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In the Name of God, the Most Gracious, the Most Merciful

Spinothalamic Tract Neurons in Laminae I, III and IV of the Rat: A Retrograde Neuronal Tracing Study

A Thesis Submitted in fulfillment of the requirements for the Degree of Doctor of Philosophy

Neuroscience and Molecular Pharmacology Research Theme

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By

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Summary

Ascending pathways in the spinal cord are very important in transmitting sensory information from the periphery to the higher brain centres. The spinothalamic tract represents an important component of these ascending pathways, and it has been traditionally described as the main pathway for conveying nociceptive and thermoceptive information. Spinothalamic neurons are widely distributed within the grey matter. Lamina I represents an important nociceptive zone and provides a significant source of projection neurons, some of which project to the thalamus. A projection from cervical superficial dorsal horn to the posterior triangular thalamic nucleus (PoT) in the rat has recently been identified. The PoT is located at the caudal end of the thalamus and was not included in injection sites in many previous quantitative retrograde tracing studies of the spinothalamic tract. Therefore, one of the reasons to conduct the present study was to estimate the number of the spinothalamic cells in lamina I in rat cervical and lumbar enlargements following injections that target the PoT with or without other thalamic nuclei known to receive input from lamina I. Neurons in this lamina are also known to project to the lateral parabrachial nucleus (LPb) and the periaqueductal grey matter (PAG). Other aims of the study were to quantify neurons in lamina I in the cervical enlargement that project to the LPb and PAG, to determine the proportion of lamina I spinothalamic neurons in lumbar and cervical enlargements that could be labelled from LPb and PAG, and to investigate morphological differences between different projection populations. Recent investigations have identified a group of neurons in lamina I of rat lumbar spinal cord that had large numbers of puncta that were immunoreactive for the glycine receptor-associated protein, gephyrin, and have a very high density of input from glutamatergic axons that contain vesicular glutamate transporter 2 (VGLUT2). These "large gephyrin-coated cells" in the lumbar cord are known to project to the LPb, but it is not yet known whether they project to thalamus and PAG. Therefore, another aim was to determine whether these cells project to these areas and also to analyse the projection pattern of cells of this type in the cervical enlargement.

Previous studies have identified a population of large neurons in laminae III and IV of rat spinal cord that express the neurokinin 1 receptor (NK1r) and have prominent dorsal dendrites that enter the superficial laminae. A substantial body of evidence points to the involvement of this population of cells in processing various types of noxious stimulus. Neurons of this type in lumbar enlargement are projection cells and form a major route

through which nociceptive information reaches the brain. The proportion of these neurons that project to thalamus was not previously known, and the projection pattern of cells of this type in cervical enlargement has not yet been investigated. Therefore, an additional aim was to elucidate more on the projection patterns of these cells in both enlargements.

Various tracers (cholera toxin B subunit, Fluorogold or fluorescent latex microspheres) were injected stereotaxically into thalamus (25 rats), into thalamus and LPb (3 rats), or into thalamus and PAG (4 rats). Rats were perfused after three days and sections from the spinal cord (cervical and lumbar enlargements) were processed immunocytochemically to reveal tracer(s) in lamina I and lamina III/IV neurons, the NK1r, neuronal nuclei and, in some cases, the glycine receptor-associated protein gephyrin. Sections from brains were processed to visualise the injection sites.

Results of this study showed that: 1) most lamina I spinothalamic neurons in the C7 and L4 segments could be labelled from injections centred on the PoT; 2) the estimated total numbers of spinothalamic cells in lamina I on the contralateral side of the C7 and L4 segments are 91 and 16 cells, respectively, and this constitutes 2-3% and 0.2% of the total neuronal population in lamina I in the C7 and L4 segments, respectively; 3) the C7 segment contained fewer lamina I spinoparabrachial cells, but a similar number of spino-PAG cells, compared to L4; 4) virtually all spinothalamic lamina I neurons at both cervical and lumbar levels were labelled from LPb and between a third and a half were labelled from PAG; 5) spinothalamic lamina I neurons differed from those labelled only from LPb in that they were generally larger, more often multipolar and (in cervical enlargement) had stronger NK1r-immunoreactivity; 6) ~39% of "large gephyrin-coated cells" in L5 project to the thalamus and this accounts for ~21% of the total thalamic projection from lamina I in this segment, even though these cells constitute only ~2.5% of projection neurons in lamina I; 7) the great majority of "large gephyrin-coated cells" in C6 project to thalamus and LPb, and at both segmental levels, some project to both of these areas; 8) only few "large gephyrin-coated cells" in L5 and some of those in C6 project to PAG; 9) ~84% of the lamina III/IV NK1r-immunoreactive neurons in C6 and C7 and 17-28% of those in L4 and L5 belong to the spinothalamic tract, and these apparently project exclusively to the caudal thalamus, including PoT; 10) most of the large NK1r-immunoreactive lamina III/IV cells at both levels project to LPb, but few were labelled from PAG, and at both segmental levels, some project to both thalamus and LPb.

Findings from the present study indicate that the PoT is one of the major targets for neurons in lamina I as well as to the population of the NK1r-immunoreactive neurons in laminae III and IV. Since the PoT projects to the second somatosensory and insular cortices, the present results suggest that these are major targets for information conveyed by both these populations of spinothalamic neurons. In addition, these results confirm that projection neurons have extensive collateral projections, and suggest that different sub-populations of lamina I cell have characteristic patterns of supraspinal projection.

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Declaration

I declare that the work presented in this thesis is my own and that this thesis has not been submitted for a degree at another institution.

Dedication

To my great parents and sons..

The light of the past and the future..

List of Abbreviations

APT	anterior pretectal nucleus
CL	central lateral thalamic nucleus
CM	central medial thalamic nucleus
CTb	cholera toxin subunit B
CVLM	caudal ventrolateral medulla
DAPI	4',6-diamidino-2-phenylindole
DLF	dorsolateral funiculus
dlPAG	dorsolateral column of periaqueductal grey matter
dmPAG	dorsomedial column of periaqueductal grey matter
FLM	fluorescent latex microspheres
HRP	horseradish peroxidase
LF	lateral funiculus
LG	lateral geniculate nucleus
lPAG	lateral column of periaqueductal grey matter
LPb	lateral parabrachial nucleus
LSN	lateral spinal nucleus
MD	mediodorsal thalamic nucleus
MG	medial geniculate nucleus
MGM	medial geniculate nucleus, medial part
MPb	medial parabrachial nucleus
NK1r	neurokinin 1 receptor
NPY	neuropeptide Y
PAG	periaqueductal grey matter
PB	0.1 M phosphate buffer
PBS	phosphate-buffered saline that contained 0.3 M NaCl
PC	paracentral thalamic nucleus
pERK	phosphorylated form of extracellular signal-related kinase
PF	parafascicular thalamic nucleus
PHA-L	Phaseolus vulgaris-leucoagglutinin
PIL	posterior intralaminar thalamic nucleus
Po	posterior thalamic nuclear group
PoT	posterior thalamic nuclear group, triangular part
Re	reuniens thalamic nucleus
S1	primary somatosensory cortex

S2	secondary somatosensory cortex
SG	suprageniculate thalamic nucleus
SPF	subparafascicular thalamic nucleus
VF	ventral funiculus
vlPAG	ventrolateral column of periaqueductal grey matter
VM	ventromedial thalamic nucleus
VMb	basal ventral medial thalamic nucleus
VMpo	ventromedial thalamic nucleus, posterior part
VPI	ventral posterior inferior thalamic nucleus
VPL	ventral posterolateral thalamic nucleus
VPM	ventral posteromedial thalamic nucleus
VPPC	ventral posterior nucleus of the thalamus, parvicellular part
WGA-HRP	horseradish peroxidase conjugated to wheat germ agglutinin

Chapter 1
Introduction

1.1 Organization of the spinal grey matter

The grey matter of the spinal cord has been conventionally divided into dorsal and ventral horns with a small intermediolateral horn in the autonomic outflow segments (the thoracic and upper lumbar segments for the sympathetic system and the middle sacral segments for the spinal parasympathetic system). The dorsal horn is the major receiving zone for primary afferents, which are organized according to modality (see below). The ventral horn contains motorneurons as well as a large number of interneurons, which are involved in control of movement. In addition, the ventral horn contains neurons that give rise to different ascending pathways.

Based on certain cytoarchitectural features in Nissl-stained sections, the dorsal horn of the spinal cord has been divided into six laminae, known as the laminae of Rexed. This division was originally described in cat (Rexed, 1952, 1954). Thereafter, it was also found to be valid in the rat (Fukuyama, 1955; Steiner and Turner, 1972; McClung and Castro, 1978; Molander et al., 1984, 1989). Laminae I and II are the most dorsal and are usually referred to as the superficial dorsal horn. Lamina I consists of small cells, with occasional large ones, distributed with a low packing density. Lamina II is also known as the substantia gelatinosa (of Rolando) because it lacks myelinated fibres, and thus appears transparent in unstained sections. Cells in lamina II are small in size and densely packed (especially in its outer part). Laminae III and IV are collectively known as the nucleus proprius. Most cells in lamina III are also small and densely packed, but some scattered large neurons are also present. Neurons in lamina IV are more heterogeneous, with cells of different sizes and this lamina has a relatively low cellular density compared to that in lamina III. Lamina V is the widest in the dorsal horn and is often referred to as forming the neck of the dorsal horn. Its lateral part is characterized by the presence of longitudinal bundles of fibres that give a reticulated appearance to this part. Cells in lamina V are of various sizes and shapes that are loosely packed. Lamina VI, which was described only in the enlargements, is a narrow band at the base of the dorsal horn and contains neurons of different sizes. Each lamina contains a mixture of interneurons, whose axons terminate in the spinal cord, and projection neurons with axons that terminate at supraspinal levels. The interneurons outnumber the projection neurons; however, due to the great functional significance of the latter, an enormous amount of research work has been carried out in order to investigate these cells.

The primary afferent axons are the central processes of primary sensory neurons that are located within dorsal root ganglia. These afferents enter the spinal cord through the dorsal roots of the spinal nerves, and end in a highly specialized pattern within the spinal grey matter. As these fibres enter the spinal cord, they are segregated according to fibre diameter and sensory modality into two main groups: lateral and medial. The lateral group consists of small-diameter thinly-myelinated (Aδ) and unmyelinated (C) fibres that mainly respond to high threshold sensory stimuli. These fibres bifurcate in the Lissauer's tract into short ascending and descending branches that synapse with secondary sensory neurons in the dorsal horn within one or two segments from their level of entrance (Szentágothai, 1964). The medial group consists of large-diameter myelinated (Aβ) fibres that mainly respond to low threshold mechanical sensory stimuli. These fibres pass into the dorsal column, where they also bifurcate into ascending and descending branches, which give rise to collaterals that enter the medial part of the dorsal horn and synapse with secondary sensory neurons at various levels. Some of these afferents also ascend to the dorsal column (gracile and cuneate) nuclei. It has been shown that most (but not all) Aδ and C fibres respond to noxious stimuli (i.e. these are nociceptive afferents). On the other hand, most Aβ afferents respond to innocuous mechanical stimuli, although some of these do respond to stimuli in the noxious range (Todd and Koerber, 2006). The Aβ afferents have been found to terminate in the deep laminae (III-VI). According to sensory modality, the Aδ afferents have been divided into two major types: nociceptive fibres that mainly terminate in laminae I and V, and D hair afferents, which innervate down hairs and terminate in dorsal lamina III and ventral lamina II (Light and Perl, 1979). According to the presence or absence of neuropeptides, the C afferents have been divided into two main populations: peptidergic and nonpeptidergic (Hunt and Rossi, 1985). Examples of peptides found in these afferents include: substance P, somatostatin and calcitonin gene-related peptide (CGRP). The nonpeptidergic afferents are identified by the presence of fluoride-resistant acid phosphatase and binding to isolectin B4 (Alvarez and Fyffe, 2000). Studies have shown that peptidergic afferents terminate mainly in lamina I and outer part of lamina II, whereas nonpeptidergic afferents mainly arborize in the middle part of lamina II (Ribeiro-da-Silva, 2004).

Ascending spinal tracts provide a pathway through which sensory information reaches the higher brain centres. There are several well documented ascending systems; the best known of which are: the dorsal column pathway, and the spinoreticular, spinoparabrachial, spinomesencephalic, spinocerebellar, spinothalamic and spinohypothalamic tracts. These pathways arise from projection neurons that are scattered all over the grey matter, as well

as in the dorsolateral funiculi of the spinal white matter. Lamina I represents an important source of neurons that contribute axons to many of these ascending systems. In addition, a significant projection is provided by a population of large neurons in laminae III and IV that express the neurokinin 1 receptor (NK1r), the receptor for substance P, and possess long dorsal dendrites that arborize in the superficial dorsal horn (laminae I and II). The present study focuses on these two groups of projection neurons (in laminae I, III and IV) since many aspects of their projection patterns are still unknown.

1.2 Review of some of the main ascending tracts

1.2.1 Methods of studying projection neurons

Before the development of modern tract tracing techniques, staining of degenerating axons (after sectioning the spinal cord) was the method of choice for tracing axons of projection neurons (e.g. Lund and Webster, 1967; Zemlan et al., 1978). However, this technique did not reveal the laminar origin of these cells. Antidromic activation of neurons from their supraspinal targets has also been used to locate projection neurons in the spinal cord (e.g. Dilly et al., 1968; Giesler et al., 1976). Nevertheless, this latter technique also showed some limitations as it samples only a small number of neurons and this sampling may be biased towards larger cells.

The introduction of the method of retrograde tract tracing in the central nervous system was a breakthrough in this field (LaVail and LaVail, 1972). This technique is valuable in identifying the cell bodies of projection neurons, and depends on the injection of a neuronal tracer substance into a target in the brain. Tracer will be taken up from the extracellular space by axon terminals and transported along the axon to reach the neuronal cell bodies in the spinal cord. Several neuronal retrograde tracer substances have been used. Horseradish peroxidase (HRP), whether free or conjugated to wheat germ agglutinin (WGA), was the first tracer substance to be used in retrograde labelling studies in the early 1970's. However, certain shortcomings were encountered with this tracer; one of these is trans-neuronal labelling; i.e. tracer can escape from the axons of some neurons and be subsequently taken up by neighbouring neurons and/or axon terminals (Gerfen et al., 1982; Klop et al., 2004a; Mouton et al., 2005). In addition, it has been reported that HRP was much less efficient in labelling lamina I projection neurons compared to other tracers (Lima and Coimbra, 1988; Craig et al., 1989a). Cholera-toxin is a bacterial toxin produced by Vibrio cholerae. It binds specifically to gangliosides (GM1) on the surface of neurons and is actively taken up and transported by the axons. The toxin was first introduced as a

retrograde neuronal tracer in 1977 (Stoeckel et al., 1977). Originally, cholera-toxin was used as a combination with HRP (CT-HRP) (Shapiro and Miselis, 1985), and it was then found that injecting free cholera-toxin yielded better results (Luppi et al., 1987). Cholera-toxin is composed of two molecular subunits: A and B. Subunit A is responsible for the toxic effect, whereas the non-toxic subunit B is responsible for the internalisation and transport of the toxin in axons and cell bodies. Consequently, researchers started to use the B subunit only of Cholera-toxin (CTb), which proved to be effective in labelling somata of neurons (Ericson and Blomqvist, 1988). Other examples of retrograde tracer substances widely used nowadays are Fluorogold (Schmued and Fallon, 1986) and fluorescent latex microspheres (FLM) (Katz et al., 1984), both of which are also very effective in labelling neurons.

Another technique widely used to determine projection targets in the brain is the anterograde transport of tracer substances. In this technique, a tracer substance (e.g. *Phaseolus vulgaris-leucoagglutinin;* PHA-L) is injected into the area of concern (e.g. the spinal cord) and then this will be transported via the axons of projection neurons to specific brain regions (e.g. Craig, 1995; Gauriau and Bernard, 2004a).

1.2.2 The spinothalamic tract

The thalamus is the best documented target for projection neurons in the spinal cord. Some of the studies that provided evidence for a projection to the thalamus were published more than a century ago (e.g. Mott, 1895). The spinothalamic ascending system has been traditionally described as the main ascending pathway for pain and temperature sensation (Dostrovsky and Craig, 2006). Behavioural experiments provided evidence for the involvement of this system in pain transmission in the rat (Peschanski et al., 1986). This pathway is generally believed to be involved in transmission of somatic pain; however, recent investigations have shown that it is also involved in visceral nociception (Palecek et al., 2003). Palecek et al. (2003) demonstrated the expression of the transcription factor Fos, a marker of neuronal activation (Hunt et al., 1987), in spinothalamic cells in rat lumbar cord after noxious visceral stimulus. In addition, it has been reported that the spinothalamic tract is involved in transmission of other modalities of sensory information, such as light touch, pressure, sensation from joints and muscles (Giesler et al., 1976; Meyer and Snow, 1982) and itching sensation (Andrew and Craig, 2001; Davidson et al., 2009). The non-nociceptive functions are generally thought to be contributed by the deep dorsal horn neurons, while the nociceptive and thermoceptive functions are attributed to lamina I neurons. However, this functional division is not always valid since some lamina I

spinothalamic neurons were reported to have non-nociceptive functions (Andrew and Craig, 2001); on the other hand, some spinothalamic neurons in the deep laminae were demonstrated to have a nociceptive role (Zhang et al., 1991). Recently, the spinothalamic system in the lumbar cord has been reported to play a role in the sexual behaviour in male rats (Truitt et al., 2003).

Early investigations confirmed the presence of this pathway by using degenerating methods (Lund and Webster, 1967; Zemlan et al., 1978). This projection has been also documented using the antidromic activation technique (Dilly et al., 1968; Giesler et al., 1976). The first studies that utilized the technique of retrograde tracing to label spinothalamic cells were performed by Trevino and Carstens (1975) in cat and monkey, and by Giesler et al. (1979) in rat. Consequently, several retrograde and anterograde tracing studies have been carried out in rat (Kevetter and Willis, 1983; Peschanski et al., 1983; Granum, 1986; Kemplay and Webster, 1986; LeDoux et al., 1987; Lima and Coimbra, 1988; Burstein et al., 1990a; Cliffer et al., 1991; Li et al., 1996; Marshall et al., 1996; Kobayashi, 1998; Kayalioglu et al., 1999; Gauriau and Bernard, 2004a; Yu et al., 2005), cat (Carstens and Trevino, 1978; Craig et al., 1989b; Zhang et al., 1996; Mouton and Holstege, 1998; Craig, 2003a; Klop et al., 2004a,b, 2005a) and monkey (Willis et al., 1979, 2001; Apkarian and Hodge, 1989; Zhang and Craig, 1997; Yu et al., 1999; Craig, 2004, 2006, 2008; Craig and Zhang, 2006).

The studies listed above have described the distribution of spinothalamic neurons in the spinal cord in different species. These cells were observed in various parts of the grey matter and in the dorsolateral funiculus in rat, cat and monkey. Nevertheless, the highest contribution is provided by the dorsal horn neurons. It has been documented that spinothalamic neurons are not uniformly distributed within the grey matter but show a laminar variation; these cells are concentrated in certain laminae, whereas only few present in other laminae. Some inter-species differences have been described with regard to the distribution of these cells in the spinal cord. In the rat, Giesler et al. (1979) identified neurons in the marginal zone, nucleus proprius, the intermediate grey zone bordering the central canal and in the ventral horn. Granum (1986) classified these spinothalamic cells into eight groups: marginal group, ventral border of substantia gelatinosa, neck of the dorsal horn, lateral cervical and spinal nuclei, ventromedial portion of the dorsal horn, intermediate grey zone, dorsal portion of the ventral horn and ventral portion of the ventral horn. Kemplay and Webster (1986) re-addressed the question of the distribution of the spinothalamic cells in the rat, and they found a similar distribution to that observed by

Granum (1986), except that they considered the ventral horn neurons as one group. The group designated as the ventral border of substantia gelatinosa by Granum (1986), and subgelatinosal population by Kemplay and Webster (1986) was not recognized by Lima and Coimbra (1988) as a localized aggregation, instead, they only observed a few cells along the border between laminae II and III. A similar distribution has been also reported in the rat by Burstein et al. (1990a) in the entire spinal cord and by Marshall et al. (1996) in the lumbar cord. Kobayashi (1998) stated that the descriptions of spinothalamic populations in the rat that were given in the studies reviewed above contained several discrepancies. Thus, depending on the location and certain morphological characteristics, Kobayashi classified these cells into: marginal zone group (corresponding to lamina I neurons), subgelatinosal group (corresponding to lamina III neurons), dorsal horn neck group (corresponding to lamina IV and superficial part of lamina V), dorsal horn base group (occupying the deep part of lamina V and lamina VI), internal basilar group (corresponding to the internal basilar nucleus and adjacent region in the medial parts of laminae V and VI), intermedio-ventral group (spread over laminae VII through IX), central group (located in lamina X), lateral cervical group (in the lateral cervical nucleus of C1-3 segments) and lateral spinal group (corresponding to neurons in the lateral spinal nucleus; LSN). Moreover, Kobayashi identified spinothalamic neurons in the posterior funiculus of the first cervical segment. He observed that these different groups did not occupy the whole length of the spinal cord, but that instead, there was a specific rostrocaudal distribution; all groups were prominent in the upper cervical cord and in the cervical and lumbar enlargements, apart from the dorsal horn base and internal basilar groups, which were rare in the cervical enlargement. Spinothalamic neurons in the dorsal funiculus of C1 and C2 have been also identified by Giesler et al. (1979), Granum (1986) and, to a lesser extent, by Kemplay and Webster (1986). These neurons were considered as the downward extension of the dorsal column nuclei (gracile and cuneate) into the upper cervical cord. Kayalioglu et al. (1999) agreed with the previous reports that described the widespread distribution of spinothalamic cells in rat spinal cord. Spinothalamic neurons were reported to be absent in lamina II, except for a few that were seen in the cervical segments (Yu et al., 2005). Attempts were made to compare the relative contribution of different laminae to the spinothalamic tract. For example, Burstein et al. (1990a) reported that 11% of the spinothalamic neurons in the rat were within the superficial dorsal horn, 38% within the deep laminae of the dorsal horn, 22% within the intermediate zone and ventral horn, 10% within lamina X and 19% within the dorsolateral funiculus.

Another well documented feature of spinothalamic cells is that these cells are not present to the same extent in all spinal segments, but tend to accumulate in certain segments and to be less common in others. Several studies reported the high concentration of spinothalamic cells in the upper cervical cord in the rat (Giesler et al., 1979; Granum, 1986; Kemplay and Webster, 1986; Lima and Coimbra, 1988; Burstein et al., 1990a; Kobayashi, 1998). However, consensus is still lacking regarding the contribution of other spinal segments to the spinothalamic tract. One of the early studies failed to locate spinothalamic cells in the lumbar cord of rat after electrical stimulation of the contralateral medial lemniscus (Dilly et al., 1968). This has led to the assumption that lumbar cord does not project directly to the thalamus. However, Giesler et al. (1976) succeeded in recording units from the lumbar cord following stimulation of the contralateral thalamus. In fact, after applying the technique of retrograde tract tracing, Giesler et al. (1979) labelled more neurons in the lumbar than in the cervical enlargement. A similar finding has been reported by Kemplay and Webster (1986), and by Lima and Coimbra (1988). Kemplay and Webster (1986) reported that approximately 34% of the spinothalamic neurons arise from T13-L5 segments, whereas C5-T1 contributed to only about 4% of the total population. Burstein et al. (1990a) presented different figures for the segmental contribution to the spinothalamic tract in two rats, these proportions were: 33% and 31% were in C1-3, 13% and 23% were in C4-8, 25% and 26% were in T1-13, 16% and 13% were in L1-5 and 13% and 7% were in L6-Co3. Another study in rat reported that spinothalamic neurons were almost equally numerous in the two enlargements (Kobayashi, 1998). It is possible that the differences in the labelling efficiency of neuronal tracers used have contributed to these discrepancies in the segmental distribution of spinothalamic cells. Alternatively, variations in the spread of the injection sites may have led to these differences, as it has been reported that involvement of different thalamic nuclei in the injection sites results in labelling of neurons in different laminae. For example, Kobayashi (1998) reported that following injections that targetted the medial thalamus, 50% of the labelled cells were in the intermedio-ventral group, whereas 40% of labelled cells after lateral thalamic injections were in the internal basilar group. Another well documented feature of the spinothalamic tract in the rat is the conspicuous accumulation of spinothalamic cells in the medial part of laminae VI and VII (an area known as the internal basilar column) in the lumbar cord (Giesler et al., 1979; Kemplay and Webster, 1986; Marshall et al., 1996).

Concerning the laminar distributions of spinothalamic neurons in the cat, early studies have shown that these neurons were concentrated in three areas: lamina I, laminae IV-VI and laminae VII-VIII (Trevino and Carstens, 1975; Carstens and Trevino, 1978; Craig et

al., 1989b). Using WGA-HRP, Klop et al. (2004a,b, 2005a) completely filled the thalamus on one side, at least in some of the cats, and found that spinothalamic cells originated from all laminae throughout the length of the spinal cord, except lamina IX, and predominantly from laminae I and V-VIII, as well as from neurons in the dorsolateral funiculus. Early studies proposed that cells in different laminae target different thalamic nuclei, and, interestingly, a thalamic injection that was restricted to the ventral posterolateral (VPL) nucleus failed to label neurons in cat spinal cord (Trevino and Carstens, 1975), whereas HRP injection into the intralaminar nuclei labelled cells mainly in laminae VII and VIII (Carstens and Trevino, 1978). These observations have led the latter authors to suggest that this segregation of spinothalamic neurons into different laminae has a functional role. The differences in the segmental distribution of the spinothalamic neurons observed in the rat have been also described in the cat. Again, several studies showed that the largest number of labelled spinothalamic neurons were located in the upper cervical cord (C1-3) (Carstens and Trevino, 1978; Klop et al., 2005a; Mouton et al., 2005). Large clusters of spinothalamic neurons have been observed in the internal basilar column of lumbar segments in cat (Trevino and Carstens, 1975; Carstens and Trevino, 1978). On the other hand, at cervical levels, these cells predominated in laminae IV-VI. By analysing sections from the entire spinal cord in two cats, Mouton et al. (2005) have reported that the mean percentages for the contribution to the spinothalamic tract in the C1-3, C5-8 and L5-S1 segments were 45%, 17% and 14%, respectively. Andrew et al. (2003) analysed the distribution of the spinothalamic cells in sections from the C5-S3 segments only and the reported proportions were 50%, 6%, 38% and 6% for the C5-8, T3-9, L5-S1 and S2-3, respectively. Klop et al. (2005a) have also reported that there are at least five separate clusters of spinothalamic neurons in cat spinal cord. These clusters, which differed in the laminar location and longitudinal distribution in the spinal cord, were designated as clusters A, B, C, D and E. Clusters A and B were located throughout the length of the spinal cord and corresponded to laminae I and V, respectively. Clusters C and D were located in the upper cervical segments and corresponded to the lateral part of laminae VI and VII and the dorsal part of lamina VIII (cluster C), and cluster D corresponded to the medial part of lamina VI. Lastly, cells in the medial portion of laminae VI-VIII in the L4-Co2 segments were designated as cluster E. Earlier reports have also documented this clustering of spinothalamic neurons (Carstens and Trevino, 1978). Klop and her co-workers proposed that each cluster projects to a specific area in the thalamus.

Trevino and Carstens (1975) reported that the distribution of spinothalamic neurons in monkey lumbar cord is different from that in the cat. They noticed that cells were mainly

concentrated in the lateral parts of laminae IV and V, the remaining cells were scattered in laminae I, VII and VIII. This last observation has been also confirmed by Willis et al. (1978, 1979). Willis et al. (1978) have also reported an occasional observation of retrogradely labelled cells within lamina II. As reported in the rat and cat, a high concentration of spinothalamic cells has been found in the upper cervical cord (Willis et al., 1979, 2001; Apkarian and Hodge, 1989). Following a large injection of WGA-HRP into the thalamus of one monkey, Apkarian and Hodge (1989) reported that the segmental distribution of spinothalamic cells at various levels of the spinal cord was as follows: 35% were in the upper cervical cord (C1-3), 18% were in the cervical enlargement (C4-8), 19% were in the thoracic cord, 19% were in the lumbar cord and 9% were in the coccygeal segments. The large clusters of spinothalamic neurons that were observed in the internal basilar column of lumbar segments of rat and cat have not been observed in the monkey (Trevino and Carstens, 1975; Willis et al., 1979, 2001). Craig has carried out extensive research in order to provide evidence for the suggestion that the primate spinothalamic tract consists of anatomically and functionally distinctive components. Most of his research interest was focused on thalamic targets of lamina I neurons (e.g. Craig, 2004, 2006), and details of these are reviewed below (section 1.3.2.1). In addition, Craig targetted the ventrolateral thalamic nucleus with CTb and reported that this nucleus received almost exclusive projection from neurons in laminae V and VII (Craig, 2008).

Based on retrograde tracing studies that involved a large injection of neuronal tracer into the thalamus, the total number of spinothalamic neurons was estimated to be 9,500 in the rat (Burstein et al., 1990a), 14,293 in the cat (Klop et al., 2005a) and 18,235 in the monkey (Apkarian and Hodge, 1989). However, Klop et al. (2004a) argued that the estimated number in the monkey in the report published by Apkarian and Hodge is inaccurate because no correction factor for over-counting of neurons was used in that study, and the injection site, in the one case in which all segments were processed, did not cover all thalamic nuclei. Therefore, Klop et al. suggested that the total number of spinothalamic neurons in the monkey still needs to be investigated. The results that were presented by Burstein et al. (1990a) contradicted those of earlier studies in rat, which estimated the total number of spinothalamic neurons to be less than 1,000 in the entire spinal cord (Granum, 1986; Kemplay and Webster, 1986). Burstein et al. explained this discrepancy to the use of Fluorogold in their study, which is a more efficient tracer substance, and to the involvement of more thalamic regions in their injection sites. It seems that the re-evaluation of the number of spinothalamic neurons in the rat has led to revision of the importance of this tract in nociception in this species.

In all three species, the spinothalamic cells were observed predominantly on the contralateral side; for example, Burstein et al. (1990a) found ~75% of the labelled spinothalamic neurons were on the contralateral side. However, a substantial population of neurons projecting to the ipsilateral side was observed in the upper cervical cord, as well as at other spinal levels (Carstens and Trevino, 1978; Apkarian and Hodge, 1989; Burstein et al., 1990a).

1.2.3 The spinoparabrachial pathway

The spinoparabrachial pathway is one of the main ascending sensory tracts in the spinal cord. It consists of fibres that project from the spinal neurons to the parabrachial area in the brainstem. The parabrachial area is defined as the region that surrounds the brachium conjunctivum (the superior cerebellar peduncle) at the junction of the mesencephalon (midbrain) with the pons. Traditionally, this area has been divided into two main parts: lateral (LPb) that lies dorsal to the brachium and medial (MPb) that lies ventral to it. The LPb nucleus in rat has been further subdivided into seven subnuclei: internal lateral, external lateral, dorsal lateral, ventral lateral, superior lateral, central lateral and the lateral crescent. The MPb nucleus is subdivided into internal and external subnuclei. The Kölliker Fuse nucleus has been also considered as part of the parabrachial complex (Kitamura et al., 1993; Slugg and Light, 1994; Saper, 1995).

Early studies described dense terminal degeneration in the region of the parabrachial complex after transection of the anterolateral spinal column at upper cervical levels in the rat (Zemlan et al., 1978). Subsequent retrograde (Cechetto et al., 1985; Bernard et al., 1989; Hylden et al., 1989; Kitamura et al., 1993; Ding et al., 1995; Todd et al., 2000, 2002, 2005; Spike et al., 2003; Almarestani et al., 2007), and anterograde (Cechetto et al., 1985; Slugg and Light, 1994; Bernard et al., 1995; Feil and Herbert, 1995) tracing studies in rat have confirmed the existence of this spinoparabrachial pathway. Furthermore, this projection has been documented using the technique of antidromic activation (Bester et al., 1995, 2000). Investigations in cat (Hylden et al., 1985; Panneton and Burton, 1985; Light et al., 1993; Craig, 1995) and monkey (Wiberg et al., 1987; Craig, 1995) have also demonstrated this spinal projection to the parabrachial complex. In each species, the great majority of the spinal input to the parabrachial area originated from lamina I. Other spinoparabrachial neurons were found in the LSN and lamina V (mainly from the reticular part), with only a few observed in laminae IV, VI-VIII and X. This projection was found to arise from the entire length of the spinal cord, with some intersegmental variation in the

number of neurons. For example, Kitamura et al. (1993) reported that the number of retrogradely labelled cells in all laminae of rat spinal cord was greatest in C1-2 followed in order by T1-13, C3-8, L1-6, the sacrococcygeal cord. After PHA-L injection into the superficial dorsal horn of different spinal levels, Feil and Herbert (1995) observed that there was more anterograde labelling in the parabrachial area following lumbar injections compared to thoracic ones. All studies have described a bilateral pattern of projection from the spinal cord into the parabrachial complex; however, the contribution from the contralateral side predominated, except in the case of the upper cervical cord when ipsilateral projection was stronger (Feil and Herbert, 1995). Against this consensus, Cechetto et al. (1985) reported that the projection from the ipsilateral side of all examined spinal segments was more prominent. Anterograde tracing studies have shown that afferents from the superficial dorsal horn in the cervical and lumbar enlargements terminate in identical areas of the parabrachial complex in the rat (Slugg and Light, 1994; Bernard et al., 1995), cat and monkey (Craig, 1995). However, somatotopy has been described with regard to the trigeminal vs. spinal afferents. Cechetto et al. (1985) reported that trigeminal afferents target the MPb nucleus, whereas the spinal afferents end mainly in the LPb nucleus. Slugg and Light (1994) confirmed this observation but in addition they reported that the medullary afferents targeted the caudal LPb, while the spinal input terminated in more rostral parts. Interestingly, the internal lateral subnucleus has been reported to receive inputs mainly from neurons in deep laminae (primarily those in lamina V), and not from lamina I, which targeted other subnuclei in the LPb nucleus, as presented below (Bernard et al., 1995; Feil and Herbert, 1995; Saper, 1995).

1.2.4 The spinomesencephalic pathway

The spinomesencephalic tract is primarily a crossed pathway that arises from several laminae of the spinal grey matter (as well as from neurons in the dorsolateral funiculus) and terminates in various regions of the mesencephalon including the periaqueductal grey matter (PAG). The mesencephalic periaqueductal grey matter, also known as the central grey, is a collection of neurons that surrounds the cerebral aqueduct in the midbrain. Based on certain cytoarchitectural characteristics, the PAG has been shown to consist of a number of longitudinal columns: dorsomedial (dmPAG), dorsolateral (dlPAG), lateral (lPAG) and ventrolateral (vlPAG) (Bandler and Shipley, 1994; Lovick and Bandler, 2005).

In the early studies, the presence of this tract was shown by using silver degenerative techniques in all species studied (Mehler et al., 1960; Yamada and Otani, 1978; Zemlan et

al., 1978). Electrophysiological investigations have also demonstrated the existence of this pathway (Menétrey et al., 1980; McMahon and Wall, 1985; Hylden et al., 1986a). Retrograde tracing studies in rat have presented anatomical evidence for the spinal origin of some PAG afferents (Beitz, 1982; Menétrey et al., 1982; Liu, 1983; Swett et al., 1985; Liu, 1986; Pechura and Liu, 1986; Lima and Coimbra, 1989; Yezierski and Mendez, 1991; Keay et al., 1997; Li et al., 1998; Kayalioglu et al., 1999). This has also been documented anatomically in the cat (Wiberg and Blomqvist, 1984; Keay and Bandler, 1992; Craig, 1995; Mouton and Holstege, 1998; Klop et al., 2005b; Mouton et al., 2005), and monkey (Mantyh, 1982; Craig, 1995). Some anterograde tracing studies have also shown that several midbrain regions (including PAG) receive afferents from the lumbosacral spinal cord in the three species (Yezierski, 1988).

There is a general consistency in the segmental and laminar distributions of spino-PAG cells in rat, cat and monkey. These cells arise primarily from lamina I, the reticular part of lamina V and from the lateral cervical and spinal nuclei; fewer cells take origin from laminae VI-VIII and X. This tract arises from all spinal levels, and as the case with most projection pathways, the upper cervical cord contributes the great majority of these cells (Menétrey et al., 1982; Yezierski and Mendez, 1991; Keay et al., 1997; Mouton et al., 2005). Yezierski and Mendez (1991) reported that the contributions of different segmental levels to the spinomesencephalic tract were: 30% (C1-4), 15% (C5-8), 9% (T1-4), 9% (T5-8), 8% (T9-13), 14% (L1-3) and 15% (L4-6). These last authors also analysed tissue from the sacral cord and Fig. 1 in their paper shows that the spinomesencephalic neurons in this part of the cord were as numerous as those in the upper segments, but it is not clear why they did not include this part in the overall percentage of segmental distribution of these cells. Keay et al. (1997) reported that the highest concentration of spino-PAG cells was observed in C1-4 (50% of the cells), followed by S1-3, where 20% of the cells were seen. They reported that fewer cells were located in the cervical and lumbar enlargements and the smallest number was found in the thoracic segments. Moreover, they reported that labelled neurons in lamina I were observed at all segmental levels and that these constituted approximately 27% of all spino-PAG neurons. Investigations have shown that cells of this tract arise from both sides of the cord but predominantly from the contralateral side; however, cells with projection to both sides have also been observed, some of which were in lamina I (Yezierski and Mendez, 1991). In the monkey, Mantyh (1982) has reported that almost all (99%) of lamina I spino-PAG cells were labelled from the contralateral side. Interestingly, some of the spinomesencephalic neurons in rat cervical and thoracic cord, including cells in lamina I, were found to send propriospinal projections

to the lumbar cord (Yezierski and Mendez, 1991). It has been reported that afferents from the spinal cord that terminate in the PAG mainly target its ventral part (Beitz, 1982); however, a projection to the dorsal part has been also described (Liu, 1983; Yezierski, 1988). Lima and Coimbra (1989) have reported that there was more retrograde labelling in various laminae of the spinal cord following injections that targetted the caudal part of the PAG and adjacent area than injections that targetted the rostral part.

For a long time, the PAG has been regarded as a source of descending inhibitory pathways that subserve an anti-nociceptive role (Beitz, 1982; Bandler and Shipley, 1994; Behbehani, 1995). The interest in this structure started 40 years ago, after the report by Reynolds (1969) who observed that electrical stimulation of the PAG led to profound analgesia in the rat. This finding has been further demonstrated in several laboratories (Mayer et al., 1971; Richardson and Akil, 1977). Therefore, it has been suggested that spinal input to the PAG (mainly that from lamina I) is involved in the activation of this inhibitory pathway, leading to a feedback modulatory effect on neurons in the spinal cord (Basbaum and Fields, 1984). The circuitry of the PAG is complex, and the spinal input forms only a minor source of afferents to this region of the midbrain (Beitz, 1982). Other sources of afferents to the PAG include: the hypothalamus, prefrontal cortex, medullary and pontine reticular formation and the parabrachial nuclei. Therefore, the PAG has been implicated in a variety of functions, including the processing and modulation of painful stimuli, processing of fear and anxiety, autonomic regulation and vocalisation (Behbehani, 1995). The longitudinal columns of the PAG have been found to differ in their response to electrical activation (Bandler and Shipley, 1994; Lovick and Bandler, 2005). Stimulation of the dorsal zone, which corresponds to the dlPAG and lPAG columns, leads to short-lasting analgesia and active reaction to pain, i.e. fight or flight behaviour as well as increase in the blood pressure and tachycardia. On the other hand, activation of the ventral zone, which corresponds to the vlPAG column, results in long-lasting analgesia and a passive response to pain, i.e. quiescence, decrease in the blood pressure and bradycardia.

1.3 Lamina I

1.3.1 General description

1.3.1.1 Structure and function

Lamina I represents a thin rim at the dorsal and dorsolateral margins of the dorsal horn (Rexed, 1952, 1954). This lamina is also known as the marginal zone or layer. Based on

the distribution of the NK1r-immunoreactivity and the location of projection neurons, it was reported that lamina I is thicker in its central part than its medial and lateral margins (Todd et al., 1998). It has been known for several decades that lamina I neurons of the spinal cord play an important role in nociceptive and thermoceptive pathways. This lamina is one of the major termination zones for Aδ and C primary afferent fibres, some of which are known to transmit nociceptive information (Light and Perl, 1979; Todd and Koerber, 2006). Some of these afferents contain substance P, a neuropeptide associated with nociception (Hökfelt et al., 1975; Lawson et al., 1997). Neurons in lamina I have been shown to respond to noxious (high threshold) thermal and mechanical stimuli mediated by Aδ and C afferent fibres (Christensen and Perl, 1970).

The circuitry in lamina I is complex and not yet fully understood. However, investigations have started to unravel some of its complexity. Two main classes of interneurons have been identified in this lamina: inhibitory and excitatory. The inhibitory interneurons use GABA and/or glycine as the main neurotransmitter, whereas the excitatory ones use glutamate. It has been reported that approximately 25-30% of neurons in lamina I are GABAergic cells (Todd and Sullivan, 1990; Polgár et al., 2003). These interneurons, along with neurons in lamina II, are thought to play a role in modulation of nociceptive processing, as originally proposed in the gate theory of pain by Melzack and Wall (1965). In this theory, it was suggested that low threshold primary afferents activate interneurons that inhibit the transmission of nociceptive inputs to central transmission (T) cells, which represented a major output from the region. Another type of modulation that occurs in lamina I is through descending axons from higher brain centres, e.g. from the PAG via nucleus raphe magnus (Behbehani, 1995).

Therefore, there is considerable evidence that lamina I plays an important role in receiving, processing and modulating nociceptive stimuli and then sending this sensory information to higher brain centres via projection neurons in this lamina. However, it has been shown that some neurons in this lamina also respond to innocuous (low threshold) mechanical or thermal stimuli (Light et al., 1993; Willis and Coggeshall, 2004), or to changes in the metabolic state of the body (Craig, 2003b). Furthermore, a response to cutaneous application of histamine (a pruritic stimulus) has been also reported (Andrew and Craig, 2001; Davidson et al., 2009).

Lamina I contains neurons of different sizes and shapes. Early studies have described the large marginal cells of Waldeyer (Gobel, 1978; Cervero and Iggo, 1980). In transverse

sections, these cells were reported to have large dorsoventrally flattened somata with long dendrites that extended in a mediolateral direction. Molander et al. (1984) reported that there are approximately three or four such large neurons in each 80 µm thick section in lamina I of rat spinal cord, and it has been reported that 6% of all lamina I neurons in the cervical and lumbar cord of cat were large cells and these were of different morphological types (Galhardo and Lima, 1999).

The great majority of lamina I neurons possess dendrites that arborize within the lamina. Therefore, morphology of these neurons is best recognized in horizontal sections. The original attempts to characterize the different morphological types of lamina I neurons were carried out using the Golgi technique on the spinal trigeminal nucleus of the cat (Gobel, 1978). Thereafter, other morphological studies have been performed in different species (Beal et al., 1981; Lima and Coimbra, 1986; Galhardo and Lima, 1999). Based on the shape of the cell bodies and the pattern of primary dendritic branching, Lima and Coimbra (1986) described four main morphological classes for lamina I neurons: fusiform, pyramidal, multipolar and flattened. This classification scheme is widely accepted nowadays, and this identifies fusiform cells as elongated bipolar cell bodies with two main dendritic trunks arising from the opposite poles. Pyramidal neurons have triangular somata with three main primary dendrites. Multipolar cells possess polygonal or ovoid cell bodies with more than three main primary dendrites, some of which extend ventrally. The flattened neurons have discoid cell bodies that are flattened dorsoventrally and possess 3-5 primary dendrites that arborize in the horizontal plane. In an attempt to unify the nomenclature, some researchers argued that since flattened neurons of Lima are basically multipolar in shape, it would be more convenient to classify them under the multipolar group (Yu et al., 2005). Subtypes of the basic forms have been also described. For example, Lima and Coimbra (1986) and Galhardo and Lima (1999) reported two varieties of fusiform cells based on the presence or absence of ventral dendrites. The latter study also reported the presence of recurrent dendrites in some fusiform cells. Two varieties of multipolar neurons have been also described according to the density of dendritic branches: compact and loose (Lima and Coimbra, 1986; Galhardo and Lima, 1999). Interestingly, Lima's group described a preferential distribution of each morphological type along the mediolateral extent of lamina I: fusiform cells predominated in the lateral third, multipolar cells were mainly located in the medial half, flattened cells were observed in the middle third, whereas the pyramidal neurons were located along the entire mediolateral extent of the lamina; however, this preferential location of specific morphological types within the lamina has not been confirmed by other investigators.

1.3.1.2 Projection targets

Lamina I constitutes one of the major sources of projection neurons in the spinal cord. Spike et al. (2003) estimated the total number of projection neurons in this lamina of the L4 segment in the rat to be approximately 400 cells on each side (equivalent to 1.6 cells per 10 µm length of the spinal cord). This constitutes ~6% of the total number of lamina I neurons which are approximately 6,238 on one side of this segment (Spike et al., 2003; Al-Khater et al., 2008). The corresponding number estimated by Bice and Beal (1997a) was 1.22 cells per 10 µm length in the L1 segment of rat spinal cord. It can be inferred from the two papers by Bice and Beal (1997a,b) that approximately 11% of lamina I neurons in the L1 segment of the rat project to supraspinal centres. However, it is unlikely that the number of projection neurons differs between the lumbar segments; probably these different results are due to different methods used in the two studies. In rat lumbar cord, it has been reported that 52% of all projection neurons in lamina I projected to rostral brain centres (dorsal thalamus and midbrain), 3% projected to caudal brainstem and 45% projected to both areas (Bice and Beal, 1997a). The percentage of projection neurons in lamina I of rat cervical cord has not yet been reported.

Using a combination of retrograde and anterograde techniques, lamina I neurons in various species have been found to terminate in several supraspinal targets. These targets are: the caudal ventrolateral medulla (CVLM) (Menétrey et al., 1983; Lima et al., 1991; Todd et al., 2002; Spike et al., 2003), LPb (Cechetto et al., 1985; Hylden et al., 1989; Ding et al., 1995; Todd et al., 2000; Spike et al., 2003), PAG (Menétrey et al., 1982; Lima and Coimbra, 1989; Craig, 1995; Keay et al., 1997; Todd et al., 2000; Spike et al., 2003), thalamus (Lima and Coimbra, 1988; Hylden et al., 1989; Burstein et al., 1990a; Marshall et al., 1996; Craig, 2003a; Gauriau and Bernard, 2004a), nucleus of the solitary tract (Menétrey and Basbaum, 1987), dorsal reticular nucleus (Lima, 1990), locus coeruleus and subcoeruleus (Craig, 1995) and limbic and striatal areas (Newman et al., 1996). A projection to the hypothalamus has also been reported (Burstein et al., 1990b). Snyder et al. (1978) described a projection to the cerebellum from lamina I neurons in cat and monkey but not in rat. In addition to these supraspinal projections, Craig (1993) has reported a projection from lamina I neurons in the cervical and lumbar cord to the thoracolumbar sympathetic nuclei in cat and monkey. Examples of the main projection targets for lamina I neurons are shown in Fig. 1-1.

Figure 1-1. Main supraspinal projections from lamina I neurons.

This diagram shows examples of the main supraspinal targets for lamina I neurons. Arrow sizes indicate the relative density of lamina I projections. CVL: caudoventrolateral reticular nucleus; Hyp: hypothalamus; LatC: lateral cervical nucleus; LPB: lateral parabrachial nucleus; Po: posterior thalamic nuclear group; Sol: nucleus of the solitary tract; SRD: subnucleus reticularis dorsalis; VLPAG: ventrolateral periaqueductal grey; VPL: ventral posterolateral thalamic nucleus. From: the multiplicity of ascending pain pathways (Villanueva and Bernard). In: Handbook of behavioral state control.

Po
VPL
Hyp

VLPAG

LPB

Sol
CVL
SRD

LatC
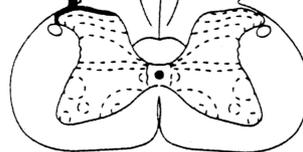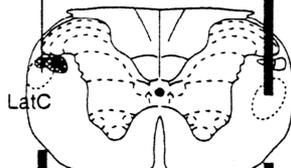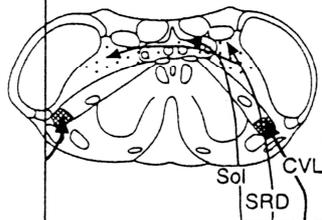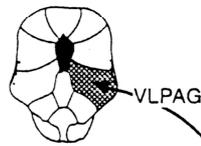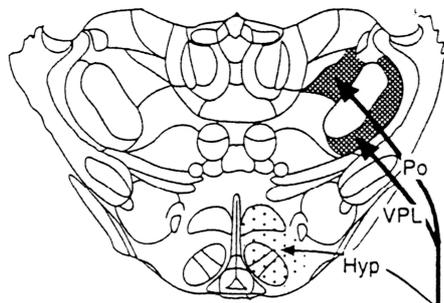
Some studies have reported that there is a preferential distribution of lamina I projection neurons within the mediolateral extent of the lamina. Bice and Beal (1997a) stated that 44% of the projection neurons were located in the lateral third of the lamina, 43% in the middle third and the remaining 13% occupied the medial third. As to the spinothalamic neurons, Lima and Coimbra (1988) reported that most of these neurons were observed in the middle third of lamina I. In a subsequent study, Lima (1990) reported that lamina I neurons that projected to the dorsal reticular nucleus were located mainly in the medial third of this lamina; however, this preferential location of a specific projection neurons within the lamina has not been confirmed by other studies.

Several studies have shown that projections from lamina I neurons are predominantly to the contralateral side of the brain, with fewer cells projecting to the ipsilateral side. However, Spike et al. (2003) provided evidence for that most of the ipsilaterally-projecting neurons actually projected to both sides of the brain. This was documented following injection of tracer into both sides of the brain to target the CVLM, LPb and PAG (Spike et al., 2003).

The course of axons of lamina I projection neurons in the spinal cord is still a matter of controversy. This topic attracted a great deal of attention due to its great clinical implications especially with regard to the ability of surgical cordotomy to alleviate pain (Nathan, 1963). Some investigators were able to visualize the course of spinothalamic axons within the grey matter, and they observed that these axons cross to the other side within a short distance from the cell body; however, this observation has not been confirmed anatomically for neurons in lamina I (Willis et al., 1979; Apkarian and Hodge, 1989). Apkarian and Hodge (1989) assumed that the reason behind the inability to trace axons of lamina I neurons within the grey matter is their small diameter compared to those from other laminae. Nonetheless, using electrophysiological methods, McMahon and Wall (1985) were able to determine the level of decussation of these axons in the rat. They found that these axons decussate within 1-5 mm from the cell body. Early degeneration studies have shown that axons of projection neurons ascend in the ventral half of the spinal cord (Mehler et al., 1960). Using HRP to label spinothalamic cells in the monkey, Willis et al. (1979) observed numerous widely dispersed spinothalamic axons in the ventral part of the lateral funiculus (LF), and some were in the ventral funiculus (VF). However, subsequent studies have reported that axons from lamina I neurons ascend via the dorsal, rather than the ventral, part of the LF. This was first documented in rat using electrophysiological methods, when McMahon and Wall (1983, 1985) traced lamina I

projections at the level of the C2 segment and reported that most, or all, recorded units were located within the dorsal half of the contralateral LF, with most axons located superficially. However, McMahon and Wall (1985) stated that these recordings were mainly for axons that terminated in the brainstem; none of the 13 lumbar lamina I neurons investigated in their study were found to project to the thalamus. Therefore, the latter authors assumed that spinothalamic axons do not ascend via the dorsolateral funiculus (DLF). However, Jones et al. (1985) demonstrated the presence of a dorsolateral spinothalamic pathway in the cat that arose from neurons in lamina I. This observation has been further extended by Apkarian et al. (1985) who reported that axons of all lamina I projection neurons ascend in the DLF. These authors reached that conclusion after injecting HRP into either the VF or DLF in cat spinal cord and observed that the labelling in lamina I neurons occurred mainly following injections in the DLF. Further evidence has been added by Hylden et al. (1986b) who reported that axons of lamina I projection neurons that terminated in the midbrain parabrachial area of the cat ascend via the DLF, and this has been also demonstrated in the rat (Hylden et al., 1989). This latter study found that the number of spinoparabrachial lamina I cells was remarkably reduced in the lumbar cord following bilateral lesion of the DLF at lower thoracic levels. In contradiction to the previous studies, Craig (1991) has reported that ascending axons of lamina I neurons in the cat mainly ascend in the middle part of the LF. In a more recent study, Craig (2000) found that his observation was also valid in the monkey. A possible explanation for the variability between these results is related to the level at which these fibres were examined, as it has been shown that spinothalamic fibres, some of which arose from lamina I, shifted from the VF to DLF as they ascended through the white matter (Dado et al., 1994a).

1.3.1.3 Immunoreactivity to the NK1 receptor

Nowadays, a substantial amount of interest has been directed towards the neurokinin 1 receptor (NK1r), through which substance P acts. The NK1r is one of the family of G protein-coupled receptors, which consist of seven membrane-spanning domains (Yokota et al., 1989; Hershey and Krause, 1990). Several studies have documented the functional role of substance P and its receptor (NK1r) in the transmission of pain sensation to higher brain centres (Iversen, 1998). Previous studies have shown that substance P is located in small-diameter primary afferents that terminate in the superficial dorsal horn (Hökfelt et al., 1975; Salt and Hill, 1983), and released into the dorsal horn following painful stimuli (Kantner et al., 1985). Earlier pharmaco-physiological studies showed that iontophoretic application of substance P on the dorsal horn of cat lumbar cord resulted in a slow-onset

and prolonged excitatory effect on neurons (Henry et al., 1975) Interestingly, substance P was found to have a preferential excitation on nociceptive neurons (Henry, 1976; Salter and Henry, 1991). Cao et al. (1998) exposed mutant mice that lack substance P-encoding gene to various types (and intensities) of noxious stimuli. They observed that, compared to the wild type, mutant mice were less responsive to the noxious stimuli when these were applied at moderate and high intensities. Similar results have been found by De Felipe et al. (1998) in mice that lacked the gene for the NK1 receptor. These mice showed depressed responses to various types of noxious stimuli when these were applied at higher intensities. In addition, the "wind-up" phenomenon that indicates the central sensitization of dorsal horn neurons was absent in the NK1r-nockout mice. Surprisingly, drugs that antagonise the NK1r proved ineffective in treating pain (Hill, 2000). This was explained by the complex nature of processing of nociceptive information in the dorsal horn since various neurotransmitters and neuropeptides are usually involved.

Immunocytochemical studies demonstrated that NK1r-immunoreactivity was densest in lamina I (Liu et al., 1994; Brown et al., 1995; Littlewood et al., 1995; Marshall et al., 1996; Todd et al., 1998, 2000). Despite this intense immunoreactivity to the NK1r in this lamina, Brown et al. (1995) have reported that only 5% of lamina I neurons expressed the receptor. However, Todd et al. (1998) found that ~45% of lamina I neurons possessed the NK1 receptor, and in another study, these authors found that the total number of NK1r-immunoreactive neurons in lamina I and the overlying dorsal white matter on one side of the L3 segment in rat was 64 cells per 70 µm thick Vibratome section (Todd et al., 2000). Todd et al. attributed the discrepancy between their results and those of Brown et al. (1995) to the sensitivity of the methods adopted by the two studies. The method used by Todd et al. (1998) was thought to be more accurate because it allowed the detection of the receptor on the plasma membrane of weakly-immunoreactive neurons. Furthermore, Todd et al. used a stereological counting method, unlike the other study. It has been reported that none of the NK1r expressing neurons in lamina I were GABA- or glycine-immunoreactive, and therefore, unlikely to be inhibitory in function (Littlewood et al., 1995).

With regard to the NK1r-immunoreactivity of projection neurons in lamina I, Li et al. (1996) reported that between one-fourth and one-third of lamina I spinothalamic neurons were immunoreactive to the receptor, whereas this was only present on one-tenth of spinothalamic neurons in laminae IV-X. However, Marshall et al. (1996) have found that the majority of lamina I spinothalamic neurons (77%) showed immunoreactivity to the NK1 receptor. It has been reported that 49% of the NK1r-immunoreactive lamina I cells in

rat spinal cord project to the thalamus (Yu et al., 2005). Moreover, investigations showed that the great majority of lamina I neurons that project to other supraspinal targets such as CVLM, LPb or PAG also expressed the receptor (Ding et al., 1995; Todd et al., 2000, 2002; Spike et al., 2003). It has been reported that the intensity of this immunoreactivity differed between the projection neurons according to the projection target. Spike et al. (2003) described three different levels of NK1r-immunoreactivity (strong, medium and weak) in lamina I projection neurons. The neurons that were labelled from the CVLM and LPb displayed all strengths of immunoreactivity, whereas those labelled from the PAG were mainly medium or weak. A comparison has been made between NK1r-immunoreactivity and morphology of projection neurons in lamina I of rat lumbar cord. This showed that significantly fewer unclassified cells were NK1r-immunoreactive compared to multipolar (81%), pyramidal (80%) and fusiform (75%) cells (Spike et al., 2003). Furthermore, pyramidal neurons had significantly stronger immunoreactivity than both fusiform and unclassified cells.

Several attempts have been made to classify the NK1r-immunoreactive neurons in lamina I. Cheunsuang and Morris (2000) and Cheunsuang et al. (2002) classified these neurons according to morphology and size into small fusiform and large multipolar or pyramidal neurons. The former study stated that the large neurons accounted for less than half of the NK1r expressing population in lamina I, and were located mainly dorsal to the small type. The dendritic trees of these neurons were found to have infrequent branches and to be oriented mainly mediolaterally or rostrocaudally with very limited ventral spread (Cheunsuang and Morris, 2000).

Evidence for the involvement of NK1r-immunoreactive lamina I neurons in the processing of nociceptive signals has been provided by anatomical studies. Noxious stimulation of the hindpaw in the rat led to internalisation of the NK1r into the cytoplasmic compartment of the cell body and the dendrites (Mantyh et al., 1995; Abbadie et al., 1997; Allen et al., 1997; Honoré et al., 1999; Polgár et al., 2007a). Furthermore, the number of neurons that showed receptor internalisation was increased in the rats that had inflamed hindpaw (Abbadie et al., 1997), which led to the suggestion that substance P and NK1r play a pivotal role in the central sensitization of dorsal horn neurons in chronic pain states. Other anatomical studies demonstrated the expression of Fos in NK1r-immunoreactive lamina I neurons in response to various forms of noxious stimulus (Doyle and Hunt, 1999 a,b; Todd et al., 2002). These NK1r expressing neurons were shown to have a role in the development of hyperalgesia, since selective ablation of these neurons by intrathecal

administration of a cytotoxic substance (saporin) conjugated to substance P resulted in attenuation of responses to highly noxious stimuli and reduction in the mechanical and thermal hyperalgesia in inflammatory (Mantyh et al., 1997) and neuropathic (Nichols et al., 1999) pain models. Another line of evidence for the nociceptive function of the NK1r expressing neurons in lamina I was provided by the activation of the phosphorylated form of extracellular signal-related kinase (pERK) in these neurons after different types of noxious stimuli (mechanical, thermal, chemical) (Ji et al., 2002; Polgár et al., 2007a). Since most lamina I projection neurons in the rat express the NK1r (Marshall et al., 1996; Spike et al., 2003), it is possible that this projection system is involved in the analysis of intense pain and hyperalgesia, as was suggested by many investigators.

1.3.1.4 Current views about the relation between function, morphology and NK1r expression

Han et al. (1998) have characterized three distinct groups of lamina I neurons in the cat. A correlation was observed between the functional types and the morphology of the neurons: nociceptive-specific cells (responding only to noxious mechanical and heat stimuli) were mainly fusiform, COOL cells (responding only to innocuous cooling) were mainly pyramidal, and polymodal nociceptive neurons (responding to noxious heat, pinch, and cold; also known as wide dynamic range neurons) were mainly multipolar in shape (Han et al., 1998). The findings by Yu et al. (1999, 2005) of low expression of NK1r in spinothalamic pyramidal cells in monkey and rat are in favour of the previous assumption. However, the situation appears to be controversial in the rat since Todd et al. (2002) and Spike et al. (2003) reported that 80% of pyramidal projection neurons in lamina I of rat lumbar cord were NK1r-immunoreactive. Moreover, it has been reported that 80% of each of the three morphological types of projection neurons in lamina I in rat showed Fos-labelling after injection of formalin into the ipsilateral hindpaw (Todd et al., 2002). Todd et al. (2002) observed that all of the three morphological types of NK1r expressing projection neurons in lamina I in rat received many contacts from substance P-containing primary afferents with no significant difference between the density of this innervation among the three groups. Therefore, most pyramidal projection neurons in rat appear to express the NK1r and to be activated by noxious stimuli. Todd et al. (2002) suggested that the reason behind the discrepancy between their results and those of Han et al. (1998) and Yu et al. (1999, 2005) is probably due to the difference between neuronal populations analysed in each case; the neuronal sample studied by Yu et al. included mainly spinothalamic neurons (and this could be also true for Han et al.), while those studied by Todd et al. belonged to

the spinomedullary system. Todd et al. (2002) concluded that most lamina I projection neurons that possess the NK1r are nociceptive and receive substance P-containing primary afferent fibres, and that if any cooling-specific neurons are present in lamina I then it is likely that these would be found among the cells that lack the receptor and do not express Fos after noxious stimulation. However, it appears that some correlation between morphology and function does occur in rat lamina I spinoparabrachial neurons; Todd et al. (2005) reported that multipolar, but not fusiform or pyramidal, neurons were activated by noxious cold stimuli. It has been reported that NK1r-immunoreactivity of lamina I projection neurons in rat, rather than the morphology, gives a better indication of the density of innervation by substance P-containing primary afferents and responsiveness to acute noxious stimuli, i.e. a better indicator of function (Todd et al., 2002).

From the previous account it appears that the subject of NK1r expression among pyramidal neurons in lamina I is a matter of controversy in the literature. Hence, one of the aims of the present study is to determine the percentage of cells that express the NK1r among the pyramidal spinothalamic neurons in lamina I of rat cervical and lumbar cord.

1.3.1.5 Large gephyrin-coated neurons

Recent investigations have identified a group of neurons in lamina I of rat lumbar spinal cord that had large numbers of puncta that were immunoreactive for the glycine receptor-associated protein, gephyrin, at inhibitory synapses (Puskár et al., 2001). These cells also receive a very dense input from axons that contain vesicular glutamate transporter 2 (VGLUT2) at excitatory synapses (Polgár et al., 2008). Puskár et al. (2001) reported that these neurons have a larger cross sectional area compared to other cells in this lamina and lacked, or only weakly expressed, the NK1 receptor. Both of these studies described these cells to be mainly multipolar with dendrites that arborize in the horizontal plane. These cells were defined as "large gephyrin-coated cells" by these authors and probably represent a subset of the classical Waldeyer cells identified in earlier studies (Gobel, 1978; Lima and Coimbra, 1986). It has been reported that there are approximately 10 of these cells on each side in the L4 segment in the rat, and these were present throughout the mediolateral extent of the most superficial part of lamina I (Polgár et al., 2008).

This group of lamina I neurons has been found to respond to noxious chemical stimuli: most (87%) expressed Fos following subcutaneous injection of formalin (Puskár et al., 2001), and some (38%) expressed Fos in response to noxious heat stimuli (Polgár et al., 2008).

Tracer injection into the LPb and adjacent regions demonstrated that these cells are projection neurons, and presumably account for 2.5% of the total population of the spinoparabrachial neurons in lamina I (Puskár et al., 2001). It is still unknown whether these neurons project to the thalamus or PAG. Thus, one of the objectives of the present study is to determine whether cells of this type in the lumbar cord project to these areas. An additional aim is to investigate these cells in the cervical enlargement since this is still unknown. The size of these cells and their special input indicate that these cells play a particular role in lamina I; therefore, investigating projection targets of these cells would lead to a better understanding of their significance in spinal cord circuitry.

1.3.2 Projection to the thalamus

1.3.2.1 Thalamic nuclei that receive input from lamina I

In spite of the general consensus regarding projections of lamina I neurons to the thalamus, debate still exists around the precise area of termination within the thalamic nuclei, especially in the monkey (Craig and Blomqvist, 2002; Jones, 2002; Treede, 2002; Willis et al., 2002). Classically, it had been believed that there was a clear distinction between medial and lateral thalamic nuclei. The medial nuclei receive spinal input mainly from deeper parts of the grey matter, whereas the lateral nuclei receive input from more superficial areas (Giesler et al., 1979; Willis et al., 1979), and each of these thalamic nuclei projects to different cortical areas: the medial nuclei project to the limbic cortex concerned with affect and motivation, whereas the lateral nuclei project to the somatosensory cortex, which is involved in the discriminative aspects of pain processing. However, this classical view is highly doubted nowadays since it has been found that lamina I projects to medial and lateral thalamic nuclei (Gauriau and Bernard, 2004a). A substantial body of evidence points to the VPL nucleus as the main spinal somatosensory relay station in the thalamus (Willis et al., 2001; Groenewegen and Witter, 2004). Gauriau and Bernard (2004a) have found that lamina I neurons in rat cervical cord project largely to the caudal part of the VPL. They also reported that projection from the deep laminae to the VPL was less prominent than that from lamina I. A projection to this nucleus from the marginal zone in rat cervical and lumbar cord has been also documented physiologically by Zhang et al. (2006). The importance of this nucleus as one of the targets for lamina I neurons in the cat has been also reported (Martin et al., 1990), although these authors observed that the projection was mainly to the periphery of the VPL, rather than the core. Some earlier studies have doubted the significance of the VPL in receiving afferents from the spinal

neurons in cat (Carstens and Trevino, 1978). Instead, this latter study revealed that lamina I neurons projected mainly to the rostral ventrobasal complex and caudal ventrolateral nucleus. The posterior complex of the thalamus (Po), which is located at its caudal part, has received considerable attention as one of the sensory relay stations that receive substantial input from the spinal cord (Gauriau and Bernard, 2004a; Groenewegen and Witter, 2004; Zhang and Giesler, 2005). Gauriau and Bernard (2004a) referred specifically to the triangular part of the Po (PoT) as receiving a significant spinal input from lamina I in rat cervical cord, and stated that their study was the first to demonstrate this projection. The spinal projection to the PoT was also documented physiologically when Zhang and Giesler (2005) demonstrated units in the marginal zone, and deep neurons, of rat cervical cord after antidromic activation of the contralateral PoT of the thalamus. In agreement with Gauriau and Bernard (2004a), the study by Zhang and Giesler (2005) reported that about two-thirds of the spinal projection to posterior thalamus originated from the superficial dorsal horn. Other regions in the posterior thalamus, apart from the PoT, were also found to receive terminals from spinal lamina I neurons (Gauriau and Bernard, 2004a; Zhang and Giesler, 2005). The parvicellular part of the ventral posterior nucleus (VPPC), a well-known gustatory and visceral centre (Groenewegen and Witter, 2004), has been also shown to receive spinal projections from lamina I in the cervical cord of the rat (Gauriau and Bernard, 2004a). These authors also described sparse terminal labelling in the mediodorsal nucleus (MD) that originated from lamina I; however, almost no projection was found from the deep laminae. The dorsal portion of the nucleus submedius was originally described by Craig and Burton (1981) as receiving afferents from the superficial layers of the spinal dorsal horn in the cat. Subsequent reports have supported this projection (Blomqvist et al., 1992). Andrew et al. (2003) observed that fewer lamina I neurons were labelled in the cat in which the injection missed the nucleus submedius, compared with other cats that successfully received injection in this nucleus. Craig (2003a) confirmed the prominent projection of lamina I neurons in cat to the nucleus submedius; in addition, he described intense projection to the dorsomedial part of the ventral posteromedial nucleus (VPM), and the ventral aspect of basal ventral medial nucleus (VMb), as well as to the lateral habenula. In the rat, the nucleus submedius has been shown to receive sparse input from spinal laminae III-V and VII but not from the superficial laminae (Dado and Giesler, 1990; Li et al., 1996; Gauriau and Bernard, 2004a). However, functional studies have demonstrated a nociceptive role for the nucleus submedius in the rat (Fu et al., 2002). The paraventicular nucleus, one of the midline thalamic nuclei, has been found to receive sparse projection from both superficial and deep laminae of the cervical dorsal horn in rat (Gauriau and Bernard, 2004a). A few terminals from lamina I in rat cervical cord have

been demonstrated in nucleus reuniens thalami (Re), another midline thalamic nucleus (Gauriau and Bernard, 2004a). The intralaminar nuclei consist of the central medial (CM), paracentral (PC) and central lateral (CL) nuclei in the rostral part, and the parafascicular (PF) and subparafascicular (SPF) nuclei in the caudal part. These are known to receive afferents from the spinal cord (Groenewegen and Witter, 2004). Gauriau and Bernard (2004a) have demonstrated projections to CL, PF, and SPF from the deep laminae, especially from neurons in laminae IV-VII to the CL nucleus, but almost none from the superficial dorsal horn. In agreement with this, Liu (1986) observed little lamina I projection to the intralaminar nuclei in the rat, and Kobayashi (1998) failed to label neurons in lamina I following medial thalamic injections. However, Li et al. (1996) observed spinothalamic neurons in lamina I retrogradely labelled from the contralateral CL nucleus in this species. Although Li et al. (1996) stated that the Fluorogold injection was centred on the CL nucleus in this rat, it is possible that some spread occurred into the adjacent MD nucleus and that this led to the labelling in lamina I in that case. In a recent study, it has been found that certain thalamic nuclei (ventrolateral, ventral anterior, intralaminar, and lateral dorsal nuclei) are not a major target for lamina I neurons in the cat (Klop et al., 2005a).

In summary, the study by Gauriau and Bernard (2004a) demonstrated for the first time the difference between thalamic projection from superficial and deep laminae in the cervical cord of the rat. Using PHA-L as an anterograde tracer, they found that superficial laminae project extensively to the caudal VPL and Po with prominent terminations in the PoT. A minor projection to the VPPC, Re and MD was also observed. In contrast, the main thalamic target for the deep laminae was the CL nucleus. According to the same authors, PoT also received a substantial projection from the deep laminae; though this projection was weaker than that from lamina I. Moderate to scarce terminal labelling was observed in the Po, PF, SPF, VPPC, VPL and ventral medial (VM) nucleus from the deep laminae. The findings of this latter study seems to obscure the classical mediolateral distinction of the thalamus (Hunt and Bester, 2005), as neurons in superficial laminae were found to terminate in both medial and lateral thalamic nuclei.

The precise area of termination of lamina I neurons in the thalamus in monkey is a highly debated issue at the present time. Using the anterograde tracing technique, Craig et al. (1994) and Craig (2004) have reported that lamina I neurons in the monkey project to three main thalamic nuclei: the posterior part of the VM nucleus (VMpo), the ventral caudal part of the medial dorsal nucleus and the ventral posterior inferior nucleus (VPI). This

projection was also confirmed using the retrograde tracing technique (Craig and Zhang, 2006). Ultrastructural evidence for this projection was also provided (Beggs et al., 2003). Craig et al. (1994) considered the VMpo as a newly-discovered thalamic pain centre. On the other hand, Craig (2006) has reported that the VPL received terminal fibres mainly from neurons in lamina V, and that the VPI is a projection target for neurons in laminae I and V. However, other studies debated these findings and provided evidence that the VPL is the main target for spinal lamina I neurons in the monkey (Jones, 2002; Willis et al., 2002; Graziano and Jones, 2004). A recent clinical report provided further evidence for the importance of the VPL in pain pathway: Montes et al. (2005) described a human case of sudden right-sided hyperaesthesia for lemniscal (touch, vibration and joint position) and spinothalamic (pain and temperature) modalities, and 3D magnetic resonance images revealed a left thalamic infarct involving mainly VPL, with no extension to the VMpo. In a recent study, Davidson et al. (2008) antidromically activated 25 neurons in monkey lumbar dorsal horn (16 of which were in lamina I) from the posterior thalamus, and reported that their study confirms that the posterior thalamus receives a relatively high proportion of projections from lamina I. These authors have also found that lamina I neurons frequently projected to VPL, a finding consistent with earlier studies.

Based on the density of terminals from lamina I neurons and other characteristics, it has been proposed that the PoT in the rat, and the ventrolateral VMb in the cat are equivalent to the VMpo in the monkey (Gauriau and Bernard, 2002; Craig, 2003a). Therefore, one of the main objectives of the present study is to investigate the magnitude of the projection from lamina I neurons in rat cervical and lumbar cord to the PoT using the retrograde tracing technique, and to see whether previous quantitative studies in the rat (see below) have underestimated this projection because of the lack of extension of tracer into the caudal thalamus. The projection to the PoT from lamina I neurons in rat lumbar cord has not been investigated previously.

The PoT is a small triangular nucleus located at the caudal end of the posterior thalamus (Paxinos and Watson, 2005). Nowadays, there has been considerable interest in this nucleus due to accumulating evidence for its role in processing of painful stimuli. Investigations have shown that the PoT receives afferents from nociceptive neurons (Zhang and Giesler, 2005), and that some of its neurons respond to noxious stimuli (Gauriau and Bernard, 2004b). In addition, it has been reported that the PoT projects to cortical areas involved in response to pain (Carvell and Simons, 1987; LeDoux et al., 1990; Linke, 1999a; Gauriau and Bernard, 2004b).

Several physiological studies have demonstrated that spinothalamic cells in lamina I respond to noxious stimuli. Giesler et al. (1976) activated two lamina I neurons from the thalamus in rat, and they found that both of these cells responded to noxious stimuli (one was nociceptive-specific, the other was wide dynamic range neuron). Dado et al. (1994b) reported that all of the superficial dorsal horn cells that were antidromically activated from thalamus (15 cells) in rat cervical enlargement responded to noxious stimuli applied to their receptive fields (nine of these were classified as wide dynamic range neurons, while the other six were nociceptive-specific). Palecek et al. (2003) have reported upregulation of Fos in some lamina I spinothalamic neurons in rat thoracolumbar cord in response to intradermal injection of capsaicin and distention of the ureter. Furthermore, Zhang and Giesler (2005) characterized marginal zone units in rat cervical cord, that were antidromically activated from the contralateral PoT, as nociceptive, a finding that implicates the PoT in nociceptive processing of spinal stimuli. Zhang et al. (2006) antidromically activated 33 lamina I cells in rat cervical (30 cells) and lumbar (three cells) cord from the contralateral VPL, and reported that all of these cells responded to noxious stimuli: nociceptive-specific, wide dynamic range or COOL cells; however, the COOL cells responded maximally to noxious cold, as well as to noxious heat and noxious mechanical stimulation. In the cat, Hylden et al. (1986a) activated 16 lamina I cells in the lumbosacral cord from the thalamus and reported that all of these were nociceptive-specific. Based on the conduction velocity of action potentials produced by electrical stimulation of the sciatic nerve, Hylden et al. (1986a) reported that spinothalamic lamina I cells in cat lumbosacral cord received input from Aδ and/or C primary afferents. In monkey, several investigations have also shown that many lamina I spinothalamic neurons responded to noxious stimuli (Willis et al., 2002; Davidson et al., 2008).

1.3.2.2 Quantitative studies

The number of spinothalamic cells in lamina I has been estimated in various species. After targetting all thalamic nuclei known to receive projections from lamina I neurons in the cat, Zhang et al. (1996) estimated the total number of spinothalamic neurons in lamina I in the C3-Co4 segments to be 1,360 cells. This estimate is very similar to that reported by Klop et al. (2005b) for the C1-Co2 segments, which was 1,398 neurons. Most of these cells were located in the enlargements with an estimated total of 575 cells in the C5-8 segments and 297 in the L4-S1 segments (Zhang et al., 1996). The latter study reported that 64% of lamina I spinothalamic neurons originated from the C3-T2 segments; large aggregations were observed in the C6-8 segments (35%) and in the L7-S1 segments (17%). Klop et al.

(2004b, 2005a) have also found that lamina I neurons were most numerous in the enlargements, estimated to be up to 44.5% in the C8 segment in one case, and more labelled in those animals in which the medial thalamus was targeted, a finding that provided evidence that many lamina I projection neurons terminate in medial thalamus in the cat (Craig et al., 1989b). These segmental differences have been also reported in the rat (Granum, 1986; Kemplay and Webster, 1986; Lima and Coimbra, 1988; Kobayashi, 1998) and monkey (Zhang and Craig, 1997). Klop et al. (2004b) found that less than 15% of spinothalamic fibres originated from lamina I in cat. Their finding is in agreement with several studies in different species (Kemplay and Webster, 1986; Apkarian and Hodge, 1989; Burstein et al., 1990a; Klop et al., 2005a). However, it conflicts with others who estimated a much higher proportion (50%) for this contribution (Craig et al., 1989a,b; Andrew et al., 2003). Klop et al. attributed this discrepancy to the differences in thalamic injection sites and/or the selection of the studied spinal segments. This explanation is quite reasonable since it is well known that specific thalamic nuclei receive more spinal input from lamina I compared to others. Furthermore, there are segmental differences regarding the percentage of contribution of lamina I to the spinothalamic tract as presented above.

In the rat, Lima and Coimbra (1988) reported that there are 24 lamina I spinothalamic cells on the contralateral side in the C7 segment, approximately 38 such cells in the combined L3 and L4 segments, and around 23 lamina I cells in the upper two lumbar segments. Burstein et al. (1990a) found that the mean number of spinothalamic neurons in the superficial dorsal horn on the contralateral side of the C7 and C8 segments combined was 110 cells with only 19 such cells in L4-5. Marshall et al. (1996) found that there are about 14 spinothalamic cells in lamina I on the contralateral side of the L4 segment in the rat.

The paucity of spinothalamic neurons in the marginal zone of rat lumbar enlargement was observed in several studies (Kevetter and Willis, 1983; Granum, 1986; Kemplay and Webster, 1986; Harmann et al., 1988; Lima and Coimbra, 1988; Hylden et al., 1989; Burstein et al., 1990a; Dado and Giesler, 1990; Kobayashi, 1998). This feature has been also described in the cat and monkey (Zhang and Craig, 1997; Klop et al., 2004b).

Yu et al. (2005) reported that 9% of the lamina I neurons in rat cervical enlargement project to the thalamus. This finding appears not to be consistent with the report by Spike et al. (2003), who estimated that only 5% of neurons in this lamina project to the brain, and proposed that only a small fraction of this 5% project to the thalamus. A possible explanation for the discrepancy between these two studies is the difference in the method

used for counting neurons. An alternative explanation is the difference in the segments investigated in the two studies; Yu et al. (2005) analysed sections from the cervical cord, whereas Spike et al. (2003) examined sections from the lumbar part. Thus, one of the aims of the current study is to estimate the proportion of the lamina I neurons in the cervical cord that belong to the spinothalamic tract using a standard stereological method.

1.3.2.3 Morphological studies

As stated earlier, the horizontal plane is ideal for examining the morphology of lamina I neurons. Therefore, this section will mainly review studies that analysed the shapes of neurons in horizontal sections.

The morphology of lamina I cells in rat that had been retrogradely labelled with CTb from the thalamus has been described by Lima and Coimbra (1988). These authors reported that spinothalamic cells were either pyramidal or flattened and these were observed in both cervical and lumbar enlargements. Yu et al. (2005) reassessed this issue in the rat by performing 3D reconstructions of spinothalamic neurons from serial confocal images of lamina I in horizontal sections. Unlike the findings by Lima and Coimbra, Yu et al. observed fusiform cells among the population of spinothalamic cells in lamina I. They found that the relative proportions of the three morphological types of spinothalamic neurons in lamina I of rat (averaged for both enlargements) were: 43% fusiform cells, 28% pyramidal cells and 26% multipolar cells, with the remainder being unclassified.

Investigations in cat have shown that the three basic morphological types of lamina I neurons were also represented within the spinothalamic population in this species. Zhang et al. (1996) looked at spinothalamic lamina I cells in horizontal sections from the entire length of the spinal cord in three cats and reported the following percentages for the different morphological types: 34% were fusiform cells, 36% were pyramidal cells, 25% were multipolar cells and 5% were unclassified. They reported that there was no difference in the proportions of the different morphological types between the two enlargements. However, the C7-8 and L6-7 segments contained more pyramidal and multipolar and fewer of the fusiform types compared to the thoracic segments. Similar results have been also reported in other studies in the cat (Andrew et al., 2003), and monkey (Yu et al., 1999; Craig and Zhang, 2006). Zhang and Craig (1997) reported a slight difference between cat and monkey; they observed more fusiform and fewer pyramidal cells in the latter. In addition, the same authors reported that multipolar neurons were more common in the lumbosacral enlargement, whereas fusiform predominated in the cervical enlargement of

the monkey. Several studies have shown that the primary dendritic arborisation of lamina I spinothalamic cells is nearly always restricted to lamina I (Zhang et al., 1996; Yu et al., 2005). However, ventral dendrites from lamina I spinothalamic neurons were reported by Marshall et al. (1996) in rat. Zhang et al. (1996) proposed that these ventral dendrites may be more common in the rat. It has been suggested that the presence or absence of ventral dendrites in lamina I neurons has a relation with the NK1r-immunoreactivity; it was commoner in non-NK1r-immunoreactive neurons (Cheunsuang et al., 2002).

From the previous account, it seems that there is disagreement about the morphology of lamina I spinothalamic neurons in the rat (Lima and Coimbra, 1988; Yu et al., 2005). Therefore, one of the aims of the present study is to analyse the morphology of the spinothalamic neurons in both enlargements in this species. Reaching a correct conclusion with this regard is important especially with the putative correlation between morphology and function of lamina I neurons.

1.3.3 Projection to the parabrachial complex

1.3.3.1 Parabrachial subnuclei that receive input from lamina I

Knowledge about the exact areas of termination of axons from lamina I neurons within the parabrachial complex primarily came from anterograde tracing studies, and to a lesser extent, from the retrograde studies. Cechetto et al. (1985) reported that retrograde labelling in lamina I neurons occurred only after targeting LPb (with the Kölliker Fuse nucleus); no labelling was observed following injections that involved only the medial group of nuclei. Results obtained by Bernard et al. (1989) supported those of Cechetto et al. and further added that retrograde labelling in lamina I occurred mainly after targeting the external portion of LPb, and not the internal part. Results obtained from the anterograde approaches are in line with previous findings of retrograde studies. After injecting PHA-L into the superficial layers of rat lumbar cord, Slugg and Light (1994) observed numerous axon terminals in the rostral half of LPb; mainly in the central lateral, dorsal lateral and external lateral subnuclei. In addition, they observed many terminals in the Kölliker Fuse nucleus. Similar findings were reported by Feil and Herbert (1995) after targeting the superficial dorsal horn of various segmental levels of the spinal cord, and also by Bernard et al. (1995) following injections of PHA-L into the superficial laminae of the cervical enlargement. Bernard et al. (1995) also observed anterograde labelling in the superior lateral and lateral crescent subnuclei. Injections of PHA-L into the superficial dorsal horn of cervical and lumbar cord in cat and monkey have also demonstrated that the lamina I neurons target

mainly the LPb in these two species, with the highest density of labelling observed in the lateral and caudal parts of this region (Craig, 1995).

Electrophysiological investigations have provided evidence that lamina I spinoparabrachial neurons have a role in processing nociceptive information. Bester et al. (2000) antidromically activated 53 lamina I neurons in rat lumbar cord from the contralateral parabrachial area and found that all of these cells responded to noxious stimuli with 75% classified as nociceptive-specific, and 25% as wide dynamic range neurons. Todd et al. (2005) have shown that many lamina I spinoparabrachial neurons in rat lumbar cord upregulated Fos in response to painful thermal stimuli (especially to noxious heat). In a recent study, Williams and Ivanusic (2008) reported that lamina I spinoparabrachial cells in rat lumbar cord upregulated Fos in response to bone drilling, i.e. these cells responded to acute bone nociception. In the cat, Hylden et al. (1985) found that all of the physiologically characterized spinoparabrachial cells (67 cells) in lamina I in the lumbar cord responded to noxious stimuli (94% nociceptive-specific and 6% wide dynamic range neurons). Similar results have been also reported by Light et al. (1993). Another reported physiological property of the spinoparabrachial neurons in spinal lamina I is the possession of small and restricted receptive fields (Bester et al., 2000). This last feature contrasts with the large receptive fields of the parabrachial neurons that sometimes cover the whole body (Bester et al., 1995). Therefore, it has been suggested that axons of many lamina I neurons converge on single parabrachial cells. In addition, this somatic convergence is augmented by afferents from the nucleus of the solitary tract that relay vagal inputs to the parabrachial complex. As a result, the parabrachial nuclei have been implicated in the integration of somatic and visceral information. Despite this high degree of convergence of afferents at the level of the parabrachial complex, it has been reported that the parabrachial neurons are modality specific. Bester et al. (1995) found that 78% of the investigated parabrachial neurons (that projected to the hypothalamus) responded exclusively or preferentially either to mechanical or thermal noxious stimuli. It has been shown that the parabrachial complex project to the central nucleus of the amygdala (Bernard et al., 1993), and to the ventromedial and retrochiasmatic hypothalamic nuclei (Bester et al., 1997). In addition, projections to the PAG and the ventrolateral medulla have been described (Gauriau and Bernard, 2002). Taking into account the functional roles of the projection targets of the parabrachial nuclei, it has been suggested that this complex is involved mainly in the affective and motivational responses to painful stimuli, as well as in regulation of autonomic responses to such an input (Hylden et al., 1985; Light et al., 1993; Craig, 1995).

1.3.3.2 Quantitative studies

Despite the great importance of the spinoparabrachial pathway from lamina I, only a few studies have been carried out in order to quantify these neurons. In the rat, the first study was performed by Hylden et al. (1989) who injected WGA-HRP into the parabrachial area of three rats. They reported that the mean number of spinoparabrachial cells in lamina I on the contralateral side per 50 µm section of the cervical enlargement was around seven cells and the corresponding mean for the lumbar cord was approximately 11 cells. Kitamura et al. (1993) observed 32 spinoparabrachial neurons on the contralateral side of lamina I per 1 mm thickness in each segment of C3-8 in one of the rats that had received tracer injection centred on the internal lateral nucleus, and a mean of only six cells in another rat that had received tracer injection targeted on other lateral parabrachial nuclei. Their corresponding means for the L1-6 segments were 26 and 13 cells, respectively. Two other quantitative studies were performed on rat lumbar cord and both of these showed rather similar results: Todd et al. (2000) found that the number of lamina I spinoparabrachial neurons on the contralateral side of the L3 segment was approximately nine cells per 70 µm thick Vibratome section. The other study was performed by Spike et al. (2003), and these authors estimated the number of these cells on the contralateral side of the L4 segment to be approximately 10 cells per 70 µm thick Vibratome section. Since the estimated numbers of spinoparabrachial lamina I cells in the cervical cord given by Hylden et al. and Kitamura et al. are very different, one of the aims of this study is to estimate this number by using a standard counting method.

Quantitative reports of the spinoparabrachial cells in lamina I in the cat are few. Klop et al. (2005b) counted these neurons from 1:4 series of sections of the entire cord and reported that there were approximately 225 and 40 cells on the contralateral side of the C7 and L4 segments, respectively (data from Fig. 4 in that paper). The estimated total number of spinoparabrachial cells in lamina I on the contralateral side of the entire spinal cord in that cat was 4,187 cells (Klop et al., 2005b).

1.3.3.3 Morphological studies

Few studies have analysed the morphology of spinoparabrachial neurons in lamina I of the rat. Spike et al. (2003) found that the three basic morphological types (multipolar, pyramidal and fusiform) were represented almost equally among this population in the L3 segment (30% were multipolar cells, 32% were pyramidal cells, 31% were fusiform cells and 7% were unclassified; data from Fig. 7 in that paper). Almarestani et al. (2007)

examined rat lumbar cord and reported generally similar results. It is generally believed that spinoparabrachial neurons in lamina I lack ventral dendrites and the dendritic trees are largely confined to lamina I (Almarestani et al., 2007). The morphology of these neurons in rat cervical cord has not been investigated thoroughly. Cechetto et al. (1985) looked at spinoparabrachial cells in horizontal sections of the C2 segment in rat and reported that these were large, flattened and elongated multipolar cells oriented in a rostrocaudal direction and lying parallel to the surface of the spinal cord. That study did not report whether pyramidal or fusiform cells present in the cervical cord. Therefore, one of the objectives of the present study is to investigate the morphology of lamina I spinoparabrachial neurons in the cervical enlargement, and to compare morphology of spinoparabrachial cells with that of spinothalamic cells in both enlargements of the spinal cord, in order to see whether these two populations of cells differ in the proportions of the three morphological types.

The morphology of the spinoparabrachial neurons in lamina I has apparently not been investigated extensively in cat or monkey. Only a brief mention of the shape of cat lamina I neurons activated from the LPb was given by Hylden et al. (1985) and Light et al. (1993). The latter study described the morphology of some intracellularly labelled cells that were reconstructed from adjacent parasagittal sections. They reported that many of these cells were "Waldeyer-like" and had dendrites mainly confined to lamina I and outer lamina II. Hylden et al. (1985) described one representative lamina I cell activated from the ipsilateral parabrachial area in the cat. This cell, which was stained intracellularly and viewed in the parasagittal plane, had an elongated soma with longitudinal dendrites that extended for 1,500 µm in the rostrocaudal direction and around 500 µm mediolaterally and contained within lamina I.

1.3.4 Projection to the periaqueductal grey matter

1.3.4.1 Areas in the PAG that receive input from lamina I

It has been reported by retrograde tracing (Swett et al., 1985; Lima and Coimbra, 1989), anterograde tracing (Bernard et al., 1995) and antidromic activation (McMahon and Wall, 1985) studies that lamina I neurons that project to the PAG terminate mainly in the caudal and ventral part of this region. In the rat, Liu (1983) targeted different columns of the PAG with retrograde tracers and observed that retrograde labelling in lamina I occurred only after injections localised to the vlPAG, and that this labelling occurred in the entire cord bilaterally with contralateral predominance. This prominent projection to the contralateral

side has been also reported by other researchers (Lima and Coimbra, 1989). Lima and Coimbra (1989) have reported that the strongest labelling in lamina I occurred following injections that extended to the vlPAG. Using electrophysiological methods, McMahon and Wall (1985) traced the areas of projection of 13 lamina I cells in rat L4 segment, and reported that one of the main areas where these axons terminated was the caudal and ventral PAG. Keay et al. (1997) were able to label neurons in lamina I of the entire rat spinal cord after targetting either the lPAG or vlPAG with either CTb or FLM. They also observed dense anterograde terminal labelling in the contralateral vlPAG after injecting PHA-L into the superficial dorsal horn of the lower thoracic or lumbar segments. In addition, a moderate amount of terminal labelling was observed in the lPAG. The pattern of projection of lamina I neurons in the upper cervical cord in cat was more specific, as Keay and Bandler (1992) reported that labelling in lamina I was mainly after injections that were restricted to the lPAG. Interestingly, Keay et al. (1997) have reported that neurons in the lPAG receive axons from the superficial dorsal horn of rat thoracolumbar cord but not from the deep neurons in this part of the cord. Thus, the latter authors suggested that lamina I input into the lPAG column offers a route by which cutaneous stimuli initiate distinct defensive responses. On the other hand, deep neurons in the spinal cord transmit stimuli from deep tissues and viscera to the vlPAG, where the depressor response is initiated. The importance of the caudal part of the PAG as a receiving zone for axons of lamina I neurons has been also observed in the cat, as Mouton and Holstege (1998) found much more labelling in lamina I following injections that targeted the intermediate and caudal PAG compared to cases in which the rostral PAG was targeted. After making several localised injections of HRP into the PAG of 13 monkeys, Mantyh (1982) reported that retrograde labelling in lamina I occurred only following involvement of the lateral part of the PAG. On the other hand, injections that targetted the medial part only labelled neurons in the deep laminae and failed to yield labelling in lamina I.

As stated above, the PAG input that originates from lamina I is thought to be involved in the activation of descending analgesic pathways. Some studies in the rat have demonstrated that spinal input from lamina I led to activation of specific PAG columns. For example, Keay and Bandler (1993) reported that cutaneous noxious stimulation (thought to be mediated by input from superficial dorsal horn) led to increased Fos expression in lPAG, whereas noxious stimulation of deep neck muscles (thought to be mediated by input from deep dorsal horn) led to Fos expression mainly in vlPAG. Hylden et al. (1986a) were able to activate some lamina I neurons in cat lumbosacral cord from the midbrain (some of which were from the PAG). They reported that the great majority of

these cells (92%) were nociceptive-specific, the remaining were wide dynamic range neurons, and most of these cells had small receptive fields restricted to one or two toe pads. Based on the conduction velocity of action potentials produced by electrical stimulation of the sciatic nerve, Hylden et al. reported that all of these cells received input from Aδ and/or C primary afferents.

1.3.4.2 Quantitative studies

Most published quantitative studies in the rat provided estimates of numbers of spino-PAG cells in lamina I in the lumbar cord only. Todd et al. (2000) estimated that there are approximately 86 such cells on the contralateral side of the L3 segment. Spike et al. (2003) concluded that the number of these cells on the contralateral side of the L4 segment was 117 cells. Li et al. (1998) reported that there were 51 spino-PAG cells in lamina I per 800 µm in the C7 segment and 42 such cells in L5; however, these data were obtained from one rat only. Thorough quantification of spino-PAG cells in lamina I at cervical levels in the rat has not been carried out previously. Therefore, one of the aims of this study is to provide an estimation of these cells in the cervical enlargement using a standard counting method.

Studies in cat reported that there are up to 1,323 lamina I spino-PAG neurons on the contralateral side in the entire spinal cord, and most of these cells were concentrated in the cervical and lumbosacral enlargements: 260 cells in the C5-8 segments and 295 such cells in L5-S1 (Mouton and Holstege, 1998). In a more recent study, Klop et al. (2005b) estimated the total number of lamina I spino-PAG cells on the contralateral side of cat spinal cord, and reported that these cells were as high as 2,784 cells in one case. In one of the cats in the latter study, the authors estimated that there were ~60 spino-PAG lamina I cells in the C7 segment and ~50 such cells in the L4 segment. There are apparently no quantitative studies of spino-PAG cells in lamina I in the monkey.

1.3.4.3 Morphological studies

Few studies have analysed the morphology of lamina I spino-PAG cells in horizontal sections. One of these was carried out by Lima and Coimbra (1989) who have reported that lamina I spino-PAG cells in rat cervical and lumbar cord were either pyramidal or fusiform. They reported that fusiform cells were predominantly located in the lateral third of the lamina, whereas pyramidal cells were present throughout its mediolateral extent. In transverse sections, Lima and Coimbra observed that some pyramidal cells had a dorsal

dendrite that emerged from the apical pole and ramified within the dorsal white matter. Spike et al. (2003) analysed the morphology of lamina I spino-PAG cells in horizontal sections of the L3 segments in six rats. They found that multipolar cells were slightly more common (39%) than pyramidal (28%) or fusiform (27%) cells. Yezierski and Mendez (1991) have analysed the morphology of lamina I neurons labelled from large tracer injections into the midbrain in rat (i.e. not only spino-PAG cells). This analysis was performed on transverse sections, which are not ideal for morphological analysis, and they reported that the cell bodies were of four major types: round, fusiform, pyramidal and multipolar. Cells with dorsally- or ventrally-directed dendrites were also observed. Keay et al. (1997) briefly referred to the shape of lamina I spino-PAG cells in transverse sections as small round or medium-sized oval cells with occasional elongated "Waldeyer-like" neurons. Similar observations have been also reported by Menétrey et al. (1982) and Swett et al. (1985). Hylden et al. (1986a) intracellularly stained some lamina I neurons, which were activated from midbrain (some of which were activated from the PAG), in sagittal sections of lumbosacral cord in cat, and reported that the morphology of these included smooth and spiny pyramidal, as well as compact and loose multipolar types. Their illustrations showed that some of the cells were fusiform with elongated rostrocaudal dendritic trees.

1.3.5 Collateralisation of lamina I projection neurons

Many lamina I neurons were found to send projections to more than one brain area in the rat (Kevetter and Willis, 1983; McMahon and Wall, 1985; Liu, 1986; Harmann et al., 1988; Hylden et al., 1989; Bice and Beal, 1997a; Spike et al., 2003; Zhang et al., 2006), cat (Hylden et al., 1985, 1986a; Light et al., 1993) and monkey (Zhang et al., 1990). Collateralisation of lamina I neurons into the LPb and thalamus was initially demonstrated in the cat by antidromic activation of single lamina I neurons from both areas (Hylden et al., 1985, 1986a). Light et al. (1993) confirmed this last observation. Collateralisation was demonstrated anatomically in the rat by Hylden et al. (1989) who injected Fluorogold into the lateral thalamus and FLM into the LPb of the same side. These authors reported that 84-88% of lamina I spinothalamic neurons in the cervical and lumbar cord were also labelled from the LPb. On the other hand, Hylden et al. reported that 31-34% of lamina I spinoparabrachial neurons in the lumbar and cervical cord were also labelled from the thalamus. However, the study by Hylden et al. (1989) might have underestimated the proportion of double-labelling in the spinothalamic neurons because of the very small injection sites in the LPb. Therefore, one of the objectives of the present study is to analyse

the dual projection of lamina I neurons in both enlargements to the thalamus and LPb after filling the LPb nucleus with tracer substance.

Another pattern of collateralisation in rat has been found in lamina I cells that were labelled from the CVLM, LPb and PAG. Spike et al. (2003) found that 80% of lamina I neurons that were labelled from the CVLM were also found to project to the LPb and vice versa, and more than 90% of spino-PAG cells were also labelled from either LPb or CVLM. Therefore, it has been suggested that the spino-PAG cells constitute a subset of the spinoparabrachial population (Spike et al., 2003). Bice and Beal (1997a) reported that 95% of lamina I spinomedullary neurons in rat lumbar cord had connections with rostral brain centres (midbrain and/or thalamus). However, Kevetter and Willis (1983) reported that very few double-labelled cells were seen in lamina I following injections that targeted the thalamus and medullary reticular formation (including CVLM, central nucleus of the medulla or nucleus paragigantocellularis lateralis) in the rat.

Collateral branches from single lamina I neurons were also reported to terminate in multiple thalamic nuclei. This last observation was demonstrated anatomically in rat cervical cord (Kevetter and Willis, 1983), and physiologically by recording from single cervical spinothalamic neurons after antidromic stimulation of terminals in three thalamic nuclei in rat (VPL, Po and dorsomedial nucleus) (Zhang et al., 2006).

Retrograde double-labelling studies in rat have shown that some lamina I neurons project to both PAG and thalamus (Liu, 1986; Harmann et al., 1988). Liu (1986) observed a few double-labelled neurons in lamina I, but did not report the proportion of labelled cells that contained both tracers. Harmann et al. (1988) reported that double-labelled neurons represented only ~10% of the spino-PAG population and less than 5% of the spinothalamic population in lamina I at cervical and lumbar levels. It is very likely that these two studies have underestimated the proportion of double labelled cells since the thalamic injection sites in Liu (1986) were small, and Harmann et al. (1988) reported that they targetted mainly the ventrobasal complex and did not include the caudal part of the thalamus in their injection sites. Therefore, one of the objectives of the present study is to investigate the magnitude of collateralisation of lamina I neurons into the thalamus and PAG in both enlargements, taking into consideration all possible thalamic targets for lamina I neurons.

However, apart from some evidence from physiological studies mentioned above, this feature of collateralisation of lamina I projection neurons has not been documented

anatomically in the cat. Andrew et al. (2003) failed to demonstrate an overlap between lamina I neurons that projected to the thalamus and ventrolateral medulla. These authors reported that lamina I spinothalamic population is different from the spinomedullary population and that the former group is located dorsal to the latter in lamina I. However, collateral branches from single lamina I neurons were reported to terminate in medial and lateral thalamic nuclei in the cat (Craig et al., 1989b). Hylden et al. (1986a) have also reported activation of three cells in cat lumbosacral lamina I from medial and lateral thalamic nuclei.

This feature of collateral projection of lamina I neurons has not been investigated thoroughly in the monkey. One study was carried out by Zhang et al. (1990), who investigated the collateral projection of lamina I neurons to the thalamus and PAG, and found some double-labelled cells in this lamina.

1.4 Laminae III and IV: the large NK1r-immuno-reactive neurons

1.4.1 General description

Lamina III corresponds to the dorsal part of the nucleus proprius and runs ventral and parallel to lamina II (Rexed, 1952, 1954). It contains a variety of neurons of different sizes and many of these possess rostrocaudally-oriented dendritic trees (Grant and Koerber, 2004). Lamina IV occupies a region ventral to lamina III and some authors refer to this lamina as forming the base of the head of the dorsal horn (Grant and Koerber, 2004). In the rat, lamina IV curves ventrally along the medial border of the dorsal horn and joins lamina IV of the opposite side in the dorsal commissure at sacral and lumbar levels, but it ends at lamina X at cervical and thoracic levels (Grant and Koerber, 2004). Cells in this lamina are often large and loosely arranged compared to those in lamina III. Some investigators have described a distinct population of neurons in laminae III and IV. These neurons are large and possess dorsally-directed dendrites that arborize in the superficial laminae. Cells of this morphological type were originally described in cat (Szentágothai, 1964), and thereafter found in rat (Todd, 1989; Liu et al., 1994; Brown et al., 1995; Littlewood et al., 1995). Indeed, the old literature has also referred to these cells (Cajal, 1909). Schoenen (1982) has identified cells of similar morphology in human dorsal horn and referred to these cells as "antenna-like neurons". Cells of this type have been also described in the monkey (Beal and Cooper, 1978). Furthermore, Scheibel and Scheibel (1968) referred to the parallel arrangement of some dendritic arbors of these neurons within lamina II,

allowing synaptic contact in several adjacent neuropil fields. Although the prominent feature of these neurons is their possession of dorsal dendrites, ventrally-, medially- and laterally-directed dendrites were also observed (Littlewood et al., 1995). Szentágothai (1964) reported that the intimate relation of the richly-branching dorsal dendrites of these cells with the fine longitudinal fibre system in the substantia gelatinosa is similar to the relation of the purkinje neurons in the cerebellar cortex to the molecular layer. This similarity was also stated by Scheibel and Scheibel (1968) but they mainly referred to neurons in lamina III. Previous Golgi studies have revealed the presence of long and complex spines projecting from the dorsal dendrites of these cells (Scheibel and Scheibel, 1968). The presence of such spiny arborisations of the dorsal dendrites within the primary afferent rich-lamina II has led to the suggestion that these dendrites have ample chance of forming synapses with the primary axon terminals in this lamina (Szentágothai, 1964). This suggestion was confirmed by Todd (1989) who sectioned two or three dorsal roots in rat and observed synapses between the degenerating primary afferent fibres and the dorsal dendrites of such cells in laminae II and III. However, Todd (1989) has found that most of the synapses that were observed in his study were formed with non-degenerating axonal elements, an observation that led him to suggest the presence of another source for the presynaptic elements for these dorsal dendrites. Therefore, it has been proposed that these cells form part of the circuitry by which input from fine primary afferents reaches the deeper laminae of the dorsal horn (Todd, 1989). Subsequent investigations have shown that some cells of this type express the NK1 receptor (Liu et al., 1994; Brown et al., 1995; Littlewood et al., 1995), and constitute a subset of the NK1r expressing neurons in these laminae. Todd et al. (1998) have reported that around 11% of lamina III neurons and approximately 28% of those in lamina IV were immunoreactive to the NK1r in the rat, but the population of large NK1r-immunoreactive neurons with long dorsal dendrites represents approximately 0.1% of all neurons in lamina III, since it has been estimated that there are 21,928 of these cells on each side in the L4 segment (Polgár et al., 2004). Based on the dendritic arborisation of the NK1r-immunoreactive neurons in rat dorsal horn, Brown et al. (1995) described four types of these cells; the first two probably correspond to those described above. The first type usually identified in lamina III and had a large pyramidal cell body with up to 300 µm long dorsally-directed dendrites. The second type was multipolar with widespread primary dendrites distributed dorsoventrally and mediolaterally. The third and fourth types were relatively small and present all over the dorsal horn, and did not possess prominent dorsal dendritic arborisation. According to Brown et al. (1995), these NK1r-immunoreactive neurons were best seen in the lumbar and sacral cord and very rarely observed in the cervical or thoracic cord. In contrast, bipolar

(fusiform) cell bodies with ventrally- and dorsally-directed dendrites predominated in the cervical cord.

Attempts to quantify the large NK1r-immunoreactive lamina III/IV neurons with long dorsal dendrites in rat lumbar cord revealed that there are approximately 20 of these cells on each side of the L4 segment (Todd et al., 2000). Reports that quantify these neurons in the cervical cord are still lacking in the literature. Therefore, two of the aims of the present study are to count these neurons in the cervical enlargement and to see whether they differ from those in the lumbar cord in terms of their number and location within laminae III and IV.

1.4.2 Role in nociception

Several lines of evidence indicate that the large NK1r-immunoreactive neurons with long dorsal dendrites in laminae III and IV are involved in pain mechanisms. It has been reported that these neurons in rat lumbar cord were selectively innervated by substance P-containing primary afferents on both their cell bodies and dendrites, with higher frequency of contact on the dorsally-directed ones (Naim et al., 1997; Todd et al., 2000). On the other hand, these neurons were demonstrated to receive few contacts from C fibres that did not contain neuropeptides (Naim et al., 1998; Sakamoto et al., 1999). In a recent study, Todd et al. (2009) found that the dendritic plasma membrane of these cells possess numerous synapses that contained the GluR2 and GluR4 subunits of the AMPA (α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptor. This last observation indicates that the lamina III/IV NK1r-immunoreactive neurons have a high density of excitatory synapses on their dendritic trees. Some studies have shown that neurons of this type responded to noxious chemical stimuli: Mantyh et al. (1995) and Honoré et al. (1999) showed internalisation of the NK1r in the dorsal dendrites after injection of capsaicin (the former study) or formalin (the latter study) into the ipsilateral hindpaw of rats. Moreover, internalisation of the NK1r in the dorsal dendrites of these neurons has been shown following mechanical and thermal stimuli (Polgár et al., 2007a). The demonstration of the phosphorylation of ERK after mechanical, thermal and chemical noxious stimuli indicates that these cells respond to a wide variety of noxious information (Polgár et al., 2007a). Furthermore, it has been suggested that these cells encode for the intensity of the noxious stimulation, because Mantyh et al. (1995) reported that acute noxious stimulation led to NK1r internalisation only in the distal dendrites, while inflammation or sectioning of the peripheral nerve led to internalisation of the receptor in the proximal dendrites as well as in

the cell body (Abbadie et al., 1997; Allen et al., 1999). Expression of Fos in these cells has been also reported following various types of noxious stimulation (Doyle and Hunt, 1999a), and following graded intensities of cutaneous cooling (Doyle and Hunt, 1999b).

The large NK1r-immunoreactive neurons in laminae III and IV have been shown to be a target for axons that contain both neuropeptide Y (NPY) and GABA that are likely to arise from local inhibitory interneurons (Polgár et al., 1999). Interestingly, the NPY contacts were present mainly on the cell bodies and the proximal parts of the dendrites, whereas those from substance P-containing afferents were concentrated on the distal dendrites. These neurons have been also shown to be innervated by serotonin-containing axons from the medulla (Stewart and Maxwell, 2000). The latter authors demonstrated that almost half of the large NK1r-immunoreactive lamina III/IV neurons with long dorsal dendrites in rat lumbar cord received a substantial number of contacts from serotonin-containing axons, and that some of these formed basket-like arrangement around the cell bodies and proximal dendrites. Stewart and Maxwell (2000) suggested that these serotoninergic contacts might have a role in anti-nociceptive mechanisms.

1.4.3 Projection targets

Retrograde tracing studies in the rat have demonstrated that virtually all of the large lamina III/IV NK1r-immunoreactive neurons with long dorsal dendrites in lumbar cord were labelled from the CVLM, many also projected to the LPb and only few projected to the PAG (Todd et al., 2000). Todd et al. (2000) also reported that about 27% of neurons of this type were retrogradely labelled from injections into the contralateral dorsal caudal medulla (dorsal reticular nucleus and nucleus of the solitary tract). Previous studies have also shown that some cells of this type in the lumbar cord projected to the thalamus, but the proportion of these cells that belong to the spinothalamic tract was not reported (Marshall et al., 1996; Naim et al., 1997). It has been documented that neurons of this type in the rat do not belong to the postsynaptic dorsal column pathway (Polgár et al., 1999; Palecek et al., 2003). Nothing is yet known about the projection patterns of cells of this type in the cervical cord. Therefore one of the aims of this study is to determine the proportion of cells in this population in cervical and lumbar enlargements that project to the thalamus. A further analysis on this thalamic projection is aimed by targetting the PoT with one tracer and other parts of the thalamus with another tracer in order to test whether these cells project to both of these regions or only to one of them. It is possible that some cells of this type send collateral projections to more than one supraspinal target, but this feature has not

been investigated before. The present study therefore aims to address this issue by injecting tracer substance into two brain regions (thalamus and either the LPb or PAG), and document whether these cells, in both enlargements, send collaterals to more than one brain target. Shedding light on the projection pattern of these cells would improve the current knowledge about their role in nociceptive mechanisms.

A neuronal population of similar morphology to the above described group has been also identified in laminae III and IV of rat dorsal horn (Polgár et al., 2007b). However, these neurons do not express the NK1r, are not labelled from the CVLM and receive a significantly fewer contacts from substance P-containing primary afferents. Thus, it has been suggested that these neurons play a different functional role compared to those expressing the NK1 receptor.

1.5 Objectives of the study

In summary, the objectives of the present study are:

1.5.1 Objectives related to lamina I neurons

1. To investigate the projection of lamina I neurons in the cervical and lumbar enlargements to the thalamus (including the PoT nucleus).

2. To estimate the proportion of the lamina I neurons in the C7 segment that belong to the spinothalamic tract.

3. To provide an estimate of the number of lamina I spinoparabrachial and spino-PAG cells in the C7 segment.

4. To investigate the extent of collateralisation of lamina I spinothalamic neurons, in the cervical and lumbar enlargements, to the LPb and PAG.

5. To compare the morphology and the NK1r expression of lamina I spinothalamic and spinoparabrachial neurons in both enlargements.

6. To quantify the large gephyrin-coated cells in the cervical enlargement and investigate the projection targets of these cells in both enlargements.

1.5.2 Objectives related to lamina III/IV NK1r-immunore-active neurons

1. To quantify the large NK1r-immunoreactive lamina III/IV neurons in the cervical enlargement and to see whether they differ from those in the lumbar cord in terms of their number and location within laminae III and IV.

2. To investigate the thalamic projections of these cells in both enlargements.

3. To determine whether the cervical population of these neurons project to the LPb or PAG, and whether cells in either enlargement send collaterals to more than one brain target (thalamus, LPb and PAG).

Chapter 2
Materials and Methods

2.1 Investigation of projection of neurons in laminae I, III and IV to the PoT of the thalamus

2.1.1 Animals and experimental procedure

All experiments were approved by the Ethical Review Process Applications Panel of the University of Glasgow, and were performed in accordance with the UK Animals (Scientific Procedures) Act 1986.

Twenty-five adult male Wistar rats were used in this series of experiments (240-330 g; Harlan, Loughborough, UK). Rats were anesthetized with ketamine and xylazine (73.3 and 7.3 mg/kg i.p., respectively) or with a volatile anaesthetic (halothane or isofluorane; 1.5-4%) in a mixture of oxygen and nitrous oxide. They were placed on a heating pad to maintain normal body temperature with the head fixed in a stereotaxic frame. Vaseline was placed on each eye in order to prevent drying of the cornea. In those cases in which halothane or isofluorane were used, the anaesthetic was administered through a mask attached to the frame. For the rats anesthetized with ketamine and xylazine, these drugs were supplemented as necessary. The level of anesthesia was monitored throughout the operation by applying a gentle pressure on the hindpaw and checking the withdrawal reflex. Under aseptic techniques, skin of the head and underlying fascia were incised and reflected laterally. The point on the skull that overlay the intended injection site was determined (with reference to atlas of Paxinos and Watson, 2005) and then a craniotomy was performed using a fine drill. Occasional bleeding from bone was stopped by applying a gentle pressure or by cauterization. Each rat received injections of one or two of the following tracers into the left thalamus: 1% CTb (Sigma, Poole, UK), 4% Fluorogold (Fluorochrome Inc, Englewood, CO, USA) or rhodamine-labelled fluorescent latex microspheres (undiluted, Lumafluor Corp., Naples, FL, USA). All injections were made under pressure through glass micropipettes. The pipette was left in place for five minutes after each injection in order to minimize the leakage of tracer substance back up the track. In those cases in which two tracers were injected, a different pipette was used for each tracer. Four different injection strategies were used: (1) injection of Fluorogold into all of the thalamic nuclei that are known to receive projections from superficial dorsal horn neurons, including PoT (experiments Thal 1-5), (2) injection of CTb or Fluorogold into the PoT (PoT 1-10), (3) injection of CTb into the PoT and of Fluorogold into other thalamic areas known to receive projections from superficial dorsal horn neurons (double injection, DI 1-7), and (4) injection of fluorescent latex microspheres into PoT (FLM 1-3). The experiments with fluorescent latex microspheres were performed as these show very little

spread (resulting in restricted injection sites) and are not taken up by undamaged fibres (Katz et al., 1984). Details of the tracer(s) used, injection coordinates and volumes injected in each experiment are given in Tables 2-1, 2-2, 2-3 and 2-4. After completion of the injection procedure, the wound was sutured with absorbable stitches, and an analgesic drug (carprofen; 5mg/Kg; s.c.) was administered. Postoperatively, the rats were kept under observation, and all made uneventful recovery from anaesthesia.

2.1.2 Perfusion and tissue processing

After a survival period of three days, all rats were re-anesthetized with pentobarbitone (300 mg, i.p.) and perfused intracardially with Ringer's solution for five seconds, followed by one litre of freshly depolymerized 4% formaldehyde in 0.1 M phosphate buffer (PB), PH 7.4. The selection of three-day survival for the rats was based on previous findings in this laboratory that perfusing rats more than three days after the injection of tracer does not lead to a significant increase in the number of the retrogradely labelled cells. Immediately after completion of the perfusion process, the skin of the back was incised in the midline along the entire length of the vertebral column. The paraspinal muscles were removed and in this way the vertebral column was exposed. Using a pair of fine rongeurs, laminectomy of all vertebrae was performed and the entire spinal cord within the dural sac was then visible. Using a fine curved blade, the dura mater was incised in the midline and reflected laterally exposing the dorsal roots of the spinal nerves. Identification of the cervical segments was done by first identifying the dorsal rootlets of the first cervical nerve, which are visible just below the junction of the spinal cord with the medulla oblongata, and then the following cervical nerves were identified from above downwards. The dorsal roots of the C6 and C7 nerves were freed from their corresponding ganglia at the intervertebral foramina and lifted up to their origin at the corresponding segments. The left dorsal roots were kept longer than the right in order to identify the two sides of the segments at subsequent stages. Using a fine straight blade, the C6 and C7 segments were separated by making a sharp cut just underneath the caudalmost dorsal rootlets of the relevant segment. In the case of the lumbar cord, the dorsal root of the T13 nerve was first identified by following it from its ganglion located in the intervertebral foramen just beneath the last rib. In this way, the first and subsequent lumbar dorsal roots were identified. The procedures of separating the roots from the ganglia, tracing them up to the corresponding segments and separation of the L4 and L5 segments were done in the same way as that described for the cervical cord. After removal of the segments concerned, these were kept in the same fixative and stored at 4 ^{o}C for 24 hours. In order to remove the brain, the skin of the head was incised in the midline and reflected laterally. Using a bone rongeurs, the bones of the

Table 2-1. Details of tracer injections for Thal experiments

Exp	FG	Injection coordinates (mm)			Targetted thalamic nuclei
		AP*	DV**	ML***	
Thal 1	100 nl	3.0	3.8	2.5	PoT (caudal part)
	100 nl	3.8	3.8	2.5	PoT (rostral part)
	100 nl	4.8	4.0	3.0	VPL+Po+VPPC
	100 nl	5.8	4.0	3.0	VPL+Po
	100 nl	6.8	4.0	3.0	VPL
Thal 2	100 nl	3.0	3.8	2.5	PoT (caudal part)
	100 nl	3.8	3.8	2.5	PoT (rostral part)
	100 nl	4.8	4.0	3.0	VPL+Po+VPPC
	100 nl	5.8	4.0	3.0	VPL+Po
	100 nl	6.8	4.0	3.0	VPL
	100 nl	5.8	4.6	1.0	MD+CL
Thal 3	100 nl	3.0	3.8	2.5	PoT (caudal part)
	100 nl	3.8	3.8	2.5	PoT (rostral part)
	100 nl	4.8	4.0	3.0	VPL+Po+VPPC
	100 nl	5.8	4.0	3.0	VPL+Po
	100 nl	6.8	4.0	3.0	VPL
	100 nl	5.8	4.6	1.0	MD+CL
Thal 4	100 nl	3.0	3.8	2.5	PoT (caudal part)
	100 nl	3.8	3.8	2.5	PoT (rostral part)
	100 nl	4.8	4.0	3.0	VPL+Po+VPPC
	100 nl	5.8	4.0	3.0	VPL+Po
	100 nl	6.8	4.0	3.0	VPL
	100 nl	5.8	4.6	1.0	MD+CL
Thal 5	100 nl	3.0	3.8	2.5	PoT (caudal part)
	100 nl	3.8	3.8	2.5	PoT (rostral part)
	100 nl	4.8	4.0	3.0	VPL+Po+VPPC
	100 nl	5.8	4.0	3.0	VPL+Po
	100 nl	5.8	4.6	1.0	MD+CL

This table shows the approximate volumes and numbers of Fluorogold (FG) injections that were targetted on different thalamic nuclei (last column). The coordinates of each injection (according to Paxinos and Watson, 2005) are presented opposite to the volume injected. For definition of thalamic nuclei, refer to list of abbreviations (pages xii-xiii).

* AP, anteroposterior coordinates, all values are anterior to the interaural plane

**DV, dorsoventral coordinates

***ML, mediolateral coordinates

Table 2-2. Details of tracer injections for PoT experiments

Exp	CTb	FG	Injection coordinates (mm)		
			AP	DV	ML
PoT 1	250 nl		3.7	3.8	2.5
PoT 2		100 nl	3.0	3.8	2.5
PoT 3		100 nl 100 nl	3.0 3.8	3.8 3.8	2.5 2.5
PoT 4		100 nl 100 nl	3.0 3.8	3.8 3.8	2.5 2.5
PoT 5		100 nl	3.0	3.8	2.5
PoT 6		50 nl	3.4	3.8	2.5
PoT 7	200 nl		3.2	3.8	2.5
PoT 8	200 nl		3.0	3.8	2.5
PoT 9	50 nl		3.5	3.8	2.5
PoT 10	50 nl		3.5	3.8	2.5

This table shows the type and approximate volumes of tracers used in each experiment. The coordinates of each injection that was targetted on the PoT nucleus (according to Paxinos and Watson, 2005) are shown opposite to the volume of tracer injected. Abbreviations as in Table 2-1.

Table 2-3. Details of tracer injections for DI experiments

Exp	CTb	FG	Injection coordinates (mm)			Targetted thalamic nuclei
			AP	DV	ML	
DI 1		100 nl	4.8	4.0	3.0	VPL+Po+VPPC
		100 nl	5.8	4.0	3.0	VPL+Po
		100 nl	5.8	4.6	1.0	MD+CL
	200 nl		3.0	3.8	2.5	PoT
DI 2		100 nl	5.0	4.0	3.0	VPL+Po+VPPC
		100 nl	5.8	4.0	3.0	VPL+Po
		100 nl	5.8	4.6	1.0	MD+CL
	200 nl		3.0	3.8	2.5	PoT
DI 3		100 nl	5.0	4.0	3.0	VPL+Po+VPPC
		100 nl	5.8	4.0	3.0	VPL+Po
		100 nl	5.8	4.6	1.0	MD+CL
	200 nl		3.0	3.8	2.5	PoT
DI 4		100 nl	5.0	4.0	3.0	VPL+Po+VPPC
		100 nl	5.8	4.0	3.0	VPL+Po
		100 nl	5.8	4.6	1.0	MD+CL
	200 nl		3.0	3.8	2.5	PoT
DI 5		100 nl	4.8	4.0	3.0	VPL+Po+VPPC
		100 nl	5.8	4.0	3.0	VPL+Po
		100 nl	5.5	3.2	1.0	MD+CL
		100 nl	5.5	4.6	1.0	MD+CL
		100 nl	6.5	4.0	2.8	VPL+Po
		100 nl	6.5	3.8	1.2	MD+CL
	200 nl		3.0	3.8	2.5	PoT
DI 6		100 nl	4.8	4.0	3.0	VPL+Po+VPPC
		100 nl	5.8	4.0	3.0	VPL+Po
		100 nl	5.5	3.2	1.0	MD+CL
		100 nl	5.5	4.6	1.0	MD+CL
		100 nl	6.5	4.0	2.8	VPL+Po
		100 nl	6.5	3.8	1.2	MD+CL
	200 nl		3.0	3.8	2.5	PoT
DI 7		100 nl	4.8	4.0	3.0	VPL+Po+VPPC
		100 nl	5.8	4.0	3.0	VPL+Po
		100 nl	5.5	3.2	1.0	MD+CL
		100 nl	5.5	4.6	1.0	MD+CL
		100 nl	6.5	4.0	2.8	VPL+Po
		100 nl	6.5	3.8	1.2	MD+CL
	200 nl		3.0	3.8	2.5	PoT

This table shows the type and approximate volumes of tracers used in each experiment. The coordinates of each injection (according to Paxinos and Watson, 2005) are shown opposite to the volume of tracer injected. In each case, CTb was targetted on PoT, while FG was injected into other thalamic nuclei as presented in the last column. Abbreviations as in Table 2-1.

Table 2-4. Details of tracer injections for FLM experiments

Exp	FLM	Injection coordinates (mm)		
		AP	DV	ML
FLM 1	50 nl	3.2	3.8	2.5
FLM 2	50 nl	3.2	3.8	2.7
	50 nl	3.6	3.8	2.7
FLM 3	50 nl	3.2	3.8	2.6
	50 nl	3.5	3.8	2.6

This table shows the approximate volumes and numbers of fluorescent latex microspheres (FLM) injections that were targetted on PoT nucleus in each case. The coordinates of each injection (according to Paxinos and Watson, 2005) are shown opposite to the volume injected. Abbreviations as in Table 2-1.

cranium were cut and removed. A sharp cut was then made at the junction of the brainstem with the spinal cord, and the brain was gently lifted up, placed in 30% sucrose in fixative (for cryoprotection) and stored at 4 °C overnight.

In order to measure the length of the C7 segment, this was photographed *in situ* in three experiments (DI 5, DI 6 and FLM 3) and the distance between the most rostral and caudal rootlets of the C7 dorsal roots was determined. The length of the C7 segment was measured in order to estimate the total number of spinothalamic neurons in the entire segment.

On the following day, the brain was cut into 100 μm thick coronal sections using a freezing microtome. Brains from animals that had received injections of CTb (with or without Fluorogold) were cut into five complete series and sections from at least one of these series were reacted with an immunoperoxidase method to reveal the spread of CTb. The sections were incubated for three days in goat antiserum against CTb (List Biological Laboratories, Campbell, CA, USA; diluted 1:50,000), then rinsed three times with phosphate-buffered saline that contained 0.3 M NaCl (PBS). After that sections were reacted overnight with biotinylated antigoat IgG (Jackson ImmunoResearch, West Grove, PA; diluted 1:500). Thereafter, three (10 min each) PBS rinses were performed and sections were reacted for 3-4 h with streptavidin-horseradish peroxidase (Sigma; diluted in PBS, 1:1000). Peroxidase activity was revealed with 0.025 M diaminobenzidine in the presence of 0.001% hydrogen peroxide. Sections were then extensively rinsed in PB, mounted on gelatinized slides and air dried. On the following day, the sections were rinsed in water, dehydrated in ascending grades of alcohol, cleared in Histo-Clear, mounted with Histomount and coverslipped. All remaining sections from animals that had received injections of Fluorogold in addition to CTb were mounted directly on slides with anti-fade medium (Vectashield, Vector laboratories, Peterborough, UK) for detection of Fluorogold with epi-fluorescent illumination and an UV filter set. All sections through the injection sites from animals that had received only Fluorogold or FLM were mounted in serial order. Those from Fluorogold-injected rats were mounted with Vectashield and viewed with UV epi-fluorescence, while sections from animals that received injections of FLM were mounted in Gel-Mount medium (Sigma-Aldrich, Poole, Dorset), as beads are sensitive to the glycerol, and viewed with bright- and dark-field illumination. In all cases the spread of tracer from the injection sites was plotted onto drawings of the thalamus (Paxinos and Watson, 2005), and representative examples were photographed.

The C7 and L4 spinal cord segments from all animals (apart from those that received injections of FLM) were used to estimate the numbers of retrogradely labelled cells in lamina I. Sections from the C7 segments from four rats (Thal 2-5) were also used to determine the proportion of lamina I cells that belonged to the spinothalamic tract. The segments were initially notched on the left side so that the two sides could subsequently be distinguished, and were cut into 60 μm thick transverse sections with a Vibratome. The sections were then rinsed three times with PB, and then treated with 50% ethanol for 30 min to enhance antibody penetration (Llewellyn-Smith and Minson, 1992). Subsequently, sections were rinsed three times with PBS. Thereafter, sections were reacted immunocytochemically to reveal neuronal nuclei, the tracer(s) and, in some cases, the NK1 receptor. Sections were incubated free-floating at 4 °C for three days in one of the following antibody combinations: (1) mouse monoclonal antibody NeuN (against a neuronal nuclear protein, Millipore, Watford, UK, diluted 1:1000), goat anti-CTb (1:5000) and rabbit anti-NK1r (Sigma-Aldrich, 1:10,000) (experiments PoT 1 and PoT 7-10); (2) NeuN, guinea-pig anti-Fluorogold (Protos Biotech Corp., New York, USA, 1:500) and rabbit anti-NK1r (experiments Thal 2-5 and PoT 4-6); or (3) NeuN, goat anti-CTb and guinea-pig anti-Fluorogold (experiments DI 1-7). The sections were then rinsed three times with PBS and incubated overnight at 4 °C in species-specific secondary antibodies that were raised in donkey and conjugated to either Alexa 488 (Invitrogen, Paisley, UK; 1:500), or to Rhodamine Red or Cy5 (Jackson ImmunoResearch, West Grove, PA, USA; 1:100). Following immunostaining, some of the sections from the C7 segment of experiments Thal 2-5 were incubated for 30 minutes in the fluorescent nuclear stain 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, 1 μg/ml in phosphate-buffered saline). All antibody solutions were made up in PBS that contained 0.3% Triton X-100. All sections were then rinsed three times with PBS, mounted in anti-fade medium (Vectashield) and stored at -20 °C until needed.

Some of the experiments (Thal 1 and PoT 2-3) were carried out before the guinea-pig antibody against Fluorogold was obtained. Therefore, sections from these experiments were incubated for three days in rabbit anti-Fluorogold, overnight in Fab' fragment of goat anti-rabbit IgG conjugated to rhodamine (Jackson ImmunoResearch; 1:100), and then for two hours in unlabelled Fab' fragment of goat anti-rabbit IgG (Jackson ImmunoResearch, 1:20). Thereafter, sections were incubated for two days in rabbit anti-NK1r, followed by one day in donkey anti-rabbit IgG conjugated to Alexa 488. Rinsing and mounting of the sections and storage of the slides were carried out as described above. This procedure was performed because both of the primary antibodies were raised in the same host species

(rabbit). If a normal (divalent) anti-rabbit secondary antibody had been used, then this would have allowed binding of the second primary antibody, resulting in inappropriate labelling with the second secondary antibody. The use of Rhodamine-labelled Fab' fragment of goat anti-rabbit IgG followed by an excess of unlabelled Fab' fragment should lead to binding to all available sites on the first primary antibody, and thus prevent the unwanted binding of the second secondary antibody.

The C6 and L5 segments from all experiments were used to analyse the large NK1r-immunoreactive neurons in laminae III and IV. In addition, these segments were used to count the retrogradely labelled lamina I neurons in experiments FLM 1-3. These segments were divided into right and left halves, and only the right half (i.e. the side contralateral to the injection site) was used in this study. In order to allow orientation of the sections, the caudal ventral region of each block was notched, and then these were cut with a Vibratome into 60 μm thick parasagittal sections (50 μm thick for experiments FLM 2-3). In order to enable mounting of the sections in serial order, the cutting was made into three serial bottles, but all bottles were processed in the same way. The sections were rinsed and treated with 50% alcohol as described above for the transverse sections except in the cases of experiments FLM 1-3 when alcohol was not used as this causes loss of the fluorescence of beads. The sections were reacted to reveal NK1r, CTb and/or Fluorogold (when these had been injected), and in some cases, NeuN. Sections were incubated for three days at 4 °C in one of the following antibody combinations: (1) rabbit anti-NK1r and goat anti-CTb (experiments PoT 1 and PoT 7-10); (2) rabbit anti-NK1r and guinea-pig anti-Fluorogold (experiments Thal 2-5 and PoT 4-6); (3) rabbit anti-NK1r, goat anti-CTb and guinea-pig anti-Fluorogold (experiments DI 1-7); or (4) rabbit anti-NK1r and NeuN (FLM 1-3). After rinsing, sections were incubated overnight at 4 °C in species-specific secondary antibodies that were raised in donkey and conjugated to Alexa 488, Rhodamine Red or Cy5. Sections from experiments Thal 1 and PoT 2-3 were processed sequentially in rabbit antibodies against Fluorogold and NK1r, as described above for the transverse sections. Sections were rinsed and mounted in serial order in Vectashield (Thal, PoT and DI experiments) or Gel-Mount (FLM experiments) and stored at -20 °C.

2.1.3 Antibody characterization

The NK1r antibody (catalogue number S8305) was raised in rabbit against a peptide corresponding to amino acids 393-407 at the C-terminus of the rat NK1 receptor, which was conjugated to keyhole limpet haemocyanin. The antibody recognises a 46 kDa band in

Western blots of rat brain extracts and this staining is specifically abolished by preabsorption of the antibody with the immunising peptide (manufacturer's specification). It has been shown that there is no immunostaining with this antibody in sections of medulla and cervical spinal cord from mice in which the NK1r has been deleted (NK1$^{-/-}$), while staining is present in sections from wild-type mice (Ptak et al., 2002).

Goat (catalogue number 703) and guinea-pig (catalogue number NM101) polyclonal antibodies against CTb and Fluorogold, respectively, were used in this study. Specificity of each of these antibodies was shown by the lack of staining in regions of the CNS that did not contain neurons that had taken up and transported the tracer, and by the presence of immunostaining in populations of neurons that are known to project to the injection sites. The specificity of the Fluorogold antibody was also directly confirmed by comparing Fluorogold fluorescence (observed with an UV filter set) with that for anti-Fluorogold in individual neurons. In all cases examined there was a perfect match between the two types of fluorescence.

2.1.4 Confocal microscopy and analysis

All analysis of lamina I neurons and of the large NK1r-immunoreactive cells in laminae III and IV was performed on the right (contralateral) dorsal horn.

Transverse sections from the rats that had received injections of CTb and/or Fluorogold were used to analyse the number of retrogradely labelled neurons in lamina I in C7 and L4 segments in each experiment. From each animal, 10 sections from the C7 segments and 20 sections from the L4 segments were randomly selected and viewed with epi-fluorescence. More sections were analysed from the L4 segment as spinothalamic lamina I neurons were far less numerous in this segment. All lamina I neurons that were labelled with CTb and/or Fluorogold were identified and then scanned sequentially (to avoid fluorescent bleed-through) with a confocal laser scanning microscope equipped with a krypton-argon laser (Bio-Rad MRC1024; Bio-Rad, Hemel Hempstead; UK) or with HeNe, red diode, blue diode, Argon lasers (Bio-Rad Radiance 2100) through dry (4×, 10×, 20×) and oil-immersion (60×) lenses. In order to correct for the over-counting that results from the presence of transected cells at the section surfaces, cells were only included in the sample if their nucleus was entirely contained within the Vibratome section, or if part of the nucleus was present in the first optical section in the z-series (corresponding to the top of the Vibratome section); they were excluded if part of the nucleus was present in the last

optical section (Spike et al., 2003). The low-magnification images were used to plot the position of the cells on an outline of the dorsal horn drawn with Neurolucida for Confocal (MicroBrightField Inc., Colchester, VT, USA). In this way the number of retrogradely labelled lamina I cells that contained CTb, Fluorogold or both tracers per 600 μm (C7) or 1,200 μm (L4) was determined for each experiment. The approximate boundaries of lamina I were distinguished using dark-field microscopy (see Results). For most of the experiments in which a single tracer was used (Thal 2-5, PoT 1, PoT 4-10), the presence or absence of NK1r was also noted for each retrogradely labelled lamina I cell.

In order to determine the proportion of lamina I neurons that belonged to the spinothalamic tract in the C7 segment, six Vibratome sections that had been immunostained and incubated in DAPI were randomly selected from four experiments in which extensive injections of Fluorogold had been made (Thal 2-5). These sections were scanned through a 40× oil-immersion lens to reveal NeuN, NK1r, Fluorogold and DAPI. In each case, z-series consisting of 16 optical sections at 1 μm spacing were obtained from the entire mediolateral extent of lamina I on the right side of the spinal cord. To determine the degree of tissue shrinkage, the thickness of each section was measured by scanning top and bottom surfaces at three different locations and then the mean distance between these surfaces was calculated (Polgár et al., 2004). Drawings of these sections were made with Neurolucida for Confocal, and the boundaries of lamina I were identified, based on the pattern of NK1r-immunostaining (see Results). A modification of the optical disector method (Sterio, 1984) was then used to determine the total number of neurons per 9 μm of lamina I, by examining all optical sections and counting all of the neurons with nuclei that had a bottom surface between the 3rd and 11th optical sections in the z-series (Todd et al., 1998; Spike et al., 2003). To avoid bias, the inclusion of neurons was made before the Fluorogold-immunoreactivity was viewed. For each neuron that was included in the disector sample, the presence or absence of Fluorogold was then noted, and in this way the number of spinothalamic neurons per 9 μm of lamina I was determined. The proportion of lamina I neurons that belonged to the spinothalamic tract was estimated in two different ways. Firstly, for each rat the number of retrogradely labelled neurons in the disector samples was divided by the total number of lamina I neurons in the samples, and this value was averaged for the four rats. Secondly, the disector samples were used to estimate the number of lamina I neurons per 600 μm in the C7 segment, and this was subsequently compared to the numbers of retrogradely labelled lamina I neurons found in this segment in the experiments in which CTb and/or Fluorogold were injected. In order to estimate the

total number of lamina I neurons using this way, the following formula was used to correct for tissue shrinkage (Polgár et al., 2004):

$$N = n \times \frac{\text{T-final}}{\text{T-cut}} \times \frac{600}{9},$$

where N is the number of lamina I neurons per 600 μm of spinal cord, n is the number counted in the 9 μm disector, T-final is the thickness of the particular section measured after any shrinkage, and T-cut the original thickness of the section (60 μm).

For each of the rats that had received injections of CTb and/or Fluorogold, the entire series of parasagittal sections through the grey matter of the right side of both the C6 and L5 segments were used to count the number of large NK1r-immunoreactive neurons that had cell bodies in laminae III or IV and dorsal dendrites that could be followed into laminae I or II, and to determine the proportion of these cells that were retrogradely labelled. This part of the analysis was not carried out for cases PoT 1 and PoT 2, as well as for L5 in the case of DI 5, since no tissue was available from these experiments. Dark-field microscopy was used to ensure that all cells had their somata ventral to lamina II. Sections were initially viewed with epi-fluorescence through a 20× lens to identify NK1r-immunoreactive cell bodies in laminae III or IV. For each cell body seen, the dorsal dendrite(s) was followed in order to check if these entered the superficial dorsal horn. For some cells, it was possible to follow the dorsal dendrites in a single section; however, in other cases, the dendrites left the section before reaching the superficial dorsal horn. Therefore, these dendrites were followed in the adjacent serial section, and when necessary, confocal microscopy was used in order to construct the course of these dendrites in two or three adjacent sections. NK1r-immunoreactive cells bodies were excluded if their dorsal dendrites failed to reach lamina II. Confocal microscopy was also used to determine whether the cells were retrogradely labelled with CTb and/or Fluorogold, to measure the distance between the cell body and the overlying dorsal white matter and to ensure that cell bodies that were cut by the Vibratome and appeared on the adjacent surfaces of serial sections were not counted twice. During the course of the study, it was found that retrogradely labelled lamina III/IV NK1r-immunoreactive neurons in the L5 segment appeared to be more common in the medial part of the dorsal horn than in the lateral part. In order to document this observation, all of the NK1r-immunoreactive cells in this segment with dorsal dendrites that entered the superficial dorsal horn were assigned into one of two groups, medial and lateral, based on the section in which they were present and if necessary, their (mediolateral) depth within that section. In experiments in which the

number of these cells was even, the two groups were of equal size, while in the other experiments the extra cell was included in the lateral group.

For the three rats that had received injections of fluorescent latex microspheres (FLM 1-3), complete series of parasagittal sections through the right side of the C6 and L5 segments were examined with epi-fluorescence through a 40× oil-immersion lens. All retrogradely labelled neurons in lamina I, and retrogradely labelled neurons in laminae III and IV that were NK1r-immunoreactive were identified. The cell was considered retrogradely labelled if it contained five or more beads in the soma and/or dendrites. Care was taken to avoid double-counting by ensuring that cells at the cut surface of a section were not counted on the adjacent section. For the retrogradely labelled lamina III/IV NK1r-immunoreactive cells, the presence or absence of dorsal dendrites that could be followed into the superficial laminae (I-II) was determined in each case.

Figures were composed with Adobe Photoshop (ver 7.0). In some cases, image brightness and contrast were adjusted by using the levels setting.

2.2 Investigation of collateralisation of projection neurons in laminae I, III and IV to the thalamus, lateral parabrachial area and periaqueductal grey matter

2.2.1 Animals and experimental procedure

Seven adult male Wistar rats (260-335 g; Harlan, Loughborough, UK) were used in this part of the study. Rats were anesthetized with ketamine and xylazine (73.3 and 7.3 mg/kg i.p., respectively) or with a volatile anaesthetic (isofluorane) in the same way as described previously. Three of these rats (experiments Pb 1-3) received injections of Fluorogold into the caudal half of the thalamus, including the PoT, together with an injection of CTb into the LPb (both on the left side). The other four rats (experiments PAG 1-4) received injections of Fluorogold targetted on the left PoT (as above) and CTb into the PAG on the left side. The PoT nucleus was targeted for thalamic injections, as this is a major termination zone for projections from the superficial dorsal horn (Gauriau and Bernard, 2004a) and results from the first part of this study showed that injections of tracer into this region label virtually all spinothalamic neurons in lamina I, together with those in laminae III and IV that express the NK1 receptor (as described in section 3.1.1.2 in Results; Al-Khater et al., 2008). Details of the tracers used, injection coordinates and volumes injected in each experiment are given in Table 2-5. The operative procedure and the method of injection were the same as that described for the first part of the study. All animals made an uneventful recovery from anaesthesia.

2.2.2 Perfusion and tissue processing

After a survival period of three days, all rats were re-anesthetized and perfused intracardially with fixative as explained above (section 2.1.2). Dissection of the brain and spinal cord was also carried out as explained previously in section 2.1.2. Cervical (C6, C7 and C8) and Lumbar (L3, L4 and L5) spinal cord segments were stored in fixative at 4 ^{o}C for 24 hours, while the brain was cryoprotected in 30% sucrose in fixative at 4 ^{o}C overnight.

Brain regions containing thalamic injection sites were cut into 100 μm thick coronal sections with a freezing microtome. These were mounted in serial order with anti-fade medium and viewed with epi-fluorescent illumination and an UV filter set. Blocks of the brainstem that contained the LPb or PAG injection sites were cut into five complete series

Table 2-5. Details of tracer injections for Pb and PAG experiments

Exp	CTb	FG	Injection coordinates (mm)			Targetted nuclei/area
			AP	DV	ML	
Pb 1		100 nl	3.3	3.8	2.5	PoT
		100 nl	4.0	3.8	2.5	Po (caudal part)
	200 nl		-0.4	3.2	2.1	LPb
Pb 2		100 nl	3.3	3.8	2.5	PoT
		100 nl	4.0	3.8	2.5	Po (caudal part)
	200 nl		-0.4	3.2	2.1	LPb
Pb 3		100 nl	3.3	3.8	2.5	PoT
		100 nl	4.0	3.8	2.5	Po (caudal part)
	200 nl		-0.4	3.2	2.1	LPb
PAG 1		100 nl	3.3	3.8	2.5	PoT
		100 nl	4.0	3.8	2.5	Po (caudal part)
	100 nl		0.7	4.4	0.8	PAG
PAG 2		100 nl	3.3	3.8	2.5	PoT
		100 nl	4.0	3.8	2.5	Po (caudal part)
	100 nl		0.7	4.4	0.8	PAG
PAG 3		100 nl	3.3	3.8	2.5	PoT
		100 nl	4.0	3.8	2.5	Po (caudal part)
	100 nl		0.7	4.4	0.8	PAG
PAG 4		100 nl	3.3	3.8	2.5	PoT
		100 nl	4.0	3.8	2.5	Po (caudal part)
	100 nl		0.7	4.4	0.8	PAG

This table shows the type and approximate volumes of tracers used in each experiment. In each case, one injection of CTb and two of Fluorogold (FG) were given. The coordinates of each injection (according to Paxinos and Watson, 2005) are shown opposite to the volume of tracer injected. CTb was targetted on LPb (in Pb 1-3) or on PAG (in PAG 1-4), while FG was targetted on the caudal thalamus (including the PoT) in the seven cases. Abbreviations as in Table 2-1.

of 100 µm thick coronal sections using a freezing microtome. Sections from at least two of these series were reacted with goat anti-CTb using the immunoperoxidase method described above. In all cases the spread of tracer from the injection sites was plotted onto drawings of the thalamus and brainstem (Paxinos and Watson, 2005), and representative examples were photographed.

The C7 and L4 segments from all animals were used to count retrogradely labelled lamina I neurons as well as the large NK1r-immunoreactive neurons with long dorsal dendrites in laminae III and IV. In order to allow serial mounting of the sections, the ipsilateral (left) ventral quadrants of these segments were notched in oblique way, and then the segments were cut into five complete series of 60 µm thick transverse sections with a Vibratome. Subsequently, the sections were reacted immunocytochemically in the same way as described above (section 2.1.2) but using the following combination of primary antibodies: guinea-pig anti-Fluorogold, goat anti-CTb and rabbit anti-NK1r. Section were reacted with species-specific secondary antibodies (as described before), rinsed and mounted in serial order in anti-fade medium and stored at -20 °C until needed.

The C6 and L5 segments from all seven rats were used to analyse the large gephyrin-coated lamina I cells, and the C8 and L3, from experiments Pb 1-3 only, were used to perform the morphological analysis of the spinothalamic and spinoparabrachial neurons in lamina I. All of these segments were notched on the caudal part of the left side and cut into 60 µm thick horizontal sections. Those from C8 and L3 were processed in the same way as described above (for the transverse sections). Immunocytochemical processing of sections from C6 and L5 was carried out as described previously and using the following combination of antibodies: guinea-pig anti-Fluorogold, goat anti-CTb and mouse monoclonal antibody against gephyrin (mAb 7a; Synaptic Systems, Göttingen, Germany; 1:1000). After rinsing, sections were incubated overnight in fluorescent secondary antibodies (as above), rinsed, mounted and stored at -20°C.

2.2.3 Antibody characterization

The mouse monoclonal antibody against gephyrin was generated against an extract of rat spinal cord synaptic membranes (Pfeiffer et al., 1984) and has been extensively characterised and shown with Western Blots to bind to a 93kDa peripheral membrane protein (gephyrin) in extracts of rat brain membranes (Becker et al., 1989; Kirsch and Betz, 1993).

Details of the other antibodies used in this part of the study were given in section 2.1.3.

2.2.4 Confocal microscopy and analysis

All analysis of lamina I neurons and of the large NK1r-immunoreactive cells in laminae III and IV was performed on the right (contralateral) dorsal horn.

Transverse sections from the C7 and L4 segments of all seven rats were used to analyse the numbers of retrogradely labelled lamina I neurons that contained one or both tracers. In each case, 10 or 20 sections (an alternate series) were scanned sequentially with the confocal microscope through dry (20×) and oil-immersion (40×) lenses. Dark-field microscopy was used to distinguish laminar boundaries and retrogradely labelled cells were judged to be in lamina I if they were very close to the dorsal border of the dorsal horn, or lay dorsal to the dark band identified as lamina II with dark-field microscopy. In order to avoid over-counting of neurons, these were included only if the bottom of the nucleus was present in the Vibratome section, as described above. Spinothalamic lamina I neurons are infrequent in the lumbar enlargement (as described in Results; Al-Khater et al., 2008), and therefore 20 sections through the L4 segment were examined to count these cells (and determine whether they were double-labelled). Twenty sections were also used to quantify spinal neurons labelled from PAG in the L4 segment, while 10 sections were used for the other parts of this analysis. In this way, the number of retrogradely labelled lamina I cells that contained CTb, Fluorogold or both tracers per 600 μm (C7 for each injection site, L4 for LPb injections) or per 1,200 μm (L4 for thalamic and PAG injections) was determined for each experiment. The presence or absence of immunoreactivity to the NK1r was also noted for each retrogradely labelled lamina I cell identified in this part of the analysis. In order to compare the mediolateral distribution of spinothalamic and spinoparabrachial neurons in lamina I, the locations of single- or double-labelled neurons in the sections analysed were plotted onto an outline drawing of the dorsal horn with Neurolucida for Confocal software. Lamina I was divided into three equal parts (medial, middle and lateral) and the numbers of spinoparabrachial and spinothalamic neurons in each part were counted. For this analysis, spinothalamic neurons in the L4 segment were analysed on 20 sections, while those in C7, together with spinoparabrachial neurons in both segments, were analysed on 10 sections.

In all seven rats, the complete series of transverse sections from C7 and L4 were also used to determine the number of NK1r-immunoreactive neurons that had cell bodies in laminae

III or IV and dorsal dendrites that could be followed into laminae I or II, and to determine the proportion of these cells that were retrogradely labelled with one or both tracers. Dark-field microscopy was used to ensure that all of these cells had their somata ventral to lamina II. Sections were initially viewed with epi-fluorescence through a 20× lens to identify NK1r-immunoreactive cell bodies in laminae III or IV. In most cases it was possible to determine with epi-fluorescence whether the dendrites of these cells entered the superficial dorsal horn (laminae I or II). However, in some cases (particularly when dendrites had to be followed into serial sections) it was necessary to scan with the confocal microscope. This was also used to determine whether the cells were retrogradely labelled with CTb and/or Fluorogold, to measure the distance between the cell bodies and the overlying dorsal white matter and to define their mediolateral location. Because it was found that retrogradely labelled lamina III/IV neurons were more frequent in the medial part of the L5 segment (see section 3.1.1.3), the locations of all of these cells in L4 were plotted with Neurolucida for Confocal onto an outline of the dorsal horn. A vertical line was then drawn mid-way through the mediolateral extent of lamina III and the cells were divided into two groups, those in the medial half and those in the lateral half of the dorsal horn.

For the rats that had received injections into thalamus and LPb, all of the horizontal sections through the C8 and L3 segments that contained retrogradely labelled lamina I neurons were scanned sequentially through dry (20×) and oil-immersion (40×) lenses to reveal NK1r, CTb and Fluorogold. These scans were used to compare the morphology and NK1r expression of neurons that were retrogradely labelled from both thalamus and LPb with those of neurons labelled only from LPb. In the C8 segment, confocal image stacks (1 μm z-separation) were obtained through cell bodies and dendritic trees of all retrogradely labelled neurons. Because a much lower number of Fluorogold-labelled cells was present in the L3 segment, only regions that contained Fluorogold-labelled cells were scanned, but all of the retrogradely labelled cells that were present in these scans were analysed. Cells were excluded from the sample if they were very close to one surface of the Vibratome section that substantial parts of their proximal dendrites (and/or cell bodies) were not present in the section, since this made it impossible to assign them to one of the three morphological classes. Drawings of the cell bodies and proximal dendrites of all of the retrogradely labelled neurons included in the sample were made with Neurolucida for Confocal software and the presence or absence of CTb and Fluorogold were recorded for each cell. The drawings were used to analyse neuronal morphology and to measure soma size. For each cell in the sample, morphology was assessed independently by two

observers who were blind to the projection target(s), and cells were provisionally allocated to one of the following classes: multipolar, pyramidal or fusiform (Zhang et al., 1996; Zhang and Craig, 1997). In cases of initial disagreement, the cells were re-examined and where possible, allocated to one of these classes. A small number of cells could not be assigned to one of the three groups, since they showed features that were transitional between two of the three morphological classes, while a few could not be allocated to any of these classes because of their atypical appearance (Zhang et al., 1996; Zhang and Craig, 1997). These cells were defined as "unclassified". The maximum cross-sectional area of the soma of all cells was measured from projected confocal images with Neurolucida for Confocal (Puskár et al., 2001; Polgár et al., 2002). Strength of NK1r-immunoreactivity on the plasma membrane was also recorded, because of the variation in staining intensity at different depths of the Vibratome section a scoring system was used (Spike et al., 2003), and each cell was assigned a score ranging from 4 (strong) to 1 (very weak) or 0 (negative). Since the sample of spinothalamic neurons in the L3 segments was small (a total of 21 cells in the three parabrachial experiments), morphology and soma size of spinothalamic neurons from the L5 segments of these experiments were also analysed. In order to allow unbiased analysis of morphology, the spinothalamic cells in this segment, together with a sample of cells labelled only from LPb, were drawn and assessed by two observers blind to their projection targets, as described above. However, only the spinothalamic cells were included in the analysis.

For all seven rats, horizontal sections through the C6 and L5 segments were examined with an oil-immersion (40×) lens to allow identification of the large gephyrin-coated lamina I cells, and these were then scanned sequentially to reveal gephyrin, CTb and Fluorogold. These scans were used to determine the number of the gephyrin-coated cells in the C6 and L5 segments and the proportions of these that were retrogradely labelled from thalamus, LPb or PAG.

Figures were composed with Adobe Photoshop as in the first part of the study.

Chapter 3
Results

3.1 Investigation of projection of neurons in laminae I, III and IV to the PoT of thalamus (Thal, PoT, DI and FLM series of experiments)

3.1.1 Retrograde labelling with Fluorogold and CTb

3.1.1.1 Analysis of the injection sites

In all 22 experiments, the spread of tracer(s) within the thalamus was plotted on diagrams from atlas of Paxinos and Watson (2005), and details of the injection sites in each of the three series of experiments (Thal, PoT and DI) are presented below. In the cases in which Fluorogold was used, the injection site was defined as the dense core and the surrounding halo, as there is no evidence that documents which of these areas is the functional uptake zone.

3.1.1.1.1 Total thalamic injections (Thal 1-5)

The spread of tracer within the thalamus in each of these five experiments is illustrated in Fig. 3-1, and representative examples of photographed injection sites are shown in Fig. 3-2. Injection sites were successful in all five cases with slight variations among them. Fluorogold did not spread into the hypothalamus, PAG or the contralateral thalamus in any of the experiments. The following account gives a brief description of Fluorogold spread in these experiments:

Thal 1: In this case, Fluorogold was targeted only on the lateral thalamus (including the PoT). Tracer filled the dorsal part of the lateral thalamic nuclei (Po and ventrobasal complex). The PoT was included in the injection site apart from a small region between interaural 3.4 and 3.6 mm. Areas surrounding the PoT such as the medial geniculate nucleus (MG), posterior intralaminar nucleus (PIL) and anterior pretectal nucleus (APT) were also included in the injection site. Little caudal extension of tracer into the superior colliculus and rostral extension into internal capsule and striatum were also observed.

Thal 2-5: The lateral thalamus was almost completely included in the injection site in these four experiments, and the PoT was completely filled. Various parts of the MD thalamic nucleus and some intralaminar nuclei (e.g. CL, PC) were also included. Fluorogold also extended to involve regions surrounding the PoT and into the lateral part of the superior colliculus. At rostral levels, tracer spread into internal capsule, and variable extension into the striatum was observed in all cases except Thal 5.

Figure 3-1. Injection sites for experiments Thal 1-5.

Drawings to show the spread of Fluorogold (shaded area) in these experiments. Each vertical column represents a single experiment, and the experiment number is shown at the bottom of the column. Numbers at the top left of each drawing give the approximate position of the section anterior to the interaural plane. Drawings are based on those in Paxinos and Watson (2005). *AM*, anteromedial thalamic nucleus; *APT*, anterior pretectal nucleus; *AV*, anteroventral thalamic nucleus; *CL*, centrolateral thalamic nucleus; *CM*, central medial thalamic nucleus; *fr*, fasciculus retroflexus; *IAM*, interanteromedial thalamic nucleus; *ic*, internal capsule; *LD*, laterodorsal thalamic nucleus; *LG*, lateral geniculate nucleus; *LP*, lateral posterior thalamic nucleus; *MD*, mediodorsal thalamic nucleus; *MG*, medial geniculate nucleus; *PC*, paracentral thalamic nucleus; *PF*, parafascicular thalamic nucleus; *PIL*, posterior intralaminar thalamic nucleus; *Po*, posterior thalamic nuclear group; *PoT*, posterior thalamic nuclear group, triangular part; *PP*, peripeduncular nucleus; *Re*, reuniens thalamic nucleus; *RRE*, retrouniens area; *Rt*, reticular thalamic nucleus; *Sub*, submedius thalamic nucleus; *SubB*, subbrachial nucleus; *VA*, ventral anterior thalamic nucleus; *VL*, ventrolateral thalamic nucleus; *VM*, ventromedial thalamic nucleus *VPL*, ventral posterolateral thalamic nucleus; *VPM*, ventral posteromedial thalamic nucleus; *VPPC*, ventral posterior thalamic nucleus, parvicellular part.

7.6

6.8

6.1

5.4

4.6

4.0

3.6

3.2

2.9

Thal1   Thal2   Thal3   Thal4   Thal5
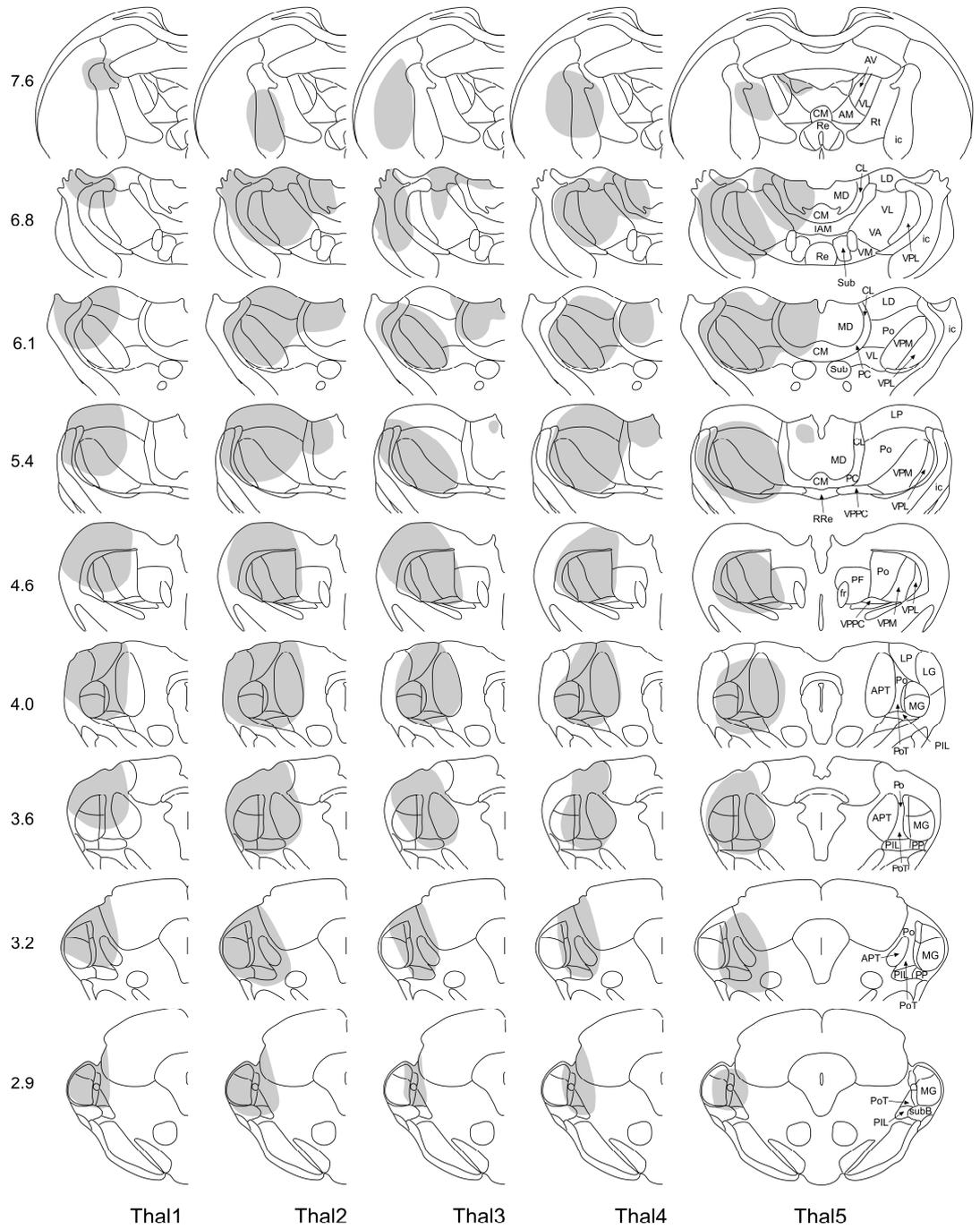
Figure 3-2. Examples of Fluorogold injection sites in Thal experiments.

a, b: brightfield and fluorescence micrographs of a section (interaural ~4.9 mm) through part of a Fluorogold injection site showing filling of the ventrobasal complex (from experiment Thal 2). **c, d:** brightfield and fluorescence micrographs of a section (interaural ~ 3.2 mm) through part of a Fluorogold injection site showing filling of the PoT area (from experiment Thal 5). Note the necrotic centre in **a** and **b** and the spread of Fluorogold fluorescence from this region. Abbreviations as in Figure 3-1. Scale bars = 1 mm.
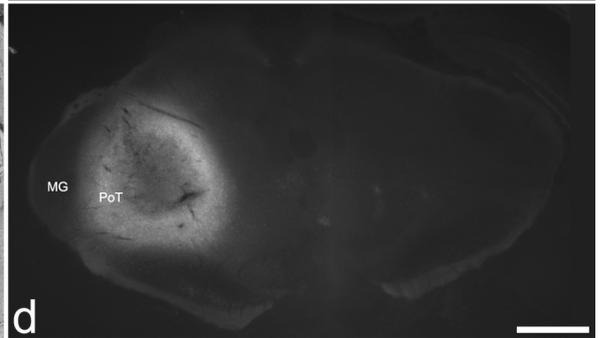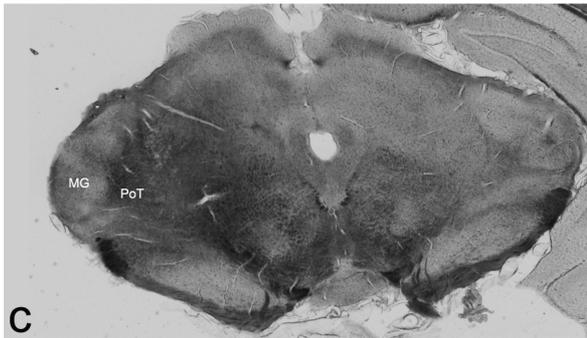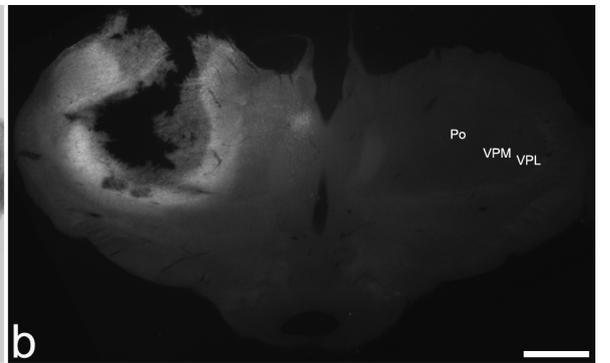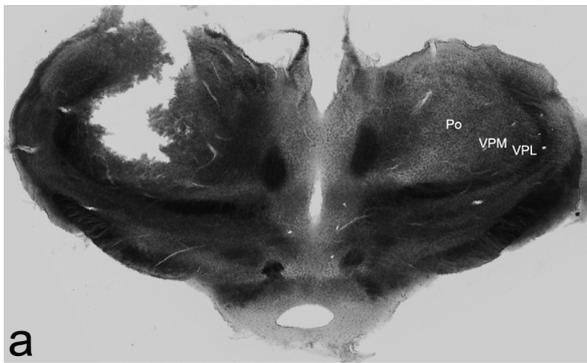
3.1.1.1.2 PoT injections (PoT 1-10)

The spread of tracer within the thalamus in each of these 10 experiments is illustrated in Fig. 3-3, and a representative example of a photographed injection site is shown in Fig. 3-4. According to the atlas of Paxinos and Watson (2005), the PoT nucleus extends from interaural 2.88 to 4.08 mm. In the present series of experiments, the tracer substance filled all or most of the PoT region in most cases. There was invariably spread of tracer into adjacent areas like the PIL, MG and APT. Tracer did not spread into the PAG in any of these cases.

The following account gives more detail about the spread of the injection site in each individual experiment. Fluorogold was used in PoT 2-6, whereas CTb was used in PoT 1 and in PoT 7-10.

PoT 1: The spread of CTb was observed between interaural 3.4 and 4.7 mm. The caudal part of the PoT was not included in this injection. There was very limited extension into adjacent nuclei, such as APT, PIL, medial part of MG (MGM), caudal part of the Po nucleus, suprageniculate thalamic nucleus (SG) and into parts of the lateral posterior thalamic nucleus.

PoT 2: In this experiment the apparent centre of the injection was displaced caudally; thus, Fluorogold spread was observed from interaural 1.5 to 3.3 mm. As a result, only the caudal part of the PoT was included in this injection site, and there was extensive spread into mesencephalic structures such as the lateral part of the superior colliculus and the deep mesencephalic nucleus. At the rostral end of the injection site, nearby nuclei such as APT, MGM, PIL and SG were also partly involved.

PoT 3: This case received two injections of Fluorogold at the region of the PoT, as a result, more rostrocaudal spread of tracer occurred. The spread was observed between interaural 2.3 and 6.0 mm. In addition, this injection site was more ventrally located than other experiments. The PoT was included in this case; however, because the injection was more ventral, the dorsalmost part of PoT at interaural 4.0 mm was not included in the tracer spread. Structures adjacent to the PoT were also involved, as in other experiments. At rostral levels, the injection had a flattened pattern of spread in and around the medial lemniscus, i.e. structures ventral and dorsal to the medial lemniscus were involved: ventral

Figure 3-3. Injection sites for experiments PoT 1-10.

Drawings to show the spread of CTb (dark shading) or Fluorogold (light shading) in these experiments. Each column represents a single experiment, and the experiment number is shown at the bottom of the column. Numbers at the top left of each drawing give the approximate position of the section anterior to the interaural plane. Drawings are based on those in Paxinos and Watson (2005). Abbreviations as in Figure 3-1.

5.4

4.6

4.0

3.6

3.2

2.9

PoT1   PoT2   PoT3   PoT4   PoT5   PoT6   PoT7   PoT8   PoT9   PoT10
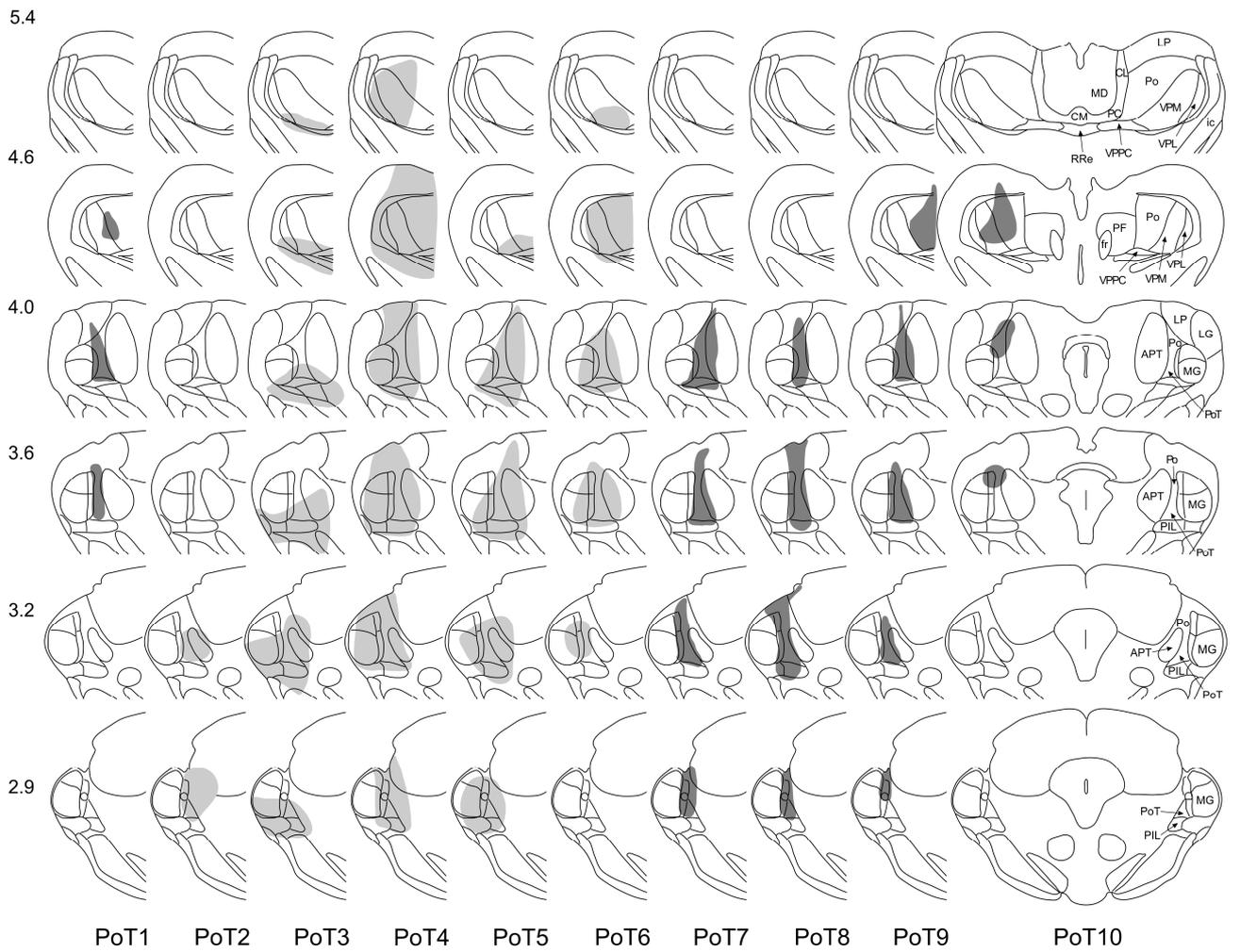
Figure 3-4. CTb injection site in PoT experiments.

A brightfield micrographs of a section (interaural ~3.4 mm) through part of an injection site of CTb that was targetted on the PoT nucleus (from experiment PoT 7). The section was reacted with an immunoperoxidase method to reveal CTb. Abbreviations as in Figure 3-1. Scale bar = 1 mm.
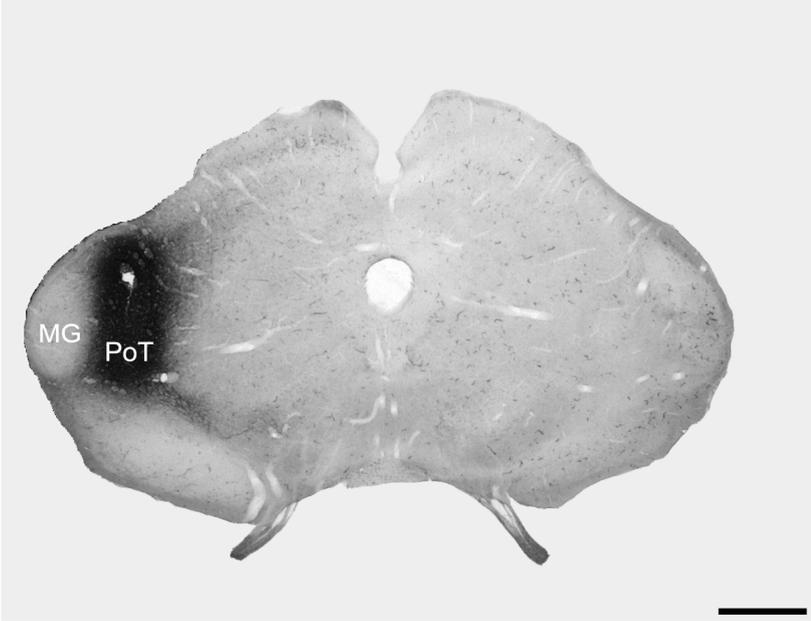
part of the Po and ventrobasal complex, and dorsal structures in the ventral thalamus like zona incerta.

PoT 4: This case also received two injections of Fluorogold. Therefore, the rostrocaudal spread was more or less similar to that in PoT 3; however, the injection site extended further dorsally in this experiment. The PoT was completely included in the injection site. Some spread into adjacent structures was also observed. The prominent feature of this injection site is the massive involvement of the Po nucleus as far rostrally as interaural 5.5 mm, and the ventrobasal complex as far rostrally as interaural 5.8 mm.

PoT 5: This case received only one injection of Fluorogold, which extended from interaural 2.5 to 4.7 mm. The PoT was entirely contained within the injection site, and nearby structures were also involved. The ventral parts of the Po and VPM nuclei were also included at rostral levels of the injection site.

PoT 6: The injection site in this experiment extended between interaural 3.1 and 5.5 mm. Only the rostral part of the PoT (the part that extends between interaural 3.5 and 4.08 mm) was included in this injection site. Fluorogold extended into areas surrounding the PoT and into the caudal part of the Po nucleus and the ventrobasal complex.

PoT 7: CTb injection in this case was centred on the middle of the PoT, and thus, this region was totally filled. Compared to cases in which Fluorogold was used, there was less spread into adjacent structures in this experiment. Dorsal spread into the caudal part of the Po nucleus was observed and this extended as far rostrally as interaural 4.5 mm.

PoT 8: CTb was also used in this case, and the spread of the injection site was similar to that seen in PoT 7; however, the rostral spread of CTb in this case was less.

PoT 9: In this experiment, CTb spread between interaural 2.9 and 4.8 mm. The PoT was completely contained within the injection site apart from the caudalmost region (between interaural 2.8 to 3.1 mm). The Po nucleus was also included in the rostral extension of the injection site.

PoT 10: In this case, the aim was to target the rostral part of the PoT at interaural 3.9 mm; however, the apparent centre of the injection occurred rostral to that level, and thus, CTb

spread included mainly the Po nucleus and VPM (between interaural 3.6 and 4.8 mm). The PoT was free from tracer spread in this experiment.

3.1.1.1.3   Double injections (DI 1-7)

The spread of tracers within the thalamus in each of these seven experiments is illustrated in Fig. 3-5, and representative photomicrographs of injection sites are shown in Fig. 3-6. In this series of experiments, Fluorogold was targetted on thalamic nuclei that are known to receive projections from lamina I, except the PoT, which was injected with CTb. The first four cases in this series (DI 1-4) received three injections of Fluorogold and therefore presented some common features in the spread of this tracer, whereas the remaining three rats (DI 5-7) received six injections of Fluorogold and thus tracer spread was more extensive in these cases. In all experiments, neither tracer spread into the hypothalamus, PAG or the contralateral thalamus. The following account gives a brief description of spread of tracers in each of these seven experiments (DI 1-7).

DI 1: Fluorogold spread in this case was observed between interaural 3.9 and 7.6 mm. The caudal extent of Fluorogold injection site reached the rostral part of the PoT and there was some overlap with the CTb injection. The Po nucleus and the ventrobasal complex were completely contained within the Fluorogold injection site. The medial injection covered most of the MD nucleus, as well as parts of the intralaminar nuclei (CL, PC, and CM). At the most rostral level of Fluorogold spread, slight involvement of internal capsule and striatum was observed. CTb covered all of the rostrocaudal extent of the PoT with some extension into surrounding structures.

DI 2: Fluorogold spread was observed as far caudally as interaural 4.2 mm, and did not extend into the PoT. The rostral spread of Fluorogold reached interaural 7.8 mm, and the pattern of the involvement of the Po nucleus and the ventrobasal complex was similar to that observed in DI 1. Parts of the MD nucleus and several intralaminar nuclei (CL, PC, and CM) were also involved. At rostral levels, some spread of Fluorogold was seen into internal capsule and striatum. CTb covered the entire extent of the PoT apart from the region at and caudal to interaural 3.3 mm. CTb also spread into areas adjacent to the PoT and extended rostrally to involve the caudal part of the Po nucleus.

DI 3: The spread of Fluorogold in this experiment involved areas in the lateral (Po and ventrobasal complex) and medial (parts of MD and intralaminar nuclei) thalamus. In this case, Fluorogold extended caudally as far as interaural 3.9 mm. This caudal extension

Figure 3-5. Injection sites for experiments DI 1-7.

Drawings to show the spread of Fluorogold (light shading) and CTb (dark shading) in these experiments. Each vertical column represents a single experiment, and the experiment number is shown at the bottom of the column. Numbers at the top left of each drawing give the approximate position of the section anterior to the interaural plane. Drawings are based on those in Paxinos and Watson (2005). For labelling of structures shown within these drawings, see Figure 3-1.

7.6

6.8

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4.0

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3.2

2.9

DI1 DI2 DI3 DI4 DI5 DI6 DI7
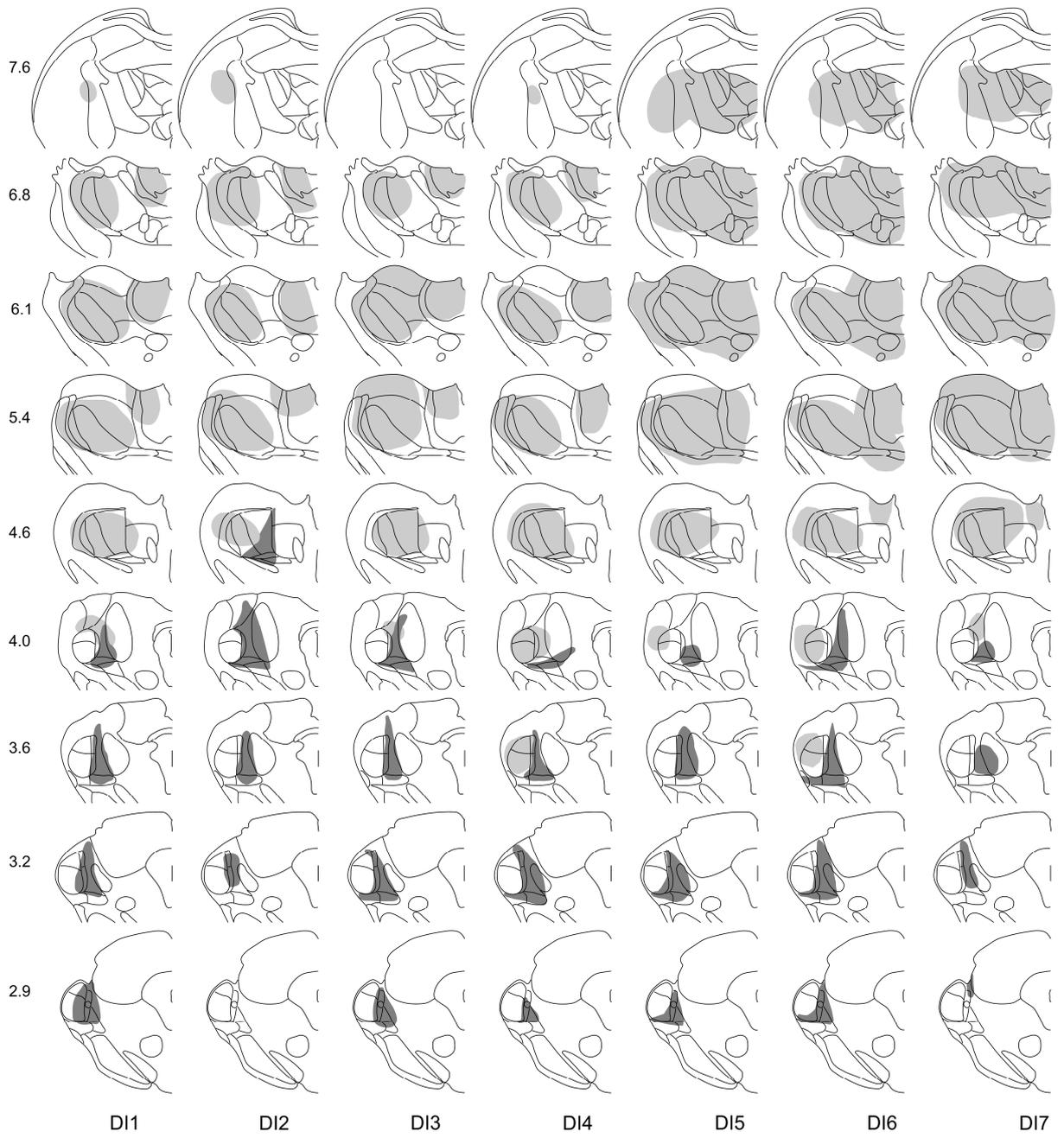
Figure 3-6. Examples of CTb and Fluorogold injection sites in DI experiments.

a, a section (interaural ~3.8 mm) through an injection of CTb that was targetted on the PoT nucleus (experiment DI 1). The section was reacted with an immunoperoxidase method to reveal CTb. **b,c:** brightfield and fluorescence micrographs of a section (interaural ~5.2 mm) through part of a Fluorogold injection site (experiment DI 7). Note the necrotic centre and the spread of Fluorogold fluorescence from this region. *3V*, 3rd ventricle; *ml*, medial lemniscus; other abbreviations as in Figure 3-1. Scale bar for all parts = 1 mm.
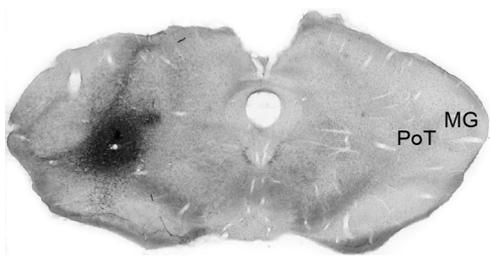
a
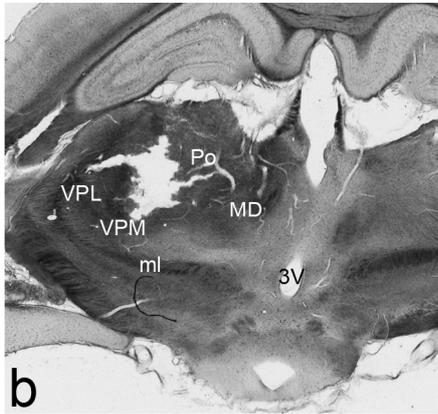
b
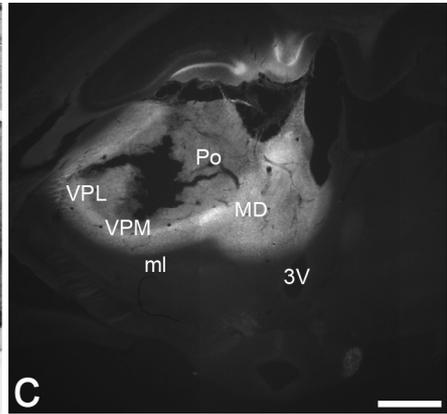
c

involved the Po nucleus, but the PoT was free from such extension. The PoT was completely included within the CTb injection site, and there was a very small area of overlap between Fluorogold and CTb in the Po nucleus at interaural 4.0 mm. Some extension of CTb into areas surrounding the PoT was also seen.

DI 4: Compared to the other experiments, Fluorogold in this case extended to more caudal levels (as far as interaural 3.3 mm). At the caudalmost level, the Fluorogold injection involved mainly the MG nucleus, but it did extend into the PoT at interaural 3.8 mm, where it included only the lateral part of this area. The rostral part of the PoT was completely contained within the Fluorogold injection. The rostral extension of Fluorogold was comparable to that in DI 1 and DI 2. CTb spread involved the entire PoT, apart from the rostral region that was included within the Fluorogold injection. At these rostral levels of PoT, both tracers were present, although there was no overlap between them. As in the other experiments, slight extension of CTb occurred into areas adjacent to the PoT.

DI 5: Fluorogold spread was observed between interaural 3.9 and 8.6 mm. Both medial and lateral nuclei of the thalamus were involved in this injection site. The caudal extension of Fluorogold involved parts of the MG and lateral geniculate (LG) nuclei with no extension into the PoT nucleus. Parts of the midline thalamic nuclei at (and around) interaural 5.4 mm were not involved within the Fluorogold injection. The internal capsule and part of the striatum were also included in Fluorogold spread. CTb completely filled the PoT, and some extension into nearby structures was also observed.

DI 6: Fluorogold spread in this case was comparable to that in DI 5; however, Fluorogold extended as far caudally as interaural 3.4 mm and again it involved parts of the MG and LG nuclei at this level with no spread into the PoT. More involvement of the midline thalamic nuclei at interaural 5.4 mm occurred in this case. CTb filled the PoT and extended into nearby structures.

DI 7: Fluorogold almost filled the entire thalamus in a pattern similar to that observed in DI 6, but the caudal extension of Fluorogold in this case reached as far as interaural 3.9 mm and mainly involved the Po nucleus. CTb filled the PoT as far caudally as interaural 3.3 mm, i.e. the injection missed the most caudal part of the PoT. Some extension of CTb into areas adjacent to the PoT was also observed.

3.1.1.2 Quantitative analysis of lamina I spinothalamic neurons in the C7 and L4 segments

This part of the analysis was performed on transverse sections from the C7 and L4 segments from all series of experiments (Thal, PoT, DI), and all data were obtained from the contralateral (right) side. With dark-field microscopy, lamina II can be identified as a dark band due to the lack of myelin (Fig. 3-7a). It has been previously reported that in the L4 segment of rat spinal cord lamina I is relatively wider in the central part of the dorsal horn, and narrower laterally and medially (Todd et al., 1998). In this study, the width of lamina I in the C7 segment (as judged by dark-field microscopy) was also found to be uneven, being somewhat greater in the central region (up to 35 μm) than in the lateral or medial parts, where it was generally between 10 and 15 μm thick. An identical pattern was seen with the plexus of NK1r-immunoreactive dendrites and cell bodies that occupies lamina I (Fig. 3-7b). Retrogradely labelled cells were judged to be in lamina I if they were very close to the dorsal border of the dorsal horn, or lay dorsal to the dark band identified as lamina II with dark-field microscopy.

The distributions of the retrogradely labelled cells were generally similar across all of the experiments in which CTb or Fluorogold were used as tracers. At both segmental levels (C7 and L4), labelled neurons were observed in lamina I and scattered throughout the deeper laminae (III-VI) of the dorsal horn, as well as in the LSN. In addition, labelled cells were seen in laminae VII, VIII and X, with only occasional cells in lamina II. However, in the L4 segment, retrogradely labelled cells in lamina I were far less numerous than those in the C7 segment; for example, most transverse 60 μm Vibratome sections from L4 contained no labelled neurons in lamina I, whereas each of these sections from C7 contained some labelled lamina I cells.

Quantitative data for the retrogradely labelled neurons in lamina I were obtained from 10 randomly selected 60 μm thick transverse sections from the C7 segment and from 20 such sections from the L4 segment in each animal, and these are shown in Tables 3-1, 3-2 and 3-3. The locations of the retrogradely labelled cells in the dorsal part of the dorsal horn that were sampled in a representative experiment are illustrated in Fig. 3-8, and examples of labelled lamina I cells are shown in Fig. 3-9. The mean numbers of retrogradely labelled cells in lamina I per 600 μm in the C7 segment were 23.2 ± 1.3 (S.D.) for experiments Thal 1-5 and 24.6 ± 5.6 for experiments PoT 1-9 (data from PoT 10 were not included in this part of the analysis, since the injection did not spread into the PoT). The corresponding

Figure 3-7. Identification of lamina I in transverse sections of C7.

a: a dark-field micrograph of the C7 segment from experiment Thal 5. The continuous white line outlines the grey matter, while the two dashed lines show the limits of the dark band that corresponds to lamina II. Note that lamina I is wider in the central part of the dorsal horn than in the lateral or medial parts. **b**: the same section scanned to reveal NK1r-immunoreactivity. The plexus of strongly immunoreactive profiles that occupies lamina I is also wider in the central part of the dorsal horn, and its dorsoventral extent closely matches the region defined as lamina I with dark-field microscopy. The confocal image in **b** is a projection of 6 optical sections at 4 μm z-spacing. Scale bar = 100 μm.

a

b
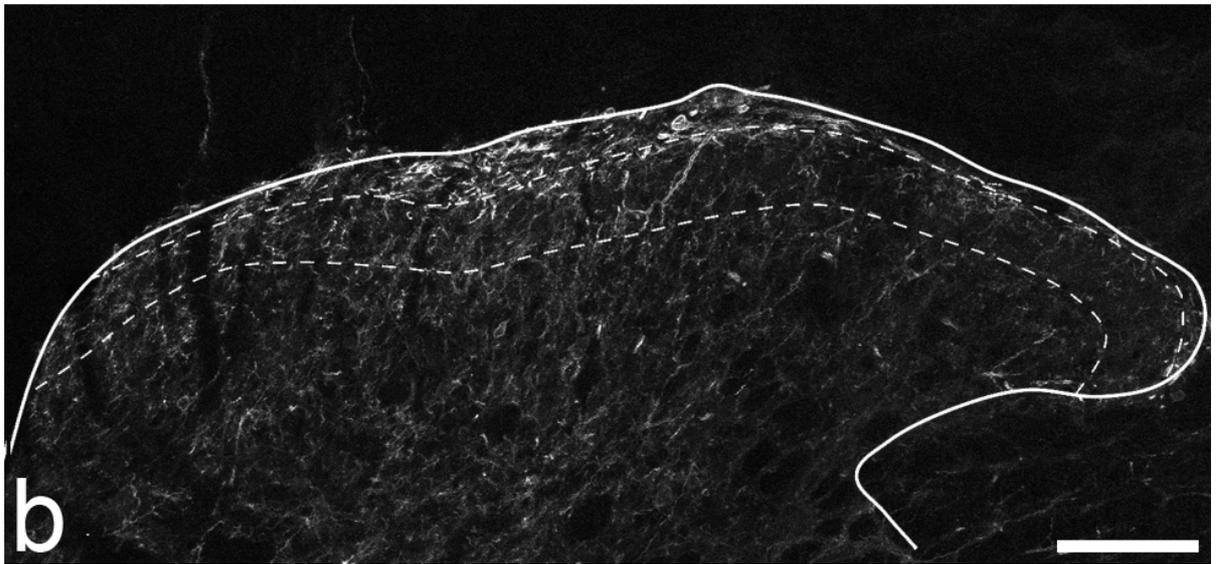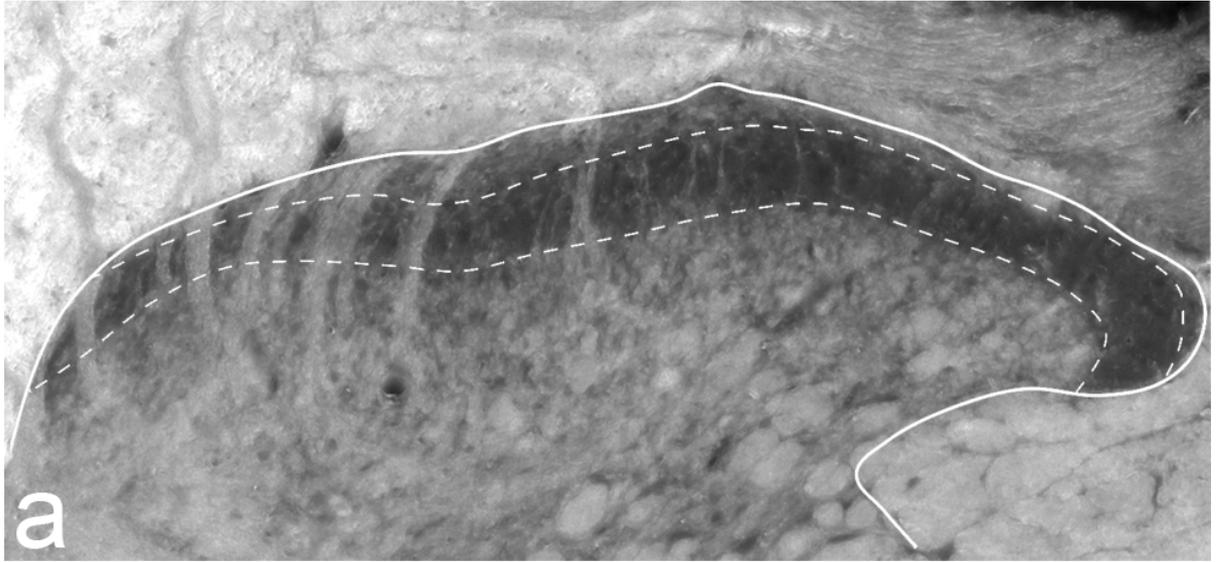
Table 3-1. Quantitative data for retrogradely labelled lamina I neurons in Thal experiments

Exp	C7 (cells per 600 μm)	L4 (cells per 1,200 μm)
Thal 1	25	8
Thal 2	22	3
Thal 3	23	5
Thal 4	24	9
Thal 5	22	10
Mean	**23.2**	**7**

This table shows numbers of retrogradely labelled lamina I cells in the experiments in which Fluorogold injections were targetted at all thalamic regions known to receive projections from superficial dorsal horn neurons. In each experiment, cells were counted in 10 randomly selected transverse 60 μm sections from the C7 segment and in 20 such sections from the L4 segment.

Table 3-2. Quantitative data for retrogradely labelled lamina I neurons in PoT experiments

Exp	C7 (cells per 600 μm)	L4 (cells per 1,200 μm)
PoT 1	20	5
PoT 2	18	5
PoT 3	18	6
PoT 4	36	6
PoT 5	26	
PoT 6	26	9
PoT 7	24	10
PoT 8	25	9
PoT 9	28	7
PoT 10	18	4
Mean*	24.6	7.1

This table shows numbers of retrogradely labelled lamina I cells in the experiments in which tracer injections were targetted on PoT. In each experiment, cells were counted in randomly selected 10 transverse 60 μm sections from the C7 segment and in 20 such sections from the L4 segment. No data were availabe from the L4 segment in experiment PoT 5.

* In experiment PoT 10, the tracer did not spread into PoT and therefore these values were not included in the calculation of the means.

The opposite table shows numbers of retrogradely labelled lamina I cells in the experiments in which CTb was targetted on PoT and Fluorogold (FG) on other thalamic regions known to receive projections from superficial dorsal horn neurons. In each experiment, cells were counted in 10 randomly selected transverse 60 μm sections from the C7 segment and in 20 such sections from the L4 segment.

*These columns show the percentage of all retrogradely labelled cells that contained CTb.

Table 3-3. Quantitative data for retrogradely labelled lamina I neurons in DI experiments

Exp	C7 (cells per 600 μm)					L4 (cells per 1,200 μm)				
	FG-only	CTb-only	FG+CTb	% with CTb*	total	FG-only	CTb-only	FG+CTb	% with CTb*	total
DI 1	1	9	19	97	29	1	6	3	90	10
DI 2	0	14	7	100	21	1	4	5	90	10
DI 3	0	17	5	100	22	1	6	2	89	9
DI 4	6	2	8	63	16	3	1	7	73	11
DI 5	4	5	12	81	21	1	1	2	75	4
DI 6	16	8	4	43	28	6	4	1	45	11
DI 7	4	1	15	80	20	6	2	2	40	10
Mean	4.4	8	10	81	22.4	2.7	3.4	3.1	72	9.3

Figure 3-8. Plots of the locations of spinothalamic tract neurons at cervical and lumbar levels.

These drawings show the location of all retrogradely-labelled cells identified in the dorsal part of the dorsal horn in the transverse sections that were used for quantitative analysis in experiment Thal 1. Each symbol represents a single neuron. The two thin lines indicate the dorsal and ventral borders of lamina II, which were determined from dark-field micrographs. The drawing on the left shows the cells seen in 10 randomly selected 60 μm sections through the C7 segment, while the drawing on the right represents the cells in 20 such sections from the L4 segment.

C7

L4
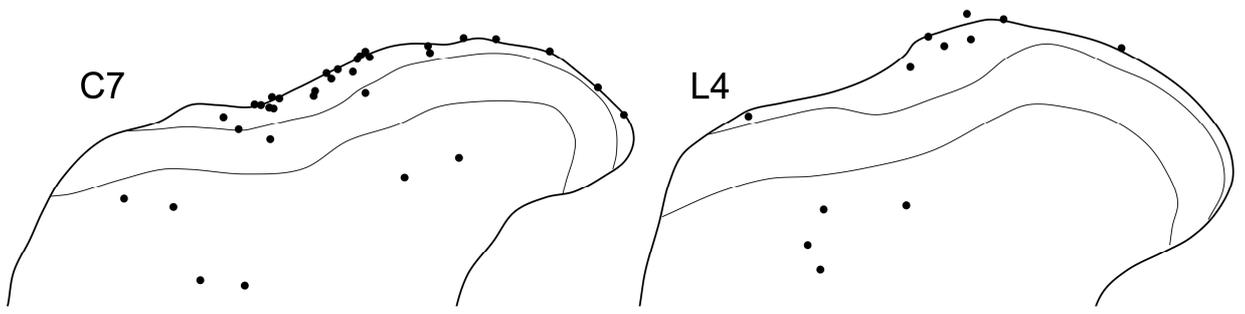
Figure 3-9. Retrograde labelling of lamina I spinothalamic neurons with CTb and Fluorogold.

The main part of the figure shows part of lamina I in a transverse section from the C7 segment of experiment DI 3 immunostained to reveal CTb (green) and Fluorogold (FG, red). A merged image is shown on the right. Several retrogradely labelled lamina I cells are present in this field. Two of these (arrows) contain both CTb and Fluorogold, while others (two of which are indicated with arrowheads) are labelled with CTb but not Fluorogold. The inset shows a single lamina I neuron in a transverse section from the L4 segment of experiment DI 5 that was labelled with both CTb and Fluorogold. The images were obtained from a projection of 19 (main part) or 18 (inset) confocal optical sections at 1 µm z-spacing. Scale bar = 20 µm.
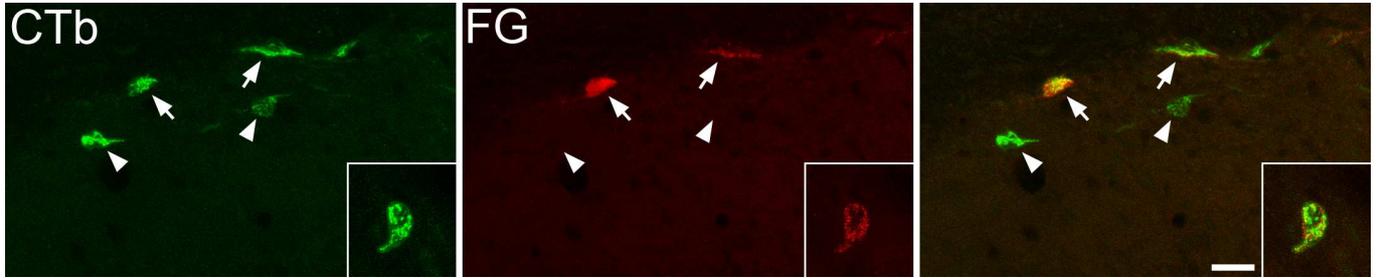
numbers per 1,200 μm for the L4 segment were 7 ± 2.9 for Thal 1-5 and 7.1 ± 2.0 for PoT 1-9 (Tables 3-1 and 3-2). In experiments DI 1-7, the mean numbers of cells that were retrogradely labelled (with either tracer) per 600 μm in the C7 and per 1,200 μm in the L4 segments were 22.4 ± 4.6 and 9.3 ± 2.4, respectively (Table 3-3). Comparison of these values with those obtained in experiments Thal 1-5 and PoT 1-9 indicates that there were no significant differences between the numbers of retrogradely labelled cells found in each series for either cervical or lumbar segments (one-way ANOVA, P > 0.05 for C7 and L4). Numbers of spinothalamic lamina I cells observed in the examined sections were extrapolated to the total number in the entire segment by multiplying that number by length of the segment (in micrometers) and then dividing the result by 600 (in the case of C7) or 1,200 (in the case of L4). The mean length of the C7 segment found in this study was 2.3 mm (range 2.2 - 2.4, n = 3), whereas that of the L4 segment was 2.4 mm (Polgár et al., 2004). Therefore, the estimated total numbers of spinothalamic cells in lamina I in the C7 and L4 segments are approximately 90 and 15 cells, respectively.

In all of the DI experiments, apart from DI 6, the majority of the retrogradely labelled lamina I cells in the C7 segment contained CTb (Table 3-3). In DI 2 and DI 3, most of the cells (≥ 66%) were only labelled with CTb, and none were only labelled with Fluorogold. Interestingly, the mean number of all Fluorogold-labelled cells in DI 1-7 was 14 cells per 600 μm, and this number is significantly lower than the mean number of retrogradely labelled cells found in Thal and PoT experiments (24/600 μm) (t-test, P < 0.05). In the L4 segment, the majority of labelled lamina I cells contained CTb, except in DI 6 and DI 7 (Table 3-3). More than one-third of the labelled cells in DI 1-3 and DI 6 were only positive for CTb. In DI 1-5, few cells were only labelled with Fluorogold.

For experiments Thal 2-5, PoT 1 and PoT 4-10, the retrogradely labelled lamina I neurons were examined for the presence of NK1 receptor. The distribution of NK1r-immunoreactivity within the spinal grey matter was the same as that reported in previous studies (Bleazard et al., 1994; Liu et al., 1994; Nakaya et al., 1994; Brown et al., 1995; Littlewood et al., 1995). As has been described by Liu et al. (1994), Mantyh et al. (1995) and Marshall et al. (1996), the location of the immunoreactivity for the NK1r was seen on the plasma membrane of cell bodies and dendrites. When data from these 12 experiments (11 experiments in the case of L4 as there was no tissue available from PoT 5) were pooled, it was found that 255 out of 294 (87%) of lamina I spinothalamic cells in the C7 segment and 65 out of 77 (84%) of those in the L4 segment were NK1r-immunoreactive (Fig. 3-10).

Figure 3-10. A lamina I spinothalamic neuron with NK1r.

a: a transverse section through the medial part of lamina I in the C7 segment of experiment Thal 3 scanned to reveal NeuN (red) and DAPI (blue). Neuronal nuclei contain both NeuN and DAPI, and therefore appear pink, while non-neuronal nuclei are blue. **b**: the same field scanned to reveal NK1r (green) and Fluorogold (FG, red). The continuous and dashed lines show the upper and lower borders of lamina I, respectively. The arrow indicates a retrogradely labelled neuron in lamina I, which is also NK1r-immunoreactive. Two other NK1r-immunoreactive neurons that are not retrogradely labelled are shown with asterisks, and several neurons that are NK1r-negative and lack Fluorogold are also present in lamina I. The upper inset shows DAPI staining in the nucleus of the spinothalamic neuron, while the lower inset shows the NK1r on its surface membrane. All images are projections of 2 optical sections at 1 μm z-spacing. Scale bar = 20 μm.

a NeuN/DAPI

b NK1r/FG
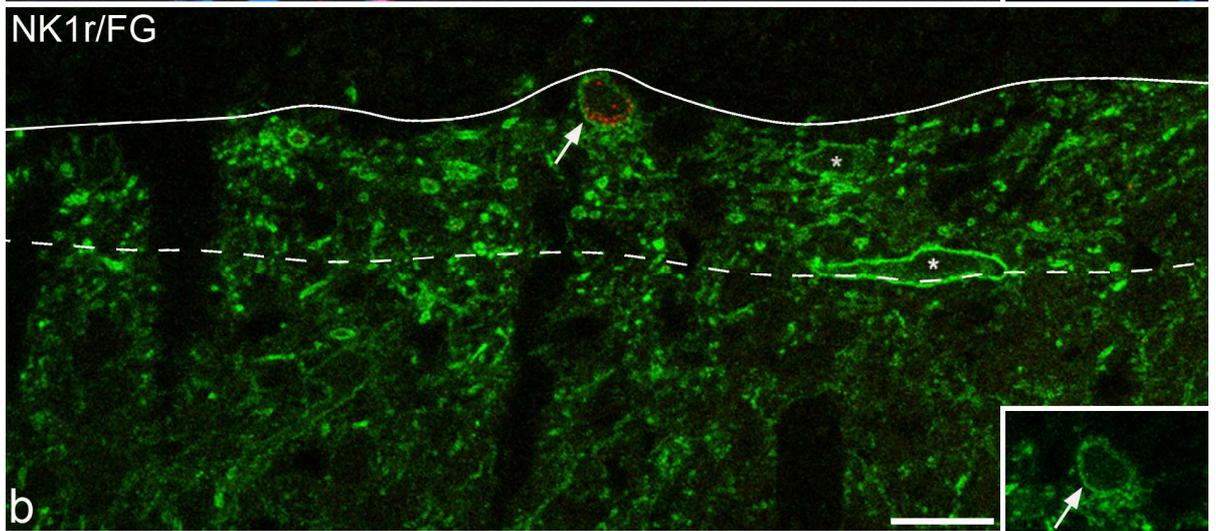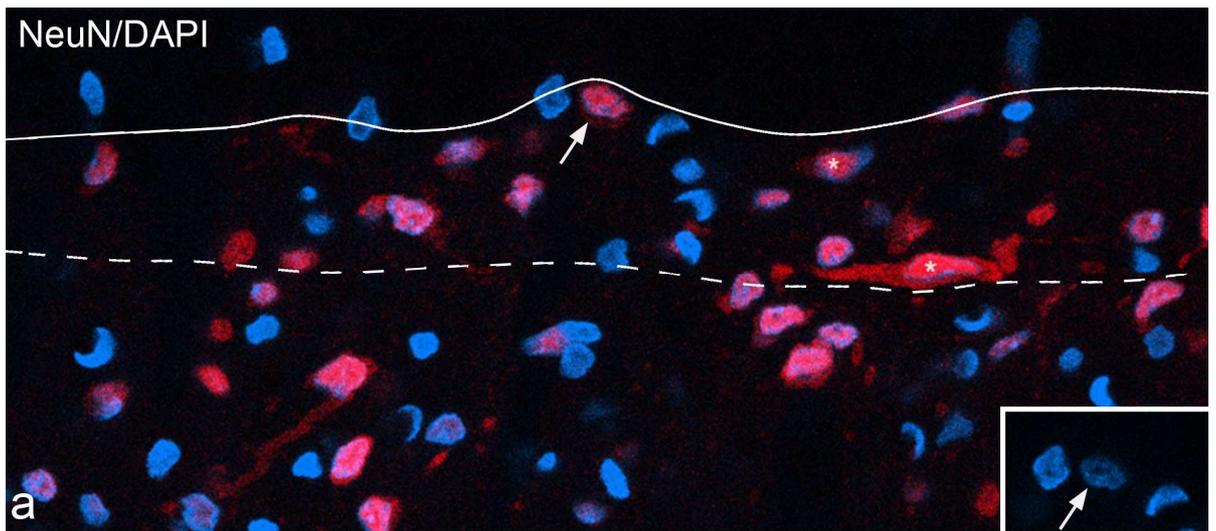
In sections from the C7 segments of experiments Thal 2-5 that were reacted to reveal NeuN, NK1r and Fluorogold, and subsequently incubated in DAPI, numerous neuronal (NeuN-positive) profiles with DAPI-labelled nuclei were visible in lamina I, and many of these were also NK1r-immunoreactive (Fig. 3-10). Although Fluorogold-positive cells were seen in lamina I in most sections, these were greatly outnumbered by neurons that were not retrogradely labelled. The total numbers of lamina I neurons that were included in the 9 µm thick samples obtained with the disector method from experiments Thal 2-5 varied from 139 to 168 (mean 150, n = 4), while the numbers that were retrogradely labelled in these samples ranged from one to seven (mean 3.5). The proportion of the sampled lamina I neurons that were retrogradely labelled in the four experiments ranged between 0.6-5% (mean 2.4%). Estimates of the total number of lamina I neurons per 600 µm length of C7 (calculated according to the formula in Methods) for the four experiments ranged from 1,028 to 1,420 (mean 1,254). The mean numbers of retrogradely labelled lamina I neurons determined for all Thal, PoT and DI experiments (excluding PoT10, as explained above) was 23.5 per 600 µm (data from Tables 3-1, 3-2 and 3-3), and this corresponds to 1.9% of the estimated total population of lamina I neurons.

3.1.1.3 The proportion of lamina III/IV NK1r-immunoreactive neurons that project to the thalamus

This part of the analysis was performed on parasagittal sections from the contralateral side of the C6 and L5 segments from experiments Thal 1-5, PoT 3-10 and DI 1-7. In experiment DI 5, the analysis was performed only on tissue from the C6 segment. It has been reported that the large NK1r-immunoreactive lamina III/IV cells with long dorsal dendrites in lumbar spinal cord are best seen in the parasagittal plane, as most of the dendritic tree of these cells could be followed in this plane (Naim et al., 1997). The pattern of immunostaining with the NK1r that was observed in these sections was similar to that reported in previous studies (e.g. Naim et al., 1997, 1998; Todd et al., 2000). Generally, there was a dense plexus of immunoreactive profiles in lamina I with scattered immunostained cell bodies and dendrites in the deeper laminae. As reported by other investigators (Brown et al., 1995; Naim et al., 1997), some of the large NK1r-immunoreactive neurons in laminae III and IV had pyramidal cell bodies, whereas others were multipolar. In line with previous reports, some of the dorsal dendrites arborized extensively in laminae I and II, whereas others reached only the ventral part of lamina II, and most of the neurons were found to have dendrites in other directions (ventral, medial and lateral) (Brown et al., 1995; Naim et al., 1997; Todd et al., 2000).

Tables 3-4, 3-5 and 3-6 summarise data obtained from the analysis of this population of neurons in the C6 and L5 segments in single- (Thal and PoT series) and double- (DI series) labelling experiments. When data from Thal, PoT and DI experiments were pooled, the mean numbers of large NK1r-immunoreactive cells with somata in laminae III and IV and dendrites that could be followed into the superficial dorsal horn were 22.1 ± 4.7 (mean ± S.D., range 15-28, n = 19) on the right side in the L5 segment and 15.8 ± 3.7 (range 10-22, n = 20) on the right side in the C6 segment. The cells were uniformly distributed along both the mediolateral and rostrocaudal extent of the dorsal horn in each segment. The cell bodies were located between 93-405 µm (mean 223 µm ± 63, n = 419 cells) below the dorsal white matter in L5 and between 82-349 µm (mean 161 µm ± 42, n = 316 cells) below the dorsal white matter in C6. The mean depth of these cells in L5 was significantly different from that in C6 (t-test, $P < 0.001$).

In order to determine the proportion of these neurons that were labelled from the thalamus, the NK1r-immunoreactivity was first checked with the epifluorescence microscope using the appropriate filter for the fluorochrome Alexa 488, then the presence of tracer substance (Fluorogold or CTb) was detected using the filter set to detect Rhodamine red. In the cases when tracer was labelled with Cy5, the confocal microscope was used for this detection. The pattern of labelling with tracer was variable; in some cases there was extensive filling of the cell body as well as primary and secondary dendrites, while in others the labelling faded along the course of the primary dendrites. The proportions of such cells that were labelled in each experiment are given in Tables 3-4, 3-5 and 3-6. In the C6 segment, most cells of this type were retrogradely labelled in all of the experiments except in PoT 10. The percentage of cells that were labelled in experiments Thal 1-5 ranged from 83-100 (mean 93), while for experiments PoT 3-9 these values were 69-100 (mean 86, data from PoT 10 were not included in this part of the analysis, since the injection did not spread into PoT). For experiments DI 1-7, the percentage of cells that contained one or both tracers varied from 60-94 (mean 79). Comparison of the percentage values obtained in each group indicated that there were no significant differences between them (one-way ANOVA, $P > 0.05$). The overall proportion of labelled cells was 85% (data pooled for all experiments apart from PoT 10). In the DI series of experiments (Table 3-6), all of the retrogradely labelled cells in the C6 segment contained CTb, apart from three cells in DI 4, which were positive for Fluorogold only. In four of the seven DI experiments (DI 2-3 and DI 6-7), none of the retrogradely labelled NK1r-immunoreactive lamina III/IV neurons contained Fluorogold, while this was found in the other three experiments. An example of a

Table 3-4. Quantitative data for lamina III/IV NK1r cells from Thal experiments

Exp	C6			L5				
	number	STT	%	number	STT	%	med %	lat %
Thal 1	13	12	92	27	7	26	38	14
Thal 2	15	15	100	24	0	0	0	0
Thal 3	12	10	83	28	7	25	43	7
Thal 4	21	20	95	23	5	22	18	25
Thal 5	16	15	94	28	3	11	21	0
Mean	**15.4**	**14.4**	**93**	**26**	**4.4**	**17**	**24**	**9**

This table shows numbers of the lamina III/IV NK1r cells with dendrites that could be followed into the superficial dorsal horn on the contralateral side of C6 and L5. For each segment, the total number of cells of this type (number), and the number (STT) and percentage (%) that were retrogradely labelled are given. For the L5 segment, the percentage of cells of this type in the medial or lateral group that were retrogradely labelled is also provided (see text for further details).

Table 3-5. Quantitative data for lamina III/IV NK1r cells from PoT experiments

Exp	C6			L5				
	number	STT	%	number	STT	%	med %	lat %
PoT 3	15	14	93	15	4	27	43	13
PoT 4	18	14	78	18	3	18	33	0
PoT 5	15	15	100	15	2	13	29	0
PoT 6	11	9	82	26	4	15	31	0
PoT 7	22	20	91	24	4	17	17	17
PoT 8	13	9	69	23	7	30	36	25
PoT 9	14	12	86	16	4	25	50	0
PoT 10	21	8	38	24	2	8	8	8
Mean*	**15.4**	**13.3**	**86**	**19.6**	**4**	**21**	**34**	**8**

This table shows numbers of the lamina III/IV NK1r cells with dendrites that could be followed into the superficial dorsal horn on the contralateral side of C6 and L5. For each segment, the total number of cells of this type (number), and the number (STT) and percentage (%) that were retrogradely labelled are given. For the L5 segment, the percentage of cells of this type in the medial or lateral group that were retrogradely labelled is also provided (see text for further details).

*The mean value excludes the results for PoT 10, since in this experiment there was no spread of tracer into the PoT.

The opposite table shows numbers of the lamina III/IV NK1r cells with dendrites that could be followed into the superficial dorsal horn on the contralateral side of C6 and L5. For each segment, the total number of cells of this type (number), and the number (STT) and percentage (%) of the lamina III/IV NK1r-immunoreactive neurons that were retrogradely labelled with either tracer are given. The retrogradely labelled cells are further divided into those that were labelled with CTb only (CTb; from PoT), Fluorogold only (FG; from thalamic nuclei other than PoT) or labelled with both tracers (double-labelled, DL). For the L5 segment, the percentage of cells of this type in the medial or lateral group that were retrogradely labelled with either tracer is also provided (see text for further details). Note that no data were available for the L5 segment in experiment DI 5.

Table 3-6. Quantitative data for lamina III/IV NK1r cells from DI experiments

Exp	C6						L5							
	number	STT	CTb	FG	DL	%	number	STT	CTb	FG	DL	%	med%	lat %
DI 1	12	10	5	0	5	83	16	2	0	0	2	13	25	0
DI 2	19	16	16	0	0	84	27	0	0	0	0	0	0	0
DI 3	20	16	16	0	0	80	24	3	3	0	0	13	17	8
DI 4	16	15	6	3	6	94	15	2	1	0	1	13	29	0
DI 5	13	9	8	0	1	69								
DI 6	20	16	16	0	0	80	25	6	5	0	1	24	33	15
DI 7	10	6	6	0	0	60	21	2	2	0	0	10	20	0
Mean	**15.7**	**12.6**	**10.4**	**0.4**	**1.7**	**79**	**21.3**	**2.5**	**1.8**	**0**	**0.7**	**12**	**21**	**4**

retrogradely labelled lamina III/IV NK1r-immunoreactive neuron in the cervical cord is shown in Fig. 3-11a-c.

In contrast, in the L5 segment, most cells of this type were not retrogradely labelled in any of the Thal, PoT or DI experiments (Tables 3-4, 3-5 and 3-6). The percentage of cells that were labelled in experiments Thal 1-5 ranged from 0-26 (mean 17), while for experiments PoT 3-9 these values were 13-30 (mean 21), and for DI 1-4 and DI 6-7 the percentage of cells that contained one or both tracers varied from 0-24 (mean 12). Comparison of the percentage values obtained in each group indicated that there were no significant differences between them (one-way ANOVA, P > 0.05). The overall proportion of retrogradely labelled cells was 17% (data pooled for all experiments apart from PoT10). In the two experiments in which no retrogradely labelled cells of this type were identified in L5 (Thal 2 and DI 2), some retrogradely labelled NK1r-immunoreactive cells were seen in laminae III and IV, but their dendrites could not be traced into the superficial dorsal horn, hence these were not included in the analysis. In the DI experiments (Table 3-6), all of the retrogradely labelled cells of this type in L5 contained CTb, and interestingly, 11 out of the 15 CTb-labelled cells (pooled from all experiments) were only labelled with this tracer. Labelling with Fluorogold was only seen in DI 1, DI 4 and DI 6. An example of a retrogradely labelled lamina III/IV NK1r-immunoreactive neuron in th lumbar cord is shown in Fig. 3-11d-f.

Interestingly, in experiment PoT 10, relatively few retrogradely labelled cells were observed among the lamina III/IV NK1r-immunoreactive population at either segmental level (38% in C6 and 8% in L5, Table 3-5).

Results of the analysis of the mediolateral distribution of retrogradely labelled lamina III/IV NK1r-immunoreactive neurons in the L5 segment are shown in Tables 3-4, 3-5 and 3-6. When data from experiments Thal 1-5, PoT 3-9, DI 1-4 and DI 6-7 were pooled, the proportion of these cells in the medial part of the dorsal horn that were retrogradely labelled from the thalamus was 51/205 (25%), while the corresponding proportion for those in the lateral part was 16/214 (7%). These values differed significantly (χ^2 test, P < 0.0001).

Figure 3-11. Lamina III NK1r-expressing spinothalamic neurons in parasagittal sections.

a,b,c: a retrogradely-labelled neuron in the C6 segment from experiment PoT 7; **d,e,f**: a retrogradely-labelled neuron in L5 from experiment DI 6. In each case, CTb is shown in red, NK1r in green and a merged image is shown on the right. The dorsal limit of the dorsal horn is near the top of the field. Note that both of the labelled neurons have extensive dorsal dendrites that pass through the superficial dorsal horn. Images built from projections of 36 (a-c) or 60 (d-f) confocal optical sections at 1 μm z-spacing. Scale bar = 20 μm.

CTb   NK1r

a   b   c

d   e   f
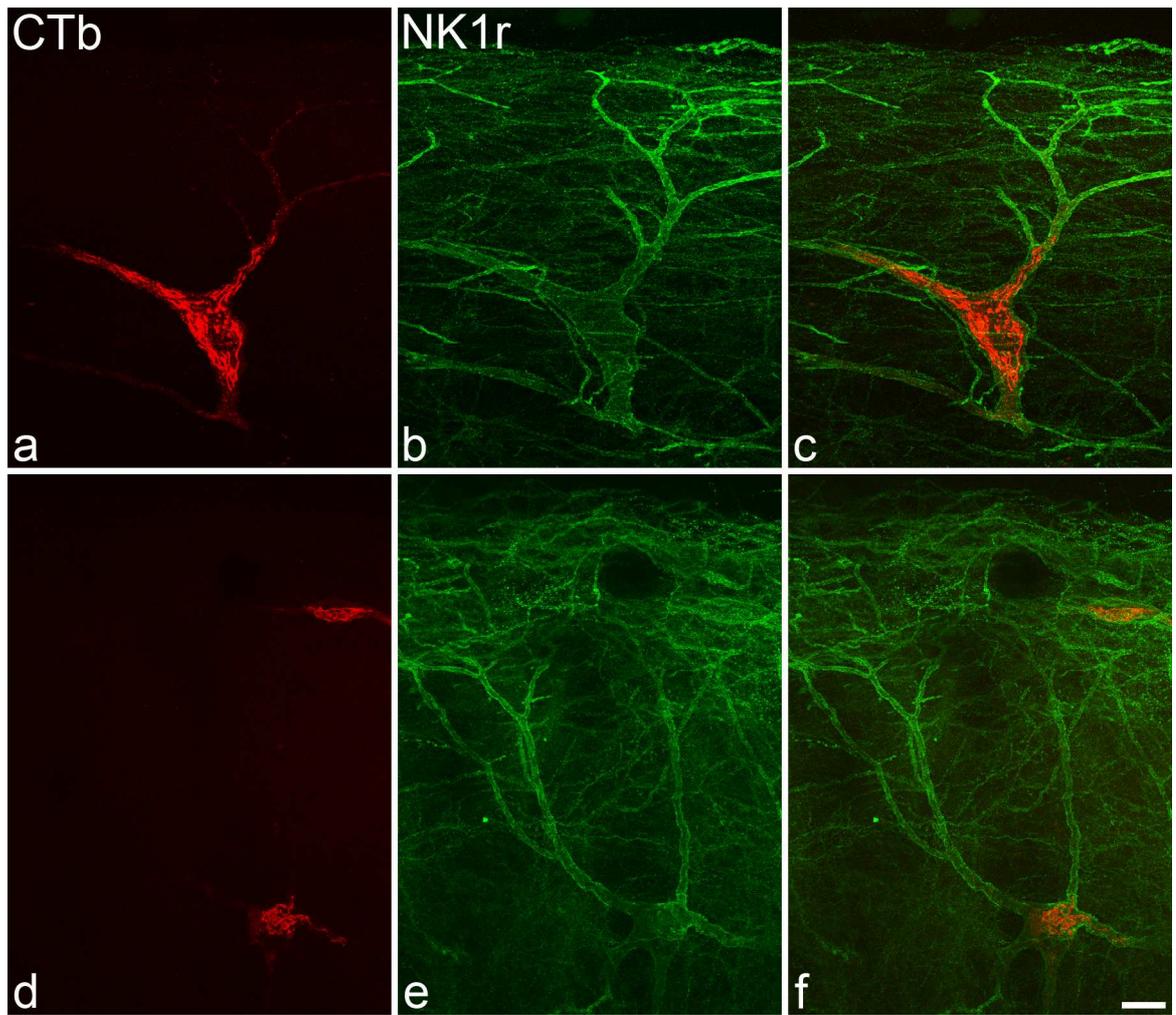
3.1.2 Retrograde labelling with fluorescent latex micro-spheres (FLM)

3.1.2.1 Analysis of the Injection sites

Drawings of the spread of fluorescent latex microspheres in experiments FLM 1-3 are illustrated in Fig. 3-12, and example of an injection site is also shown. In all cases, the spread of tracer was very limited, compared to that seen with Fluorogold or CTb. Tracer spread in FLM 1 was restricted to the caudal half of the PoT (between interaural 2.9 and 3.7 mm), and was mainly confined to its medial part. Some spread was also noted along the needle track into the region of Po that lies dorsal to PoT (at and around interaural 3.4 mm). Tracer spread in FLM 2 was greater than that in FLM 1 because this rat received two injections at different rostrocaudal levels. The spread covered most of the rostrocaudal extent of PoT and occurred between interaural 3.2 and 4.1 mm. However, beads mainly occupied the lateral part of PoT (area adjacent to the MG nucleus). Dorsal spread of tracer along the needle track to the Po nucleus at (and around) interaural 3.3 and 3.7 mm was also observed. In FLM 3, the tracer occupied the medial part of the PoT from interaural 3.5 to 4.1 mm, with slight extension into the APT, the ethmoid thalamic nucleus and the extreme caudal end of Po.

3.1.2.2 Retrograde labelling in laminae I, III and IV

This part of the analysis was carried out on complete series of parasagittal sections from the contralateral side of the C6 and L5 segments from the three experiments (FLM 1-3). Retrogradely labelled neurons in the spinal cord were identified by the presence of small red fluorescent granules (microspheres/beads) that occupied the cell body and, in some cases, spread into proximal dendrites. Examples of retrogradely labelled neurons are shown in Figs. 3-13 and 3-14, and quantitative data are provided in Table 3-7. The numbers of the microspheres in the labelled neurons varied considerably: only cells that contained five or more microspheres were considered to be retrogradely labelled, and most of the labelled cells contained more than ten microspheres. Since fluorescent latex microspheres are destroyed by glycerol, Gel-Mount was used to mount the sections in these experiments, and the optical properties of this medium were not as good as those of the glycerol-based medium used for other experiments. Although fluorescent latex microspheres could be identified through the whole depth of the section, it was difficult to follow NK1r-immunoreactive profiles deep within the section, even when 50 μm thick parasagittal sections were used (experiments FLM 2 and FLM 3).

Figure 3-12. Injection sites for experiments FLM 1-3.

Drawings to show the spread of fluorescent latex microspheres (red) in these experiments. Each column represents a single experiment, and the experiment number is shown at the bottom of the column. Numbers at the top left of each drawing give the approximate position of the section anterior to the interaural plane. Drawings are based on those in Paxinos and Watson (2005). *Eth*, ethmoid thalamic nucleus; *MGD*, medial geniculate nucleus, dorsal part; *MGV*, medial geniculate nucleus, ventral part; other abbreviations as in Figure 3-1. The image below shows a dark-field micrograph (interaural ~3.9 mm) from experiment FLM 3. Note the limited spread of the red fluorescent latex microspheres. Scale bars = 1 mm.

4.6

4.3

4.1

4.0

3.7

3.5

3.4

3.1

2.9

Po   Eth
VPM

APT
VPM   Po
        Eth

Po   APT
MGV
PIL   PoT

MGD   Po   APT
MGV   PoT

MGD   Po
MGV   APT
PIL   PoT

MGD   Po
MGV   APT
PoT

MGD   Po
MGV   APT
PoT

MGD
MGV
PoT

MGV
PoT

FLM1   FLM2   FLM3
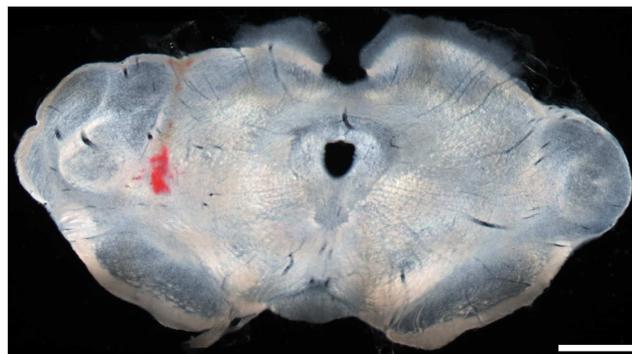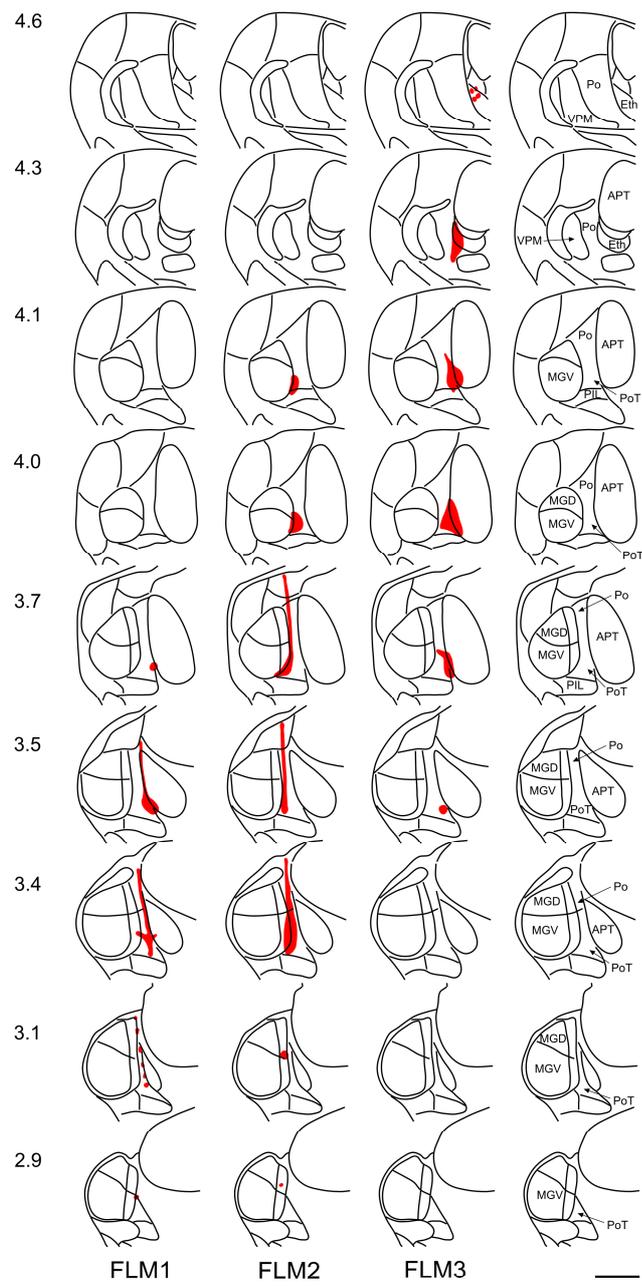
Figure 3-13. Retrograde labelling of lamina I neurons with fluorescent latex microspheres in parasagittal sections.

The top row of images shows an example of a labelled lamina I neuron from C6 in experiment FLM 3, while the bottom row shows a labelled neuron in L5 from experiment FLM 2. In each case, separate images show the fluorescent latex microspheres (beads, red), NK1r (green) and NeuN (blue), with a merged image on the right. Note the presence of numerous beads in each neuron, and that both neurons are NK1r-immunoreactive (arrow). Images are projections of 7 (top row) or 5 (bottom row) confocal optical sections at 1 μm z-spacing. Scale bar = 20 μm.
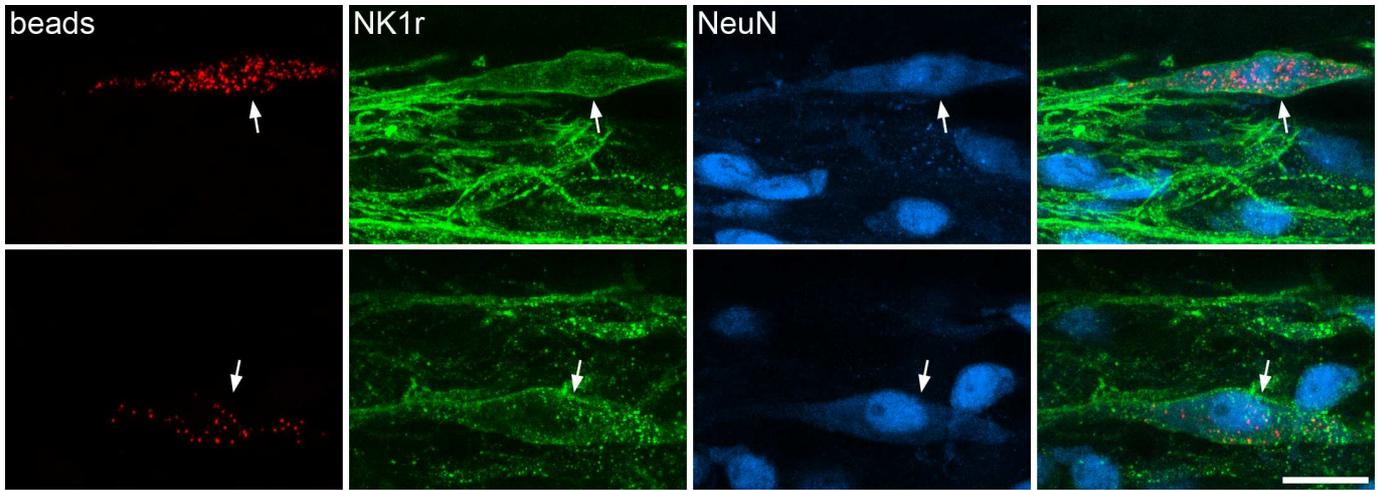
Figure 3-14. Retrograde labelling of NK1r-immunoreactive neurons in lamina III with fluorescent latex microspheres in parasagittal sections.

a and **b** show NK1r-immunoreactive lamina III cells in C6 (**a**) and L5 (**b**) from experiment FLM 3. In each case, the soma is marked with an asterisk and dorsal dendrites (arrowheads) can be seen passing into the superficial dorsal horn. The insets (**a'**, **b'**) show the region of the soma of each cell scanned to reveal fluorescent latex microspheres (red) and NK1r (green), together with a merged image. Note the presence of numerous microspheres in the soma of each cell. **a** is a projection of 14 confocal optical sections at 1 μm z-spacing, while **b** is a montage of two fields projected from 14 and 9 optical sections at the same spacing. **a'** and **b'** are projections of 6 and 11 optical sections at 1 μm z-spacing, respectively. Scale bar = 20 μm.
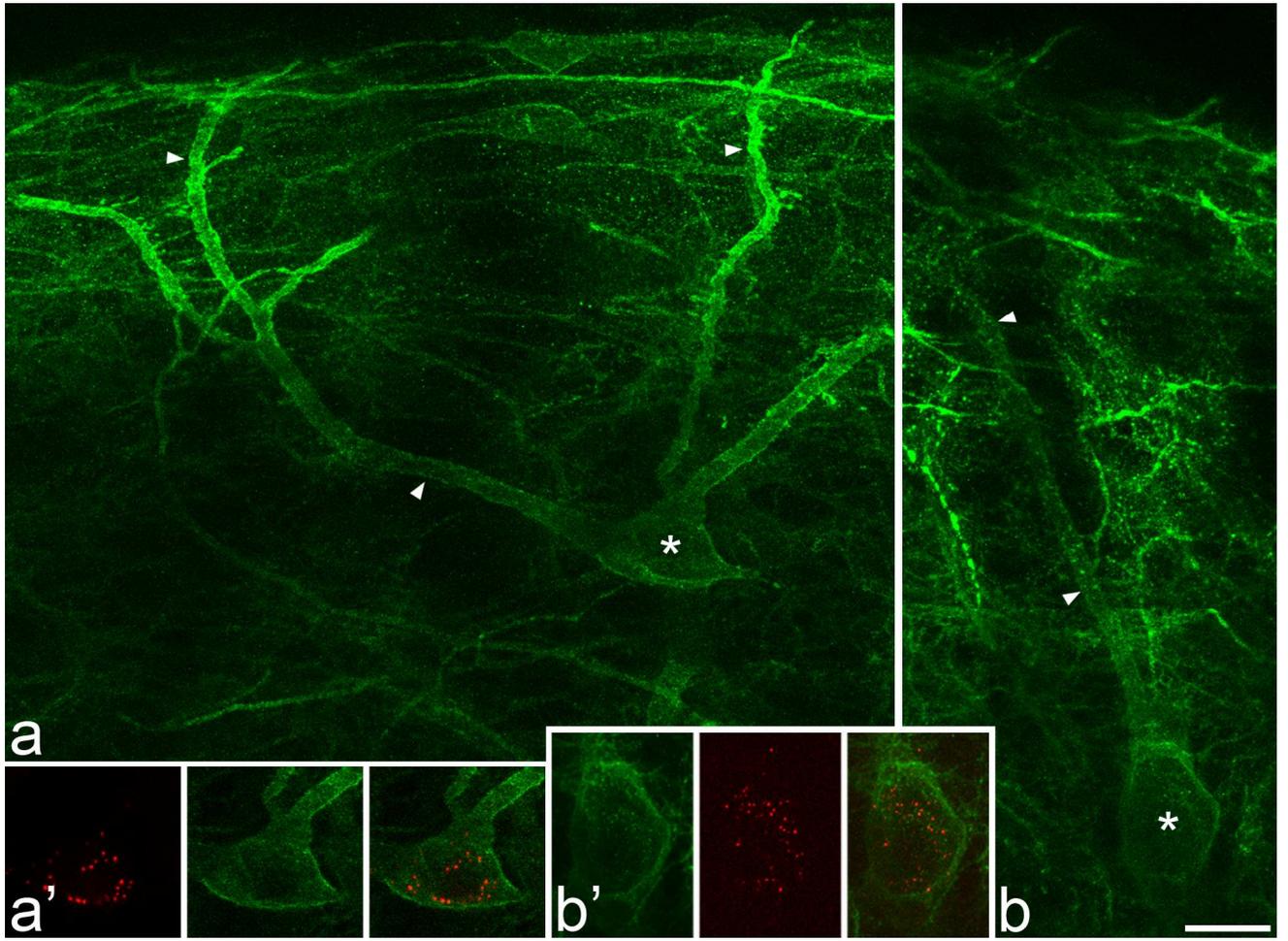
Table 3-7. Quantitative data from FLM experiments

Exp	lamina I		lamina III/IV NK1r-immunoreactive	
	C6	L5	C6	L5
FLM 1	15	0	5 (1)	0
FLM 2	37	4	10 (6)	4 (0)
FLM 3	46	5	7 (3)	6 (2)

This table shows numbers of retrogradely labelled neurons in cervical and lumbar segments from experiments in which injections of fluorescent latex microspheres (FLM) were targetted on the PoT. Complete series of parasagittal sections through the dorsal horn of C6 and L5 segments were examined, and the total number of retrogradely labelled cells was counted. For lamina III/IV NK1r-immunoreactive cells, the number of retrogradely labelled neurons for which dorsal dendrites could be followed into lamina II is given in brackets (see text for further details).

In the C6 segment, lamina I was found to contain between 15 and 46 retrogradely labelled cells, while 5-10 retrogradely labelled lamina III/IV NK1r-immunoreactive cells were observed, of which 1-6 had dendrites that could be followed into the superficial dorsal horn (Fig. 3-14a). Among the large NK1r-immunoreactive lamina III/IV cells, only those that had dorsal dendrites that entered lamina II in the superficial part of the same section could be unequivocally identified as belonging to this type. This was because of the difficulty in following NK1r-immunoreactive dendrites in these sections. In the L5 segment, no retrogradely labelled cells were detected in laminae I or III/IV in experiment FLM 1, while in experiments FLM 2 and FLM 3, four and five lamina I cells (respectively) and four and six lamina III/IV NK1r-immunoreactive cells (respectively) were seen. Dorsal dendrites of two of the lamina III/IV NK1r-immunoreactive cells in experiment FLM 3 could be followed into the superficial dorsal horn and one of these is illustrated in Fig. 3-14b.

When data from all three experiments were pooled, it was found that most of the retrogradely labelled cells in lamina I of the C6 segment were positive for the NK1 receptor (73 out of 98; 74%). NK1r-immunoreactivity in the remaining cells was either difficult to assess (13 cells) because of their depth in the section, or absent (12 cells). Five out of the nine retrogradely labelled cells in lamina I of L5 expressed the receptor, while it was difficult to assess in two cells and absent in other two.

3.2 Investigation of collateralisation of projection neurons in laminae I, III and IV to the thalamus, lateral parabrachial area and periaqueductal grey matter (Pb and PAG series of experiments)

3.2.1 Analysis of the injection sites

In all seven experiments, the spread of tracer within the thalamus and brainstem was plotted on diagrams from atlas of Paxinos and Watson (2005), and details of the injection sites in the two series of experiments (Pb 1-3 and PAG 1-4) are presented below.

3.2.1.1 Injections into the thalamus and parabrachial area (Pb 1-3)

The spread of Fluorogold within the thalamus, and of CTb within the parabrachial area, in each of these three experiments is illustrated in Fig. 3-15. A representative example of an injection site in the parabrachial area is shown in Fig. 3-16. The following account describes the features of the injection sites in the thalamus and parabrachial nuclei.

Thalamic injection sites: In all cases, the region of the PoT was completely filled with Fluorogold. In addition, variable extension into areas surrounding the PoT (e.g. APT, PIL, and MG) was observed. Fluorogold also extended rostrally to involve the caudal part of the Po and ventrobasal complex, as well as some intralaminar nuclei (e.g. PF, PC, and to a lesser extent CL). There was no spread of Fluorogold into the hypothalamus, PAG or LPb in any of these experiments.

Parabrachial injection sites: The pattern of CTb spread in these experiments was more or less the same, except that CTb extended into more ventral structures in Pb 3. CTb filled the entire rostrocaudal extent of the LPb and involved its various parts: the internal, external, dorsal, ventral, central, lateral crescent, and superior subnuclei. In addition, CTb spread ventrally across the superior cerebellar peduncle and involved the MPb nucleus. The involvement of MPb was greater in Pb 3, where CTb filled this region, while this spread occurred only into the dorsal part of MPb in the other two experiments. The involvement of the Kölliker Fuse nucleus was variable among the three cases: this region was almost completely filled in Pb 3 (apart from the most rostral part), only its caudal half was filled in Pb 2, and only minimal spread occurred into its most caudal part in Pb 1. At the caudal extent of the injection site, some spread occurred into the locus coeruleus (in Pb 1 and Pb 3) and ventral spinocerebellar tract (in all cases). At the rostral end of the injection site,

Figure 3-15. Fluorogold and CTb injection sites in experiments Pb 1-3.

Drawings to show the spread of tracer (shaded area) in these experiments. Each vertical column represents a single experiment, and the experiment number is shown at the bottom of the column. Numbers to the left give the approximate position of the section relative to the interaural plane. Drawings are based on those in Paxinos and Watson (2005). The upper six outlines in each column represent the Fluorogold injection (targetted on caudal thalamus), while the lower four show the spread of CTb (targetted on LPb). *CnF*, cuneiform nucleus; *IC*, inferior colliculus; *KF*, Kölliker-Fuse nucleus; *LPB*, lateral parabrachial nucleus; *MPB*, medial parabrachial nucleus; *PAG*, periaqueductal grey matter; *SC*, superior colliculus; *scp*, superior cerebellar peduncle. Other abbreviations as in Figure 3-1.

5.4

4.6

4.0

3.6

3.2

2.9

0.7

0.2

-0.2

-0.7

Pb1   Pb2   Pb3
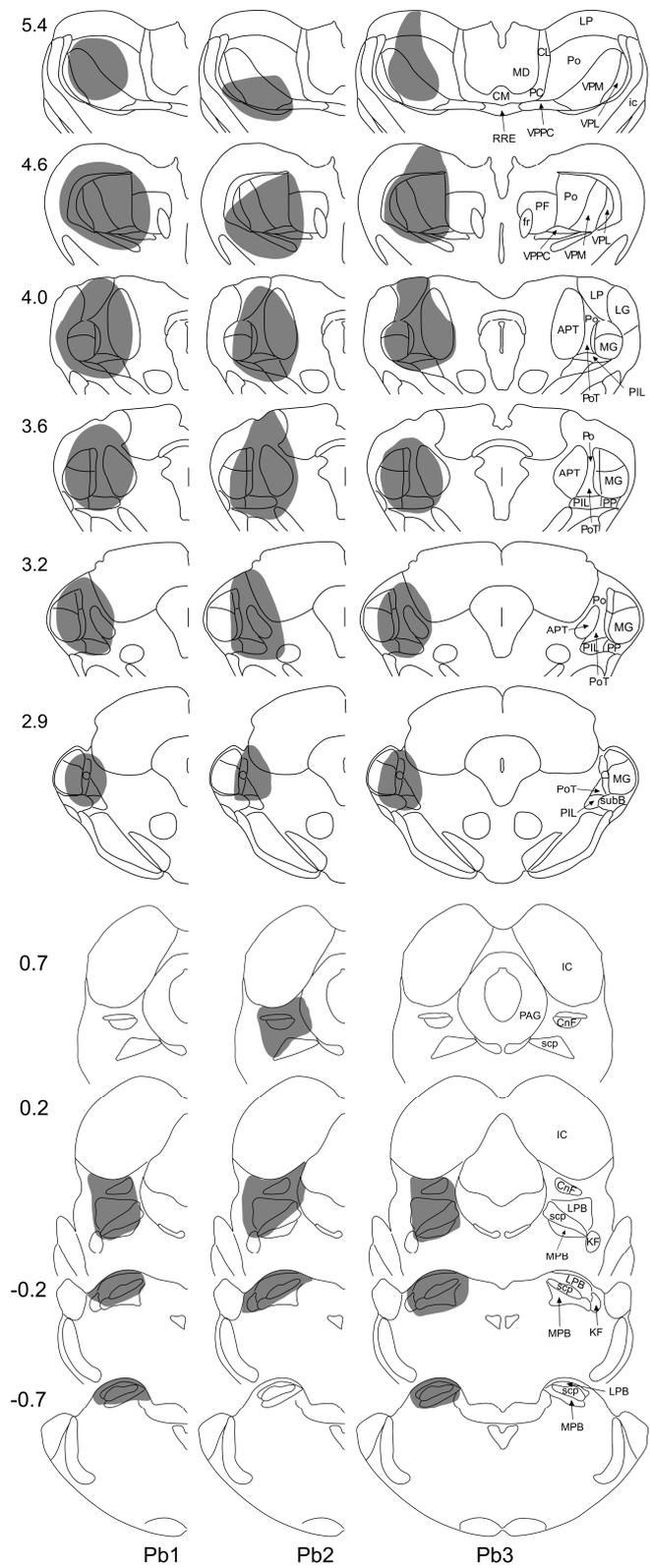
Figure 3-16. Examples of Fluorogold and CTb injection sites in LPb experiments.

a: a brightfield micrograph showing a section through an injection of CTb that was targetted on the parabrachial area at interaural 0.5 mm from experiment Pb 2. The section was reacted with an immunoperoxidase method to reveal CTb. **b**, **c**: fluorescence and brightfield micrographs through the thalamic injection site (interaural ~ 4.2) from Pb 2. Scale bar = 50 µm.

a

b
MG Po

c
MG Po
°
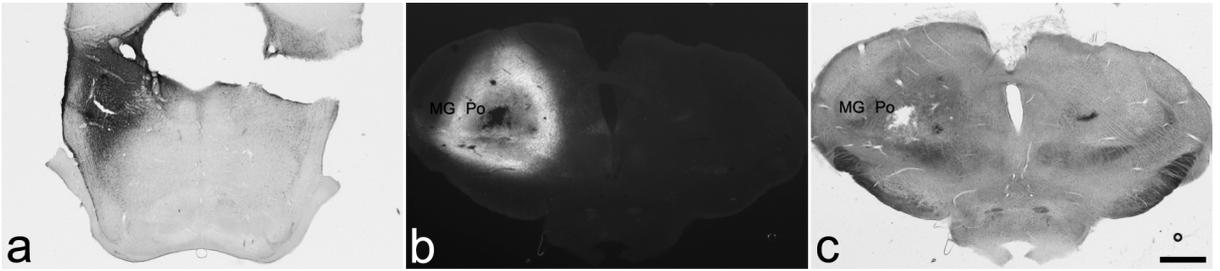
there was variable involvement of the cuneiform nucleus, and there was slight extension into the extreme caudal part of the vlPAG in Pb 2.

3.2.1.2 Injections into thalamus and PAG (PAG 1-4)

The spread of Fluorogold within the thalamus, and of CTb within the PAG, in each of these four experiments is illustrated in Fig. 3-17. A representative example of an injection site is shown in Fig. 3-18. The following account describes the features of the injection sites in the thalamus and PAG.

Thalamic injection sites: In all four cases, the injection completely filled the PoT, and there was variable spread into areas around the PoT (e.g. APT, PIL, MG). In addition, Fluorogold extended rostrally to include parts of the Po nucleus and ventrobasal complex. Some involvement of the intralaminar nuclei (e.g. CL, PF) was also seen. There was no spread of Fluorogold into the hypothalamus, PAG or LPb in any of the four cases.

PAG injection sites: In all four experiments, CTb filled various parts of the four columns of PAG: vlPAG, lPAG, dlPAG and dmPAG. This spread occurred as far caudally as interaural 0.2 mm (PAG 1), 0.4 mm (PAG 2 and PAG 3), and 0.7 mm (PAG 4), and as far rostrally as interaural 2.6 mm (in PAG 1-3) and interaural 2.3 mm (in PAG 4). Tracer did not spread across the midline, but a little lateral spread occurred into the deep mesencephalic nucleus in PAG 2. In PAG 1 and PAG 3 (and to a lesser extent in PAG 2), dorsal spread into the overlying superior colliculus was observed. In all cases, tracer did not spread into the LPb.

3.2.2 Quantitative analysis of projection neurons in lamina I

This part of the study was performed on transverse sections from the C7 and L4 segments from all seven experiments, and all data were obtained from the contralateral side. The distributions of cells retrogradely labelled from each target were generally similar across each of the experiments. This distribution was consistent with that previously reported for spinothalamic (Giesler et al., 1979; Lima and Coimbra, 1988; Burstein et al., 1990a; Li et al., 1996; Marshall et al., 1996; Kobayashi, 1998), spinoparabrachial (Cechetto et al., 1985; Hylden et al., 1989; Ding et al., 1995), and spino-PAG (Liu, 1986; Keay et al., 1997; Li et

Figure 3-17. Fluorogold and CTb injection sites in experiments PAG 1-4.

Drawings to show the spread of tracer (shaded area) in these experiments. Each vertical column represents a single experiment, and the experiment number is shown at the bottom of the column. Numbers to the left give the approximate position of the section anterior to the interaural plane. Drawings are based on those in Paxinos and Watson (2005). The upper six outlines in each column represent the Fluorogold injection (targetted on caudal thalamus), while the lower five show the spread of CTb (targetted on PAG). *4V*, fourth ventricle; *Aq*, aqueduct. Other abbreviations as in Figures 3-1 and 3-15.

5.4

4.6

4.0

3.6

3.2

2.9

2.2   SC   PAG

1.7   SC   PAG

1.2   IC   PAG   scp

0.7   IC   PAG   Aq   CnF   scp

0.2   IC   4V   CnF   LPB   scp   KF   MPB

PAG1   PAG2   PAG3   PAG4
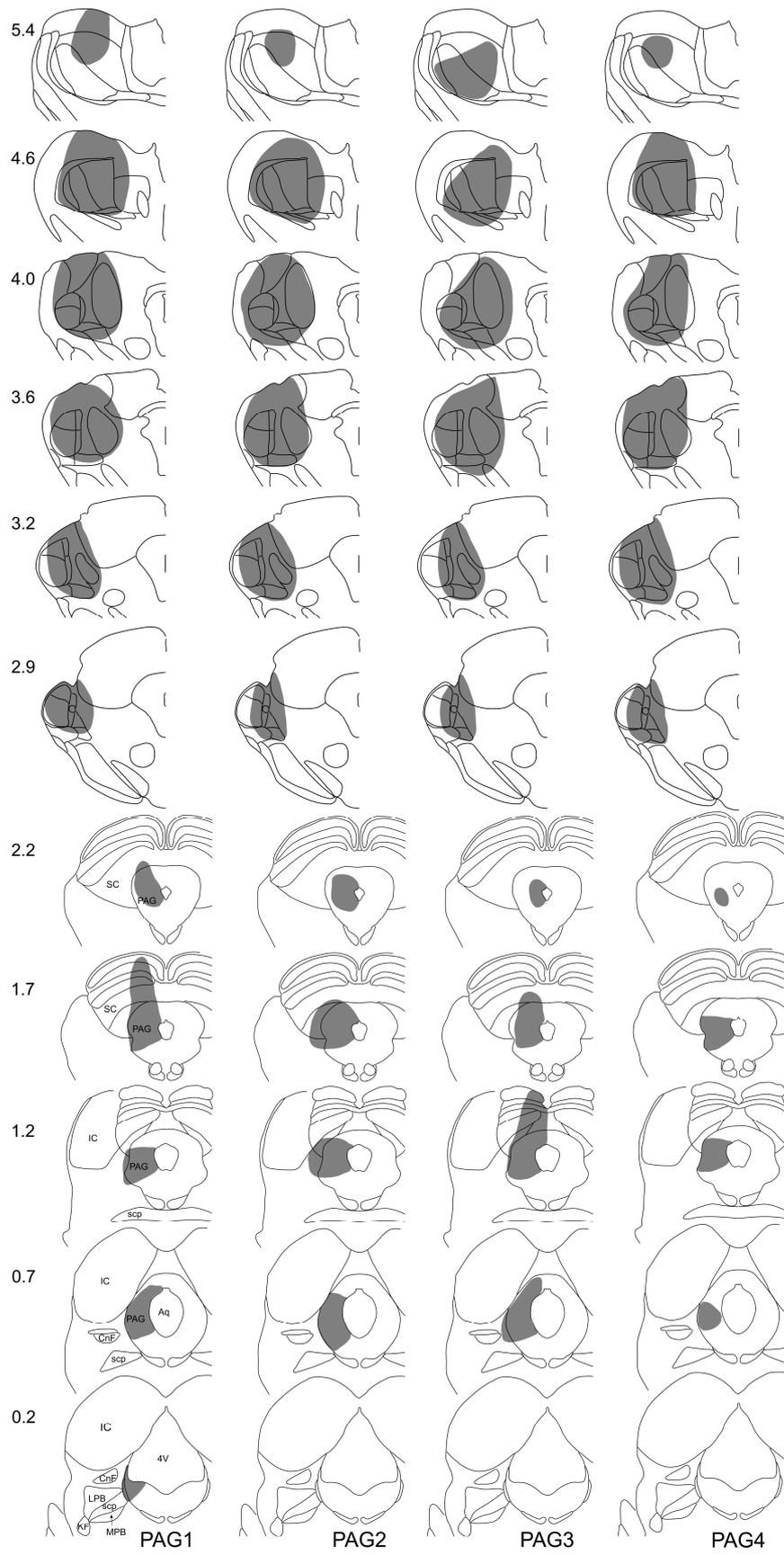
Figure 3-18. Examples of Fluorogold and CTb injection sites in PAG experiments.

a, **b:** fluorescence and brightfield micrographs of a section (interaural ~4.0 mm) through the thalamic injection site in PAG 3. **c**: section (interaural ~0.9 mm) through the CTb injection in PAG 4. The section was reacted with an immunoperoxidase method to reveal CTb. Scale bar = 1 mm (applies to all).
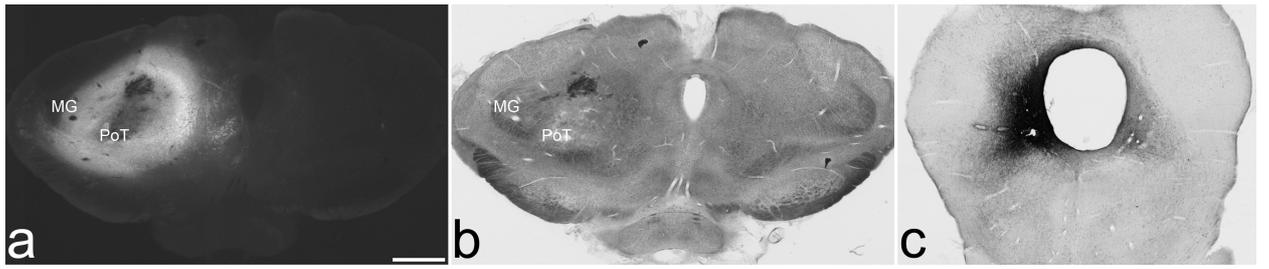
al., 1998; Todd et al., 2000; Spike et al., 2003) neurons in the rat. In the C7 segment, spinothalamic cells were frequently observed in lamina I and were scattered throughout the deeper laminae of the dorsal horn; some were also found in the LSN, and in the intermediate grey matter and ventral horn. Spinoparabrachial cells were observed mainly in lamina I and the LSN with some present in the reticular part of lamina V. Occasional cells were seen scattered elsewhere (laminae III, IV, VII, VIII and X). Spino-PAG cells were mainly observed in lamina I, LSN and the reticular part of lamina V; some were seen in lamina X, as well as in the medial parts of the intermediate grey matter and ventral horn. In the L4 segment, the spinothalamic neurons were mainly seen in the internal basilar column, and the deep laminae of the dorsal horn; only a few cells were present in lamina I. In addition, some spinothalamic cells were found in the LSN and lamina X. The distribution of spinoparabrachial cells was generally similar to that in the C7 segment; however, cells in lamina I were much more numerous. Spino-PAG cells were observed predominantly in lamina I and medial parts of the intermediate grey matter and ventral horn; some were also found in the LSN, reticular part of lamina V and lamina X.

Quantitative data for retrogradely labelled neurons in lamina I in all seven experiments were obtained from ten 60 µm thick Vibratome sections (alternate series) in the case of the C7 segment. For the L4 segment, 20 such sections were used to count spinothalamic and spino-PAG cells, but only 10 sections were used to count spinoparabrachial cells. These data are presented in Tables 3-8 and 3-9. When data from all seven experiments were pooled, the mean numbers of neurons retrogradely labelled from the thalamus were 24.3 per 600 µm for C7 (corresponding to 93 cells in the entire segment) and 9.3 per 1,200 µm for L4 (corresponding to 19 cells in the entire segment; data from Tables 3-8 and 3-9). In experiments Pb 1-3, the numbers of lamina I neurons labelled from the LPb were considerably higher in L4 (80.3 cells/600 µm, corresponding to 335 in the entire segment) than in C7 (46 cells/600 µm, corresponding to 176 cells in the entire segment). In the PAG series, the numbers of spino-PAG cells were similar in both segments (21.8/600 µm, corresponding to 83 cells in C7; 41.3/1,200 µm, corresponding to 86 cells in L4).

In experiments Pb 1-3, all of the Fluorogold-positive (spinothalamic) lamina I neurons in C7 were also labelled with CTb (i.e. they were spinoparabrachial), while the great majority (93%) of Fluorogold-labelled cells in L4 were also CTb-labelled (Table 3-8). Double-labelled cells made up 45% of those labelled from LPb in C7, but only 6% of those labelled from LPb in L4 (Table 3-8). In the PAG series, many of the lamina I spinothalamic cells (47%) in C7 were also labelled from the PAG, and these cells

Table 3-8. Quantitative data for lamina I neurons in experiments Pb 1-3

Exp	C7					L4				
	SPb cells (600 μm)	ST cells (600 μm)	DL cells (600 μm)	DL as % of SPb	DL as % of ST	SPb cells (600 μm)	ST cells (1,200 μm)	DL cells (1,200 μm)	DL as % of SPb	DL as % of ST
Pb 1	46	26	26	56.5	100	78	13	13	5.1	100
Pb 2	42	18	18	42.9	100	75	8	7	8	87.5
Pb 3	50	17	17	34	100	88	10	9	3.4	90
Mean	**46**	**20.3**	**20.3**	**44.5**	**100**	**80.3**	**10.3**	**9.7**	**5.5**	**92.5**

This table shows numbers of retrogradely labelled cells in the C7 and L4 segments from experiments in which Fluorogold was injected into thalamus and CTb into LPb. Cells labelled with Fluorogold were classified as spinothalamic (ST), those labelled with CTb as spinoparabrachial (SPb). Cells that contained both tracers are shown as double-labelled (DL). In each experiment cells were counted in 10 (SPb, ST, DL in C7, SPb in L4) or 20 (ST, DL in L4) alternate 60 μm transverse sections. Note that in the L4 segment the percentage of DL cells among the SPb population was determined for 10 sections, while the percentage of DL within the ST cells was determined for 20 sections.

Table 3-9. Quantitative data for lamina I neurons in experiments PAG 1-4

Exp	C7					L4				
	SPAG cells (600 μm)	ST cells (600 μm)	DL cells (600 μm)	DL as % of SPAG	DL as % of ST	SPAG cells (1,200 μm)	ST cells (1,200 μm)	DL cells (1,200 μm)	DL as % of SPAG	DL as % of ST
PAG 1	25	20	13	52	65	46	12	8	17.4	66.7
PAG 2	20	29	14	70	48.3	29	5	1	3.4	20
PAG 3	20	29	8	40	27.6	51	15	7	13.7	46.7
PAG 4	22	31	15	68.2	48.4	39	2	0	0	0
Mean	21.8	27.3	12.5	57.5	47.3	41.3	8.5	4	8.6	33.3

This table shows numbers of retrogradely labelled cells in the C7 and L4 segments from experiments in which Fluorogold was injected into thalamus and CTb into PAG. Cells labelled with Fluorogold were classified as spinothalamic (ST), those labelled with CTb as spino-PAG (SPAG). Cells that contained both tracers are shown as double-labelled (DL). In each experiment cells were counted in 10 (C7) or 20 (L4) alternate 60 μm transverse sections.

represented 58% of the spino-PAG population (Table 3-9). In the L4 segment, 33% of lamina I spinothalamic neurons were labelled from PAG, and these constituted 9% of spino-PAG cells (Table 3-9). Examples of single- and double-labelled lamina I neurons are illustrated in Fig. 3-19.

In experiments Pb 1-3, retrogradely labelled cells positive for one or both tracers were present throughout the mediolateral extent of lamina I, but were concentrated in its middle third (Fig. 3-20). The numbers of spinothalamic cells and of cells labelled only from LPb that were present in the medial, middle and lateral thirds of lamina I in these three experiments are shown in Table 3-10. For each of these regions, there was no difference in the proportion of cells labelled from thalamus or only from LPb in either segment (χ^2 test, P = 0.29 for C7, P = 0.18 for L4).

In the C7 segment, when data from these seven experiments were pooled, it was found that 147 out of the 170 spinothalamic cells (86%), 113 out of the 138 spinoparabrachial cells (82%) and 80 out of the 87 spino-PAG cells (92%) were NK1r-immunoreactive. This percentage of NK1r-immunoreactive cells was also found to be the same for neurons in L4; in this segment, 57 out of the 65 spinothalamic cells (88%), 199 out of the 241 spinoparabrachial cells (83%) and 150 out of the 165 spino-PAG cells (91%) expressed the NK1 receptor. In both segments, some of the labelled cells that lacked the NK1r (or only very weakly expressed the receptor) were large, and these probably belong to the class of "large gephyrin-coated cells" that were recently identified (section 3.2.4; Puskár et al., 2001; Polgár et al., 2008).

3.2.3 Morphology, soma size and NK1r expression of lamina I spinothalamic and spinoparabrachial neurons

This analysis was carried out on horizontal sections from the C8 and L3 segments of experiments Pb 1-3. In addition, for the morphological analysis, spinothalamic cells from the L5 segments of these experiments were added to the L3 sample. All data were obtained from the contralateral side of lamina I. Identification of lamina I neurons in horizontal sections requires knowledge about orientation of this lamina in the segments analyzed. For example, the slope in the dorsal surfaces of the C8 and L3 segments makes lamina I appear gradually in a number of adjacent horizontal sections. Furthermore, because the medial ~2/3-3/4 of the superficial dorsal horn is oriented at an angle from the horizontal plane, lamina I neurons appear gradually as sections go deeper. In addition, other criteria were

Figure 3-19. Retrograde labelling of lamina I neurons in transverse sections.

In all cases Fluorogold (FG, transported from thalamus) is shown in red, while CTb (transported from LPb or PAG) is green. Arrows indicate double-labelled cells, filled arrowheads indicate cells labelled only with CTb, and the open arrowhead shows a cell labelled only with Fluorogold. **a**-**c**: part of a section from C7 of experiment Pb 1 contains several spinoparabrachial neurons, two of which are also labelled from the thalamus. **d**-**f**: a section from L4 of the same experiment shows spinoparabrachial neurons, one of which is labelled from thalamus. **g**-**i**: this field from C7 of experiment PAG 3 contains single-labelled spinothalamic and spino-PAG neurons, as well as a cell labelled from both sites. **j**-**l**: a section through L4 of the same experiment contains two spino-PAG cells, one of which is labelled from the thalamus. All images are obtained from 10 optical sections at 2 µm z-spacing. Scale bar = 20 µm (applies to all).

FG

CTb
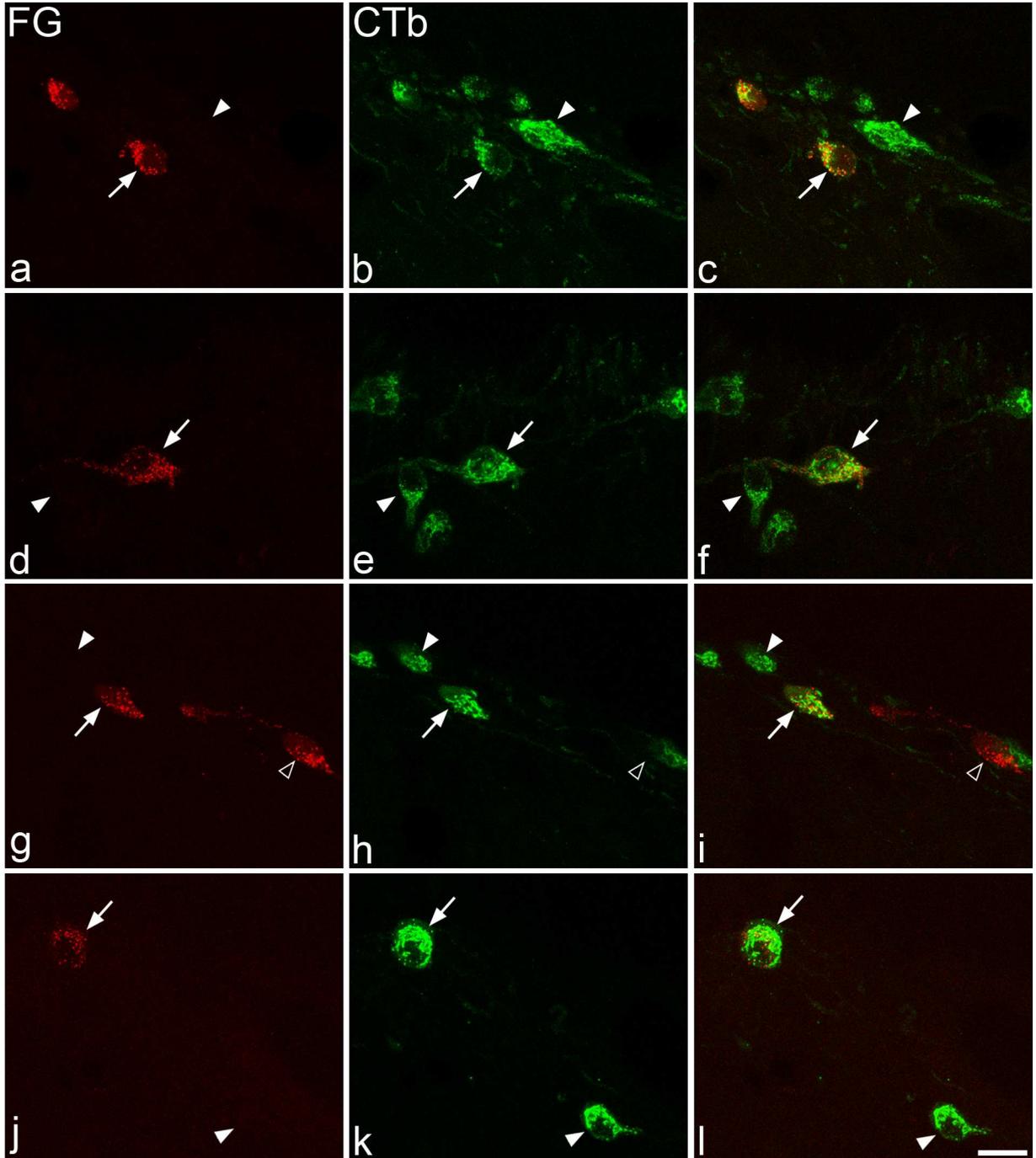
Figure 3-20. Plots of the locations of spinothalamic and spinoparabrachial neurons at cervical and lumbar levels.

These drawings show the locations of all retrogradely labelled lamina I neurons in ten alternate 60 µm sections through the C7 segment of experiment Pb 2 and the L4 segment of Pb 3. Open circles indicate cells labelled only from the LPb, filled circles are double-labelled cells and the filled square is a cell that was labelled only from thalamus. The lower line indicates the approximate border between laminae I and II. There are more spinoparabrachial cells and many fewer spinothalamic cells in L4, compared with C7. Note that these drawings show 10 sections through the L4 segment (to allow direct comparison with C7), although the quantitative analysis of spinothalamic cells in L4 in these experiments presented in Table 3-10 was performed on 20 sections.
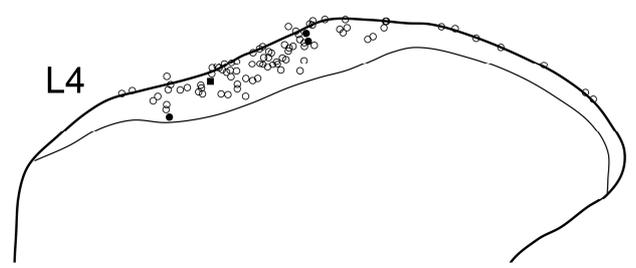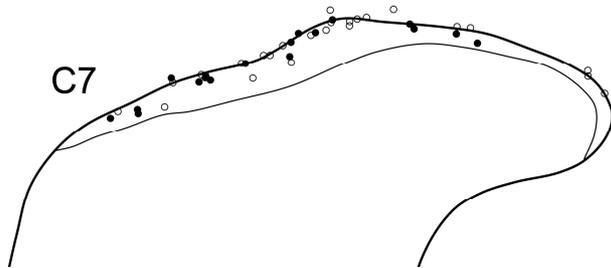
Table 3-10. Mediolateral distribution of spinothalamic and spinoparabrachial lamina I neurons

	C7			L4		
Projection population	medial	middle	lateral	medial	middle	lateral
Spinothalamic	**5 (0-5)**	**34 (9-16)**	**22 (4-10)**	**7 (1-3)**	**16 (2-7)**	**8 (2-3)**
Spinopara-brachial only	**10 (3-4)**	**48 (11-22)**	**19 (6-7)**	**45 (13-17)**	**151 (40-63)**	**32 (7-16)**

This table shows total numbers of contralateral spinothalamic neurons, and neurons labelled only from LPb (spinoparabrachial only) in medial, middle or lateral thirds of lamina I in Pb 1-3. Ranges between brackets represent the number of cells found in each experiment. Spinothalamic neurons in L4 were counted from 20 sections, while all other groups were counted in 10 sections. See text for further details.

used to identify cells in lamina I: these cells lie close to the dorsal surface of the dorsal horn within a band that contains numerous NK1r-immunoreactive profiles, as lamina II contains very few NK1r-immunoreactive cells. The most lateral part of lamina I curves around the lateral margin of the dorsal horn, as a result, in horizontal sections, neurons in this part will be cut at a perpendicular angle rather than tangentional; therefore, neurons in this region were not included in this part of the analysis.

The CTb filled cell bodies of labelled neurons and extended for a variable distance into the dendritic tree. Labelling with Fluorogold was granular and seldom filled the somata of labelled neurons completely. Therefore, allocation of lamina I neurons into a specific morphological class was mainly based on labelling with CTb, as well as on the immuno-reactivity for the NK1 receptor, when possible.

Altogether, 347 projection neurons were examined in the C8 segments (between 97 and 137 per experiment) and 239 neurons (53-98 per experiment) in the L3/L5 segments. Of these, 142 neurons (41-52 per experiment) in C8 and 55 neurons (16-20 per experiment) in L3/L5 were retrogradely labelled from the thalamus. All except one of the spinothalamic neurons in the lumbar segments and 140/142 of those in C8 were also labelled from LPb.

Lamina I cells were classified according to certain morphological criteria that depend on the soma shape and the number of major primary dendritic trunks (Zhang et al., 1996; Zhang and Craig, 1997). Most of lamina I cells analysed in this part of the study showed features characteristic of one of the three main morphological classes, i.e. multipolar, pyramidal or fusiform. However, some cells showed features transitional between two classes, and therefore, these were defined as "unclassified". Very occasionally, cells had atypical features and could not be classified into any of the three types; these were also defined as "unclassified". Cells were classified as multipolar if they possessed polygonal somata and more than three primary dendritic trunks. However, cells with varieties of shapes were seen, and the number of the primary dendrites ranged from four to seven. Two of the multipolar cells observed in the C8 segment had only three dendrites but these cells could not be classified as pyramidal because the somata were not triangular in shape. As observed in the cat (Zhang et al., 1996), and monkey (Zhang and Craig, 1997), several subtypes of the multipolar class were seen: (a) quadrilateral soma with four primary dendrites, (b) stellate soma with five or more radiating primary dendrites, (c) elongated cell body (tubular) with multiple dendrites and (d) T-like soma with a major dendritic trunk that issued at right angle to the cell body. Examples of these subtypes are illustrated in the

drawings in Fig. 3-21a-d. Cells were considered as pyramidal if they possessed a triangular soma and three major primary dendrites. Occasionally, cells with triangular somata and four primary dendrites, two of which arose from one angle, were seen. In these cases, the original z-stack was checked and if these two dendrites emerged at the same level then the cell was considered as pyramidal but if they emerged at different levels then the cell was classified as multipolar. Sometimes one or two slender dendrites arose from one side of the soma of a pyramidal cell. The total number of primary dendrites for the pyramidal cells observed in this study ranged from three to five. Examples of pyramidal cells are illustrated in the drawings in Fig. 3-21e-f. Cells were classified as fusiform if they had bipolar elongated somata with two tapering ends from which primary dendrites arose. Some atypical fusiform cells were also seen, in which the cell body was more globular, or which had extra slender dendrites that emerged from the ends or the side of the cell body. A variety of fusiform cells that had a recurrent dendrite or a T-like protrusion from the soma was also observed. The number of primary dendrites for the fusiform cells observed in the present study ranged from two to four. Examples of fusiform cells are illustrated in the drawings in Fig. 3-21g-h.

Although neurons belonging to each morphological type were seen within each projection population in both segments (Table 3-11), the distribution of the three morphological types differed significantly between spinothalamic neurons and neurons labelled only from LPb (χ^2 test, $P < 0.05$ for C8, $P < 0.001$ for L3/L5). Spinothalamic neurons were more often multipolar and less often fusiform than those labelled only from the LPb. This was particularly evident for the lumbar segments, where 32 out of 55 (58%) of the spinothalamic neurons were classified as multipolar. Examples of lamina I neurons belonging to different morphological classes are illustrated in Fig. 3-22.

The cross-sectional areas of cell bodies of spinothalamic neurons and those labelled only from LPb in the two segments are presented in histograms in Fig. 3-23. The soma areas of spinothalamic neurons ranged from 142-1,132 μm^2 (median 434, n = 142) for those in C8 and 173-1,350 μm^2 (median 380, n = 55) for those in L3/L5, compared with 159-1,016 μm^2 (median 354, n = 205) and 149-1,230 μm^2 (median 313, n = 184) for neurons labelled only from LPb in C8 and L3, respectively. These differences were highly significant (Mann-Whitney Rank Sum test, $P < 0.001$ for both segments). Since the large gephyrin-coated lamina I neurons (which are either non-immunoreactive or very weakly-immunoreactive for the NK1r) make up ~21% of the spinothalamic population in the L5 segment (see

Figure 3-21. Drawings of lamina I neurons in horizontal sections.

These drawings show representative examples of the three morphological types of lamina I neurons observed in this study. Cells were drawn with Neurolucida for Confocal software based on their CTb and/or NK1r-immunoreactivity. **a-d** show different subtypes of multipolar cells: **a**, stellate, **b**, quadrilateral, **c**, T-like, and **d**, tubular. **e** and **f** illustrate two forms of pyramidal cells. **g** and **h** show two forms of fusiform cells, **h** had two dendrites that emerged from each pole, one of these is a recurrent dendrite. Scale bar = 100 µm.
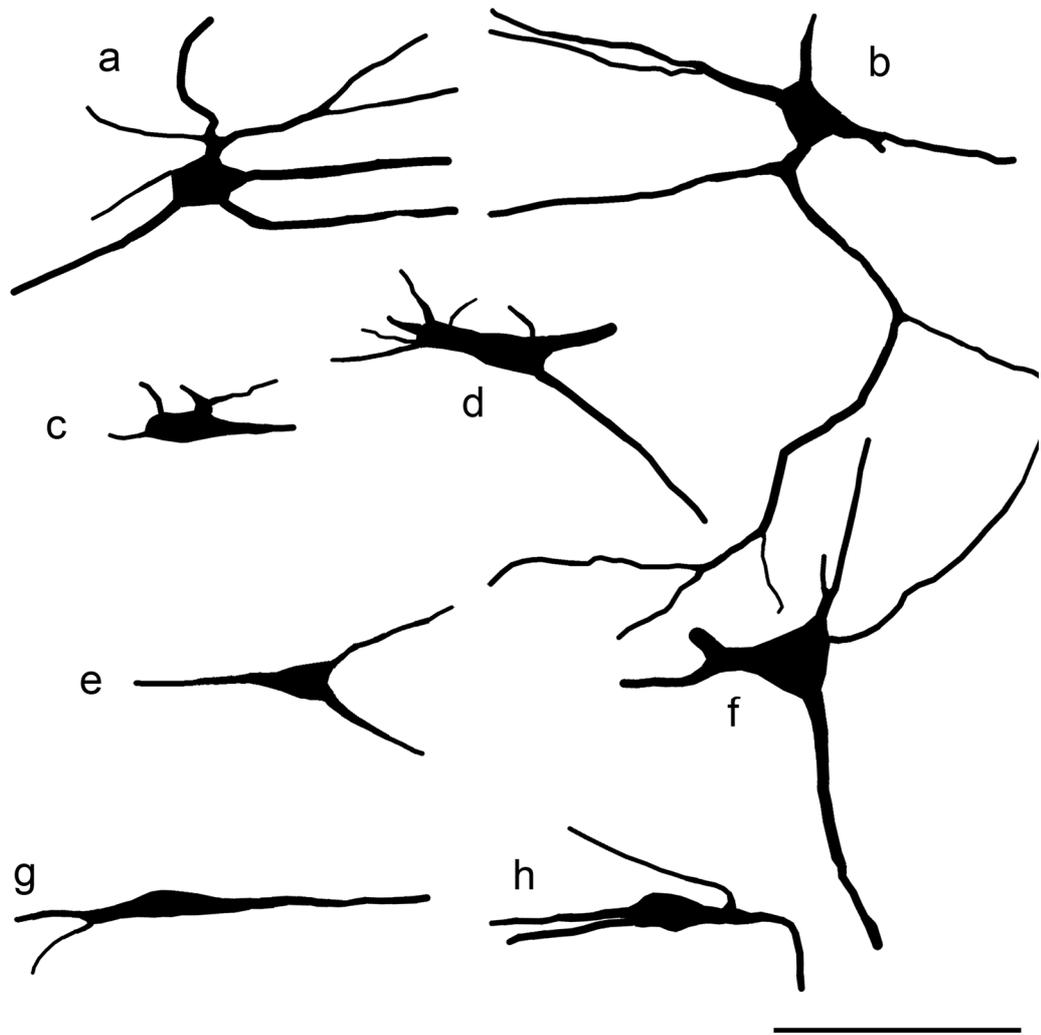
Table 3-11. Morphology of spinothalamic and spinoparabrachial neurons

Morphology	C8			L3/L5*		
	ST	SPb only	SPb total	ST	SPb only	SPb total
Multipolar	53 (37)	54 (26)	106 (31)	32 (58)	49 (27)	64 (31)
Pyramidal	35 (25)	42 (20)	77 (22)	12 (22)	50 (27)	55 (27)
Fusiform	35 (25)	74 (36)	108 (31)	9 (16)	70 (38)	71 (35)
Unclassified	19 (13)	35 (17)	54 (16)	2 (4)	15 (8)	15 (7)
Total	**142**	**205**	**345**	**55**	**184**	**205**

This table shows numbers (and percentages) of contralateral lamina I neurons belonging to the spinothalamic (ST) or spinoparabrachial (SPb) tracts that were assigned to each morphological class. Cells labelled only from LPb are shown as SPb only, while all of those labelled from LPb are shown as SPb total. Data were pooled from experiments Pb 1-3. All of the spinothalamic neurons in L3, and all but two of those in C8, were also labelled from LPb, and are therefore classified as spinoparabrachial.

*Note that the morphology of spinothalamic neurons in the L5 segment was also analysed, and these are included in the ST column. The SPb only and SPb total columns show data from L3 only.

Figure 3-22. Lamina I spinoparabrachial and spinothalamic neurons in horizontal sections.

a,c,e: part of a section through the C8 segment of experiment Pb 1. **a**: CTb (transported from LPb) is shown in green, **c**: Fluorogold (FG, transported from thalamus) is red. **e**: shows a merged image. Several spinoparabrachial neurons (three of which are indicated with arrows) are visible, and some of these are also retrogradely labelled from the thalamus. These cells have various shapes: those labelled P, F and M are of pyramidal, fusiform and multipolar types, respectively. **b,d,f**: part of a section through L3 from Pb 1 (colours as in a,c,e). Several spinoparabrachial neurons are visible and two of these (arrows) are labelled from the thalamus. Both of these cells are multipolar (M). **g-u**: higher magnification views through the five neurons marked with arrows in a-f, scanned to reveal Fluorogold (g-k), CTb (l-p) and NK1r (q-u). **g,l,q**: the pyramidal cell shows moderate (+++) NK1r-immunoreactivity. **h,m,r**: the fusiform cell is very weakly immunoreactive (+) for NK1r. **i,n,s**: the multipolar cell was also classified as moderately (+++) NK1r-immunoreactive. **j,o,t**: the upper marked multipolar cell seen in f shows weak (++) NK1r-immunostaining. **k,p,u**: the lower multipolar cell in f was classified as non-immunoreactive (-) for NK1r. a,c,e and b,d,f are projections of 20 and 26 optical sections at 2 µm z-spacing, respectively. g-u are projections of 2 optical sections at 1 µm z-spacing. Scale bars a-f = 50 µm, g-u = 10 µm.

CTb   CTb

FG   FG

e P F   M   f M

M

FG
g   h   i   j   k

CTb
l   m   n   o   p

NK1r
q   r   s   t   u
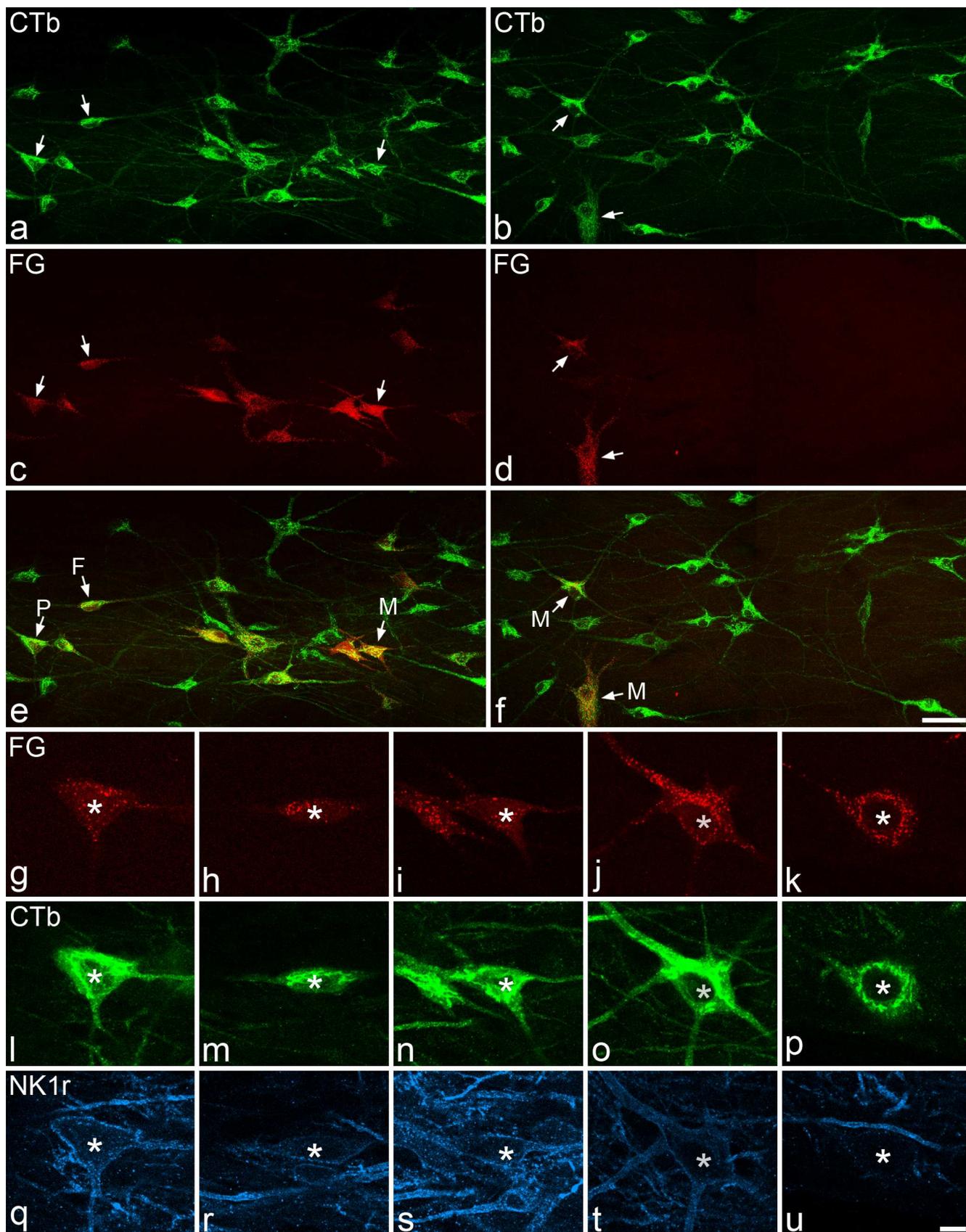
Figure 3-23. Soma areas in spinothalamic and other spinoparabrachial lamina I neurons.

a: A histogram showing the distribution of cross-sectional areas of lamina I neurons labelled from the thalamus (grey bars) and of neurons that were labelled from LPb but not thalamus (black bars) in the C8 segment. **b**: Histogram showing the equivalent data for the lumbar enlargement. In this case, the sizes of spinothalamic neurons in both L3 and L5 segments are included (grey bars), while the sizes of neurons labelled from LPb but not thalamus (black bars) are from the L3 segment only.

C8

L3

a

b
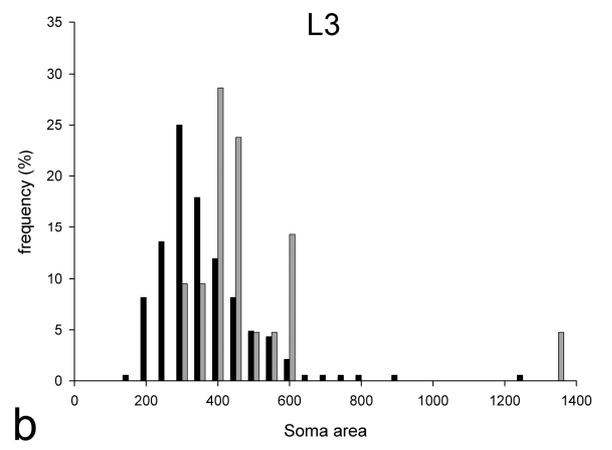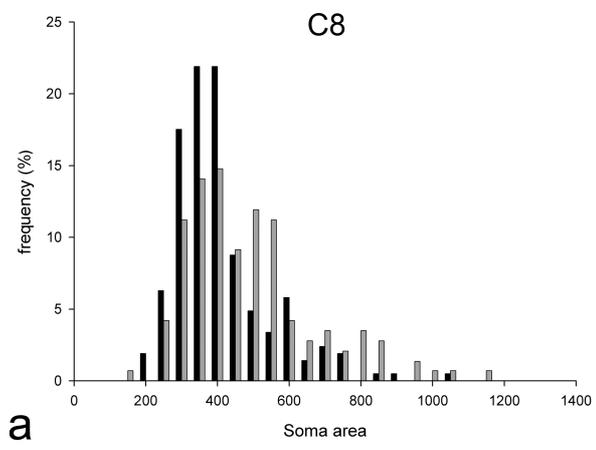
section 3.2.4), the soma size of cells that were assigned a strength of 2-4 for NK1r-immunoreactivity in the C8 and L3 segments was also analysed. For these cells, areas of spinothalamic neurons were 253-1,132 μm^2 (median 477, n = 108) and 274-577 μm^2 (median 398, n = 14) for C8 and L3, respectively. Corresponding values for spinoparabrachial neurons that were not labelled from thalamus were 241-1,016 μm^2 (median 374, n = 137) for C8 and 163-1,230 μm^2 (median 319, n = 115) for L3. The differences between the two projection populations were still highly significant (Mann-Whitney Rank Sum test, $P < 0.005$ for both segments).

Results of the analysis of NK1r expression are summarised in Table 3-12 and examples of immunostaining are shown in Fig. 3-22. NK1r-immunoreactivity was detected on 82% and 81% of spinothalamic neurons in C8 and L3, respectively, and on 79% and 72% of spinoparabrachial neurons in these segments. Neurons with NK1r scores of 0 (negative) to 4 (strong) were found within each projection population in both segments. However, in the C8 segment, the strength of NK1r expression was significantly higher among spinothalamic neurons than among neurons labelled only from LPb (Mann-Whitney Rank Sum test, $P < 0.05$). Within this segment, NK1r-immunoreactivity was scored 3 or 4 in 59% of spinothalamic neurons, but only in 43% of the other spinoparabrachial cells. However, there was no significant difference in NK1r strength among the two projection populations in L3 (Mann-Whitney Rank Sum test, $P = 0.9$). Since it has been reported that only 6% of the NK1r-immunoreactive spinothalamic cells in the rat were pyramidal (Yu et al., 2005), NK1r expression among pyramidal spinothalamic neurons was analysed, and it was found that 25% and 18% of all NK1r-immunoreactive spinothalamic cells in lamina I in the C8 and L3 segments, respectively, were pyramidal. Furthermore, 76% of pyramidal spinothalamic cells in C8 were NK1r-immunoreactive, and 64% of them were scored 3 or 4 for NK1r strength. This last analysis was not performed on the pyramidal cells in L3 because of the small sample size of the spinothalamic cells in this segment.

3.2.4 Large gephyrin-coated lamina I neurons

These cells were identified in horizontal sections through the L5 and C6 segments in the seven experiments. Cells of this type were characterized by the large size of their cell bodies and by the high density of gephyrin-immunoreactive puncta on their cell bodies and dendrites. As described previously (Puskár et al., 2001; Polgár et al., 2008), these cells were generally multipolar and were located throughout the mediolateral extent of lamina I.

Table 3-12. Strength of NK1r-immunoreactivity in spinothalamic and spinoparabrachial neurons

NK1r-ir strength	C8			L3		
	ST	SPb only	SPb total	ST	SPb only	SPb total
4	49 (35)	50 (24)	99 (29)	3 (14)	44 (24)	47 (23)
3	35 (25)	39 (19)	74 (21)	7 (33)	38 (21)	45 (22)
2	24 (17)	48 (23)	71 (21)	4 (19)	33 (18)	37 (18)
1	9 (6)	22 (11)	30 (9)	3 (14)	16 (9)	19 (9)
0	25 (18)	46 (22)	71 (21)	4 (19)	53 (29)	57 (28)
Total	142	205	345	21	184	205

This table shows numbers (and percentages) of contralateral lamina I neurons belonging to the spinothalamic (ST) or spinoparabrachial (SPb) tracts that were assigned to different groups based on strength of NK1r-immunoreactivity (NK1r-ir): 4 = strong, 3 = moderate, 2 = weak, 1 = very weak, 0 = negative. Data were pooled from experiments Pb 1-3. Note that all of the spinothalamic neurons in L3, and all but two of those in C8, were also labelled from LPb, and are therefore classified as spinoparabrachial.

Table 3-13 summarises data obtained from the contralateral side of the C6 and L5 segments in the Pb and PAG series. In the seven experiments, 56 large gephyrin-coated lamina I cells were identified on the right side of the L5 segment (6-12 per experiment; mean 8), and 22 of these (1-5 per experiment; mean 3.3; 39%) were retrogradely labelled with Fluorogold (i.e. were spinothalamic cells). The total numbers of lamina I spino-thalamic neurons that were identified on the right side of the L5 segment in these experiments varied from 8 to 22 (mean 15.4); therefore, the large gephyrin-coated cells constituted ~21% of the spinothalamic population in this segment. All of the large gephyrin-coated spinothalamic cells that were identified in experiments Pb 1-3 (12 cells) were also labelled with CTb (i.e. they were spinoparabrachial cells), whereas none of the 10 large gephyrin-coated spinothalamic cells that were identified in experiments PAG 1-4 were labelled with CTb. In experiments Pb 1-3, 24 large gephyrin-coated lamina I neurons were identified on the right side in L5 (7-9 per experiment; mean 8), and 21 of these (6-8 per experiment; 87.5%) were retrogradely labelled from the LPb. In PAG 1-4, 32 large gephyrin-coated lamina I neurons were identified on the right side in L5 (6-12 per experiment; mean 8), and only two of these (0-1 per experiment; 6%) were retrogradely labelled from the PAG.

In the C6 segment, 33 large gephyrin-coated lamina I cells were identified on the right side in sections from all seven experiments (2-6 per experiment; mean 4.7), and 27 of these (2-6 per experiment; mean 3.9; 82%) were labelled with Fluorogold (i.e. were spinothalamic neurons). All except one of the large gephyrin-coated spinothalamic cells in experiments Pb 1-3 were also labelled from the LPb, whereas only seven of the 15 such cells that were identified in experiments PAG 1-4 were also labelled from the PAG. In cases Pb 1-3, 12 of 16 cells (75%) were CTb-labelled (i.e. spinoparabrachial cells), while in the PAG experiments: 9 of 17 cells (53%) were CTb-labelled (i.e. spino-PAG cells).

Examples of retrogradely labelled large gephyrin-coated lamina I neurons in the L5 and C6 segments are shown in Figs. 3-24 and 3-25, respectively.

3.2.5 Projection pattern of the large NK1r-immunoreactive lamina III/IV neurons to the thalamus, lateral parabrachial area and PAG

This part of the analysis was performed on the contralateral side of serially mounted transverse sections through the C7 and L4 segments in each experiment and results are

Table 3-13. Quantitative data for large gephyrin-coated lamina I cells in Pb and PAG experiments

	C6				L5			
Exp	**number**	**FG only**	**CTb only**	**DL**	**number**	**FG only**	**CTb only**	**DL**
Pb 1	6	0	0	5	9	0	3	5
Pb 2	5	1	0	3	8	0	2	5
Pb 3	5	0	1	3	7	0	4	2
PAG 1	6	2	1	3	6	1	0	0
PAG 2	3	2	1	0	12	3	1	0
PAG 3	2	0	0	2	6	3	0	0
PAG 4	6	4	0	2	8	3	1	0

This table shows numbers of large gephyrin-coated lamina I cells observed on the contralateral side in the C6 and L5 segments. Cells that were labelled with Fluorogold only (FG only, from thalamus), CTb only (from LPb or PAG) or double-labelled (DL) are shown.

Figure 3-24. Large gephyrin-coated lamina I cells in L5 retrogradely labelled from thalamus, and LPb.

Retrograde labelling of large gephyrin-coated lamina I cells. **a**-**d** show part of a horizontal section through the contralateral side of the L5 segment from a rat that had received injections of CTb into the lateral parabrachial area and of Fluorogold centred on the PoT nucleus of the thalamus. **a**-**d** have been scanned to reveal gephyrin (green), CTb (blue) and Fluorogold (FG, red). This field contains three of the large gephyrin-coated cells (arrows). All of these are retrogradely labelled with CTb, as are numerous other smaller neurons in lamina I. Although only a small number of cells are labelled with Fluorogold, these include two of the gephyrin-coated cells (the ones on the left and right of the field). **a**-**d** are projections of 15 optical sections at 1 μm z-spacing. Scale bar = 50 μm.

gephyrin

CTb

FG

a

b

c

d
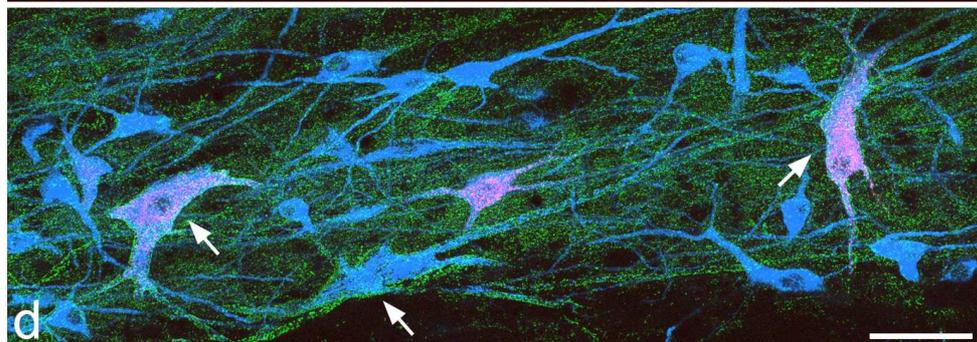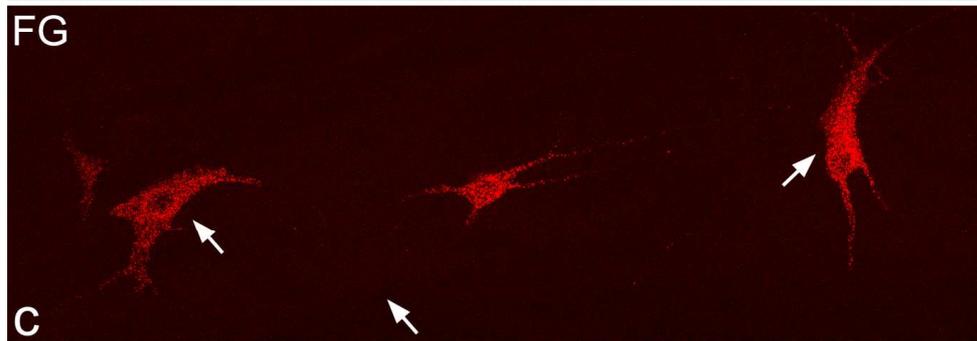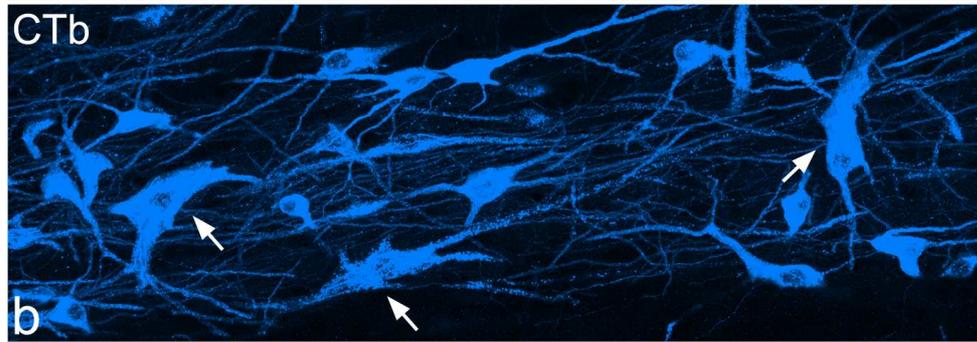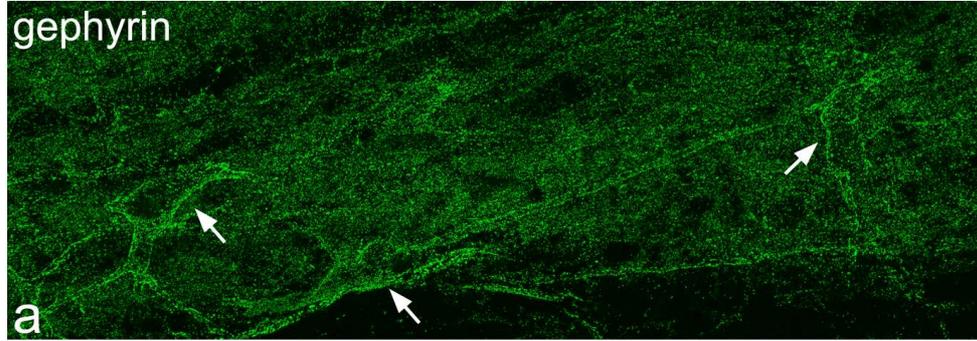
Figure 3-25. Large gephyrin-coated lamina I cells in C6 retrogradely labelled from thalamus, LPb and PAG.

Images from confocal scans through horizontal sections of C6 that show Fluorogold (FG, transported from thalamus, red), CTb (transported from LPb or PAG, blue) and gephyrin (green). **a-d**: This field from experiment Pb 2 shows a large gephyrin-coated cell (centre) that is retrogradely labelled from both thalamus and LPb. Several other CTb-labelled (spinoparabrachial) cells are also visible, and some of these also contain Fluorogold. **e-h**: Part of a section from experiment PAG 4 shows a large gephyrin-coated cell (centre) that is retrogradely labelled from thalamus and PAG. Other cells that are labelled from one or both of these regions are also visible. Images are projected from 24 (a-d) and 12 (e-h) optical sections at 2 µm z-spacing. Scale bar = 50 µm.

FG

CTb

gephyrin

FG

CTb

gephyrin

a

b

c

d

e

f

g

h
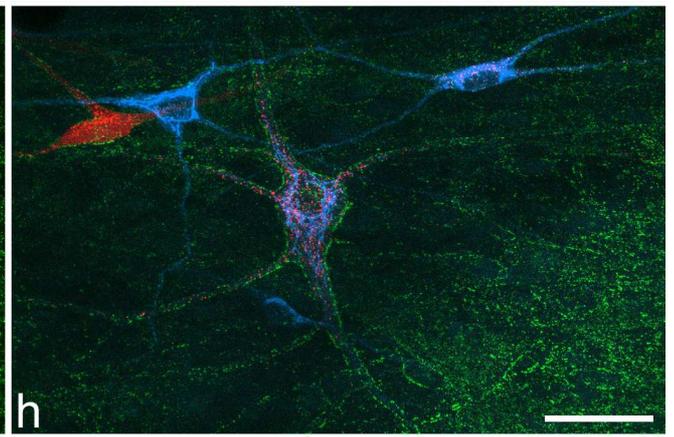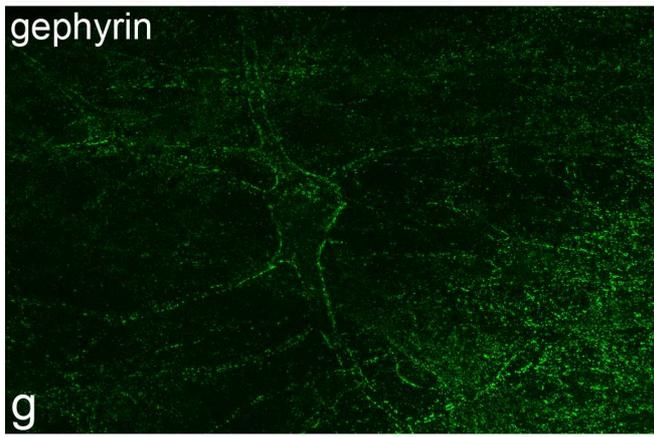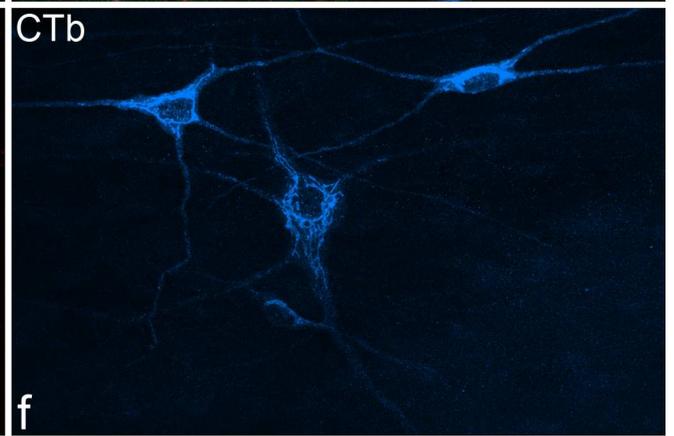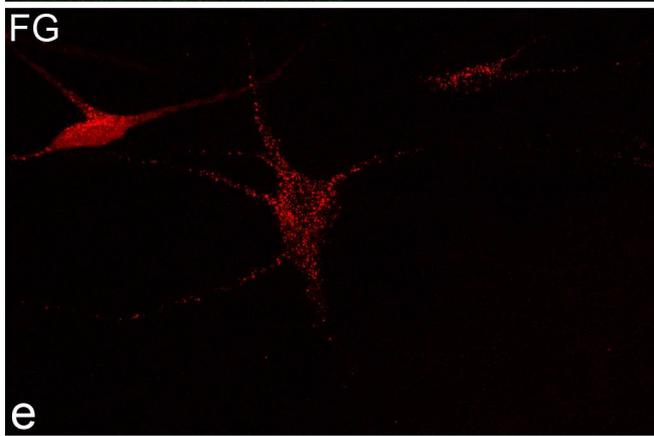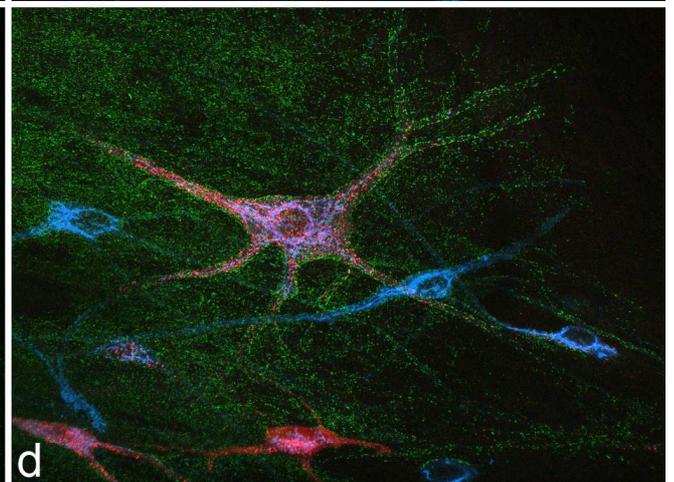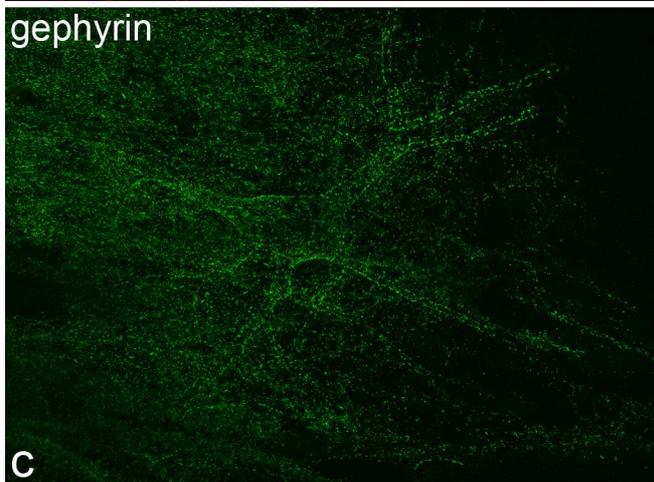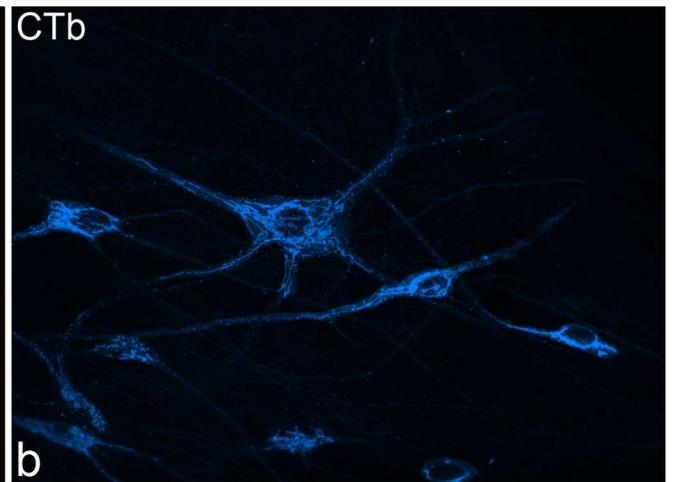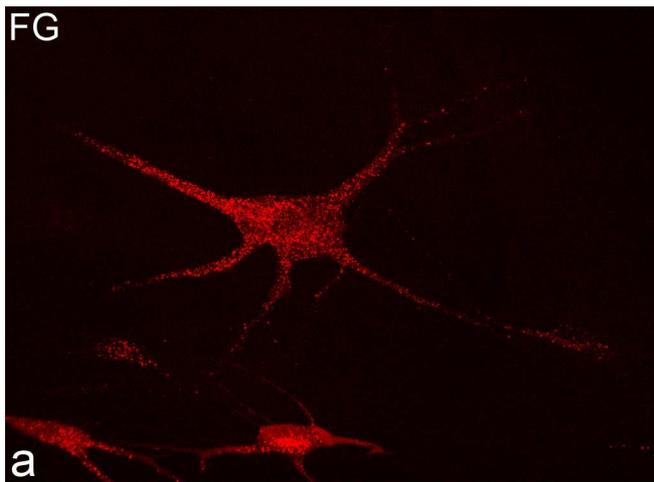
shown in Tables 3-14 and 3-15. Although it has been reported that the parasagittal plane is optimal for the analysis of the large NK1r-immunoreactive lamina III/IV neurons (Naim et al., 1997), neurons of this type could be readily identified in the transverse plane. As reported previously (Bleazard et al., 1994; Liu et al., 1994; Nakaya et al., 1994; Brown et al., 1995; Littlewood et al., 1995; Mantyh et al., 1997; Naim et al., 1997, 1998; Todd et al., 2000), neurons in this population are characterized by the large NK1r-immunoreactive somata and the dorsally directed dendrites that arborize in the superficial dorsal horn (laminae I-II). In addition, mediolateral and ventral dendrites were also observed. Neurons in this population were distributed along the rostrocaudal and mediolateral extent of laminae III and IV in both segments; however, they tended to be rare at the extreme medial and lateral parts. Because of variations in the lengths of the blocks that were used from each experiment, the number of the 60 µm thick Vibratome sections included in each series varied from 30-50 (corresponding to lengths of 1.8-3.0 mm). To allow a direct comparison between the numbers of cells of this type in the C7 and L4 segments, a correction was made by multiplying the observed total number of cells (Tables 3-14 and 3-15) by the expected length of the segment (2.5 mm for L4, 2.3 mm for C7) and dividing this by the actual length of the series (number of sections × 60 µm), as presented in Table 3-16 . This gave mean values of 16 cells for C7 (range 12-19, n = 7) and 23 cells for L4 (range 18-28, n = 7), and these were significantly different (t-test, P < 0.005). The cell bodies were located between 70 and 286 µm (mean 164 µm ± 47, S.D., n = 101 cells) below the dorsal white matter in C7, and between 100 and 367 µm (mean 239 µm ± 60, n = 170 cells) below the dorsal white matter in L4. The mean depth of these cells in L4 was significantly different from that in C7 (t-test, P < 0.001).

In experiments Pb 1-3, virtually all of the lamina III/IV NK1r-immunoreactive neurons (98%) in the C7 segment were retrogradely labelled with either or both tracers. Data showed that 87% of these cells were retrogradely labelled from LPb, 75% were labelled from thalamus, and 64% were labelled from both targets. In the L4 segment, most of these neurons were also retrogradely labelled; with the proportion of retrogradely labelled cells from one or both targets being 79%. Approximately 64% of the cells were labelled from the LPb. However, unlike the C7 segment, the projection to the thalamus was considerably smaller since only 29% of these cells were labelled with Fluorogold. Nearly half of those cells that were retrogradely labelled from the thalamus were also labelled from the parabrachial area, and this double labelling occurred in 14% of the entire population (Table 3-14).

Table 3-14. Quantitative data for lamina III/IV NK1r cells from experiments Pb 1-3

Exp	C7							L4						
	number	FG-only	CTb-only	DL	% SPb	% ST	% DL	number	FG-only	CTb-only	DL	% SPb	% ST	% DL
Pb 1	12	0	1	11	100	91.7	91.7	26	4	10	3	50	26.9	11.5
Pb 2	14	2	5	6	78.6	57.1	42.9	26	5	14	4	69.2	34.6	15.4
Pb 3	12	2	3	7	83.3	75	58.3	19	2	11	3	73.7	26.3	15.8
Mean	**12.7**	**1.3**	**3**	**8**	**87.3**	**74.6**	**64.3**	**23.7**	**3.7**	**11.7**	**3.3**	**63.8**	**29.3**	**14.2**

This table shows numbers of large lamina III/IV NK1r-immunoreactive neurons on the contralateral side of the C7 and L4 segments in these experiments. Cells were classified according to whether they contained only Fluorogold (FG-only), only CTb (CTb-only) or were double-labelled (DL). The percentages of cells belonging to spinoparabrachial (SPb) or spinothalamic (ST) tracts, and the percentage that were double-labelled are also provided.

Table 3-15. Quantitative data for lamina III/IV NK1r cells from experiments PAG 1-4

Exp	C7							L4						
	number	FG-only	CTb-only	DL	% SPAG	% ST	% DL	number	FG-only	CTb-only	DL	% SPAG	% ST	% DL
PAG 1	16	14	1	0	6.3	87.5	0	26	3	1	1	7.7	15.4	3.8
PAG 2	14	9	0	2	14.3	78.6	14.3	27	6	1	2	11.1	29.6	7.4
PAG 3	20	17	0	1	5	90	5	26	12	1	1	7.7	50	3.8
PAG 4	13	13	0	0	0	100	0	20	3	1	0	5	15	0
Mean	**15.8**	**13.3**	**0.3**	**0.8**	**6.4**	**89**	**4.8**	**24.8**	**6**	**1**	**1**	**7.9**	**27.5**	**3.8**

This table shows numbers of large lamina III/IV NK1r-immunoreactive neurons on the contralateral side of the C7 and L4 segments in these experiments. Cells were classified according to whether they contained only Fluorogold (FG-only), only CTb (CTb-only) or were double-labelled (DL). The percentages of cells belonging to spino-PAG (SPAG) or spinothalamic (ST) tracts, and the percentage that were double-labelled are also provided.

Table 3-16. Counts of NK1r-immunoreactive lamina III/IV neurons in Pb and PAG series

Exp	segment	No.	AL	EL	CF	Observed No.	Corrected No.
Pb1	C7	30	1,800	2,300	1.277	12	15.3
Pb2	C7	34	2,040	2,300	1.127	14	15.8
Pb3	C7	38	2,280	2,300	1.008	12	12.1
PAG1	C7	32	1,920	2,300	1.197	16	19.2
PAG2	C7	35	2,100	2,300	1.095	14	15.3
PAG3	C7	40	2,400	2,300	0.958	20	19.2
PAG4	C7	34	2,040	2,300	1.127	13	14.7
Pb1	L4	39	2,340	2,500	1.068	26	27.7
Pb2	L4	41	2,460	2,500	1.016	26	26.4
Pb3	L4	40	2,400	2,500	1.044	19	19.8
PAG1	L4	44	2,640	2,500	0.945	26	24.6
PAG2	L4	50	3,000	2,500	0.833	27	22.5
PAG3	L4	50	3,000	2,500	0.833	26	21.7
PAG4	L4	47	2,820	2,500	0.886	20	17.7

This table shows the number of 60 µm thick sections (No.) that were analysed in the C7 and L4 segments in these seven experiments. The observed number of large NK1r-immunoreactive lamina III/IV neurons (before correction for length) and the corrected number are presented in the last two columns. AL: actual length of segment analysed in that experiment (µm); EL: expected length of segment (µm); CF: correction factor (EL/AL). See text for further details.

In the PAG series, most of cells of this type in the C7 segment (approximately 91%) were retrogradely labelled (Table 3-15); however, this labelling was mainly from the thalamus (89%) with only very small proportion retrogradely labelled from the PAG (6%). This small percentage of labelling from the PAG is represented by one cell each in PAG 1 and PAG 3, two cells in PAG 2 and none in PAG 4. Three out of these four PAG neurons were also retrogradely labelled from the thalamus. In the L4 segment, less than one third of the cells in this population (32%) were retrogradely labelled. The percentage of cells that were labelled from the thalamus was 28%, whereas only 8% of the cells were labelled from the PAG. There were two spino-PAG cells in PAG 1 and PAG 3, three in PAG 2 and one in PAG 4. Four of these eight spino-PAG cells were also retrogradely labelled from the thalamus.

When results from all seven experiments were combined, the proportion of cells of this type that were retrogradely labelled from thalamus was 83% for C7 and 28% for L4. Representative examples of double labelled NK1r-immunoreactive neurons in laminae III and IV in the C7 and L4 segments from the Pb series of experiments are shown in Fig. 3-26.

In the L4 segment, the proportion of lamina III/IV NK1r-immunoreactive cells in the medial half of the dorsal horn that were retrogradely labelled from the thalamus was 35/72 (49%) (data pooled from all seven experiments), while the corresponding proportion for those in the lateral half was 14/98 (14%). These differed significantly (χ^2 test, P < 0.001).

Plots of the locations of the lamina III/IV NK1r-immunoreactive neurons within the dorsal horn in the C7 and L4 segments in representative experiments from the LPb series are shown in Fig. 3-27. It can be seen that there is a widespread distribution of these neurons within laminae III and IV with a tendency for them to be absent from the extreme medial and lateral regions. The spinothalamic cells within this population in the L4 segment were found in the medial half of the dorsal horn, but these were seen in both halves in the case of Pb 2. In the C7 segment, spinothalamic cells were observed in various regions of laminae III and IV. No consistent pattern was seen for cells labelled from the LPb in both regions of the cord.

Fig. 3-28 shows plots of the locations of the NK1r-immunoreactive neurons in laminae III and IV in the C7 and L4 segments in representative experiments from the PAG series. In the L4 segment, the spinothalamic cells were observed mainly in the medial half of the

dorsal horn. Interestingly, the rare PAG cells (whether labelled from the thalamus or not) were also observed in the medial half. In the C7 segment, the spinothalamic neurons were widely distributed (as most of these cells were labelled from the thalamus), and the occasional PAG cells were observed in the medial part in PAG 1 and PAG 3 but in the middle of the laminae in PAG 2.

Figure 3-26. Retrograde labelling of lamina III/IV NK1r-immunoreactive neurons in LPb experiments.

These images are from confocal scans that show immunoreactivity for NK1r (green), CTb (transported from LPb, blue) and Fluorogold (FG, transported from thalamus, red) in transverse sections through C7 (**a-d**) and L4 (**e-h**) from experiment Pb 3. In each case, a single large NK1r-immunoreactive cell with its soma in lamina III is labelled with both retrograde tracers (arrows). Images are projections of 20 (a-d) or 18 (e-h) confocal optical sections at 2 µm z-spacing. Scale bar = 50 µm.

NK1r   CTb   FG

a   b   c   d

e   f   g   h
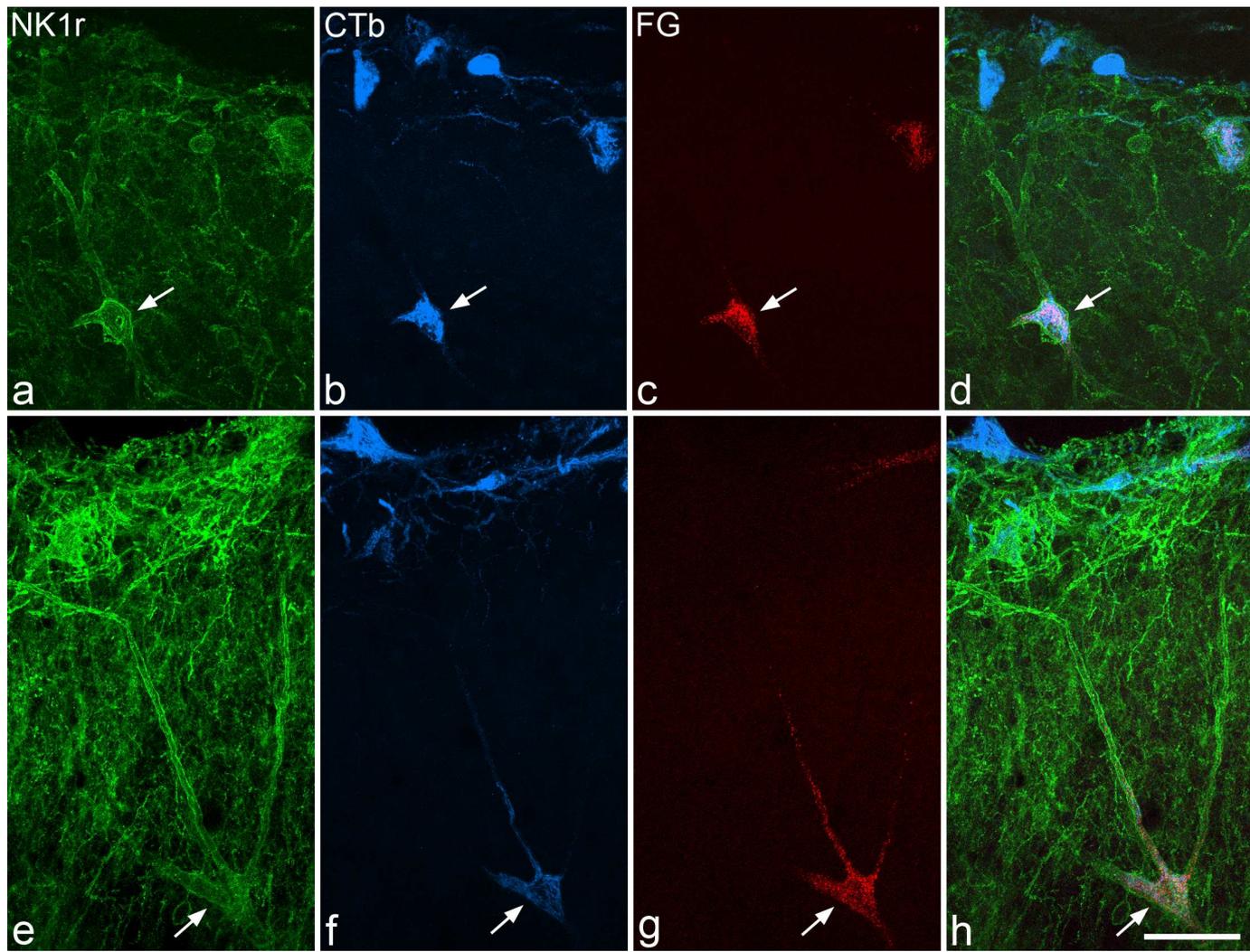
Figure 3-27. Plots of the locations of large NK1r-immunoreactive lamina III/IV neurons in transverse sections in Pb series.

These drawings show the location of all large NK1r-immunoreactive lamina III/IV neurons observed in representative examples from the Pb series. Each drawing is a composite of all cells seen in the entire segment in one experiment (from Pb 1 for the L4 segment and from Pb 2 for the C7 segment). Each symbol represents a single neuron: filled circles indicate cells only labelled from thalamus, filled squares indicate cells labelled from thalamus and LPb, open squares indicate cells only labelled from LPb and open circles indicate non-labelled cells. Note the medial location of cells labelled from thalamus in the case of the L4 segment.
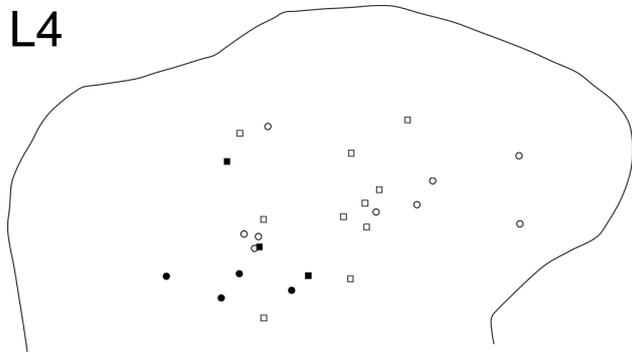
L4   C7
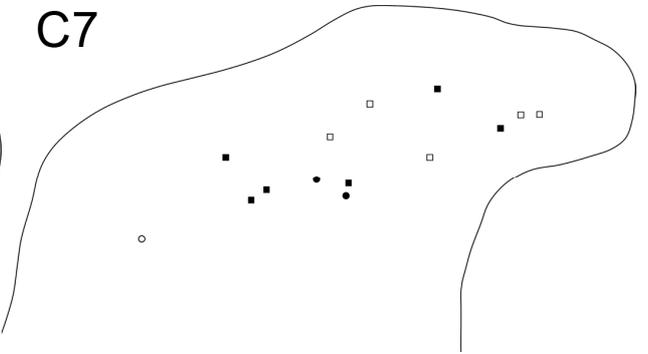
Figure 3-28. Plots of the locations of large NK1r-immunoreactive lamina III/IV neurons in transverse sections in PAG series.

These drawings show the location of all large NK1r-immunoreactive lamina III/IV neurons observed in representative examples from the PAG series. Each drawing is a composite of all cells seen in the entire segment in one experiment (from PAG 2 for the L4 segment and from PAG 1 for the C7 segment). Each symbol represents a single neuron: filled circles indicate cells only labelled from thalamus, filled squares indicate cells labelled from thalamus and PAG, open squares indicate cells only labelled from PAG and open circles indicate non-labelled cells. Note the widespread distribution of cells labelled from thalamus in C7 and the medial location of these cells in L4.
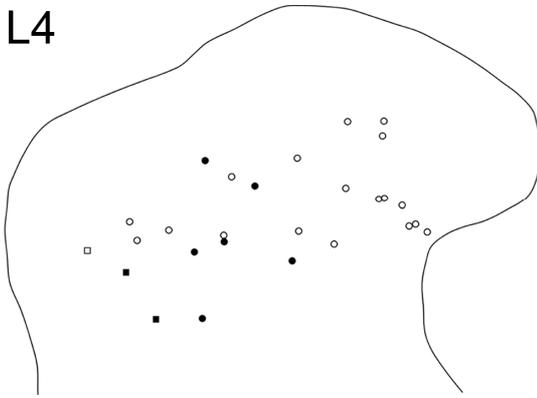
L4   C7
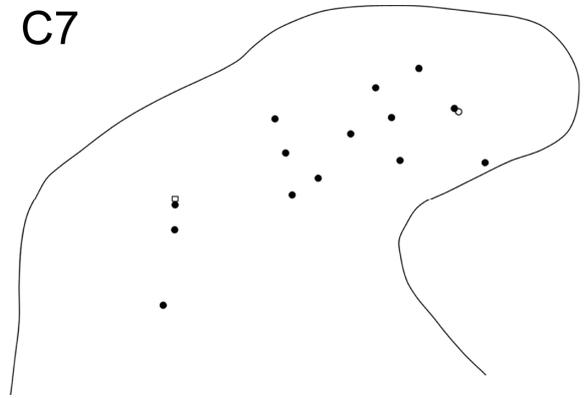
Chapter 4
Discussion

The main findings of this study can be summarised as follows: (1) injections of CTb or Fluorogold targeted on the PoT can label most (if not all) lamina I spinothalamic neurons in lumbar and cervical enlargements; (2) the estimated total numbers of spinothalamic cells in lamina I on the contralateral side of the C7 and L4 segments are 91 and 16 cells, respectively, and this constitutes 2-3% and 0.2% of the total neuronal population in lamina I in the C7 and L4 segments, respectively; (3) the number of lamina I neurons retrogradely labelled from the LPb is considerably lower in C7 than in L4, while numbers labelled from the PAG in these segments are similar; (4) >95% of spinothalamic lamina I neurons in both enlargements are labelled from the LPb, and between a third and a half are also labelled from the PAG; (5) lamina I spinothalamic neurons differ from other neurons in this lamina that are labelled from the LPb in morphology, soma size and, for the C8 segment, in strength of NK1r expression; (6) ~39% of "large gephyrin-coated cells" in L5 project to the thalamus and this accounts for ~21% of the total thalamic projection from lamina I in this segment, even though these cells constitute only ~2.5% of projection neurons in lamina I; (7) the great majority of "large gephyrin-coated cells" in C6 project to thalamus and LPb, and at both segmental levels, some project to both of these areas; (8) only few "large gephyrin-coated cells" in L5 and some of those in C6 project to PAG; (9) ~17-28% of the large lamina III/IV NK1r-immunoreactive neurons with long dorsal dendrites in the lumbar segments and ~84% of those in the middle of the cervical enlargement belong to the spinothalamic tract; (10) the large lamina III/IV NK1r-immunoreactive cells are not retrogradely labelled by injections of Fluorogold that occupied the ventrobasal complex, the rostral part of Po and the medial thalamus; and (11) many lamina III/IV NK1r-immunoreactive projection neurons in cervical enlargement, and some of those in lumbar enlargement are labelled from both thalamus and LPb.

4.1 Technical considerations

4.1.1 Selection of tracer substances

In the present study, the retrograde tract tracing technique was used to investigate projection neurons in laminae I, III and IV in rat spinal cord. It has been reported that this technique is the best choice to determine the location of cell bodies of origin of long neuronal pathways (Kristensson and Olsson, 1971; LaVail and LaVail, 1972; Vercelli et al., 2000). The main tracer substances used in the current study are Fluorogold and CTb. Both tracers were reported to be effective in labelling somata of neurons (Sawchenko and Gerfen, 1985; Schmued and Fallon, 1986; Ericson and Blomqvist, 1988). Lima and Coimbra (1988) found that CTb was superior to HRP and WGA-HRP in labelling

spinothalamic neurons in the rat, as it labelled a greater number of cells in the spinal cord compared to the latter two tracers, although the injection sites were similar in all cases. Findings from other studies agree with this observation (Marshall et al., 1996; Yu et al., 2005). Burstein et al. (1990a) used Fluorogold to retrogradely label spinothalamic neurons in rat and reported that this tracer was also very effective in labelling these cells.

It has been reported that Fluorogold and CTb have a similar efficiency in labelling spinal projection neurons in the rat (Spike et al., 2003). Spike et al. observed that injecting either Fluorogold or CTb into the CVLM or LPb led to retrograde labelling of a similar number of cells in lamina I from each target. Thus, it is unlikely that the use of different tracer substances in experiments PoT 1-10 contributed to variation in the observed numbers of retrogradely labelled neurons. However, an important difference between the two tracers is related to the pattern of spread within the injection site; Fluorogold has been found to spread widely, whereas CTb was reported to produce a more restricted spread. This feature of CTb is advantageous especially if a specific area in the brain has to be targeted. Therefore, in the single tracer-injection experiments (Thal and PoT series), Fluorogold was used to target the thalamus in experiments Thal 1-5, whereas CTb was used to target the PoT in some of the cases in the PoT series. However, Fluorogold was also used in some experiments in the PoT series. In the double tracer-injection experiments (DI, Pb and PAG series), the combination of Fluorogold and CTb was selected as this has proved to be effective in previous retrograde double-labelling studies (Spike et al., 2003). In these cases, CTb was used to target the more caudal structure (i.e. PoT in DI 1-7, LPb in Pb 1-3 and PAG in PAG 1-4). This is because it has been reported that if Fluorogold is used as one of the tracers in double-labelling experiments, then it should be injected into the more rostral site (Akintunde and Buxton, 1992; Bice and Beal, 1997b). This observation was attributed to the tendency of Fluorogold to cause necrosis within the tissue; then if Fluorogold was injected into the more caudal site, this would impede the transport of the other tracer (if the main bundle of that pathway is passing through or close to that necrotic region). It has been reported that CTb gives extensive filling of the retrogradely labelled neurons (Lima and Coimbra, 1988; Marshall et al., 1996; Zhang and Craig, 1997; Spike et al., 2003), which is an advantage for the morphological analysis of lamina I neurons in horizontal sections.

4.1.2 Selection of the examined segments

In the present investigation, analysis of data was carried out on segments selected from the middle regions of the cervical (C6-8) and lumbar (L3-5) enlargements. The cervical

enlargement in the rat extends from the C5 segment to the T1 segment, and the lumbar enlargement extends from the T13 segment to the L5 segment (Kemplay and Webster, 1986). The choice of these segments was made for a number of reasons. Firstly, most of the receptive fields of cutaneous afferents from the forelimb and hindlimb are located within the C6-8 and L3-5 segments, respectively (Swett and Woolf, 1985; Takahashi and Nakajima, 1996; King and Apps, 2000). Secondly, apart from the upper cervical cord, studies have shown that projection neurons in lamina I are mainly concentrated within the middle parts of the enlargements (Menétrey et al., 1982; Lima and Coimbra, 1988; Burstein et al., 1990a; Yezierski and Mendez, 1991; Kobayashi, 1998; Li et al., 1998). Thus, the larger number of labelled lamina I cells that would be expected in these segments is likely to provide more consistent results than would be obtained from segments that contain only a small number of these cells (e.g. thoracic segments). Thirdly, studies that have not analysed tissue from the entire spinal cord have restricted their analysis to segments from the spinal enlargements (e.g. Hylden et al., 1989; Marshall et al., 1996; Spike et al., 2003; Yu et al., 2005; Almarestani et al., 2007). Therefore, it is possible to compare present findings with most available studies in the literature.

The sectioning strategy adopted in the first part of present study was to cut the L4 and C7 segments in the transverse plane and the L5 and C6 segments in the parasagittal plane. The L4 segment was selected for that plane of cutting because previous quantitative studies on rat lumbar cord that were undertaken in this laboratory were carried out on this segment (Marshall et al., 1996; Spike et al., 2003), and the best plane to perform quantitative analysis on lamina I cells is the transverse plane, in which the entire mediolateral extent of the lamina can be easily seen. From the two cervical segments, C7 was selected for the quantitative analysis, and therefore cut in the transverse plane. The remaining two segments in this series (L5 and C6) were cut in the parasagittal plane, because the aim was to analyse the population of the large NK1r-immunoreactive neurons in laminae III and IV and it has been reported that these neurons are best seen in the parasagittal plane (Naim et al., 1997). Throughout the first part of the study, these segments were consistently cut in these planes, and the analysis of lamina I or lamina III/IV neurons was carried out on the transverse or parasagittal sections, respectively. However, in the FLM series, the quantitative analysis of lamina I cells was performed on parasagittal sections from the C6 and L5 segments. This is because the aim was to count the total number of retrogradely labelled cells in lamina I in the entire segment, and this requires mounting of all sections in serial order. This is more easily carried out for sections cut in the parasagittal plane, because this yields fewer sections than cutting in the transverse plane. Moreover, the shape

of the dorsal horn changes gradually from lateral to medial and this helps in the identification of the order of these sections. The same sagittal sections were also used to investigate the large NK1r-immunoreactive neurons in laminae III and IV in this series of experiments.

In the Pb and PAG series, the C7 and L4 segments were also selected for the quantitative analysis of lamina I (as in the first part of the study), and therefore cut transversely. However, these same sections were also used to analyse the large NK1r-immunoreactive neurons in laminae III and IV, because the C6 and L5 segments in this series had been cut in the horizontal, rather than the parasagittal, plane in order to study the large gephyrin-coated cells in lamina I, which are best studied in horizontal sections. The L3 and C8 segments were also cut in the horizontal plane because this plane is the best to perform morphological analysis of the projection populations in lamina I.

4.2 Projection of lamina I neurons to the PoT

4.2.1 Issues related to the Injection sites

A major aim of the present study was to investigate the projection from spinal lamina I neurons to the PoT in thalamus, since this has been shown to be a major target for axons from cervical superficial dorsal horn in the rat (Gauriau and Bernard, 2004a), and to compare this with the overall thalamic projection from this lamina. Thus, the decision was made to include in the injection sites of the Thal and DI series every thalamic nucleus reported to receive terminals from lamina I neurons in the rat (Lima and Coimbra, 1988; Burstein et al., 1990a; Li et al., 1996; Marshall et al., 1996; Kobayashi, 1998; Kayalioglu et al., 1999; Gauriau and Bernard, 2004a; Yu et al., 2005).

The PoT is a small region in the caudal thalamus, and it is difficult to target this region precisely with no spread into adjacent structures. Therefore, areas surrounding the PoT were included in the injection site in most cases. However, this should not create problems in the interpretation of the results because, in most cases, tracer spread did not include any regions that have been shown to receive significant input from the superficial dorsal horn, except as an extension of the terminal field centred on the PoT into PIL and APT that was described by Gauriau and Bernard (2004a).

One significant concern in the present study is the potential labelling of fibres of passage, since axons of the spinothalamic tract pass through the area of the PoT before entering

more rostral nuclei in the thalamus (LeDoux et al., 1987; Cliffer et al., 1991; Gauriau and Bernard, 2004a). In addition, Gauriau and Bernard (2004a) reported that axons of the spinohypothalamic tract ascend through the caudal thalamus before reaching their target. However, the presence of projection from lamina I neurons to the hypothalamus is a matter of controversy. A recent anterograde tracing study did not demonstrate a significant projection from the superficial dorsal horn in rat cervical cord to the hypothalamus (Gauriau and Bernard, 2004a). On the other hand, some retrograde tracing and physiological studies have identified such a projection in the rat (Burstein et al., 1990b; Dado et al., 1994a,b,c). However, it appears that even if such a projection exists, many of the axons send collateral branches to the thalamus before terminating in the hypothalamus (Dado et al., 1994a,b,c). Therefore, it is unlikely that labelling of the spinohypothalamic axons from the caudal thalamus had a major effect on the present results. The issue of labelling ascending fibres of the spinothalamic tract is discussed below (section 4.2.6).

4.2.2 PoT in the literature

The posterior triangular nucleus (PoT) is a term recently introduced to describe a small region in the posterior thalamic nucleus in the rat, which is triangular in outline. Its narrow apex points dorsally and its wider base faces ventrally. According to the atlas of Paxinos and Watson (2005), this region extends in the anteroposterior axis from interaural 2.88 to 4.08 mm. It is bounded laterally by the MG complex, and is in close contact with the MGM. Ventrally it is bounded by the PIL, medially by the APT, posterior limitans thalamic nucleus and, at its caudal end, by the deep mesencephalic nucleus. Dorsally it is continuous with the Po nucleus. Jones (2007) defined this region as the backward extension of the posterior medial nucleus that extends along the medial edge of the MGM and even merges with it to form the small-celled component of MGM. According to Jones, this nucleus is present in all mammalian species examined; however, it is more evident in rodents. Different authors have referred to this area by different names, for example: pars medialis of the MG complex (Lund and Webster, 1967), the magnocellular area of the MG body (Giesler et al., 1976; Carstens and Trevino, 1978), and posterior intralaminar nucleus (LeDoux et al., 1987; Linke, 1999a).

Although some early studies have described a spinal projection to areas equivalent to the PoT, many recent retrograde tracing studies of the spinothalamic tract did not include this area in their injection sites. An example of the early studies is that conducted by Lund and Webster (1967), who observed heavy terminal degeneration in this region following spinal

cord hemisection. Giesler et al. (1976) antidromically activated neurons in lumbar spinal cord, some of which were in lamina I, after stimulation of the magnocellular area of the MG complex, an area that corresponds to the PoT in the new nomenclature. Following injection of WGA-HRP into the cervical or lumbar enlargements in the rat, LeDoux et al. (1987) observed anterogradely labelled terminals in the region of the PoT, at levels corresponding to interaural 3.5 to 4.1 mm. This last finding was also confirmed by the retrograde tracing technique, when LeDoux et al. (1987) injected the WGA-HRP into an area that corresponded to PoT and observed many retrogradely labelled cell bodies in the cervical segments and the caudal lumbar and upper sacral regions. Most labelled neurons were detected in the neck of the dorsal horn (the reticular part of lamina V and to a lesser extent in lamina IV); some neurons were also seen in other laminae including lamina I. In cat and monkey, spinothalamic terminations in areas equivalent to PoT in rat have also been described (Jones, 2007). However, it seems that the subcortical inputs to the PoT do not only come from somatosensory pathways because LeDoux et al. (1987) also described convergent inputs from the ascending auditory pathways. Linke (1999b) reported that input to an area equivalent to PoT was predominantly from the external nucleus of the inferior colliculus and deep layers of the superior colliculus.

4.2.3 Thalamic injections that included the PoT labelled more lamina I neurons than had been reported

Since the PoT was not apparently included in the injection sites in many of the previous retrograde tracing studies, the first aim of this investigation was to determine whether injections that included this region would label a larger number of lamina I spinothalamic neurons than had been seen in the previous studies. In experiments Thal 1-5, the injections included all of the main thalamic regions that are known to receive inputs from the superficial dorsal horn: VPL, PO, PoT and VPPC (Gauriau and Bernard, 2004a). Although there was some variation between injection sites (e.g. VPL was only partially filled in Thal 1 and Thal 3, and there was variable spread into the striatum), the numbers of retrogradely labelled lamina I neurons in C7 were very consistent, averaging 23.2 cells (range 22-25) per 600 μm. The mean number in L4 was considerably lower: 7 cells (range 3-10) per 1,200 μm. The observed consistency in number of labelled cells between the five experiments suggests that excluding the MD nucleus (in Thal 1) did not lead to a significant reduction in the number of retrogradely labelled cells. In support of this, Gauriau and Bernard (2004a) observed only sparse projection from lamina I neurons to the MD nucleus in the rat. It is unlikely that spread of Fluorogold into the striatum influenced

these numbers as this only occurred to a significant extent in Thal 3 and Thal 4, and the numbers of retrogradely labelled cells were no higher in these experiments. Giesler et al. (1979) reported that following control injections of HRP into the caudate nucleus in the rat, no retrograde labelling was observed in the spinal cord. In addition, Gauriau and Bernard (2004a) found no evidence of a significant projection from superficial dorsal horn to the striatum. Since the length of the C7 segment is 2.3 mm (present study), while that of L4 is ~2.5 mm (Polgár et al., 2004), it was estimated that there are ~90 lamina I spinothalamic neurons on the contralateral side in C7 and ~15 such cells in L4.

Early studies that used HRP to retrogradely label spinothalamic cells (Granum, 1986; Kemplay and Webster, 1986) found only a small number of these cells below the second cervical segment, presumably because of the lack of sensitivity of this tracer; therefore, these studies will not be discussed in relation to the present findings. Following injection of CTb into the ventrobasal thalamic complex of the contralateral side, Lima and Coimbra (1988) observed 24 spinothalamic cells in lamina I of the C7 segment, and 38 such cells in the combined L3 and L4 segments (i.e. ~19 cells in the L4 segment, assuming that L3 and L4 contain equal numbers of spinothalamic cells in lamina I). However, it appears that the PoT was not included in that injection site since Fig. 1A, presented in Lima and Coimbra (1988), shows that spread of tracer did not extend as far backward as the PoT region. Burstein et al. (1990a) found 68-152 lamina I spinothalamic cells in the combined C7-8 segments after injecting Fluorogold into the contralateral thalamus. They reported that their injection sites included the ventrobasal complex and Po in all cases, as well as various midline and intralaminar thalamic nuclei. Assuming that the C7 and C8 segments contain equal numbers of spinothalamic neurons in lamina I, then each segment would contain between 34 and 76 labelled cells. This variability in their results may be explained by variations in the spread of Fluorogold in the five analysed cases. As presented in Table 2 in their paper, the injection site extended to the MG nucleus in only two of the five cases. Spread of tracer into MG would probably fill the adjacent PoT region, and it is possible that the highest number of labelled cells was encountered in these two cases, although this was not reported by the authors. As to the lumbar segments, Burstein et al. (1990a) reported a range of 6-32 spinothalamic lamina I cells in the combined L4 and L5 segments (i.e. 3-16 cells in the L4 segment). Marshall et al. (1996) found approximately 14 labelled cells in lamina I in the L4 segment after entire thalamic injection of the contralateral side. Yu et al. (2005) conducted a quantitative study of rat spinothalamic lamina I neurons; however, they did not estimate the total number of spinothalamic cells in lamina I per segment in that study. Surprisingly, Li et al. (1996) found a very high number of

spinothalamic cells in lamina I of the C7 segment. They reported that the thalamus of the contralateral side was almost filled with Fluorogold, and their illustration showed that large part of the Po and ventrobasal complex were included within the injection site, but they did not illustrate more caudal levels, so it is not known whether that injection site extended to the PoT. They reported finding 81 retrogradely labelled lamina I cells per 800 µm, and this is more than twice as high as that seen in the present study. However, this finding was only obtained from one rat, and the authors did not provide details of how sections were selected for analysis or whether they corrected for over-counting. Interestingly, Dado and Giesler (1990) found a high number of retrogradely labelled neurons in lamina I of the cervical enlargement following a small Fluorogold injection that was restricted to the caudal part of Po, and their illustration showed filling of the rostral part of PoT. In conclusion, the number of spinothalamic cells in lamina I estimated in the present study is considerably higher than those of Lima and Coimbra or Burstein et al. for the C7 segment. Since the injection sites in these two previous studies did not consistently extend far enough caudally to include PoT, this suggests that inclusion of the PoT in the injection sites increases the number of labelled cells in lamina I in the cervical enlargement. However, for the L4 segment, the present finding is generally similar to that reported by previous studies, even though the PoT was not apparently included in the injection sites in their cases. Nevertheless, injections that targeted the PoT area in the present study (PoT series) were able, in some cases, to label all or most of the spinothalamic cells in lamina I in this segment (e.g. PoT 6-8; see section 4.2.4). Furthermore, many of the cells were retrogradely labelled with CTb (i.e. were labelled from the PoT) in the DI series of experiments. There are two possible explanations for this observation: 1) all lamina I cells that were labelled from PoT in the L4 segment also project to more rostral parts of the thalamus; or 2) the labelling of fibres of passage in the region of PoT led to this observation.

4.2.4 Injections that target the PoT are capable of labelling all spinothalamic neurons in lamina I

Since the Fluorogold injections in the Thal experiments included several known targets for lamina I neurons, the next aim was to target the tracer injections on the PoT. Surprisingly, although there was some variation in numbers of retrogradely labelled lamina I neurons, the mean values for those experiments in which PoT was included in the injection site (PoT 1-9) were very similar to those for the Thal series: 24.6 cells/600 µm in C7 and 7.1 cells/1,200 µm in L4.

The following is a detailed analysis of individual experiments in the PoT series in the C7 segment: First of all, some degree of variability was observed in the numbers of lamina I spinothalamic cells that were labelled in PoT 1-9. This could be explained by variations in the extent of the injection sites, though PoT was included, totally or partially, in all nine experiments. Experiments PoT 7 and PoT 8 were the most successful experiments in this series, in terms of filling the PoT with relatively little spread into surrounding structures. These two experiments resulted in labelling of 24 and 25 cells per 600 µm length of the C7 segment, respectively, and these were close to the mean value for the Thal series. The slightly lower numbers of retrogradely labelled cells found in PoT 2 and PoT 3 could be due to partial filling of the PoT in these cases (only the caudal part of PoT was included in the injection site in PoT 2 and the dorsal part of the PoT at interaural 3.9 - 4.1 mm was not included in the injection site in PoT 3). It is unlikely that the involvement of the superior colliculus or the deep mesencephalic nucleus in PoT 2 contributed to the observed number of labelled cells in this case because these areas have not been documented to be major targets for lamina I neurons in the rat (Gauriau and Bernard, 2004a). Therefore, it is very interesting to find that including only the caudal part of the PoT in the injection site (in PoT 2) was capable of labelling a high proportion of the spinothalamic cells in lamina I. An interesting observation from experiments PoT 3, PoT 5, PoT 6 and PoT 9 is that the rostral extension of tracer into the ventral parts of the Po/ventrobasal complex in the former two experiments, into the Po nucleus in PoT 9, or the massive extension of tracer into large parts of the Po/ventrobasal complex in PoT 6 did not lead to an increase in the number of labelled lamina I neurons in these experiments. This probably indicates that these nuclei do not receive terminals from lamina I in the C7 segment apart from any that send collaterals to the PoT or pass through it. The possibility of collateral projection to the Po/ventrobasal complex and the PoT was addressed in the double tracer injection experiments, as presented below. The finding in PoT 10, in which 18 lamina I cells were labelled per 600 µm in the C7 segment, indicates that some lamina I neurons could be labelled from the Po nucleus. The highest number of retrogradely labelled neurons in lamina I of the C7 segment was observed in experiment PoT 4. It is obvious that tracer spread in this experiment was massive but this result is difficult to interpret especially after comparing this observation with results obtained after total thalamic injections. The mean number of retrogradely labelled neurons in the C7 segment following a total thalamic injection was 23 cells per 600 µm (data from Thal and DI series), and this number is smaller than the one obtained in experiment PoT 4, which is 36 cells per 600 µm. Probably the random variation in the numbers of spinothalamic cells between sections contributed to this odd finding.

In the L4 segment, because of the very small numbers of spinothalamic cells in lamina I, it is difficult to interpret the differences between the different experiments. In spite of that, it is very clear that experiments PoT 6-8 labelled the highest number of cells in this series, and this was similar to the mean number observed in the Thal series. However, as suggested above, retrograde labelling in lamina I in the L4 segment from the PoT is likely to be due to labelling of collaterals or of fibres of passage.

A comparison of the results from the Thal and PoT experiments suggests that injections of tracer targetted on PoT can label most (if not all) spinothalamic neurons in lamina I in both segments. This finding has an important practical implication: investigations that aim to label all spinothalamic cells in lamina I could restrict tracer injection to the PoT area. This would save time during the surgery and is a very cost-effective approach, in terms of the amount of tracer used, compared to injections that fill the entire thalamus.

4.2.5 Retrograde labelling in lamina I observed in the DI experiments

4.2.5.1 The C7 segment

If the difference between the numbers of labelled lamina I neurons in the C7 segment in the present study and those reported by Lima and Coimbra (1988) and Burstein et al. (1990a) is due to inclusion of the PoT in injections in this study, this would suggest that some lamina I cells in this segment project to the PoT, but do not have axons that extend further rostrally. This possibility was explored in the DI experiments, in which CTb was injected into the PoT and Fluorogold into other thalamic regions that receive projections from superficial dorsal horn. If that interpretation was correct, then it would be expected that most lamina I cells in the C7 segment were either double-labelled or only contained CTb. The total numbers of spinothalamic lamina I cells observed in these experiments were similar to those seen in Thal or PoT experiments, and (as expected) the mean number of lamina I cells that were labelled with Fluorogold in C7 (14/600 μm) was significantly lower than the mean number of retrogradely labelled neurons seen in Thal or PoT experiments (24/600 μm) (t-test; $P < 0.05$).

The first group of experiments in the DI series (DI 1-4) presented some common features in the spread of tracer substances; however, slight variations were observed and these rendered the interpretation of the results rather interesting. First of all, the total number of

retrogradely labelled cells varied slightly between the four cases. This variation might be insignificant and could be due to the random variation in the distribution of cells between sections, or it could be due to differences in the spread of tracer substances. If the second assumption is correct, then it would be more informative to compare the two extreme data sets, i.e. data from DI 1 and DI 4. By analyzing results presented in Table 3-3, it can be seen that the difference in the total number of retrogradely labelled cells between these two cases is mainly due to the number of CTb-labelled cells. Experiment DI 4 had a much lower number of these cells than DI 1 (10 compared to 28). This might be explained by the lack of spread of CTb into the PoT at interaural 3.9 - 4.1 mm in experiment DI 4. Instead, this area was filled with Fluorogold, and this could explain the higher number of cells that were only labelled with Fluorogold in this case compared to the other three experiments. This observation suggests that the rostral part of PoT (or the Po at this level) receives terminals from lamina I neurons in the C7 segment. Another observation that can be obtained from experiments DI 1-4 is that the number of Fluorogold-labelled cells is higher in DI 1 and DI 4 than that in DI 2 and DI 3. The logical explanation is that Fluorogold extended in the former two experiments into areas, from which lamina I neurons could be labelled, but these were not included in the latter two cases. After examining the spread of Fluorogold in the thalamus in these four experiments, the most likely explanation is the extension of Fluorogold into the rostral part of the PoT in DI 1 and DI 4, which did not occur in DI 2 and DI 3. One difference between the caudal extension of Fluorogold in DI 1 and DI 4 is that some overlap occurred between the two tracers at rostral PoT in DI 1, but this did not occur in DI 4. Correspondingly, the number of double-labelled cells in DI 1 was higher than that in the other experiments. On the other hand, the number of cells that were only labelled with CTb was lower in DI 1 compared to DI 2 and DI 3. In other words, some of the cells that were only labelled with CTb in DI 2 and DI 3 probably correspond to the double-labelled cells in DI 1. The last observation on this series is related to the proportion of the double-labelled cells out of all Fluorogold-labelled cells in lamina I. It is obvious that all (in DI 2 and DI 3) or most (in DI 1) of the lamina I cells that were labelled from the rostral thalamus could be also labelled from the PoT. This proportion was smaller in DI 4, and this is probably for the reason presented above, i.e. lack of spread of CTb into the rostral part of the PoT in this case. If data from experiments DI 2 and DI 3 are considered, as the results here are more consistent with each other, the pattern of thalamic projection from lamina I cells in C7 could be summarised as follows: about two-thirds of the cells could only be labelled from the area of the PoT, whereas the remaining third could be labelled from both the PoT and the rostral thalamus. Therefore, this group of experiments suggests that injection of tracer substance into the PoT is capable of labelling

all lamina I spinothalamic cells in the C7 segment, provided that the rostral PoT is contained within the injection site.

As to experiments DI 5-7, the mean number of retrogradely labelled cells in these three cases did not differ much from that observed in the other series of experiments (Thal 1-5, PoT 1-9 and DI 1-4). Therefore, inclusion of more thalamic nuclei (e.g. SPF, Re, rhomboid nucleus, retroreuniens nucleus, nucleus submedius, VM, in addition to the other parts of VPPC, CM, MD, CL and PF nuclei that were not included in the other experiments) in the injection site did not lead to an increase in the number of retrogradely labelled cells in lamina I. However, the number of cells that were only labelled with CTb in this group is relatively lower than that seen in DI 1-4, apparently being replaced mainly with double-labelled cells (in DI 5 and DI 7) or with cells only labelled with Fluorogold (in DI 6). It is obvious that the dorsal PoT/Po at interaural 4.0 mm was not filled with CTb in cases DI 5 and DI 7. Thus, it is possible that the dorsal PoT/Po at interaural 4.0 mm receives projection from lamina I cells, as suggested above. However, it is difficult to explain the low number of cells labelled with CTb in DI 6, since in this case the CTb injection filled much of the PoT and extended dorsally into Po at interaural 3.2 to 4.0 mm. Nevertheless, in this experiment (DI 6), the Fluorogold injection spread caudally to enter the MG at interaural 3.6 mm, and was thus close to the region that contains projections from cervical superficial dorsal horn (Gauriau and Bernard, 2004a). It has been reported that injecting Fluorogold proximal to other tracers in double-labelling experiments interferes with the uptake of the other tracer (Akintunde and Buxton, 1992; Bice and Beal, 1997b), and it is therefore possible that the proximity of the Fluorogold injection site to the PoT prevented uptake of CTb from this region by some spinothalamic lamina I neurons. Interestingly, only one cell in DI 7 was only labelled with CTb. Thus, most of the cells that were labelled from the parts of PoT that were included in the injection site of DI 7 were also labelled from more rostral parts of the thalamus. In DI 5, it was found that five cells were only labelled with CTb. By comparing the spread of CTb in DI 5 with that in DI 7, it was found that the additional areas that were included in the injection site in DI 5 were: caudal PoT (around interaural 2.9 mm) and Po at interaural 3.6 mm. The filling of these areas with CTb in DI 6 may account for the eight cells that were only labelled with CTb in this case. The reason for the higher number of cells that were only labelled with CTb in experiments DI 1-3 is probably because these cells had collateral projections to the additional thalamic nuclei included in DI 5-7 but not in DI 1-3; hence corresponding cells would have appeared as single labelled in the latter. The numbers of Fluorogold-labelled cells in experiments DI 5-7 were high and were similar to those observed in DI 1 and DI 4.

However, this high number in DI 5-7 can not be accounted for the explanation suggested for DI 1 and DI 4, i.e. Fluorogold extension into rostral PoT/Po at interaural 4.0 mm. Little caudal extension of Fluorogold into rostral PoT/Po occurred in DI 7 but this is more or less as much as that observed in DI 3, and this latter extension did not lead to an increase in the number of Fluorogold-labelled cells. The most likely explanation is that the additional thalamic nuclei included in this series of experiments receive collaterals from lamina I cells that were labelled from the PoT. Some of the retrogradely labelled cells in experiments DI 5 and DI 7 were found to be positive for Fluorogold only, i.e. these cells were labelled only from rostral nuclei of the thalamus, and were responsible for about 20% of the total thalamic projection from lamina I in C7. Probably, the deficient spread of CTb in the region of dorsal PoT/Po at interaural 4.0 mm led to the appearance of cells that were only labelled with Fluorogold instead of double-labelled cells. Surprisingly, the number of cells that were only labelled with Fluorogold in DI 6 was much higher than that in the other two experiments in this series, and this is difficult to interpret. The major difference in the Fluorogold injection site between DI 6 and the other two cases (DI 5 and DI 7) was the spread into the MG nucleus in DI 6. However, this is unlikely to account for the observed high number of cells only labelled with Fluorogold in DI 6 since this region of MG is not thought to be a major target for lamina I axons (Gauriau and Bernard, 2004a). Probably, the reason behind this observation is the reduced labelling with CTb mentioned above.

Although Gauriau and Bernard (2004a) described a significant projection from lamina I neurons to the PoT, they considered the VPL nucleus as the main thalamic target for lamina I neurons in the cervical enlargement in the rat. Results of the present study suggest that rostral thalamic nuclei (presumably mainly the VPL) received projections from a lower number of lamina I cells compared to caudal nuclei (probably mainly the PoT). However, this does not contradict the finding by Gauriau and Bernard because they based their conclusion on the sizes and packing densities of the terminals they observed in these areas, whereas this conclusion (from present study) is based on numbers of lamina I neurons that project to each of these targets.

4.2.5.2 The L4 segment

Some observations could be made from the double-labelling (DI) experiments in the L4 segment, although the interpretation of data in this case might be less clear than that in the C7 segment due to the lower number of spinothalamic cells at this level. First of all, it is obvious that including additional thalamic nuclei in the injection site in DI 5-7 did not lead

to any increase in the total number of labelled cells in these cases compared to that seen in DI 1-4. The observed total number in DI 5 was surprisingly low. One of the differences between tracer spread in DI 5 and that in DI 6 and DI 7 is that filling of midline thalamic nuclei was greater in the latter two cases. However, missing these nuclei is unlikely to be the reason for the lower number of cells retrogradely labelled in DI 5 because these nuclei were not included in all other experiments (Thal and PoT series); nevertheless, the total number of retrogradely labelled cells in many of these cases was as many as that observed in DI 6 and DI 7. It is possible that the lack of tracer spread into dorsal PoT/Po at interaural 4.0 mm in DI 5 led to this lower number of labelled cells; however, this did not lead to reduction in the number of labelled cells in the C7 segment. It is therefore likely that this difference is not significant and merely due to the randomness in selection of the analysed sections. In most DI experiments, the majority of the retrogradely labelled cells contained CTb, and indeed some were only positive for CTb. This might indicate that these cells project only to the PoT. However, this is unlikely because other studies have reported as many spinothalamic cells in lamina I of L4 as the present study even though the PoT was not included in their injection sites, as presented above. The mean number of lamina I cells that were retrogradely labelled with Fluorogold in all seven DI experiments is six cells per 1,200 µm, equivalent to approximately 13 cells in the entire L4 segment, i.e. similar to that seen in Thal experiments. This suggests that lamina I cells in the L4 segment that were labelled from the PoT also project to more rostral parts of the thalamus. The number of cells that were labelled with Fluorogold varied between the seven cases. The highest number was observed in DI 4. Probably the reason for this is the same as that suggested for the C7 segment, i.e. the caudal extension of Fluorogold into the rostral PoT/Po at interaural 4.0 mm. This could also explain the lower number of cells only labelled with CTb in this case. More than half of the retrogradely labelled cells in experiments DI 6 and DI 7 were positive for Fluorogold only. Probably, this is due to the spread of tracer into Po at interaural 4.0 mm in DI 7, but this explanation is not valid for DI 6. Interestingly, this was also observed in lamina I cells in the C7 segment in DI 6, but again there was no obvious explanation for this observation.

4.2.6 Evidence for lamina I projection to the PoT from FLM experiments

The present results indicate that most lamina I spinothalamic neurons can be labelled by injection of CTb or Fluorogold into the PoT. However, as mentioned above, this might have resulted from uptake of tracer by fibres of passage, since the main ascending bundle

of spinothalamic axons from lamina I passes through this region (LeDoux et al., 1987; Cliffer et al., 1991; Gauriau and Bernard, 2004a). In order to demonstrate that labelling from the PoT, or at least part of this labelling, is due to uptake of tracer by terminals rather than by passing fibres, three more experiments (FLM 1-3) were carried out using another tracer substance: rhodamine-labelled fluorescent latex microspheres (red beads), which were injected into the PoT. This tracer substance has two main advantages over other tracers: (1) it gives a highly restricted injection site that could be almost entirely contained within the PoT, and (2) it has been reported that the beads are not taken up by intact axons near the injection site (Katz et al., 1984; Pu and Amthor, 1990; Krug et al., 1998). Katz et al. (1984) observed that when they placed the fluorescent latex microspheres on top of the corpus callosum of rats, no retrograde labelling occurred in cortical cells. Pu and Amthor (1990) reported that injection of this tracer into areas known to contain passing retinal fibres, such as the deep layers of the superior colliculus or the brachium of the superior colliculus, did not result in retrograde labelling in the retina. A similar conclusion was reached by Krug et al. (1998) who made multiple injections of fluorescent latex microspheres into cortical areas, through which geniculate fibres pass before their termination in the visual cortex. These authors found no retrograde labelling in the lateral geniculate nucleus after these injections.

All three FLM experiments resulted in labelling of lamina I neurons in the C6 segment, and in FLM 3, 46 of these cells were labelled. If the number of lamina I spinothalamic neurons in C6 is similar to that in C7, this would represent approximately half of the spinothalamic population in this lamina, and since the injection site in this animal occupied much less than half of the entire PoT, it is likely that the number of cells that project there has been considerably underestimated. This suggests that most (if not all) lamina I spinothalamic neurons in the cervical enlargement have axons that arborise in the PoT or immediately surrounding areas. Retrogradely labelled lamina I neurons were also seen in the L5 segment in two animals (FLM 2 and FLM 3). Although the numbers were small, they represent approximately one third of the population of spinothalamic neurons in this lamina (assuming that the number of cells in L5 is similar to that in L4). This suggests that at least some lamina I spinothalamic cells from the lumbar enlargement also project to the PoT.

Taken together with previous reports, findings of the present study suggest that the great majority of lamina I spinothalamic neurons have axons that pass through or close to the PoT. Some of those in the cervical enlargement continue rostrally to terminate in VPL and

Po (in many cases giving off collaterals to PoT), while others terminate in PoT. In the lumbar segments, all lamina I spinothalamic cells appear to terminate at rostral thalamic nuclei (VPL and Po), with some sending collaterals to the PoT, but none terminating exclusively in this nucleus.

4.2.7 Evidence for a projection to the PoT from physiological studies

The present finding that some lamina I cells in the C7 segment project only to the PoT is consistent with the results of a recent physiological study by Zhang and Giesler (2005), who reported that a significant number of lamina I spinothalamic cells in the rat cervical enlargement projected to the PoT and surrounding regions, but no further rostrally. Furthermore, Zhang and Giesler (2005) reported that they found more spinothalamic axons that terminated within PoT than within any other area of the posterior thalamus. Dado et al. (1994c) also reported that some neurons in the superficial dorsal horn in rat cervical enlargement were activated from the PoT, but not from further rostral sites.

4.2.8 Is the PoT in the rat equivalent to the VMpo in the monkey?

Based on a number of similarities, Gauriau and Bernard (2004a) suggested that the rostral part of the PoT in the rat corresponds to the VMpo nucleus in monkey. This suggestion is very likely to be correct because exclusion of this part from injection sites in the present study led to reduction in the number of labelled cells, as presented above. Some of the reported similarities between PoT and VMpo are that: 1) both nuclei are located in the caudal part of the thalamus, close to the MG and SG nuclei; 2) both receive a major input from the superficial dorsal horn; 3) both contain calbindin-immunoreactive profiles (Craig et al., 1994; Coolen et al., 2003; Craig, 2004); and 4) both contain nociceptive-specific neurons and project to similar areas in the cerebral cortex (see below).

4.2.9 Functional role of the PoT

Several studies have implicated the PoT in the processing of nociceptive information. This was based on finding that it receives afferents from nociceptive neurons, that some of its neurons respond to noxious stimuli, and that it projects to areas involved in response to pain (see below). Zhang and Giesler (2005) reported that all of the eight lamina I cells in rat cervical enlargement that were activated from the PoT responded to noxious stimuli:

seven cells were nociceptive-specific and only one cell was classified as a wide dynamic range neuron. Gauriau and Bernard (2004b) recently reported that about 45% of the 108 neurons recorded in and around the PoT in the rat were nociceptive-specific, 19% responded to both noxious and innocuous stimuli, and 36% responded to low threshold tactile stimuli. Because most lamina I neurons are activated by noxious, but not tactile, stimuli (Christensen and Perl, 1970; Bester et al., 2000), Gauriau and Bernard (2004b) proposed that input from lamina I mainly targets the nociceptive neurons (either the specific or non-specific type), whereas the low threshold tactile neurons in the PoT receive their afferents from dorsal column neurons and some deep dorsal horn neurons. Since the receptive fields of the PoT cells were found to be large (often one to three paws), it has been suggested that axons of many lamina I neurons converge onto single PoT cells in the thalamus (Gauriau and Bernard, 2004b).

Several studies have shown that PoT projects to several areas and mainly to the secondary somatosensory cortex (S2) (Carvell and Simons, 1987; Spreafico et al., 1987; Shi and Cassell, 1998; Linke, 1999a; Linke and Schwegler, 2000; Gauriau and Bernard, 2004b). Projections to the insular cortex (Linke, 1999a; Linke and Schwegler, 2000; Gauriau and Bernard, 2004b) and lateral nucleus of the amygdala (Ottersen and Ben-Ari, 1979; LeDoux et al., 1990; Gauriau and Bernard, 2004b) have been also described. Interestingly, Gauriau and Bernard (2004b) observed a correlation between the physiological properties of PoT neurons and the cortical projection target. They found that nociceptive-specific neurons projected to S2 cortex, whereas other neurons projected to the insular cortex and/or amygdala. In close resemblance to the PoT, it has been reported that the VMpo nucleus in monkey sends projections to the insular cortex (Craig et al., 2000).

The secondary somatosensory cortex (S2) is located lateral to the primary somatosensory cortex (S1) and contains a complete representation of the face and body (Tracey, 2004). The function of the S2 cortex is poorly understood; however, the extensive research work that has been done during the last two decades started to clarify some of its functional roles. There is growing evidence that S2 differs from S1 in some aspects: for example, S2 is characterized by a high degree of convergence of afferent input, thus the receptive fields of its neurons are larger than those in S1, and sometimes of mixed modalities (Carvell and Simons, 1986). Furthermore, unlike S1, it has been shown that S2 of both sides respond to unilateral sensory stimuli (Carvell and Simons, 1986; Timmermann et al., 2001; Lin and Forss, 2002; Ploner et al., 2002). Therefore, it has been suggested that S2 might play a role in the integration of sensory information from both halves of the body, which might be

important for the maintenance of a unified body image (Lin and Forss, 2002). S2 is also thought to play a role in sensorimotor integration as the response to sensory stimuli (e.g. median nerve stimulation) was found to be influenced by muscular contraction (Lin and Forss, 2002), and also in multisensory integration (auditory and somatosensory stimuli) (Menzel and Barth, 2005). Like S1, it has been reported that S2 responds to painful stimuli in human imaging studies (Coghill et al., 1994; Kakigi et al., 1995; Casey et al., 1996), as well as in the rat (Chang and Shyu, 2001). However, in certain aspects, S2 differs from S1 in the response to the noxious stimuli. For example, Timmermann et al. (2001) have provided evidence for a differential coding of nociceptive stimuli in human S1 and S2. They used whole-head magnetoencephalography to record cortical responses within the S1 and S2 cortices to different intensities of nociceptive laser stimuli applied to the dorsum of the hand of human subjects. They noticed a strong correlation between pain intensity and activation in S1 and S2, but the increase in the amplitude of activation in S2 was only at stimulus intensity well above pain threshold, whereas, in S1, this relation was exponential and closely corresponded to the patient's pain rating. As a result, this observation added further evidence for the importance of the S1 cortex in the sensory-discriminative aspects of pain processing (Schnitzler and Ploner, 2000; Bushnell and Apkarian, 2006). On the other hand, the activity of S2 neurons reflects poorly the intensity of nociceptive stimuli (Dong et al., 1994) and points toward an involvement of S2 in recognition, learning, and memory of painful events (Dong et al., 1994; Kwan et al., 2000; Schintzler and ploner, 2000). Another reported difference between S1 and S2 (and also the anterior cingulate cortex) is related to the differential temporal activation patterns of these areas in response to painful cutaneous laser stimuli (Ploner et al., 2002). Ploner et al. found that S1 showed a strong predominance of first pain-related activation (in response to activity of Aδ fibres), whereas the anterior cingulate cortex displayed a strong predominance of second pain-related activation (in response to activity of C fibres). However, S2 was almost equally activated during first and second pain. This finding led Ploner et al. (2002) to support the suggestion that S2 is involved in cognitive-evaluative components of pain perception, as stated above. Interestingly, Ploner et al. (1999) described a clinical case of a patient who had an ischemic lesion that affected S1 and S2. This patient was able to describe a vague unpleasantness (i.e. affect was intact probably due to sparing of the anterior cingulate cortex), but not the intensity and location of the applied stimulus (most probably due to the lesion of S1). The patient also failed to recognize the noxious nature of the stimulus, and this was attributed to lesion of S2. This case added further support to the possible role of S2 in the cognitive detection of the "painful" nature of nociceptive stimuli.

The insular cortex in the rat is located on the lateral surface of the cerebral hemisphere. Several functions have been attributed to the insular cortex; for example: regulation of autonomic activities (Cechetto and Saper, 1987; King et al., 1999), auditory and multisensory processing (Rodgers et al., 2008), and affective aspects of nociceptive processing (Berthier et al., 1988). Berthier et al. (1988) described interesting clinical cases of six patients with insular lesion, who were able to recognize the painful nature of the noxious stimulus but showed no reaction to it (i.e. no emotional response), a condition described as "asymbolia for pain".

The amygdala is a complex nucleus located deep within the temporal lobe of the brain and defined as part of the limbic system. Considerable evidence points to the involvement of the amygdala in fear conditioning, i.e. repeated pairing of a neutral visual or auditory stimulus (conditioned stimulus) with a painful stimulus (e.g. foot shock; unconditioned stimulus) leads to production of autonomic and emotional changes in response to the neutral stimulus alone (LeDoux et al., 1990; Paré et al., 2004).

Therefore, it appears that the PoT could be involved in various functions through projections to the abovementioned areas. The nociceptive input that probably reaches the PoT mainly from lamina I is likely to mediate the functions related to processing of painful stimuli, as suggested by Gauriau and Bernard (2004b) and Zhang and Giesler (2005), i.e. the recognition of noxious stimuli as a specific "painful" sensation (through projection to the S2 cortex), recognition of the meaning and threat of painful stimuli (through projection to the insula), and providing the nociceptive information necessary for the fear conditioning through projection to the amygdala.

4.3 The proportion of lamina I neurons that project to the thalamus

4.3.1 The C7 segment

To determine the percentage of lamina I neurons in the C7 segment that belong to the spinothalamic tract, the disector method was used on sections reacted with NeuN (to reveal all neurons) and DAPI (to stain nuclei) from four rats. The combination of NeuN and DAPI allowed top and bottom surfaces of neuronal nuclei to be identified, which is necessary for the disector method (Todd et al., 1998). Although spinothalamic neurons were present throughout the mediolateral extent of lamina I, their distribution was often not uniform, with clustering in the central part. Therefore, the entire extent of the lamina was

analysed in each section. In the disector method, the separation between reference and look-up sections is normally set so that none of the structures to be examined could lie entirely between the two, and thus be missed during counting (Polgár et al., 2004). However, in the present study, every optical section in the stack was examined to ensure that no neurons were missed, and therefore a wider separation was used (9 μm), thus increasing the size of the sample analysed for each Vibratome section.

Two methods were used to calculate the percentage of retrogradely labelled lamina I cells. First, the number of the retrogradely labelled neurons that were sampled with the disector in each animal was divided by the total number of neurons in the disector sample to obtain a mean value for each experiment. Although this method is simple to calculate, its accuracy is limited by the small number of retrogradely labelled neurons included in the disector samples (between one and seven for the four experiments), which reflects their relative scarcity. To provide a more accurate value, the disector sample (after correcting for tissue shrinkage) was used to estimate the total number of lamina I neurons per 600 μm in C7, and then this was compared with the mean number of retrogradely labelled neurons per 600 μm from all experiments (except PoT 10). This gave a slightly lower value of 1.9% (compared to 2.4% obtained with the first method).

Yu et al. (2005) also used the disector method on confocal images and estimated that 9% of lamina I neurons in the cervical enlargement belong to the spinothalamic tract. It is difficult to explain the discrepancy between results of the present study and those of Yu et al. One possible source of difference is the definition of the lamina I/II border in the two studies. The position of this border was estimated from the NK1r plexus (which was found to be co-extensive with lamina I), whereas Yu et al. assumed a uniform width of 20 μm for lamina I. To determine whether this could explain the discrepancy between these results, the percentages were re-calculated based on the assumption that lamina I had a uniform width of 20 μm. This gave slightly higher values: 3.2% (instead of 2.4%) of the neurons in the new disector sample were retrogradely labelled, while with the second method, an estimate of 2.1% (instead of 1.9%) was obtained. These values are still far below that of Yu et al. (2005), and therefore the method of placing the lamina I/II border does not account for the difference in these conclusions. Probably the sampling method used by Yu et al. led to the difference between the two studies.

4.3.2 The L4 segment

The proportion of lamina I neurons in the L4 segment that belong to the spinothalamic tract was not directly determined in the present study, but this can be estimated by comparison with previously published data. Spike et al. (2003) used a similar disector method and concluded that there were 8,318 lamina I neurons on each side in the L4 segment. However, in that study correction for tissue shrinkage was not performed, and this value will therefore be an overestimate. In the present study the mean thickness of sections when scanned with the confocal microscope was found to be 45 μm, which corresponds to a shrinkage in thickness of 25%. The tissue used by Spike et al. was prepared in the same way, and is therefore likely to have undergone similar shrinkage. If this correction is applied retrospectively to the data from Spike et al., the number of lamina I neurons on one side of L4 would be 6,238. Since a mean of 15 spinothalamic lamina I cells on one side of L4 was found, these would constitute 0.2% of the neuronal population in this lamina.

In the monkey, Yu et al. (1999) suggested that the proportion of the spinothalamic cells among all neurons in lamina I in both spinal enlargements could be ~50%. They did not use the disector method to determine this percentage, but instead they used a formula to calculate it. Their calculations were based on the report that 45% of all neurons in lamina I of the rat express the NK1r (Todd et al., 1998), and their own findings that 69% of all NK1r-immunoreactive neurons in lamina I project to the thalamus, and that 62% of all spinothalamic cells in lamina I express the NK1 receptor.

4.4 Expression of the NK1r by spinothalamic cells in lamina I

Marshall et al. (1996) reported that 77% of lamina I spinothalamic neurons in the lumbar cord were NK1r-immunoreactive, and ~80% of lumbar lamina I neurons that were labelled from CVLM, LPb or PAG also expressed the receptor (Todd et al., 2000). In this study, a similar result was obtained for spinothalamic lamina I neurons in L4 (84%) and C7 (87%). It has been shown that NK1r-immunoreactive lamina I projection neurons in lumbar cord receive strong monosynaptic input from substance P-containing (nociceptive) primary afferents (Todd et al., 2002), and the NK1r-expressing spinothalamic lamina I neurons presumably provide a powerful input from these afferents to the PoT, Po and VPL nuclei of the thalamus.

4.5 Projection of lamina I neurons to the LPb, PAG and thalamus

4.5.1 Issues related to the injection sites

In the Pb and PAG series, the PoT was targetted for the thalamic injections since results of the first part of the present study showed that most lamina I spinothalamic neurons in the C7 and L4 segments could be labelled from this region (section 3.1.1.2 in Results; Al-Khater et al., 2008). Spread of Fluorogold into other thalamic nuclei, which occurred in all cases, did not cause problems in interpretation of results, since the aim was to label all lamina I spinothalamic cells. As expected, it was found that extension into other thalamic nuclei did not significantly influence the number of spinothalamic neurons in lamina I as the mean numbers of spinothalamic cells observed in the seven experiments (24 cells/600 µm in C7 and 9 cells/1,200 µm in L4) were similar to those obtained in the PoT series (25/600 µm in C7 and 7/1,200 µm in L4).

In the LPb and PAG injection sites, it is necessary to consider the possibility of uptake of CTb by fibres of passage (especially by axons of the spinothalamic tract). Ascending axons from the spinal cord are located ventrally in the medulla (Lund and Webster, 1967; Mehler, 1969). In the pons, axons of lamina I spinoparabrachial neurons, which probably represent collateral branches of the main ascending bundle (McMahon and Wall, 1985), ascend dorsally near the lateral aspect of the brainstem at a rostrocaudal level close to interaural 0, to enter the lateral parabrachial area. These give rise to a substantial plexus that occupies the LPb, together with a smaller projection to the Kölliker-Fuse nucleus (Slugg and Light, 1994; Bernard et al., 1995; Feil and Herbert, 1995). From here, axons travel dorsomedially into the PAG and arborise extensively within its caudal part (Bernard et al., 1995; Feil and Herbert, 1995; Keay et al., 1997). Since injections of CTb into rostral PAG and surrounding regions label very few lamina I neurons (Lima and Coimbra, 1989), it is unlikely that spinothalamic axons reach the thalamus by passing rostrally from PAG. Anterograde tracing studies have not reported a rostral continuation of axons that have entered LPb from the superficial dorsal horn, apart from those that pass into the PAG, and it appears that the parent axons of lamina I spinothalamic tract neurons are located ~500 µm lateral to the external lateral nucleus of the LPb (J.F. Bernard, personal communication). Therefore, it is very unlikely that CTb could have been taken up by spinothalamic axons in experiments PAG 1-4, since these axons are located a considerable distance lateral to the injection sites within the PAG in these experiments.

Because the spinoparabrachial projection from the superficial dorsal horn occupies most of the mediolateral extent of the LPb and extends to its lateral edge, the entire LPb was filled with tracer in experiments Pb 1-3. In Pb 2, there was some extension of CTb lateral to LPb, and it is possible that in this case tracer was taken up by some spinothalamic axons. However, it is unlikely that this had a significant effect on the results, since CTb did not spread lateral to the LPb in the region occupied by these axons in experiments Pb 1 and Pb 3, and both the numbers of spinoparabrachial cells and the proportion of spinothalamic neurons labelled with CTb were consistent between the three experiments.

The projection from the superficial dorsal horn to the PAG travels through the rostral part of the parabrachial area, as stated above, and it is very likely that these axons give collaterals to the LPb before their final termination within the PAG. However, it is possible that axons of some lamina I cells project to the PAG without first arborising in LPb. If this is the case, and if these cells were labelled through uptake of CTb into their axons within LPb, this would result in an over-estimate of the proportion of spinothalamic neurons that projected to LPb. However, since many fewer cells are labelled from the PAG than from LPb, this is unlikely to have had a major impact on the present results.

Bernard et al. (1995) reported that the main areas in the lateral parabrachial complex that received significant input from lamina I of the C7 segment were: the lateral crescent, dorsal lateral, external lateral (outer part only) and superior lateral subnuclei, and this extended between interaural -0.1 and -0.9 mm. According to the same authors, the caudal and rostral continuation of these subnuclei received a medium to low density of terminals. Slugg and Light (1994) also reported similar results from the superficial dorsal horn of the lumbar cord in the rat. In experiments Pb 1-3, all of the abovementioned lamina I target areas were included in the injection sites; therefore, it is very likely that all (or virtually all) spinoparabrachial neurons in lamina I in the C7 and L4 segments were labelled. In these experiments, some extension of CTb occurred into structures adjacent to the lateral parabrachial area, such as the medial parabrachial, cuneiform and Kölliker Fuse nuclei and locus coeruleus. The Kölliker Fuse nucleus is considered as part of the parabrachial complex (Saper, 1995), and the variable extension of tracer into this region in the present study would therefore not alter the interpretation of the present results. Furthermore, it has been found that the spinal afferents to the Kölliker Fuse nucleus are usually collaterals from axons terminating in the parabrachial nuclei (Bernard et al., 1995). It is unlikely that the spread of tracer into the other structures has affected the results since Bernard et al. (1995) and Slugg and Light (1994) did not find significant anterograde labelling in these

areas after injecting PHA-L into the superficial laminae of rat cervical and lumbar enlargements. Furthermore, injection of WGA-HRP into regions surrounding the lateral parabrachial area (including the locus coeruleus) failed to label lamina I cells in the spinal cord of the rat (Cechetto et al., 1985). The slight spread of tracer into the PAG in Pb 2 is likely to have labelled some spino-PAG cells in lamina I. However, it has been found that 97% of the spino-PAG cells in rat lumbar lamina I were also labelled from the LPb (Spike et al., 2003), and it is therefore unlikely that this spread has led to an overestimation of the number of spinoparabrachial cells in this case.

It has been reported that the main PAG termination zone for lamina I neurons in rat is the ventral part of the lateral quadrants (Bernard et al., 1995). This projection was found to target mainly the caudal part of the PAG (Bernard et al., 1995; Craig, 1995; Mouton and Holstege, 1998). In PAG 1-4, this area in the PAG was adequately filled. The slight dorsal extension of CTb into the superior colliculus in PAG 1 and PAG 3 should not result in a detrimental effect on the present findings because spinotectal projection from lamina I neurons has not been described in the rat, and injections restricted to superior colliculus failed to label neurons in the spinal cord (Beitz, 1982; Menétrey et al., 1982). Moreover, Mouton and Holstege (1998) injected WGA-HRP into the deep layers of the superior colliculus in cat and found no significant retrograde labelling in lamina I.

It is important to emphasize that although all measures and precautions were taken in order to label all neurons (by selecting the efficient tracers and filling the intended targets), underestimation of the numbers of retrogradely labelled neurons can not be ruled out. This is because there is no guarantee that all afferents will be labelled by the tracer, as pointed out by Schofield et al. (2007).

4.5.2 Quantitative comparison of the spinothalamic, spinoparabrachial and spino-PAG lamina I neurons in the two enlargements

The results of the present study suggest that compared to L4, C7 contains fewer spinopara-brachial cells, a similar number of spino-PAG cells and many more spinothalamic cells in lamina I.

4.5.2.1 Numbers of spinothalamic lamina I cells

Data from the second part of this study (Pb and PAG series) showed that the estimated total number of spinothalamic neurons in lamina I on the contralateral side of the C7 segment is 93 cells, and 19 such cells in the L4 segment. This estimation is close to what was found in the first part of the study (Thal, PoT and DI series; 90 cells in C7 and 15 in L4; Al-Khater et al., 2008). If data from all 28 experiments were pooled, then these numbers will be 91 and 16 cells in the C7 and L4 segments, respectively.

The present finding of higher numbers of spinothalamic lamina I cells in the cervical than lumbar enlargement has been documented previously in the rat (Kevetter and Willis, 1983; Harmann et al., 1988; Burstein et al., 1990a; Dado and Giesler, 1990; Kobayashi, 1998), cat (Zhang et al,. 1996; Klop et al., 2005a) and monkey (Zhang and Craig, 1997). In part, this presumably reflects the greater representation of forelimb compared to hindlimb in the somatosensory cortex (Kaas, 1983; Remple et al., 2003). However, the difference appears to be considerably greater in the rat, since Zhang and Craig (1997) found approximately twice as many lamina I spinothalamic cells in C6-8 as in L5-7 in the monkey, while the present results indicate that the difference between C7 and L4 in rat is more than four-fold. The difference between cervical and lumbar enlargements may be less dramatic for neurons that project to the VPL nucleus and thus convey information to S1 cortex, since some lamina I spinothalamic neurons in rat cervical enlargement project only to PoT or surrounding regions (Zhang and Giesler, 2005; present study), while it is unlikely to be the case for those in the lumbar enlargement, as described above.

Zhang et al. (2006) reported that cervical lamina I neurons projecting to VPL often had large receptive fields, covering several digits and extending proximally on the limb. If this is also the case for lumbar cells, it may explain why so few are needed to provide input to S1 cortex from the entire dermatome. Nonetheless, the larger number of spinothalamic lamina I cells in cervical segments presumably results in more accurate stimulus localisation in forelimb compared to hindlimb. Nociceptive information from the hindlimb is also thought to reach the brain through short-fibre mutlisynaptic pathways (Basbaum, 1973), and these presumably supplement direct pathways, such as the spinothalamic tract. In addition, it has been suggested that lamina I spinothalamic cells in the lumbar cord project indirectly to thalamus by first relaying in the upper cervical segments (Kemplay and Webster, 1986). Liu (1986) suggested that spinothalamic neurons in rat lamina I are far less numerous in the lumbar cord because these first relay in the reticular formation in the

brainstem, which project to the PAG and then to the thalamus. Direct projection from caudal medulla to the thalamus has been also demonstrated by Villanueva et al. (1998).

4.5.2.2 Numbers of spinoparabrachial lamina I cells

The present data showed that there are approximately 46 spinoparabrachial neurons on the contralateral side of lamina I per 600 µm of the C7 segment, and the estimated total number of these cells in the entire segment is therefore 176 cells. Interestingly, this number is half that found in the lumbar cord (see below). Hylden et al. (1989) reported a mean of 7.2 spinoparabrachial lamina I cells per 50 µm section (approximately equivalent to 331 neurons per side in the C7 segment). On the other hand, in the current study, 80 spinoparabrachial neurons were found on the contralateral side of lamina I per 600 µm in L4, equivalent to 335 cells in the entire segment. This estimation is in agreement with previous reports by Todd et al. (2000) and Spike et al. (2003), but different from that found by Hylden et al. (1989). The latter authors reported a mean number of 11 retrogradely labelled cells on the contralateral side of lamina I per 50 µm sections, which is equivalent to 550 neurons in the entire L4 segment. Spike et al. (2003) attributed the discrepancy between their results and that of Hylden et al. to technical factors, as Hylden et al. did not correct for the presence of transected neurons at section surfaces. This latter explanation could have attributed to the higher number of spinoparabrachial cells in the cervical cord that was reported by Hylden et al. compared to the present study. Data presented by Kitamura et al. (1993) are difficult to compare with present results as they estimated the total number of spinoparabrachial lamina I cells in a total of six adjacent segments (C3-8 and L1-6), and it is incorrect to assume that this total is contributed equally by the six segments because plots of spinoparabrachial cells in Figs. 3 and 5 in their paper show that the contribution by C3 was more than that by C6. Nonetheless, assuming that the reported mean is valid for the C7 and L4 segments, then the present estimation is much higher than that reported by Kitamura et al. (1993). They found 32 spinoparabrachial neurons on the contralateral side of lamina I per 1 mm thickness in each segment of C3-8 in one rat that had received tracer injection centred on the internal lateral nucleus and a mean of six cells only in another rat that had received an injection targeted on other lateral parabrachial nuclei. Their corresponding means in the L1-6 segments were 26 and 13 cells per 1 mm, respectively, in these two rats. Probably CTb (used in the present study) is a more efficient tracer than Fast blue used by Kitamura et al. (1993); therefore, more retrograde labelling was found in lamina I in both segments in this study. Furthermore, the findings by Kitamura et al. (1993) are not in accord with other studies (Slugg and Light, 1994; Bernard

et al., 1995; Feil and Herbert, 1995), as the quantitative data presented by Kitamura et al. showed that there were more spinoparabrachial cells in lamina I following injections that targeted the internal lateral subnucleus than following injections that were centred on other subnuclei (see above). Bernard et al. (1995) disagreed with this interpretation and attributed the findings by Kitamura et al. to the spread of tracer into adjacent parabrachial nuclei that receive input from lamina I.

It is unlikely that the lower number of spinoparabrachial cells in the C7 segment (compared to L4) found in this study is due to failure to label a significant number of these cells, since projections from superficial dorsal horn of lumbar and cervical enlargements terminate in similar areas of LPb (Slugg and Light, 1994; Bernard et al., 1995; Feil and Herbert, 1995; Saper, 1995), which were included in the injection sites in this study. It is therefore likely that the great majority of lamina I spinoparabrachial neurons were labelled in these experiments, and that the lower number in C7 is genuine. In support of this, it was found that virtually all of the lamina I spinothalamic neurons in C7 were also labelled from LPb. If a significant number of spinoparabrachial cells had been missed, then a much larger number of spinothalamic cells would have been expected to be retrogradely labelled only from thalamus in these experiments. The lower number of spinoparabrachial cells in C7 is not due to a difference in the size of lamina I, as the mediolateral extent of the dorsal horn is similar in C7 and L4. The present observation is consistent with that of Hylden et al. (1989), who reported a lower number of spinoparabrachial lamina I cells in cervical than in lumbar enlargement. It seems that the situation is different in the cat since Klop et al. (2005b) reported higher numbers of retrogradely labelled lamina I cells in cervical enlargement compared to lumbosacral enlargement in two cats that had received tracer injections into LPb.

The difference between numbers of spinoparabrachial lamina I cells in the two enlargements may reflect relative sizes of dermatomes, since the L4 dermatome is considerably larger than that of C7 (Takahashi and Nakajima, 1996). The main outputs from regions of LPb innervated by lamina I neurons are the amygdala and hypothalamus, which are thought to play a role in affective and autonomic aspects of pain (Bernard et al., 1996). Although the major targets of PoT are S2 and insular cortices, it also projects to the amygdala (Ottersen and Ben-Ari, 1979; Gauriau and Bernard, 2004b), which will presumably receive a much smaller input through the spinothalamic tract from the lumbar enlargement. In addition, it has been reported that the number of spinohypothalamic lamina I neurons is considerably lower in lumbar than in cervical segments (Burstein et al.,

1990b); although the existence of this projection is debated (see section 4.2.1). The larger number of spinoparabrachial cells in lumbar cord may therefore compensate for the reduced input that this region provides to both amygdala and hypothalamus through spinothalamic and spinohypothalamic tracts.

In agreement with other studies (Ding et al., 1995; Todd et al., 2000; Spike et al., 2003), it was found that most of spinoparabrachial neurons in lamina I expressed the NK1 receptor.

4.5.2.3 Numbers of spino-PAG lamina I cells

The present data showed that there are 22 spino-PAG lamina I cells on the contralateral side per 600 µm of the C7 segment. This is equivalent to 83 cells in the entire segment. Interestingly, a similar number was found in L4: 41 spino-PAG lamina I cells were observed on the contralateral side per 1,200 µm, this is equivalent to 86 cells in the entire segment. Data for the lumbar cord are consistent with those of Todd et al. (2000). However, these estimations are approximately half those reported by Li et al. (1998). The latter authors found 51 cells on the contralateral side of lamina I per 800 µm of the rat C7 segment and 42 such cells were observed in the L5 segment. It is likely that Li et al. (1998) overestimated these numbers as they did not state whether they corrected for over-counting of labelled cells. Spike et al. (2003) estimated the total number of spino-PAG cells in lamina I per side of the L4 segment in the rat to be 117 cells. This estimation is not likely to be significantly different from the present result because individual values for each experiment in their study are generally similar to the present data, apart from one rat that yielded an unexpectedly high number (49 cells per 700 µm); conversely, one of the rats of this study (PAG 2) had a relatively low number of spino-PAG cells (17 cells per 700 µm). The reason for this variability between results may be the random distribution of the spino-PAG cells in the analysed sections.

The present observation that similar numbers of lamina I spino-PAG cells were present in C7 and L4 is consistent with the report by Keay et al. (1997) who found a similar density of labelled superficial dorsal horn neurons per section in C5-8 and L4-5 regions following injections of retrograde tracer into either ventrolateral or lateral columns of PAG. The similarity in numbers of spino-PAG cells in C7 and L4 presumably reflects an equivalent importance of the two segments in activating anti-nociceptive and other coping mechanisms. Mouton and Holstege (2000) analysed numbers of spino-PAG neurons throughout the cat spinal cord and identified large numbers of labelled lamina I cells in

both cervical and lumbosacral enlargements, although in several cases the cells were more frequent in the lumbar segments.

In agreement with other studies (Todd et al., 2000; Spike et al., 2003), it was found that most of spino-PAG neurons in lamina I expressed the NK1 receptor. Surprisingly, Li et al. (1998) reported that only 13% of spino-PAG cells in lamina I of rat spinal cord showed immunoreactivity to the NK1 receptor.

4.5.2.4 Quantitative comparison of the different projection populations

At both segmental levels, spinothalamic cells in lamina I are much less numerous than spinoparabrachial cells in this lamina. This difference is well documented by previous studies (Hylden et al., 1989; Feil and Herbert, 1995). After targetting the LPb, PAG or thalamus in six different cats, Klop et al. (2005b) found that there are four times as many lamina I neurons projecting to the LPb and twice as many to the PAG as to the thalamus. Their estimated numbers for the entire spinal cord on both sides were 5,939; 3,026; and 1,398 lamina I neurons projecting to the LPb, PAG and to the thalamus, respectively. In a previous report, Mouton and Holstege (1998) reported that there are three times as many lamina I neurons projecting to the PAG as to the thalamus. Klop et al. (2005b) argued that their study is more accurate since the numbers of lamina I cells projecting to the PAG and thalamus have been compared using the same tracer, the same histological procedure and the same counting method, unlike that of Mouton and Holstege (1998) who compared their own results for spino-PAG projection with those of Zhang et al. (1996) for the spinothalamic cells. Nevertheless, this conclusion was reached by comparing the total numbers of the projection populations in the entire cord, a situation that might not be true for each individual segment. In the present study, there was very little difference between the numbers of spino-PAG and spinothalamic lamina I cells in the cervical cord. However, there were more than four times as many spino-PAG as spinothalamic cells in the lumbar cord. It has been reported that retrograde labelling of lamina I neurons following injections that targetted the CVLM was similar to that seen after LPb injections in the rat (Spike et al., 2003). However, because the main ascending bundle of axons belonging to lamina I projection neurons is located near the region of the CVLM, uptake of tracer by fibres of passage can not be ruled out in the study by Spike et al. (2003). The prominent projection of lamina I neurons to the brainstem, compared to the thalamus, has led Klop et al. (2005b) to support the notion that pain has a homeostatic emotional component, as suggested by

Gauriau and Bernard (2002) and Craig (2003b), and they recommended that more attention be given to this pathway as an important part of the nociceptive system.

4.5.3 Collateral projection from lamina I spinothalamic neurons to the LPb and PAG

4.5.3.1 Projection to thalamus and LPb

The present study showed that there is almost complete overlap between the spinothalamic and spinoparabrachial populations in lamina I in the C7 and L4 segments; i.e. the spinothalamic neurons represented a subset of the spinoparabrachial population. The proportions of spinothalamic cells that were also labelled from LPb in the present study were somewhat higher than those reported by Hylden et al. (1989), presumably because they injected the LPb with fluorescent latex microspheres, which give very restricted injection sites, and therefore, it is likely that they did not label all spinoparabrachial cells.

In the present study, a great discrepancy was found between the cervical and lumbar cord in the proportion of the spinoparabrachial population that also belonged to the spinothalamic tract. In the C7 segment, the spinothalamic population represented 45% of the spinoparabrachial neurons in lamina I, whereas it constituted only 6% of those in the L4 segment. This is not surprising since the number of spinothalamic cells in lamina I in rat lumbar cord is very small compared to that of spinoparabrachial cells. Hylden et al. (1989) found that 34% of spinoparabrachial cells in the cervical enlargement were labelled from thalamus. This is slightly lower than the proportion observed in the present study and may have resulted from incomplete labelling of the spinothalamic neurons in their study. Surprisingly, Hylden et al. reported that 31% of lumbar spinoparabrachial lamina I neurons projected to thalamus, and this is difficult to explain, probably this have resulted from the sampling method used by these authors. The present finding that relatively few spinoparabrachial cells in this region project to thalamus is consistent with the report by McMahon and Wall (1985), who examined axons of 13 lamina I projection neurons in rat lumbar enlargement and found that all of them gave collaterals to midbrain (including PAG and an area that corresponds to LPb), while none could be activated from more rostral areas.

4.5.3.2 Projection to thalamus and PAG

Previous studies in rat have indicated that some lamina I cells project to both thalamus and PAG (Liu, 1986; Harmann et al., 1988). In the present study, a much higher degree of double-labelling was observed in the C7 segment (58% of spino-PAG cells and 47% of spinothalamic cells were double-labelled), and a higher proportion of spinothalamic cells labelled from PAG (33%) was found in the L4 segment than had been observed in these earlier studies. These discrepancies are probably due to differences in the injection sites.

Although a degree of collateralisation to the thalamus and PAG was found in the lumbar lamina I neurons, the exact proportion of cells that project to both areas in this segment is difficult to determine because of the low number of lamina I spinothalamic cells in rat lumbar cord. Concerning the proportion of spino-PAG cells among the spinothalamic population in lamina I, although a significant proportion was found in PAG 1 and PAG 3 (67% and 47%, respectively, a mean of 57%), this percentage dropped to 20% and 0% in PAG 2 and PAG 4, respectively. This high variability was also observed in the converse situation, i.e. the proportion of the spinothalamic cells among the entire population of spino-PAG neurons in lamina I. These percentages were 17% and 14% for PAG 1 and PAG 3, but 3% and 0% for PAG 2 and PAG 4, respectively. It is very likely that the reason for the low proportions seen in PAG 2 and PAG 4 is the extremely low number of spinothalamic cells found in these two experiments. However, this finding of a low number of spinothalamic cells is not likely to be due to failure of the injection to cover the PoT, because the expected number of spinothalamic lamina I cells were observed in the C7 segment in these two cases. It is therefore likely to be due to the sampling process. Despite this variability in results, this series of experiments showed that a significant proportion of the spinothalamic cells in rat lumbar lamina I possess collateral projection to the PAG of the same side as the thalamus. On the other hand, only a minority of the spino-PAG cells in rat lumbar lamina I send collaterals to the thalamus.

In the C7 segment, despite the high degree of consistency in the numbers of spino-PAG cells seen in lamina I (especially in PAG 2-4), the proportion of the spinothalamic cells that was also labelled from the PAG was relatively lower in PAG 3 (28% only). This lower proportion might reflect random variation due to the sampling process, or could be a genuine difference due to difference in PAG injection site. After examination of the PAG injection sites, it was found that the CTb did not spread into the lateral portion of the PAG at rostral levels (interaural 1.9 to 2.1 mm) in PAG 3, unlike the situation in the other three cases. It is possible that this region is selectively targetted by spino-PAG cells that also

belong to the spinothalamic population, and lack of tracer spread into this region in PAG 3 has led to the smaller number of double-labelled cells seen in this experiment. This would suggest that some lamina I cells that project to both thalamus and PAG target a different region of the PAG compared to lamina I cells that project only to the PAG; however, there is no evidence to support this suggestion.

The present finding that virtually all spinothalamic lamina I neurons were labelled from LPb and that a significant proportion were labelled from PAG suggests that nearly half of the spinothalamic lamina I cells in C7 and almost a third of those in L4 send collateral branches to both LPb and PAG.

In the cat, Hylden et al. (1986a) antidromically activated eight cells in lamina I in the lumbosacral cord from midbrain (lateral PAG/ nucleus cuneiformis) and thalamus, and eight other cells were activated from the thalamus but not from the midbrain. Interestingly, in the monkey, Yezierski et al. (1987) found that the axonal conduction velocities and the receptive field properties of cells that projected to both thalamus and midbrain were different from those that projected to midbrain only. They reported that the mean conduction velocity of cells activated from the midbrain only was lower than that for cells activated from both thalamus and midbrain. The receptive fields for cells activated from midbrain only were complex, whereas those of cells activated from thalamus and midbrain were confined to a single limb.

4.5.3.3 Functional significance of collateral projections

Processing of painful stimuli at higher brain centres is poorly understood. Studies referred to the parallel processing of painful stimuli by more than one set of central neurons, which eventually leads to changes in affect, level of alertness, behaviour and memory. In line with this, it has been known that individual lamina I neurons send axons to multiple brain areas, in other words, these cells distribute information to several higher brain centres. The present findings of collateral projection of lamina I neurons to more than one brain target are in agreement with this view. Collateral projection of lamina I neurons to the thalamus, LPb and PAG indicates that individual lamina I neurons may contribute to several different functions, including discriminative and affective aspects of pain (through projections to thalamus and LPb), as well as activation of descending modulatory influences (through projections to PAG).

Since it was found that virtually all spinothalamic neurons in lamina I were labelled from the parabrachial area, then it follows that all reported functional properties of spinoparabrachial cells (e.g. responsiveness to stimuli, receptive fields properties) are likely to be valid for the spinothalamic neurons in this lamina, and this has also been suggested previously by Hylden et al. (1989).

4.5.4 Morphology, soma size and NK1r expression of spinothalamic and spinoparabrachial neurons in lamina I

One of the goals in recent research work has been to classify lamina I neurons into recognisable populations that share specific features based on morphology, functional properties, synaptic inputs or projection targets. Therefore, there have been several attempts to correlate the morphology of lamina I neurons with projection targets (Lima and Coimbra, 1989; Lima et al., 1991), or with functional properties (Han et al., 1998).

One of the aims of the present study was to determine whether spinothalamic lamina I cells in rat cervical and lumbar cord differ from spinoparabrachial cells that do not project to the thalamus in terms of morphology, soma size and strength of NK1r expression. Previous studies have already shown that all three morphological types of lamina I neurons (fusiform, pyramidal and multipolar) in the rat do project to each of these two targets (thalamus and parabrachial area) (Spike et al., 2003; Todd et al., 2005; Yu et al., 2005; Almarestani et al., 2007). However, none of these studies have addressed this question directly by carrying out double tracer injection experiments that target thalamus and parabrachial area in the same animal. Comparing these two populations of projection neurons in the same rat is the best approach to answer this question, since it ensures that similar classification criteria were used, in addition to the advantage of the exposure of cells to the same conditions of fixation and tissue processing.

The present study added further evidence against the notion that each morphological type projects primarily to a specific supraspinal target, as proposed by Lima and colleagues (see below). However, this study does show that the contribution of neurons of a specific morphology varies among different pathways. Lima and Coimbra (1988, 1989) and Lima et al. (1991) reported that there is a correlation between the morphology of lamina I projection neurons and the area of termination of their axons within the brain. They reported that fusiform cells project to the LPb or CVLM but not to the thalamus. On the other hand, they found that pyramidal cells project to thalamus, bulbar reticular formation

and the PAG, and the location of these pyramidal cells differs according to the projection target: spinothalamic cells were in the middle third, those projecting to the bulbar reticular formation were in the medial third and spino-PAG cells were present along the entire mediolateral extent of the lamina. This correlation between neuronal morphology and projection target was rejected by Spike et al. (2003) who did not find any significant difference in the morphology of projection neurons retrogradely labelled from CVLM, LPb, or PAG. For example, while Lima et al. (1991) reported that 80% of lamina I neurons that project to the CVLM were fusiform, Spike et al. (2003) reported that 35-36% of cells labelled from CVLM in rat were of the fusiform type. There is no clear explanation for the differences in the observations of these studies.

4.5.4.1 Morphology of spinoparabrachial cells

The present study is apparently the first to analyse the morphological types of spinoparabrachial lamina I cells in the cervical cord in the rat. The proportions of the multipolar, pyramidal and fusiform cells in this population were 31%, 22% and 31%, respectively. Very similar results were found in the lumbar cord with proportions of 31%, 27% and 35%, respectively. This finding in the lumbar cord is in good agreement with that reported by Spike et al. (2003) and Almarestani et al. (2007).

4.5.4.2 Morphology of spinothalamic cells

The proportions of the multipolar, pyramidal and fusiform spinothalamic lamina I cells in the cervical enlargement found in the present study were 37%, 25% and 25%, respectively. With regards to the proportion of fusiform cells, the present finding is in between two extremes: Lima and Coimbra (1988) reported that almost none of the spinothalamic cells were fusiform, while Yu et al. (2005) found that approximately half of the spinothalamic cells were of this type. A possible explanation for the discrepancy between results of these three studies is the subjective nature of the morphological classification of neurons. A specific problem is that many neurons appear borderline between two classes. In order to overcome this potential problem, these proportions were re-calculated considering only cells with a definite morphological type and ignoring those that appeared equivocal. Interestingly, this demonstrated that the three basic morphological types had almost equal contribution to the spinothalamic population in the cervical enlargement: 34% were multipolar, 32% were pyramidal and 32% were fusiform. Therefore, it was concluded that the fusiform class is neither absent, as reported by Lima and Coimbra (1988), nor forms the major morphological type, as concluded by Yu et al. (2005). This agrees with findings

in cat since Zhang et al. (1996) reported that fusiform cells constituted between 20-30% of all spinothalamic cells in lamina I of the enlargements. Similarly, Andrew et al. (2003) have shown that fusiform cells represented 36% of lamina I spinothalamic neurons in cat, whereas the pyramidal and multipolar formed 30% and 26% of these cells, respectively. An interesting observation made by Zhang et al. (1996) concerns the longitudinal distribution of the three morphological types of lamina I spinothalamic cells along the spinal cord. They observed that fusiform cells formed the great majority (about 60%) of those in the upper cervical and thoracic cord, whereas this cell type constituted only 20-30% of the spinothalamic population in the cervical and lumbar enlargements. As a result these authors suggested that this differential distribution of the three morphological types has a functional significance. Findings in the monkey agree with the latter statement concerning the differential longitudinal distribution of the three morphological types of lamina I spinothalamic neurons (Zhang and Craig, 1997). Although the morphology of lamina I spinothalamic neurons in the upper cervical or thoracic cord was not analysed in this study, the present observation of relatively low percentage of fusiform spinothalamic cells in lamina I (especially in the lumbar cord) suggests that this longitudinal distribution of the three morphological types could also be present in the rat.

As to the lumbar cord, during the course of the analysis, the sample size of the spinothalamic cells in the L3 segment was found to be small (a total of 21 cells in the three LPb experiments). Therefore, the sample was increased by adding lamina I spinothalamic cells from horizontal sections of the L5 segment. The multipolar, pyramidal and fusiform cells constituted 58%, 22% and 16%, respectively, of the spinothalamic population in lamina I in the L3 and L5 segments combined. This result differs from that reported by Yu et al. (2005), who found an almost equal representation of the three types in rat lumbar cord. Interestingly, the proportion of the multipolar spinothalamic cells in lumbar lamina I was higher than that in the cervical enlargement. In the monkey, Zhang and Craig (1997) observed that the multipolar lamina I spinothalamic cells were more frequent in the lumbar than in the cervical enlargement. However, this was not found in the cat, in which the proportion of the multipolar cells was the same in both enlargements (Zhang et al., 1996).

4.5.4.3 Comparison of the spinothalamic and spinoparabrachial cells

The present results indicate that spinothalamic lamina I cells differed from other neurons in this lamina labelled from LPb in terms of morphology, soma size and (in the C8 segment)

strength of NK1r expression. A significantly greater proportion of the spinothalamic cells were multipolar, particularly in the lumbar region, where multipolar cells made up 58% of the population. One reason for the high proportion of multipolar spinothalamic cells in lumbar enlargement is that large gephyrin-coated cells (most of which are multipolar) make up ~21% of the lamina I spinothalamic population at this level. These are also present in cervical enlargement, but are greatly outnumbered by other spinothalamic cells, and can not therefore account for the relatively high proportion of multipolar cells in this region. Spinothalamic lamina I cells also had significantly larger somata than other cells labelled from LPb. Again, this is partly due to the presence of large gephyrin-coated neurons within this population, but when cells that were negative or very weak for NK1r (which should account for all of the gephyrin-coated cells) were excluded, a significant difference in soma size was still found. Electrophysiological studies in the rat suggest that spinothalamic lamina I neurons generally have larger receptive fields than those of spinoparabrachial neurons (Bester et al., 2000; Zhang and Giesler, 2005; Zhang et al., 2006). Bester et al. (2000), who analysed 53 spinoparabrachial neurons in lumbar enlargement, reported that these had receptive fields restricted to one or two digits and the present results suggest that few of these would have belonged to the spinothalamic tract. Since the primary afferent input to the dorsal horn is somatotopically organised, receptive field size of a lamina I neuron is probably correlated with the extent of the dendritic tree. It is therefore likely that the larger size of cell bodies of spinothalamic neurons is at least partly accounted for by the larger dendritic trees of these cells compared to those that project only to LPb.

In the C8 segment, the strength of NK1r-immunoreactivity was significantly higher among spinothalamic neurons. It has been reported that lamina I cells with strong NK1r-immunoreactivity are under-represented among the population that project to the PAG (Spike et al., 2003). Taken together, these results indicate that different sub-populations of lamina I cells differ in their supraspinal projections. Willcockson et al. (1984) investigated the actions of substance P on primate spinothalamic cells in the dorsal horn, and reported that this peptide produced complex effects on these cells (excitatory, inhibitory, no effect, biphasic effect or multiple effects). These authors assumed that this observation was due to the presence of multiple substance P receptors in the spinal cord. It is possible that the effect of substance P observed in that study was influenced by the differences in the strength of expression of NK1 receptor on spinothalamic cells.

Han et al. (1998) reported that there was a close relationship between morphology and function for lamina I neurons in cat spinal cord, with pyramidal cells responding only to innocuous cooling, and those in the other two morphological classes being activated by noxious stimuli. Consistent with this suggestion, Yu et al. (2005) and Almarestani et al. (2007) reported that pyramidal cells projecting to thalamus or LPb in the rat were seldom NK1r-immunoreactive. However, although cooling-sensitive lamina I spinothalamic neurons have been identified in the rat, these cells also responded to noxious mechanical stimuli (Zhang et al., 2006). If these are included among the pyramidal class, these may correspond to some of the NK1r-positive pyramidal spinothalamic neurons seen in the present study. However, it is unlikely that all pyramidal lamina I neurons in the rat respond to innocuous cooling, since 23-30% of lumbar spinoparabrachial cells are pyramidal (Spike et al., 2003; Almarestani et al., 2007; present study), and it has been reported that lamina I spinoparabrachial neurons were not activated by innocuous cooling (Bester et al., 2000). Nevertheless, Todd et al. (2005) have obtained evidence for functional differences related to morphology in lamina I of rat spinal cord: among NK1r-positive projection neurons, multipolar cells were more likely to express Fos in response to noxious cold stimuli than cells belonging to the other morphological classes. However, functional differences among multipolar lamina I cells have been also found: although the great majority of both gephyrin-coated and NK1r-expressing multipolar projection neurons up-regulated Fos following subcutaneous formalin injection (Puskár et al., 2001; Todd et al., 2002), only 38% of the gephyrin-coated cells did so in response to a noxious heat stimulus that caused Fos expression in 85% of the NK1r-immunoreactive multipolar cells (Polgár et al., 2008). This suggests that while the great majority of lamina I projection neurons in the rat respond to noxious stimuli, there may be differences in the types of information transmitted to different supraspinal targets by sub-populations of these cells.

4.6 Large gephyrin-coated cells

The present study showed that the "large gephyrin-coated cells" are also present in the cervical enlargement but these were fewer than those in the lumbar part of the cord. It is not clear whether this difference in number has a functional significance. As reported previously (Puskár et al., 2001), most cells of this type in the lumbar cord were labelled from the parabrachial area, and the present study showed that most of these cells in the cervical enlargement also project to the parabrachial area. Therefore, it is likely that the parabrachial region, an area thought to be important in the affective, emotional and autonomic responses to noxious stimulation, plays an important role in the circuitry

involving these cells at both spinal levels. However, an interesting difference between the two levels, with regard to the supraspinal projection of these cells, is related to the projection to the PAG: while approximately half of the "large gephyrin-coated cells" in the C6 segment projected to the PAG, only a minority (6%) did so in L5. The functional significance of this differential projection deserves further investigations in the future. The proportion of the cervical gephyrin-coated cells that projected to the thalamus was also higher than that observed in the lumbar cord, and this is in line with the magnitude of thalamic projection from lamina I at both spinal levels. An interesting finding is that the "large gephyrin-coated cells" constitute approximately 21% of the spinothalamic population in lamina I in the lumbar cord. Therefore, although the gephyrin-coated neurons make up only 0.2% of the total neuronal population in lamina I, which is estimated to be ~6200 cells in L4 (Spike et al., 2003; Al-Khater et al., 2008), and approximately 2.5% of projection neurons in this lamina, they constitute a much higher proportion (~21%) of the spinothalamic population. Given the significance of the spinothalamic tract in pain transmission, it is likely that despite the small number of these cells, they play an important role in transmission of nociceptive information to cortical areas involved in pain perception. The gephyrin-coated cells might contribute to the normal responses to acute noxious stimuli in the model used by Mantyh et al. (1997), in which they gave intrathecal injection of substance P conjugated to saporin in order to destroy NK1r-expressing neurons, because these cells lack the NK1 receptor, and thus probably survive this treatment.

The "large gephyrin-coated cells" represent a distinctive population within lamina I because of the highly specific input that they receive from different sources. Puskár et al. (2001) found that these cells receive a large number of contacts from glutamic acid decarboxylase (GAD)-immunoreactive boutons (at GABAergic synapses). Some of these boutons were immunoreactive for the neuronal glycine transporter (GLYT2) and/or the neuronal isoform of nitric oxide synthase (NOS). The innervation by NOS-containing axons was selective for these cells, compared to other neurons in the dorsal horn (Puskár et al., 2001). Polgár et al. (2008) reported that these cells receive a very large number of contacts from VGLUT2-immunoreactive boutons that are associated with GluR4-containing puncta (i.e. these were glutamatergic synapses). It has been suggested that the main source of VGLUT2-immunoreactive boutons is likely to be excitatory interneurons because these express VGLUT2 (Todd et al., 2003). VGLUT2 is also expressed by Aδ nociceptive primary afferents in lamina I (Todd et al., 2003), but these are unlikely to be the source of these boutons because injection of CTb into sciatic nerve showed that

gephyrin-coated cells received few contacts from this class of afferents. However, indirect input from Aδ afferents, by relaying first on interneurons, can not be ruled out. The gephyrin-coated cells also differ from NK1r-positive projection neurons in this lamina in having a much lower density of contacts from peptidergic primary afferents (Polgár et al., 2008).

The "large gephyrin-coated cells" have large, widespread dendritic trees, and since the primary afferent input to the dorsal horn is somatotopically arranged (Willis and Coggeshall, 2004), they are likely to have large peripheral receptive fields. This suggests that they may have an important role in the affective and autonomic responses to pain, rather than in providing information about accurate stimulus localization. The fact that they form a relatively small population and project to the parabrachial area is in favor of this suggestion.

Investigations that used anatomical markers for detection of neuronal activation, such as Fos, have shown that the gephyrin-coated neurons respond to noxious stimuli, but this activation depends on the type of the noxious stimulus. Most cells of this type upregulated Fos following subcutaneous formalin injection (Puskár et al., 2001). However, noxious heat stimulation caused Fos upregulation in only 38% of these cells (Polgár et al., 2008). Interestingly, this latter study showed that the same noxious heat stimulus led to activation of 85% of the NK1r-immunoreactive multipolar cells in lamina I. This suggests that the gephyrin-coated cells subserve a specific function within lamina I different from other multipolar cells in this lamina.

4.7 The large NK1r-immunoreactive lamina III/IV neurons

4.7.1 General remarks

Lamina III/IV NK1r-immunoreactive neurons with long dorsal dendrites represent a distinctive population within rat dorsal horn. Cells of this type in the lumbar cord receive strong monosynaptic input from substance P-containing (nociceptive) primary afferents (Naim et al., 1997), and respond to various types of noxious stimuli (Polgár et al., 2007a). In addition, it was found that this population of cells in the cervical enlargement also receive contacts from substance P-containing primary afferents (Al-Khater K.M., unpublished observation).

Quantitative data in the present study showed that these neurons were more numerous in the lumbar (23 cells in L4; 22 cells in L5) compared to the cervical enlargement (16 cells in C6 and C7), and this confirms previous observations reported by Brown et al. (1995). The observed numbers in the lumbar cord are generally in agreement with those reported by Todd et al. (2000). In addition, it appears that the cell bodies of these neurons tend to be located at deeper levels in the lumbar cord (mean 239 µm and 223 µm below dorsal white matter in L4 and L5, respectively) compared to the cervical (mean 161 µm and 164 µm below dorsal white matter in C6 and C7, respectively). Ribeiro-da-Silva and Coimbra (1982) reported that the mean depth of the superficial dorsal horn (laminae I and II) in the rat is 100 µm and that for lamina III is 90 µm. Therefore, most of cells in this study were located in laminae III and IV; however, a few cells were located less than 100 µm below the dorsal white matter. Ten of these were seen in C6, seven in C7 and three in L5 (pooled from all experiments). These cells are unlikely to belong to lamina II because the location of the somata was checked using dark field optics. The border between laminae II and III can be seen clearly using dark-field illumination because of the lack of myelin in the ventral part of lamina II, and these cell bodies were observed at, or just ventral to, this border; therefore, these were identified as lamina III cells. The deepest cells in this group (deeper than 300 µm) are likely to be in lamina V. Only three cells were found at such a depth in C6 (out of the 317 cells analysed in all 20 experiments, i.e. 0.95%), whereas 48 such cells were found in L5 (out of the 417 neurons analysed in 19 experiments, i.e.11.5%), and 26 cells were observed in L4 (out of the 170 neurons analysed in the seven experiments, i.e. 15.3%). It is likely that lumbar lamina V contains more neurons of this type than the cervical cord. It has been reported that lamina V in rat dorsal horn does contain some large NK1r-immunoreactive neurons with long dorsal dendrites; however, these are much less numerous than those in laminae III and IV, and are more common at caudal spinal levels (Brown et al., 1995).

Another observation in the present study is related to the size of these neurons. It appeared that the cervical population had smaller somata than the lumbar ones, though this size difference was not analysed. This observation is in accordance with findings by Brown et al. (1995). It is plausible to explain this size difference to the fact that the lumbar neurons have longer axons compared to the cervical, a feature that necessitates a larger cell body, although this assumption is not supported by finding that there is no size difference between lamina I projection neurons in the two parts of the cord.

4.7.2 Projection to the thalamus

The present study showed that the great majority of cells in this population in the C6 (85%) and C7 (83%) segments project to the contralateral thalamus. The proportion that project to the thalamus among neurons of this type in the lumbar cord was much lower than that in the cervical cord since only 28% and 17% of these cells in the L4 and L5 segments, respectively, were labelled from the contralateral thalamus. Interestingly, in both segments, this projection was found to target mainly the PoT region.

4.7.2.1 The cervical enlargement

The present finding of a prominent thalamic projection from the large NK1r-immunoreactive lamina III/IV neurons in rat cervical cord has not been documented before. In addition, this study showed that all or most of cells of this type in the C6 segment were labelled from the PoT. This conclusion was reached after finding that the mean values of the percentages of retrogradely labelled cells did not differ significantly between the three series of experiments (Thal, PoT and DI), that some labelled cells were observed in the FLM series and that most cells were not labelled with Fluorogold in the DI series (see below).

In the Thal series, some variability in the proportion of retrogradely labelled cells was observed between the five experiments. All cells of this type were retrogradely labelled in Thal 2, whereas the percentage of the cells that were retrogradely labelled in the other cases was slightly lower. In Thal 1, this could be due to lack of tracer spread into the middle part of PoT; no clear explanation for the lower proportion of retrograde labelling in cells of this type in the other cases (Thal 3-5). As to the PoT series, the proportion of cells that were retrogradely labelled varied from 69-100% (PoT 3-9). This could be explained by variation in the spread of tracer substance. Interestingly, most of cells of this type were retrogradely labelled in experiments PoT 7 and PoT 8, in which only minimal spread of tracer occurred into structures adjacent to the PoT.

Results from the DI series showed that 79% of these cells in the C6 segment project to the contralateral thalamus, consistent with results from Thal and PoT series. The labelling of 85 cells with CTb (out of the 88 cells of this type pooled from all experiments) in the DI series strongly supports the previous observations that these cells could be labelled from the PoT. On the other hand, very few cells of this type were labelled with Fluorogold, except in DI 1 and DI 4, in which the Fluorogold spread into the rostral part of PoT. The

other experiments in this series had extensive filling of the thalamus extending rostrally from interaural 4.6 mm, and including the ventrobasal complex, and variable parts of Po, MD and CL nuclei. The absence of Fluorogold labelling in these cells in these experiments suggests that they do not project significantly to the ventrobasal complex, rostral Po or medial thalamus, and that PoT is their major target. Since only a small number of cells were retrogradely labelled with CTb in experiment PoT 10 (eight out of 21 cells in C6), this suggests that some of these cells presumably have projections that extend into the caudal part of Po. Surprisingly, the pattern of labelling seen in DI 1-4 resembled to a large extent that observed in lamina I cells in the C7 segment in these four experiments. In both cases, all but a few cells were positive for CTb, and cells only labelled with Fluorogold were mainly observed in DI 4. Therefore, a plausible explanation of this variation in the labelling between the four experiments is the same as that suggested for lamina I, i.e. the caudal extension of Fluorogold into the rostral PoT/Po at interaural 4.0 mm is responsible for the labelling with Fluorogold in DI 4. A notable difference between the pattern of labelling in lamina I and that in the population of large lamina III/IV NK1r-immunoreactive cells is that none of the latter were positive for Fluorogold in experiments DI 2 and DI 3, whereas some of the CTb-labelled cells in lamina I of C7 were also labelled with Fluorogold in these two experiments.

Further evidence that cells of this type in the C6 segment project to the PoT was obtained from the FLM experiments, in which 5-10 large NK1r-immunoreactive cells in laminae III and IV were retrogradely labelled in this segment. In most cases the dorsal dendrites of these cells could not be followed into the superficial dorsal horn due to the inferior optical properties of the Gel-Mount medium that was used in these experiments.

Zhang and Giesler (2005) identified spinothalamic neurons in the region corresponding to laminae III and IV in rat cervical enlargement that projected only to caudal thalamus (mainly PoT and PIL), and their sample is likely to have included some of the NK1r-expressing cells with long dorsal dendrites.

These cells have dendrites that arborise extensively in laminae I and II, and some of them would therefore presumably have been labelled following injections of anterograde tracers into the superficial dorsal horn in the study by Gauriau and Bernard (2004a). Although they are outnumbered by lamina I spinothalamic neurons (~91 lamina I spinothalamic neurons in C7 and ~14 lamina III/IV spinothalamic neurons in C6), they are likely to have contributed to the terminal labelling seen in the thalamus following such injections, and

this is consistent with the present finding that they could be labelled following injections into the PoT.

4.7.2.2 The lumbar enlargement

The picture is different in the L5 segment, since the present study showed that only 17% of neurons in this population in L5 projected to the contralateral thalamus. This result agrees with the observations by Marshall et al. (1996) and Naim et al. (1997) who observed that only a few of these neurons were labelled after extensive filling of the contralateral thalamus with tracer substance. Interestingly, as in the C6 segment, this thalamic projection mainly targetted the PoT region. Evidence for this is provided by the finding that the mean percentage of cells that were retrogradely labelled in the PoT series did not differ significantly from that in the Thal experiments. The findings in the DI experiments also support this conclusion, since in these cases very few cells of this type were labelled with Fluorogold, and this labelling occurred in DI 1 and DI 4, in which the Fluorogold spread into the rostral part of PoT. One cell in DI 6 was positive for Fluorogold, but there is no clear explanation for this labelling. The absence of Fluorogold labelling in cells of this type in the other experiments suggests that, like the equivalent cells in C6, they do not project significantly to the ventrobasal complex, rostral Po or medial thalamus, and that PoT is their major target. Since a few cells were labelled with CTb in experiment PoT10 (two out of 24 cells in L5), some of them presumably have projections that extend into the caudal part of Po. Further evidence that cells of this type in the L5 segment project to the PoT was obtained from the FLM experiments, in which four and six NK1r-immunoreactive cells in laminae III and IV were retrogradely labelled in this segment in FLM 2 and FLM 3, respectively.

Several studies have reported that cells of this type in the rat were labelled after tracer injection into different supraspinal areas. Giesler et al. (1979) have recognized dorso-ventrally elongated spinothalamic cells in the nucleus proprius (laminae III and IV) of rat spinal cord, but it is not known whether these cells expressed the NK1 receptor. Li et al. (1996) reported that almost one third of the spinothalamic neurons in lamina III of the C7 and L5 segments in the rat were immunoreactive for the NK1 receptor. They observed NK1r-immunoreactive spinothalamic cells in laminae III and IV after injections that filled the entire thalamus of the contralateral side or injections that targeted the posterior nuclear complex. Injections into the CL nucleus labelled neurons in lamina IV but not in III, whereas injections into nucleus submedius labelled neurons in both laminae. Interestingly, injections that targeted the VPL labelled no NK1r-immunoreactive neurons in laminae III

or IV. However, Li et al. (1996) did not report whether these labelled cells in laminae III and IV possessed long dorsal dendrites. Kobayashi (1998) designated the dorsoventrally-elongated lamina III spinothalamic cells in rat as "large vertical neurons".

This thalamic projection from the large NK1r-immunoreactive neurons in laminae III and IV of rat lumbar cord has been described already, as summarised above. However, the difference between the present results and those of previous reports is the finding that this thalamic projection targets mainly the PoT region. Naim et al. (1997) provided an illustration of the spread of CTb in the thalamus in one rat, and this showed that the PoT was not included in the injection site. Naim et al. reported that the spread of CTb in the other two rats was similar to this one. Nevertheless, they were able to label 2-5 neurons of this type in each rat (total of 10 cells in the three rats). It is possible that tracer extended slightly into the PoT region in the other two rats that were not illustrated in that paper, and this has led to retrograde labelling in three and five cells in these two rats, whereas the illustrated injection site led only to labelling of two cells. Marshall et al. (1996) also conducted their study on three rats and provided illustration of tracer spread in only one rat. The caudal spread of CTb in their diagram reached the rostral PoT at interaural 3.8 mm, but the authors did not comment on tracer spread in the other two rats except by stating that extensive filling of the thalamus was noted. Furthermore, no data were given for the number of labelled cells in this population. Hence, it is difficult to compare their study with the present one.

The present data showed that most of the NK1r-immunoreactive lamina III/IV neurons with long dorsal dendrites that projected to the thalamus were located in the medial half of the dorsal horn in the L4 and L5 segments. It has been documented that the receptive fields of the cutaneous afferents from the hindlimb of the rat are topographically organized in the lumbar cord (Swett and Woolf, 1985). The medial parts of the L4 and L5 segments receive afferents from the plantar surface of the foot and back of the ankle region via the tibial nerve. The lateral parts of these two segments receive input from the lateral side of the leg and thigh through the sural nerve, and from the medial and posterior aspects of the leg and thigh through the posterior cutaneous nerve of the thigh. Afferents from the dorsal surface of the foot that pass through the superficial peroneal nerve, end in the middle area of L4 and middle of the rostral part of L5. Thus, the medial parts of the L4 and L5 segments mainly receive sensation from distal structures in the hindlimb, whereas more proximal regions end in more lateral parts of these segments. It is possible that the tendency of the spinothalamic cells in the population of the NK1r-immunoreactive neurons in laminae III

and IV to lie within the medial half of L4 and L5 has a functional correlation, and this suggests that these distal limb inputs would have preferential access to the thalamus through spinothalamic cells of this type.

4.7.3 Collateral projection to the thalamus, LPb and PAG

In agreement with previous reports (Todd et al., 2000), it was found that approximately 64% of the NK1r-immunoreactive lamina III/IV cells in the L4 segment were labelled from the contralateral parabrachial area. The new information that has been added by this study concerns the extent of overlap between the two projection groups (parabrachial and spinothalamic neurons). Interestingly, the pattern of projection is a mixture of three types: one group projected to the thalamus only, another projected to the parabrachial area only and the third group sent collaterals to both regions. This latter group constituted about half of the spinothalamic cells, and approximately 22% of the spinoparabrachial cells in this population. It is known that virtually all cells of this type in the lumbar cord were labelled from the contralateral CVLM (Todd et al., 2000). However, labelling from the CVLM could be due to uptake of tracer by fibres of passage rather than by terminals, as stated earlier.

Concerning the C7 segment, the present study showed that 87% of neurons in this population projected to the parabrachial area. Because of this high percentage of projection to the parabrachial area and thalamus, it would be expected that some cells send axonal projections to both regions, and indeed this was found in more than 64% of neurons in this population. Interestingly, the size of the projection to the parabrachial area in the cervical enlargement is more than that in the lumbar and this is in line with the projection to the thalamus. It is surprising that Bernard et al. (1995) did not find anterograde labelling in the parabrachial complex after making tracer injection into laminae III and IV of cervical cord in two rats. However, their illustrated diagram shows that these two injections were very small and, as a result, it is unlikely that adequate number of these cells were included in that injection site. However, these authors described a bilateral projection to the mesencephalic portion of the internal lateral subnucleus of LPb area (and to a lesser extent to the PAG) from the lateral part of lamina V. Kitamura et al. (1993) have concurred in this last finding on the basis of their observations that deep dorsal horn neurons (reticular part of lamina V, laminae VII and X) projected mainly to the internal lateral parabrachial nucleus. Because of the complete filling of the parabrachial area in the present experiments, it is not

possible to determine whether the large NK1r-immunoreactive neurons in laminae III and IV targeted a specific subnucleus in the parabrachial complex.

The present study showed that the PAG did not receive significant projection from the NK1r-immunoreactive lamina III/IV neurons in either region of the cord. The observed percentage of projection to the PAG was less than 10%, and this is again in agreement with Todd et al. (2000).

4.7.4 Functional significance

There is a substantial body of evidence that points to the functional significance of the large NK1r-immunoreactive lamina III/IV cells in processing of nociceptive information as elaborated in the Introduction. Therefore, demonstrating that most of these cells in rat cervical cord, and some of those in the lumbar cord, belong to the spinothalamic system would certainly support their nociceptive role. Moreover, the present finding of a prominent projection to the PoT region provides further support for this nociceptive function especially with the accumulating evidence for the role of PoT in pain processing. Lamina III/IV NK1r-immunoreactive neurons receive monosynaptic inputs from myelinated (presumed low-threshold mechanoreceptive) primary afferents in lamina III, although these are far less numerous than those from substance P-containing (nociceptive) afferents (Naim et al., 1997, 1998). These cells are therefore likely to have wide dynamic range receptive fields, and may provide input to those neurons in PoT that respond to noxious and tactile stimuli and project to insular cortex and amygdala (Gauriau and Bernard, 2004b). Since lamina III/IV NK1r-immunoreactive neurons do not appear to project significantly to the ventrobasal complex, it is not likely that their activity is transmitted directly to the primary somatosensory cortex. This is consistent with the small size of this population of cells, which means that they are unlikely to provide accurate information about stimulus localisation, an important function of the primary somatosensory cortex.

The present finding of collateral projection of the large NK1r-immunoreactive lamina III/IV neurons to thalamus and parabrachial area suggests that these cells are capable of distributing information concerning various types of noxious stimuli to more than one brain target. Projection to the parabrachial area allows this type of information to reach the hypothalamus and amygdala, areas thought to be involved mainly in the autonomic and affective emotional aspects of pain processing (Bernard et al., 1996).

Conclusions and Future Directions

The present study highlighted some important aspects regarding the supraspinal projection of neurons in laminae I, III and IV. Using the retrograde neuronal tracing technique, this study showed that the area of the PoT forms an important termination zone not only for lamina I neurons (in the cervical enlargement) but also for the population of large NK1r-immunoreactive neurons in laminae III and IV that possess long dorsal dendrites. However, consistent with previous reports, this study also showed that other thalamic nuclei (presumably mainly the VPL) receive input from lamina I. Fluorogold injections in the DI series of the current study involved several thalamic nuclei; therefore, it was not possible to determine which of these were the targets for axons of lamina I neurons. A recommendation for future studies is to target individual thalamic nuclei (mainly VPL and Po) with very restricted injections using FLM (or other tracer known to give limited spread), as well as targetting the PoT with another tracer in the same experiment. The aim of these studies would be to investigate whether cells that project to VPL or Po are similar or different from those that project to the PoT. This detailed mapping of thalamic projection from lamina I is important for better understanding of the spinothalamic pathway.

The present study showed that virtually all spinothalamic neurons in lamina I in both enlargements project to LPb, but only partial overlap was observed between the spino-PAG and spinothalamic populations in this lamina. Based on the quantitative results, this study hypothesized that lamina I cells that project to the PAG and thalamus target specific areas in the PAG that are different from areas targetted by cells that project to PAG only. This hypothesis could be tested in future studies by performing dual-tracer injection experiments in which one tracer specifically target one column of PAG (and probably at certain rostrocaudal levels) and injecting another tracer into thalamus, and then looking for single- and double-labelled cells in lamina I.

One of the interesting findings in the present study is that the number of the spinoparabrachial cells in lamina I found in the cervical enlargement is smaller than that observed in the lumbar enlargement (unlike the situation with spinothalamic cells). Several explanations have been presented; however, the functional significance of this difference calls for further studies.

The mediolateral distribution of spinothalamic cells among the population of large NK1r-immunoreactive lamina III/IV neurons in the lumbar cord is interesting. This observation need to be confirmed and extended by further studies. For example, retrograde tracing from the thalamus could be combined with induction of pERK or Fos in these cells following noxious stimulation of distal part of the hindlimb to allow comparison of the mediolateral location of double-labelled cells within the dorsal horn to that of double-labedded cells seen following stimulation of more proximal parts of the limb. This would demonstrate whether the somatotopic arrangement of nociceptive input seen in the superficial dorsal horn also applies to NK1r-expressing projection neurons in laminae III and IV. Once this has been confirmed, then a further step is to investigate the functional significance of this differential projection to the thalamus among cells of this type in the dorsal horn.

Results from the present and other studies suggest that the "large gephyrin-coated cells" play a special role in the circuitry of lamina I. Many aspects of this circuitry are still unknown. Therefore, future studies are recommended to address various issues such as:

1) significance of the projection to the PAG from cells of this type in the cervical enlargement compared to the lack of such a projection from those in the lumbar enlargement. Would painful stimuli that activate gephyrin-coated cells from forelimb activate descending modulatory pathways through PAG while inputs from hindlimb use another route to activate these pathways?

2) the suggestion that these cells possess large receptive fields necessitates performing studies that explore receptive field sizes of these cells using pERK or Fos as markers of activation following stimulation (using an effective stimulus) of different cutaneous areas of the body.

3) the response of these cells to other types of noxious stimuli such as noxious cold and mechanical stimuli is still unknown; therefore, these need to be investigated in the future.

4) the precise area of termination of gephyrin-coated cells within the thalamus also deserves special attention in the future.

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