1982) A note on the transneuronal transport of wheat germ agglutinin-conjugated horseradish peroxidase in the avian and rodent visual system. Experimental Brain Research, 48, 443-448.

Gibbins, I,L., Furness, J.B. & Costa, M. (1987) Pathway-specific patterns of the co-existence of substance P, calcitonin gene-related peptide, cholesystokinin and dynorphin in neurons of the dorsal ganglia of the guinea-pig. Cell and Tissue Research, 248, 417-437.

Gibbins, I,L., Furness, J.B., Costa, M., MacIntyre, I., Hillyard, C.J. & Girgis, S. (1985) Co-localization of calcitonin gene-related peptide-like immunoreactivity with substance P in cutaneous vascular anc visceral sensory neurons of guinea pigs. Neuroscience Letters, 57, 125-130.

Gibson, S.J., Polak, J.M., Allen, J.M., Adrian, T.E., Kelly, J.S. & Bloom, S.R. (1984a) The distribution and origin of a novel brain peptide, neuropeptide Y, in the spinal cord of several mammals. Journal of Comparative Neurology, 227, 78-91.

Gibson, S.J., Polak, J.M., Bloom, S.R. & Wall, P.D. (1981) The distribution of nine peptides in rat spinal cord with special emphasis on the substantia gelatinosa and on

the area around the central canal (lamina X). Journal of Comparative Neurology, 201, 65-79.

Gibson, S.J., Polak, J.M., Bloom, S.R., Sabate, I.M., Mulderry, P.M., Ghatei, M.A., McGregor, G.P., Morrison, J.F., Kelly, J.S., Evans, R.M. & Rosenfeld, M.G. (1984b) Calcitonin gene-related peptide immunoreactivity in the spinal cord of man and eight other species. Journal of Neuroscience, 4, 3101-3111.

Gibson, S.J., Polak, J.M., Giaid, A., Hamid, Q.A., Kar, S., Jones, P.M., Denny, P., Legon, S., Amara, S.G., Craig, R.K., Bloom, S.R., Penketh, R.J.A., Rodek, C., Ibrahim, N.B.N. & Dowson, A. (1988) Calcitonin gene-related peptide messenger RNA is expressed in sensory neurons of the dorsal root ganglia and also in spinal motoneurons in man and rat. Neuroscience Letters, 91, 283-288.

Giesler G.J. & Cliffer, K.D. (1985) Postsynaptic dorsal column pathway in the rat. II. evidence against an important role in nociception. Brain Research, 326, 347-356.

Giesler, G.J., Menetrey, D. & Basbaum, A.I. (1979) Differential origins of spinothalamic tract projections to medial and lateral thalamus in the rat. Journal of Comparative Neurology, 184, 107-126.

Giesler, G.J., Menetrey, D., Guilbaud, G. & Besson, J.M. (1976) Lumbar cord neurons at the origin of the spinothalamic tract in the rat. Brain Research, 118, 320-324.

Giesler, G.J., Nahin, R.L. & Madsen, A.M. (1984) Postsynaptic dorsal column pathway of the rat. I. anatomical studies. Journal of Neurophysiology, 51, 260-275.

Gilbert, R.F., Emson, P.C., Hunt, S.P., Bennet, G.W., Marsden, C.A., Sandberg, B.E., Steinbusch, H.W. & Verhofstad, A.A. (1982) The effects of monoamine neurotoxins on peptides in the rat spinal cord. Neuroscience, 7, 69-87.

Go, V.L. & Yaksh, T.L. (1987) Release of substance P from the cat spinal cord. Journal of Physiology, 391, 141-167.

Gobel, S. (1978) Golgi studies of the neurons in layer II of the dorsal horn of the medulla (trigeminal nucleus caudalis). Journal of Comparative Neurology, 180, 395-414.

Gonatas, N.K., Harper, C., Mizutani, T. & Gonatas, J.O. (1979) Superior sensitivity of conjugates of horseradish peroxidase with wheat germ agglutinin for studies of retrograde axonal transport. Journal of Histochemistry and Cytochemistry, 27, 728-734.

Granum, S.L. (1986) The spinothalamic system of the rat. I. locations of cells of origin. Journal of Comparative Neurology, 247, 159-180.

Gray, E.G. (1962) A morphological basis for presynaptic inhibition?. Nature, 193, 82-83.

Gregor, M. & Zimmermann, M. (1972) Characteristics of spinal neurons responding to cutaneous myelinated and unmyelinated fibres. Journal of Physiology, 221, 555-576.

Greves, P.L., Nyberg, F., Terenius, L. & Hokfelt, T. (1985) Calcitonin gene-related peptide is a potent inhibitor of substance P degradation. European Journal of Pharmacology, 115, 309-311.

Guyton, A.C. & Hall, J.E. (1996) Text Book of Medical Physiology. 9th ed. Philadelphia, London: W.B. Saunders.

Haber, L.H., Moore, B.D. & Willis, W.D. (1982) Electrophysiological response properties of spinoreticular neurons in the monkey. Journal of Comparative Neurology, 207, 75-84.

Handwerker, H.O., Iggo, A. & Zimmermann, M. (1975) Segmental and supraspinal actions on dorsal horn neurons responding to noxious and non-noxious skin stimuli. Pain, 1, 147-165.

Handwerker, H.O., Kilo, S. & Reeh, P.W. (1991) Unresponsive afferent nerve fibres in the sural nerve of the rat. Journal of Physiology, 435, 229-242.

Harber, A.A. & Lawson, S.N. (1985) Electrical properties of rat dorsal root ganglion neurons with different peripheral nerve conduction velocities. Journal of Physiology, 359, 47-63.

Harmann, P.A., Chung, K., Briner, R.P., Westlund, K.N. & Carlton, S.M. (1988) Calcitinin gene-related peptide (CGRP) in the human spinal cord: a light and electron microscopic analysis. Journal of Comparative Neurology, 269, 371-380.

Helke, C.J., Charlton, C.G. & Wiley, R.G. (1986) Studies on the cellular localization of spinal cord substance P receptors. Neuroscience, 19, 523-533.

Helke, C.J., Krause, J.E., Mantyh, P.W., Couture, R. & Bannon, M.J. (1990) Diversity of tachykinin peptidergic neurons: multiple peptides, receptors and regulatory mechanisms. FASEB Journal, 4, 1606-1615.

Helke, C.J., Neil, J.J., Massari, V.J. & Loewy, A.D. (1982) Substance P neurons project from the ventral medulla to the intermediolateral cell column and ventral horn in the rat. Brain Research, 243, 147-152.

Henry, M.A., Johnson, L.R., Nousek-Goeble, N. & Westrum, L.E. (1996) Light microscopic localization of calcitonin gene-related peptide in the normal feline trigeminal system and following retrogasserian rhizotomy. Journal of Comparative Neurology, 365, 526-540.

Hillman, P. & Wall, P.D. (1969) Inhibitory and excitatory factors influencing the receptive fields of lamina 5 spinal cord cells. Experimental Brain Research, 9, 284-306.

Hokfelt, T., Elde, R., Johansson, O., Luft, R., Nilsson, G., & Arimura, A. (1976) Immunocytochemical evidence for separate populations of somatostatin-containing

and substance P-containing primary afferent neurons in the rat. Neuroscience, 1, 132-136.

Hokfelt, T., Kellerth, J.O., Nilsson, G. & Pernow, B. (1975) Substance P: localization in the central nervous system and in some primary sensory neurons. Science, 190, 889-890.

Hokfelt, T., Ljungdahl, A., Steinbusch, H., Verhofstad, A., Nilsson, G., Brodin, E., Oernow, B. & Goldstein, M. (1978) Immunohistochemical evidence of substance P-like immunoreacivity in some 5-hydroxy-tryptamine-containing neurons in the rat central nervous system. Neuroscience, 3, 517-538.

Hokfelt, T., Ljungdahl, A., Terenius, L., Elde, R. & Nilsson, G. (1977) Immunohistochemical analysis of peptide pathways possibly related to pain and analgesia: enkephalin and substance P. Proceedings of the National Academy of Sciences of the U.S.A., 74, 3081-3085.

Hokfelt, T., Wiesenfeld-Hallin, Z., Villar, M.J. & Molander, C. (1987) Increase of galanin-like immunoreactivity in rat dorsal root ganglion cells after peripheral axotomy. Neuroscience Letters, 83, 217-220.

Holets, V.R., Hokfelt, T., Rokaeus, A., Terenius, L. & Goldstein, M. (1988) Locus coeruleus neurons in the rat containing neuropeptide Y, tyrosine hydroxylase or galanin and their different projections to the spinal cord, cerebral cortex and hypothalamus. Neuroscience, 24, 893-906.

Holmgren, J., Lonnrot, I. & Svennerholm, L. (1973) Tissue receptor for cholera exotoxin: postulated structure from studies with GM1 ganglioside and related glycolipids. Infection and Immunity, 8, 208-214.

Hu, H-Z., Li, Z-W. & Si, J-Q. (1997) Evidence for the existence of substance P autoreceptor in the membrane of rat dorsal root ganglion neurons. Neuroscience, 77, 535-541.

Hunt, S.P. & Rossi, J. (1985) Peptide-and non-peptide-containing unmyelinated primary afferents: the parallel processing of nociceptive information. Philosophical Transactions of the Royal Society of London B, 308, 283-289.

Hunt, S.P., Kelly, J.S., Emson, P.C., Kimmel, J.R., Miller, R.J., & Wu, J.Y. (1981) An immunohistochemical study of neuronal populations containing neuropeptides or γ-aminobutyrate within the superficial layers of the rat dorsal horn. Neuroscience, 6, 1883-1898.

Hunt, S.P., Mantyh, P.W. & Priestley, J.V. (1992) The organization of biochemically characterized sensory neurons. In Sensory Neurons: Diversity, Deveopment and Plasticity, ed. Scott, S.A., pp 60-76. New York: Oxford University Press.

Hylden, J.L. & Wilcox, G.L. (1981) Intrathecal substance P elicits a caudally-directed biting and scratching behaviour in mice. Brain Research, 217, 212-215.

Iversen, L. (1998) Substance P equals pain substance P? Nature, 392, 334-335.

Jessell, T., Tsunoo, A., Kanazawa, I. & Otsuka, M. (1979) Substance P: depletion in the dorsal horn of rat spinal cord after section of the peripheral processes of primary sensory neurons. Brain Research, 168, 247-259.

Jessell, T.M., Iversen, L.L. & Cuello, A.C. (1978) Capsaicin-induced depletion of substance P from primary sensory neurons. Brain Research, 152, 183-188.

Johansson, O., Hokfelt, T., Pernow, b., Jeffcoate, S.L., White, N., Steinbusch, H.W.M., Verhofstad, A.A.J., Emson, P. C. & Spindel, E. (1981) Immunohistochemical support for three putative transmitters in one neuron: coexistence of 5-hydroxytryptamine, substance P, and thyrotropin releasing hormone-like immunoreactivity in medullary neurons projecting to the spinal cord. Neuroscience, 6, 1857-1881.

Ju, G., Hokfelt, T., Brodin, E., Fahrenkrug, J., Fischer, J.A., Frey, P., Elde, R.P. & Brown, J.C. (1987) P rimary sensory neurons of the rat showing calcitonin gene-

related peptide immunoreactivity and their relation to substance P- and somatostatin-, galanin-, vasoactive intestinal polypeptide-, and cholecystokinin-immunoreactive ganglion cells. Cell and Tissue Research, 247, 417-431.

Ju, G., Hokfelt, T., Fischer, J.A., Frey, P., Rehfeld, J.F. & Dockray, G.J. (1986) Does cholecystokinin-like immunoreactivity in rat primary sensory neurons represent calcitonin gene-related peptide? Neuroscience Letters, 68, 305-310.

Kamogawa, H. & Bennett, G.J. (1986) Dorsal column postsynaptic neurons in the cat are excited by myelinated nociceptors. Brain Research, 364, 386-390.

Kawamura, J. & Dyck, P.J. (1978) Evidence for 3 populations by size in L5 spinal ganglion in man. Journal of Neuropathology and Experimental Neurology, 37, 269-272.

Kemplay, S.K. & Webster, K.E. (1986) A qualitative and quantitative analysis of the distributions of cells in the spinal cord and spinomedullary junction projecting to the thalamus of the rat. Neuroscience, 17, 769-789.

Kevetter, G.A. & Willis, W.D. (1982) Spinothalamic cells in the rat lumbar cord with collaterals to the medullary reticular formation. Brain Research, 238, 181-185.

Kevetter, G.A. & Willis, W.D. (1983) Collaterals of spinothalamic cells in the rat. Journal of Comparative Neurology, 215, 453-464.

Kevetter, G.A., Haber, L.H., Yezierski, R.P., Chung, J.M., Martin, R.F. & Willis, W.D. (1982) Cells of origin of the spinoreticular tract in the monkey. Journal of Comparative Neurology, 207, 61-74.

Kitao, Y., Robertson, B., Kudo, M. & Grant, G. (1996) Neurogenesis of subpopulations of rat lumbar dorsal root ganglion neurons including neurons projecting to the dorsal column nuclei. Journal of Comparative Neurology, 371, 249-257.

Kitchener, P.D., Wilson, P. & Snow, P.J. (1993) Selective labeling of primary sensory afferent terminals in lamina II of the dorsal born by injection of *Bandeiraea*

Simplicifolia isolectin B4 into peripheral nerves. Neuroscience, 54, 545-551.

Kiyama, H. & Emson, P.C. (1991) Colchicine-induced expression of proneurotensin mRNA in rat striatum and hypothalamus. Molecular Brain Research, 9, 353-358.

Kiyama, H., Maeno, H. & Tohyama, M. (1993) Substance P receptor (NK-1) in the central nervous system: possible functions from a morphological aspects. Regulatory Peptides, 46, 114-123.

Knyihar, E., Laszlo, I. & Tornyos, S. (1974) Fine structure and fluoride resistant acid phosphatase activity of electron dense sinusoid terminals in the substantia gelatinosa Rolandi of the rat after dorsal root transection. Experimental Brain Research, 19, 529-544.

Knyihar-Csillik, E. & Csillik, B. (1981) FRAP: histochemistry of the primary sensory nociceptive neurons. Progress in Histochemistry and Cytochemistry, 14, 1-137.

Knyihar-Csillik, E., Bezzegh, A., Boti, S., & Csillid, B. (1986) Thiamine monophosphatase: A genuine marker for transganglionic regulation of primary sensory neurons. Journal of Histochemistry and Cytochemistry, 34, 363-371.

Kobayashi, Y. & Matsumura, G. (1996) Central projections of primary afferent fibres from the rat trigeminal nerve labeled with isolectin B4-HRP. Neuroscience Letters, 217, 89-92.

Kunze, W.A.A., Wilson, P. & Snow, P.J. (1987) Response of lumbar spinocervical tract cells to natural and electrical stimulation of the hindlimb footpads in cats. Neuroscience Letters, 75, 253-258.

Laing, I., Todd, A.J., Heizmann, C.W. & Schmidt, H.H.H.W. (1994) Subpopulations of GABAergic neurons in laminae I-III of rat spinal dorsal horn defined by coexistence with classical transmitters, peptides, nitric oxide synthase or parvalbumin. Neuroscience, 61, 123-132.

LaMotte, C.C., Carlton, S.M., Honda, C.N., Surmeier, D.J. & Willis, W.D. (1988) Innervation of identified primate spinothalamic tract neurons: ultrastructure of serotonergic and other synaptic profiles. Society for Neuroscience Abstracts, 14, 852.

LaMotte, C.C., Kapadia, S.E. & Shapiro, C.M. (1991) Central projections of the sciatic, saphenous, median and ulnar nerves of the rat demonstrated by transganglionic transport of choleragenoid-HRP (B-HRP) and wheat germ agglutinin-HRP (WGA-HRP). Journal of Comparative Neurology, 311, 546-562.

Lawson, S.N, & Biscoe, T.J. (1979) Development of mouse dorsal root ganglia: An autoradiographic and quantitative study. Journal of Neurocytology, 8, 265-274.

Lawson, S.N. & Waddell, P.J. (1991) Soma neurofilament immunoreactivity is related to cell size and fibre conduction velocity in rat primary sensory neurons. Journal of Physiology, 435, 41-63.

Lawson, S.N. (1979) The postnatal development of large light and small dark neurons in mouse dorsal root ganglia: A statistical analysis of cell numbers and size. Journal of Neurocytology, 8, 275-294.

Lawson, S.N. (1992) Morphological and biochemical cell types of sensory neurons. In Sensory Neurons: Diversity, Development and Plasticity, ed. Scott, S.A., pp 27-59. New York: Oxford University Press.

Lawson, S.N. (1995) Neuropeptides in morpholocically and functionally identified primary afferent neurons in dorsal root ganglia: substance P, CGRP and somatostatin. In Progress in Brain Research, ed. Nyberg, F, Sharma, H.S. & Wiesenfeld-Hallin, Z., 104, 161-173.

Lawson, S.N., Caddy, K.W.T. & Biscoe, T.J. (1974) Development of rat dorsal root ganglion neurons. Studies of cell birthdays and changes in mean cell diameter. Cell and Tissue Research, 153, 399-413.

Lawson, S.N., Crepps, B.A. & Perl, E.R. (1997) Relationship of substance P to afferent characteristics of dorsal root ganglion neurons in guinea-pig. Journal of Physiology, 505, 177-191.

Lawson, S.N., Harber, E.I., Harber, A.A., Garson, J.A., Coakham, H.B. & Randle, B.J. (1985) Monoclonal antibody 2C5: A marker for a subpopulation of small neurons in rat dorsal root ganglia. Neuroscience, 16, 365-374.

Lawson, S.N., Harper, A.A., Harber, E.I., Garson, J.A. & Anderton, B.H. (1984) A monoclonal antibody against neurofilament protein specifically labels a subpopulation of rat sensory neurons. Journal of Comparative Neurology, 228, 263-272.

Lawson, S.N., Perry, M.J., Prabhakar, E. & McCarthy, P.W. (1993) Primary sensory neurons: neurofilament, neuropeptides and conduction velocity. Brain Research Bulletin, 30, 239-243.

Leah, J., Menetrey, D. & De Pommery, J. (1988) Neuropeptides in long ascending spinal tract cells in the rat: evidence for parallel processing of ascending information. Neuroscience, 24, 195-207.

Leah, J.D., Cameron, A.A. & Snow, P.J. (1985b) Neuropeptides in physiologically identified mammalian sensory neurons. Neuroscience Letters, 56, 257-263.

Leah, J.D., Cameron, A.A., Kelly, W.L. & Snow, P.J. (1985a) Co-existence of peptide immunoreactivity in sensory neurons of the cat. Neuroscience, 16, 683-690.

Lee, K.H., Chung, K., Chung, J.M. & Coggeshall, R.E. (1986) Correlation of cell body size, axon size and signal conduction velocity for individually labeled dorsal root ganglion cells in the rat. Journal of Comparative Neurology, 243, 335-346.

Leem, J.W., Willis, W.D. & Chung, J.M. (1993) Cutaneous sensory receptors in the rat foot. Journal of Neurophysiology, 69, 16841699.

Lembeck, F. (1953) Zur frage der zentralen ubertragung afferenter impulse. III. mitteilung. Das vorkommen und die bedeutung der substanz Pin den dorsalen wurzeln des ruckenmarks. Archiv Fuer Experimentelle Pathologie und Pharmakologie, 219, 197-213.

Light, A.R. & Kavookjian, A.M. (1988) Morphology and ultrastructure of physiolocially identified substantia gelatinosa (lamina II) neurons with axons that terminate in deeper dorsal horn laminae (III-V). Journal of Comparative Neurology, 267, 172-189.

Light, A.R. & Perl, E.R. (1979) Spinal termination of functionally identified primary afferent neurons with slowly conducting myelinated fibres. Journal of Comparative Neurology, 186, 133-150.

Lima, D. & Coimbra, A. (1988) The spinothalamic system of the rat: structural types of retrogradely labelled neurons in the marginal zone (lamina I). Neuroscience, 27, 215-230.

Lima, D. & Coimbra, A. (1990) Structural types of marginal (lamina I) neurons projecting to the dorsal reticular nucleus of the medulla oblongata. Neuroscience, 34, 591-606.

Lima, D. (1990) A spinomedullary projection terminating in the dorsal reticular nucleus of the rat. Neuroscience, 34, 577-589.

Lima, D., Mendes-Ribeiro, J.A. & Coimbra, A. (1991) The spino-latero-reticular system of the rat: projections from the superficial dorsal horn and structural charaterization of marginal neurons involved. Neuroscience, 45, 137-152.

Lindh, B., Aldskogius, H. & Hokfelt, T. (1989) Simultaneous immunohistochemical demonstration of intraaxonally transported markers and neuropeptides in the peripheral nervous system of the guinea pig. Histochemistry, 92, 367-376.

Littlewood, N.K., Todd, A.J., Spike, R.C., Watt, C. & Shehab, S.A.S. (1995) The types of neurons in spinal dorsal horn which possess neurokinin-1 receptors. Neuroscience, 66, 597-608.

Liu, H., Brown, J.L., Jasmin, L., Maggio, J.E., Vigna, S.R., Mantyh, P.W. & Basbaum, A.I. (1994) Synaptic relationship between substance P and the substance P receptor: light and electron microscopic characterization of the mismatch between neuropeptides and their receptors. Proceedings of the National Academy of Sciences of the U.S.A., 91, 1009-1013.

Ljungdahl, A., Hokfelt, T. & Nilsson, G. (1978) Distribution of substance P-like immunoreactivity in the central nervous system of the rat. I. Cell bodies and nerve terminals. Neuroscience, 3, 861-943.

Llewellyn-smith, I.J. & Minson, J.B. (1992) Complete penetration of antibodies into Vibratome sections after glutaraldehyde fixation and ethanol treatment: light and electron microscopy for neuropeptides. Journal of Histochemistry and Cytochemistry, 40, 1741-1749.

Lund, R.D. & Webster, K.E. (1967) Thalamic afferents from the spinal cord and trigeminal nuclei. An experimental anatomical study in the rat. Journal of Comparative Neurology, 130, 313-328.

Lynn, B. & Carpenter, S.E. (1982) Primary afferent units from the hairy skin of the rat hind limb. Brain Research, 238, 29-43.

Lynn, B. & Hunt, S.P. (1984) Afferent C-fibres: Physiological and biochemical correlations. Trends in Neurosciences, 7, 186-188.

Ma, Q-P. & Woolf, C.J. (1995) Involvement of neurokinin receptors in the induction but not the maintenance of mechanical allodynia in rat flexor motoneurons. Journal of Physiology, 486, 769-777.

Ma, W., Ribeiro-da-Silva, A., De Koninck, Y., Radhakrishnan, V., Henry, J.L. & Cuello, A.C. (1996) Quantitative analysis of substnace P-immunoreactive boutons on physiologically characterized dorsal horn neurons in the cat lumbar spinal cord. Journal of Comparative Neurology, 376, 45-64.

Ma, W., Ribeiro-da-Silva, A., De Koninck, Y., Radhakrishnan, V., Cuello, A.C. & Henry, J.L. (1997) Substance P and enkephalin immunoreactivities in axonal boutons presynaptic to physiologically identified dorsal horn neurons. An ultrstructural multiple-labelling study in the cat. Neuroscience, 77, 793-811.

Maeno, H., Kiyama, H. & Tohyama, M. (1993) Distribution of the substance P receptor (NK-1 receptor) in the central nervous system. Molecular Brain Research, 18, 43-58.

Mannen, H. (1975) Reconstruction of axonal trajectory of individual neurons in the spinal cord using Golgi-stained serial sections. Journal of Comparative Neurology, 159, 357-374.

Mannen, N. & Sugiura, Y. (1976) Construction of neruons of dorsal horn proper using Golgi-stained serial sections. Journal of Comparative Neurology, 168, 303-312.

Mantyh, P.W. & Hunt, S.P. (1985) The autoradiographic localization of substance P receptors in the rat and bovine spinal cord and the rat and cat spinal trigeminal nucleus pars caudalis and the effects of neonatal capsaicin. Brain Research, 332, 315-324.

Mantyh, P.W., DeMaster, E., Malhorta, A., Ghilardi, J.R., Rogers, S.D., Mantyh, C.R., Liu, H., Basbaum, A.I, Vigna, S.R., Maggio, J.E. & Simone, D.A. (1995) Receptor endocytosis and dendrite reshaping in spinal neurons after somatosensory stimulation. Science, 268, 1629-1632.

Mantyh, P.W., Hunt, S.P. & Maggio, J.E. (1984) Substance P receptors: localization by light microscopic autoradiography in rat brain using [^{3}H]SP as the radioligand.

Brain Research, 309, 147-165.

Mantyh, P.W., Rogers, S.D., Ghilardi, J.R., Maggio, J.E., Mantyh, C.R. & Vigna, S.R. (1996) Differential expression of two isoforms of the neurokinin-1 (substance P) receptor *in vivo*. Brain Research, 719, 8-13.

Marshall, G.E., Shehab, S.A.S., Spike, R.C. & Todd, A.J. (1996) Neurokinin-1 receptors on lumbar spinothalamic neurons in the rat. Neuroscience, 72, 255-263.

Masu, Y., Nakayama, K., Tamaki, H., Harada, Y., Kuno, M. & Nakanishi, S. (1987) cDNA cloning of substance K receptor through oocyte expression system. Nature, 329, 836-838.

Maxwell, D.J. (1985) Combined light and electron microscopy of Golgi-labeled neurons in lamina III of feline spinal cord. Journal of Anatomy, 141, 155-169.

Maxwell, D.J., Bannatyne, B.A., Fyffe, R.E.W. & Brown, A.G. (1982a) Ultrastructure of hair follicle afferent fibre terminations in the spinal cord of the cat. Journal of Neurocytology, 11, 571-582.

Maxwell, D.J., Bannatyne, B.A., Fyffe, R.E.W. & Brown, A.G. (1984a) Fine structure of primary afferent axon terminals projecting from rapidly adapting mechanoreceptors of the toe and foot pads of the cat. Quarterly Journal of Experimental Physiology, 69, 381-392.

Maxwell, D.J., Fyffe, R.E.W. & Brown, A.G. (1982b) Fine structure of spinocervical tract neurons and the synaptic boutons in contact with them. Brain Research, 233, 394-399.

Maxwell, D.J., Fyffe, R.E.W. & Brown, A.G. (1984b) Fine structure of normal and degenerating primary afferent boutons associated with characterized spinocervical tract neurons in the cat. Neuroscience, 12, 151-163.

Maxwell, D.J., Fyffe, R.E.W. & Rethelyi, M. (1983) Morphological properties of physiologically characterized lamina III neurons in the cat spinal cord. Neuroscience, 10, 1-22.

Maxwell, L., Maxwell, D.J., Neilson, M. & Kerr, R. (1996) A confocal microscopic survey of serotoninergic axons in the lumbar spinal cord of the rat: colocalization with glutamate decarboxylase and neuropeptides. Neuroscience, 75, 471-480.

McCarthy, P.W. & Lawson, S.N. (1989). Cell type and conduction velocity of rat primary sensory neurons with substance P-like immunoreactivity. Neuroscience, 28, 745-753.

McCarthy, P.W. & Lawson, S.N. (1990) Cell type and conduction velocity of rat primary sensory neurons with calcitonin gene-related peptide like immunoreactivity. Neuroscience, 34, 623-632.

McCarthy, P.W. & Lawson, S.N. (1997) Differing action potential shapes in rat dorsal root ganglion neurons related to their substance P and calcitonin gene-related peptide immunoreactivity. Journal of Comparative Neurology, 388, 541-549.

McGregor, G.P., Gibson, S.J., Sabate, I.M., Blank, M.A., Christofides, N.D., Wall, P.D., Polak, J.M. & Bloom, S.R. (1984) Effect of peripheral nerve section and nerve crush on spinal cord neuropeptides in the rat: increased VIP and PHI in the dorsal horn. Neuroscience, 13, 207-216.

McMahon, S.B., Sykova, E., Wall, P.D., Woolf, C.J. & Gibson, S.J. (1984) Neurogenic extravasation and substance P levels are low in muscle as compared to skin in the rat hindlimb. Neuroscience Letters, 52, 235-240.

McNeill, D.L., Coggeshall, R.E. & Carlton, S,M. (1988) A light and electron microscopic study of calcitonin gene-related peptide in the spinal cord of the rat. Experimental Neurology, 99, 699-708.

Melander, T., Hokfelt, T. & Rokaeus, A. (1986) Distribution of galanin-like immunoreactivity in the rat central nervous system. Journal of Comparative Neurology, 248, 475-517.

Mendell, L.M. & Wall, P.D. (1965) Responses of single dorsal cord cells to peripheral cutaneous unmyelinated fibres. Nature, 206, 97-99.

Mendell, L.M. (1966) Physiological properties of unmyelinated fibre projection to the spinal cord. Experimental Neurology, 16, 316-332.

Menetrey, D. & Basbaum, A.I. (1987) The distribution of substance P-, enkephalin- and dynorphin-immunoreactive neurons in the medulla of the rat and their contribution to bulbospinal pathways. Neuroscience, 23, 173-188.

Menetrey, D., Giesler, G.J. & Besson, J.M. (1977) An analysis of response profiles of spinal cord dorsal horn neurons to non-noxious and noxious stimuli in the spinal rat. Experimental Brain Research, 27, 15-33.

Merighi, A., Kar, S., Gibson, S.J., Ghidella, S., Gobetto, A., Peirone, S.M. & Polak, J.M. (1990) The immunocytochemical distribution of seven peptides in the spinal cord and dorsal root ganglia of horse and pig. Anatomy and Embryology, 181, 271-280.

Merighi, A., Polak, J.M. & Theodosis, D.T. (1991) Ultrastructural visualization of glutamate and aspartate immunoreactivities in the rat spinal dorsal horn, with special reference to the colocalization of glutamate, substance P and calcitonin gene-related peptide. Neuroscience, 40, 67-80.

Millhorn, D.E., Seroogy, K., Hokfelt, T., Schmued, L.C., Terenius, L., Buchan, A. & Brown, J.C. (1987) Neurons of the ventral medulla oblongata that contain both somatostatin and enkephalin immunoreactivities project to nucleus tractus solitarii and spinal cord. Brain Research, 424, 99-108.

Molander, C. & Grant, C. (1985) Cutaneous projections from the hindlimb foot to the substantia gelatinosa of the spinal cord studied by transganglionic transport of WGA-HRP conjugate. Journal of Comparative Neurology, 237, 476-484.

Molander, C. & Grant, C. (1986) Laminar distribution and somatotopic organization of primary afferent fibres from hindlimb nerves in the dorsal horn. A study by transganglionic trasport of horseradish peroxidase in the rat. Neuroscience, 19, 297-312.

Molander, C. & Grant, G. (1995) Spinal cord cytoarchitecture. In the Rat Nervous System. 2nd ed. Paxinos, G., pp 39-45. Sydney: Academic Press.

Molander, C., Xu, Q. & Grant, G. (1984) The cytoarchitectonic organization of the spinal cord in the rat. I. The lower thoracic and lumbosacral cord. Journal of Comparative Neurology, 230, 133-141.

Molander, C., Yegge, Y. & Daalsgaard, C-J. (1987) Substance P, somatostatin and calcitonin gene-related peptide-like immunoreactivity and fluoride resistant acid phosphatase activity in relation to retrogradely labeled cutaneous, muscular and visceral primary sensory neurons in the rat. Neuroscience Letters, 74, 37-42.

Morris, R. (1989) Responses of spinal dorsal horn neurons evoked by myelinated primary afferent stimulation are blocked by excitatory amino acid antagonists acting at kainate/quisqualate receptors. Neuroscience Letters, 105, 79-85.

Moussaoui, S.M., Hermans, E., Mathieu, A.M., Bonici, B., Clerc, F., Guinet, F., Garret, C. & Laduron, P.M. (1992a) Polyclonal antibodies against the rat NK-1 receptor: characterization and localization within the spinal cord. Neuroreport, 3, 1073-1076.

Moussaoui, S.M., Le Prado, N., Bonici, B., Faucher, D.C., Cuine, F., Laduron, P.M. & Garret, C. (1992b) Distribution of neurokinin B in rat spinal cord and peripheral tissues: comparison with neurokinin A and substance P and effects of neonatal capsaicin treatment. Neuroscience, 48, 969-978.

Munglani, R., Bond, A., Smith, G.D., Harrison, S.M., Elliot, P.J., Birch, P.J. & Hunt, S.P. (1995) Changes in neuronal markers in a mononeuropathic rat model: relationship between neuropeptide Y, pre-emptive drug treatment and long-term mechanical hyperalgesia. Pain, 63, 21-31.

Nagy, J.I. & Hunt, S.P. (1982) Fluoride-resistant acid phosphatase-containing neurons in dorsal root ganglia are separate from those containing substance P or somatostatin. Neuroscience, 7, 89-97.

Nagy, J.I. & Hunt, S.P. (1983) The termination of primary afferents within the rat dorsal horn: evidence for rearrangement following capsaicin treatment. Journal of Comparative Neurology, 218, 145-158.

Nagy, J.I., Hunt, S.P., Iverson, L.L. & Emson, P.C. (1981) Biochemical and anatomical observations on the degeneration of peptide containing primary afferent neurons after neonatal capsaicin. Neuroscience, 6, 1923-1934.

Nagy, J.I., Iversen, L.L., Goedert, M., Chapman, D. & Hunt, S.P. (1983) Dose-dependent effect of capsaicin on primary sensory neurons in the neonatal rat. Journal of Neuroscience, 3, 399-406.

Nahin, R.L. & Micevych, P.E. (1986) A long ascending pathway of enkephalin-like immunoreactive spinoreticular neurons in the rat. Neuroscience Letters, 65, 271-276.

Nahin, R.L. (1987) Immunocytochemical identification of long ascending peptidergic neurons contributing to the spinoreticular tract in the rat. Neuroscience, 23, 859-869.

Nahin, R.L. (1988) Immunocytochemical identification of long ascending, peptidergic lumbar spinal neurons terminating in either the medial or lateral thalamus in the rat. Brain Research, 443, 345-349.

Naim, M.M., Spike, R.C., Watt, C., Shehab, S.A.S. & Todd, A.J. (1997) Cells in laminae III and IV of the rat spinal cord that possess the neurokinin-1 receptor and have dorsally directed dendrites receive a major synaptic input from tachykinin-containing primay afferents. Journal of Neuroscience, 17, 5536-5548.

Nakanishi, S. (1991) Mammalian tachykinin receptors. Annual Review of Neuroscience, 14, 123-136.

Nakaya, Y., Kaneko, T., Shigemoto, R., Nakanishi, S. & Mizuno, N. (1994) Immunocytochemical localization of substance P receptor in the central nervous system of the adult rat. Journal of Comparative Neurology, 347, 249-274.

Nawa, H., Hirose, T., Takashima, H., Inayama, S., & Nakanishi, S. (1983) Nucleotide sequences of cloned cDNAs for two types of bovine brain substance P precursor. Nature, 306, 32-36.

Neumann, S., Doubell, T.P., Leslie, T. & Woolf, C.J. (1996) Inflammatory pain hypersensitivity mediated by phenotypic switch in myelinated primary sensory neurons. Nature, 384, 360-364.

Noguchi, K., Dubner, R., De Leon, M., Senba, E. & Ruda, M.A. (1994) Axotomy induces preprotachykinin gene expression in a subpopulation of dorsal root ganglion neurons. Journal of Neuroscience Research, 37, 596-603.

Noguchi, K., Senba, E., Morita, Y, Sato, M. & Tohyama, M. (1990a) α-CGRP and β -CGRP mRNAs are differentially regulated in the rat spinal cord and dorsal root ganglion. Molecular Brain Research, 7, 299-304.

Noguchi, K., Senba, E., Morita, Y., Sato, M. & Tohyama, M. (1990b) Co-expression of α-CGRP and β-CGRP mRNA in the rat dorsal root ganglion cells. Neuroscience Letters, 108, 1-5.

O'Brien, C., Woolf, C.J., Fitzgerald, M., Lindsay, R.M. & Molander, C. (1989) Differences in the chemical expression of rat primary afferent neurons which innervate skin, muscle or joint. Neuroscience, 32, 493-502.

Ogawa, T., Kanazawa, I. & Kimura, S. (1985) Regional distribution of substance P, neurokinin α and neurokinin β in rat spinal cord, nerve roots and dorsal root ganglia, and the effects of dorsal root section or spinal transection. Brain Research, 359, 152-157.

Ohnishi, A. & Ogawa, M. (1986) Preferential loss of large lubar primary sensory neurons in carcinomatous sensory neuropathy. Annals of Neurology, 20, 102-104.

Orazzo, C., Pieribone, V.A., Ceccatelli, S., Terenius, L. & Hokfelt, T. (1993) CGRP-like immunoreactivity in all dopamine neurons projecting to the spinal cord and a note on CGRP-CCK cross-reactivity. Brain Research, 600, 39-48.

Ositelu, D.O., Morris, R. & Vaillant, V. (1987) Innervation of facial skin but not masticatory muscles or the tongue by trigeminal primary afferents containing somatostatin in the rat. Neuroscience Letters, 78, 271-276.

Otsuka, M. & Konishi, S. (1976) Release of substance P-like immunoreactivity from isolated spinal cord of newborn rat. Nature, 264, 83-84.

Otsuka, M. & Yanagisawa, , M. (1988) Effect of a tachykinin antagonist on a nociceptive reflex in the isolated spinal cord-tail preparation of the newborn rat. Journal of Physiology, 395, 255-270.

Otsuka, M. & Yoshioka, K. (1993) Neurotransmitter functions of mammalian tachykinins. Physiological Review, 73, 229-308.

Otsuka, M., Konishi, S. & Takahashi, T. (1972) A further study of the motoneuron depolarizing peptide extracted from dorsal roots of bovine spinal nerves. Proceedings. Japan Academy, 48, 747-752.

Palecek, J., Paleckova, V., Dougherty, P.M., Carlton. S.M. & Willis, W.D. (1992) Responses of spinothalamic tract cells to mechanical and thermal stimulation of skin in rats with experimental peripheral neuropathy. Journal of Neurophysiology, 67, 1562-1573.

Paxinos, G. & Watson, C. (1982) The Rat Brain in Stereotaxic Coordinates. Sydney: Academic Press.

Perry, M.J. & Lawson, S.N. (1998) Differences in expression of oligosaccarides, neuropeptides, carbonic anhydrase and neurofilament in rat primary afferent neurons retrogradely labelled via skin, muscle or visceral nerves. Neuroscience, 85, 293-310.

Plenderleith, M.B. & Snow, P.J.(1993) The plant lectin *Bandeiraea simplicifolia* I-B4 identifies a subpopulation of small diameter primary sensory neurons which innervate the skin in the rat. Neuroscience Letters, 159, 17-20.

Plenderleith, M.B., Cameron, A.A., Key, B. & Snow, P.J. (1989) The plant lectin soybean agglutinin binds to the soma, axon and central terminals of a subpopulation of small-diameter primary sensory neurons in the rat and cat. Neuroscience, 31, 683-695.

Polgar, E. & Todd, A.J. (1997) Inhibitory inputs onto lamina III/IV cells in rat spinal cord which express neurokinin-1 receptor. Society for Neuroscience Abstracts 23, 1803.

Pollock, R., Kerr, R. & Maxwell, D.J. (1997) An immunocytochemical investigation of the relationship between substance P and neurokinin-1 receptor in the lateral horn of the rat thoracic spinal cord. Brain Research, 777, 22-30.

Price, D.D. & Browe, A.C. (1973) Responses of spinal cord neurons to graded noxious and non-noxious stimuli. Brain Research, 64, 425-429.

Price, D.D. & Mayer, D.J. (1974) Physiological laminar organization of the dorsal horn of M. mulatta. Brain Research, 79, 321-325.

Proshansky, E. & Egger, M.D. (1977) Dendritic spread of dorsal horn neurons in cats. Experimental Brain Research, 28, 153-166.

Radhakrishnan, V. & Henry, J.L. (1993) Excitatory amino acid receptor mediation of sensory inputs to functionally identified dorsal horn neurons in cat spinal cord. Neuroscience, 55, 531-544.

Radhakrishnan, V. & Henry, J.L. (1995) Antagonism of nociceptive responses of cat spinal dorsal horn neurons *in vivo* by the NK-1 receptor antagonists CP 96,345 and CP 99,994 but not by CP 96,344. Neuroscience, 64, 943-958.

Ralston, H.J. (1979) The fine structure of lamina I, II and III of the macaque spinal cord. Journal of Comparative Neurology, 184, 619-642.

Rambourg, A., Clermont, Y. & Beaudet, A. (1983) Ultrastructural features of six types of neurons in rat dorsal root ganglia. Journal of Neurocytology, 12, 47-66.

Ramon Y Cajal, S. (1909) Histologie du systeme nerveux de l'homme et des vertebres. 1, 908-911. Madrid: Inst. Cajal, reprinted in 1952.

Regoli, D., Boudon, A. & Fauchere, J-L. (1994) Receptors and antagonists for substance P and related peptides. Pharmacological Review, 46, 551-599.

Regoli, D., Drapeau, G., Dion, S. & Couture, R. (1988) New selective agonists for neurokinin receptors: pharmacological tools for receptor characterization. Trends in Pharmacological Sciences, 9, 290-295.

Rethelyi, M. & Szentagothai, J. (1973) Distribution and connections of afferent fibres in the spinal cord. In Handbook of Sensory Physiology, vol. II. Somatosensory System, ed. Iggo, A., pp. 207-252. New York: Springer-Verlag.

Rethelyi, M. (1977) Preterminal and terminal axon arborizations in the substantia gelatinosa of cat's spinal cord. Journal of Comparative Neurology, 172, 511-528.

Rethelyi, M., Light, A.R. & Perl, E.R. (1982) Synaptic complexes formed by fuctionally defined primary afferent units with fine myelinated fibres. Journal of Comparative Neurology, 207, 381-393.

Rethelyi, M., Metz, C.B. & Lund, P.K. (1989) Distribution of neurons expressing calcitonin gene-related peptide mRNA in the brain stem, spinal cord and dorsal root ganglia of rat and guinea-pig. Neuroscience, 29, 225-239.

Rethelyi, M., Trevino, D.L. & Perl, E.R. (1979) Distribution of primary afferent fibres within the sacrococcygeal dorsal horn. An autoradiographic study. Journal of Comparative Neurology, 185, 603-622.

Rexed, B. (1952) The cytoarchitectonic organization of the spinal cord in the cat. Journal of Comparative Neurology, 96, 415-466.

Rexed, B. (1954) A cytoarchitectonic atlas of the spinal cord in the cat. Journal of Comparative Neurology, 100, 297-380.

Ribeiro-da-Silva, A. & Coimbra, A. (1982) Two types of synaptic glomeruli and their distribution in laminae I-III of the rat spinal cord. Journal of Comparative Neurology, 209, 176-186.

Ribeiro-da-Silva, A. & Cuello, A.C. (1990) Ultrastructural evidence for the occurrence of two distinct somatostatin-containing systems in the substantia gelatinosa of rat spinal cord. Journal of Chemical Neuroanatomy, 3, 141-153.

Ribeiro-da-Silva, A. (1995) Ultrastructural features of the colocalization of calcitonin gene-related peptide with substance P or somatostatin in the dorsal horn of the spinal cord. Canadian Journal of Physiology and Pharmacology, 73, 940-944.

Ribeiro-da-Silva, A., Castro-Lopes, J.M. & Coimbra, A. (1986) Distribution of glomeruli with fluoride-resistant acid phosphatase (FRAP)-containing terminals in the substantia gelatinosa of the rat. Brain Research, 377, 323-329.

Ribeiro-da-Silva, A., Pioro, E.P. & Cuello. A.C. (1991) Substance P- and enkephalin-like immunoreactivities are colocalized in certain neurons of the substantia gelatinosa of the rat spinal cord: an ultrastructural double-labelling study. Journal of Neuroscience, 11, 1068-1080.

Ribeiro-da-Silva, A., Tagari, P. & Cuello, A.C. (1989) Morphological characterization of substance P-like immunoreactive glomeruli in the superficial dorsal horn of the rat spinal cord and trigeminal subnucleus caudalis: a quantitative study. Journal of Comparative Neurology, 281, 497-515.

Rivero-Melian, C. & Grant, G. (1990) Distribution of lumbar dorsal root fibres in the lower thoracic and lumbosacral spinal cord of the rat studied with choleragenoid horseradish peroxidase conjugate. Journal of Comparative Neurology, 299, 470-481.

Rivero-Melian, C. & Grant, G. (1991) Choleragenoid horseradish peroxidase used for studying projections of some hindlimb cutaneous nerves and planter foot afferents to the dorsal horn and Clarke's column in the rat. Experimental Brain Research, 84, 125-132.

Rivero-Melian, C., Rosario, C. & Grant, G. (1992) Demonstration of transganglionically transported choleragenoid in rat spinal cord by immunofluorescence cytochemistry. Neuroscience Letters, 145, 114-117.

Robertson, B. & Arvidsson, J. (1985) Transganglionic transport of wheat germ agglutinin-HRP and choleragenoid-HRP in rat trigeminal primary sensory neurons. Brain Research, 348, 44-51.

Robertson, B. & Grant, G. (1985) A comparison between wheat germ agglutinin and choleragenoid-horseradish peroxidase as anterogradely transported markers in central branches of primary sensory neurons in the rat with some observations in the cat. Neuroscience, 14, 895-905.

Robertson, B. & Grant, G. (1989) Immunocytochemical evidence for the localization of the GM1 ganglioside in carbonic anhydrase-containing and RT97-immunoreactive rat primary sensory neurons. Journal of Neurocytology, 18, 77-86.

Robertson, B. (1990) Wheat germ agglutinin binding in rat primary sensory neurons: a histochemical study. Histochemistry, 94, 81-85.

Robertson, B., Lindh, B. & Aldskogius, H. (1992) WGA-HRP and choleragenoid-HRP as anterogradely transported tracers in vagal visceral afferents and binding of WGA and choleragenoid to nodose ganglion neurons in rodents. Brain Research, 590, 207-212.

Robertson, B., Perry, M.J. & Lawson, S.N. (1991) Populations of rat spinal primary afferent neurons with choleragenoid binding compared with those labelled by markers for neurofilament and carbohydrate groups: a quantitative immunocytochemical study. Journal of Neurocytology, 20, 387-395.

Routh, V.H. & Helke, C.J. (1995) Tachykinin receptors in the spinal cord. In Progress in Brain Research, ed. Nyberg, F, Sharma, H.S. & Wiesenfeld-Hallin, Z., 104, , 93-108.

Rowan, S., Todd, A.J. & Spike, R.C. (1993) Evidence that neuropeptide Y is present in GABAergic neurons in the superficial dorsal horn of the rat spinal cord. Neuroscience, 53, 537-545.

Rubniak, N..M.J., Carlson, E., Boyce, S., Webb, J.K. & Hill, R.G. (1996) Enantioselective inhibition of the formalin paw late phase by the NK-1 receptor antagonist L-733,060 in gerbils. Pain, 67, 189-195.

Ruda, M. & Coulter, J.D. (1982) Axonal and transneuronal transport of wheat germ agglutinin demonstrated by immunohistochemistry. Brain Research, 249, 237-246.

Ruda, M.A., Bennett, G.J. & Dubner, R. (1986) Neurochemistry and neural circuitry in the dorsal horn. In Progress in Brain Research, ed. Emson, P.C., Rossor, M.N. & Tonyama M., 66, 219-268. Amsterdam: Elsevier

Ruda, M.A., Coffield, J. & Dubner, R. (1984) Demonstration of postsynaptic opioid modulation of thalamic projection neurons by the combined techniques of retrograde horseradish peroxidase and enkephalin immunocytochemistry. Journal of Neuroscience, 4, 2117-2132.

Rustioni, A. & Kaufman, A. B. (1977) Identification of cells of origin of non-primary afferents to the dorsal column nuclei of the cat. Experimental Brain Research, 27, 1-14.

Rustioni, A., Hayes, N.L. & O'Neill, S. (1979) Dorsal column nuclei and ascending spinal afferents in macaques. Brain, 102, 95-125.

Salt, T.E. & Eaton, S.A. (1989) Function of non-NMDA receptors and NMDA receptors in synaptic responses to natural somatsensory stimulation in the ventrobasal thalamus. Experimental Brain Research, 77, 646-652.

Salt, T.E. & Hill, R.G. (1983) Pharmacological differentiation between responses of rat medullary dorsal horn neurons to noxious mechanical and noxious thermal cutaneous stimuli. Brain Research, 263, 167-171.

Schaible, H.G., Jarrott, B., Hope, P.J. & Duggan, A.W. (1990) Release of immunoreactive substance P in the spinal cord during development of acute arthritis in the knee joint of the cat: a study with antibody microprobes. Brain Research, 529, 214-223.

Scheibel, M.E. & Scheibel, A.B. (1968) Terminal axon patterns in cat spinal cord. II: the dorsal horn. Brain Research, 9, 32-58.

Schiffman, S.N., Teugels, E., Halleux, P., Menu, R. & Vanderhaeghen, J.J. (1991) cholecystokinin mRNA detection in rat spinal cord motoneurons but not in dorsal root ganglia neurons. Neuroscience Letters, 123, 123-126.

Schoenen, J. (1982) The dendritic organization of the human spinal cord: The dorsal horn. Neuroscience, 7, 2057-2087.

Schroder, H.D. (1984) Somatostatin in the caudal spinal cord: an immunohistochemical study of the spinal centres involved in the innervation of pelvic organs. Journal of Comparative Neurology, 223, 400-414.

Sedivec, M.J., Capowski, J.J. & Mendell, L.M. (1986) Morphology of HRP-injected spinocervical tract neurons: effect of dorsal rhizotomy. Journal of Neuroscience, 6, 661-672.

Senba, E., Yanaihara, C., Yanaihara, N. & Tohyama, M. (1988) Colocalization of substance P and met-enkephalin-Arg^{6}-Gly^{7}-Leu^{8} in the intraspinal neurons of the rat, with special reference to the neurons in the substantia gelatinosa. Brain Research, 453, 110-116.

Seroogy, K.B., Mohapatra, N.K., Lund, P.K., Rethelyi, M., McGehee, D.S. & Perl, E.R. (1990) Species-specific expression of cholecystokinin messenger RNA in rodent dorsal root ganglia. Molecular Brain Research, 7, 171-176.

Seybold, V. & Elde, R. (1980) Immunocytochemical studies of peptidergic neurons in the dorsal horn of the spinal cord. Journal of Histochemistry and Cytochemistry, 28, 367-370.

Seybold, V.S., Grkovic, I., Portbury, A.L., Ding, Y-Q., Shigemoto, R., Mizuno, N., Furness, J.B. & Southwell, B.R. (1997) Relationship of NK-3 receptor-immunoreactivity to subpopulations of neurons in rat spinal cord. Journal of Comparative Neurology, 381, 439-448.

Seybold, V.S., Tuchserer, M.M. & Garry, M.G. (1989) Coexistence of peptides in primary afferent neurons. In Processing of Sensory Information in the Superficial Dorsal Horn of the Spinal Cord, ed. Cervero, F., Benette, G.J. & Headley, P.M., pp 75-81. New York: Plenum.

Sharp, G.A., Shaw, G. & Weber, K. (1982) Immunoelectronmicroscopial localization of the three neurofilament triplet proteins along neurofilaments of cultured dorsal root ganglion neurons. Experimental Cell Research, 137, 403-413.

Shehab, S.A.S. & Atkinson, M.E. (1984) Sciatic nerve section has variable effects on primary afferent neuropeptides. Journal of Anatomy, 139, 725.

Shehab, S.A.S. & Atkinson, M.E. (1986a) Vasoactive intestinal polypeptide increases in areas of the dorsal horn of the spinal cord from which other neuropeptides are depleted following peripheral axotomy. Experimental Brain Research, 62, 422-430.

Shehab, S.A.S. & Atkinson, M.E. (1986b) Vasoactive intestinal polypeptide (VIP) increases in the spinal cord after peripheral axotomy of the sciatic nerve originate from primary afferent neurons. Brain Research, 372, 37-44.

Shigemoto, R., Nakaya, Y., Nomura, S., Ogawa-Meguro, R., Ohishi, H., Kaneko, T., Nakanishi, S. & Mizuno, N. (1993) Immunocytochemical localization of rat substance P receptor in the striatum. Neuroscience Letters, 153, 157-160.

Shortland, P., Woolf, C.J. & Fitzgerald, M. (1989) Morphology and somatotopic organization of the central terminals of hindlimb hair follicle afferents in the rat lumbar spinal cord. Journal of Comparative Neurology, 298, 416-433.

Silverman, J.D. & Kruger, L. (1988) Acid phosphatase as a selective marker for a class of small sensory ganglion cells in several mammals: spinal cord distribution, histochemical properties and relation to fluoride-resistant acid phosphatase (FRAP) of rodents. Somatosensory Research, 5, 219-246.

Silverman, J.D. & Kruger, L. (1990) Selective neuronal glycoconjugate expression in sensory and autonomic ganglia: relation of lectin reactivity to peptide and enzyme markers. Journal of Neurocytology, 19, 789-801.

Silverstein, C.C., Steinman, R.M. & Cohn, Z.A. (1977) Endocytosis. Annual Review of Biochemistry, 46, 669-722.

Simmons, D.R., Spike, R.C. & Todd, A.J. (1995) Galanin is contained in GABAergic neurons in the rat spinal dorsal horn. Neuroscience Letters, 187, 119-122.

Skofitsch, G. & Jacobwitz, D.M. (1985) Calcitonin gene-related peptide coexists with substance P in capsaicin sensitive neurons and sensory ganglia of the rat. Peptieds, 6, 747-754.

Spike, R.C., Todd, A.J. & Johnston, H.M. (1993) Coexistence of NADPH diaphorase with GABA, glycine and acetylcholine in rat spinal cord. Journal of Comparative Neurology, 335, 320-333.

Sterling, p. & Kuypers, H.G.J.M. (1967) Anatomical organization of the brachial spinal cord of the cat. I. the distribution of dorsal root fibres. Brain Research, 4, 1-15.

Sugiura, Y., Lee, C.L. & Perl, E.R. (1986) Central projections of identified unmyelinated (C) afferent fibres innervating mammalian skin. Science, 234, 358-361.

Sugiura, Y., Terui, N. & Hosoya, Y. (1989) Difference in distribution of central terminals between visceral and somatic unmyelinated (C) primary afferent fibres. Journal of Neurophysiology, 62, 834-840.

Surmeier, D.J., Honda, C.N. & Willis, W.D. (1988) Natural grouping of primate spinothalamic neurons based on cutaneous stimulation. Physiological and anatomical features. Journal of Neurophysiology, 59, 833-860.

Swett, J.E. & Woolf, C.J. (1985) The somatotopic organization of primary afferent terminals in the superficial laminae of the dorsal horn of the rat spinal cord. Journal of Comparative Neurology, 231, 66-77.

Szentagothai, J. (1964) Neuronal and synaptic arrangement in the substantia gelatinosa Rolandi. Journal of Comparative Neurology, 122, 219-239.

Tajti, J., Fischer, J., Knyihar-Csillik, E. & Csillik, B. (1988) Transganglionic regulation and fine structural localization of lectin-reactive carbohydrate epitopes in primary sensory neurons of the rat. Histochemisty, 88, 213-218.

Takahashi, T. & Otsuka, M. (1975) Regional distribution of substance P in the spinal cord and nerve roots of the cat and the effect of dorsal root section. Brain Research, 87, 1-11.

Tessler, A., Gazer, E., Artymyshyn, R., Murray, M & Goldberger, M.E. (1980) Recovery of substance P in the cat spinal cord after unilateral lumbosacral deafferentation. Brain Research, 191, 459-470.

Tessler, A., Himes, B.T., Artymyshyn, R., Murray, M & Goldberger, M.E. (1981) Spinal neurons mediate return of substance P following deafferentation of cat spinal cord. Brain Research, 230, 263-281.

Todd, A.J. & Spike, R.C. (1993) The localization of classical transmitters and neuropeptides within neurons in laminae I-III of the mammalian spinal dorsal horn. Progress in Neurobiology, 41, 609-645.

Todd, A.J. (1988) Electron microscope study of Golgi-stained cells in lamina II of the rat spinal dorsal horn. Journal of Comparative Neurology, 275, 145-157.

Todd, A.J. (1989) Cells in laminae III and IV of rat spinal dordal horn receive monosynaptic primary afferent input in lamina II. Journal of Comparative Neurology, 289, 676-686.

Todd, A.J. (1997) A method for combining confocal and electron microscopic examination of sections processed for double- or tirple-labelling immunocytochemistry. Journal of Neuroscience Methods, 73, 149-157.

Todd, A.J., Spike, R.C. & Polgar, E. (1998) A quantitative study of neurons which express neurokinin-1 or somatostatin sst_{2a} receptor in rat spinal dorsal horn. Neuroscience, 85, 459-473.

Too, H-P., Cordova, J.L. & Maggio, J.E. (1989) Heterogeneity of tachykinin-like immunoreactive peptides in rat spinal cord and dorsal root ganglia. Peptides, 10, 25-30.

Traub, R.J., Iadarola, M.J. & Ruda, M.A. (1989) Effect of multiple dorsal rhizotomies on calcitonin gene-related peptide-like immunoreactivity in the lumbosacral dorsal spinal cord of cat: a radioimmunoassay analysis. Peptides, 10, 979-938.

Tregear, G., Niall, H.D., Potts, J.T., Leeman, S.E. & Chang. M.M. (1971) Synthesis of substance P. Nature, 232, 87-89.

Trojanowski, J.Q. & Schmidt, M.L. (1984) Interneuronal transfer of axonally transported proteins: studies with HRP and HRP conjugates of wheat germ agglutinin, cholera toxin and the B subunit of cholera toxin. Brain Research, 311, 366-369.

Tuchscherer, M.M. & Seybold, V.S. (1985) Immunocytochemical studies of substance P, cholecystokinin-octapeptide and somatostatin in dorsal root ganglia of the rat. Neuroscience, 14, 593-605.

Vigna, S.R., Bowden, J.J., McDonald, D.M., Fisher, J., Okamoto, A., McVey, D.C., Payan, D.G. & Bunnett, N.W. (1994) Characterization of antibodies to the rat substance P (NK-1) receptor and to a chimeric substance P receptor expressed in mammalian cells. Journal of Neuroscience, 14, 834-845.

Von Euler, U.S. & Gaddum, J.H. (1931) An unidentified depressor substance in certain tissue extract. Journal of Physiology, 72, 74-87.

Wakisaka, S., Kajander, K.C. & Bennett, G.J. (1991) Increased neuropeptide Y (NPY)-like immunoreactivity in rat sensory neurons following peripheral axotomy. Neuroscience Letters, 124, 200-203.

Wakisaka, S., Kajander, K.C. & Bennett, G.J. (1992) Effects of peripheral nerve injuries and tissue inflammation on the levels of neuropeptide Y-like immunoreactivity in rat primary afferent neurons. Brain Research, 598, 349-352.

Wall, P.D. (1960) Cord cells responding to touch, damage and temperature of skin. Journal of Neurophysiology, 23, 197-210.

Wall, P.D. (1967) The laminar organization of dorsal horn and effects of descending impulses. Journal of Physiology, 188, 403-423.

Wan, X-C.S., Trojanowski, J.Q. & Gonatas, J.O. (1982) Cholera toxin and wheat germ agglutinin conjugates as neuroanatomical probes: their uptake and clearance, transganglionic and retrograde trasport and sensitivity. Brain Research, 243, 215-224.

Wang, H., Rivero-Melian, C., Robertson, B. & Grant, G. (1994) Transganglionic transport and binding of the isolectin B4 from Griffonia Simplicifolia I in rat primary sensory neurons. Neuroscience, 62, 539-551.

Warden, M.K. & Young, W.S. (1988) Distribution of cells containing mRNA encoding substance P and neurokinin B in the rat central nervous system. Journal of Comparative Neurology, 272, 90-113.

Warrington, W.G. & Griffith, F. (1904) On the cells of the spinal ganglia and on the relationship of their histological structure to the axonal distribution. Brain, 27, 297-325.

Westlund, K.N., Carlton, S.M., Zhang, D. & Willis, W.D. (1990) Direct catecholaminegic innervation of primate spinothalamic tract neurons. Journal of Comparative Neurology, 299, 178-186.

Westlund, K.N., Carlton, S.M., Zhang, D. & Willis, W.D. (1992) Glutamate-immunoreactive terminals synapse on primate spinothalamic tract cells. Journal of Comparative Neurology, 322, 519-527.

White, J.C. & Swett, W.H. (1969) Pain and the Neurosurgeon, Springfield: Thomas.

Wiesenfeld-Hallin, Z. (1986) Substance P and somatostatin modulate spinal cord excitability via physiologically different sensory pathways. Brain Research, 372, 172-175.

Willis, W.D. & Coggeshall, R.E. (1991) Sensory Mechanisms of the Spinal Cord. New York: Plenum.

Willis, W.D., Trevino, D.L., Coulter, J.D. & Maunz, R.A. (1974) Responses of primate spinothalamic tract neurons to natural stimulation of hindlimb. Journal of Neurophysiology, 37, 358-372.

Willis, W.D., Westlund, K.N. & Carlton, S.M. (1995) Pain. In the Rat Nervous System. 2nd ed. Paxinos, G., pp 725-750. Sydney: Academic Press.

Woolf, C.J. & Fitzgerald, M. (1986) Somatotopic organization of cutaneous afferent terminals and dorsal horn receptive fields in the superficial and deep laminae of the rat lumbar spinal cord. Journal of Comparative Neurology, 251, 517-531.

Woolf, C.J. & King, A.E. (1987) Physiology and morphology of multireceptive neurons with C-afferent inputs in the deep dorsal horn of the rat lumbar spinal cord. Journal of Neurophysiology, 58, 460-479.

Woolf, C.J. (1987) Central terminations of cutaneous mechanoreceptive afferents in the rat lumbar spinal cord. Journal of Comparative Neurology, 261, 105-119.

Woolf, C.J., Shortland, P. & Coggeshall, R.E. (1992) Peripheral nerve injury triggers central sprouting of myelinated afferents. Nature, 355, 75-78.

Woolf, C.J., Shortland, P., Reynolds, M., Ridings, J., Doublell, T. & Coggeshall, R.E. (1995) Reorganization of central terminals of myelinated primary afferents in the rat dorsal horn following peripheral axotomy. Journal of Comparative Neurology, 360, 121-134.

Yashpal, K. & Henry, J.L. (1983) Endorphins mediate overshoot of substance P-induced facilitation of a spinal nociceptive reflex. Canadian Journal of Physiology and Pharmacology, 61, 303-307.

Yashpal, K., Dam, T-V, & Quirion, R. (1990) Quantitative autoradiographic distribution of multiple neurokinin binding sites in rat spinal cord. Brain Research, 506, 259-266.

Yashpal, K., Dam, T-V, & Quirion, R. (1991) Effects of dorsal rhizotomy on neurokinin receptor subtypes in the rat spinal cord: a quantitative autoradiographic study. Brain Research, 552, 240-247.

Yoshida, S. & Matsuda, Y. (1979) Studies on sensory neurons of the mouse with intracellular recording and dye injection techniques. Journal of Neurophysiology, 42, 1134-1145.

Yoshida, S., Senba, E., Kubota, Y., Hagihira, S., Yoshiya, I., Emson, P.C. & Tohyama, M. (1990) Calcium-binding proteins calbindin and parvalbumin in the superficial dorsal horn of the rat spinal cord. Neuroscience, 37, 839-848.

Zhang, E-T., Han, Z-S. & Graig, A.D. (1996) Morphological classes of spinothalamic lamina I neurons in the cat. Journal of Comparative Neurology, 367, 537-549.

Zhang, X., Nicholas, A.P. & Hokfelt, T. (1993) Ultrastructure studies on peptides in the dorsal horn of the spinal cord.1. Coexistence of galanin with othe peptides in primary afferents in normal rats. Neuroscience, 57, 365-384.

Zhu, P.C., Thureson-Klein, A. & Klein, R.L. (1986) Exocytosis from large dense cored vesicles outside the active synaptic zones of terminals within the trigeminal subnucleus caudalis: a possible mechanism for neuropeptide release. Neuroscience, 19, 43-54.

Zimmer, A., Zimmer, A.M., Baffi, J., Usdin, T., Reynolds, K., Konig, M., Palkovits, M. & Mezey, E. (1998) Hypoalgesia in mice with a targeted deletion of the tachykinin 1 gene. Proceedings of the National Academy of Sciences of the U.S.A., 95, 2630-2535.