Spinal Cord Neuronal Circuitry Involving Dorsal Horn Projection Cells

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Summary

The spinal cord dorsal horn is involved in the processing and transmission of sensory information to the brain. There are several distinct populations of dorsal horn projection cells that constitute the major output of the spinal cord. These cells are mostly found in lamina I and are scattered throughout the deep dorsal horn. There is a population of large lamina III projection cells that expresses the neurokinin 1 receptor (NK1r), which is the main target for substance P released by nociceptive primary afferents. These cells are densely innervated by peptidergic nociceptive afferents and more sparsely by low-threshold myelinated afferents. In addition, they also receive selective innervation from neuropeptide Y-containing inhibitory interneurons. However, not much is known about their input from glutamatergic spinal neurons. It has already been reported that the great majority of large lamina III NK1r expressing cells project to caudal ventrolateral medulla (CVLM) therefore in this study these cells were easily identified without retrograde tracer injection. Preliminary observations showed that these cells received contacts from preprodynorphin (PPD)-containing excitatory axons. The first part of the study tested the hypothesis that lamina III projection cells are selectively targeted by PPD-containing excitatory spinal neurons. Spinal cord sections from lumbar segments of the rat underwent immunocytochemical processing including combined confocal and electron microscopy to look for the presence of synapses at the sites of contact. The results showed that lamina III NK1r cells received numerous contacts from non-primary boutons that expressed vesicular glutamate transporter 2 (VGLUT2), and formed asymmetrical synapses on their dendrites and cell bodies. These synapses were significantly smaller than those formed by peptidergic afferents but provided a substantial proportion of the glutamatergic input to lamina III NK1r projection cells. Furthermore, it was observed that PPD was found to be present in ~58% of the VGLUT2 boutons that contacted these cells while a considerably smaller proportion of (5-7%) VGLUT2 boutons in laminae I-IV expressed PPD. These results indicate a highly selective targeting of the lamina III projection neurons by glutamatergic neurons that express PPD.

Fine myelinated (Aδ) nociceptors are responsible for the perception of fast, well-localised pain. Very little is known about their postsynaptic targets in the spinal cord, and therefore about their roles in the neuronal circuits that process nociceptive information. In the second part of the study, Fluorogold injections were made into the lateral parabrachial region (LPb) of the rat brain on one side and cholera toxin B subunit (CTb) was injected into the sciatic nerve on the contralateral side to assess whether Aδ nociceptors provide input to

lamina I projection cells. The vast majority of lamina I projection neurons belong to the spinoparabrachial tract, and these can be divided into two major groups: those that express NK1r, and those that do not. The results suggested that CTb labelled a distinct set of Aδ nociceptors, most of which lack neuropeptides. CTb-labelled Aδ afferents formed contacts on 43% of the spinoparabrachial lamina I neurons that lacked the NK1r, but on a significantly smaller proportion (26%) of NK1r projection cells. Combined confocal and electron microscopy established that the contacts were associated with synapses. Furthermore, the contact density of CTb labelled boutons was considerably higher on the $NK1r^-$ cells than on those with the NK1r. These results provide further evidence that primary afferents input to projection cells is organized in a specialized way and that both $NK1r^+$ and $NK1r^-$ lamina I projection neurons are directly innervated by Aδ nociceptors, thus may have an important role in the perception of fast pain.

Lamina I of the rat spinal cord dorsal horn contains a population of large spinoparabrachial projection neurons (giant cells) that receive numerous synapses from both excitatory (VGLUT2) and inhibitory (VGAT) interneurons. The giant cells are selectively innervated by GABAergic axons that express neuronal-nitric oxide synthase (nNOS) and are thought to originate from local inhibitory interneurons. In the rat, the nNOS inhibitory cells belong to a distinct functional population that differs from other inhibitory interneurons in terms of somatostatin receptor (sst_{2A}) expression and also in responsiveness to painful stimuli. There is a population of inhibitory interneurons that express green fluorescent protein (GFP) in lamina II of mice in which GFP is under control of the prion promoter (PrP) and the great majority of these cells also express nNOS. In this part of the study, the inhibitory synaptic input from nNOS-containing GFP boutons to giant lamina I cells was investigated. The great majority of lamina I projection neurons express NK1 receptor; therefore, the possibility that lamina I NK1r-expressing projection neurons received innervation from $GFP^+/nNOS^+$ axons was also tested. Since retrograde tracing technique was not used in this part of the study, lamina I projection cells were identified based on the observations made in the previous studies in the rat. Lamina I giant cells were recognized with antibodies against glycine receptor associated protein gephyrin as well as VGLUT2 and VGAT boutons, all of which provide dense innervation to these cells while only those lamina I NK1cells were included in the sample that were large and strongly immunoreactive for NK1r. The results indicated that although GFP axons accounted for only 7-9% of the GABAergic boutons in superficial dorsal horn, they provided over 70% of the inhibitory synapses on most of the giant cells in the PrP-GFP mouse and the great majority of these boutons also contained nNOS. Moreover, a subset of large lamina I

NK1r-expressing cells (18/60) received a substantial inhibitory input (> 30%) from GFP^+ boutons while the majority of these neurons showed sparse (< 15%) synaptic input.

Recently, it has been reported that loss of some inhibitory interneurons in mice lacking the transcription factor *Bhlhb5* results in exaggerated itch, and the cells that are lost include many of those that would normally express nNOS. Therefore, in the final set of experiments was designed to test whether there is a reduction in the inhibitory synaptic input to the giant cells in *Bhlhb5*$^{-/-}$ mouse. Spinal cord sections from *Bhlhb5*$^{-/-}$ mice and the wild type littermates were processed and analysed to determine any difference in the inhibitory nNOS input to lamina I giant cells belonging to either group. The giant cells from the knockout mice showed a substantial reduction (~80%) in their inhibitory nNOS input; with a moderate reduction in their overall GABAergic input (~35%). There was a considerable increase in $nNOS^-/VGAT^+$ boutons in the *Bhlhb5*$^{-/-}$ mouse (18 ± 4.6 and 37.7 ± 8.2/100 µm of the dendrite in WT and KO, respectively), suggesting some compensation from other nNOS-negative inhibitory interneurons. These results suggest that the loss of nNOS-containing inhibitory synaptic input to lamina I projection cells may contribute to the abnormal scratching behaviour seen in the *Bhlhb5*$^{-/-}$ mouse. This raises the possibility that the giant cells and a subset of large lamina I NK1r-expressing cells are involved in perception of itch.

Acknowledgements

بِسْمِ اللهِ الرَّحْمنِ الرَّحِيمِ

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ربِّ زِدْنِي عِلْما **(20:114)**

My Lord increase me in my knowledge

Author's declaration

I hereby declare that the work presented in this thesis is my own, except the figures 1-2 and 1-3 that have been modified and presented with permission from the review article published by Todd (2010). Furthermore, explicit reference is made to the contribution of others.

The retrograde tracing surgery and sciatic nerve injections were performed by Dr Safa A. Shehab of Al-Ain University, UAE. This thesis has not been submitted in any previous application for any other degree in the University of Glasgow or any other institution.

Dr Najma Baseer

June 2014

Dedication

To my beloved parents & husband; who believed in me more than I did.

List of abbreviations

ALT	anterolateral tract
AMC	A mechano-cold receptors
AMH	A-mechano heat nociceptors
AMPA	α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
Bhlhb5	basic helix-loop-helix
BS IB4	*Bandeiraea simplicifolia* isolectin B4
CGRP	calcitonin gene related peptide
C-LTMRs	C-low threshold mechanoreceptors
CMH	C-mechano-heat sensitive
CTb	cholera toxin subunit B
CVLM	caudal ventrolateral medulla
DRG	dorsal root ganglion
ERK	extracellular signal-related kinase
GABA	gamma-aminobutyric acid
GAD	glutamic acid decarboxylase
GalR	galanin receptor
GFP	green fluorescent protein
GlyT2	glycine transporter 2
HRP	horseradish peroxidase
HTM	high threshold mechanoreceptors
KOR	kappa opioid receptor
LPb	lateral parabrachial nucleus

LTMs	low threshold mechanoreceptors
MIAs	mechanically insensitive afferents
MOR	mu-opioid receptor
Mrgprd	mas-related G-protein coupled receptor member D
NADPH-d	nicotineamide adenine dinucleotide phosphate diaphorase
NF200	neurofilament 200
NK1r	neurokinin 1 receptor
NMDA	N-methyl-D-aspartate receptor
nNOS	neuronal nitric oxide synthase
NTS	nucleus of the solitary tract
PAG	periaqueductal grey matter
PB	phosphate buffer
PBS	phosphate-buffered saline
pERK	phosphorylated form of extracellular signal-related kinase
PKC	protein kinase C
PoT	posterior triangular nucleus
PPD	preprodynorphin
PrP	prion-promoter
PSDC	post-synaptic dorsal column
PV	parvalbumin
SCT	spino-cervicothalamic tract
SDH	superficial dorsal horn
SP	substance P

SP-SAP	substance P conjugated to the cytotoxin saporin
sst_{2A}	somatostatin receptor 2_A
STT	spinothalamic tract
TRPs	transient receptor potential receptors
TSA	tyramide signal amplification
VGAT	vesicular GABA transporter
VGLUT	vesicular glutamate transporter
WGA	wheat germ agglutinin
WT	wild type

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Chapter 1

Introduction

1 Structure and function of spinal dorsal horn

1.1 Primary Afferent fibres

The initial step in somatosensory perception is the activation of primary sensory neurons. The primary sensory neurons of the trunk and limbs have their pseudo-unipolar cell bodies in the dorsal root ganglion. These cell bodies give rise to a single axon that divides into two branches, the central and the peripheral branch. The central branch enters the spinal cord via the dorsal root. Its subsequent course and target site depends on the type of fibre and the sensory information it carries while the peripheral branch communicates with peripheral tissues in the form of free or specialized nerve endings.

The central axons of all the primary afferents split into multiple branches that terminate within the dorsal horn and synapse with second order neurons. In addition, the large myelinated afferents give off projection fibres extending supraspinally or inter-segmentally. The primary afferent fibres can be classified on the basis of their respective target sites, conduction velocity, presence or absence of myelination, diameter, neurochemical characteristics and responses to various stimuli. Based on the peripheral target sites, they are categorized as deep afferents which include visceral, articular and muscle afferents and the superficial afferents that consists of cutaneous fibres innervating skin. The cutaneous afferents are further classified as mechanoreceptors, nociceptors, thermoreceptors and chemoreceptors, depending upon the sensory modality they carry.

The primary afferent fibres are either myelinated or unmyelinated. The myelin sheath allows the action potential to travel with a high velocity. However, about 80% of the cutaneous primary afferent fibres are unmyelinated (Lynn, 1984). On the basis of electrical thresholds, conduction velocity and fibre diameter, primary afferents are classified as:

Large myelinated (Aβ) fibres have a diameter of 6-12μm and they transmit sensory information at a velocity of 35-75m/s in humans. These fibres are very sensitive to mechanical non-painful stimuli; therefore they are also named as low threshold mechanoreceptors (LTM). These cutaneous sensory afferents respond to innocuous mechanical stimuli such as light touch, hair movement and propioception. The firing pattern of LTMRs to a sustained mechanical stimulus varies considerably. Some cutaneous LTMRs are rapidly adapting (RA) while others are intermediate (IA) or slow adapting (SA) (Leem *et al.*, 1993). These afferents target both the glabrous and hairy skin. Glabrous

skin has 4 different types of specialized mechanosensory end organs, namely Pacinian corpuscles, Ruffini endings, Meissner corpuscles and Merkel's discs, while on the hairy skin, the hair follicles function as specialized mechanosensory organs. Some of these fibres also conduct pain signals (Djouhri and Lawson, 2004). They respond to the mechanical stimuli of nociceptive range and exhibit adaptation properties similar to slow adapting LTMRs (Burgess and Perl, 1967, Woodbury and Koerber, 2003). It has been suggested that Aβ fibres may contribute to certain pain states such as allodynia after peripheral nerve injury (Campbell *et al.*, 1988). These observations suggest that the functions of primary afferent fibres are closely related to their sizes; however some degree of overlap exists between the different sub groups on the basis of type and intensity of the stimuli they respond to.

The **A-delta (Aδ) afferents** consist of thinly myelinated fibres that conduct with a velocity of 5-30m/s in humans. They include afferents that have small receptive fields and respond to mechanical and thermal stimuli of nociceptive range (high threshold mechanoreceptors HTM) (Burgess and Perl, 1967) and those that innervate hair follicles (D-hair afferents). The HTM fibres conduct fast pricking or sharp pain and temperature, while the hair afferents are responsive to the slow movement of the hair. The Aδ-mechanoresponsive fibres respond to high threshold mechanical stimulation such as deep pressure, pinch and stretch. These fibres are also called mechano-heat (AMH) nociceptors as they respond to a both noxious heat and mechanical stimuli, and the threshold for heat is higher than that of un-myelinated C-afferents (LaMotte *et al.*, 1983). The Aδ fibres are slowly adapting and they have free nerve endings in the skin. Based on their discharge frequencies, the Aδ mechano-heat nociceptors have been further classified into type I and type II Aδ mechano-heat nociceptors. Type I fibres are present in glabrous and hairy skin. They respond to prolonged heat impulses (>53^{o}) but are irresponsive to capsaicin. Their peak discharge is delayed and is not reached abruptly, while type II mechano-heat nociceptors, which are present in hairy skin, respond quickly to capsaicin and noxious heat stimuli (>46^{o}) and are rapidly adapting in nature (Treede *et al.*, 1998). Since type I fibres also respond to chemical stimuli, they have been regarded as A-fibre polymodal nociceptors (Davis *et al.*, 1993). A subset of these fibres called mechano-cold (AMC) receptors respond to noxious mechanical and cold stimulus of high intensity (temperature ranging between 14 to -18^{o}C) (Simone and Kajander, 1996). In contrast, the D-hair afferents are excited by the movement of the body hair or gentle brushing over the skin (Brown and Iggo, 1967, Burgess *et al.*, 1968). However, they differ from the true hair follicles, at least in primates (Merzenich, 1968).

Small unmyelinated fibres or **C-fibres** are unmyelinated, thin and have the slowest conduction velocity (0.5-2m/s) of all the afferent fibres in humans. These afferents constitute the majority of cutaneous nociceptive innervation. They innervate multiple structures such as skin, joints, muscles and internal viscera and form free nerve endings extending into the skin. They vary significantly in their function as well as neurochemical properties and conduct sensory modalities like slow pain or second pain (burning sensation), temperature and itch. They respond to a wide range of stimulus intensity. Some fibres are activated in response to gentle brushing while others respond to intense noxious mechanical and thermal stimulation (Bessou and Perl, 1969). These fibres also respond to chemical agents such as capsaicin and histamine as well as to pruritogens, hence they are also categorized as chemoreceptors. Their response changes after the exposure to inflammatory chemical mediators (Davis *et al.*, 1993). Those afferents, which respond to innocuous stimuli, are called C low threshold mechanoreceptors (C-LTMRs). They are only found in the hairy skin and are sensitive to skin indentation, slow moving stimuli across the receptive field and rapid cooling (Kumazawa and Perl, 1977, Willis and Coggeshall, 2004). It has been suggested that these afferents not only mediate emotional touch and therefore may take part in affiliative behaviour (Kumazawa and Perl, 1977, Vallbo *et al.*, 1993, Olausson *et al.*, 2003) but are also involved in the injury induced mechanical hypersensitivity (Seal *et al.*, 2009). Another group of C-fibres are mechanically insensitive and are non-responsive to peripheral stimulation but they are activated only in response to high threshold mechanical stimuli like prolonged noxious stimulation, tissue injury or inflammation (Meyer and Campbell, 1988, Ringkamp *et al.*, 2013). They are known as silent nociceptors or mechanically insensitive afferents (MIAs) (Lynn and Carpenter, 1982, Meyer *et al.*, 1991). A specific group of C-afferents is also activated by cooling stimuli such as menthol (Dhaka *et al.*, 2008).

Some afferents respond to a single type of stimulus while others are polymodal in nature. The polymodal nociceptors are called mechano-heat sensitive (MH) receptors that are further divided into C-mechano-heat sensitive nociceptors (CMH) and A-mechano-heat sensitive (AMH). Some polymodal C-nociceptors are also activated by chemical (itch provoking) stimuli such as cowhage and histamine (Tuckett and Wei, 1987, Schmelz *et al.*, 1997) but their response is not as much as A-fibre nociceptors (Davis *et al.*, 1993).

1.1.1 Spinal terminations of primary afferents

Primary afferents terminate within the spinal dorsal horn in an orderly pattern. This arrangement is based on their fibre diameters, functions and somatotopic distribution (fig 1. It has been suggested that the fibre diameter or conduction velocities alone does not define the functional category of the primary afferents (Burgess and Perl, 1973). Instead, it is the response to the relevant stimulus that seems to determine the arrangement of their central projections (Light and Perl, 1979a). Broadly, these afferents are either non-nociceptive or nociceptive in nature. The non-nociceptive afferents are mostly myelinated (with an exception of C-LTMRs) and are grouped under low threshold mechanoreceptors while nociceptive afferents are either myelinated or un-myelinated. Primary afferents enter the spinal cord via dorsal root. Before entering the dorsal horn they divide into rostral and caudal branches within the dorsal column. Several techniques have been used to determine the termination pattern of primary afferent fibres in the spinal dorsal horn. These include Golgi technique that was initially performed by Cajal (1909) to detect the termination pattern of fine afferents, intra axonal labelling, detection of degenerating axon terminals and bulk labelling method. The latter technique has been used to identify target sites of myelinated afferents. In this technique, neuroanatomical tracers such as cholera toxin B subunit (CTb) is injected in to the nerve (Robertson and Grant, 1985). CTb binds to GM1 ganglioside, which is specifically expressed by myelinated afferents (Ganser *et al.*, 1983, Lamotte *et al.*, 1991). CTb is selectively taken up by these afferents through absorptive endocytosis (more specific uptake) (Robertson and Grant, 1985, Wang *et al.*, 1998) and is transported transganglionically. With this method it has been shown that CTb labelled terminals are distributed in lamina I and in the region between lamina II-III border to lamina IX with sparse labelling in lamina IIo (Robertson and Grant, 1985, Willis and Coggeshall, 1991). Similarly, the spinal targets of primary afferents in the cat were determined by injecting horseradish peroxidise (HRP) intra-axonally (Brown *et al.*, 1977, Brown, 1981). It was further suggested that free HRP was less potent than its conjugated forms with choleragenoid or wheat germ agglutinin. HRP is internalized by the fluid phase endocytosis (less specific uptake). LaMotte *et al.* (1991) identified the central projection of rat sciatic, saphenous, median and ulnar nerves using choleragenoid-HRP (B-HRP) and wheat germ agglutinin-HRP (WGA-HRP) or their mixture. In this study, the B-HRP labelled a great majority of large myelinated afferents, which were distributed in lamina I, III, IV and V as well in the intermediate grey and ventral horn. On the contrary, the WGA-HRP labelled the non-myelinated afferents, and their staining was limited to superficial dorsal horn and the regions in the dorsal column nuclei. A similar approach was adapted to

determine the spinal distribution of Aδ fibres (Light and Perl, 1979b). Recent advancement in the transgenic technology has resulted in the identification of sub classes of sensory fibres by combining intracellular recordings with fluorescent reporter constructs (Zylka *et al.*, 2005, Seal *et al.*, 2009).

1.1.1.1 Low threshold mechanoreceptors

The low threshold mechanoreceptors include the both the myelinated and unmyelinated afferents. The myelinated afferents comprise of Aβ cutaneous fibres and Aδ D-hair afferents while the unmyelinated fibres consist of C-low threshold mechanoreceptors (C-LTMRs). These fibres on entering the dorsal column move medially, away from their point of entry, and bifurcate into main caudal and rostral branches. Both these branches extend inter segmentally and give off many collaterals. Some fibres, from the rostral branch of Aβ cutaneous fibres, extend through the dorsal column to synapse in the dorsal column nuclei in the medulla (Brown and Fyffe, 1981). The myelinated afferents terminate mainly in the deeper laminae of the dorsal horn (lamina III-VI). The axon collaterals of Aβ cutaneous fibres terminate in the deep dorsal horn (III-V), with some arbors entering lamina IIi (Brown *et al.*, 1981). In the cat, the slow adapting LTM fibres terminate deeper than those innervating hair follicles, while in the rat they form a narrow band that extends between lamina IIi to lamina IV (Shortland *et al.*, 1989) or may also extend dorsally to lamina IIi (Hughes *et al.*, 2003). Unlike the Aβ afferents, the Aδ and C-LTMRs do not bifurcate into rostral and caudal branches, instead they travel one or two segments rostrally before entering the dorsal horn. The Aδ D-hair afferents terminate most superficially among all the myelinated LTMs. They project till lamina IIi and arborize extensively at the border of lamina II-III (Light and Perl, 1979a, Sugiura *et al.*, 1986, Woodbury and Koerber, 2003), while the C-LTMR afferents have a more restricted distribution and they occupy a characteristics location in lamina IIi of the spinal dorsal horn (Sugiura, 1996, Seal *et al.*, 2009). The cold C-fibres, which also belong to C-LTMRs, terminate in lamina I of the spinal dorsal horn (Seal *et al.*, 2009).

1.1.1.2 Myelinated nociceptors

The myelinated nociceptors include fibres that have a very large range of conduction velocities varying from Aβ to Aδ (Campbell *et al.*, 1979, Koerber and Mendell, 1988). The Aβ-afferents that carry nociceptive information arborize throughout lamina I-V (Woodbury *et al.*, 2008). Most nociceptive Aδ afferent branches are found in lateral dorsal column and in or near Lissauer's tract. According to Light and Perl (1979b) these myelinated

nociceptors have their central projections terminating in lamina I and IIo, with some extending ventrally to the deep dorsal horn (lamina V). Some of these fibres give off collateral that penetrate deep into the dorsal horn and then curve dorsally to enter into ventral part of lamina IV. Woodbury and Koerber (2003) and Woodbury *et al.* (2004) identified 2 different morphological types of myelinated nociceptive afferents using ex vivo preparation of neonatal and adult mice spinal cord. The first type was similar to the Aδ afferents reported by Light and Perl (1979b). These axons, on entering the spinal cord, gave rise to ascending and descending branches that extended inter segmentally. Some fibres ascended in the dorsal column while the others terminated mostly in lamina I and IIo. The second type gave rise to numerous collaterals that penetrated deep into the dorsal horn before curving back to the superficial laminae and extended branches from lamina I-V, as seen with LTMs of Aβ range. Interestingly, not much is known about the synaptic targets of Aδ-nociceptive afferent, therefore; one aim of this project was to determine the potential targets of Aδ nociceptors in lamina I of the rat spinal dorsal horn.

1.1.1.3 Unmyelinated fibres

The termination patterns of unmyelinated afferents were identified using intracellular labelling techniques (Sugiura *et al.*, 1986, 1989). The central arborisations of unmyelinated C-afferents are limited to lamina I-II of the spinal dorsal horn. These fibres, on entering the spinal cord, extend rostrally and/or caudally for a short distance before terminating into the spinal grey matter. On the basis of neurochemical properties, these unmyelinated C fibres have been classified into peptidergic and non-peptidergic afferents (Hunt *et al.*, 1992, Lawson *et al.*, 1997). These sub types differ also in terms of their functions and termination pattern within the spinal dorsal horn. This will be discussed later in detail. The peptidergic afferents innervate lamina I and IIo (Sugiura *et al.*, 1986) with sparse distribution in the deep dorsal horn (IIi-V), (Ribeiro-Da-Silva *et al.*, 1986, Silverman J.D and Kruger L, 1988) while the central terminals of non-peptidergic afferents preferentially innervate central part of lamina II (dorsal lamina IIi).

The primary afferent fibres also differ in terms of their ultrastructural appearances and synaptic arrangements within the dorsal horn. Two types of synaptic arrangements have been identified, which include type I and type II synaptic glomeruli (Ribeiro-da-Silva and Coimbra, 1982). The non-peptidergic C fibres and Aδ hair follicle afferents form central axons of synaptic glomeruli type I and II, respectively. In contrast, most peptidergic afferents in the rat form simple synaptic arrangements (Ribeiro-da-Silva A *et al.*, 1989),

where as in monkey, they may form synaptic glomeruli. These afferents also receive quite a low number of axoaxonic synapses. Both Aβ afferents and Aδ nociceptors form non-glomerular synaptic organizations (Light *et al.*, 1982, Ribeiro-da-Silva and Coimbra, 1982, 1989). In lamina I, Aδ nociceptors form simple axo-dendritic synapses and at times they are post synaptic to GABAergic axons, while in lamina V they form simple synaptic arrangements.

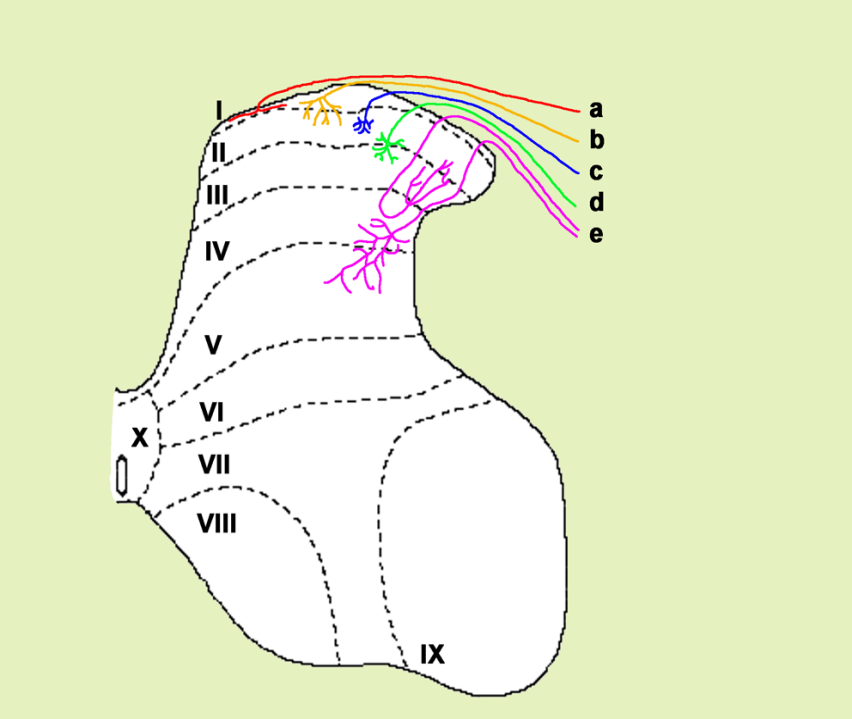


Figure 1-1 Rexed's laminae and the spinal termination pattern of primary afferents.
The image shows the central termination pattern of different groups of primary afferents. The dashed lines mark laminar boundaries. **a** Aδ nociceptors (I) **b** Peptidergic primary afferents (C or Aδ) (I-IIo) **c** Non-peptidergic afferents (II) **d** Aδ hair follicle afferents (IIi-III) **e** Aβ hair follicle and tactile afferents (IIi-V). Modified from Todd (2010).

1.1.2 Neurochemical properties of primary afferents

All primary afferents are excitatory in nature and express glutamate, which is a principal excitatory neurotransmitter. Glutamate is involved in the perception of acute and chronic pain as well as mechanical, thermal and chemical stimuli (Haley *et al.*, 1990, Dougherty *et al.*, 1992, Dickenson *et al.*, 1997, Garry and Fleetwood-Walker, 2004). Before its release, glutamate is accumulated in the synaptic vesicles by means of vesicular glutamate transporters (VGLUTs) (Naito and Ueda, 1985, Maycox *et al.*, 1988). Three distinct populations of glutamatergic vesicles have been identified, namely vesicular glutamate transporter 1, 2 and 3 (VGLUT1, VGLUT2 and VGLUT3) (Takamori *et al.*, 2000, Fremeau *et al.*, 2001, Varoqui *et al.*, 2002). Glutamatergic neurons are difficult to detect due to lack of an appropriate marker. However, glutamatergic axon terminals can be identified with antibodies against VGLUTs. Both VGLUT1 and VGLUT2 are distributed throughout the nervous system with some degree of co-localization (Fremeau *et al.*, 2001, Varoqui *et al.*, 2002). VGLUT1 is expressed by myelinated primary afferents of mechanoreceptive origin that terminate in the deeper laminae (IIi-III), intermediate grey matter, the central canal and the ventral horn. These primary afferents are not the only source of VGLUT1 in the spinal dorsal horn. The descending axons from the pyramidal cells of neocortex and corticospinal tract also express VGLUT1 (Fremeau *et al.*, 2001, Du Beau *et al.*, 2012). VGLUT2 is mostly expressed by the axon terminals of spinal origin; with an exception of myelinated nociceptive afferents (Aδ fibres) that also contain VGLUT2 and terminate in lamina I (Varoqui *et al.*, 2002, Todd *et al.*, 2003). In addition, peptidergic primary afferents and LTMRs also express low levels of VGLUT2 in their axon terminals. VGLUT3 is associated with C-LTMRs (Seal *et al.*, 2009). These fibres are involved in the generation of hypersensitivity in response to spared nerve injury. The distribution pattern of VGLUT3 is quite restricted in dorsal horn. It is expressed in lamina I and IIi (Gras *et al.*, 2002), while some GABAergic cells, astrocytes, progenitor cells also contain VGLUT3 (Fremeau *et al.*, 2002, Gras *et al.*, 2002).

Maxwell and colleagues (1990) divided glutamate containing presynaptic terminals cytologically into 3 categories; (1) terminals containing clear vesicles in an electron dense axoplasm, (2) uniform looking vesicles in a relatively clear axoplasm and (3) large dense-core vesicles that were scattered among a collection of clear small vesicles. The electron dense terminals were associated with the endings of unmyelinated fibres in the superficial laminae and the clear endings, which were located relatively deep, belonged to myelinated sensory axons. The GABAergic axons of spinal origin form synapses with the

glutamatergic terminals, suggesting that excitatory synapses are under a strong inhibitory control (Basbaum *et al.*, 1986, Carlton and Hayes, 1990).

As described previously, unmyelinated C-afferents are categorized on the basis of expression of neuropeptides into peptidergic and non-peptidergic (Lawson *et al.*, 1997, Snider and McMahon, 1998, Lawson 1992, Hunt *et al.*, 1992). Some myelinated Aδ nociceptive afferents are also peptidergic in nature (Lawson *et al.*, 1997). Peptidergic afferents innervate deeper regions in the skin and various tissues while the non-peptidergic afferents innervate the epidermis. In the rat, most if not all the peptidergic primary afferents contain calcitonin gene related peptide (CGRP**)** (Ju *et al.*, 1987, Chung *et al.*, 1988). It is released by the peptidergic afferents following noxious stimulation (Morton and Hutchison, 1989). CGRP expression in the spinal dorsal horn disappears after dorsal rhizotomy (Noguchi *et al.*, 1990, Hökfelt *et al.*, 1994), which indicates its primary afferent origin. Apart from glutamate and CGRP, the peptidergic afferents also contain neuropeptides such as substance P, somatostatin and galanin (Wiesenfeld-Hallin *et al.*, 1984, Ju *et al.*, 1987, Lawson *et al.*, 1997). These peptides are stored in large dense-cored vesicles and are released by a calcium dependent method (Zhu *et al.*, 1986). Mostly, peptides are expressed by the axonal boutons but in some cases the perikaryal cytoplasm of some neurons also contains detectable levels of neuropeptides. Peptidergic afferents form dense plexus in the superficial dorsal horn, with sparse distribution in the deeper laminae (Hokfelt *et al.*, 1975, Chung *et al.*, 1988, Tuchscherer and Seybold, 1989, Alvarez FJ *et al.*, 1993). CGRP-containing peptidergic afferents preferentially innervate 2 different populations of NK1 receptor expressing dorsal horn projection neurons (Naim *et al.*, 1997, Todd *et al.*, 2002) that will be described later in detail.

Substance P is a tachykinin peptide and acts on a G-protein coupled receptor, with seven membrane spanning domains, called neurokinin 1 receptor (NK1r) (Ogawa *et al.*, 1985), which is widely distributed in the spinal dorsal horn (Vigna *et al.*, 1994, Bleazard *et al.*, 1994, Liu *et al.*, 1994). Substance P-containing fibres are either small myelinated Aδ afferents or unmyelinated C fibres that terminate mainly in the superficial dorsal horn (Kantner *et al.*, 1985). Substance P is released into the dorsal horn in response to painful stimuli (Duggan and Hendry, 1986) and there are strong evidences to suggest that both substance P and NK1r are involved in the spinal pain mechanisms (Lawson *et al.*, 1997, Mantyh *et al.*, 1997). Substance P, when released from primary afferent terminals at extra synaptic sites, acts on its target receptors through volume transmission, while glutamatergic transmission occurs at the sites of asymmetrical synapses (Todd and

Koerber, 2005). Although, primary afferent fibres are the main source of substance P (Hokfelt *et al.*, 1975, Barber *et al.*, 1979, Ogawa *et al.*, 1985, Moussaoui *et al.*, 1992), some locally occurring interneurons as well as axons descending from the brain stem also express the peptide (Hunt *et al.*, 1981).

Somatostain containing fibres constitute a distinct population of peptidergic afferents (Hökfelt *et al.*, 1975, 1976). Somatostatin is an inhibitory peptide. In the spinal dorsal horn, somatostatin-expressing boutons are distributed in lamina I and II. Most of these boutons are derived from the local neurons while axons from primary afferents constitute a minority (Alvarez and Priestley, 1990). These afferents provide sparse input to projection cells in lamina III of the spinal dorsal horn (Sakamoto *et al.*, 1999) (described later).

Galanin is expressed by a subset of primary afferents and a distinct population of inhibitory interneurons within the dorsal horn (Simmons *et al.*, 1995, Tuchscherer and Seybold, 1989, Zhang *et al.*, 1993a). Galanin immunoreactivity is substantially reduced after dorsal rhizotomy, which suggests that most of the galanin in the spinal dorsal horn is of primary afferent origin (Tuchscherer and Seybold, 1989). It colocalizes considerably with substance P and CGRP-containing peptidergic afferents and these fibres terminate in lamina I-II (Zhang *et al.*, 1993a). It has been suggested that galanin has both anti- and pro-nociceptive roles, which are thought to result from its action on receptors expressed by spinal neurons and central terminal of primary afferents (Malkmus *et al.*, 2005, Xu *et al.*, 2008a). Three different types of galanin receptors (GalR1-3) are present at the primary afferent terminals. GalR1 is also found in the dorsal horn and is involved in anti nociceptive actions while the pro-nociceptive effect is mediated by GalR2 (Ju *et al.*, 1987, Antal *et al.*, 1996). It is suggested that GalR2 is also involved in the neuroprotective and developmental functions of galanin (Shi *et al.*, 2006). At an ultrastructural level, galanin is found in both type I and type II glomeruli but mostly in type I glomeruli, which also show strong galanin immunoreactivity as compared to type II.

The non-peptidergic afferents include the C-LTMRs and fibres that express Mas-related G-protein coupled receptor member D (Mrgprd), which is a sensory neuron specific G-protein coupled receptor (Zylka *et al.*, 2005). These 2 groups differ in their neurochemical properties and functions. The Mrgprd-afferents show affinity towards lectin *Bandeiraea simplicifolia* isolectin B4 (BS IB4) while C-LTMRs are non-IB4 in nature (Seal *et al.*, 2009). IB4 affinity towards unmyelinated afferents is suggested by the lack of RT97 expression by IB4-immunoreactive neurons. RT97 is a neurofilament antibody and a

specific marker for myelinated axons (Silverman J.D and Kruger L, 1988, Lawson *et al.*, 1993). However, peptidergic afferents also express it at low levels. When injected directly into the peripheral nerve, IB4 is transported transganglionically towards the central terminals (Kitchener *et al.*, 1993, Wang *et al.*, 1994, Silverman J.D and Kruger L, 1988). IB4 can also be applied directly on the ganglia or dorsal horn histological sections to label neurons and axon terminals (Ambalavanar and Morris, 1992). The non-peptidergic C-fibres innervate the central region of lamina II. The function of non-peptidergic primary afferents is not fully known but it is suggested that some of them may contribute to nociception (Gerke and Plenderleith, 2001). Studies based on intracellular recordings have reported that Mrgprd expressing fibres respond to mechanical stimulation while a majority are also sensitive to heat and rarely to cold stimuli (Rau *et al.*, 2009). In addition, Mrgprd ablation results in a selective loss of sensitivity to noxious mechanical stimuli, while the perception of thermal stimuli remains intact (Cavanaugh *et al.*, 2009). This suggests that both peptidergic and non-peptidergic populations of C-afferents vary considerably in terms of their functions (Zylka *et al.*, 2005, Todd, 2010). Peptidergic and the IB4 expressing non-peptidergic fibres either do not express VGLUTs or have low levels of VGLUT2 (Todd *et al.*, 2003). This observation stands contrary to the fact that all primary afferents are glutamatergic (Broman *et al.*, 1993). This discrepancy can be explained by the presence of glutamate transporter other than VGLUT1 and 2 such as VGLUT3 (Gras *et al.*, 2002, Seal *et al.*, 2009) within the nerve terminals of these afferents. However, this possibility is yet to be determined.

Despite all the differences, it is not yet clear whether peptidergic and non-peptidergic afferents constitute different functional sub types. For instance in the rat, capsaicin receptor TRPV1 that responds to noxious heat stimuli (Caterina *et al.*, 1997) is expressed by a number of sensory neurons including fibres belonging to both peptidergic and non-peptidergic afferents (Guo *et al.*, 1999, Michael and Priestley, 1999). However, unlike in the rat, the non-peptidergic IB4-binding afferents in the mouse do not express TRPV1 (Zwick *et al.*, 2002).

From the earlier discussion it is apparent that primary afferents have a characteristic arrangement in the spinal dorsal horn. The fibres with fine diameter terminate mostly in the superficial dorsal horn and those with larger diameter target deeper laminae (Light and Perl, 1979b). This is further supported by the fact that the neurons located in the superficial dorsal horn are nociceptive in nature while those in the deep dorsal are of wide dynamic range and respond to noxious as well as tactile stimuli (Gauriau and Bernard, 2002).

Primary afferent inputs to the selective populations of dorsal horn neurons will be discussed later in detail.

1.2 Spinal cord dorsal horn

At the spinal cord level, the nociceptive information is dealt in several ways. It is either processed by the intricate circuitry of the spinal dorsal horn, before being carried to brain or it is transmitted to ventral horn, where spinally mediated nocifensive reflexes are generated. In addition, this information is also modified by the axons descending from brain. Rexed (1952) divided the spinal cord into a series of parallel laminae. This scheme was based on the Nissl-staining that was performed on the transverse sections of cat spinal cord. This organization has also been widely accepted for mouse (Sidman *et al.*, 1971) and rat (Fukuyama, 1955, Molander *et al.*, 1984). It was suggested that even though the basic scheme was similar for both cat and rat, the laminae shaped somewhat differently in the rat. Moreover, the exact demarcation between the laminar boundaries could not be defined. These delineations among the laminae were more or less like transition zones rather than strict margins. There are 10 spinal cord laminae, which are numbered from dorsal to ventral. The first 6 laminae constitute the spinal cord dorsal horn while lamina I and II are collectively called the superficial dorsal horn (SDH). These laminae will be described briefly.

1.2.1 Dorsal horn cytoarchitecture

Lamina I is also called the marginal layer. It makes the dorsal or dorsolateral margin of the dorsal horn and is thinnest of all the laminae. The reticulated appearance of this lamina is due to the penetration of several small and large nerve fibres. Neurons with supraspinal projections are mostly found in lamina I and they constitute 6% of all the cells in this region (Todd *et al.*, 2002, Spike *et al.*, 2003). Lamina II is also called substantia gelatinosa of Rolando (Rolando, 1824). It lacks myelinated fibres and appears transparent in unstained sections. It is characterized by the presence of large number of small neurons, which give it dark appearance. Lamina II is further divided into a highly cellular and denser looking outer zone (lamina IIo) and a less compact inner zone (lamina IIi). Interneurons make up the vast majority of SDH cells (Lima and Coimbra, 1983, Lima and Coimbra, 1986). Lamina III appears quite similar to lamina II with less densely packed cells of more variable sizes. Unlike lamina II, lamina III also consists of large projection cells. It runs parallel to lamina II occupying a more ventral position. Lamina III and IV are

collectively known as nucleus proprius. Lamina IV stretches uniformly throughout its length and doesn't bend at its ventrolateral edge. Lamina V appears more variable in its appearance while lamina IV forms the base of the dorsal horn. It is prominent only in the cervical and lumbosacral enlargements of spinal cord.

Several methods have been used in the past to identify superficial dorsal horn cells on the basis of their morphology, since morphology of neurons is often closely related to functions of the cells in other parts of the nervous system. One of the methods was Golgi impregnation in which Golgi stain was used to reveal the soma as well as dendrites and axons of dorsal horn cells (Gobel, 1978). Based on the anatomical or electrophysiological studies, it has been reported that most dorsal horn interneurons extend their axons either locally or have propiospinal axons extending inter segmentally (Scheibel and Scheibel, 1968, Gobel, 1978, Beal and Cooper, 1978, Lima and Coimbra, 1986, Schneider, 1992, Bice and Beal, 1997, Yasaka *et al.*, 2010). Lamina I receives nociceptive primary afferents and thus plays an important role in pain transmission. The cytoarchitecture of lamina I is complicated due to the heterogeneity of different neuronal populations. Many attempts have been made to classify lamina I neurons on the basis of their dendritic architecture and three dimensional soma shapes (Lima and Coimbra, 1983, Lima and Coimbra, 1986, Heise *et al.*, 2009). However, there is a lack of universally accepted single classification, as a significant number of cells remain unclassified due to their variable structure. Functionally, all projection neurons are thought to be excitatory while the interneurons are categorized into excitatory and inhibitory cells. This classification is based on the expression of glutamate or GABA/glycine as their principal neurotransmitters (Todd and Spike, 1993). In the earlier studies, lamina I neurons were classified into 4 major morphological types, which included fusiform, multipolar, flattened aspiny and pyramidal/ prismatic wedge shaped cells (Lima and Coimbra, 1983, Lima and Coimbra, 1986). These cells differed not only in their structure but also in their neurochemical properties and functions. Both interneurons and projection cells in this region were collectively classified into these 4 morphological classes. According to Lima and Coimbra, the fusiform cells were the most numerous and constituted approximately 1/3rd of lamina I neurons. These cells were mostly inhibitory and also contained neurochemical substances such as dynorphin (Light *et al.*, 1993). Some fusiform cells were projection neurons (Lima *et al.*, 1991). The multipolar cells were mostly confined to medial portion of the lamina I. The dendrites of some large cells extended into lamina III (Lima and Coimbra, 1986). Some of these neurons were also inhibitory in nature. Flattened aspiny neurons had a flattened disc appearance. These cells extended axons as a constituent of spinothalamic tract (Lima and Coimbra, 1983). The

fourth class of neurons was the pyramidal cells, which made up around 25% of lamina I neurons. These cells constituted a majority of lamina I projection neurons in the rat and projected to thalamus and PAG (Lima and Coimbra, 1989, Lima and Coimbra, 1990, Lima *et al.*, 1991). Some of these cells also expressed endogenous opioid peptides i.e. enkephalin and dynorphin (Lima and Coimbra, 1983, Lima *et al.*, 1993).

In the cat, some fusiform cells responded to the noxious stimuli while the multipolar neurons were polymodal nociceptive in nature and the innocuous cooling was interpreted by pyramidal cells (Han *et al.*, 1998). This classification has largely been modified due to a great degree of overlap between the functions and neurochemical characteristics of these cells. In addition, subsequent studies have shown no obvious correlation between the morphological subtypes, projection targets or functions of these cells. Many cells exhibited functional variability or remained unclassified in terms of their morphology.(Andrew and Craig, 2001, Spike *et al.*, 2003). For instance, almost all lamina I spinoparabrachial projection cells that belong to different morphological sub groups, respond to noxious stimuli and none of these cells is activated by innocuous mechanical stimulation (Bester *et al.*, 2000).

In lamina II of the spinal dorsal horn, Golgi studies have identified 2 major types of cells on the basis of their axonal spread (Gobel, 1978, Todd and Lewis, 1986) namely; Golgi type I neurons that project their long axons beyond the vicinity of their soma or into the adjacent laminae. At times they extend further into the white matter and brain. The Golgi type II neurons have limited axons. Gobel (1975, 1978) defined these cells as islet or stalked cells, but many cells did not belong to either of the two. Islet cells were inhibitory with limited axon arbors while stalked cells were excitatory interneurons with axons entering lamina I. It was further suggested that the axons of stalked cells were responsible for the transmission of sensory information from primary afferents in lamina II to lamina I projection cells. Similar observations were made by Todd and Lewis (1986) in the rat, where stalked cells were found to be distributed in the dorsal part of lamina II while islet cells were present throughout this region. The most widely accepted morphological classification of lamina II neurons was suggested by Grudt and Perl (2002). They classified lamina II interneurons within the hamster superficial dorsal horn into 4 categories by correlating their morphology and electrophysiological features. These include islet, central, vertical and radial cells. Islet cells have their elongated dendrites (>400 µm) and axons extending locally in rostrocaudal direction. Central cells mimicked islet cells but their dendritic tree was less extensive (<400 µm). Radial cells were very small and compact

with dendrites radiating towards all directions while vertical cells have marginal cell body with dendrites passing deep and axons reaching up to lamina I. Since vertical cells resembled Gobel's stalked cells, in the literature, they are sometimes also referred as stalked cells. It has been reported that islet cells are inhibitory while radial and most vertical cells are excitatory in nature. This was later supported by many other studies (Todd and McKenzie, 1989, Todd *et al.*, 1998, Lu and Perl, 2003, Maxwell *et al.*, 2007, Yasaka *et al.*, 2010). Still, around 30% of the interneurons do not fall into any of these categories and remain unclassified (Grudt and Perl, 2002, Brelje *et al.*, 2002, Maxwell *et al.*, 2007).

Not much is known about the morphological classes of interneurons in other laminae of the dorsal horn. Laminae III-IV exhibit heterogeneity in their cytoarchitecture. Neurons belonging to these laminae were initially discussed in the cat dorsal horn (Szentagothai, 1964), and their fine dendritic arrangement was compared to purkinje neurons in the molecular layer of cerebellum. This idea was later supported by Scheibel (1968). Subsequently, similar neurons were identified in other species like rat (Todd, 1989, Liu *et al.*, 1994, Brown *et al.*, 1995, Littlewood *et al.*, 1995), monkey (Beal and Cooper, 1978) and humans, where they were referred to as antenna like neurons (Schoenen, 1982). Todd and Spike (1993) reported that many lamina III cells with rostrocaudally oriented dendrites were GABAergic while those with dorsally directed dendrites were not. All these findings suggest that morphology has a limited value in the identification as well as classification of different neuronal populations in the spinal dorsal horn. Although morphology is related to the transmitter content of lamina II cells, this association is not very clear.

1.2.2 Dorsal horn interneurons

Majority of cells in lamina I-III of the spinal dorsal horn are interneurons. These cells are densely packed and have axons that terminate either locally or into the adjacent laminae and at times intersegmentally (Todd, 2010). Spinal cord interneurons are divided into two major functional classes: excitatory cells that express glutamate and inhibitory cells, which use GABA as their principal neurotransmitter (Todd and Sullivan, 1990). In the rat spinal dorsal horn, approximately 25-30% of lamina I-II and around 40% of lamina III interneurons express GABA and/or glycine while the remaining cells contain glutamate (Todd and Sullivan, 1990, Polgár *et al.*, 2003). Nearly all glycinergic cells are GABAergic but not all the GABAergic cells contain glycine (Todd and Sullivan, 1990). The cell bodies of inhibitory neurons can be identified with antibodies against GABA, while GABA

antagonists have been used to determine the functions of these cells (Yaksh, 1989). These cells are involved in regulating the transmission of somatosensory information. Recently, it has been suggested that the inhibitory interneurons prevent hyperalgesia and allodynia (Sandkühler, 2009) while their role in the suppression of itch has also been established (Ross *et al.*, 2010). Sandkühler (2009) described potential mechanisms underlying the functions of inhibitory interneurons. According to his observations, inhibitory interneurons regulate the level of activity of nociceptive projection cells to ensure an appropriate response to noxious stimuli. In addition, these cells prevent the spontaneous activity of projection cells in the absence of noxious stimuli. They are also involved in minimizing a cross talk between various sensory modalities at the spinal cord level and thus limit the spatial spread of activity to the somatotopically appropriate regions of the spinal dorsal horn.

Unlike inhibitory cells, there is no reliable immunocytochemical marker for glutamatergic cells. Glutamate is also expressed by primary afferents and it is difficult to distinguish glutamate of spinal origin with that from primary afferents or to block its activity to determine the functions of excitatory interneurons. Therefore, it has been suggested that most if not all cells that do not express GABA or glycine are glutamatergic. These cells are involved in the transmission of sensory information between the dorsal horn laminae. This observation is further supported by the presence of polysynaptic excitatory pathways between lamina I projection cells and the primary afferents terminating in lamina II of the spinal dorsal horn (Grudt and Perl, 2002, Torsney and MacDermott, 2006, Yasaka *et al.*, 2014).

It is now possible to identify the excitatory and inhibitory axons and to determine their synaptic connections using neurotransmitter specific antibodies. The expression of various VGLUTs by excitatory boutons has already been mentioned in the previous section. These axons make asymmetrical synapses at their post synaptic target sites. These asymmetrical synapses are more common in the central nervous system. They are associated with spherical vesicles and contain a thickened post synaptic density. These synapses are associated with AMPA receptors, which are tetramers having 4 subunits, the GluR1-GluR4. (α-Amino-3-hydroxy-5-methyl-4-isoxazoleprpionic acid) (Yoshimura and Jessell, 1990). Recent studies have suggested that AMPA receptor subunit composition is important since it determines the properties of the receptor. These receptors underlie the fast excitatory synaptic transmission and their expression varies in the spinal dorsal horn (Watanabe *et al.*, 1998, Nagy *et al.*, 2004, Polgár *et al.*, 2008b). However, these receptors

are not detectable in fixed tissue using routine immunocytochemical methods. The cross-linking of proteins prevents the access of antibodies to the synaptic cleft and post synaptic density; therefore, these receptors are retrieved using pepsin treatment (an antigen retrieval method) (Watanabe *et al*., 1998, Polgár *et al*., 2008b, Polgár *et al*., 2010a). In spinal cord dorsal horn, GluR1 and GluR4-containing receptors are found in 2 distinct non-overlapping populations of cells. GluR4 is found mostly on large projection neurons in lamina I and III (Todd *et al.,* 2009) while GluR1 is present at synapses on small projection neurons and interneurons (Polgár *et al*., 2008b).

Like glutamate, GABA is also stored in the vesicles by means of vesicular GABA transporters (VGAT) at axon terminals. GABA is released from these vesicles exocytotically in response to an appropriate stimulus (Chaudhry *et al.,* 1998). Antibody against GABA-synthesizing enzyme glutamic acid decarboxylase (GAD) can be used to identify inhibitory boutons. GAD catalyzes the decarboxylation of glutamate to GABA (Barber *et al.,* 1978, Hunt *et al.,* 1981). GAD exists in 2 different isoforms, GAD65 and GAD67 (Erlande and Tobin, 1991, Mackie *et al.,* 2003). Both these isoforms differ not only in their expression pattern but also in intensity of immunostaining, therefore, antibody against VGAT has been used widely to identify inhibitory axon terminals (McLaughlin *et al.,* 1975, Todd *et al.,* 1996, Mackie *et al.,* 2003).

Similarly, glycinergic axons are identified with antibody against glycine transporter 2 (GlyT2), which is a sodium/chloride-dependent transporter (Spike *et al.,* 1997). Glycinergic receptors are associated with α (ligand-binding), β (structural) subunits and a peripheral membrane protein called gephyrin. Gephyrin binds to β-subunit and anchors the glycinergic receptors to the postsynaptic membrane (Maxwell *et al.,* 1995, Todd *et al.,* 1996). These receptors are also found with $GABA_A$-receptor β3 subunit throughout the cord (Todd *et al.,* 1996). Since gephyrin is associated with inhibitory synapses, therefore monoclonal antibody against gephyrin can be used to reveal the sites of inhibitory synapses (Pfeiffer *et al.,* 1984, Puskár *et al.,* 2001). The distribution of gephyrin is quite complex. It is relatively infrequent in the superficial dorsal horn as compared to rest of the spinal grey matter. However, a distinct population of lamina I projection neurons, called the giant cells, are heavily coated with it (Puskár *et al.,* 2001). These cells will be discussed later in detail. The great majority of GABAergic and glycinergic axons generate post synaptic inhibition by forming axodendritic or axoaxonic synapses with dorsal horn cells as well as with primary afferent terminals (Todd and Koerber, 2005).

Several attempts have been made to categorize interneurons on the basis of different criteria such as morphology, electrophysiology, developmental evidences and neurochemistry. Although certain morphological, electrophysiological and neurochemical subtypes have been identified, there is still no universally accepted classification that can account for all dorsal horn interneurons (Graham *et al.*, 2007a, Todd, 2010). Morphological classification has already been described in the previous section. Electrophysiologically, interneurons have been classified on the basis of their firing patterns, pharmacological properties and types of primary afferent input. Based on the firing pattern, these cells have been categorized as tonic, delayed, phasic and single spike cells (Ruscheweyh and Sandkühler, 2002, Prescott and Koninck, 2002, Graham *et al.*, 2007b). Yasaka *et al.* (2010) identified functional populations of lamina II excitatory and inhibitory interneurons by comparing electrophysiological properties with morphology and neurotransmitter content of the cells. Their findings suggested that although the two major groups of interneurons were quite heterogeneous in their properties, certain morphological subtypes were typically associated with only one group. The great majority of inhibitory cells had a characteristic tonic firing pattern, while most excitatory cells and few inhibitory cells showed delayed-, gap- and reluctant-firing patterns. In addition, the responses to pharmacological agents such as noradrenaline, serotonin and somatostatin were also tested. Although somatostatin was present exclusively in different morphological types of excitatory cells; only inhibitory cells were selectively hyperpolarized in response to somatostatin. Later, Polgár *et al.* (2013b) reported that approximately half of the inhibitory interneurons in superficial dorsal horn expressed somatostatin receptor sst_{2A}. Furthermore, it has been reported that different morphological types of lamina II interneurons are associated with characteristic synaptic input (Yasaka *et al.*, 2007). Islet, central and vertical cells received GABAergic input while radial cells were associated with glycine rich afferents.

Few studies have been carried out on developmental evidences regarding dorsal horn interneurons. Brohl *et al.* (2008) reported that the expression of distinct peptides by subgroups of inhibitory interneurons is controlled by specific transcription factors (such as Neurod1/2/6 for dynorphin and galanin). Furthermore, these transcription factors differ from the one that controls the expression of excitatory interneuron (Lhx1/5 for neuropeptide Y). Recently, Ross *et al.* (2010) reported that loss of inhibitory interneurons in the mice lacking transcription factor *Bhlhb5* resulted in increased itching in these animals. In addition, this genetically altered phenotype resulted from a substantial loss of

two specific, partly overlapping inhibitory interneurons population in such mice (Kardon *et al.*, 2014) (see later).

It has been suggested that neurochemistry provides an alternative but a more useful way for the identification and classification of dorsal horn interneurons (Todd, 2010). It is possible that a single cell contains more than one peptide and often these peptides co-exist with classical neurotransmitters (i.e. GABA and glutamate). Among many neurochemical markers (neuropeptides and various proteins) that have been identified in the spinal dorsal horn, some are found exclusively in the excitatory interneurons such as neurotensin, somatostatin and neurokinin B while others like galanin and neuropeptide Y are restricted to inhibitory cells. In addition, some peptides like endogenous opioids (dynorphin and enkephalin) are found in both excitatory and inhibitory cells (Todd and Spike, 1993, Todd *et al.*, 2003, Polgár *et al.*, 2006, Sardella *et al.*, 2011a). Apart from neuropeptides, there are other neurochemical markers such as nitric oxide synthase (nNOS) and parvalbumin (PV) (Sardella *et al*., 2011b, Hughes *et al*., 2012, Polgár *et al*., 2013a) that are found in restricted populations of dorsal horn interneurons, predominantly among the inhibitory cells while others such as calcium binding proteins (calbindin and calretinin) (Antal *et al.*, 1991) and the γ-isoform of protein kinase C (PKCγ) (Polgár *et al*., 1999a) are expressed exclusively by excitatory cells. Since some of these compounds are expressed by both excitatory and inhibitory cells, it again suggests that the neurochemical approach alone cannot define discrete populations. However, some markers show a more restricted distribution and are expressed by non-overlapping populations of inhibitory cells and may therefore represent different functional populations.

There are several neurochemically-defined populations of dorsal horn cells. This part of the introduction describes only those neuronal populations that are relevant to this study. Opioid peptides such as **dynorphin** are widely distributed in the spinal dorsal horn. In the superficial dorsal horn, dynorphin exists in two major forms, dynorphin A and dynorphin B (Khachaturian *et al.*, 1982, Vincent *et al.*, 1982, Ruda *et al.*, 1989). Both forms of dynorphin are derived from a common precursor **preprodynorphin (PPD)** (Lee *et al.*, 1997, Marvizón *et al.*, 2009, Sardella *et al.*, 2011a). Dynorphin is more potent than other opioids peptides (Goldstein *et al.*, 1979) and is expressed quite distinctively (Cruz and Basbaum, 1985, Standaert *et al.*, 1986, Marvizón *et al.*, 2009). Dynorphin expression is mostly of spinal origin as spinal cord transection does not alter its immunoreactivity below the level of injury (Cho and Basbaum, 1989). Since dynorphin and its precursor PPD, co-localize extensively in the dorsal horn, dynorphin-containing cells and axon terminals can

be identified using antibody against PPD (Li *et al.*, 1999, Lee *et al.*, 1997, Marvizón *et al.*, 2009). PPD is seen mostly in lamina I-II with few deep cells in lamina III (Ruda *et al.*, 1989). It is expressed by both inhibitory (Sardella *et al.*, 2011a) and excitatory interneurons (Marvizón *et al.*, 2009) and their respective axon terminals. Some weakly-immunoreactive PPD profiles colocalize with substance P and CGRP in the SDH and dorsal root ganglion fibres, respectively (Tuchscherer and Seybold, 1989, Marvizón *et al.*, 2009). Some dynorphin cells in lamina I have supraspinal projections that target parabrachial nucleus (Standaert *et al.*, 1986) and nucleus of solitary tract (Leah *et al.*, 1988). The role of dynorphin inhibitory interneurons in the prevention of itch has been proposed (Ross *et al.*, 2010, Sardella *et al.*, 2011a, Kardon *et al.*, 2014), while an increased expression of dynorphin cells following a chronic noxious stimulation has also been reported (Cho and Basbaum, 1988, Wang *et al.*, 2001). It is therefore suggested that dynorphin, together with other co-transmitters, contributes to nociception, while action on non-opioid receptors accounts for its role in neuropathic pain (Lai *et al.*, 2006).

It has been suggested that dynorphin performs its inhibitory action via opioid receptors while the mechanism underlying its excitatory functions is not known (Lai *et al.*, 2006). Opioid receptors are G-protein coupled (Janecka *et al.*, 2004, Waldhoer *et al.*, 2004) and are expressed by interneurons as well as by C-and A-δ primary afferents (Lamotte *et al.*, 1976, Wamsley *et al.*, 1982). The three classes of opioid receptors include μ-opioid receptor (MOR), kappa-opioid receptor (κ-opioid receptor or KOR) and δ-opioid receptor (DOR) (Goodman *et al.*, 1980, Fields *et al.*, 1980, Arvidsson *et al.*, 1995, Spike *et al.*, 2002). Dynorphin acts on kappa opioid (κ-opioid or KOR) (James *et al.*, 1982) and glutamate specific NMDA receptors (Lai *et al.*, 2006, Drake *et al.*, 2007). In the spinal dorsal horn, μ-opioid receptors are involved in opioids induced analgesia (Jessell and Iversen, 1977, Yoshimura and North, 1983). It has been estimated that around 10% of lamina II neurons are MOR-1-immunoreactive and they are mostly excitatory in nature (Kemp *et al.*, 1996). MOR-1 immunoreactive cells receive numerous contacts from substance P-containing afferents but most of them are found to be non-synaptic suggesting the importance of electron microscopy in identifying synapses at the point of contacts.

In addition to a large number of neuropeptides, there are several neuropeptide receptors that are also expressed by dorsal horn cells. One of these is the **neurokinin 1 receptor (NK1r)**, which is the main target for substance P containing afferents and is widely distributed in the spinal dorsal horn. NK1r-expressing cells are identified using the specific antibodies directed against the receptor (Vigna *et al.*, 1994, Bleazard *et al.*, 1994, Liu *et*

al., 1994, Brown *et al.*, 1995, Littlewood *et al.*, 1995). Initially it was suggested that only 5% of all the lamina I neurons expressed NK1r (Brown *et al.*, 1995). Later, it was shown that approximately half of lamina I neurons and around a quarter of lamina IV-VI neurons express NK1r-immunoreactivity (Moussaoui *et al.*, 1992, Nakaya Y *et al.*, 1994, Todd *et al.*, 1998). Todd *et al.* (1998) used NeuN antibody to exclude the non-neuronal cells and confocal microscope with improved spatial resolution, thus making it possible to identify weak NK1r-immunoreactive neurons. The great majority of lamina I, III projection cells and many excitatory interneurons also express NK1r in this region (Littlewood *et al.*, 1995). However, the receptor expression of interneurons is weak as compared to projection neurons (Al Ghamdi *et al.*, 2009). NK1r-expressing projection cells will be discussed later in detail.

Recently 4 non-overlapping populations of inhibitory interneurons have been identified in lamina II-III of the rat dorsal horn (Sardella *et al.*, 2011b, Polgár *et al.*, 2011, 2013). These cells have been classified on the basis of nNOS, galanin, NPY or Parvalbumin expression (Sardella *et al.*, 2011a, 2011b, Tiong *et al.*, 2011, Polgár *et al.*, 2011, 2013). NPY, nNOS and galanin expressing cells together account for ~50% of lamina I-II inhibitory interneurons (Sardella *et al.*, 2011b). The great majority of nNOS and galanin inhibitory cells while a small proportion of NPY cells and none of the PV cells express sst_{2A} receptor (Polgár *et al*., 2013b). sst_{2A} receptor is restricted to inhibitory interneurons while receptors like neurokinin-1 (NK1r) and μ-opioid receptor (MOR1) are exclusively found on excitatory cells. Five different G-protein coupled-**somatostatin receptors** (sst_{1-5}) have been identified. One subtype, sst_2 exists as a short (sst_{2B}) and a long form (sst_{2A}). Somatostain binds to sst_{2A} receptor in the spinal dorsal horn (Schindler *et al.*, 1996). The peptide itself is expressed by excitatory cells but the receptor is exclusively found to be associated with inhibitory cells (Todd *et al.*, 1998). Recently, it has been reported that approximately half of the inhibitory interneurons in lamina II of the rat as well as mouse spinal dorsal horn express sst_{2A} receptor (Polgár *et al.*, 2013, Iwagaki *et al.*, 2013).

Nitric oxide synthase (NOS) is an enzyme which is involved in the synthesis of a nitric oxide (NO) gas in both physiological and pathological conditions (Blottner *et al.*, 1995). NOS catalyzes the conversion of $_{L}$-arginine to $_{L}$-cirtulline (Radomski *et al.*, 1990). This enzyme exists in 3 isoforms; the neuronal nitric oxide synthase (nNOS), endothelial nitric oxide synthase (eNOS) and the inducible nitric oxide (iNOS) (Förstermann *et al.*, 1995). Both nNOS and eNOS are calcium/calmodulin dependent and are found in the central nervous system while iNOS is calcium independent and is mostly present in macrophages

and inflammatory cells (Wei *et al.*, 1999, Tao *et al.*, 1999). nNOS is the most abundant isoform in the CNS. It plays an important role in the development and maintenance of inflammatory hyperalgesia and neuropathic pain (Schmidtko *et al.*, 2009). NO acts both pre-and post-synaptically. The pre-synaptic action involves the release of nNOS from the pre-synaptic axon terminals that either depolarizes or hyperpolarizes the post synaptic target (Garthwaite, 2008) while the post synaptic action is brought about by the activation of nNOS-containing cell bodies and dendrites by calcium entering through NMDA receptor (Brenman *et al.*, 1996). nNOS can be identified using histochemical reaction against neuronal nicotinamide adenine dinucleotide phosphate (NADPH) diaphorase (Valtschanoff JG *et al.*, 1992, Spike *et al.*, 1993) and more recently with antibodies against nNOS (Valtschanoff JG *et al.*, 1992, Zhang *et al.*, 1993b). Initially it was suggested that approximately 90% of NADPH and nNOS positive profiles in laminae I-III are GABAergic (Spike *et al.*, 1993, Valtschanoff JG *et al.*, 1992). However, a recent study reported that nNOS cells constitute around 4-5% of the total neuronal population in laminae I and III and around 18% in lamina II (Sardella *et al.*, 2011b). The great majority of these cells (around 2/3rd in lamina II and >50% in lamina III) are excitatory. This observation is contradictory to what has been reported previously. A probable explanation is that previous studies used post-embedding immunocytochemistry for NADPH diaphorase to identify nNOS cells (Spike *et al.*, 1993) and therefore, might not have included weak nNOS cells, which are readily identifiable using nNOS antibody (Sardella *et al.*, 2011b). Neurons with strong nNOS-immunoreactivity are mostly GABAergic and it is likely that only cells with high concentration of nNOS were identified as NADPH positive. The excitatory nNOS cells colocalize considerably with glutamatergic lamina II interneurons that are PKC γ-immunoreactive, however nNOS-immunoreactivity in these cells is weak. Although in the rat, inhibitory nNOS cells constitute a non-overlapping population of dorsal horn cells; recently, it has been shown that there is considerable overlap between nNOS and galanin inhibitory cells in lamina II-III of the spinal dorsal horn of the rat and mouse that expresses green fluorescent protein under the control of prion-promoter. (Polgár *et al.*, 2013, Iwagaki *et al.*, 2013). These cells will be described in detail in chapter 5. Many inhibitory nNOS cells get activated in response to formalin injection and noxious heat by up-regulating the protein product of immediate-early gene called c-*fos* while only few cells express phosphorylated extracellular regulatory kinase (pERK). In contrast, they do not respond to capsaicin injection (Polgár *et al.*, 2013), Both c-*fos* and pERK are the reliable markers of neuronal activity (Ji *et al.*, 1999, Doyle and Hunt, 1999a).

It has been shown that 7% of all lamina I and 3% of lamina II neurons express **galanin** (Tiong *et al.*, 2011). These cells are mostly concentrated in lamina I-IIo with fewer deep cells (lamina IIi-III). In another observation, it was reported that PPD co-localises extensively in both cells bodies and axons of galanin-immunoreactive neurons (Sardella *et al.*, 2011a). The nociceptive role of galanin is also supported by the fact that around 40% of galanin cells phosphorylate ERK in response to capsaicin injection in the rat (Polgár *et al.*, 2013). **Neuropeptide (NPY)** is expressed only by GABAergic cells and it rarely colocalizes with glycine (Hunt *et al.*, 1981, Gibson *et al.*, 1984, Rowan *et al.*, 1993). NPY boutons seldom show VGLUT2 immunoreactivity, which further supports its inhibitory nature (Todd *et al.*, 2003). In the rat, NPY cells constitute approximately 4-6% of lamina I-III neuronal population (Polgár *et al.*, 2011). Dorsal rhizotomy decreases the NPY-binding sites in the dorsal horn, suggesting its primary afferent origin (Kar and Quirion, 1992). Recently it has been shown that NPY cells express pERK in response to mechanical, thermal or chemical noxious stimulation (Polgár *et al.*, 2013). **Parvalbumin** expressing cells belong to a different population of GABA and glycine expressing inhibitory interneurons (Antal *et al.*, 1991, Laing *et al.*, 1994). Most of these cells are found in the ventral lamina II, while others are scattered in lamina II- III. Unlike the other three populations of inhibitory interneurons, PV cells neither express sst_{2A} receptor nor they respond to nociceptive stimuli (Polgár *et al.*, 2013).

Studies have shown that some populations of dorsal horn interneurons are quite selective in their post synaptic targets. In the rat, the inhibitory nNOS boutons selectively target giant lamina I projection cells and constitute around 27% of their total inhibitory input, whereas they make up only 3% of overall inhibitory input to lamina I NK1r projection neurons (Puskár *et al.*, 2001). Similarly, the axons of NPY cells selectively target large lamina III NK1 receptor-expressing projection cells (Polgár *et al*., 1999b). They also provide selective input to PKC γ-expressing excitatory interneurons in lamina II of the rat spinal dorsal horn (Polgár *et al.*, 2011). Recently it has been reported that PV cells receive inputs from myelinated primary fibres and form axoaxonic symmetrical synapses with central terminals of these afferents in ventral lamina II (Hughes *et al.*, 2012).

All these observations suggest that dorsal horn interneurons are selective in their synaptic interactions and they probably perform distinct functions, however, there is no single universally accepted scheme to classify these cells. Although electrophysiological, pharmacological and developmental evidences are helpful in the classification of dorsal horn interneurons, these observations suggest that a single criteria is not useful in defining

any specific population and that a combinational approach should be utilized in categorizing these cells.

1.2.3 Dorsal horn projection cells

Projection neurons constitute a principal source of dorsal horn output to the brain. Several anatomical and electrophysiological studies have been carried out to determine the potential brain targets of dorsal horn cells. These cells can be labelled retrogradely by injecting different tracer substances into their supraspinal target sites while the termination patterns of their axons in the brain can be determined by anterograde tracing method (Todd, 2010). The sensory information from spinal cord dorsal horn is projected via several ascending tracts including anterolateral tract (ALT), post-synaptic dorsal column (PSDC) system and spino-cervicothalamic (SCT) pathway. Since ALT is considered as the main ascending pathway for nociception, it will be explained in detail while PSDC and SCT will be described briefly in this section.

1.2.3.1 Anterolateral tract

Most lamina I and some lamina III dorsal horn projection cells convey nociceptive information through their axons ascending as a component of **anterolateral tract (ALT).** These axons project to one or more brain regions such as lateral parabrachial area (LPb), thalamus, periaqueductal brain region (PAG), caudal ventrolateral medulla (CVLM) (that constitutes a part of reticular formation) and nucleus of solitary tract (NTS) (Giesler *et al.*, 1979, Lima and Coimbra, 1991, Lima *et al.*, 1991, Todd *et al.*, 2000, Spike *et al.*, 2003, Al-Khater *et al.*, 2008, Al-Khater and Todd, 2009). Most ALT neurons in lamina I and III project contralaterally while ~25% also give bilateral projections. (Spike *et al.*, 2003). Dostrovsky and Craig (2006) categorized these projections on the basis of their brain targets as fibres projecting to thalamus (spinothalamic tract STT), direct projections to medulla (brainstem) and hypothalamus as spino-medullary (spino-bulbar) and spino-hypothalamic tract, respectively.

Projection neurons in the rat spinal dorsal horn are mostly concentrated in lamina I and scattered throughout the deeper laminae (III-IV). At the level of 4th lumbar vertebra, lamina I and III projection cells constitute around 400 and 24 cells on each side of the cord, which is approximately 5% and 0.1% of all lamina I and III cells, respectively (Bice and Beal, 1997, Spike *et al.*, 2003, Polgár *et al.*, 2004, Al-Khater *et al.*, 2008). The great majority of these cells (>80% of lamina I and around 70-90% of lamina III-IV), in the

lumbar 4 segment of the spinal cord, target contralateral LPb and CVLM (lateral reticular nucleus). These observations suggest that both LPb and CVLM constitute the major target sites of dorsal horn projection cells in the rat. Furthermore, over 80% of the cells that are retrogradely filled from either of these brain regions also get labelled from the other.

Around 1/3rd of these cells project to PAG, a quarter extend their axons to NTS, while <5% of lamina I and one-fourth of lamina III projection cells target thalamus (Todd *et al*., 2000, Spike *et al*., 2003, Al-Khater *et al*., 2008, Polgár *et al*., 2010). The great majority (~97%) of contralateral lamina I neurons that are retrogradely filled from PAG are also labelled from either LPb or from CVLM (Spike *et al.*, 2003). A considerably smaller proportion of lamina I neurons projecting to thalamus is surprising since spinothalamic component of ALT is considered as the major ascending pathway for pain perception in cat and monkey (primates) (Willis and Coggeshall, 1991, Zhang *et al.*, 1996, Zhang and Craig, 1997). Subsequent studies have reported that this pattern of thalamic projections is limited to lumbar segment of the rat spinal cord since large number (~85%) of lamina I cells in the rat cervical enlargement project exclusively to the caudal part of thalamus, the posterior triangular nucleus (PoT) (Gauriau and Bernard, 2004, Al-Khater *et al*., 2008, Polgár *et al*., 2010). Al-Khater *et al.* further reported that the retrograde injection into PoT labelled a higher proportion of lamina I STT cells not only in the cervical but also in lumbar segment of the cord (~17% as compared to <5% in the previous studies). The majority of projection cells also extend axon collaterals to dorsal and/or ventral horn (Szucs *et al.*, 2010) that contribute not only to the processing of sensory information within the spinal dorsal horn but also to segmental reflex pathways (Todd and Koerber, 2005).

Around 80% of lamina I and ~30% of laminae III-V projection cells express NK1 receptor (Ding *et al.*, 1995, Marshall *et al.*, 1996, Todd *et al.*, 1998), while >90% of lamina III-IV NK1r-expressing cells with dorsally directed dendrites are projection cells (Todd *et al.*, 2000). Al Ghamdi *et al.* (2009) reported that lamina I NK1r projection cells differed from NK1r-expressing interneurons in terms of their soma sizes and NK1r immunoreactivity. Their results showed that more than 90% of the projection neurons had soma cross-sectional areas greater than 200µm^2 and they expressed strong NK1r immunoreactivity while almost all interneurons had soma size of less than 200µm^2 and they were significantly paler in terms of NK1r-expression. Therefore, soma size and NK1 receptor expression can be used to differentiate lamina I NK1r projection cells from NK1r-expressing interneurons, without the need for retrograde tracing methods.

Lamina I projection cells in rat, cat and monkey have been categorized into 3 morphological classes: fusiform, pyramidal and multipolar/flattened (Lima and Coimbra, 1991, Zhang *et al.*, 1996, Zhang and Craig, 1997) and according to Han *et al*. (1998) they represent different functional sub groups. In the cat, pyramidal cells respond to innocuous cooling while fusiform and multipolar cells are activated in response to noxious mechanical and heat stimuli. In addition to that multipolar cells are also responsive to noxious cold (Han *et al.*, 1998). However, in the rat, the majority of cells belonging to all the morphological subtypes express c-*fos* in response to noxious heat, while the same proportion of multipolar cells gets activated in response to noxious cold (Todd *et al.*, 2005). These findings suggest that there is some degree of correlation between the morphology and function of lamina I projection cells in the rat.

Lima & Coimbra (1989) and Lima *et al.* (1991) observed significant differences in the proportion of morphological subtypes of projection neurons that were labelled from the various brain targets. In contrast, Spike *et al.* (2003) reported that both morphology and NK1 receptor-expression did not differ significantly between the cells that were retrogradely labelled from different regions of the brain. The only difference observed was among PAG cells that expressed relatively weak NK1r immunoreactivity as compared to those cells that were labelled from the CVLM or LPb. This discrepancy was attributed to the methodological differences between these studies. In another observation, it was reported that in the L3 segment of the rat, the proportion of lamina I spinoparabrachial cells of different morphological classes was similar, however; lamina I STT cells differed from spinoparabrachial cells in terms of their morphology, soma size and receptive fields (Al-Khater and Todd, 2009). Most lamina I STT cells (~58%) were multipolar with large soma size and receptive fields as compared to LPb projection cells. STT cells in the cervical segment were strongly immunoreactive for NK1 receptor as compared to spinoparabrachial cells (Al-Khater and Todd, 2009). A population of large gephyrin coated lamina I projection neurons was included in among these cells that made up ~20% of lamina I STT cells. These cells were also present in the cervical segment; however, they were outnumbered by STT cells of other morphological subgroups. They will be described later in detail.

Both lamina I and lamina III NK1r projection cells receive substantial synaptic input from substance P containing nociceptive primary afferents (Naim *et al.*, 1997, Todd *et al.*, 2002). This peptidergic input is so dense that at times their boutons outline the dendrites. These afferents constitute approximately 50% of glutamatergic input to NK1r-expressing

lamina I projection cells (Polgár *et al.*, 2010a). Lamina I cells also receive a poly-synaptic input from primary afferents, via lamina II excitatory interneurons, called the vertical cells (Lu and Perl, 2005). The peptidergic input to lamina III NK1r-expressing projection cells is mostly concentrated on their dorsal dendrites their deep dendrites and cell bodies also receive significant input from these afferents. In addition to peptidergic afferents input, lamina III cells also receive synaptic input from myelinated LTMs and very few contacts from somatostain containing peptidergic and IB4-binding C-fibres (Naim *et al.*, 1997, Sakamoto *et al.*, 1999). This suggests that lamina I projection cells are exclusively nociceptive in nature while some deep projection cells have wide dynamic range since they receive input from different primary afferents. Furthermore, lamina III NK1r projection cells also receive a high density of inhibitory input and a significant proportion of this synaptic input is constituted by a subset of inhibitory interneurons that contain neuropeptide Y (NPY) (Polgár *et al*., 1999b). This synaptic input is quite selective as both PSDC and lamina I NK1r cells receive relatively fewer contacts from NPY boutons. The NPY inhibitory boutons make up around 30% of overall inhibitory input to lamina III NK1r-pressing projection cells. This input is concentrated mainly on soma and proximal dendrites, while the input from peptidergic afferents is present mainly on the dorsally directed distal dendrites. Another source of input to lamina III-IV NK1r-expressing projection cells comes from the serotoninergic axons descending from the raphe nuclei at the higher brain centres (Stewart and Maxwell, 2000, Polgár *et al.*, 2002) and noradrenergic fibres from locus coeruleus and various pontine regions (Stewart, 2001). Although lamina III NK1r projection cells are not numerous, their large dendritic tree and dense innervations from peptidergic afferents and inhibitory spinal neurons make them a conspicuous population of dorsal horn projection cells. Recent studies have also shown that the dendrites of NK1r-expressing lamina III projection cells have multiple synapses that contain the GluR2 and GluR4 subunits of AMPA receptor, which indicate a high density of excitatory input to these cells (Polgár *et al*., 2008a, Todd *et al*., 2009). However, not much is known about the source of non-primary glutamatergic input to these cells. It would be interesting to see whether lamina III NK1r–expressing projection neurons receive synaptic input from glutamatergic spinal neurons. It has already been suggested that there is a wide variation in the sizes of glutamatergic synapses formed on lamina III NK1r projection neurons and that the source of some of these synapses is the peptidergic primary afferents. However, the exact proportion of these contacts among the overall excitatory input to these cells is not known (Naim *et al.*, 1997, Todd *et al.*, 2009). In addition to that, whether these synapses vary in sizes from the ones formed by glutamatergic dorsal horn neurons is yet to be determined.

Electrophysiological studies have shown that most lamina I cells are activated in response to noxious stimulation (Light and Perl, 1979, Han *et al.*, 1998, Bester *et al.*, 2000, Craig *et al.*, 2001) and that STT cells have larger receptive fields as compared to spinoparabrachial neurons (Bester *et al.*, 2000, Zhang *et al.*, 2006). Activation of lamina I and III-IV projection cells by noxious stimulation is indicated by the induction of transcription factors such as c-*fos* (Hunt *et al.*, 1987) and pERK (Polgár *et al*., 2007a) as well as by internalization of anatomical markers such as NK1 receptor (Mantyh *et al.*, 1995, Abbadie *et al.*, 1997). Spinothalamic tract is involved in the transmission of light touch, pressure and itch sensation (Giesler *et al.*, 1976, Andrew and Craig, 2001, Davidson *et al.*, 2009). Palecek *et al.*(2003) reported the expression of c-*fos* in STT cells in response to visceral and cutaneous noxious stimuli. Furthermore, all lamina I and some lamina III-IV projection cells, some of which also contribute to STT, are nociceptive in nature (Craig *et al.*, 2001, Andrew and Craig, 2001). They up regulate Fos and show ERK phosphorylation in response to mechanical, thermal or chemical noxious stimuli (Doyle and Hunt, 1999a, Todd *et al*., 2002, Polgár *et al*., 2007a).

The NK1-receptors are activated in response to noxious stimulation and undergo substance P induced phosphorylation and internalization into the cytoplasm of soma and dendrites (Mantyh *et al*., 1995, Abbadie *et al*., 1997, Polgár *et al*., 2007b). The degree of NK1 receptor internalization directly corresponds to the severity of noxious stimulus and is important for the intracellular signalling (Wang and Marvizón, 2002). Mantyh and colleagues (1997) used the capsaicin model of hyperalgesia and reported that an intrathecal injection of substance P conjugated to cytotoxin saporin (SP-SAP) caused selective destruction of NK1 receptor-expressing cells in lamina I of the spinal dorsal horn. The Rats that underwent this procedure showed significantly reduced responses to intense noxious stimuli as well as to mechanical and thermal hyperalgesia produced by capsaicin injection while the responses to mild noxious stimuli remained intact. Similarly, the effects of SP-SAP in inflammatory and neuropathic pain models resulted in the attenuation of thermal hyperalgesia and mechanical allodynia (Nichols *et al.*, 1999). However, the responses to mild noxious stimuli and morphine induced analgesia remained unaffected. These results support the role of substance P and NK1 receptor-expressing cells in the development of chronic hyperalgesia and further suggest that the cells responsible for the transmission of acute painful stimuli are different from NK1r projection cells. However, studies based on genetically modified mice that either lack NK1 receptor (Felipe *et al.*, 1998) or the gene that encodes for substance P (preprotachykinin gene) (Cao *et al.*, 1998), reported minimally reduced signs of hyperalgesia in inflammatory pain models while the responses

to moderate to intensive painful stimulation were significantly reduced. These results are contradictory to what Mantyh (1997) reported and imply the role of neurochemical substances other than substance P (such as glutamate) in the development of hyperalgesia as well as responses to acute noxious stimuli (De Biasi and Rustioni, 1988). Previous studies have shown that the response of NK1r cells to substance P and noxious stimuli is dramatically reduced by NK1r antagonists (Laird *et al.*, 1993, Jung *et al.*, 1994). Surprisingly, NK1r antagonist drugs are unsuccessful in treating pain (Hill, 2000). These observations suggest that a more intricate circuitry is responsible for the spinal dorsal horn pain processing.

Apart from lamina I NK1r-expressing projection cells, there are other populations of projection neurons in lamina I of the spinal dorsal horn. They include gephyrin enriched giant spino-parabrachial projection cells and a population of projection neurons that do not express NK1r or belong to the giant cells. Lamina I giant cells possess gephyrin coated cell bodies (Puskár *et al.*, 2001) and multipolar dendritic tree that arborize in the horizontal plane. These giant cells form a subset of classical marginal cells of Waldeyer (1888) that were identified in the earlier studies (Gobel, 1978, Lima and Coimbra, 1986). It has been suggested that the Waldeyer cells are usually sparse and twice as large as the adjacent cells in lamina I of the spinal dorsal horn (Ralston, 1968). The light microscopic studies have shown that these cells are mostly located at the border between the dorsal columns and the spinal gray matter (Rexed, 1952, Ralston, 1968, Brown, 1982). Furthermore, their dendritic processes are oriented in the mediolateral axis along the horizontal plane (Lima and Coimbra, 1983, 1986, Han *et al.*, 1998, Galhardo and Lima, 1999) while their axons project intersegmentally as well as supraspinally (Brown, 1982, Puskár *et al.*, 2001). Some large lamina I NK1r-expressing neurons have also been included among Waldeyer cells (Brown *et al.*, 1995, Puskár *et al.*, 2001), therefore lamina I giant cells and large NK1r-expressing neurons constitute 2 different sub-groups of marginal cells of Waldeyer.

The lamina I giant cells are sparse with 10 cells on each side of the 4th lumbar segment in the rat and they constitute only 2.5% of all the lamina I projection neurons (Puskár *et al*., 2001, Polgár *et al*., 2008b). The great majority of giant cells project to lateral parabrachial region (LPb) (Polgár *et al*., 2008b), while only 5% in lumbar and over 50% in the cervical segments also project to periaqueductal grey region (PAG) and few target thalamus (Al-Khater and Todd, 2009). They either lack NK1 receptor or show a very weak expression. Another characteristic feature of these cells is that they receive a very dense innervation from both excitatory (VGLUT2) and inhibitory (VGAT) spinal boutons (Puskár *et al.*,

2001). This input is so dense that it almost outlines the soma and most of the dendritic tree of these cells. VGLUT2 boutons on these cells are associated with GluR4-containing puncta, which indicate glutamatergic synapses. GluR4 containing synapses are relatively sparse in the superficial laminae but are present at high concentration on giant cells and other lamina I projection neurons (Polgár *et al*., 2008a, Polgár *et al*., 2010a). These excitatory and inhibitory boutons are derived exclusively from interneurons as lamina I giant cells do not seem to receive primary afferent input (Todd *et al*., 2003, Polgár *et al*., 2008b). In the rat, these cells receive a selective innervation from a subset of inhibitory interneurons that express neuronal nitric oxide synthase (nNOS) (Puskár *et al*., 2001, Polgár *et al*., 2008b). They also express c-*fos* in response to formalin injection and noxious heat, however the response to noxious heat is relatively weak (Puskár *et al*., 2001, Polgár *et al*., 2008b). Their responsiveness to noxious stimuli suggests that this population of projection neurons may contribute to the normal response to acute noxious stimuli seen after the selective destruction of NK1r cells by SP-SAP injection (Mantyh *et al.*, 1997). These cells mostly project to the parabrachial region in the pons, which is known to participate in the autonomic adaptations and emotional component of pain processing. These findings support the idea that the giant lamina I cells are involved in the processing of affective and emotional aspect of pain (Gauriau and Bernard, 2002, Craig, 2003). However, it is not known whether giant cells also exist in the mouse and if so whether they also receive a selective innervation from NOS-containing inhibitory axons.

Not much is known about the remaining projection neurons in lamina I that constitute around 20% of all lamina I projection cells (Marshall *et al.*, 1996). Very few studies have been done in the past to determine the morphology of NK1r-negative cells (Almarestani *et al.*, 2007, Al Ghamdi *et al.*, 2009). In the latter study, a comparison was made between the soma sizes of $NK1r^{+}$ and $NK1r^{-}$ projection cells. The results suggested that the NK1r-expressing projection cells were larger than the $NK1r^{-}$cells but the difference was statistically insignificant. There have been different opinions about the functions of lamina I $NK1r^{-}$cells. Some of these cells are activated only by noxious stimuli (Todd *et al.*, 2002) while others respond to noxious as well as innocuous thermal stimulation (Dostrovsky and Craig, 1996). The pyramidal shaped $NK1r^{-}$cells have been related to the cooling specific cells in the cat (Han *et al.*, 1998, Almarestani *et al.*, 2007). Studies based on Fos as an activation marker have reported that lamina I projection cells that do not express NK1 receptor are less likely to express Fos in response to noxious heat or sub-cutaneous formalin, as compared to NK1r-expressing projection cells (Todd *et al.*, 2002, 2005). Todd *et al.* (2005) reported that only 14% of lamina I $NK1r^{-}$ projection cells showed Fos

following immersion of foot in water at 52^{o}C for 20 sec. In contrast, the majority (63%) of lamina I NK1r projection cells expressed Fos in response to the same stimulus. Since, the great majority of nociceptive afferents terminate in lamina I and its projection cells are retrogradely labelled from brain regions known to be involved in nociception, it is likely that the remaining projection cells are also involved in nociception. One aim of this study was to determine the pattern of nociceptive primary afferent input to lamina NK1r^{-} projection cells.

Not all the large neurons of lamina III-IV with dorsally directed dendrites project supraspinally or express NK1 receptors. There is another population of large neurons in lamina III, which extend their dorsal dendrites to the superficial laminae and contain NF200, however they do not express NK1 receptor and receive very few contacts from peptidergic afferents (Polgár *et al*., 2007b). Unlike other projection cells in this region, they are not labelled retrogradely from contralateral CVLM, thalamus or gracile nucleus. It has been suggested that these cells might be involved in transmitting information to the deep dorsal horn from non-peptidergic afferents (Polgár *et al*., 2007b).

It has been reported that not all the cells in lamina III-IV that are retrogradely labelled from injection into gracile nucleus are NK1r-immunoreactive (Marshall *et al.*, 1996). Some deep dorsal horn (lamina III-IV) projection neurons belong to **post synaptic dorsal column cells (PSDC)** (Giesler *et al.*, 1984). Their cell bodies are located quite ventral to lamina II-III border. They extend their fibres uninterruptedly via dorsal column to synapse with second order neurons in the ipsilateral dorsal column nuclei in the medulla (Uddenberg, 1968, Giesler *et al.*, 1984). Brown and Fyffe (1981) suggested that in the cat, these cells have dendrites that extend dorsally as far as lamina I and therefore seem to mimic large lamina III projection cells in the rat. However, other studies have reported that some of these cells do have dendrites extending dorsally to superficial dorsal horn with only a few entering lamina II (Polgár *et al*., 1999b, Palecek *et al*., 2003). In addition, these cells lack NK1r and receive fewer contacts from NPY containing inhibitory boutons (Polgár *et al*., 1999b). In the cat, these cells receive innervation from cutaneous and LTMRs through mono- or di-synaptic pathways (Jankowska *et al.*, 1979). It has been suggested that these cells respond to noxious stimulation and therefore might be involved in visceral nociception (Uddenberg, 1968, Ness *et al.*, 1998).

The spino-cervicothalamic tract (SCT) consists of lamina III-IV projection cells in the lumbar segment that project to synapse with second order neurons in the lateral cervical

nuclei in the cervical segment. The cells in the lateral cervical nucleus then extend their axons in the most lateral part of dorsolateral funniculus that eventually terminate in the midbrain (Giesler *et al.*, 1988). These cells form a di-synaptic (indirect) route to thalamus from spinal dorsal horn. Brown *et al.* (1976) suggested that most SCT cells have dorsally directed dendrites that terminate at lamina II-III border, however, none of the dendrites seem to enter lamina I. Nearly all SCT cells receive input from mechanoreceptors and most of these cells also respond to noxious stimulation (Brown and Franz, 1969).

1.3 Projection targets

Several studies have determined the brain targets of dorsal horn projection neurons. Most lamina I and III-IV projection cells can be labelled retrogradely from **LPb** (Cechetto *et al.*, 1985, Ding *et al.*, 1995, Todd *et al.*, 2000, Spike *et al.*, 2003), and **CVLM** (Menétrey *et al.*, 1983, Lima and Coimbra, 1991, Todd *et al.*, 2002, Spike *et al.*, 2003), while some project to **PAG** (Menétrey *et al.*, 1983, Lima and Coimbra, 1989, Todd *et al.*, 2000) (Spike *et al.*, 2003) and a smaller proportion to certain **thalamic nuclei** (Lima and Coimbra, 1988, Burstein *et al.*, 1990, Marshall *et al.*, 1996). Other potential targets for these cells include nucleus of solitary tract (NTS) (Menétrey and Basbaum, 1987), locus coeruleus and sub-coeruleus (Craig, 1995), limbic and striatal areas (Newman *et al.*, 1996).

The **lateral parabrachial (LPb)** region lies at the border of midbrain and pons in the lateral reticular formation. The fibres projecting to this region are collectively known as spino-parabrachial tract. In the rat, the great majority (95%) of lamina I projection neurons target LPb (Spike *et al.*, 2003).Studies based on retrograde and anterograde labelling methods have shown that most lamina III NK1r-expressing projection cells in the lumbar of the spinal cord also target LPb (Slugg and Light, 1994, Todd *et al.*, 2000, Al-Khater and Todd, 2009). Bernard *et al.* (1995) reported that lamina III-IV cells in the cervical segment did not project to LPb while Al-Khater and Todd showed that LPb did receive projections from lamina III-IV cells in the cervical segment, however, these cells were fewer in number as compared to those in the lumbar enlargement. The discrepancy in the results might be due to the different labelling technique and small injections used by Bernard *et al.*, which would have failed to label the cells.

The **caudal ventrolateral medulla (CVLM)** is not a very well defined region but is often regarded as an area of medulla between the lateral reticular and spinal trigeminal nuclei (Lima *et al.*, 1991, Tavares and Lima, 1994, Todd *et al.*, 2000, Spike *et al.*, 2003). CVLM

is a major target site for lamina I projection neurons in cat and monkey (Craig, 1995) and lamina I and III projection cells in the rat (Todd *et al.*, 2000). Since the main bundle of ALT axons, projecting to midbrain, lies very close to this region, therefore, there is a possibility that the retrograde labelling seen with CVLM injection may result from the uptake of tracer by nearby ALT fibres (Todd *et al.*, 2000). However, Craig (1995) evidently showed direct projections of spinal dorsal horn cells to CVLM using anterograde tracing method. CVLM not only receives the projecting axons but its neurons also send descending fibres to the cord. It appears that the cells in CVLM and lamina I projection neurons are reciprocally connected by a closed circuit that controls supraspinally projected nociceptive information (Tavares and Lima, 2002). The great majority of lamina III NK1r-expressing projection cells (>90%) target contralateral CVLM while many (>60%) send axon collaterals to LPb. Some of these cells also project to dorsal part of caudal medulla, solitary tract nuclei and PAG (Todd *et al.*, 2000). CVLM is also involved in the integration of nociceptive and cardiovascular responses (Lima and Coimbra, 1991, Lima *et al.*, 2002).

The **periaqueductal grey PAG** is the region of the grey matter which surrounds the aqueduct of sylvius, within the tegmentum of the midbrain. Around 1/3rd of lamina I projection neurons target PAG (Spike *et al.*, 2003). The number of lamina I projection cells that are labelled from PAG is similar in cervical and lumbar enlargement (Polgár *et al.*, 2010b), however, >50% of lamina I giant cells in the cervical segment project to PAG as compared to ~5% from the lumbar segment (Al-Khater and Todd, 2009). PAG also receives some projections from lamina III NK1r-expressing projection (Al-Khater and Todd, 2009). PAG plays an important role in the modulation of dorsal horn transmission through fibres that descend from rostral ventromedial medulla. It is also involved the management of the stressful situations including pain (Todd *et al.*, 2000, Spike *et al.*, 2003, Al-Khater and Todd, 2009, Drew *et al.*, 2009).

The **thalamus** is a main relay station for almost all the sensory and motor information to the somatosensory regions in the cerebral cortex. It comprises of several nuclei that receive axons from dorsal horn projection neurons. Among them are ventral posterolateral nucleus (VPL) and posterior triangular nucleus (PoT) (Al-Khater *et al.*, 2008, Todd, 2010). VPL is connected to somatosensory cortex reciprocally while PoT sends projections to second somatosensory region and insular cortex (Gauriau and Bernard, 2004). Both these regions take part in sensory-discriminative as well as affective motivational aspects of pain. Most lumbar enlargement cells in cat (Zhang *et al.*, 1996) and monkey (Zhang and Craig, 1997) project to thalamus. However, STT cells make up a small proportion of lamina I projection

neurons in the lumbar segment of the rat spinal cord (Al-Khater and Todd, 2009). As in PAG, thalamus also receives a much higher number of lamina I projection neurons from the cervical segment (C7) (Polgár *et al.*, 2010b), while only a small proportion project to hypothalamus as spinohypothalamic tract (Todd *et al.*, 2000).

The proportion of lamina I and lamina III-IV projection neurons projecting to various brain regions is illustrated in figure 1-2.

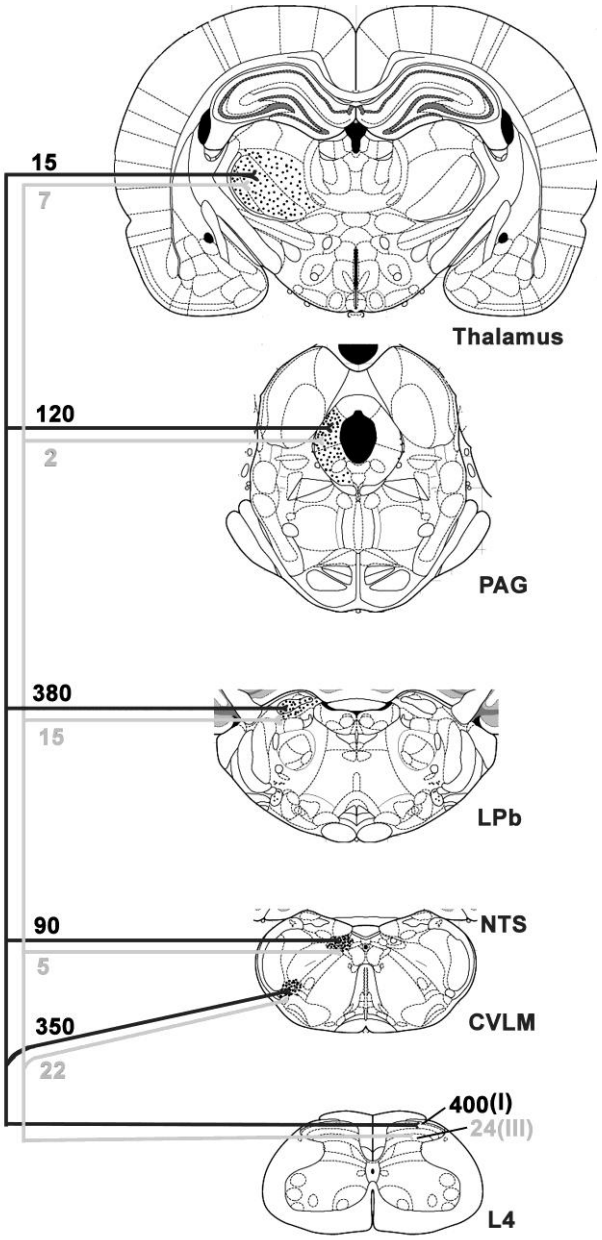


Figure 1-2 Summary of the quantitative data from a number of studies of projection neurons in L4 segment of spinal dorsal horn.

The great majority of lamina I and III cells (5% and 0.1% of all the cells in lamina I and III, respectively), project to lateral parabrachial (LPb) and caudal ventrolateral medulla (CVLM), with some projecting to other regions. Numbers along the lines in black and grey indicate the total number of lamina I and III NK1r-expressing projection cells terminating in these regions, respectively . Almost all the cells projecting to thalamus, PAG and NTS can be retrogradely labelled from the tracer injected into LPb and CVLM. Modified from Todd (2010).

1.4 Functional implications of dorsal horn neuronal circuitry

Spinal cord dorsal horn receives sensory information from different types of primary afferents, including those that perceive noxious stimuli. This incoming sensory information is processed and integrated by the intricate spinal circuitry that further transmits it to several brain areas (Todd, 2010). Since this neuronal circuitry includes various excitatory and inhibitory cells, it is imperative to understand the balance between excitation and inhibition in order to maintain normal sensory functions. Furthermore, the information about the normal morphological and physiological characteristics of dorsal horn is vital in understanding the mechanisms underlying certain pathological conditions such as acute and chronic pain as well as pathological itch. Despite several studies, we still lack the knowledge about the neuronal circuits that link sensory afferents and interneurons to dorsal horn projection cells. These projection cells constitute the major output of spinal dorsal horn. The different experiments performed in this study provide evidences regarding the preferential innervation of various populations of dorsal horn projection cells by nociceptive primary afferents as well as spinal excitatory and inhibitory cells, thus adding to the better understanding of dorsal horn circuitry.

Studies in the past have suggested that certain pathological conditions (tissue inflammation and nerve injury) can evoke abnormal pain sensations such as hyperalgesia, allodynia and spontaneous pain (Sandkühler, 2009, Todd, 2010). Several changes taking place in the spinal cord could underlie these phenomenons, for instance the lack of expression of hyperalgesia may result from selective destruction of lamina I NK1r-expressing cells by cytotoxic SP-SAP in capsaicin (Mantyh *et al.*, 1997) and inflammatory/neuropathic chronic pain model (Nichols *et al.*, 1999). One of the proposed cellular mechanisms underlying NK1r- induced hyperalgesia is long term potentiation (LTP), which is a long lasting but not necessarily an irreversible increase in synaptic strength (Sandkühler, 2009). In case of NK1r-expressing cells, LTP is brought about by calcium-dependant post-synaptic mechanism induced by dorsal root stimulation at C-fibre strength (Ikeda *et al.*, 2003). This observation is further supported by the dense innervation of dorsal horn lamina I-III NK1r projection cells by peptidergic afferents (Naim *et al.*, 1997, Todd *et al.*, 2002). Furthermore, insertion of AMPAr subunits into the excitatory synapse on NK1r projection cells also contribute to LTP (Polgár *et al.*, 2008a). However, it is not known whether these cells receive glutamatergic synapses of spinal origin and if so, whether they belong to a discrete population of dorsal horn excitatory cells. Furthermore, it is not known whether

synapses of this type are anatomically different from those formed by peptidergic afferents and contribute to any pain state. This observation is important since studies have shown that dorsal horn excitatory cells have a tendency to undergo intrinsic plasticity that changes the neuronal excitability (Yasaka *et al.*, 2010). This increased excitatory transmission could enhance the activity of dorsal horn projection cells through polysynaptic pathways and may contribute to hyperalgesia in inflammatory pain states.

Not much is known about the transmission of fast pain. An interesting observation made by both Mantyh *et al.* (1997) and Nichols *et al.* (1999) was that the responses to acute noxious stimuli were retained by their respective experimental models after the treatment with SP-SAP. This suggested that the cells responsible for the transmission of acute noxious stimuli are different from NK1r-expressing projection cells. It would be interesting to determine whether those lamina I projection cell that do not express NK1 receptor are targeted by afferents (Aδ-nociceptors) responsible for the perception of fast pain. This observation could unravel the spinal circuits involved in the transmission of fast pain.

According to the Gate Control Theory of Pain (Malzack and Wall, 1965), cells in lamina II of the spinal dorsal horn are involved in the modulation of the afferent inputs. Furthermore, disinhibition caused by a reduction in the GABAergic/ glycinergic neurotransmission could also contribute to allodynia, hyperalgesia and spontaneous pain (Sandkühler, 2009). It is therefore important to determine the potential targets of spinal inhibitory cells within the dorsal horn as alteration of their inputs or intrinsic properties could lead to disinhibition. It has also been suggested that in inflammatory and neuropathic pain states, disinhibition may also result in the increased transmission of Aβ-polysynaptic input to the nociceptive cells in the superficial dorsal horn (Baba *et al.*, 1999, Torsney and MacDermott, 2006, Schoffnegger *et al.*, 2008), there by modifying nociceptive specific cells into wide dynamic range cells. It is therefore necessary to identify the neuronal circuits involved in these pathological conditions in order to interpret their underling mechanisms.

Similar to pain, itch is also an aversive sensation that warns the body against the potential threats. Conditions such as chronic itch are as debilitating as chronic pain (Ross, 2011, Bautista *et al.*, 2014). Unfortunately, there are fewer therapeutic therapies to relieve pathological itch due to the lack of clear understanding of itch transmission. There is no single neuronal mechanism that explains all the features of itch. This is generally attributed to the overlapping morphological and functional elements of central nervous system, which

contribute to the transmission of both pain and itch. Several theories have been proposed regarding the neural basis of itch transmission by McMahon and Koltzenburg (1992). One explanation is based on intensity theory that suggests that the neurons for both pain and itch are the same and the intensity with which they are stimulated determines the type of sensation perceived. Weak stimulation of nociceptive fibres signals itch while pain is felt when these afferents are triggered strongly. Another proposed model of itch transmission called the spatial contrast model suggests that itch and pain-related cells are polymodal in nature but their distribution varies. Therefore it is the location of the afferents that differentiates itch from pain. Selectivity model of itch proposes that both itch and pain sensitive cells are polymodal; however itch is perceived when only itch sensitive cells are activated while pain is felt when both itch and pain sensitive neurons are stimulated. Recently the specificity theory/ labelled line theory of itch has gained much popularity. Based on this theory, itch is considered as a functionally and anatomically distinct sensory modality from pain. This is supported by the presence of itch specific unmyelinated primary afferents in humans (Schmelz *et al.*, 1997) and mice (Han *et al.*, 2013). In addition to that, itch specific spinothalamic tract cells (Andrew and Craig, 2001) and GRPR-expressing dorsal horn cells (Sun *et al.*, 2009) have also been identified. Based on the recent studies, it has been shown that distinct subsets of dorsal horn inhibitory cells that express nNOS and/or galanin are required for the normal perception of itch (Ross *et al.*, 2010, Kardon *et al.*, 2014). However the potential targets of these inhibitory interneurons in the spinal dorsal horn are not known. This information is important since it would provide an insight to the changes at the level of dorsal horn circuitry that would contribute to refractory itch seen in a number of clinical conditions. In such cases, the responses to painful stimuli usually remain intact. These findings suggest that different sensory modalities are probably transmitted through distinct pathways, there by supporting the labelled line theory of itch.

1.5 **Project Aims**

The overall aim of this project was to determine the inputs to different populations of dorsal horn projection cells by discrete subgroups of spinal neurons (both inhibitory and excitatory) and primary afferents. The first two parts of this study were carried out on rat while the last set of experiments was performed on transgenic mice. The individual aims of each part of the study will be discussed in detail in the corresponding chapters. Broadly, this project is based on the following outlines:

1. It has already been mentioned that lamina III of the spinal dorsal horn contains a distinct population of NK1r-expressing projection neurons with dorsally directed dendrites. These projection cells are extensively innervated by substance P-containing peptidergic primary afferents and receive modest synaptic input from low threshold mechanoreceptors. In addition, they also receive selective inhibitory input from NPY-containing axons of spinal origin. However, not much is known about the input that these cells receive from excitatory spinal neurons. The first aim of this study is to compare the frequency of excitatory input to the lamina III NK1r^{+} projection neurons from non-primary VGLUT2 boutons (which are likely to originate from local neurons) with that from primary afferents (both CGRP-expressing peptidergic and VGLUT1 expressing LTMRs afferents).

2. Some excitatory interneurons in dorsal horn express dynorphin precursor preprodynorphin (PPD) and preliminary observations suggest that the lamina III projection neurons receive numerous contacts from VGLUT2 boutons that contained PPD. The second aim is therefore to determine whether this input to lamina III NK1r-expressing projection neurons represents a selective innervation.

3. It has been shown that there is a wide variation in the sizes of glutamatergic synapses on the lamina III projection cells so another aim is to test whether synapses formed by peptidergic primary afferents are larger than those from glutamatergic dorsal horn neurons.

4. Aδ-nociceptors conduct fast/sharp pain. These afferents terminate in lamina I of the spinal dorsal horn and can be identified with CTb injected into sciatic nerve. Little is known about their neurochemistry (with respect to peptide expression) and potential targets in lamina I of the spinal dorsal horn. Therefore, another aim is to

compare the expression pattern of Aδ-nociceptors with peptidergic afferents in rat spinal dorsal horn.

5. Since lamina I of the spinal dorsal horn is the region that contains a high proportion of projection cells, it will be interesting to observe whether CTb-labelled Aδ-nociceptors innervate lamina I projection neurons that project to LPb and if so, the proportion of this input in comparison to excitatory inputs from glutamatergic interneurons and peptidergic afferents.

6. Since in the rat, nNOS inhibitory boutons selectively target lamina I giant projection cells, it is not known whether the axons of GFP^{+}/$nNOS^{+}$ cells provide inhibitory synaptic input to lamina I giant cells in the PrP-GFP mouse. This part of the study will be based on 2 different types of genetically modified mice. In the first group, the green fluorescent protein (GFP) is expressed, under the control of prion promoter (PrP-GFP), by a distinct population of lamina II inhibitory interneurons (Hantman *et al.*, 2004) while the second group consists of mice that lack transcription factor *Bhlhb5*, and exhibit an altered phenotype (Ross *et al.*, 2010)

7. In order to establish whether the PrP-GFP input to giant cells represent a selective innervation, the distribution of PrP-GFP expressing axons among the inhibitory boutons in superficial dorsal horn of the PrP-GFP mouse and GFP input to lamina I NK1r-expressing neurons is also determined.

8. It has been reported that cells belonging to nNOS and galanin containing inhibitory interneurons are substantially depleted in *Bhlhb5*$^{-/-}$ mouse. Since, lamina I giant cells in the rat receive a selective innervation from nNOS inhibitory boutons, therefore, the possibility that the synapses formed by nNOS-containing inhibitory boutons on lamina I giant cells are also lost in the *Bhlhb5*$^{-/-}$ mice is also tested.

2 General Methods

This chapter provides a brief description of general methods that were used during this study. All experiments were approved by the Ethical Review Process Application Panel of the University of Glasgow and were performed in accordance with the UK Animals (Scientific Procedures) Act 1986. The surgical procedures performed by Dr S. A. Shehab of UAE University were in accordance with Animal Ethics Committee of the College of Medicine and Health Science of the United Arab Emirates University. The animal perfusions were carried out with Dr. Erika Polgár and Mr. Robert Kerr, while the tissue processing for electron microscopy was made possible with the technical assistance from Mrs. Christine Watt. Details about some specific techniques, species, number and weights of the animals that were used in various parts of this study will be provided in the corresponding chapters.

2.1 Perfusion fixation

Animals were deeply anesthetized with pentobarbitone (300 mg for rats, 30 mg for mice intraperitoneally). A midline thoraco-abdominal incision was made and extended to the diaphragm. Animals were perfused through the left ventricle with Ringer solution for approximately 5 seconds, to flush out the blood. This was followed by the perfusion with one litre of fixative for the rats and 250ml for the mice. The fixative contained 4% freshly depolymerised formaldehyde or 4% formaldehyde with 0.2% or 1 % glutaraldehyde in 0.1M phosphate buffer (PB). The latter combination was used for combined confocal and electron microscopy. After fixation, animals were dissected by incising the skin of the back in the midline along the entire length of the vertebral column. Tissue underlying the skin was dissected in layers and paraspinal muscles were removed to expose the vertebral column. Laminectomy of the lumbar region was carried using a pair of fine rongeurs. Care was taken to avoid any damage to the underlying dura and spinal cord. The spinal cord within the dural sac was exposed and dura was excised using fine curved blades. The dura was reflected laterally to allow maximum exposure of the spinal cord and the dorsal roots of the spinal nerves. The last rib was identified and used to locate the dorsal root and ganglion of the 13^{th} thoracic segment, which lies beneath the last rib in the intervertebral foramen. The dorsal roots of the spinal nerves were then followed caudally and lumbar segments were identified sequentially. The roots were separated from the ganglia and those on the left hand side were kept intact to allow the identification of the two sides of the segment. Lumbar spinal cord was removed and the mid lumbar segments (L3-L5) were separated using a fine straight blade.

The detailed account of the brain surgery will be described in chapter 4 of this thesis. For the animals that received brain injections, the skin of the head was incised in the midline and reflected laterally. With the help of a bone rongeurs, the bones of the cranium were cut and removed. At the junction of brainstem and spinal cord, a sharp cut was made and the brain was carefully lifted after separating it from the surrounding dura. Brain was placed in fixative solution containing 30% sucrose for cryoprotection. The spinal cord segments and brain were kept in fixative over night at 4^{o}C. In these animals, spinal cord segments were notched on the ipsilateral side for the identification purpose.

2.2 Tissue processing and immunocytochemistry

All the experiments were carried out on the mid lumbar (L3-L5) segments of the spinal cord. Binocular dissecting microscope was used to remove the dorsal and ventral roots of the spinal cord segments, which were then laid flat in a petri dish. A low-melting-point agarose was prepared in distilled water (concentration of 3gms agarose in 100ml of distilled water), cooled and slowly poured over the cord until it was fully embedded. Segments were kept in the fridge at 4^{o}C for approximately 15-20 minutes, until the agar was completely solidified. These agar blocks-containing spinal cord segments were then cut into either 50µm or 60µm-thick sections using vibrating microtome (VT 1000S, Vibrating blade microtome, Lieca microsystems Ltd Milton Keynes, UK). Sections were cut in different planes (parasagittal, horizontal or transverse) depending upon the cells of interest, type of experiment and corresponding immunocytochemistry protocol. For instance, the dendrites of lamina I projection neurons are restricted within the lamina and are oriented mainly rostrocaudally and/or mediolateraly, while lamina III contains a mixed population of large neurons with dorsally directed dendrites, these cells are therefore best studied in horizontal (Cheunsuang and Morris, 2000) and parasagittal sections (Todd, 1989), respectively. In addition, the laminar boundaries and the distribution of cells or their boutons with in different laminae are best studied in transverse sections of the cord. All the sections were treated with 50% ethanol for 30 minutes to improve antibody penetration (Llewellyn-Smith and Minson, 1992). This was followed by 3 rinses of 10 minutes each in 0.3M NaCl containing phosphate buffer saline (PBS). Sections for combined confocal and electron microscopy were processed differently. This will be discussed in the method section of the corresponding chapters.

The immunocytochemical technique was used to visualize and determine the distribution of neurochemical markers of interest. This method has been widely applied to identify antigenic substance on histological specimens. In all the cases, incubation in primary antibodies was at 4^{o}C for 3 days. Apart from the reaction that was done for combined confocal and electron microscopy, antibodies were diluted in PBS that contained 0.3% Triton® X-100. The details of the sources and concentrations of the antibodies used in this project are listed in Table 2-1. After 3 days, sections were rinsed in PBS for 30 minutes, followed by overnight incubation in the species-specific secondary antibodies at 4^{o}C. All secondary antibodies were raised in donkey. Fluorescent secondary antibodies were conjugated to Rhodamine Red, DyLight 649 (1:100 and 1:500, respectively; both from Jackson ImmunoResearch), Alexa 488, Alexa 555 or Alexa 568 (1:500; Invitrogen). Secondary antibodies labelled with biotin (1:500) or horseradish peroxidase (HRP; 1:1000, both from Jackson Immunoresearch) were also used. The biotinylated antibodies were revealed with either avidin-Pacific Blue (Invitrogen; 1:1000), or with avidin-HRP (Sigma; 1:1000). The various antibody combinations will be discussed in detail in each chapter. All the sections were then rinsed three times in PBS, unless otherwise stated and were mounted on glass slides in anti-fade medium and covered with glass cover slips.

2.3 Antibody characterization

The antibody against NK1 receptor was raised in rabbit against a peptide that was constituted by amino acids 393–407 of the rat NK1r and corresponded to 46 kDa bands in Western blots of rat brain extracts. This antibody showed no staining in mice in which NK1r was deleted (Ptak *et al.*, 2002). The guinea pig antibody against NK1 receptor was raised commercially against a synthetic peptide that was used in Vigna *et al.* (1994). The specificity of NK1r staining was established previously by performing immunofluorescence with both rabbit and guinea pig anti serum against NK1 receptor (Polgár *et al*., 1999b).

The guinea pig and goat anti-VGLUT2 were raised against peptides that corresponded to amino acids 565–582 of rat VGLUT2 and amino acids 550–582 of mouse VGLUT2, respectively while the guinea pig VGLUT1 antibody was raised against amino acids 541–560 of the rat protein. It has been shown that the guinea pig antibodies against VGLUT1 and VGLUT2 stain equivalent structures, which are also stained, by a well-characterized VGLUT1 and VGLUT2 rabbit antibodies (Todd *et al.*, 2003). The goat anti-VGLUT2

identifies a single protein band of the appropriate molecular weight (60 kDa) (Kawamura *et al.*, 2006).

The rabbit and guinea pig antibodies against PPD were raised against a peptide corresponding to amino acids 229–248 at the C-terminus of rat PPD. They have been shown to be specific to PPD and do not label dynorphin or enkephalin. It has also been reported that the immunizing peptide blocks the tissue staining due to the pre-absorption of the antibody (Lee *et al.*, 1997).

The guinea pig and goat CGRP antibodies identify both α and β forms of the peptide.

The GFP antibodies were raised against recombinant GFP, and the distribution of GFP-immunostaining corresponded to cells that expressed GFP (Iwagaki *et al.*, 2013).

The mouse monoclonal antibody against gephyrin (mAb 7a) was raised against an extract of rat spinal cord synaptic membranes. It recognizes a 93 kDa peripheral membrane protein (gephyrin) in extracts of rat brain membranes (Pfeiffer *et al.*, 1984).

The nNOS antibody recognizes a band of 155 kDa in western blots of rat hypothalamus, and the staining disappears by pre-incubation with nNOS (Herbison *et al.*, 1996).

Dorsal horn immunostaining with galanin antibodies can be abolished by pre-treatment with the corresponding peptide (Simmons *et al.*, 1995) and the staining of galanin containing neurons is absent from the brains of galanin knock-out mice (Makwana *et al.*, 2010).

The VGAT antibody corresponded to amino acids 75-87 of the rat VGAT. Its immunostaining is also blocked by pre-incubation with the immunising peptide at 10-6 M (Polgár *et al.*, 2011). It stains a band of the appropriate molecular weight on western blots (Takamori *et al.*, 2000).

The rat substance P antibody (mAb NC1/34 HL) appears selective for substance P and neurokinin A when used with immunocytochemistry (McLeod *et al.*, 2000; Polgár *et al.*, 2006). In addition, it recognizes a sequence common to substance P and neurokinins A and B (Cuello *et al.*, 1979).

The specificity of goat, mouse anti-CTb and guinea pig anti-Fluorogold were indicated by the absence of staining in the regions of CNS that were devoid of cells. On the contrary, immunostaining with these tracer filled cells were found in the neuronal populations,

which are known to project to the corresponding injection sites (Al-Khater and Todd, 2009). In all the cases, a perfect match was found between the two types of immunofluorescence.

2.4 Confocal microscopy and analysis

These sections were scanned through their full thickness, unless otherwise stated, with a Zeiss LSM710 confocal microscope (equipped with Argon multiline, 405 nm diode, 561 nm solid-state and 633 nm HeNe lasers). Scans were done through dry (10×, 20×) and oil immersion (40×, 63×) lenses (NAs: 0.3, 0.8, 1.3 and 1.4, respectively). For all scans obtained with the 63× lens the pinhole was set to 1.0 Airy unit. This was done to allow the optimal brightness of each optical section and to prevent the out of focus light or flare. Different regions of the tissue were scanned depending upon the cells and boutons of interest. For transverse sections, transmitted light and dark field images of the dorsal horn were taken through dry lens (10×) using a sensitive transmitted photomultiplier tube (TPMT). This was done to identify the outline of grey and white matter along with laminar boundaries. Following the acquisition of the images, these scans were stored as Ziess laser scanning microscope 5 (LSM 5) files and were analyzed with Neurolucida for Confocal software (Microbrightfield). Images were made using Adobe Photoshop® CS version 8.0.

2.5 Statistical analysis

The statistical tests that were used in this study include; a student's t test, Mann–Whitney-U test, one-way ANOVA and two-way ANOVA on ranks with *post hoc* all-pairwise multiple comparisons (Holm-Sidak method). A p-value of <0.05 was considered significant. The detailed account of statistical analysis performed in this project will be described in the corresponding chapters.

Table 2-1Primary antibodies used in this study

Antibody	Species	Dilution	Source
NK1r	Rabbit	1:1000* or 1:10,000*	Sigma Aldrich
NK1r	Guinea pig	1:1000	ATD3 (Chemicon)
VGLUT2	Goat	1:500	M.Watanabe
VGLUT2	Guinea pig	1:1000	Millipore
VGLUT2	Rabbit	1:5000	Synaptic systems
PPD*	Guinea pig	1:5000	T.Kaneko
PPD	Rabbit	1:10,000	T.Kaneko
CGRP	Guinea pig	1:10,000	Bachem
CGRP	Sheep	1:5000	Enzo Life Sciences
VGLUT1	Guinea pig	1:5000	Millipore
GFP	Chicken	1:1000	Abcam
VGAT	Goat	1:1000	M.Watanabe
VGAT	Rabbit	1:1000	Chemicon
VGAT	Mouse	1:1000	Chemicon
Gephyrin	Mouse	1:500	Synaptic Systems
nNOS	Sheep	1:2000	Gift from P.C Emson
nNOS	Rabbit	1:5000	Millipore
Galanin	Rabbit	1:1000	Peninsula
CTb	Goat	1:5000	Sigma-Aldrich, Poole, UK
CTb	Mouse	1:5000	Abcam
Fluorogold	Guinea pig	1:500	Protos Biotech Corp., NY, USA
Substance P	Rat	1:200	Oxford Biotech

* Different concentrations of antibodies against NK1r were used for immunocytochemistry and combined confocal and electron microscopy. A high concentration (1:1000) of NK1r antibody was used in a reaction in which same species of PPD antibody and TSA reaction were performed (see chapter 3 methods). Dilute NK1r antibody (1:10,000) was used in all the remaining experiments.

3 Projection neurons in lamina III of the rat spinal cord are selectively innervated by local dynorphin-containing excitatory neurons

3.1 Introduction

Despite the fact that excitatory interneurons make up the majority of dorsal horn interneurons, not much is known about their inputs, potential targets, responses to different neuromodulators and synaptic circuits. Certain technical issues, such as lack of specific and appropriate neurochemical markers for excitatory spinal neurons, make it difficult to define functional populations among these cells. In addition to that, unlike inhibitory contacts where gephyrin can be used to identify the sites of synapses, there is a lack of neurochemically defined synaptic marker for the identification of excitatory synapses. Therefore, although glutamatergic contacts can be seen with confocal microscope, electron microscopy is required additionally to establish the presence of synapses at the sites of excitatory contacts. Regardless of these issues, several studies based on neurochemistry, electrophysiology and morphology have revealed certain features that are exclusively associated with excitatory cells (Todd, 2010). Neurochemical studies have reported that a number of neurochemical markers such as neurotensin (Todd *et al.*, 1992), somatostatin (Todd *et al.*, 2003), neurokinin B (Polgár *et al.*, 2006), calcium binding proteins (calbindin and calretinin) (Antal *et al.*, 1991), mu opioid receptor (MOR) (Kemp *et al.*, 1996, Spike *et al.*, 2002) and the γ-isoform of protein kinase C (PKCγ) (Polgár *et al*., 1999a) are found essentially within the excitatory cells. In contrast, some peptides like endogenous opioids (dynorphin and enkephalin) are expressed by both excitatory and inhibitory cells (Todd and Spike, 1993, Todd *et al.*, 2003, Polgár *et al.*, 2006, Sardella *et al.*, 2011a). Electrophysiological studies have shown that most excitatory cells have delayed-, gap- and reluctant firing patterns in response to depolarizing current injections (Yasaka *et al.*, 2010). This electrical activity is thought to correspond to I_A type currents that regulate the overall excitability of these cells.

Studies based on combined electrophysiological recordings and morphology have identified certain morphological subtypes such as vertical and radial cells that are primarily excitatory in nature and broadly account for a considerable fraction of excitatory interneurons (Yasaka *et al.*, 2010). Vertical cells are found in lamina II of the spinal dorsal horn and extend their axons into lamina I (Grudt and Perl, 2002). These cells receive inputs from TRPA1- and TRPV1- expressing C-afferents (Uta *et al.*, 2010) as well as their spines receive contacts from VGLUT1-containing LTMRs (Yasaka *et al.*, 2014). There are evidences suggesting glutamatergic synapses between vertical cells and lamina I projection neurons (Lu and Perl, 2005, Cordero-Erausquin *et al.*, 2009). In addition, genetic fate

mapping study has confirmed that glutamatergic interneurons are quite heterogeneous in terms of their neuropeptide expression and are developed under the control of a specific transcription factor (Xu *et al.*, 2008b).

Functionally, it has been suggested that excitatory interneurons are responsible for transmitting information between dorsal horn laminae (Grudt and Perl, 2002, Torsney and MacDermott, 2006, Yasaka *et al.*, 2014). Some cells have the ability to develop intrinsic plasticity, thereby altering their discharge patterns at the post synaptic target sites (Hu *et al.*, 2006, Yasaka *et al.*, 2010). In addition to that, certain excitatory interneurons contribute to tissue and nerve injury-induced heat and mechanical pain (Wang *et al.*, 2013). It has been reported that excitatory interneurons are under a strong inhibitory control (Yasaka *et al.*, 2007) and loss of this control may cause generation of hyperalgesia in response to noxious stimuli or allodynia (Basbaum *et al.*, 2009). In addition to that, subsets of presumably excitatory cells that express gastrin releasing peptide (GRP) (Mishra and Hoon, 2013) and gastrin releasing peptide receptor (GRPR) (Sun and Chen, 2007, Sun *et al.*, 2009) have been identified and their role in the perception of itch has been suggested. However, further studies are required to understand the organization and role of spinal excitatory cells in the dorsal horn circuitry.

As described previously, lamina III NK1r-expressing projection cells are densely innervated by substance P-containing nociceptive primary afferents (Naim *et al.*, 1997, Lawson *et al.*, 1997). These cells receive a modest synaptic input from VGLUT1-expressing myelinated low-threshold afferents that terminate in the deeper laminae (IIi-VI) (Naim *et al.*, 1998, Torsney and MacDermott, 2006) and considerably dense input from NPY-containing inhibitory axons (Polgár *et al*., 1999b, 2011). Peptidergic afferents generally form simple synaptic arrangements and are characterized by the presence of large neuropeptidergic dense-core vesicles (LCDVs) (Ribeiro-da-Silva A *et al.*, 1989, Lawson *et al.*, 1997, 2002). It has also been suggested that the terminals of peptidergic primary afferents vary in terms of their sizes and are often associated with large synapses at their postsynaptic target sites (Naim *et al.*, 1997, Todd *et al.*, 2009). In addition to that, lamina III NK1r-expressing projection cells also receive contacts from non-peptidergic unmyelinated afferents,(Sakamoto *et al.*, 1999). However, these contacts are far less numerous than those formed by substance P-containing peptidergic afferents. It has been shown that the non-peptidergic C-fibres form central axons of synaptic glomeruli type I

with indented contour, few mitochondria and several synaptic vesicles of varying diameter (Ribeiro-Da-Silva *et al.*, 1986).

Several studies have reported selective targeting of dorsal horn projection cells by nociceptive primary afferents (Naim *et al.*, 1997, 1998) and inhibitory axons of spinal origin (Polgár *et al*., 1999b, Puskár *et al*., 2001), however, currently there is no evidence for the selective innervation of projection cells by specific types of excitatory spinal neurons. Furthermore, if such input exists, it is not known whether their synapses differ from the ones formed by peptidergic afferents in terms of synaptic lengths.

During the preliminary study, it was tested whether any of the known populations of excitatory interneurons target dorsal horn projection cells. The findings suggested that glutamatergic axons of dynorphin-expressing interneurons appeared to innervate large lamina III NK1r-expressing cells. As already mentioned, dynorphin is an endogenous opioid peptide and it exists in two different isoforms, dynorphin A and dynorphin B, both of which are derived from their common precursor preprodynorphin (PPD) (Lee *et al.*, 1997, Marvizón *et al.*, 2009, Sardella *et al.*, 2011a). PPD is widely expressed by dynorphin containing interneurons and axons (Marvizón *et al.*, 2009, Sardella *et al.*, 2011a). Dynorphin-containing axon terminals can be revealed with antibody against PPD (Li *et al.*, 1999, Lee *et al.*, 1997). Furthermore, the boutons of inhibitory and excitatory PPD cells also express VGAT and VGLUT2 (Marvizón *et al.*, 2009, Sardella *et al.*, 2011a), respectively; therefore, in this part of the project, antibody against PPD has been used to detect the axonal expression of this peptide in the dorsal horn.

Dynorphin performs both opioid and non-opioid functions. The opioid functions are brought about by its action on Kappa opioid receptor (KOR) while its non-opioid actions such as decreased blood flow and paralysis involves NMDA receptor (Shukla and Lemaire, 1992) and may contribute neuropathic pain (Laughlin *et al.*, 1997, Koetzner *et al.*, 2004, Tan-No *et al.*, 2009). Recently, the role of dynorphin-containing inhibitory interneurons in the prevention of itch has been suggested (Kardon *et al.*, 2014). However, not much is known about the PPD-containing excitatory interneurons and their postsynaptic targets. Therefore, in the first stage of this study, the frequency of input from non-primary-VGLUT2-expressing excitatory axons (which are likely to originate from local interneurons) was compared with that from primary afferents on to the lamina III $NK1r^{+}$ projection neurons. Since lamina III projection neurons received numerous contacts from

VGLUT2 boutons that contained PPD, a quantitative analysis was carried out to determine whether this represented a selective innervation. Since a wide variation in the sizes of glutamatergic synapses on the lamina III projection cells has been reported, the third aim was to test the hypothesis that synapses formed by peptidergic primary afferents were larger than those from excitatory interneurons (Todd *et al.*, 2009).

3.2 Materials and methods

3.2.1 Animals and tissue processing

Eight adult male Wistar rats (300–390 g; Harlan) were deeply anaesthetised with pentobarbitone (300 mg i.p.) and perfused with a fixative containing 4% freshly de-polymerised formaldehyde (6 rats) or 4% formaldehyde/0.2% glutaraldehyde (2 rats). Mid-lumbar (L3-L5) segments were removed and cut into 60 µm thick parasagittal sections with a vibrating microtome. Sections were treated for 30 minutes with 50% ethanol to improve antibody penetration (Llewellyn-Smith and Minson, 1992) and then processed for immunocytochemistry, as described below. In all cases sections were incubated in primary antibodies at 4°C for 3 days and overnight in secondary antibodies. Antibodies were diluted in PBS that contained 0.3% Triton-X100, unless otherwise stated, and details of the antibody characterization, sources and concentrations as well as species specific fluorescent secondary antibodies that were used in this part of the study are described in chapter 2 (general methods). The secondary antibodies labelled with biotin or horseradish peroxidase (HRP) were also used. These antibodies were revealed with avidin-Pacific Blue or with avidin-HRP followed by either tyramide signal amplification (TSA; tetramethylrhodamine kit; PerkinElmer Life Sciences) or by reaction with 3, 3'-diaminobenzidine (DAB) in the presence of hydrogen peroxide. TSA technique was used to reveal HRP antibody since it allows the identification of antigens that are not detectable by routine immunocytochemistry (Toda *et al.*, 1999). It can be helpful in protocols in which same species antibodies are used to label different antigens within the same experiment. It results in high density labelling and amplification of the target protein in live or fixed tissue. In addition, it increases the antigen-detection sensitivity up to 100-fold even with a very dilute antibody concentration.

3.2.2 Analysis of contacts from VGLUT2-expressing and primary afferent boutons on lamina III NK1r cells

It has already been mentioned that VGLUT2 is expressed at high levels in axonal boutons of spinal origin (Todd *et al.*, 2003, Alvarez *et al.*, 2004) and in some of those from descending axons (Llewellyn-Smith *et al.*, 2007, Shrestha *et al.*, 2012, Du Beau *et al.*, 2012). Furthermore, it is also detected at very low levels in myelinated low-threshold afferents (laminae IIi-IV), as well as in peptidergic primary afferents (I-II), all of which also express VGLUT1 and CGRP in the rat, respectively (Alvarez *et al.*, 2004, Todd *et al.*, 2003, Persson *et al.*, 2006). As described previously, both these distinct populations of primary afferents provide synaptic innervation to lamina III NK1r-expressing projection neurons (Naim *et al.*, 1997, 1998). In order to identify the proportion of VGLUT2 boutons that contact large lamina III NK1r cells and did not originate from either of these types of primary afferent, sections from 3 of the rats that perfused with only 4% formaldehyde, were incubated in the following primary antibody cocktail; rabbit anti-NK1r to identify lamina IIINK1r-expressing cells, guinea pig anti-CGRP to label peptidergic primary afferents contacting lamina III NK1r cells and goat anti VGLUT2 (Miyazaki *et al.*, 2003, Kawamura *et al.*, 2006) to identify glutamatergic boutons of spinal origin. These primary antibodies were revealed with Alexa 488, Pacific Blue and Rhodamine Red, respectively.

Sections were viewed with a confocal microscope and 15 large lamina III NK1r-immunoreactive neurons with dorsal dendrites entering into the superficial dorsal horn were selected for analysis (5 from each rat). These cells were then scanned through dry (10×, 20×) and oil-immersion (63×) lenses and through the full thickness of the section. For the 63× lens, several overlapping fields were scanned (with a z-step of 0.5 µm) in order to include as much of the dendritic tree as was present in the section, and these were then analysed with Neurolucida for Confocal software. The cell bodies and dendritic trees of the neurons were drawn. The surface areas of cell bodies were measured and the surface areas of the dendrites were estimated with reference to their lengths and diameters, based on the assumption that they were cylindrical (Todd *et al.*, 2002). Dark-field scans through the 10× lens were used to identify border between laminae II and III, which was then plotted onto the drawings. It has already known that the termination pattern of primary afferents varies throughout the dorsal horn. Peptidergic afferents are concentrated in the superficial laminae while the myelinated afferents preferentially innervate deep dorsal horn, therefore regions of dendrite that lay within the superficial dorsal horn (laminae I and II) were analysed separately from those in deeper laminae. The locations of contacts from

CGRP-immunoreactive varicosities, and from those that were strongly VGLUT2-immunoreactive but lacked CGRP were plotted onto the cells. These sections were then removed from the slides and re-incubated in guinea pig anti-VGLUT1 antibody, which was also revealed with Pacific Blue. It was then possible to identify VGLUT1-containing LTMRs that contacted lamina III NK1r cells. They were then re-scanned through the 63× lens and the locations of contacts from VGLUT1-immunoreactive boutons (i.e. those that were only stained with Pacific Blue after the second immunoreaction) were plotted. Although, both CGRP and VGLUT1 boutons were revealed with Pacific Blue, it was possible to identify VGLUT1 boutons since CGRP labelled afferents do not express VGLUT1. With this approach, VGLUT2 boutons of primary afferents origin were excluded from the sample. The densities of contacts per 1000 μm^2 of dendrite surface were then determined for boutons that contained CGRP or VGLUT1, as well as from those that were VGLUT2-immunoreactive and lacked CGRP and VGLUT1.

3.2.3 Analysis of contacts from PPD boutons

It is already known that excitatory PPD boutons in the spinal dorsal horn co-express VGLUT2 (Marvizón *et al.*, 2009, Sardella *et al.*, 2011a). In order to determine the coexistence of PPD with VGLUT2 in non-primary boutons that contact lamina III NK1r projection neurons, sections from 3 rats were reacted to reveal NK1r, PPD, VGLUT2, VGLUT1 and CGRP. This was done by initially incubating the sections in rabbit anti-PPD to label PPD-immunoreactive boutons (Lee *et al.*, 1997), goat anti-VGLUT2, and guinea pig antibodies against both CGRP and VGLUT1, as described previously. PPD boutons were labelled with rhodamine by TSA method, while VGLUT2 was revealed with DyLight 649 and both CGRP and VGLUT1 with Pacific Blue. The sections were then incubated in rabbit anti-NK1r, which was then detected with Alexa 488. Although both PPD and NK1r antibodies were raised in rabbit, the TSA method allowed the PPD antibody to be used at a low concentration (1:10,000) (as described before). As a result, PPD antibody was only weakly detected by the Alexa 488- secondary antibody, which was used to reveal NK1r.

Fifteen large lamina III NK1r-immunoreactive neurons (5 from each of the 3 rats) were selected and scanned through the confocal microscope, as described before, and analysed with Neurolucida. Initially, only the channels corresponding to Pacific Blue, Alexa 488 and DyLight 649 were viewed, and the locations of all contacts from VGLUT2-immunoreactive boutons that lacked CGRP or VGLUT1 were plotted onto the cell bodies

and dendrites of the selected neurons. The rhodamine channel was then revealed, and the presence or absence of PPD in each of the VGLUT2 boutons in contact with the NK1r cells was recorded. Again, contacts on dendrites in laminae I-II were analysed separately from those on cell bodies and dendrites in deeper laminae (III-IV).

To allow the comparison with lamina III cells, the frequency with which PPD was present in VGLUT2 boutons that contacted large NK1r-expressing lamina I neurons was also assessed in these sections. Fifteen lamina I NK1r-immunoreactive neurons (5 from each of the 3 rats) were selected, scanned and analysed as described previously.

The next step was to determine the extent to which PPD was expressed in the general population of VGLUT2-immunoreactive non-primary boutons in superficial and deep laminae. For this, sets of confocal scans from two sections from each of the three rats were analyzed. Each set of scans included an area that extended from the dorsal aspect of lamina I to a depth of 300 μm below the lamina II/III border. This depth was chosen as it corresponded to the most ventral level at which VGLUT2 contacts were identified on the dendrites of the lamina III NK1r projection neurons. From each scan, 100 boutons that were strongly immunoreactive for VGLUT2 and lacked CGRP or VGLUT1 were selected in both superficial and deep regions by placing a big square grid that in turn consisted of several small square grids (5 × 5 μm) covering the whole dorsal horn on the images in Neurolucida (Polgár *et al.*, 2011). For each region, the eighth optical section in the z-series was taken as a reference level and the first bouton was obtained from one of the most dorsal squares. The selection process then continued in a dorsal-to-ventral, followed by left-to-right direction, until 100 boutons had been selected. This was done to ensure that boutons were sampled from all dorsoventral locations within each region. The PPD channel was then viewed and the presence or absence of immunostaining was recorded for each of the selected boutons. Since this selection method will be biased towards boutons that were more extensive in the z-axis (Guillery, 2002), the z-axis lengths of VGLUT2 boutons that contained PPD were also compared with those of $VGLUT2^{+}/PPD^{-}$ boutons by determining the number of optical sections on which each bouton was visible (Polgár *et al.*, 2011).

3.2.4 Dynorphin expression in the descending axons

Several previous studies that showed the distribution of dynorphin-immunoreactive neurons in the rat brain (Khachaturian *et al.*, 1982, Vincent *et al.*, 1982, Guthrie and

Basbaum, 1984) and PPD mRNA-positive cells in the mouse brain from the Allen Brain atlas (http://www.brain-map.org/) suggest that the only regions that contain both spinally-projecting cells and dynorphin/PPD^{+} cells were the nucleus of the solitary tract (NTS) and the locus coeruleus. Since the descending projection from the locus coeruleus is noradrenergic, these axons will not express VGLUT2 (Willis and Coggeshall, 2004). In order to determine dynorphin expression among axons projecting to the spinal cord from the nucleus of the solitary tract (NTS), transverse sections from the L1 segment were obtained from 3 rats that had received injections of cholera toxin B subunit (CTb) into the NTS in a previous study (Polgár *et al*., 2010). These sections were immunostained for PPD to determine the co expression of CTb and PPD boutons in the spinal dorsal horn. Two sections from each rat were scanned at 40 × lens and a detailed survey of CTb labelled boutons was carried out to determine PPD expression by descending CTb-labelled axons.

3.2.5 Combined confocal and electron microscopy

With the recent advances in the field of immunocytochemistry and confocal microscopy, it is possible to distinguish various fluorochromes with in the same immunocytochemical reaction (Brelje *et al.*, 2002). In addition, the high spatial resolution and narrow depth of focus of confocal microscope allows the identification of contacts between different neuronal profiles. However, due to the absence of a reliable marker for glutamatergic synapses, it is not possible to discern whether synapses are present at the points of contact (Todd, 1997, Naim *et al.*, 1997). In such cases, a method of combined confocal and electron microscopy allows to perform electron microscopy on sections that have been processed with immunofluorescence methods and viewed with the confocal microscope (Todd, 1997), thus making it possible to determine synapses at the sites of glutamatergic contacts. In case of inhibitory contacts, synaptic protein markers such as gephyrin can be used to determine the sites of inhibitory synapses without the need for electron microscopy (Puskár *et al.*, 2001). Similarly, the excitatory synapses can be located by identifying synaptic proteins such as PSD95. However, identification of PSD95 requires antigen retrieval method, which is not only technically difficult but is also associated with high failure rates. It has been reported that PSD95 does not work well with pre-and post-synaptic markers such as VGLUTs and NK1r, respectively. One probable explanation is that to identify corresponding markers, antigen retrieval method requires a double TSA reaction, which in turn causes obliteration of synaptic cleft, thus making the detection of synapses even more difficult.

In this part of the study, combined confocal and electron microscopy was used to reveal PPD labelled glutamatergic synapses on large lamina III NK1r cells. In the preliminary experiment, it was observed that the use of 4 primary antibodies in a single experiment affected the staining quality considerably; therefore this part of the study was carried out in 2 steps, each comprising of 2 different antibody combinations. Immunostaining was performed as described before, except that the sections were treated for 30 minutes with 1% sodium borohydride (to minimise non-specific staining resulting from glutaraldehyde fixation) and antibodies were diluted in PBS that did not contain detergent to avoid any damage to the ultrastructure (Figure 3-1).

Sections from the two rats that were fixed with glutaraldehyde/formaldehyde underwent one of two different immunostaining protocols consisting of 3 different antibodies combinations. In one protocol, sections were incubated in rabbit anti-NK1r, guinea pig anti-VGLUT2 and goat anti-CGRP, followed by secondary antibodies conjugated to Alexa 488, Rhodamine Red and DyLight 649, respectively. The secondary antibody cocktail also included biotinylated anti-guinea pig IgG, and the sections were then incubated in avidin-horseradish peroxidase (HRP) and mounted. The purpose of this combination was to determine whether the VGLUT2 boutons that made contacts with lamina III NK1r-expressing projection cells are associated with glutamatergic synapses. A large lamina III NK1r-immunoreactive cell was identified and scanned with the confocal microscope to reveal contacts from VGLUT2- and CGRP-containing boutons. The section containing the cell was then removed from the slide and reacted with DAB in the presence of hydrogen peroxidase to reveal the HRP-labelled VGLUT2-immunoreactive profiles. It was then osmicated in 1% osmium tetraoxide (OsO_4) for 30 minutes, stained with uranyl acetate, dehydrated in acetone and flat embedded in Durcupan resin between acetate sheets (fig 3.1) (Todd, 1997, Naim *et al.*, 1997, Naim *et al.*, 1998, Todd *et al.*, 2002). A series of ~130 ultrathin sections (silver interference colour, ~ 70 nm thicknesses) was cut through part of the cell with a diamond knife. The sections were collected in serial order on Formvar-coated slot grids and stained with lead citrate and viewed with a Philips CM100 electron microscope (EM) equipped with digital camera. The regions of dendrite that appeared in the ultrathin sections were identified based on their location with reference to landmarks (e.g., capillaries) that could be recognized in the confocal image stacks. Since avidin-peroxidase product does not penetrate that well as compared to primary and secondary fluorescence antibodies, the penetration of 3,3′ -diaminobenzidine reaction product in the sections processed for combined confocal and electron microscopy is quite limited (Todd,

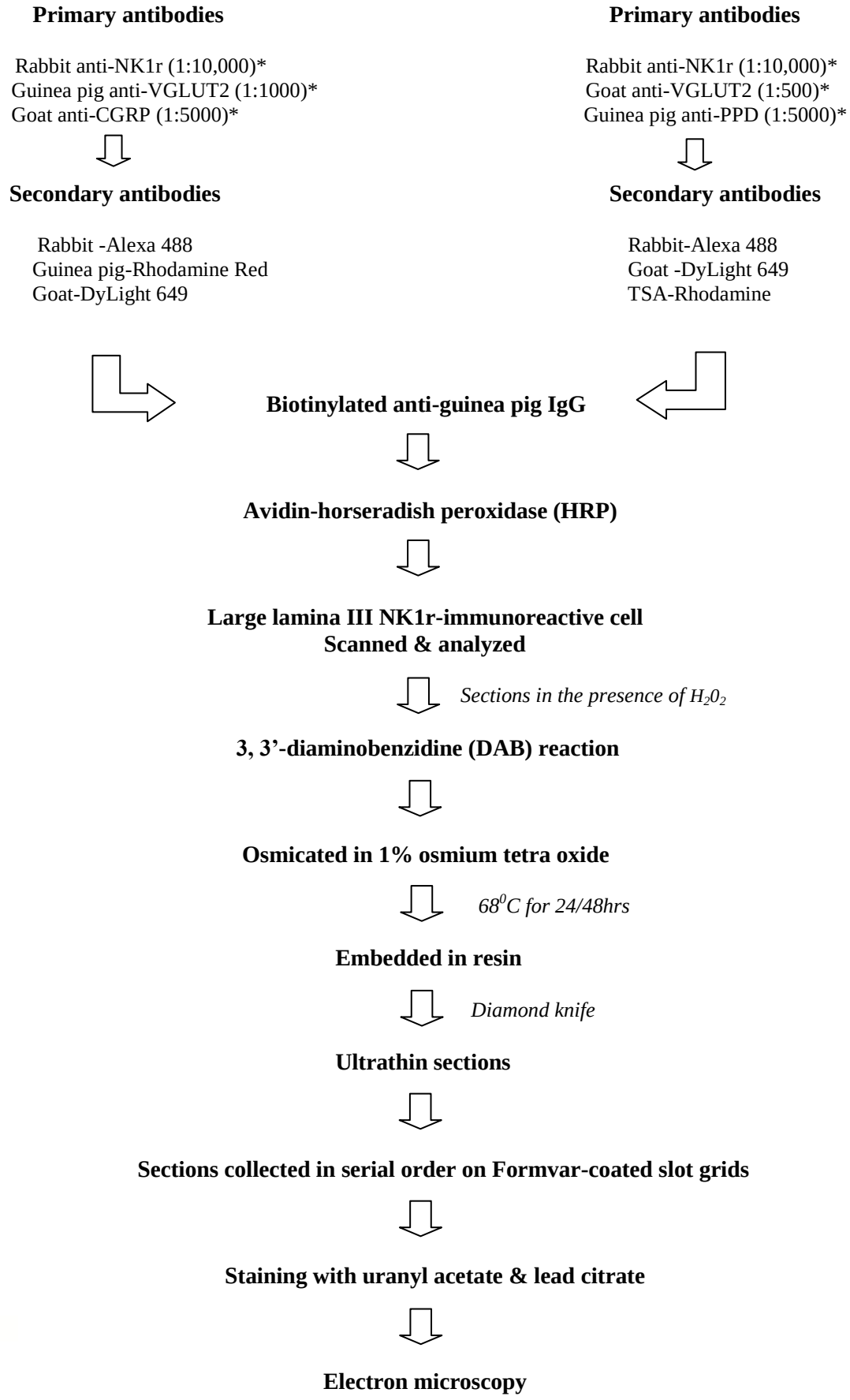
1997). Therefore, the profiles that were relatively superficial in the sections were included for this part of the analysis.

In the next protocol, sections from the other rat were incubated in rabbit anti-NK1r, guinea pig anti-PPD (Lee *et al.*, 1997) and goat anti-VGLUT2. The NK1r was revealed with Alexa 488, PPD with TSA-rhodamine and VGLUT2 with a mixture of biotin- and DyLight 649-conjugated anti-guinea pig IgG. They were then incubated in avidin-HRP and mounted. A large lamina III NK1r neuron near the surface of the tissue was selected and scanned to reveal contacts from PPD- and VGLUT2-immunoreactive boutons and the section was then reacted with the DAB method to reveal VGLUT2, osmicated, embedded in resin and processed as described previously, which was then followed by electron microscopy. Here the aim was to determine whether PPD-containing VGLUT2 boutons that contacted lamina III NK1r-expressing cells are also associated with glutamatergic synapses.

3.2.6 Statistics

The density of contacts of different types of bouton (CGRP, VGLUT2 and VGLUT1) on superficial and deep dendrites of lamina III NK1r cell was compared using Two-way ANOVA on ranks. In addition, this statistical test was also used to compare the percentage of PPD-immunoreactive VGLUT2 boutons contacting these cells with those in the general population in both deep and superficial laminae. *Post hoc* all-pairwise multiple comparisons (Holm-Sidak method) were used to determine whether the results differed significantly between superficial and deep dendrites. Differences in PPD expression among VGLUT2 boutons on superficial and deep dendrites of lamina III cells, and between those on lamina I and lamina III NK1r neurons, were analyzed with Mann-Whitney U tests. This test was also used to compare the sizes of PPD^+ and PPD^- VGLUT2 boutons in the general population. Lengths of different types of synapses were compared using *t* tests. *p* values of < 0.05 were considered significant.

Figure 3-1 Tissue processing for Combined Confocal and Electron microscopy



**Primary antibody dilutions*

3.3 Results

3.3.1 Contacts on lamina III NK1r neurons from VGLUT2, VGLUT1 or CGRP boutons

In this part of the study, parasagittal sections were examined after immunostaining for NK1r, VGLUT2 and CGRP and several large lamina III NK1r-immunoreactive neurons were readily identified in these sections. Of these, 15 large lamina III NK1r cells that were analysed, received many contacts from CGRP-immunoreactive boutons (labelled with Pacific Blue). These were particularly frequent on their dorsal dendrites within laminae I-IIo (Naim *et al.*, 1997), and some of them showed very weak VGLUT2 immunostaining, as suggested previously (Todd *et al.*, 2003). In addition, all of the cells received numerous contacts from boutons that were strongly labelled with the VGLUT2 antibody. These were seen on both superficial and deep dendrites, and also on cell bodies (Figure 3-2).

This part of the analysis was followed by incubation in VGLUT1 antibody (which was also revealed with Pacific Blue). With this approach, it was possible to identify VGLUT1-immunoreactive profiles, as these had initially been unlabelled and showed Pacific Blue labelling only after subsequent immunoreaction with VGLUT1 antibody (Figure 3-2a inset). As reported previously (Naim *et al.*, 1998), all of the 15 cells received some contacts from VGLUT1 boutons, and these were most numerous on their deep dendrites. Penetration of antibodies was complete, since the numbers of profiles that were immunostained with each of these antibodies were similar throughout the depth of the sections.

The results from the quantitative analysis of this part of the study are shown in Table 3-1 and Figure 3-3. The lengths of superficial and deep dendrites analysed for each cell ranged from 393-2189 μm (mean 1168 μm) and 119-1180 μm (mean 560 μm), respectively. Two-way analysis of variance on ranks revealed significant differences between the densities of these three types of axonal bouton on superficial (laminae I-II) and deep (laminae III-IV) regions of the cells ($p < 0.01$). *Post hoc* all-pairwise multiple comparisons (Holm-Sidak method) revealed that the density of contacts from CGRP boutons was significantly higher on superficial dendrites than on deep dendrites and cell bodies (21.1 compared to 11.1 per 1000 μm^2, $p < 0.01$), while the density of VGLUT1 contacts was higher on deep dendrites and cell bodies than on superficial dendrites (0.5 and 2.9 per 1000 μm^2; $p < 0.01$). In contrast, the density of contacts from VGLUT2 boutons that lacked CGRP and VGLUT1

did not differ significantly between superficial and deep regions (13.6 and 15.5 per 1000 μm^2, p = 0.27). In order to allow comparison with previous reports (Naim *et al.*, 1997, 1998), the numbers of contacts from CGRP and VGLUT1 boutons per unit length of dendrite were also determined. For CGRP, there were 17.4 (11.1-28) and 12.4 (3.1-27.9) contacts/100 µm for superficial and deep dendrites, respectively. The corresponding values for VGLUT1 were 0.4 (0-1.1) and 3.7 (2-6.7) contacts/100 µm.

Figure 3-2 Contacts from VGLUT2 (VG2) or CGRP- immunoreactive boutons on to a large lamina III neuron that expressed NK1r.

a A confocal image showing part of the soma and dorsal dendrites of the cell. These receive several contacts from boutons that contain VGLUT2 (labelled with rhodamine) or CGRP (labelled with Pacific Blue). Some of these contacts are indicated with arrows (VGLUT2) and arrowheads (CGRP). The inset (which corresponds to the area outlined by the box) shows the same region scanned after the section had been further incubated in anti-VGLUT1 (VG1), which was also revealed with Pacific Blue. The two profiles indicated with arrowheads are now Pacific Blue labelled, indicating that they are VGLUT1-immunoreactive. **b–e** Part of a dorsal dendrite of this cell receives several contacts from VGLUT2 boutons (some shown with arrows) and CGRP boutons (some shown with arrowheads). All images are projections of three optical sections at 0.5 µm z-spacing. Scale bar: 10 µm.

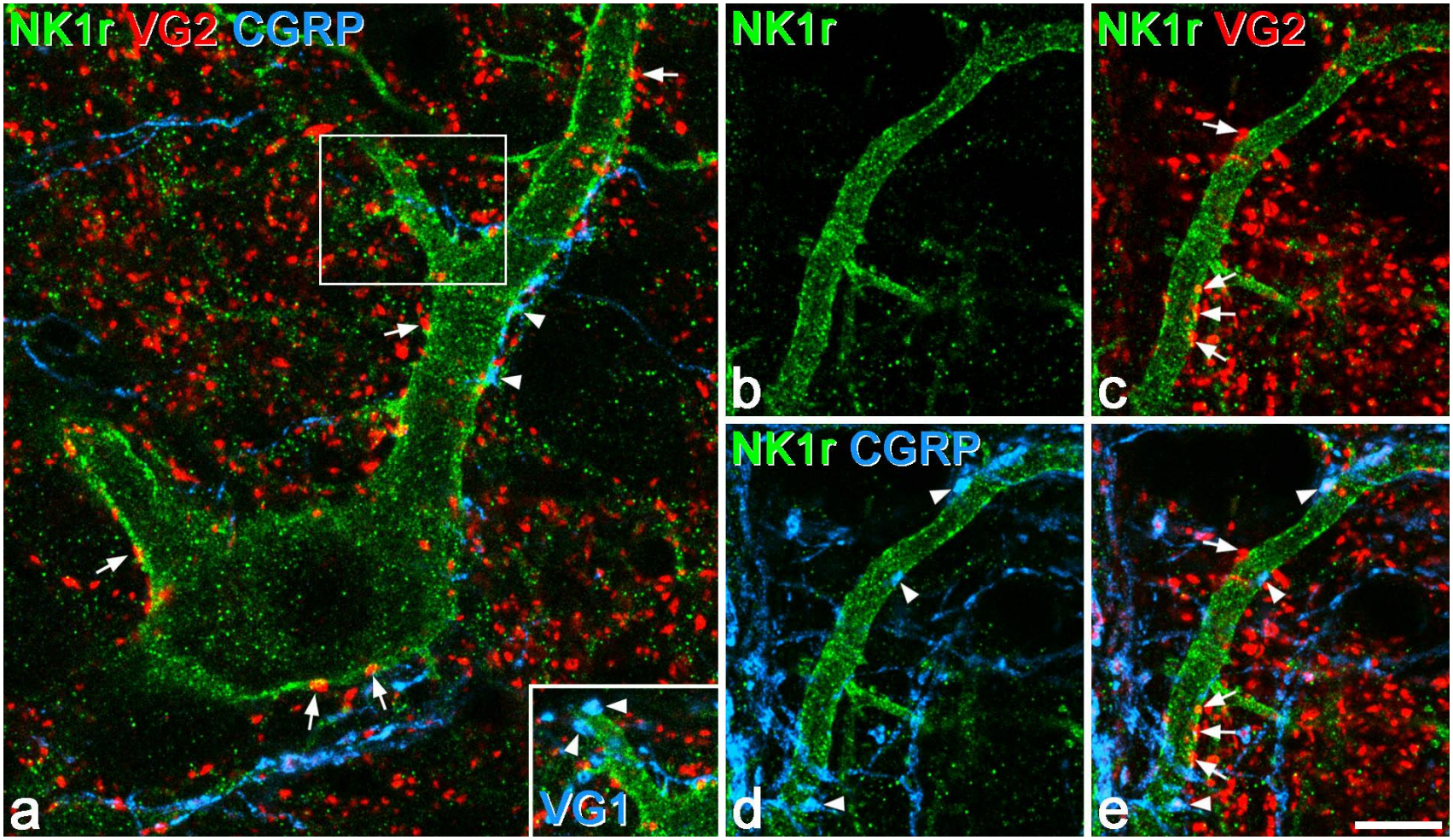

NK1r VG2 CGRP
a
VG1
NK1r
b
NK1r VG2
c
NK1r CGRP
d
e

Table 3-1 Density of contacts on large lamina III NK1r neurons from different types of glutamatergic boutons.

	CGRP		VGLUT2		VGLUT1	
	Number	**Density**	**Number**	**Density**	**Number**	**Density**
Superficial dendrites (laminae I-II)	187.7 (104-284)	21.1 (12-52.6)	125.3 (40-247)	13.6 (5.5-25.8)	4.5 (0-9)	0.5 (0-1.1)
Deep dendrites (laminae III-IV) and soma	74.5 (18-216)	11.1 (3.4-24.6)	112 (21-310)	15.5 (7.8-26.3)	18 (4-33)	2.9 (1.5-4.5)

Mean numbers of different types of boutons that contacted the 15 large lamina III NK1r cells, together with the density of contacts (per 1000μm^2 of cell surface). Ranges are given in brackets. VGLUT2 boutons are those that showed strong VGLUT2 labelling and lacked CGRP and VGLUT1.

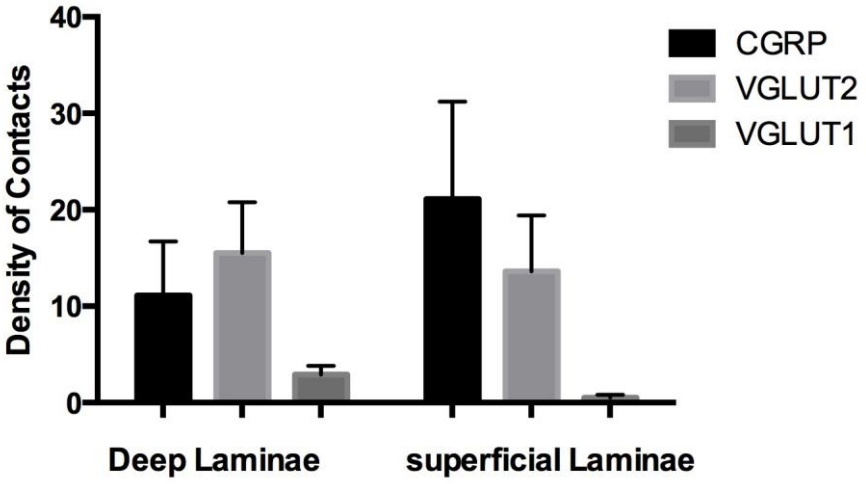


Figure 3-3 Graphical representation of contact densities of different types of boutons on lamina III NK1r-expressing cells. Two-way ANOVA on ranks revealed significant differences between the densities of CGRP, VGLUT2 and VGLUT1 boutons on the regions of the cells in superficial (I-II) and deep (III-IV) laminae ($p < 0.01$). The black bars, dark grey and light grey bars represent CGRP, VGLUT2 and VGLUT1 contact densities/ 1000 µm2 of cell surface, respectively. The graph further suggests that the contact density of CGRP boutons was significantly higher on the superficial dendrites than on the deep dendrites and soma ($p < 0.01$) while VGLUT1 boutons density was higher on the deeper parts of the cells as compared to the superficial dendrites ($p < 0.01$). Furthermore, VGLUT2 boutons were evenly distributed throughout these cells ($p = 0.27$).

3.3.2 PPD expression by VGLUT2 boutons contacting large NK1r neurons in lamina I and III

The basic aim of this part of the study was to determine the proportion of VGLUT2 boutons that contacted large lamina III NK1r cells and that how many of these boutons expressed PPD immunoreactivity. In order to exclude peptidergic or low-threshold myelinated primary afferents, which can show low levels of VGLUT2-immunoreactivity, only those boutons were included in the sample that showed strong VGLUT2 immunoreactivity and lacked CGRP or VGLUT1. Between 97 and 251 (mean 164) such boutons were identified in contact with the 15 NK1r lamina III cells that were analysed, and PPD-immunoreactivity was present in 58% (41-68%) of these (Figures 3-4 and 3-5 Table 3-2). The proportion of VGLUT2 boutons containing PPD on deep dendrites and cell bodies was 63% (range 39-76%), while for those that contacted superficial dendrites was 51% (range 46-71%), and these values differed significantly (Mann-Whitney U test, $p < 0.01$). As reported previously (Marvizón *et al.*, 2009), some boutons that were labelled with both PPD and CGRP were also observed within the general population of PPD boutons in the dorsal horn, however, these were never seen in contact with large lamina III NK1r projection cells.

In order to determine the selectivity of input from PPD-labelled VGLUT2 boutons on to large lamina III NK1r cells, the proportion of PPD boutons expressed within the general population of VGLUT2 boutons was carried out. The analysis showed that 6.5% (mean 4-10%) of VGLUT2 boutons that were selected in laminae I-II and 4.7% (mean 4-6%) of those selected in laminae III-IV were PPD-immunoreactive (Figure 3-4, 3-5). The mean z-axis lengths of PPD^+ and PPD^- VGLUT2-immunoreactive boutons did not differ in laminae I-II (1.64 µm and 1.76 µm, respectively), whereas for laminae III-IV the mean length of the PPD^+ boutons (2.02 µm) was significantly larger than that of the PPD^- boutons (1.62 µm) ($p < 0.01$; Mann-Whitney U-test). This indicates that the selection process was not biased for the VGLUT2 boutons in laminae I-II, but that it was biased towards the PPD^+ boutons in laminae III-IV. These boutons were on average 1.25× longer (2.02/1.62) in the z-axis. Since the extent of this bias is directly related to the difference in z-axis length, the true proportion of VGLUT2 non-primary boutons that express PPD in laminae III-IV was determined and was found to be 3.7%. This observation is based on the assumption that the "corrected" number of PPD^+ boutons in each sample would have been 0.8 (1.62/2.02) of the actual number, with the same number of PPD^- being included. Two-

way analysis of variance on ranks with *post hoc* all-pairwise multiple comparisons (Holm-Sidak method) revealed that in both superficial and deep regions, the proportion of VGLUT2 boutons immunoreactive for PPD was significantly higher for those contacting lamina III NK1r cells than for those in the general population ($p < 0.001$) (Table 3-2).

To test whether selective innervation by PPD-containing glutamatergic terminals was a general feature of NK1r-expressing projection neurons, 15 large NK1r-positive neurons in lamina I were also analysed. Although these also received some contacts from PPD-containing VGLUT2 boutons, these only constituted 11% of the $VGLUT2^{+}$ boutons in contact with these cells (Figure 3-6). This was significantly different from the corresponding value for the lamina III NK1r cells ($p < 0.001$; Mann-Whitney U-test).

3.3.3 Lack of PPD-expression among CTb labelled descending axons

CTb labelled boutons were distributed throughout the spinal dorsal horn. However, they were apparently denser in the deep dorsal horn. PPD-immunoreactivity was found to be similar to what has been reported before (Marvizón *et al.*, 2009, Sardella *et al.*, 2011a). Although quantitative analysis of this part of the study was not performed, a detailed survey of spinal cord sections from 3 rats that had received CTb injection into NTS showed no evidence of co localization between CTb- and PPD-immunoreactive boutons. This suggests that PPD labelled VGLUT2 boutons that were found in contact with large lamina III NKI1r-expressing cells were possibly of spinal origin.

Figure 3-4 Contacts between glutamatergic axons that contain PPD and a lamina III neuron that expressed the NK1r.

a–c Low-magnification image through the soma and dorsal dendrites of the lamina III cell, showing its association with PPD axons. The two areas in boxes are shown at higher magnification in subsequent parts. **d–f** The region in the upper box in **a**. This part of the dorsal dendrite receives several contacts from boutons that are labelled with both PPD (red) and VGLUT2 (VG2, blue), and therefore appear magenta (some marked with arrows). Note that most VGLUT2 boutons in this field are not PPD immunoreactive. **g–i** Several axosomatic contacts from boutons labelled with both PPD and VGLUT2 are visible (some marked with arrows). Images are from projections of 22 **(a– c)**, 3 **(d–f)**, or 11 **(g–i)** optical sections at 0.5 µm z-spacing. Scale bars: **a– c**, 50 µm, **d–i**, 10 µm.

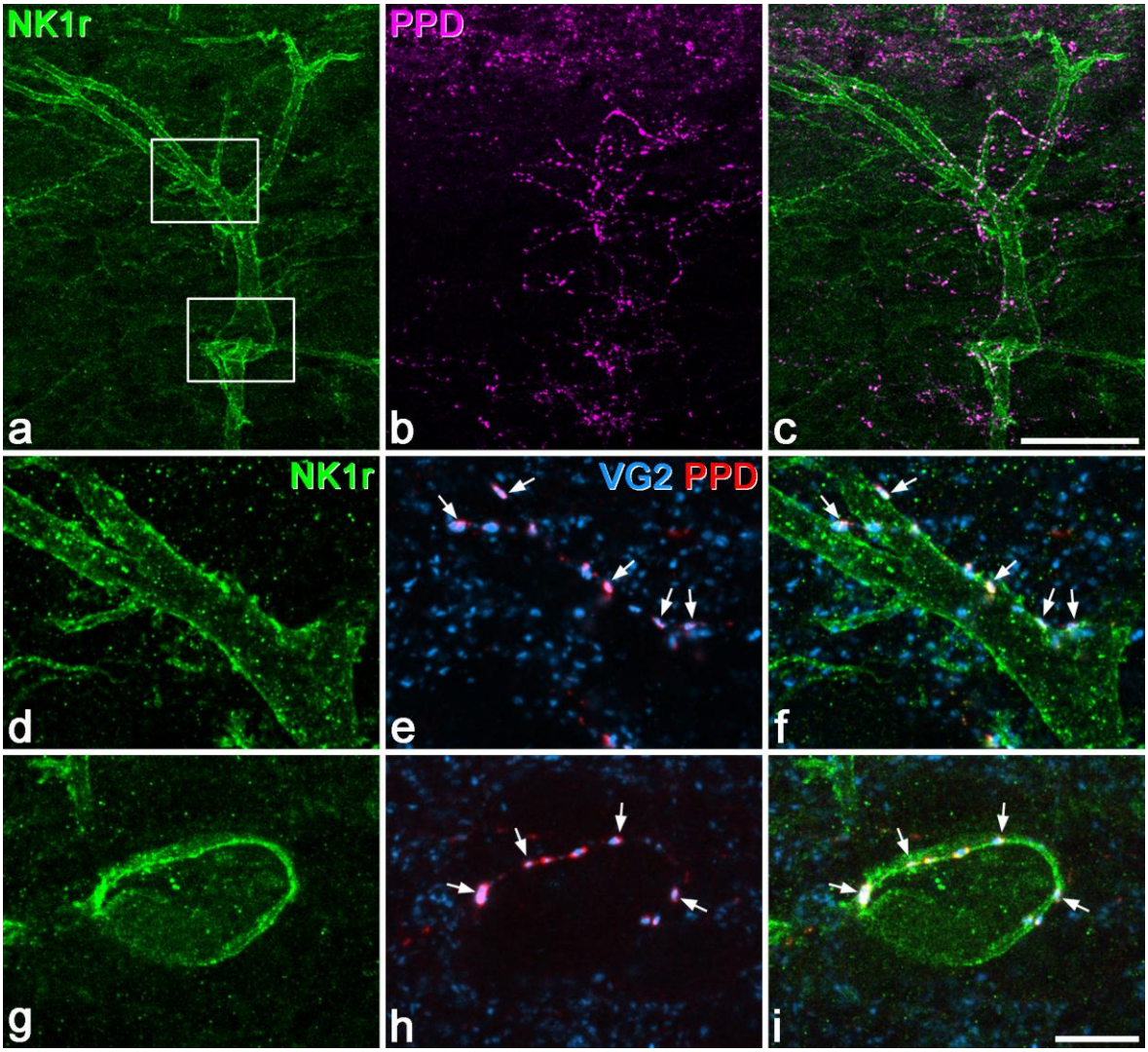

NK1r
PPD
a
b
c
NK1r
VG2 PPD
d
e
f
g
h
i

Table 3-2 Percentages of different types of VGLUT2 boutons that were PPD-immunoreactive.

		Number of VGLUT2$^+$ boutons	% of VGLUT2 that were PPD$^+$
VGLUT2 boutons contacting lamina III NK1r neurons	laminae I-II	60.1 (25-112)	51.2 (39.4-76)
	laminae III-IV	103.7 (34-211)	62.5 (46.2-70.6)
	Total	163.7 (97-251)	57.8 (41.3-67.7)
General population of VGLUT2 boutons	laminae I-II	100 (100)	6.5 (4-10)
	laminae III-IV	100 (100)	4.7 (4-6)
VGLUT2 boutons contacting lamina I NK1r neurons		129 (73-184)	10.8 (5.7-14.9)

The mean numbers of VGLUT2 boutons and the percentage that were PPD-immunoreactive for each analysis are shown in the second and third columns, with the ranges in brackets. The boutons in contact with the two different types of NK1r neuron were analyzed on 15 cells in each case. VGLUT2 boutons in the general population were selected from six sections (two from each of three rats) with 100 boutons per region analyzed on each section. Only VGLUT2 boutons that lacked CGRP or VGLUT1 were included in this quantitative analysis. Two-way ANOVA on ranks with *post hoc* all-pairwise multiple comparisons (Holm-Sidak method) showed that in both superficial and deep laminae, the proportion of PPD-expressing VGLUT2 boutons was significantly higher for those contacting lamina III NK1r cells than for those in the general population of VGLUT2 in this region ($p < 0.001$). Similarly Mann-Whitney U-test revealed that the proportion of PPD-containing VGLUT2 contacting lamina I NK1r cells was significantly lower than the corresponding value for lamina III NK1r cells ($p < 0.001$).

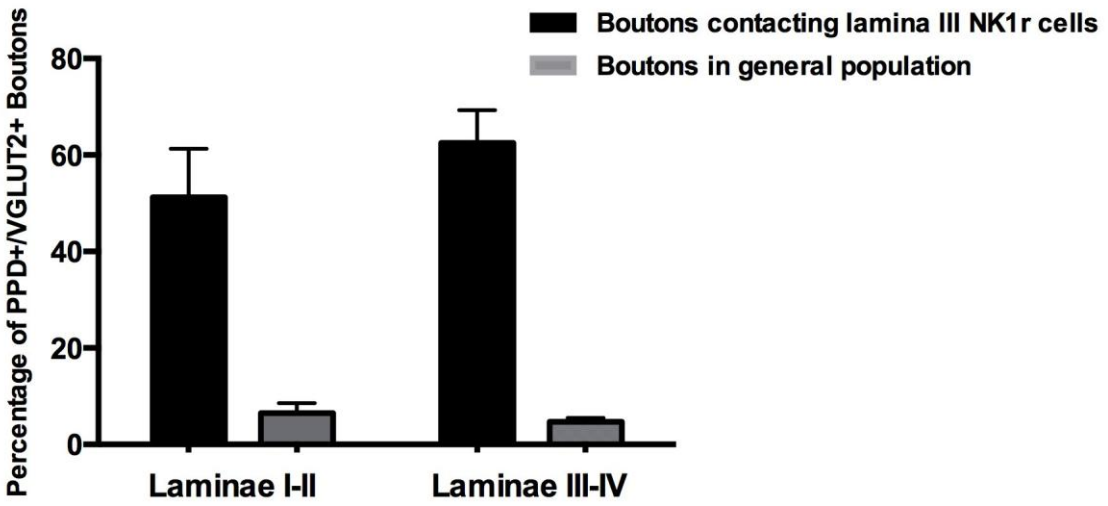
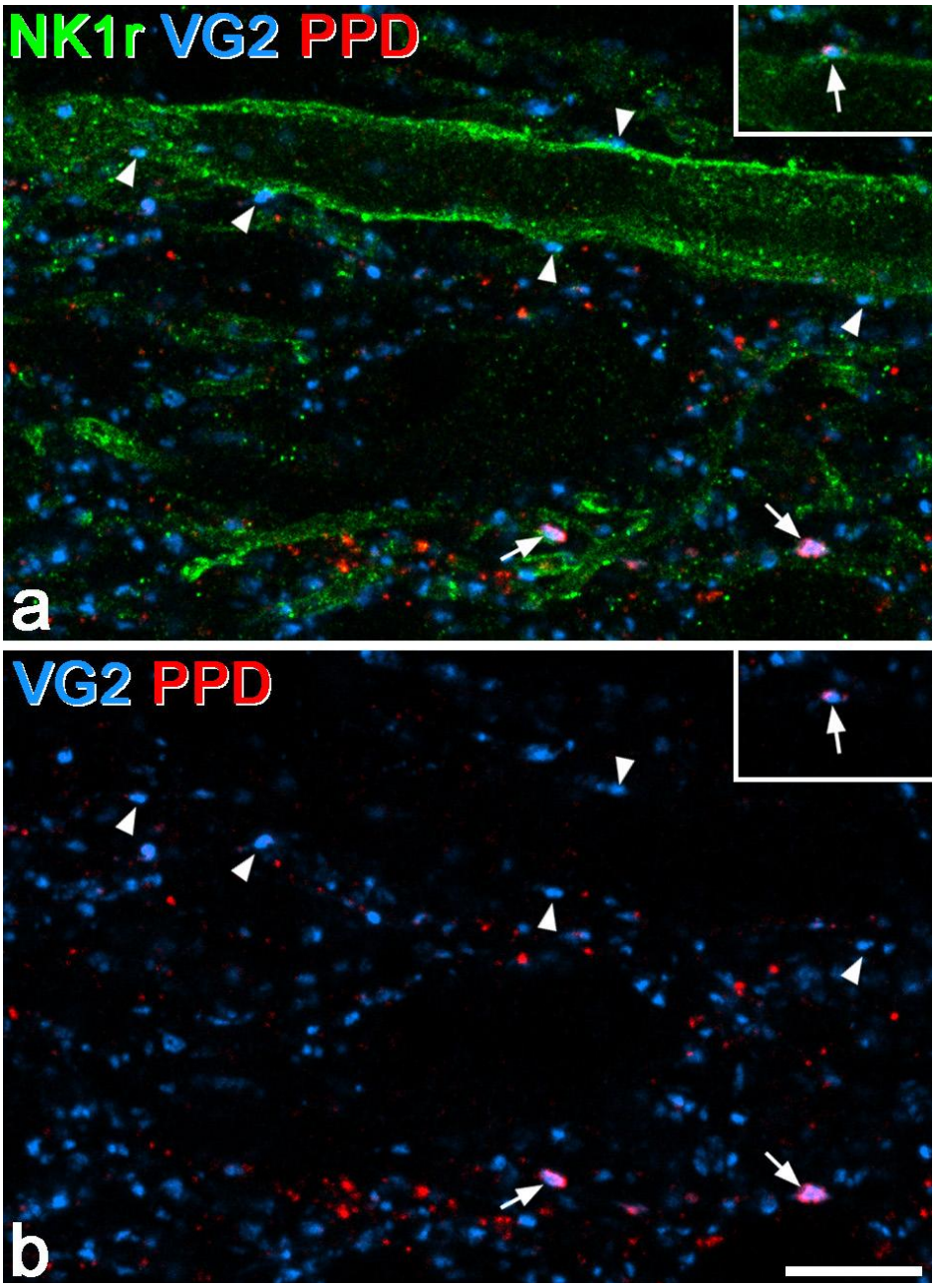


Figure 3-5 Graphical presentation of proportion of PPD-immunoreactive VGLUT2 boutons contacting lamina III NK1r cells and among general population in superficial and deep laminae. The black bars represent percentages of PPD+/VGLUT+ boutons contacting lamina III NK1r cells superficial dendrites as well as deep dendrites and soma, while grey bars represent the proportion with in the general population of VGLUT2 boutons in lamina I-II and III-IV. Two-way ANOVA on ranks with *post hoc* all-pairwise multiple comparisons (Holm-Sidak method) showed that in both superficial and deep laminae, the proportion of PPD-expressing VGLUT2 boutons was significantly higher for those contacting lamina III NK1r cells than for those in the general population of VGLUT2 in this region ($p < 0.001$).

Figure 3-6 VGLUT2 contacts on large NK1r-immunoreactive neuron in lamina I

a A proximal dendrite of the cell receives several contacts from VGLUT2-immunoreactive (VG2) boutons that lack PPD (arrowheads). Arrows indicate two nearby boutons that contain both VGLUT2 and PPD. The inset shows **a** contact that this dendrite received from a $PPD^{+}/VGLUT2^{+}$ bouton at a different depth within the section (arrow). **b** The same field scanned to reveal only VGLUT2 and PPD shows that the great majority of VGLUT2 boutons in this region do not contain PPD. Projections of four (main image) or two (inset) optical sections at 0.5 µm z-spacing. Scale bar: 10 µm.


NK1r VG2 PPD
a
VG2 PPD
b

3.3.4 Combined confocal and electron microscopy

With this approach, approximately 22 contacts between VGLUT2-immunoreactive boutons and the lamina III NK1r cell were identified in the tissue reacted for NK1r, VGLUT2 and CGRP. These contacts were identified by comparing the location of DAB-positive profiles in low magnification electron micrographs with Rhodamine-labelled profiles in corresponding optical sections from the confocal image stack. The dendritic shafts of the NK1r-expressing cell were readily identified with the EM based on their size, shape and nearby landmarks even though the NK1r, which outlined them, was not revealed with DAB (Figure 3-7). At 20 of the 22 contacts involving VGLUT2-immunoreactive boutons an asymmetrical synapse could be identified (Figure 3-7 f, h, i). Although, CGRP was not revealed with DAB, CGRP-immunoreactive boutons that contacted the NK1r cell could also be recognised on the basis of their location (Figure 3-7 c-e). Fourteen CGRP boutons that contacted the cell were identified in this way, and all were found to contain dense-cored vesicles and formed asymmetrical synapses with the NK1r cell (Figure 3-7g).

The other cell examined with EM was from a section that had been reacted with NK1r, PPD and VGLUT2. Although in this case all VGLUT2 boutons contained DAB reaction product, it was possible to identify those that were also PPD-immunoreactive, based on the co-localisation of PPD and VGLUT2-immunoreactivity seen in the confocal images (Figure 3-7a). Twenty three VGLUT2 boutons that contacted the cell were found with the EM, and 13 of these were also PPD-immunoreactive. Synapses, all of which were asymmetrical, were identified at 20 of these contacts (10 of the 13 at which the bouton was PPD-immunoreactive and all 10 of those at which the bouton lacked PPD) (Figure 3-8)In addition to that, 12 asymmetrical synapses that the NK1r cell received from axonal boutons that were not labelled with DAB (and which were neither VGLUT2- nor PPD-immunoreactive in the confocal image stacks), and which contained at least 5 dense-cored vesicles, were also identified. Since these were found at the same depths in the Vibratome section as boutons with strong VGLUT2 immunoreactivity it is unlikely that the lack of VGLUT2 staining represented a false-negative result. The lamina III NK1r cells are known to receive a high density of synaptic input from peptidergic primary afferents (Naim *et al.*, 1997) and it is likely that the VGLUT2-negative boutons with dense core vesicles were derived from these peptidergic afferents.

Another aim of this part of the study was to compare the lengths of the synapses formed by peptidergic primary afferents to those formed by glutamatergic boutons of spinal origin. The lengths of the synapses on both cells were measured from the electron micrographs and are shown in Figure 3-9. Lengths of the 40 synapses associated with VGLUT2 boutons ranged from 0.2 to 1.1 µm (mean 0.55 µm). The lengths of synapses formed by CGRP-immunoreactive boutons on the first cell were compared with those formed by VGLUT2-negative boutons with dense cored vesicles on the second cell. The results suggested that these synapses did not differ significantly (CGRP: mean 0.88 ± 0.26 µm, n = 14; $VGLUT2^-$: mean 1.19 ± 0.62µm, n = 12; p = 0.1, *t* test). Since the latter two groups are both likely to originate from peptidergic primary afferents, they were pooled together and were compared to the synapses formed by VGLUT2-immunoreactive boutons on the two cells (Figure 3-9). The results suggested a highly significant difference between the synapses belonging to 2 different populations of contacts on large lamina III NK1r-expressing cells (VGLUT2-positive: mean 0.55 ± 0.24 µm, n = 40; peptidergic afferent: mean 1.02 ± 0.48 µm, n = 26; $p < 0.001$, *t* test).

Figure 3-7 Synapses from boutons that contain VGLUT2 (VG2) or CGRP on the dorsal dendrite of a lamina III neurons with NK1r

a Single confocal optical section through the dorsal dendrite of the cell scanned to reveal NK1r (green), VGLUT2 (red), and CGRP (blue). Three contacts from VGLUT2-immunoreactive boutons are indicated with numbered arrows. **b** An electron micrograph from an ultrathin section through the cell at a level corresponding to the confocal image in **a**. The dendritic shaft is visible, and the three VGLUT2 boutons, which are labelled with DAB, are also seen. **c, d** Confocal images from an optical section slightly deeper in the z-series than that shown in **a**. These show the contact from one of the VGLUT2 boutons (numbered 1 in **a**), as well as a contact from a nearby CGRP-immunoreactive bouton (arrowhead). **e** Electron micrograph from an ultrathin section corresponding to the region shown in **c** and **d**. The VGLUT2 bouton is visible due to the DAB reaction product. The CGRP bouton is not labelled with DAB, but can be identified from its size and position relative to the dendrite and the VGLUT2 bouton. **f** High-magnification electron micrograph through the CGRP-immunoreactive bouton seen in **c–e**, which forms a synapse (between arrowheads) with the dendrite of the NK1r cell. Three dense-cored vesicles within the bouton are indicated with arrows, and these are shown at higher magnification in the insets. **g–i** Synapses (between arrowheads) formed by the three VGLUT2 boutons. Scale bars **a– e** 5 µm, **f–i** 0.25 µm.

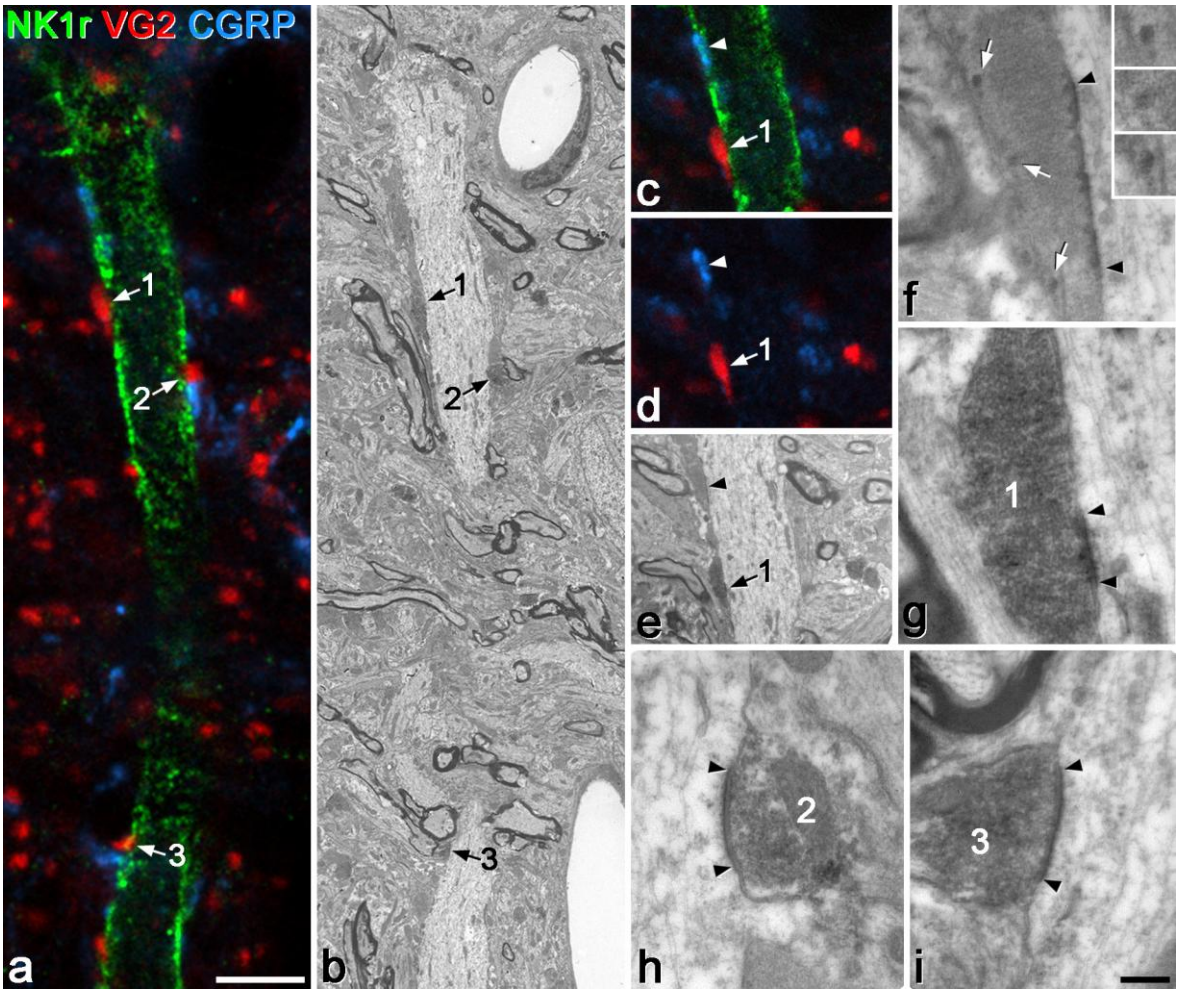
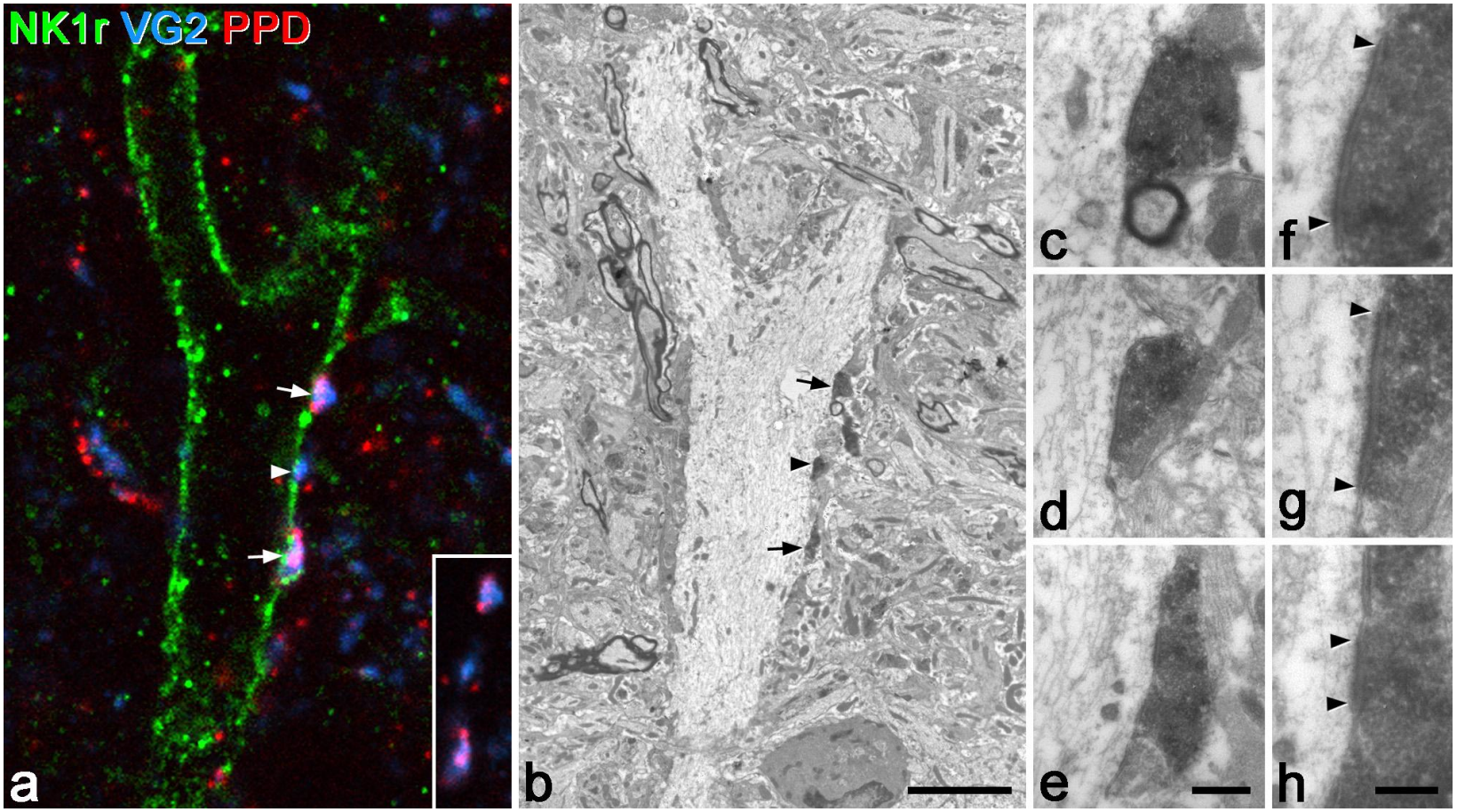

NK1r VG2 CGRP
a
b
c
d
e
f
g
h
i
1
2
3

Figure 3-8 Synapses formed by boutons containing PPD and VGLUT2 (VG2) on the dorsal dendrite of a lamina III neuron with NK1r

a Single confocal optical section scanned to reveal NK1r (green), VGLUT2 (blue), and PPD (red). The dorsal dendrite of the NK1r-immunoreactive neuron receives three contacts from VGLUT2-immunoreactive boutons. Two of these also contain PPD, and therefore appear magenta (arrows), while the other one does not (arrowhead). The boutons are shown without NK1r immunostaining in the inset. **b** Low-magnification electron micrograph through the corresponding region of the dendrite. All three VGLUT2 boutons are visible, as the immunoperoxidase reaction was linked to the VGLUT2 antibody**. c–e, f–h** Higher magnification views to show the presence of a synapse (between arrowheads) in each case. Scale bars **a-b** 5 µm, **c– e** 0.5µm, **f– h** 0.25 µm.


NK1r VG2 PPD
a
b
c
d
e
f
g
h

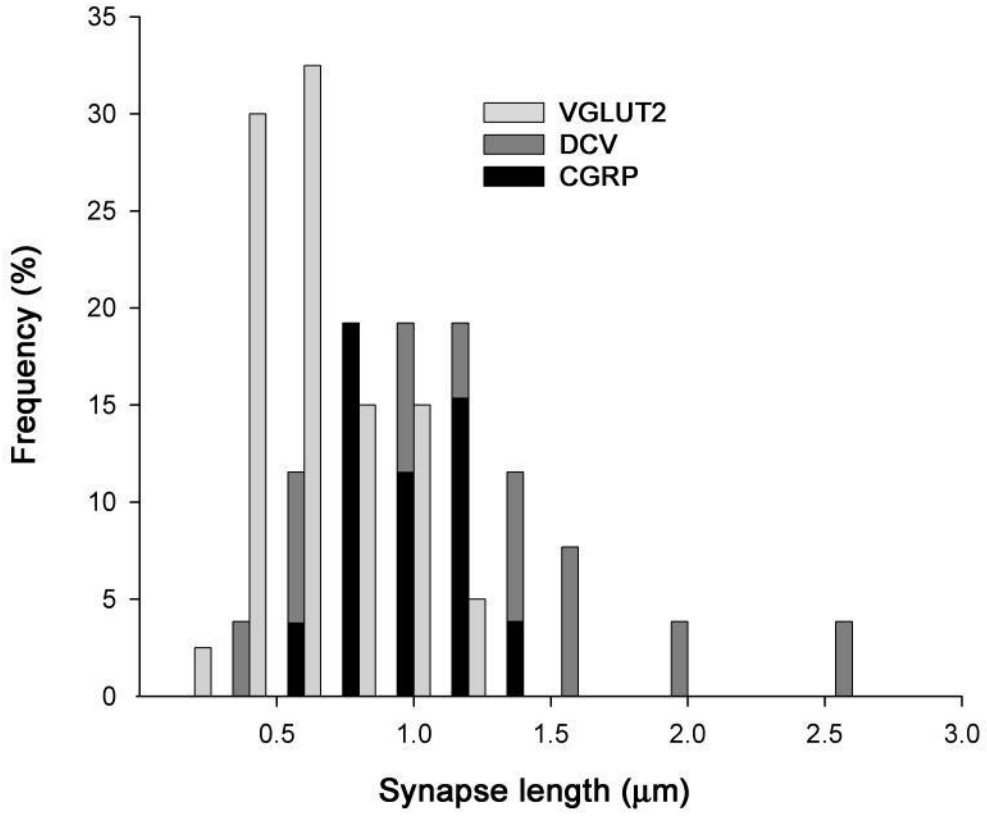


Figure 3-9 Synapse sizes for VGLUT2-immunoreactive and putative primary afferent boutons on lamina III NK1 receptor-immunoreactive cells.

Frequency histogram showing the lengths of synapses from the VGLUT2 boutons identified on the two cells examined with electron microscopy (pale grey bars, n = 40) and those of the putative primary afferents. The latter were identified by CGRP-immunoreactivity in the first cell (n = 14, black bars) or the presence of dense cored vesicles in the second cell (n = 12, dark grey bars).

3.4 Discussion

The main findings of this study are that the large lamina III NK1r-expressing cells receive numerous contacts from VGLUT2-immunoreactive boutons that are distributed evenly across their dendritic trees and over 50% of these contain the dynorphin precursor PPD. This suggests a very selective innervation since a considerably smaller proportion of VGLUT2 boutons in lamina I-IV and only around 11% of those contacting large lamina I NK1r cells express PPD. In addition, electron microscopy revealed that the great majority of contacts were shown to be associated with asymmetrical synapses. However, these synapses were significantly smaller than the ones formed by peptidergic primary afferents on these projection cells.

3.4.1 Sources of glutamatergic input to lamina III NK1r projection neurons

Previously, it has been reported that all large lamina III NK1r-expressing cells are projection neurons and that almost all the cells on one side of the spinal dorsal horn are retrogradely labelled from a tracer injected into contralateral CVLM (Todd *et al.*, 2000), therefore, retrograde tracing method was not used in this part of the study to label lamina III NK1r projection cells. These cells receive dense innervation from peptidergic primary afferents (Naim *et al.*, 1997) all of which can be detected by antibody against CGRP (Ju *et al.*, 1987). In this study, the density of contacts from CGRP boutons, on lamina III NK1r cells was very similar to that reported by Naim *et al.* (1997) for substance P-immunoreactive boutons (mean values for superficial and deep dorsal dendrites in Naim *et al.* were 18.8 and 13.2 contacts/100 µm, respectively, while the corresponding values in this study are 21.1 and 11.1 contacts/100 µm). These observations were also consistent with the finding by Naim *et al.* that 95% of the substance P boutons contacting the cells were of primary afferent origin, and with the report by Sakamoto *et al.* (1999) that another population of peptidergic afferents, those that express somatostatin (Hökfelt *et al.*, 1976), seldom contact these cells. This suggests that only peptidergic primary afferents that contain substance P innervate lamina III large NK1r-expressing cells.

It has been shown previously that large lamina III NK1r-expressing neurons receive some synapses on their deep dendrites from low-threshold myelinated afferents, which were identified following injection of cholera toxin B subunit (CTb) into a peripheral nerve (Naim *et al.*, 1998). All low-threshold myelinated afferents that are transganglionically

labelled with CTb, express high levels of VGLUT1 (Todd *et al.*, 2003) and the only other source of VGLUT1-containing boutons in this region is the corticospinal tract (Casale *et al.*, 1988, Alvarez *et al.*, 2004, Du Beau *et al.*, 2012). In this study, the density of contacts from VGLUT1 boutons was very low in the superficial laminae, and that the density on deep dendrites was no higher than that of CTb-labelled primary afferent boutons (5.9 ± 2 contacts/100 µm) (Naim *et al.*, 1998). These findings suggest that large lamina III NK1r cells do not receive significant input from corticospinal axons.

Several studies have suggested that the great majority of boutons that express strong VGLUT2 immunoreactivity in the dorsal horn are of spinal origin (Todd *et al.*, 2003, Alvarez *et al.*, 2004, Persson *et al.*, 2006). In contrast, Li *et al.* (2003) reported the depletion of VGLUT2 immunoreactivity following dorsal rhizotomy and suggested that significant number of VGLUT2-immunoreactive boutons were of primary afferent origin. They also reported somewhat different distribution of VGLUT2 boutons in spinal dorsal horn. However, subsequent studies obtained a better staining of VGLUT2 boutons and failed to detect VGLUT2 depletion after dorsal rhizotomy (Oliveira *et al.*, 2003, Brumovsky *et al.*, 2007). All these contradictory findings were attributed to the difference in the technique and sensitivity of VGLUT2 antibodies that were used in the corresponding studies (Alvarez *et al.*, 2004).

It has been reported that boutons belonging to peptidergic and low-threshold myelinated afferents can also express low levels of VGLUT2 (Todd *et al.*, 2003, Alvarez *et al.*, 2004, Persson *et al.*, 2006). In order to include only those VGLUT2 boutons that were of spinal origin, sections were therefore immunostained for both CGRP and VGLUT1. In this way those primary afferent boutons that expressed relatively weak VGLUT2 immunoreactivity were excluded. Although non-peptidergic C nociceptors, which bind the lectin BS-IB4, also show low levels of VGLUT2 (Todd *et al.*, 2003), these afferents do not innervate lamina III projection neurons (Sakamoto *et al.*, 1999) and therefore will not contribute to the overall excitatory input to these projection cells..

The large lamina III NK1r cells received many contacts from strong VGLUT2-immunoreactive boutons, most of which were associated with synapses. The main source of these boutons is likely to be local excitatory neurons, which express high levels of VGLUT2 and generate substantial local axonal arbors (Todd *et al.*, 2003, Schneider and Walker, 2007, Yasaka *et al.*, 2010). However, there may also be a contribution from

brainstem neurons with descending axons (Du Beau *et al.*, 2012). In addition to that, some VGLUT2 boutons in lamina I that made contacts with the dorsally directed distal dendrites of lamina III NK1r cells could originate from non-peptidergic Aδ nociceptors (Naim *et al.*, 1998, Todd *et al.*, 2003).

Seal *et al.* (2009) have recently identified a population of C low-threshold mechanoreceptive afferents (C-LTMRs) in mice, which express VGLUT3 and terminate in lamina IIi. It has been further suggested that these afferents might be involved in the injury-induced mechanical hypersensitivity. This observation was made in *Vglut3* knockout mice that showed impaired mechanical pain sensation and particularly absence of mechanical hypersensitivity in the inflammatory pain models. Recently it has been reported that although there is a plexus of VGLUT3-immunoreactive boutons in this region in the rat (A.J. Todd, unpublished observations), it is not known whether they correspond to the same population of VGLUT3-expressing C-LTMRs observed in the mouse and if so whether they provide input to large lamina III NK1r-expressing cells.

3.4.2 Input from boutons that contained VGLUT2 and PPD

A major finding of this study is that while PPD is expressed in 5-7% of all VGLUT2-immunoreactive boutons in the dorsal horn and around 11% of VGLUT2 boutons contacting lamina I NK1r cells, it is present in 58% of those that contacted the lamina III projection neurons. This indicates a dramatic (~10 fold) enrichment of input from PPD^{+}/VGLUT2-containing boutons on to large lamina III NK1r-expressing cells. Additionally, it also demonstrates that PPD-containing glutamatergic neurons preferentially innervate lamina III NK1r cells, providing much of their non-primary excitatory input. Dynorphin is widely distributed in the spinal dorsal horn and it acts on both kappa opioids receptor (KOR) (James *et al.*, 1982) and NMDA type of glutamate receptors (Lai *et al.*, 2006, Drake *et al.*, 2007). Although KOR are present in the dorsal horn (Arvidsson *et al.*, 1995), it is not known whether they are expressed by the lamina III projection neurons, and therefore whether dynorphin will act directly on these cells. Asymmetrical synapses at the site of contacts between PPD/VGLUT2-containing boutons and NK1r –expressing cells indicate the presence of NMDA receptors. This further suggests that dynorphin may enhance NMDA receptor activity at these synapses

Dynorphin is difficult to detect within the soma of the cells that express the peptide, therefore, earlier studies, based on the distribution of dynorphin within the spinal cord,

used colchicine to block the axonal transport of dynorphin (Khachaturian *et al.*, 1982, Cruz and Basbaum, 1985). It has been speculated that colchicine can alter the levels of neuropeptides mRNA or may result in the *de novo* synthesis of the peptides by the cells that do not normally express them (Réthelyi *et al.*, 1991). Therefore, in this study, antibody against the precursor protein, PPD, was used to detect the expression of dynorphin within the spinal dorsal horn. Potential sources of the PPD boutons that innervate lamina III projection cells are primary afferents, descending axons and local spinal neurons. Although PPD is present in some peptidergic (CGRP-containing) primary afferents (Marvizón *et al.*, 2009), observations made in this study suggest that these peptidergic PPD boutons do not contact the lamina III projection cells. PPD is expressed by several populations of neurons throughout the brainstem (Khachaturian *et al.*, 1982, Vincent *et al.*, 1982, Guthrie and Basbaum, 1984); however, the only regions reported to contain significant numbers of both PPD cells and neurons that project to the spinal cord are the locus coeruleus and nucleus of the solitary tract (NTS) (Willis and Coggeshall, 2004). Axons descending from locus coeruleus are norepinephrinergic and do not contain dynorphin (Patel *et al.*, 1997). Since CTb-labelled descending axons in the spinal dorsal horn do not express PPD, it is likely that the PPD^{+}/$VGLUT2^{+}$ boutons that innervate the lamina III projection cells are derived exclusively from local spinal neurons.

Within the spinal cord, PPD-expressing neurons are densely packed in the superficial laminae and scattered throughout the deep dorsal horn (Khachaturian *et al.*, 1982, Vincent *et al.*, 1982, Cruz and Basbaum, 1985, Standaert *et al.*, 1986, Sardella *et al.*, 2011a). Most of the PPD cells in laminae I-II are GABAergic, while ~30% of those in this region, and almost all the cells in the deeper laminae are thought to be excitatory in nature (Sardella *et al.*, 2011a). Some studies based on *in situ* hybridization (Ruda *et al.*, 1989) and genome-wide expression profiling (Wildner *et al.*, 2013) have reported considerable number of PPD-containing excitatory cells in the deeper laminae while Sardella *et al.* (2011a) reported that PPD cells in the deeper laminae were mostly sporadic as compared to those in the superficial dorsal horn. One likely explanation is that glutamatergic PPD cells in this region express relatively low levels of the peptide and are therefore not readily seen with immunocytochemistry. While most of the excitatory PPD cells are presumably interneurons, some of those in lamina I are projection cells (Standaert *et al.*, 1986, Nahin *et al.*, 1989). It is likely that both interneurons and projection neurons contribute to the local plexus of PPD^{+}/$VGLUT2^{+}$ axons, since many lamina I projection neurons also generate local axon collaterals (Szucs *et al.*, 2010).

Previously, several examples of selective targeting of projection cells by populations of inhibitory interneurons have been reported. Specifically, GABAergic neurons containing NPY innervate the lamina III NK1r projection cells (Polgár *et al*., 1999b, Polgár *et al*., 2011) while those that contain neuronal nitric oxide synthase (nNOS) innervate giant lamina I projection cells that lack the NK1r (Puskár *et al*., 2001). However, this is the first evidence that excitatory neurons in the dorsal horn are also selective in the projection neurons with which they form synapses. In addition, this represents a far more dramatic enrichment (~10 times greater than would occur by chance), compared to those found for NPY-containing GABAergic boutons on these cells (~2-3-fold enrichment in laminae I-II) (Polgár *et al.*, 2011) or for nNOS/GABA boutons on the giant lamina I cells (2-fold enrichment) (Sardella *et al.*, 2011b).

3.4.3 Functional significance of primary and non-primary glutamatergic input to lamina III projection neurons

The results of this study suggest that the lamina III projection cells receive substantial excitatory input from local glutamatergic neurons, many of which also express dynorphin. Virtually all the glutamatergic boutons that contacted lamina III NK1r-expressing projection cells are likely to have been detected by the combination of CGRP, VGLUT1 and VGLUT2 antibodies. Based on the results observed in this study, VGLUT2 boutons accounted for ~39% of the glutamatergic boutons on dorsal dendrites of lamina III NK1r cells in lamina I-II and ~53% of the contacts in lamina III-IV (Table 3-1). These VGLUT2 boutons, therefore, constituted somewhat less than half of the glutamatergic synapses that these cells received in the superficial laminae, but over half of those in deep dorsal horn. It has previously been reported that although, most glutamatergic synapses on these projection cells were <1 μm long, some were relatively elongated. It was further suggested that these synapses were associated with peptidergic primary afferents (Todd *et al.*, 2009). Studies based on electron microscopy also reported that although, the synaptic sizes of peptidergic afferents onto these cells varied considerably, they were mostly elongated (Naim *et al.*, 1997). The findings of this part of the study confirm this by showing that peptidergic afferent synapses are significantly larger than those formed by non-primary $VGLUT2^{+}$ boutons of spinal origin. Since these large synapses are associated with more postsynaptic glutamate receptors (Todd *et al.*, 2009), they will presumably generate relatively large EPSPs (Polgár *et al*., 2008a, Todd *et al*., 2009). This will provide a highly secure monosynaptic input from nociceptive primary afferents that will activate these cells

in response to noxious stimuli. Although lamina III NK1r cells receive a strong input from the peptidergic nociceptors, combined confocal and electron microscopy study has shown that they also receive some monosynaptic input from myelinated primary afferents (Naim *et al.*, 1998). Furthermore, Torsney and MacDermott (2006) suggested that mechanical hypersensitivity seen in the chronic pain states was probably due to disinhibition-induced NMDA receptor-dependant changes in the mono-synaptic Aβ input to lamina III NK1r cells. Thus, large lamina III NK1r-expressing cells are innervated by both nociceptive and LTM primary afferents that are responsive to variety peripheral stimuli, suggesting that cells of this type have a wide dynamic range receptive fields with a considerably strong input from nociceptors (Naim *et al.*, 1998).

Relatively little is known about the functions of local glutamatergic neurons that innervate the lamina III projection cells. Many of the dynorphin-containing cells are likely to be activated by noxious stimuli. A study based on in-situ hybridization and immunocytochemistry showed that cells with PPD mRNA, located throughout the dorsal horn, expressed c-*fos* after noxious stimulation (Noguchi *et al.*, 1991). This suggests that nociceptive primary afferents activate lamina III projection neurons through both mono- and polysynaptic pathways. A recent study has shown that excitatory interneurons in lamina II show firing patterns associated with A-type potassium (I_A) currents (Yasaka *et al.*, 2010). This type of currents is responsible for controlling the neuronal excitability by delaying the first action potential and causing reduction in the discharge frequency. This results in a delayed-, gap or reluctant-firing pattern in the excitatory interneurons. These observations suggest that glutamatergic cells are normally relatively inexcitable, but can undergo a form of activity-dependent intrinsic plasticity (Sandkühler, 2009) that results from inactivation of I_A currents due to phosphorylation of channel proteins (Hu *et al.*, 2006). If dynorphin-containing excitatory neurons are among those possessing I_A currents, acute noxious stimuli may normally activate the lamina III projection neurons mainly through monosynaptic inputs from the nociceptive primary afferents that make elongated synapses with large EPSPs (Polgár *et al*., 2008a, Todd *et al*., 2009). However, prolonged noxious stimulation such as that during a chronic pain state, will suppress I_A currents in the glutamatergic dynorphin-containing cells (Hu *et al.*, 2006), leading to increased excitability of the polysynaptic nociceptive input to the projection cells. This, together with the activation of NMDA receptors by locally released dynorphin could contribute to the development of inflammatory pain states (Shukla and Lemaire, 1992, Laughlin *et al.*, 1997).

4 Preferential innervation of NK1r-lacking spinoparabrachial lamina I projection neurons by non-peptidergic Aδ nociceptors in the rat spinal dorsal horn

4.1 Introduction

The myelinated nociceptive primary afferents, most of which conduct in the Aδ range, convey information that is perceived as fast pain (pricking pain, sharpness and aching pain) (Ringkamp *et al.*, 2013). These afferents are more robust in their response to noxious stimuli as compared to C-fibre nociceptors. Studies based on the hairy skin of monkey have classified these afferents into 2 subtypes, based on their location and functional differences (Treede *et al.*, 1995, 1998). The type I fibres have conduction velocity that ranges between the velocity for Aβ and Aδ fibres. These afferents have also been categorized among high-threshold mechanoreceptors (HTM) (Burgess and Perl, 1967, Lawson *et al.*, 1997). They are present in hairy and glabrous skin (Campbell *et al.*, 1979). They are polymodal in nature and respond to mechanical, thermal and chemical stimuli. The type II fibres conduct at Aδ range. They are mostly insensitive to mechanical stimulus or have significantly higher mechanical threshold. They are not found on the glabrous skin of the hand (Ringkamp *et al.*, 2013). In terms of responses to an intense heat stimulus, type I fibres appeared to have a long latency and a late peak discharge while type II afferents showed a short latency and an early peak discharge (Treede *et al.*, 1998). Furthermore, type I A-fibre nociceptors may contribute to the signalling of long duration heat stimulus and first pain sensation to mechanical stimuli while type II A-fibre nociceptors serve to signal the very first pain sensation to heat.

Many of Aδ afferents terminate in lamina I of the spinal dorsal horn (Light and Perl, 1979a, Woodbury and Koerber, 2003), a region that contains a relatively high density of dorsal horn projection neurons, nearly all of which belong to spinoparabrachial tract (Todd, 2010). The termination sites of myelinated afferents can be detected with a bulk labelling technique. In this method a neuroanatomical tracer such as Cholera toxin B subunit (CTb) is injected into the nerve. Several studies have suggested that CTb can be used as a highly sensitive immunocytochemically-detectable transganglionic tracer for the primary afferents (Wan *et al.*, 1982, Lamotte *et al.*, 1991, Rivero-Melián *et al.*, 1992). The β-subunit of cholera toxin, when injected into intact somatic peripheral nerves, binds to the GM1 ganglioside and is taken up and transported characteristically by myelinated (but not unmyelinated) primary afferents. This results in labelling of axonal boutons in lamina I and in a large region of the dorsal horn that extends ventrally from the inner half of lamina II (IIi) (Robertson and Grant, 1985, Lamotte *et al.*, 1991, Woolf *et al.*, 1992, Hughes *et al.*, 2003). The labelling in lamina I is thought to correspond to the central terminals of Aδ

nociceptors, while that in deeper laminae is mainly in low-threshold mechanoreceptive Aδ and Aβ afferents (Robertson and Grant, 1985, Willis and Coggeshall, 1991).

Previously, it has been reported that ~80% of CTb-labelled Aδ boutons in lamina I express VGLUT2 (Todd *et al.*, 2003). Although some Aδ nociceptors have been shown to express the neuropeptides CGRP and substance P (Lawson *et al.*, 1997, 2002), a preliminary immunofluorescence study suggested that there was little or no transport of CTb by peptidergic afferents (Rivero-Melián *et al.*, 1992). Lawson and her colleagues observed a relationship between the afferent properties and SP like-immunoreactivity in the dorsal root ganglion cells in guinea-pigs. They made intracellular recordings to label DRG neurons and detect the afferent receptive properties. In addition, they used avidin-biotin complex method to determine SP-like immunoreactivity. Their results suggested that around half of the Aδ- nociceptors exhibited SP-like immunoreactivity and that substance P expression was related to the afferent receptive properties, soma size and the site of peripheral receptive terminals. On the other hand, Rivero-Milan *et al.* (1992) used double immunofluorescence labelling technique with anterograde transganglionic transport of CTb to detect the expression of peptides by CTb boutons in the rat spinal dorsal horn.

It has already been mentioned that lamina I of the spinal cord dorsal horn has a relatively high density of projection neurons belonging to the anterolateral tract (Todd, 2010). These projection neurons respond to activity in Aδ afferents (Bester *et al.*, 2000, Torsney and MacDermott, 2006), and it has been reported that for at least some cells this is mediated through monosynaptic inputs (Andrew, 2010). Both neurochemical and electrophysiological studies have shown that virtually all lamina I projection neurons in the rat spinal dorsal horn respond to noxious stimuli (Bester *et al.*, 2000, Keller *et al.*, 2007, Andrew, 2009). However, anatomical studies have identified specific populations of projection neurons that differ considerably in their synaptic inputs. As described previously, the majority (75-80%) of lamina I projection cells express the neurokinin 1 receptor (NK1r), and these are densely innervated by CGRP-containing peptidergic primary afferents that constitute approximately half of their excitatory synapses (Todd *et al.*, 2002, Polgár *et al.*, 2010a). A small but distinctive population of giant cells have also been identified that either lack NK1r or express it weakly. These cells constitute approximately 3% of lamina I projection cells. They are densely innervated by both excitatory and inhibitory interneurons, but appear to receive little (if any) direct primary afferent input (Puskár *et al.*, 2001, Polgár *et al.*, 2008b). Very little is known about the

morphology, synaptic inputs and functions of the remaining ~20% (NK1r-negative) projection neurons in this lamina (Marshall *et al.*, 1996). Al Ghamdi *et al.*(2009) reported that the NK1r-negative cells are smaller in size as compared to NK1r-expressing projection cells in lamina I of the spinal dorsal horn. It has been shown that they receive a lower density of contacts from peptidergic primary afferents than the $NK1r^+$ projection neurons (Todd *et al.*, 2002). Lamina I non-NK1r cells predominantly receive polysynaptic input as compared to lamina I NK1r-expressing cells that receive mono-synaptic high-threshold input (C > Aδ) (Torsney and MacDermott, 2006). Some studies have associated the pyramidal shaped non-NK1r cells to the cooling specific cells in the cat (Han *et al.*, 1998, Almarestani *et al.*, 2007). However, not much is known about the primary afferent inputs to lamina I $NK1r^-$ projection cells.

The post-synaptic targets of CTb-labelled Aδ nociceptive afferents in lamina I have apparently not been identified. Therefore the main aim of this study was to test the hypothesis that CTb-labelled Aδ afferents were presynaptic to projection neurons in this lamina, and to determine whether any such inputs preferentially targeted specific types of projection cell. The second aim of this study was to determine whether CGRP or substance P were present in CTb-labelled boutons in lamina I and to relate this to the expression of VGLUT2 (Todd *et al.*, 2003).

4.2 Materials and methods

4.2.1 Surgical procedures, sciatic nerve and brain injections

Nine adult male Wistar rats (body weight range: 240-255g; UAE university) were anaesthetised with a mixture of ketamine and xylazine (25 mg and 5 mg i.m., respectively) that was injected intraperitoneally. These animals were placed on a heating pad in order to maintain the normal body temperature. The level of anaesthesia was supervised throughout the surgery by examining the corneal and/or withdrawal reflex as well as by applying a gentle pressure on the hind paw. Hair on the incision sites was shaved and the skin of the right thigh and head were cleaned using aseptic techniques. For all the rats, the right sciatic nerve was exposed by incising the skin of the back of the thigh and 2 μl of 1 or 2% CTb (Sigma-Aldrich, Poole, UK) was injected into the right sciatic nerve as described previously (Shehab *et al.*, 2004). The tracer was injected proximal to the major divisions of the sciatic nerve at the mid thigh level. In order to ensure the complete filling of the whole nerve, a finely drawn glass micro-pipette was inserted gently into the nerve for few

millimetres, in at least 3-4 different positions. In one of the rats, the sciatic nerve was bifurcated; therefore the injection was made into both branches with a total volume of 2.5ul of CTb. Tracers were prepared in Fast Green to make the injection visible. The skin and the muscles were then sutured in layers.

Six of the rats also received brain injections. Before fixing the head in a stereotactic frame, the coordinates were calculated for the zero position of the ear bars. This was followed by the head fixation in a stereotactic frame. Vaseline was applied on each eye to avoid dryness of the cornea. The skin and underlying fascia of the head were incised and reflected laterally to allow proper exposure. The region of the skull that overlay the site of injection was identified and calculated using atlas of Paxinos and Watson, 2005. Craniotomy was performed using a fine drill and occasional bleeding was stopped by applying gentle pressure at the bleeder site. All the animals received an injection of 50 nl of 4% Fluorogold (Fluorochrome Inc, Englewood, CO, USA) into the lateral parabrachial region of the mid brain on the left side (contralateral to the sciatic nerve injection) to label lamina I spinoparabrachial neurons (Todd *et al.*, 2000), as majority of these cells project contralaterally. Injections were made using glass micropipette. At the end of each injection, the pipette was left in place for 5 minutes to prevent the leakage of the tracer back up the track. After the completion of the surgical procedure, the wound was sutured in layers with absorbable stitches. All animals received postoperative analgesia subcutaneously (5mg/kg carprofen) and made an uneventful recovery from general anaesthesia. It has been suggested that perfusing animals later than 3rd post operative day does not increase the efficiency of the tracer significantly; therefore animals were allowed to survive for 3-4 days in order to achieve the optimal tracer spread. These animals were then terminally anesthetized with an overdose of urethane (625 mg i.p.) and were perfused using a solution of 4% freshly depolymerised formaldehyde through the left cardiac ventricle. Lumbar cords from these animals were removed and kept in a fixative for 4 hours while brains were cryoprotected with 30% sucrose as described previously.

4.2.2 Tissue processing and Immunocytochemistry

The lumbar segments were initially notched on the right hand side (contralateral to the side of sciatic nerve injection), to allow the identification of the two sides. The tissue sections (60 µm thick) of L4 spinal segments were cut into transverse and horizontal sections using vibrating microtome. They were then treated with 50% ethanol for 30 minutes as described

previously, following extensive rinsing. Sections were then processed for multiple labelling immunofluorescence and the details of the sources and concentrations of primary antibodies are given in the Table 2-1. This was followed by incubation in species-specific secondary antibodies conjugated to rhodamine red and DyLight 649 or Alexa 488. In all the cases where a secondary antibody labelled with biotin was used, it was revealed with avidin conjugated to Pacific Blue or with avidin-HRP. Primary antibody incubations were for 3 days while the incubation in the secondary antibodies was overnight (both were incubated free-floating at 4°C). Antibodies were diluted in PBS that contained 0.3% Triton-X100. All sections were mounted in anti-fade medium and stored at -20^{o}C until needed.

The brain region that received the injection was identified and a thick portion of brain tissue containing the injection site was cut transversely. This thick block of brain tissue was placed and stabilized on the stage of freezing microtome using the cryo-embedding compound called tissue teck (O.C.T™ compound). It was then cut into 100µm thick coronal sections using a freezing microtome into five complete series and one section from each series was mounted directly on the glass slide in serial order with anti-fade mounting medium and viewed under the bright and dark field illumination as well as an UV filter set to assess the spread of Fluorogold. The sections with the maximum tissue damage resulting from the injection at the lateral parabrachial region were identified for each rat and representative examples were photographed (Fig 4-2).

4.2.3 Neurochemical analysis of CTb-labelled boutons in lamina I

Transverse spinal cord sections from the 3 rats that received only sciatic nerve injections were incubated for 3 days with goat anti-CTb, rabbit anti-VGLUT2, rat anti-substance P and guinea pig anti-CGRP. The sections were then incubated overnight in species-specific secondary antibodies raised in donkey and conjugated to Alexa 488 or to Rhodamine Red, DyLight 649 or biotin. The biotinylated antibody was revealed with Pacific Blue conjugated to avidin. Sections were mounted and stored as mentioned before.

Three sections were selected from each rat before CTb immunostaining was viewed. The sections were scanned with a confocal microscope through a 63× oil-immersion lens (NA 1.4) and a pinhole of 1 Airy unit. Several overlapping z-stacks (20 optical sections at 0.5 µm z-separation) were scanned so as to include the whole of lamina I. Sections were analysed with Neurolucida, and from each section, 100 CTb-immunoreactive boutons were

selected from across the full mediolateral extent of CTb labelling in lamina I. This selection was made before other channels were viewed. The remaining channels were then switched on and the neurochemical phenotype of each of the selected CTb boutons was assessed.

In order to determine whether variations in the sizes of different types of boutons could have caused a sampling bias, the z-axis lengths of a sample of boutons belonging to each of the major neurochemical types were measured. This was done by determining the number of optical section on which they appeared and multiplying this by 0.5 μm (the z-spacing) (Sardella *et al.*, 2011b). A total of 900 CTb boutons (300 boutons from each animal) were identified and a sample of 20 boutons each was selected for CGRP-containing peptidergic CTb boutons with and without substance P and 40 boutons each were selected for CTb only and CTb^{+}/$VGLUT2^{+}$ boutons starting from medial to lateral extent of sciatic territory in lamina I. The sample size and the selection method was based on the proportion of different neurochemical sub-types of CTb-labelled boutons obtained in the results (see later).

4.2.4 Contacts between CTb-labelled Aδ afferents and spinoparabrachial neurons

Horizontal sections of spinal cord from 4 of the rats that received sciatic nerve injections on one side and LPb injections on the contralateral side were incubated in mouse anti-CTb, rabbit anti-NK1r, goat anti-VGLUT2 and guinea pig anti-Fluorogold, which were reacted with fluorescent secondary antibodies as mentioned before.

In order to estimate the proportion of projection neurons with or without the NK1r that received a significant number of contacts from CTb-labelled Aδ afferents, one or two horizontal sections that contained the largest number of lamina I projection neurons were selected from each rat and scanned through the 40× objective lens (NA 1.3) with a z-step of 1 μm. A set of overlapping fields was scanned to include the entire mediolateral and rostrocaudal extent of lamina I within each of these sections. All retrogradely labelled cells, apart from those that had substantial parts of the soma or dendritic tree missing from the section were identified and classified into one of three types: (1) NK1r-immunoreactive cells ($NK1r^{+}$), (2) giant cells (identified by the high density of VGLUT2 boutons on their cell bodies and proximal dendrites (Polgár *et al*., 2008b) and (3) projection neurons that were not giant cells and that lacked the NK1r ($NK1r^{-}$ cells). For each cell, the dendritic tree

was followed as far as possible through the z-stack and the presence or absence of contacts from CTb-labelled boutons was recorded.

This analysis revealed that relatively few $NK1r^+$ cells received contacts from CTb boutons, and these were generally at a low density. In contrast, a higher proportion of $NK1r^-$ cells were contacted, and in many cases these contacts were numerous. Since, the degree of CTb labelling varies between experiments due to the differences in the number of axons that have taken up the injected tracer, random sampling method was not used to select projection neurons for this part of the analysis. Instead, 20 Fluorogold labelled $NK1r^-$ lamina I cells that seemed to receive numerous contacts (between 4 and 6 cells from each rat) were selected and cell bodies as well as their dendritic tree with in the section were scanned through 63× oil-immersion lens to generate z-stacks (z-separation 0.5 μm). Similarly, 18 Fluorogold labelled $NK1r^+$ cells that received relatively high numbers of CTb contacts (3-8 cells per rat) were also selected and scanned in the same way. For both populations of cells, Neurolucida software was used to plot the locations of boutons in contact with the soma and dendrites of each cell that were immunoreactive for CTb, VGLUT2 or both.

During the course of this study, it was observed that a small proportion of CTb-labelled boutons in lamina I were CGRP-immunoreactive (see Results), therefore, after the completion of previously mentioned analysis, the sections were incubated with guinea pig anti-CGRP and were revealed with Pacific Blue (the same fluorochrome as was used to reveal Fluorogold) and rescanned the cells as described previously. Although both Fluorogold and CGRP were now labelled with Pacific Blue, they could easily be discriminated by comparison with the initial scans, which did not show CGRP immunoreactivity. All CTb boutons contacting the cells were re-examined to determine whether or not they contained CGRP. In addition, the locations of contacts that the cells received from CGRP-immunoreactive boutons that lacked CTb were plotted. Cell body surface areas were measured and the surface areas of dendrites were estimated from their lengths and diameters, based on the assumption that they were cylindrical (Todd *et al.*, 2002). For all types of contact, the density per 1000 μm^2 of the cell (combined soma and dendritic) surface was evaluated.

4.2.5 Combined confocal and electron microscopy

A combined confocal and electron microscopic technique was used to confirm that the contacts between CTb-labelled afferents and lamina I spinoparabrachial neurons were associated with synapses (Naim *et al.*, 1997). An observation that was made in the preliminary studies suggested that CTb was highly sensitive to glutaraldehyde fixation, and in particular the relatively light staining that was normally seen in lamina I was not detected even with very low concentrations of glutaraldehyde in the primary fixative. For this reason, the animals that have been used in this part of the study were perfused with 4% formaldehyde (i.e. without glutaraldehyde).

Horizontal sections from two rats that had received injections of CTb into the sciatic nerve and Fluorogold into the contralateral LPb were reacted with goat anti-CTb, rabbit anti-NK1r and guinea pig anti-Fluorogold. Antibodies were made in double salt phosphate buffer and the reaction was performed as described before, except that: (a) Triton was omitted to prevent any damage to the ultra structure (b) the secondary antibody cocktail contained both biotinylated and fluorescent-labelled anti-goat antibodies, and (c) the sections were incubated in avidin conjugated to horseradish peroxidase (HRP; Sigma) before being mounted and scanned (Naim *et al*., 1997, Polgár *et al*., 2008b). Three retrogradely labelled cells (two $NK1r^-$ from different animals, and one $NK1r^+$) that received contacts from CTb axons were selected and scanned. Low magnification confocal z-stacks were obtained in order to allow subsequent identification of the cells during the preparation of tissue for electron microscopy. High magnification z-series were obtained with the 63× oil-immersion objective (z step 0.5 μm) through the cell bodies and dendritic trees of each of these cells, and the locations of contacts that the cells received from CTb-immunoreactive boutons were noted. The sections containing these cells were then further fixed with 1% glutaraldehyde in PB overnight. They were then reacted with 3, 3'-diaminobenzidine (DAB) in the presence of hydrogen peroxide (Naim *et al*., 1997, Polgár *et al*., 2008b) to reveal CTb. The sections were then osmicated, block-stained with uranyl acetate and embedded in resin in the same way as described in chapter 3. Series of ultrathin sections through the cells were cut with a diamond knife and collected in serial order on Formvar-coated single-slot grids. These were contrasted with lead citrate and viewed with the electron microscope. As described previously, the DAB reaction product allowed the identification of CTb- immunoreactive boutons while the dendrites of the selected lamina I

projection cells were located with respect to their position in relation to these CTb boutons and nearby landmarks (e.g. capillaries).

4.2.6 Statistics

One-way ANOVA was used to test for differences in the z-axis lengths of different neurochemical types of CTb-labelled bouton in lamina I. A Chi-square test was used to determine whether there was a significant difference in the proportions of $NK1r^+$ and $NK1r^-$ projection neurons that received contacts from CTb-labelled boutons. Mann-Whitney U tests were used to compare densities of contacts from different types of axonal boutons onto these two different populations of projection neurons.

4.3 Results

4.3.1 VGLUT2 and neuropeptide expression by CTb boutons in lamina I

Following CTb injections into the sciatic nerve, the distribution of CTb-immunoreactive boutons was similar to what has been reported before in the previous studies. These boutons were densely located throughout the sciatic territory in the deep part of the dorsal horn (distributed ventrally from lamina IIi) with sparse plexus of labelled boutons in lamina I (Robertson and Grant, 1985, Lamotte *et al.*, 1991, Woolf *et al.*, 1992, Shehab *et al.*, 2003, Todd *et al.*, 2003, Shehab *et al.*, 2004). The distribution of staining for VGLUT2, CGRP and substance P was also the same as that described previously (Hökfelt *et al.*, 1975, Gibson *et al.*, 1984, Oliveira *et al.*, 2003, Todd *et al.*, 2003, Alvarez *et al.*, 2004, Landry *et al.*, 2004) and in all cases the antibodies penetration was complete and immunostaining was detected throughout the full thickness of the sections.

The majority of CTb-labelled boutons in lamina I were VGLUT2-immunoreactive (mean 75%, Figure 4-1a-c, Table 4-1), which is consistent with the previous reports by Todd *et al.* (2003) who reported that approximately 82% of CTb-labelled boutons in lamina I expressed VGLUT2. In addition, the strength of immunostaining varied considerably between the boutons in both the studies. Since some peptidergic primary afferents have myelinated axons (Lawson *et al.*, 1997, 2002) and that many peptidergic primary afferent terminals do not have detectable levels of VGLUT2 (Todd *et al.*, 2003), one aim of this part of the study was to test whether the remaining CTb^+/$VGLUT2^-$ boutons in lamina I

corresponded to peptidergic terminals. Although numerous boutons containing CGRP were observed in lamina I, only 11.4% (range 10-13%) of the CTb boutons in this lamina expressed CGRP immunoreactivity (Figure 4-1), and most of these (65-93%, mean 76%) were also VGLUT2^{+} (Figure 4-1d, Table 4-1). Substance P was found in an even lower proportion of CTb-labelled boutons (mean 2.3%), and all of these were CGRP^{+} (Fig 4-1d inset). In order to determine whether there was a selection bias among different neurochemical subtypes, their mean z-axis lengths were measured. A total of 900 CTb boutons (300 boutons from each animal) were identified and a sample of 20 boutons each was selected for CGRP-containing peptidergic CTb boutons with and without substance P by including every 5th bouton starting from medial to lateral extent of sciatic territory in lamina I. Similarly, 40 boutons each were selected for CTb only and CTb^{+}/VGLUT2^{+} boutons, by including every 5th and 15th bouton into the sample, respectively, as described previously. The mean z-axis lengths of non-peptidergic CTb boutons with and without VGLUT2 were 2.72 ± 0.65 μm and 2.69 ± 0.54 μm, respectively (n = 40 boutons in each case). The corresponding values for CTb boutons with CGRP but not substance P and for those with both peptides were 2.68 ± 0.58 μm and 2.71 ± 0.56 μm, respectively (n = 20 boutons in each case). These values did not differ significantly (ANOVA, p = 0.99), indicating that the estimation of the proportion of each neurochemical type is unlikely to have been affected by sampling bias.

Figure 4-1 Expression of neuropeptides by CTb-labelled afferents in lamina I.
a low magnification view of the upper part of the dorsal horn showing the general distribution of CTb-labelled profiles seen in a transverse section. Arrows point to the plexus of labelled axons in lamina I, and below this there are very few labelled structures in the outer part of lamina II. **b- d:** Confocal images from a section scanned to reveal **b** CTb (red), **c** CGRP (blue) and **d** VGLUT2 (VG2, green). A merged image is shown in **e**. Several CTb-immunoreactive boutons are visible. Two of these are $CGRP^{+}$, and these are marked with arrowheads. Arrows indicate two CTb-immunoreactive boutons that lack CGRP. Although most of the CTb-labelled boutons in this field contain VGLUT2, the level of expression of the transporter varies considerably between boutons. The inset in e (corresponding to the area in the box) shows the lower of the 2 CTb-labelled boutons that are marked with an arrowhead. This has been scanned to reveal CTb (red), CGRP (blue) and substance P (yellow), and the bouton can be seen to contain both peptides. a is a projection of 2 optical sections at 1 μm z separation, and b-e are a projection of 2 optical sections at 0.5 μm z separation. Scale bars: a 100 μm, b-e 10 μm.

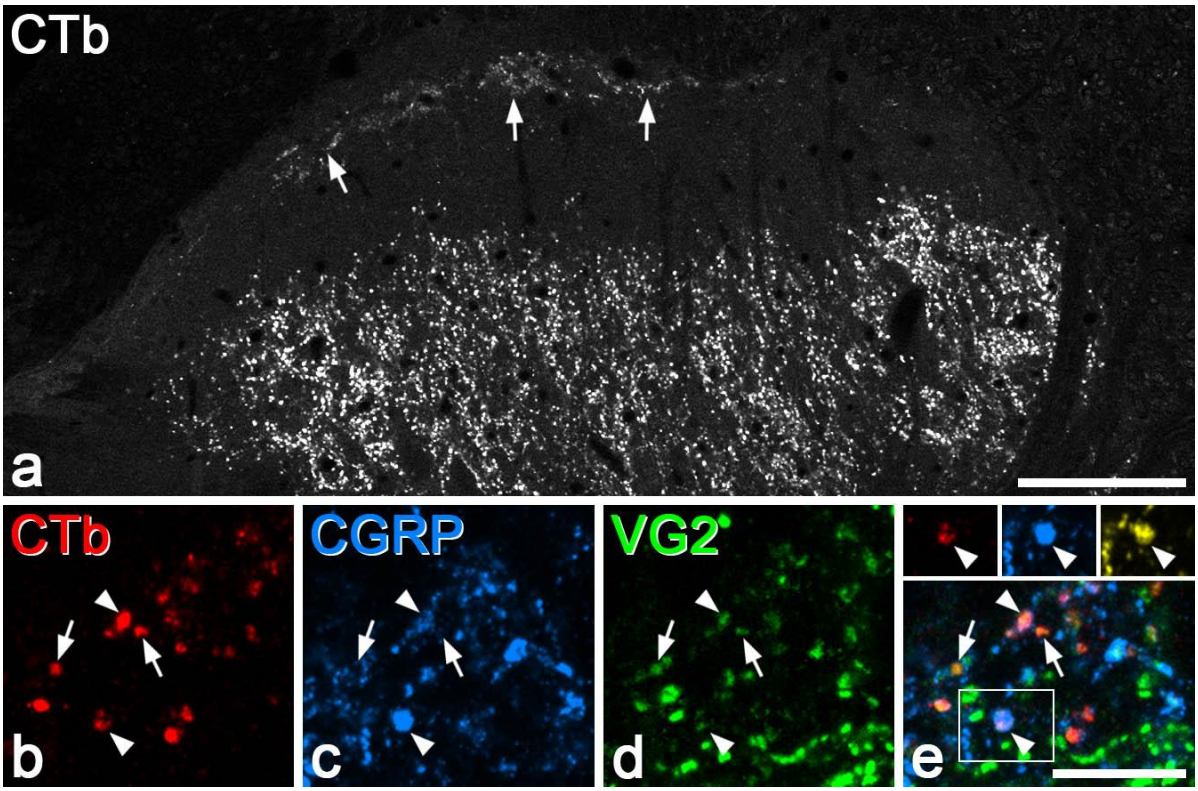

CTb
a
CTb
b
CGRP
c
VG2
d
e

Table 4-1 Neurochemistry of CTb boutons in lamina I

Rat	VGLUT2$^+$	CGRP$^+$	SP$^+$	VGLUT2$^+$/CGRP$^+$	CGRP$^+$ with VGLUT2
1	74	13.3	3.7	9.3	70
2	78.7	9.7	1.7	9	93.1
3	72.3	11.3	1.7	7.3	64.7
Mean	**75**	**11.4**	**2.3**	**8.6**	**75.9**

The columns second to fifth shows the percentages of lamina I CTb-labelled boutons in each of the 3 rats that expressed VGLUT2, CGRP, substance P, or both VGLUT2 and CGRP. The sixth column represents the percentage of CGRP containing CTb boutons that were also VGLUT2-immunoreactive. The great majority of CTb boutons were VGLUT2-immunoreactive while few were peptidergic.

4.3.2 Contacts between CTb boutons and lamina I projection neurons

In all the cases, the Fluorogold injection site included the whole of the LPb, with variable spread into surrounding areas. An example is shown in Figure 4-2. Horizontal sections containing Fluorogold-labelled lamina I projection neurons were assessed for the presence or absence of contacts from CTb-immunoreactive boutons on to these projection cells in this region. The quantitative data is shown in Table 4-2. The mean number of projection neurons identified in each rat was 208, of which 72% were $NK1r^+$, 2% were giant cells, and 26% were NK1r-negative non-giant cells (defined as $NK1r^-$ cells for convenience). The slight difference between the proportion of spinoparabrachial cells with the NK1r seen here and that reported previously (Todd *et al.*, 2000, Spike *et al.*, 2003) is likely to result from the use of horizontal sections in the present study. As reported previously (Polgár *et al*., 2008b), the giant lamina I cells did not receive contacts from CTb boutons. In contrast, 26% of the $NK1r^+$ cells and 43% of the $NK1r^-$ cells were found to receive contacts from CTb-labelled boutons. The proportions of neurons in these two populations that received contacts were significantly different ($p < 0.0001$, Chi-square test).

Results of the analysis of contacts onto the 20 $NK1r^-$ cells and 18 $NK1r^+$ cells are shown in Table 4-3, and examples are illustrated in Figures 4-3 and 4-4. The results indicated that $NK1r^-$ cells in this sample received a far higher density of contacts from CTb^+ boutons (31.7/1000 μm^2) than the $NK1r^+$ cells (9.1/1000 μm^2) ($p < 0.001$, Mann-Whitney U test). Virtually all (99.7%) of the CTb^+ boutons in contact with the $NK1r^-$ projection cells lacked CGRP, whereas 7% of CTb boutons contacting the NK1r-expressing projection cells were $CGRP^+$. However, the latter accounted for only 2% of all the CGRP boutons in contact with the $NK1r^+$ cells. The contact densities for the CTb boutons that lacked CGRP are shown in Fig. 4-5, and this indicates that virtually all of the $NK1r^+$ cells had a lower density in comparison to the $NK1r^-$ cells. In contrast, the densities of contacts from both the $CGRP^+$ and $VGLUT2^+/CGRP^-$ boutons that lacked CTb were considerably higher on the $NK1r^+$ cells (26.6 and 16.8/1000 μm^2, respectively) than on the $NK1r^-$ cells (3.1 and 9.7/1000 μm^2), and these differences were both significant ($p<0.001$, Mann-Whitney U test).

Since, the NK1r-immunostaining outlined the dendrites of $NK1r^+$ projection neurons, these projection cells could be traced until they terminated or left the section. However, for the $NK1r^-$ cells, while Fluorogold filling allowed considerable lengths of dendrite to be

identified, it is unlikely that these dendrites could be followed to their terminations. If contacts were highly concentrated on proximal dendrites of projection neurons, then the identification of more distal dendrites for the $NK1r^+$ cells could have contributed to the difference in contact density that was observed for the $CTb^+/CGRP^-$ boutons. To test this possibility, Sholl analysis was performed using shells with incremental separations of 20 μm (Figure 4-6). The results of this analysis showed that although contact density varied considerably within each projection neuron population, there was no indication of clustering of contacts on proximal dendrites of either population. It is therefore unlikely that a lower number of $CTb^+/CGRP^-$ contacts seen on distal dendrites of NK1r-expressing projection cells contributed to the lower overall contact density that was observed on these cells.

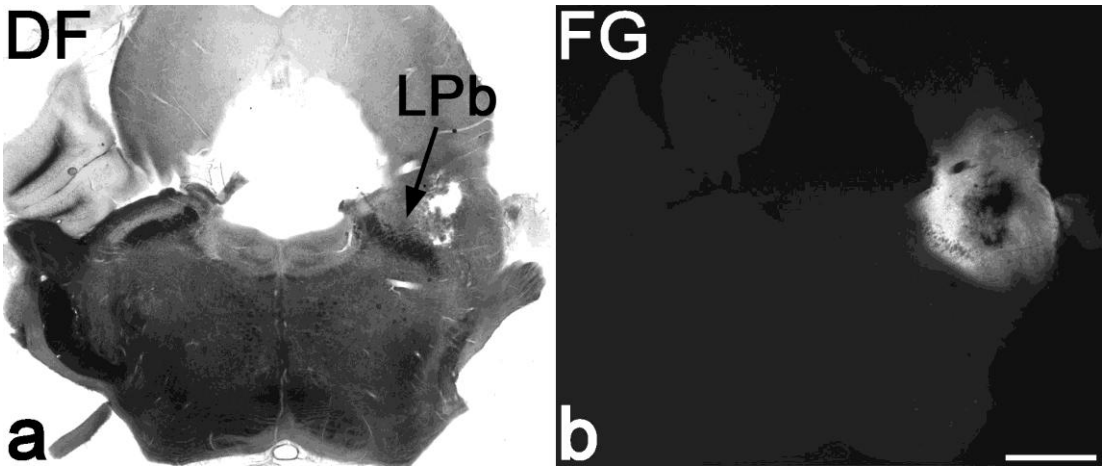


Figure 4-2 Example of a Fluorogold injection site.

a, **b** show dark field (DF) transmitted and fluorescent images of a section through the brainstem of one of the rats used in this study. The Fluorogold (FG) has spread throughout the entire lateral parabrachial area (LPb) on the left hand side, with some degree of spread into nearby structures. Scale bar: 1 mm.

Table 4-2 Analysis of contacts onto different types of projection neurons.

Rat	Total projection cells	NK1r$^+$ cells	Giant cells	NK1r$^-$ cells	% NK1r$^+$ cells with contacts	% NK1r$^-$ cells with contacts
1	254	183 (72%)	7 (2.8%)	64 (25.2%)	28	40.6
2	190	134 (70.5%)	3 (1.6%)	53 (27.9%)	26.9	47.2
3	200	128 (64%)	5 (2.5%)	67 (33.5%)	26.6	44.8
4	187	149 (80%)	3 (1.6%)	35 (18.7%)	22.8	40
Mean	**207.8**	**148.5 (71.6%)**	**4.5 (2.1%)**	**54.8 (26.3%)**	**26.1**	**43.1**

The total number of projection neurons sampled and the number belonging to each class for the 4 rats is presented in column second to fourth, while the respective percentages are shown in brackets. The last 2 columns represent the percentages of the corresponding cell types that received contacts from CTb-labelled boutons. No contacts were seen on the giant cells.

Figure 4-3 NK1r$^-$ lamina I spinoparabrachial neuron that receives numerous contacts from CTb-labelled boutons.

Confocal images from a section reacted to reveal Fluorogold (FG), CTb, VGLUT2 (VG2) and the NK1r. **a**-**c**: a projection of 3 optical sections at 0.5 μm z separation through the cell body and proximal dendrites of a lamina I neuron retrogradely labelled with Fluorogold (blue), which receives numerous contacts from CTb-immunoreactive boutons (red), some of which are indicated with arrowheads. Several of these boutons are also labelled with the VGLUT2 antibody (green), and therefore appear orange in the merged images. **b** Inset shows couple of VGLUT2-immunoreactive (green) boutons that also contain CTb (red) indicated with arrowheads. The lamina I neuron received relatively few contacts from other VGLUT2$^+$ boutons. **d** An equivalent projected image through the same section after it had been reacted to reveal CGRP (which appears in the same colour channel as Fluorogold). CGRP is represented by blue profiles that were not visible in **a**, and two of these are indicated with arrows. Note that since the section was re-mounted before scanning, its orientation is not exactly the same as in **a**-**c**, and therefore not all of the CTb-labelled profiles are visible. However, it is clear that none of those contacting the cell are CGRP-immunoreactive. **e** a projection of 3 different z-sections through the same cell, to show the relationship between NK1r (green) and Fluorogold (blue). The retrogradely labelled cell lacks the receptor, but there are two nearby dendrites that are NK1r$^+$ (arrows). Scale bar: 20 μm.

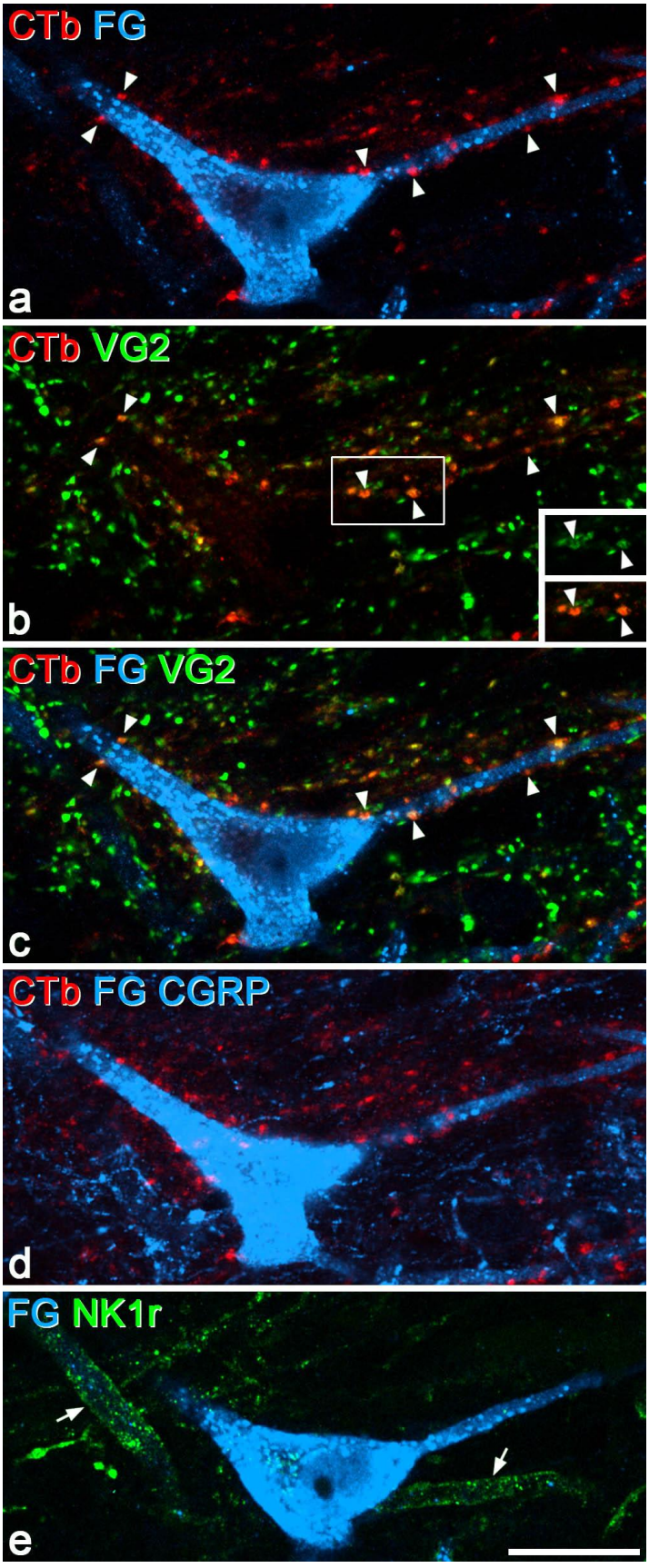

CTb FG
a
CTb VG2
b
CTb FG VG2
c
CTb FG CGRP
d
FG NK1r
e

Figure 4-4 NK1r$^+$ lamina I spinoparabrachial neuron that receives a few contacts from CTb-labelled boutons.

a-d show various combinations of staining for Fluorogold (FG, white), NK1r (green), CTb (red) and VGLUT2 (VG2, blue) in a projection of 6 optical sections (0.5 μm z separation) through the cell body and proximal dendrites of a lamina I NK1r-expressing neuron labelled with Fluorogold from the lateral parabrachial area (LPb). **b** The cell receives 4 contacts from CTb-labelled boutons, which show weak VGLUT2 **c-d.** It also receives several other contacts from VGLUT2-immunoreactive boutons, three of which are indicated with arrows. **d** The area outlined by the box is shown at a higher magnification from **e-f**. Three CTb-labelled boutons (red) are also VGLUT2 immunoreactive (blue) as indicated by arrowheads. Scale bar **a-d**: 20 μm **e-h**: 10 μm.

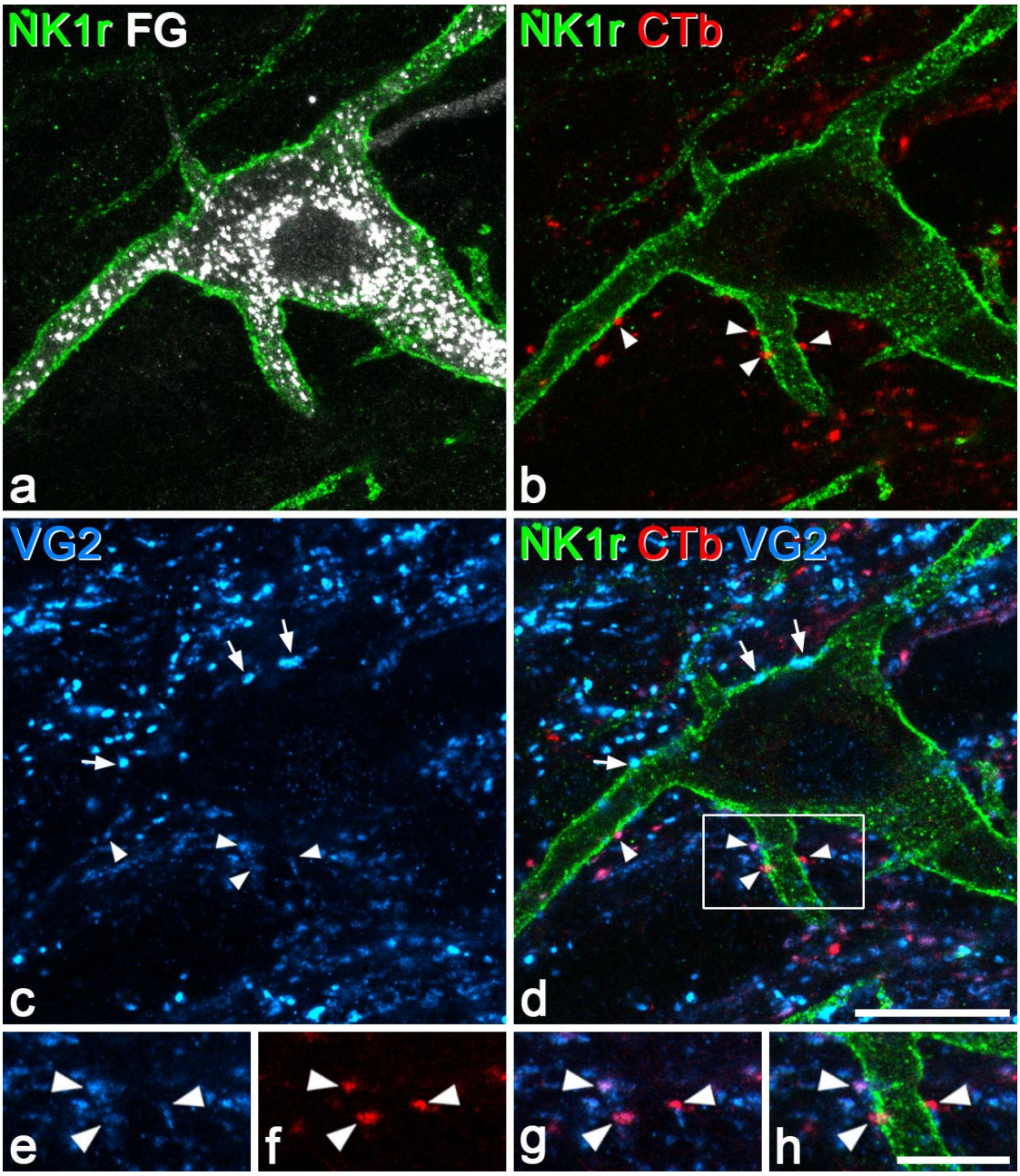

NK1r FG
NK1r CTb
a
b
VG2
NK1r CTb VG2
c
d
e
f
g
h

Table 4-3 Contact density on NK1$^+$ and NK1$^-$ projection neurons

	CTb$^+$/CGRP$^-$		**CTb$^+$/ CGRP$^+$**		**CTb$^-$/CGRP$^+$**		**CTb$^-$/VGLUT2$^+$/CGRP$^-$**	
	Number	**Density**	**Number**	**Density**	**Number**	**Density**	**Number**	**Density**
NK1r$^+$ (n=18)	43.6 (14-145)	8.5 (2.8-24.3)	3.2 (0-7)	0.6 (0-1.5)	140.7 (62-266)	26.6 (16.7-43.1)	87.7 (23-137)	16.8 (9.3-31.8)
NK1r$^-$ (n=20)	79.9 (30-210)	31.6 (20.8-45.9)	0.2 (0-2)	0.1 (0-0.5)	7.5 (2-15)	3.1 (1.2-8)	26.5 (5-99)	9.7 (4-22.4)

The mean numbers and density of contacts (per 1000 μm^2 of combined somatic and dendritic surface) of different neurochemical types of CTb-labelled boutons that contacted the 18 NK1r$^+$ and 20 NK1r$^-$ lamina I projection neurons are shown in this table. Ranges are given in brackets.

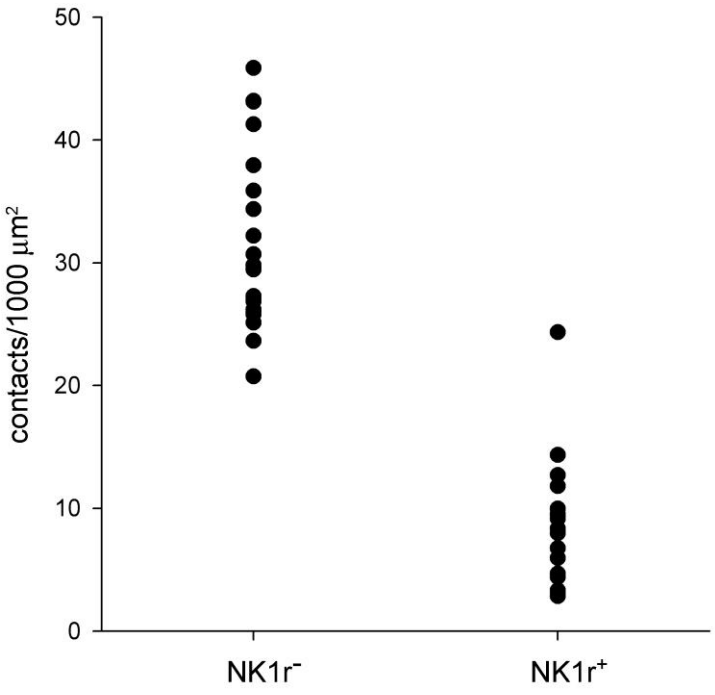


Figure 4-5 Density of contacts from non-peptidergic CTb-labelled boutons on lamina I projection neurons.

A plot of the contact densities for CTb^+ boutons that lacked CGRP onto projection neurons without ($NK1r^-$, n=20) and with ($NK1r^+$, n=18) the NK1 receptor. Only those projection cells that seemed to receive a considerably dense CTb input were included in the sample. Density is expressed as the number of contacts from $CTb^+/CGRP^-$ boutons per 1000 μm^2 combined somatic and dendritic surface area and each filled circle represents a single cell.

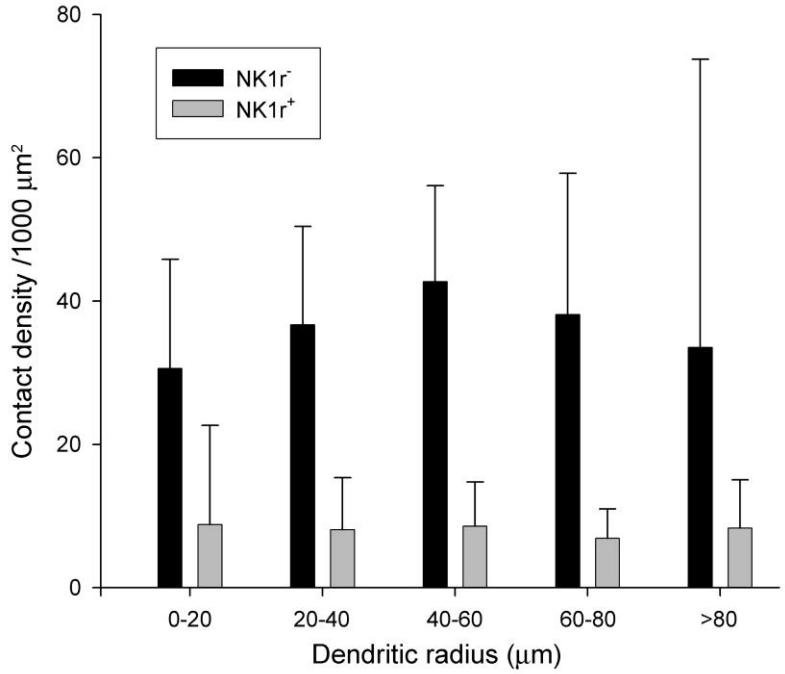


Figure 4-6 Sholl analysis of the contact densities of non-peptidergic boutons on dendrites of projection neurons.

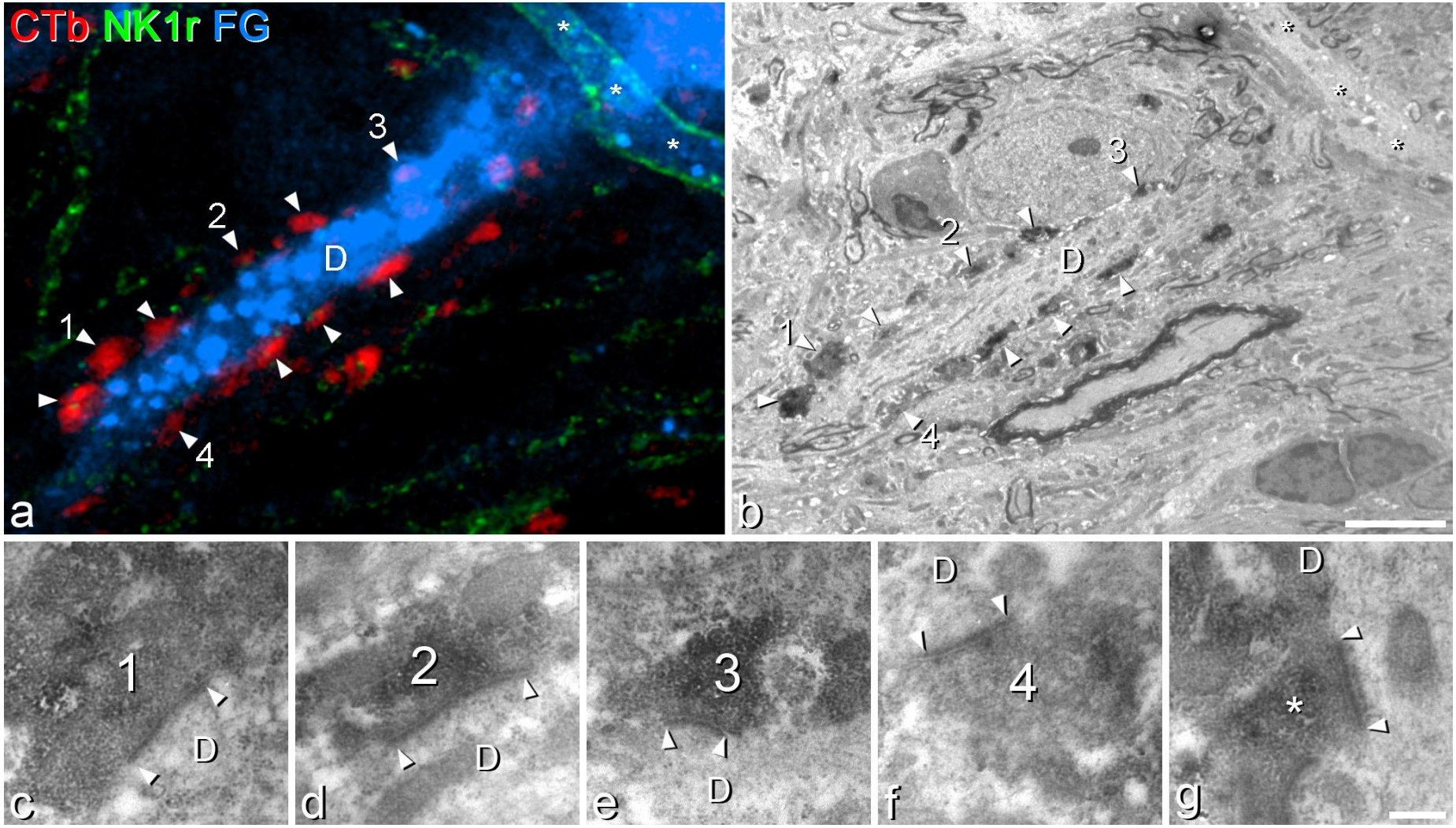
The mean densities (+ standard deviation) of contacts from $CTb^{+}/CGRP^{-}$ boutons on cells without ($NK1r^{-}$, n=20) or with ($NK1r^{+}$, n=18) the NK1. For each cell 20 µm shells were centred on the midpoint of the soma and the contact density on dendrites occurring within each cell were measured. Note that although there is considerable variability within each shell, there is no clear trend towards either increasing or decreasing density with distance from the soma for either projection neuron population.

4.3.3 Combined confocal and electron microscopy

Since, preliminary tests showed that CTb injected into sciatic nerve does not tolerate glutaraldehyde; therefore, the ultrastructural preservation of the tissue was compromised by the lack of glutaraldehyde in the primary fixative. Despite that the CTb-labelled boutons and the dendrites of the selected projection neurons could easily be recognised due to the presence of DAB product with in the CTb boutons and with the help of nearby anatomical landmarks (Figure 4-7a, b) and asymmetrical synapses could be identified. A total of 32 CTb^{+} boutons that were in contact with the two $NK1r^{-}$ spinoparabrachial cells (19 on one cell and 13 on the other) were identified. In the great majority of cases (17/19 on the first cell and all 13 on the second cell) the bouton was seen to form an asymmetrical synapse with the $NK1r^{-}$ projection neuron (Figure 4-7c, f). Six CTb-labelled boutons in contact with the $NK1r^{+}$ projection neuron were also identified, and 5 of these were associated with asymmetrical synapses (Figure 4-7g).

Figure 4-7 Combined confocal and electron microscopy.

a High magnification confocal image (single optical section) through part of a dendrite (D) of one of the two $NK1r^-$ spinoparabrachial lamina I cells that was analysed with combined confocal and electron microscopy. It has been scanned to reveal CTb (red), NK1 receptor (green) and Fluorogold (FG, blue). The dendrite receives numerous contacts from CTb-labelled boutons (some marked with arrowheads). The numbers correspond to boutons that are illustrated in the high magnification EM images (**c**-**f**). Note that the bouton numbered 3 is partially obscured by Fluorogold in this confocal image. A small part of a dendrite belonging to a different spinoparabrachial cell is visible in the top right corner, and is indicated with asterisks. This cell expressed the NK1 receptor, which can be seen outlining the dendrite. **b** A low magnification EM image of the region illustrated in **a**, with corresponding structures marked. **c**-**f** High magnification EM images of the 4 boutons indicated in **a** and **b**. In each case the synaptic specialisation is indicated (between arrowheads). **f** One of the synapses formed by a CTb-labelled bouton (*) onto a dendrite of the $NK1r^+$ lamina I spinoparabrachial neuron that was analysed. Again, the synaptic specialisation is between the arrowheads. Scale bars: **b** 5 μm (also applies to **a**); **g** 0.5 μm (also applies to **c**-**f**).


CTb NK1r FG
a
b
c
d
e
f
g
1
2
3
4
D
*

4.4 Discussion

The main findings of this study are: (1) that the great majority of transganglionically labelled sciatic afferents in lamina I are non-peptidergic, with only 2% containing substance P, and (2) that these afferents preferentially innervate a subset of $NK1r^-$ projection neurons in this lamina, providing a major component of their excitatory synaptic input. These afferents also appear to innervate a subset of lamina I NK1r-expressing projection cells and constitute only a small fraction of their glutamatergic input.

4.4.1 CTb-labelling of Aδ nociceptors

Studies based on nerve injections have suggested that CTb can be transported by unmyelinated visceral primary afferents (Robertson *et al.*, 1992, Wang *et al.*, 1998), and following nerve injury it is also taken up by axotomised somatic C fibres (Tong *et al.*, 1999, Shehab *et al.*, 2003). However, it is generally believed that among intact somatic afferents transganglionic transport is restricted to those with myelinated axons (Robertson and Grant, 1985, 1989, Lamotte *et al.*, 1991, Woolf *et al.*, 1992). Several lines of evidence support this observation. Firstly, the great majority (94-97%) of neurons in the L5 dorsal root ganglion that bind CTb stain with the anti-neurofilament antibody RT97 (Robertson and Grant, 1989, Robertson *et al.*, 1991), which is a marker for cells with myelinated axons. Secondly, dorsal horn labelling after sciatic injection of CTb or CTb conjugated to horseradish peroxidase (HRP) is present in laminae I and IIi-VI (Lamotte *et al.*, 1991, Rivero-Melián *et al.*, 1992, Woolf *et al.*, 1992, Shehab *et al.*, 2003, 2004), corresponding to the known distribution of myelinated primary afferents identified in various species by intra-axonal injections (Light and Perl, 1979a, Brown and Fyffe, 1981, Shortland *et al.*, 1989) or with neurochemical approaches (Li *et al.*, 2011). Thirdly, LaMotte *et al.* (1991) showed with electron microscopy that virtually all axons in the L4 dorsal root that contained CTb-HRP transported from the sciatic nerve were myelinated.

The pattern of CTb labelling observed in this study was similar to what has been reported before (Robertson and Grant, 1985, Lamotte *et al.*, 1991, Shehab *et al.*, 2004). This pattern changes when CTb is injected into a chronically injured nerve. CTb labelled boutons appear in the outer part of lamina II (IIo). It has been suggested that this results from the up regulation of GM1 ganglioside by axotomised C fibres that become capable of transporting CTb post injury (Tong *et al.*, 1999, Bao *et al.*, 2002, Shehab *et al.*, 2003). There is a possibility that some primary afferents get damaged by the sciatic nerve injections, it is

very unlikely that they would result in CTb transport by axotomised C-fibres since the change in central labelling takes longer than 3 days post injury to develop (Woolf *et al.*, 1995, Bao *et al.*, 2002). It is therefore suggested that the CTb labelling in lamina I that was observed in this study belonged to Aδ nociceptive primary afferents.

However, although some myelinated nociceptors transport CTb, it is likely that many do not. LaMotte *et al.* (1991) reported that while the majority of large myelinated axons (>2 μm diameter) in the L4 dorsal root were labelled after sciatic nerve injection of CTb-HRP, label was seen in a considerably lower proportion of fine myelinated axons. In addition, a population of myelinated nociceptors that arborise throughout laminae I-IV has been identified in the mouse (Woodbury and Koerber, 2003, Woodbury *et al.*, 2008). These have conduction velocities spanning the Aβ/Aδ range, mechanical thresholds that are generally lower than those of typical Aδ nociceptors and may function as "moderate pressure receptors" (Burgess and Perl, 1967, Woodbury *et al.*, 2008). If afferents of this type are present in the rat, it is highly unlikely that they transport CTb, since very few CTb-labelled boutons are seen in the outer part of lamina II following sciatic injection.

4.4.2 Neuropeptide expression in Aδ nociceptors

Electrophysiological studies based on the dorsal root ganglion cells in several species have reported that many myelinated primary afferents express neuropeptides (Lawson *et al.*, 1996, 1997, 2002, Koerber and Woodbury, 2002). Lawson *et al.* (2002), in their study based on guinea pigs, reported that approximately 5 of 12 Aδ nociceptors were CGRP-immunoreactive while in another observation, 8 of 16 contained substance P (Lawson *et al.*, 1997). It has been shown previously (Todd *et al.*, 2003) as well as in this study that approximately 20% of CTb-labelled boutons in lamina I lack detectable levels of VGLUT2 and since many peptidergic afferents are not VGLUT2-immunoreactive (Todd *et al.*, 2003, Landry *et al.*, 2004, Morris *et al.*, 2004), there was a possibility that $VGLUT2^-/CTb^+$ boutons would correspond to peptidergic afferents. However, the results of this study suggest that only 11% of CTb labelled boutons in lamina I contained CGRP and the great majority of these were VGLUT2-immunoreactive. In addition to that, $CGRP^+$ boutons only accounted for ~10% of the $VGLUT2^-/CTb^+$ boutons in lamina I and substance P was present in an even lower proportion (2%) of the CTb-labelled boutons in this region.

However, Lawson *et al.* (1997) reported that 10 out of 31 myelinated nociceptors recorded in the guinea pig were substance P-immunoreactive. Apart from a species differences,

there are various possible explanations for this discrepancy between the number of neuropeptide –expressing Aδ nociceptors in electrophysiological studies (Lawson *et al.*, 1996, 1997) and the low proportion of CTb-labelled peptidergic boutons seen in this study: (1) these central terminals of these afferents seldom terminate in lamina I, (2) they do not transport significant amounts of peptide to their central terminals, and thus it remains undetectable or (3) they lack the GM1 ganglioside, and are therefore unable to transport CTb. The third explanation seems most likely, since Robertson and Grant (1989) reported that only 3% of L5 dorsal root ganglion cells that bound CTb were substance P-immunoreactive. Because all substance P primary afferents also contain CGRP (Ju *et al.*, 1987), the central boutons of substance P-containing myelinated nociceptors will be among those immunoreactive for both peptides (Todd *et al.*, 2002). However, it is not yet possible to distinguish them from substance P-containing unmyelinated afferents.

Another observation made by Lawson *et al.* (1997) suggested that substance P was present in most Aδ nociceptors that responded to noxious heat and mechanical stimulation. In addition to that, these afferents had deep receptive fields. However, they further reported that none of the 20 Aδ high threshold mechanoreceptors (AHTM) with superficial cutaneous receptive fields contained the peptide. Based on these observations, it is proposed that many Aδ afferents that were originally classified as AHTM (Burgess and Perl, 1967) and respond to noxious heat but with high thresholds (>53^{o}) and long latencies, also correspond to central terminals of type I Aδ afferents reported in several studies (Treede *et al.*, 1995, 1998, Ringkamp *et al.*, 2013). This is further supported by an almost complete lack of coexistence of substance P and CTb among the CTb-labelled boutons in lamina I of the spinal dorsal horn seen in the present study.

The results further suggest that peptidergic afferents only account for a small proportion of the CTb^{+}/VGLUT2^{-} boutons in lamina I. The discrepancy between the findings of this study and those of (Rivero-Melián *et al.*, 1992), who did not observe colocalisation of CTb with either CGRP or substance P, is probably explained by the use of confocal microscopy in this study. This allows more accurate resolution of small profiles within a dense plexus of immunoreactive axons, as well as the detection of weakly labelled boutons.

4.4.3 Synaptic input from Aδ nociceptors to lamina I projection neurons

It has already been mentioned that a vast majority (between 75-80%) of lamina I projection cells express NK1 receptor (Ding *et al.*, 1995, Todd *et al.*, 2000, Spike *et al.*, 2003). Previous studies have shown that they are densely innervated by substance P-containing (nociceptive) primary afferents (Todd *et al.*, 2002), which account for around half of their excitatory synapses (Polgár *et al.*, 2010a). It is likely that some of the input from substance P-containing afferents is derived from Aδ nociceptors that lack the GM1 ganglioside and therefore are not identifiable with CTb injected into sciatic nerve. Around a quarter of these cells also receive contacts from CTb-labelled afferents, most of which lack neuropeptides. The EM findings for one of these cells indicate that at least some of these non-peptidergic Aδ nociceptors form synapses. However, this is at a relatively low density compared to their peptidergic input. Torsney and MacDermott reported that approximately 30% of lamina I NK1r-expressing neurons, most of which were likely to be projection cells, received monosynaptic Aδ input, presumably from Aδ nociceptors (Torsney and MacDermott, 2006, Torsney, 2011). Furthermore, hind paw inflammation increased the proportion of cells with monosynaptic Aδ input to ~60%, resulting from activation of previously silent synapses (Torsney, 2011). Although the Aδ input seen in these experiments may have involved non-peptidergic afferents, it is likely that some of the substance P^+/ $CGRP^+$ boutons that synapse on NK1r-immunoreactive projection neurons (Todd *et al.*, 2002) belong to peptidergic Aδ nociceptors, and that these contributed to the monosynaptic input.

Giant lamina I projection neurons have a very characteristic pattern of inhibitory and excitatory synaptic input (Puskár *et al*., 2001, Polgár *et al*., 2008b). The latter is derived from boutons showing strong VGLUT2 immunoreactivity, which are thought to be almost exclusively of non-primary origin (Polgár *et al*., 2008b). Although very distinctive, these cells are rare, accounting for ~3% of lamina I projection neurons. Therefore, the remaining NK1r-negative neurons constitute ~20% of the projection cells in this lamina. Until now, little was known about the synaptic inputs to these cells, but the results of this part of the study show that >40% of them receive contacts from presumed type I Aδ nociceptors that lack neuropeptides. Furthermore, these contacts can be extremely numerous, and are associated with synapses.

Excitatory synapses in lamina I originate from local neurons, primary afferents and descending axons. The VGLUT2 antibody is likely to reveal the axons of all local excitatory interneurons (Todd *et al.*, 2003, Yasaka *et al.*, 2010), as well as any descending axons, except from those of the cortico-spinal tract, which are sparse in this lamina (Du Beau *et al.*, 2012). Apart from Aδ nociceptors, most primary afferents in lamina I are peptidergic C fibres and will have been revealed with the CGRP antibody (Ju *et al.*, 1987). There is also a population of non-peptidergic TRPM8$^+$ fibres, that belong to both C and Aδ afferents (Dhaka *et al.*, 2008) and it not known whether these express VGLUT2. With the possible exception of the TRPM8$^+$ afferents, it is therefore likely that most other glutamatergic boutons in lamina I would be detected by the combination of CGRP and VGLUT2 antibodies. The mean density of contacts from CTb$^+$/CGRP$^-$ boutons on the 20 NK1r$^-$ projection neurons sampled was 32/1000 μm^2 (Table 4-3), while the mean density of contacts from all other immunostained boutons (i.e. those with CGRP and/or VGLUT2) was 13/1000 μm^2. For this group of NK1r$^-$ projection cells, non-peptidergic Aδ nociceptors (type I) (Treede *et al.*, 1998, Ringkamp *et al.*, 2013) could therefore provide up to 70% of their excitatory synapses. Interestingly, the contact density from Aδ nociceptors to the NK1r$^-$ cells is even higher than that of peptidergic primary afferent input to the NK1r$^+$ cells (Todd *et al.*, 2002), indicating that the NK1r$^-$ projection neurons are likely to be powerfully activated by Aδ nociceptors.

4.4.4 Functional significance of CTb-labelled Aδ nociceptors input to lamina I projection cells

Studies performed by Bester *et al.* (2000) and David Andrew (2009) tested the responses of lamina I spinoparabrachial neurons to mechanical and thermal stimuli. All 53 cells recorded by Bester *et al.* were activated by noxious heat of varying thresholds, and 92% responded to noxious mechanical stimuli. Andrew reported that most spinoparabrachial neurons were driven by both noxious heat and mechanical stimuli, although he suggested that 5% were cooling-specific. Cooling-specific cells are likely to be among those that lack the NK1r. The results of these two studies indicate that the vast majority of lamina I spinoparabrachial neurons respond to noxious stimuli, and these must therefore include most of the NK1r$^-$ cells. Interestingly, studies using Fos as an activation marker (Hunt *et al.*, 1987) have reported that NK1r$^-$ lamina I projection neurons are significantly less likely to show c-*fos* than those with the receptor, following noxious heat or subcutaneous injection of formalin (Todd *et al.*, 2002, Todd *et al.*, 2005). For example, immersion of the foot in water at 52°C for 20 sec evoked Fos in 63% of NK1r$^+$ lamina I spinoparabrachial

cells, but only in 14% of those without the receptor (Todd *et al.*, 2005). The discrepancy between this result and the reports that virtually all spinoparabrachial lamina I cells respond to noxious heat (Bester *et al.*, 2000, Andrew, 2009) may be because the Aδ nociceptors that innervate many of the $NK1r^-$ cells have higher heat thresholds (>53^oC) (Treede *et al.*, 1998, Ringkamp *et al.*, 2013) than peptidergic nociceptors, which provide the major primary afferent input to $NK1r^+$ lamina I projection neurons (Ringkamp *et al.*, 2013). Similarly, the difference in Fos expression after formalin injection may be because TRPA1 receptors, which are activated by formalin (Julius, 2013) are present on peptidergic afferents, but not non-peptidergic Aδ nociceptors (Story *et al.*, 2003, Bautista *et al.*, 2005, Kobayashi *et al.*, 2005).

The finding that over 40% of $NK1r^-$ spinoparabrachial cells are innervated by Aδ nociceptors suggests that these cells have an important role in the perception of fast pain. Ablation of $NK1r^+$ lamina I neurons with substance P-saporin reduced hyperalgesia in chronic pain states, but left acute pain thresholds intact (Mantyh *et al.*, 1997, Suzuki *et al.*, 2002). $NK1r^-$ lamina I projection cells may have played an important part in maintaining acute nociception in these animals.

These results provide further evidence that the primary afferent input to different classes of projection neuron are organised in a highly specific way. This implies that functional differences between nociceptors subtypes are, to some extent, maintained at the level of the projection neurons that form the major output from the superficial dorsal horn and these different classes of projection cells therefore play distinct roles in pain perception.

5 Inhibitory input to lamina I projection neurons in the mouse. A possible role in itch perception

5.1 Introduction

Most studies on spinal pain mechanisms have been carried out on the rat. However, the availability of a number of genetically modified mice has contributed to the increased use of mouse as a research model. Recent advancements in genetics have made genetic manipulation of these mice possible. Despite few inter-species differences, such as variation in the expression of TRPV1 on nociceptive afferents (Zwick *et al.*, 2002) and NK1r-expression in lamina I (Polgár *et al*., 2013a) as well as lack of NK1r-expression by lamina III projection cells in the mouse dorsal horn (A. J. Todd un-published observation), the overall neuronal organization of rat and mouse is quite similar. As in the rat, most neurons in the mouse superficial dorsal horn are also interneurons with axons arborizing locally (Polgár *et al*., 2013a). Lamina II of the mouse dorsal horn is densely packed with interneurons and among them inhibitory interneurons constitutes around 26% and 38% of all the cells in lamina I-II and III, respectively. These neurons use GABA and/or glycine as their principal neurotransmitter (Polgár *et al*., 2013a). Studies based on the rat have suggested that dorsal horn interneurons are involved in modulating sensory information. This sensory information is projected to brain and processed to cells in the deep laminae of the spinal cord. The loss of inhibition mediated by dorsal horn inhibitory interneurons has been implicated in the development of both hyperalgesia and allodynia (Sandkühler, 2009, Zeilhofer *et al.*, 2012a) and it has been proposed that these cells also take part in the suppression of itch (Ross *et al.*, 2010, Kardon *et al.*, 2014). Consistent with the findings that inhibitory interneurons suppress pain, it has been reported that a reduced GABA synthesis and/or post synaptic inhibition may contribute to neuropathic pain (Moore *et al.*, 2002, Polgár *et al.*, 2005).

Several attempts have been made to categorize distinct functional populations of interneurons in the superficial dorsal horn. Recently, 4 non-overlapping populations of inhibitory interneurons have been identified in lamina I and II of the rat dorsal horn, based on the expression of galanin, neuropeptide Y (NPY), neuronal nitric oxide synthase (nNOS) and parvalbumin (PV) (Polgár *et al.*, 2011, Sardella *et al.*, 2011b, Tiong *et al.*, 2011). Together, these cells constitute around half of the inhibitory interneurons in superficial dorsal horn (Sardella *et al.*, 2011b). Approximately 50% of inhibitory interneurons express somatostatin receptor 2_A (sst_{2A}) (Polgár *et al.*, 2013), which is widely distributed in the superficial dorsal horn (Schindler *et al.*, 1996, Todd *et al.*, 1998) and is involved in disinhibition (Todd *et al.*, 1998, Yasaka *et al*., 2010, Zeilhofer *et al*., 2012b,

Polgár *et al*., 2013a). Similar populations of inhibitory interneurons have also been identified in the mouse dorsal horn (Iwagaki *et al.*, 2013). These non- overlapping populations of inhibitory interneuron vary in terms of sst_{2A} receptor expression, responses to painful stimuli (Polgár *et al.*, 2013), developmental lineage and in their post synaptic targets. sst_{2A} receptor is expressed by virtually all nNOS and galanin containing cells, but by few NPY cells and none of the PV cells (Polgár *et al.*, 2013). In addition, many galanin- and NPY-containing cells and very few nNOS cells phosphorylate extracellular signal-regulated kinases (pERK) after noxious thermal (immersion of hind paw in water at 52^{o}C for 20 seconds), mechanical (pinch) or chemical (capsaicin or formalin injections) stimuli, while many nNOS containing inhibitory cells up regulate c-*fos* in response to noxious thermal stimulation or formalin injection but not to capsaicin. However, parvalbumin cells do not express these activity dependant markers following the noxious stimulation. Recently, it has been suggested that NPY- and galanin containing cells belong to different lineage (Bröhl *et al.*, 2008) while nNOS and galanin neurons are developmentally linked (Kardon *et al.*, 2014). These inhibitory interneurons also vary in their post synaptic targets. Many of these cells provide axodendritic or axosomatic inputs to superficial dorsal horn cells and generate post synaptic inhibition while the axons of some cells presynaptically inhibit primary afferents in this region (Ribeiro-da-Silva and Coimbra, 1982, Maxwell and Réthelyi, 1987). PV cells make axoaxonic contacts with low-threshold mechanoreceptors in mouse while NPY cells provide selective innervation to lamina III NK1r projection cells and to PKCγ-expressing excitatory interneurons in lamina II. Similarly, nNOS containing cells selectively innervate lamina I giant cells in the rat (Polgár *et al*., 1999b, 2011, Puskár *et al*., 2001, Hughes *et al*., 2012). The inhibitory input of PV cells on LTM is probably responsible for maintaining tactile acuity (Hughes *et al.*, 2012) while nNOS and NPY inhibitory axons may limit the response of their target cells to noxious stimulus (Sandkühler, 2009).

As mentioned previously, 6% of lamina I neurons in the rat mid lumbar region are projection cells (Spike *et al.*, 2003, Al-Khater *et al.*, 2008) and majority (~80%) of them express neurokinin I receptor (NK1r) (Marshall *et al.*, 1996). The distribution of NK1 receptor in the mouse somewhat differs to that in the rat. In the rat, approximately half of lamina I cells express NK1r (Todd *et al.*, 1998). However, although NK1 receptor is concentrated in lamina I of the mouse dorsal horn, it has been reported that only ~20% of all the neurons in this region express the receptor (Polgár *et al*., 2013a). The two possible explanations of this discrepancy are; 1) genuine inter species differences between rat and

mouse, 2) NK1 receptor labelling in the mouse also varies in terms of strength of immunostaining, therefore weakly labelled cells were not identified. Not many anatomical studies have been done on dorsal horn projection cells in the mouse. In a recent study, in which Fluorogold was injected into LPb on one side of the mouse brain, approximately 100 cells per 500 µm of lamina I of lumbar spinal segment were retrogradely labelled (Wang *et al.*, 2013). This number included both contralateral and ipsilateral projection cells. Since 30% of spinoparabrachial cells project bilaterally in the rat, considering a similar arrangement in the mouse, the overall number of lamina I spinoparabrachial projection cells would be around 220 on one side of the lumbar spinal segment. Polgár *et al.* (2013a) reported that the total number of neurons in lamina I of L4 segment of the mouse spinal cord was around 4500. Based on these observations, Polgár *et al.* further suggested that projection neurons constitute around 5% of lamina I cells in the mouse dorsal horn, which is similar to the proportion reported in the rat (Spike *et al.*, 2003, Al-Khater *et al.*, 2008).

As described previously, another distinctive population of lamina I spinoparabrachial projection neurons (also named as giant cells) has also been identified in rat spinal dorsal horn. These cells either lack NK1r or express it weakly (Puskár *et al.*, 2001) and receive dense excitatory and inhibitory inputs that almost completely outline their soma and dendrites. Since retrograde tracing technique was not used in this part of the study, lamina I projection cells were identified based on the observations made in the previous studies in the rat. Lamina I giant cells were recognized with antibodies against glycine receptor associated protein gephyrin as well as VGLUT2 and VGAT boutons, all of which provide dense innervation to these cells. They are selectively innervated by nNOS-containing GABAergic axons, which constitute around 30% of their overall inhibitory input and are thought to originate from local interneurons (Puskár *et al.*, 2001). However, it is not known whether equivalent cells also exist in lamina I of mouse dorsal horn and if so, whether they also receive nNOS-containing inhibitory input.

Recently, several studies have been performed on mice that express fluorescent proteins in distinct populations of interneurons (Heinke *et al.*, 2004, Hantman and Perl, 2005, Labrakakis *et al.*, 2009, Punnakkal *et al.*, 2014). One such example includes mice that express green fluorescent protein (GFP) (Van Den Pol *et al.*, 2002). Perl and colleagues identified a physiologically homogenous population of inhibitory interneurons in lamina II that express GFP reporter gene under the control of the prion promoter (PrP) (Hantman *et*

al., 2004, Hantman and Perl, 2005), hence was called PrP-GFP mouse. The underlying mechanism of selective expression of GFP is not entirely known but it has been suggested that it might result from a combination of promoter and integration site effects (Van Den Pol *et al.*, 2002). In the PrP-GFP mouse, prion promoter is under the influence of local gene sequences in the chromosomal region where the transgene gets incorporated. This transgene is randomly integrated into the receptor protein tyrosine phosphatise kappa (RPTP-k) and the laminin receptor 1 pseudogene 1 locus. Transgene and the RPTP-k locus interact through an unknown mechanism, resulting in the selective expression of GFP in lamina II of mouse dorsal horn (Hantman *et al.*, 2004, Hantman and Perl, 2005).

According to Hantman *et al.* (2004) and Hantman and Perl (2005), PrP-GFP cells are quite homogenous in their morphology, electrophysiological and molecular properties. Morphologically, these cells are mostly located in lamina IIo and resemble central cells, with axons limited to lamina II. Initially, it was reported that around 80% of these cells are GABA-immunoreactive (Hantman *et al.*, 2004). Later, it was shown that almost all PrP-GFP cells expressed sst_{2A} receptor, which is restricted to inhibitory cells in the spinal dorsal horn (Iwagaki *et al.*, 2013). This discrepancy can be due to limited penetration of GABA antibody that would have resulted in the underestimation of the proportion of GABA-expressing PrP-GFP cells in Hantman *et al.* These cells fire tonically in response to depolarizing current impulse, which is a characteristic feature of inhibitory cells (Yasaka *et al.*, 2010) and are hyperpolarized in response to somatostatin (Iwagaki *et al.*, 2013). Neurochemically, these PrP-GFP cells lack calbindin D-28k, parvalbumin and protein kinase Cγ (PKCγ) (Hantman *et al.*, 2004). All these findings further suggest that PrP-GFP cells are inhibitory nature. Recently it has been shown that these cells make up ~4% of the neurons in lamina II, corresponding to 10-15% of all the inhibitory interneurons in this region (Iwagaki *et al.*, 2013). Virtually all (98%) contained nNOS and/or galanin and some cells co-express nNOS and galanin (Iwagaki *et al.*, 2013). This observation is contrary to what was observed in the rat, where nNOS and galanin cells constituted two non-overlapping populations in this region (Tiong *et al.*, 2011, Polgár *et al.*, 2013). PrP-GFP cells receive monosynaptic inputs from C-afferents (Hantman *et al.*, 2004) but not from Aδ afferents and contain PK CβII, which is a specific isoform of PKC underlying nociception. In addition to that, they also express c-*fos* in response to formalin injection (Hantman *et al.*, 2004). These cells are reciprocally connected to GABAergic islet cells and are probably presynaptic to excitatory vertical cells (Zheng *et al.*, 2010). However, other post-

synaptic targets of this distinct population of lamina II inhibitory interneurons are not yet known.

It is already an established fact that certain proteins such as transcription factors play a key role in regulating the flow of genetic information between various genes. These transcription factors can be added, ablated, blocked or modified to demonstrate mechanisms necessary for the development of neuronal circuits. Ross *et al.* (2010, 2012) identified an important role of neural specific transcription factor called basic helix-loop-helix (*Bhlhb5*) in the development and regulation of neuronal itch circuits. This transcription factor is expressed briefly during the embryonic and early post natal developmental phases in several neuronal subtypes. The selective ablation of *Bhlhb5* resulted in a phenotypically distinct mouse that exhibited exaggerated and pathological chronic itch, resulting in self inflicted skin lesions. Furthermore, the genetic fate mapping approaches have shown that the pathological itch in *Bhlhb5* mutant mouse is due to the loss of a subset of inhibitory interneurons in superficial dorsal horn that would have normally expressed *Bhlhb5* (Ross *et al.*, 2010). Based on these observations, it is suggested that *Bhlhb5* is important for the survival of a subset of inhibitory interneurons that are responsible for normal itch sensation. Lately, it has been shown that *Bhlhb5* is expressed in around 7% of all the cells in the mouse dorsal horn during the late embryonic to early postnatal days (embryonic day 13.5- post natal day 10) and approximately three quarters of these cells are inhibitory in nature (Kardon *et al.*, 2014). This population of interneurons corresponds to specific neurochemically defined sub-group of nNOS-and galanin–containing inhibitory cells, because many nNOS and almost all galanin-containing cells express *Bhlhb5*. Both these subsets of inhibitory cells are found to be substantially depleted in the *Bhlhb5* mutant mouse, while the NPY and PV populations appeared to be normal. Loss of nNOS- and or galanin- containing interneurons is therefore likely to underlie the abnormal itching behaviour demonstrated by *Bhlhb5*$^{-/-}$ mouse.

In the rat, nNOS containing inhibitory cells provide selective synaptic input to lamina I giant cells (Puskár *et al.*, 2001). However, whether the equivalent population of cells in the PrP-GFP mouse also receive selective innervation from nNOS-containing inhibitory axons is yet to be determined and if so, whether this input is derived from PrP-GFP cell is not known. In the course of this study, it was observed that lamina I giant cells did receive this input, therefore, in order to test whether the PrP-GFP input to giant cells represented a selective innervation, the distribution of PrP-GFP expressing axons among the inhibitory

boutons in superficial dorsal horn was also determined. In addition to that, GFP input to other potential targets such as NK1r-expressing cells in lamina I of the spinal dorsal horn was also assessed. Since nNOS containing inhibitory interneurons are depleted in the *Bhlhb5*$^{-/-}$ mouse, another important aim of this study was to test whether the synapses formed by nNOS-containing inhibitory boutons on lamina I giant cells are also lost in the *Bhlhb5*$^{-/-}$ mouse.

5.2 Materials and Methods

5.2.1 Animals and tissue processing

Spinal cord sections from 10 PrP-GFP mice of either sex (20-26gms) (Hantman *et al.*, 2004, Van Den Pol *et al.*, 2002) and from 6 *Bhlhb5*$^{-/-}$ mice and 6 wild-type littermates (Ross *et al.*, 2010) were deeply anaesthetized with pentobarbitone (30 mg i.p.) and perfused with the fixative (4% formaldehyde). Following perfusion fixation, mid-lumbar (L3-5) segments were removed from all animals and cut into 60 µm thick sections with a vibratome. Horizontal sections were used for all parts of the study, except for the quantitative analyses of inhibitory axons in superficial dorsal horn that was carried out on transverse sections. Sections were processed for multiple labelling immunofluorescence, as described below. Details of the incubation periods, sources and concentrations of antibodies are mentioned in Table 2-1.

5.2.2 Confocal microscopy

For the quantitative analysis of inhibitory nNOS input to lamina I cells, horizontal sections were scanned at 63 × at 0.3 µm z-separation. Several fields were scanned to include the maximum dendritic tree of lamina I cells visible in the section. To assess the frequency of nNOS- and galanin- immunoreactive boutons among over all inhibitory and PrP-GFP boutons in superficial dorsal horn, overlapping fields of transverse sections were scanned at 40 × to include the whole of laminae I-II at 0.3 µm z-separation through the full thickness of the section. The position of the lamina I/II border was determined from dark field scans through 10 × lens and the outline of grey matter and the border between laminae I and II were drawn, and the locations of immunoreactive boutons were plotted onto these outlines using Neurolucida software.

5.2.3 Inputs to giant lamina I cells in the PrP-GFP mouse

It has been shown that many of the GFP-immunoreactive cells in superficial dorsal horn also express nNOS (Iwagaki *et al.*, 2013). In order to determine whether the $GFP^+/nNOS^+$ cells provide a synaptic input to giant cells in the mouse, horizontal sections from L3-5 segments of 3 PrP-GFP mice that had been reacted with a cocktail of 5 primary antibodies: chicken anti-GFP to label PrP-GFP boutons, guinea pig anti-VGLUT2 and rabbit anti-VGAT to mark the spinal excitatory and inhibitory axons that outline lamina I giant cells, sheep anti-nNOS and mouse antibody against the glycine receptor-associated protein gephyrin to indicate the sites of inhibitory synapses, were examined. These primary antibodies were revealed with Alexa 488, Rhodamine Red, Alexa 568, DyLight 649 and Pacific Blue, respectively. The spectral separation of these dyes was achieved with online fingerprinting method for the LSM confocal microscope. In the preliminary observation, it was observed that Alexa 568 affected the staining quality of Rhodamine Red due to their overlapping emission spectrum. Therefore VGLUT2 antibody, which was used only to identify lamina I giant cells, was revealed with Rhodamine Red while VGAT antibody was detected with Alexa 568 secondary antibody. The VGAT boutons were located adjacent to gephyrin puncta and confocal image stacks through the cells were scanned as described before. Although, retrograde tracers were not used to label lamina I giant cells, these cells were identified based on their location and neurochemical characteristics as described previously in the studies performed in the rat (Puskár *et al*., 2001, Polgár *et al*., 2008b).Confocal images were initially viewed for VGLUT2 and VGAT and the locations of VGLUT2 and VGAT boutons on these cells were plotted onto a Neurolucida drawing. The presence or absence of nNOS and GFP in each of these VGAT boutons was then noted.

5.2.4 Neurochemistry and quantitative analysis of VGAT labelled inhibitory axons in SDH of PrP-GFP mouse

To determine the extent to which PrP-GFP, nNOS and galanin were expressed in the general population of inhibitory boutons in superficial dorsal horn, transverse sections from each of 4 PrP-GFP mice were analysed. Antibody against VGAT was used to identify GABAergic axons (Polgár *et al.*, 2011). These sections were incubated in a cocktail of primary antibodies that included chicken anti-GFP, mouse anti-VGAT, sheep anti-nNOS and rabbit anti-galanin. These were revealed with Alexa 488, Rhodamine Red, DyLight 649 and Pacific Blue, respectively.

One section from each animal was selected before viewing immunostaining for PrP-GFP, nNOS or galanin boutons. The superficial dorsal horn on one side of the section was scanned as described previously. The quantification of VGAT boutons was carried out separately for both laminae I and II. For each region a single optical section in the z-series was viewed as a reference point and the bouton located nearest to the bottom right corner of the grid was chosen (Polgár *et al.*, 2011). The first bouton was selected from the most dorsal square and then this process was followed from dorsal to ventral and left to right direction until 100 boutons in lamina I and 150 boutons in lamina II had been selected. These boutons were selected by placing a square grid (10×10µm in lamina I and 20×20µm in lamina II) on the images in Neurolucida. Different grid sizes where used to include the maximum region from both laminae. The channels for PrP-GFP, nNOS and galanin were then viewed sequentially to detect the immunostaining pattern for each of the selected VGAT boutons. Since this method will inevitably be biased towards those boutons which were more extensive in the z-axis (Guillery, 2002, Polgár *et al.*, 2011), z-axis lengths of VGAT boutons that contained GFP, nNOS and galanin were compared with those that did not, by determining the number of optical sections for which each bouton was visible.

The analysis of VGAT boutons in superficial dorsal horn resulted in a sample of considerably small number of GFP boutons (see results). Therefore, a second stage of analysis was carried out in the similar manner on 50 VGAT labelled PrP-GFP boutons in superficial dorsal horn. This was done to yield a relatively large sample size and to estimate the proportion of GFP boutons that expressed nNOS and/or galanin.

5.2.5 PrP-GFP in VGAT axons that were pre-synaptic to lamina I NK1r-expressing neurons

The frequency with which VGAT labelled PrP-GFP axons contacted large NK1r-expressing lamina I cells was also determined. This was done to allow the comparison with lamina I giant cells and to identify other potential targets of PrP-GFP cells. Horizontal sections from three PrP-GFP mice were incubated in the following cocktail of primary antibodies: chicken anti-GFP, rabbit anti-NK1r, goat anti-VGAT and mouse anti-gephyrin. These were revealed with Alexa 488, Rhodamine Red, DyLight 649 and Pacific Blue, respectively.

In the rat, cells with strong NK1r- expression and soma size of more than 200 μm^2 are almost always projection neurons (Al Ghamdi *et al.*, 2009). Based on recent observations

in the mouse (see discussion), only those cells with strong NK1r-immunoreactivity were chosen for this part of the study. In order to avoid bias, cells were selected from various regions of the horizontal sections and before viewing GFP-immunoreactivity. Several scans were obtained to include as much of the dendritic tree as was present in the section and the cell bodies and dendrites of these cells were drawn and analyzed as mentioned before. Synapses involving VGAT-labelled PrP-GFP boutons were identified by the presence of gephyrin puncta in the membrane of NK1r cells at the point of contacts (Polgár *et al*., 2008b). Initially the overall inhibitory input to these cells was determined by visualizing the channels for VGAT and gephyrin. Later, the proportion of VGAT boutons expressing PrP-GFP was analyzed.

5.2.6 PrP-GFP and nNOS axons synaptic input to lamina I NK1r-expressing neurons

The next step was to determine whether the GFP boutons that innervate lamina I NK1r cells also express nNOS. This analysis could have been done with the previous experiment where PrP-GFP input to lamina I NK1r cells was determined. However, preliminary observation suggested that using 5 antibodies combination (by adding anti-nNOS antibody to the protocol described in the previous section) affected the quality of staining considerably. Therefore, this part of the analysis was performed separately. Horizontal sections from three PrP-GFP mice were incubated in the following cocktail of primary antibodies: chicken anti-GFP, guinea pig anti-NK1r, rabbit anti-nNOS and mouse anti-gephyrin. These were revealed with Alexa 488, Rhodamine Red, DyLight 649 and Pacific Blue, respectively.

Eleven large lamina I NK1r-expressing cells that received relatively dense innervation from PrP-GFP axons were identified from horizontal sections of three PrP-GFP mice (3-5 cells from each animal). These cells were scanned and the analysis was carried out in the same way as described before. It was possible to identify the sites of synapses due to the presence of gephyrin puncta adjacent to the PrP-GFP bouton. Later nNOS-immunoreactivity was revealed and the proportion of nNOS- expressing PrP-GFP boutons was assessed.

5.2.7 Synaptic input from nNOS-containing axons to lamina I giant cells in the *Bhlhb5*$^{-/-}$ and their wild type littermates

Recently, it has been shown that selective loss of a subset of inhibitory interneurons in the *Bhlhb5*$^{-/-}$ mouse results in exaggerated itch (Ross *et al.*, 2010). Cells that are lost include many that would normally express nNOS (Kardon *et al.*, 2014). Previous studies done in the rat (Puskár *et al.*, 2001) together with the results of the present study indicate that lamina I giant cells are selectively innervated by nNOS-expressing inhibitory axons. We therefore wanted to test whether there is a reduction of inhibitory synaptic input to the giant cells in the *Bhlhb5*$^{-/-}$ mouse.

This set of experiments was carried out in two steps due to the following reasons. Firstly, two different mouse primary antibodies were used in the same immunocytochemical protocol so in order to avoid any cross-reaction; the incubation with 2nd mouse antibody was done sequentially. Secondly, it has been shown that lamina I giant cell contain neurofilament 200 (NF200), which is a major component of neuronal cytoskeleton (Polgár *et al*., 2007b, Polgár *et al*., 2008b) and preliminary study suggested that NF200 was helpful in identifying distal dendrites of the giant cells. However, the bright NF200 staining obscured VGLUT2 boutons, thereby making it considerably difficult to identify lamina I giant cells. Therefore, lumbar cords of 6 *Bhlhb5*$^{-/-}$ mice and 6 wild type littermates were initially processed with rabbit anti-VGAT, guinea pig anti-VGLUT2, sheep anti-NOS and mouse anti-gephyrin that were then revealed with Alexa 488, Rhodamine Red, DyLight 649 and Pacific Blue, respectively. Lamina I giant cells were identified by the presence of VGLUT2 and VGAT boutons and before the nNOS channel was viewed. Low power scans (at 10×) were taken to locate the cells after the subsequent reaction with mouse anti-NF200 that was later revealed with corresponding specie specific secondary antibody conjugated to Alexa 555. In this way the input from nNOS containing axons to all parts of the dendritic tree was obtained (Polgár *et al*., 2008b). This analysis was performed without knowing the genotype of the mouse tissue to avoid bias towards the variations in synaptic inputs to the cells belonging to either population.

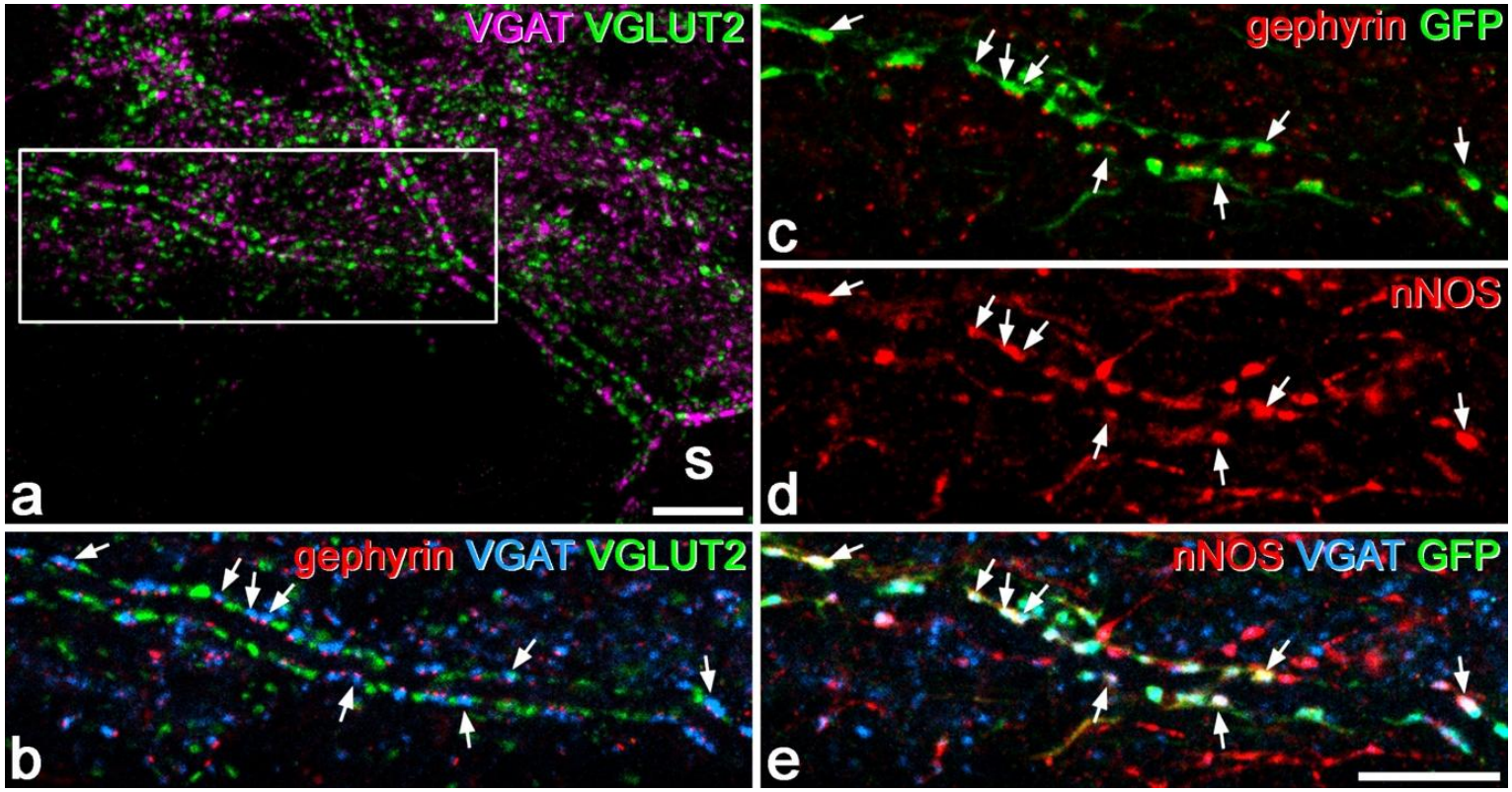
5.3 Results

5.3.1 Contacts on giant lamina I neurons in the PrP-GFP mouse

Examination of horizontal sections through lamina I revealed that, as in the rat, there was a distinct population of very large neurons with cell bodies and dendrites that were outlined by VGLUT2 and VGAT-immunoreactive boutons (Figure 5-1a) (Puskár *et al.*, 2001). The VGAT boutons were associated with gephyrin puncta, which indicated the sites of inhibitory synapses (Figure 5-1b, c). Twenty-five of these cells were identified in sections from 3 PrP-GFP mice (7-9 cells from each mouse), and between 104-509 (mean 234) VGAT boutons that were associated with gephyrin puncta were analyzed for each cell. The majority of these cells (21/25) were associated with large numbers of VGAT boutons that contained GFP and/or nNOS (Figure 5-1b-e). For these 21 cells, a high proportion (62-82%) of the VGAT boutons were labelled with both GFP and nNOS antibodies, while a few were labelled only for GFP (1-5%) or for nNOS (2-11%) (Figure5-2). Although the remaining four cells also received contacts from VGAT boutons with GFP and/or nNOS, these made up a much smaller proportion of the total VGAT population (27-44%). However, this proportion was quite similar to that observed in the rat (~30%) (Puskár *et al.*, 2001).

Figure 5-1 The association between GFP and nNOS-containing inhibitory boutons and lamina I giant cell in the PrP-GFP mouse.

a Soma of lamina I giant cell (s) and its dendritic tree is outlined by numerous VGAT and VGLUT2 boutons. Boxed area in **a** is shown at higher magnification in **b-e. b** Dendrite belonging to the cell is surrounded by VGLUT2 and VGAT boutons, associated with gephyrin puncta, indicating the position of inhibitory synapses. **c-e** The same field scanned to reveal gephyrin, GFP and nNOS. Many of the VGAT boutons that are adjacent to gephyrin puncta in **b** are immunoreactive for GFP and nNOS as indicated with arrows. Confocal images at 0.3 μm z-separation. Scale bar: 10 μm.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s
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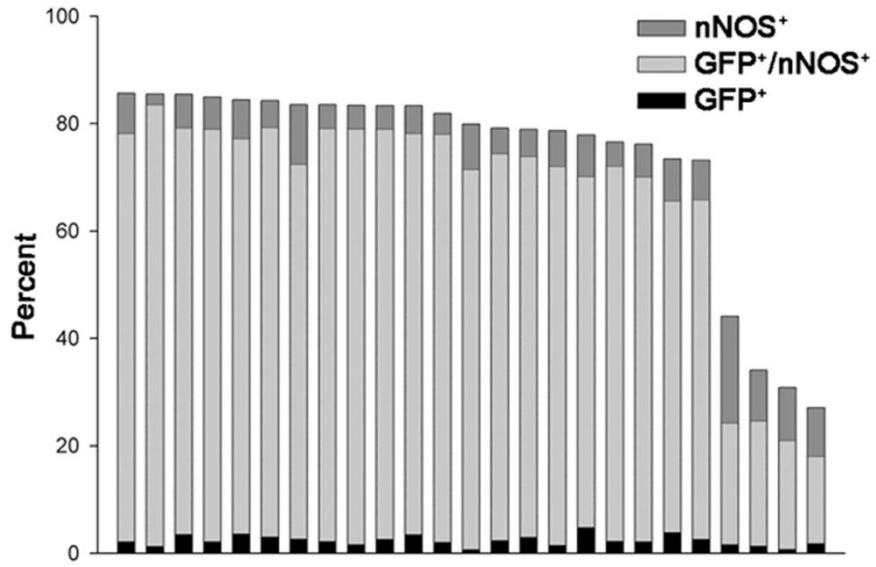


Figure 5-2 Percentages of VGAT boutons that were immunoreactive for GFP and/or nNOS and made synapses with lamina I giant cells

A total of 25 cells were analyzed and each vertical bar represents single cell. In the majority of cells (21/25), most VGAT boutons (~70%) that made synapses with lamina I giant cells in PrP-GFP mouse were immunoreactive for both GFP and nNOS (indicated by the light grey region in each vertical bar). A minority of VGAT boutons were immunoreactive for only nNOS or GFP (indicated by dark grey and black regions in each vertical bar, respectively).

5.3.2 Quantification of inhibitory axons in the superficial dorsal horn of PrP-GFP mouse

The laminar distribution for PrP-GFP, nNOS and galanin immunoreactivity seen in these sections were similar to that observed previously (Hantman *et al.*, 2004, Zheng *et al.*, 2010, Sardella *et al.*, 2011b, Tiong *et al.*, 2011). Many GFP cells were present in lamina II, with occasional cells in lamina I and III. nNOS-immunoreactive dendrites and axons formed a dense plexus in superficial dorsal horn, especially in lamina II with numerous immunoreactive cells in this region (Sardella *et al.*, 2011b). Galanin immunoreactive profiles where present in lamina I-IIo (Tiong *et al.*, 2011) (Figure 5-4).

The analysis of VGAT boutons in lamina I-II showed that only 9% of inhibitory boutons in lamina I and 7% of those in lamina II expressed GFP immunoreactivity (Table 5-1). For both nNOS and galanin this proportion was 16.3% and 6.5%, respectively, in lamina I while 19% contained nNOS and 6.3% expressed galanin in lamina II (Figure 5-3, Table 5-1). The mean z-axis lengths of GFP^+ and GFP^-/ VGAT-immunoreactive boutons did not differ significantly in both the laminae. For lamina I the mean z-axis lengths of GFP^+/VGAT and GFP^-/ VGAT-immunoreactive boutons were 0.99 µm and 0.95 µm, respectively ($p=0.2$; Mann-Whitney U test). For lamina II these values were 0.90 µm and 0.91 µm, respectively ($p=0.2$; Mann-Whitney U test). This indicates that selection process based on the size of VGAT boutons was not biased in superficial dorsal horn.

In the second stage, the quantitative analysis of 50 GFP boutons in lamina I and II from each animal was carried out to determine the expression of nNOS and/or galanin among these boutons (Figure 5-4). Quantitative results of this part of the study are shown in Table 5-2. For lamina I, 58% of GFP boutons contained only nNOS while 8% expressed only galanin, and 15% of the boutons were immunoreactive for both. However, 19.5% did not express either nNOS or galanin (Figure 5-3 and 5-4). For lamina II, 62% were $nNOS^+$ only, 14.5% were immunoreactive for $galanin^+$ only, 15.5% expressed both and 23.4 % were negative for nNOS and galanin. The mean z-axis lengths for these different neurochemical types of GFP boutons did not differ significantly in both lamina I and II ($p=0.4$ for lamina I and $p=0.6$ for lamina II; Kruskal-Wallis test). These results suggest that once again the selection process was not biased in terms of size of the boutons.

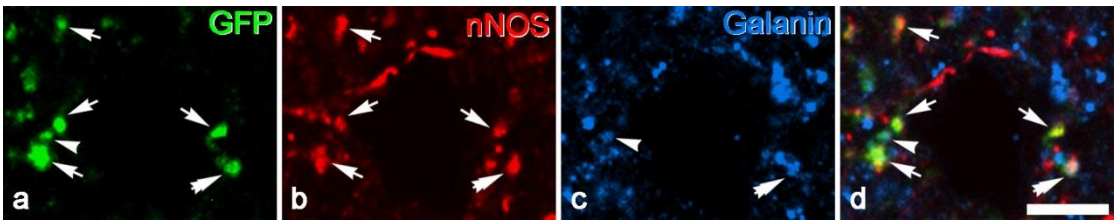


Figure 5-3 Distribution of VGAT labelled GFP boutons in superficial dorsal horn. Confocal images from lamina I in a transverse section to reveal **(a)** GFP (green**) (b)** nNOS (red) and **(c)** galanin (blue) together with a merged image **(d).** Four boutons that are immunoreactive with both GFP and nNOS antibodies are indicated with arrows, while a single bouton containing both GFP and galanin is shown with an arrow head. Double arrow heads indicate a single GFP bouton which is positive for both nNOS and galanin. Single confocal optical section scanned at 40×. Scale bar: 5µm.

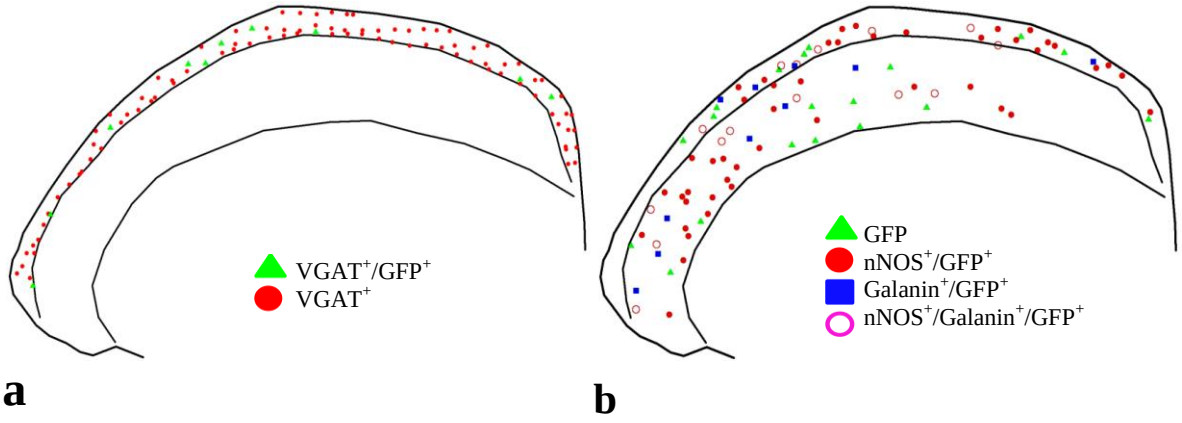


Figure 5-4 Distribution and neurochemistry of VGAT labelled GFP boutons in superficial dorsal horn of PrP GFP mouse.

a Illustrates the proportion of PrP-GFP boutons among 100 VGAT boutons in lamina I of the spinal dorsal horn. **b** Boutons that are immunoreactive for GFP, nNOS and galanin various combinations are plotted in the superficial dorsal horn of the PrP-GFP mouse. In these plots, the upper line represents the edge of dorsal horn; the middle and lower line represent laminae I-II and II-III borders, respectively.

Table 5-1 Percentages of VGAT boutons in laminae I-II that were GFP, nNOS-and galanin–immunoreactive

Lamina	Number of VGAT boutons	% of GFP^+ boutons	% of $nNOS^+$ boutons	% of $Galanin^+$ boutons	% of $nNOS^+/Galanin^+$ boutons	$nNOS^+$ only	$Galanin^+$ only
I	100	9 ± 2.2	16 ± 3.6	6.5 ± 0.5	2 ± 0.7	8 ± 4.4	3 ± 1.5
II	150	7.5 ± 2.1	19 ± 2.7	6 ± 3.4	2.3 ± 1.3	12 ± 2.7	4 ± 1.9

In each case the mean ± SD values of the VGAT boutons that were either GFP, nNOS and/or galanin-immunoreactive in laminae I and II, from 4 PrP-GFP mice are shown.

Table 5-2 Percentages of GFP boutons in laminae I-II that were nNOS-and/or galanin–immunoreactive

Lamina	Number of GFP boutons	% of $nNOS^+$ boutons	% $Galanin^+$ boutons	% $nNOS^-/Gal^-$ boutons	% $nNOS^+/Gal^+$ boutons	% $nNOS^+/Gal^-$ boutons	% $Gal^+/nNOS^-$ boutons
I	50	72.5 ± 3.6	22 ± 5	19.5 ± 4.3	15 ± 4.6	57.5 ± 5	8 ± 2
II	50	62 ± 4.2	30 ± 9	23.5 ± 2.3	15.5 ± 8	46.5 ± 9.9	14.5 ± 1.7

Percentages ± SD values of 50 GFP boutons from lamina I and II, that were nNOS-and/or galanin–immunoreactive. Data is obtained from spinal cord sections from 4 PrP-GFP mice. Column 3-8 represent average percentages of GFP boutons belonging to various neurochemical subgroups.

5.3.3 PrP-GFP in VGAT axons target a subset of lamina I NK1r-expressing cells

In this part of the study, the density of PrP-GFP synaptic input to 60 large lamina I NK1r-expressing cells (20 from each PrP-GFP mouse) was analyzed. As NK1 receptor completely outlines the soma and dendrites of these cells, it was possible to determine the density of synaptic input that they received from PrP-GFP axons. In the rat, lamina I giant cells either lack NK1 receptor or stain weakly with NK1r antibody (Puskár *et al.*, 2001). The great majority of lamina I giant cell received substantial input from GFP boutons, therefore, only cells with strong NK1r-immunoreactivity were selected in order to avoid lamina I giant cells (Figure 5-5). In the rat, NK1r-expressing lamina I projection cells can be differentiated from NK1r interneurons on the basis of their soma sizes and the intensity of NK1r (Al Ghamdi *et al.*, 2009). In the preliminary study in the mouse, similar relationship was observed between lamina I NK1r-expressing projection cells and interneurons (Baseer, Mcintyre and Todd unpublished observations). Cells with strong NK1r immunoreactivity and large soma sizes (>200 μm^2) were almost always retrogradely labelled while those with relatively weak NK1r-staining and small soma sizes (<200 μm^2) were not. Although, the soma sizes of the selected lamina I NK1r expressing cells were not measured, the GFP boutons appeared to target NK1r cells that expressed strong NK1r-immunoreactivity and had either large or medium soma sizes (Figure 5-6).

All NK1r-expressing neurons possessed gephyrin puncta, but their density varied considerably (Puskár *et al.*, 2001). These gephyrin puncta were almost always associated with VGAT boutons. The lengths of dendrites analyzed for each cell ranged from 111.5-2803.1µm (mean 615µm). These cells varied in terms of density of VGAT input (mean ± SD; 15.8 ± 7 boutons/100µm of the dendrite). The quantitative analysis of PrP-GFP-labelled VGAT boutons on these cells demonstrated that majority (42/60) of NK1r cells had few synapses from GFP boutons (Figure 5-5). They constituted only 6.3% ± 3.5 (mean ± SD) of their overall inhibitory input with a contact density of 0.7 ± 0.5/100 µm (mean ± SD) of the dendrite. However, there was a subset of large lamina I NK1r cells (18/60) that received substantial input from GFP neurons (Figure 5-6). For these 18 cells, the contacts were so numerous that GFP boutons outlined the soma and dendrites (Figure 5-6) and constituted 45.4% ± 12.5 of all the VGAT boutons synapsing with these cells (range: 25.2-59.6%), averaging a contact density of 11.5 ± 4.2/100 µm (mean ± SD). The frequency histogram of VGAT-labelled GFP synapses for 60 lamina I NK1r cells indicating a bimodal distribution is shown in Figure 5-7.

Figure 5-5 GFP input onto lamina I NK1r-expressing neuron and a nearby cell with weak NK1r-immunoreactivity.

a Soma and proximal dendrite of a large lamina I NK1r-expressing neuron (n), with strong NK1r immunoreactivity (red) and virtually no GFP input suggested by the absence of PrP-GFP boutons adjacent to gephyrin puncta on large lamina I NK1r cells (arrow heads). **b** Also present is a soma of another lamina I cell (g) (probably a giant lamina I cell), expressing very weak NK1r immunoreactivity (red) that receives substantial GFP input (green) (arrows). **c** GFP axons are lying adjacent to gephyrin puncta (blue). Images are from projection of 2 optical sections at 0.3 µm z-spacing and scale bar: 10µm.

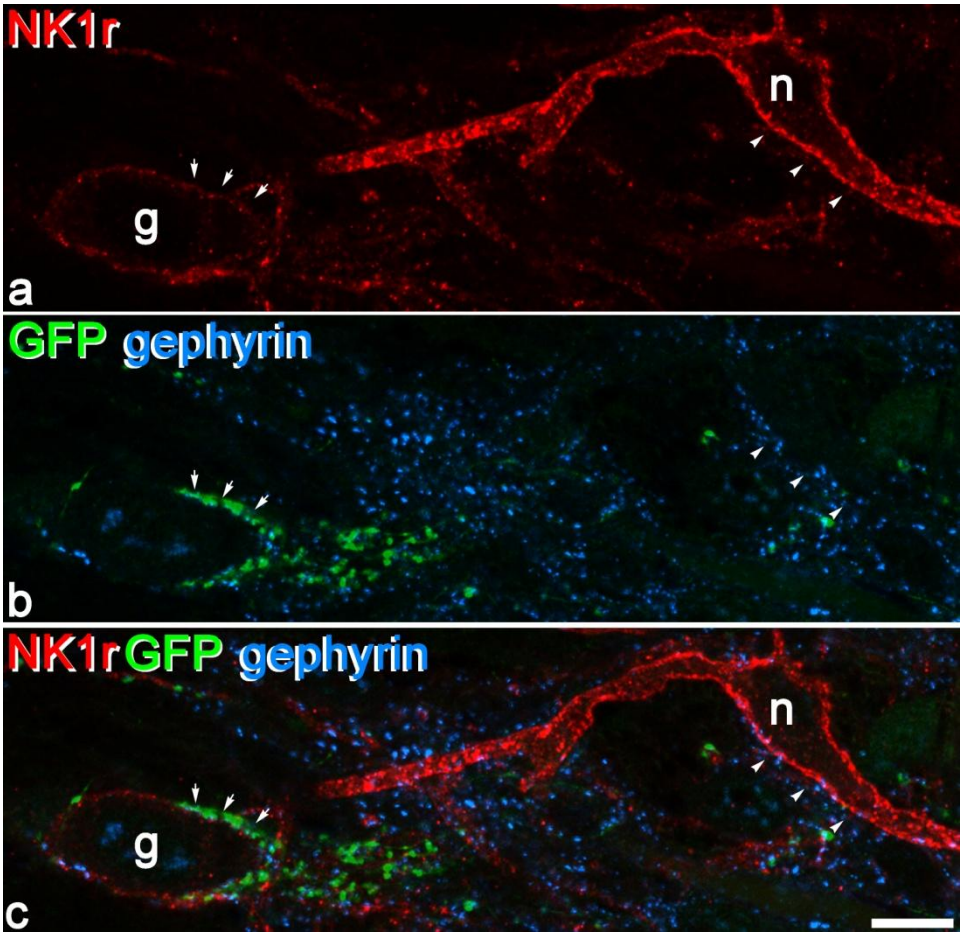
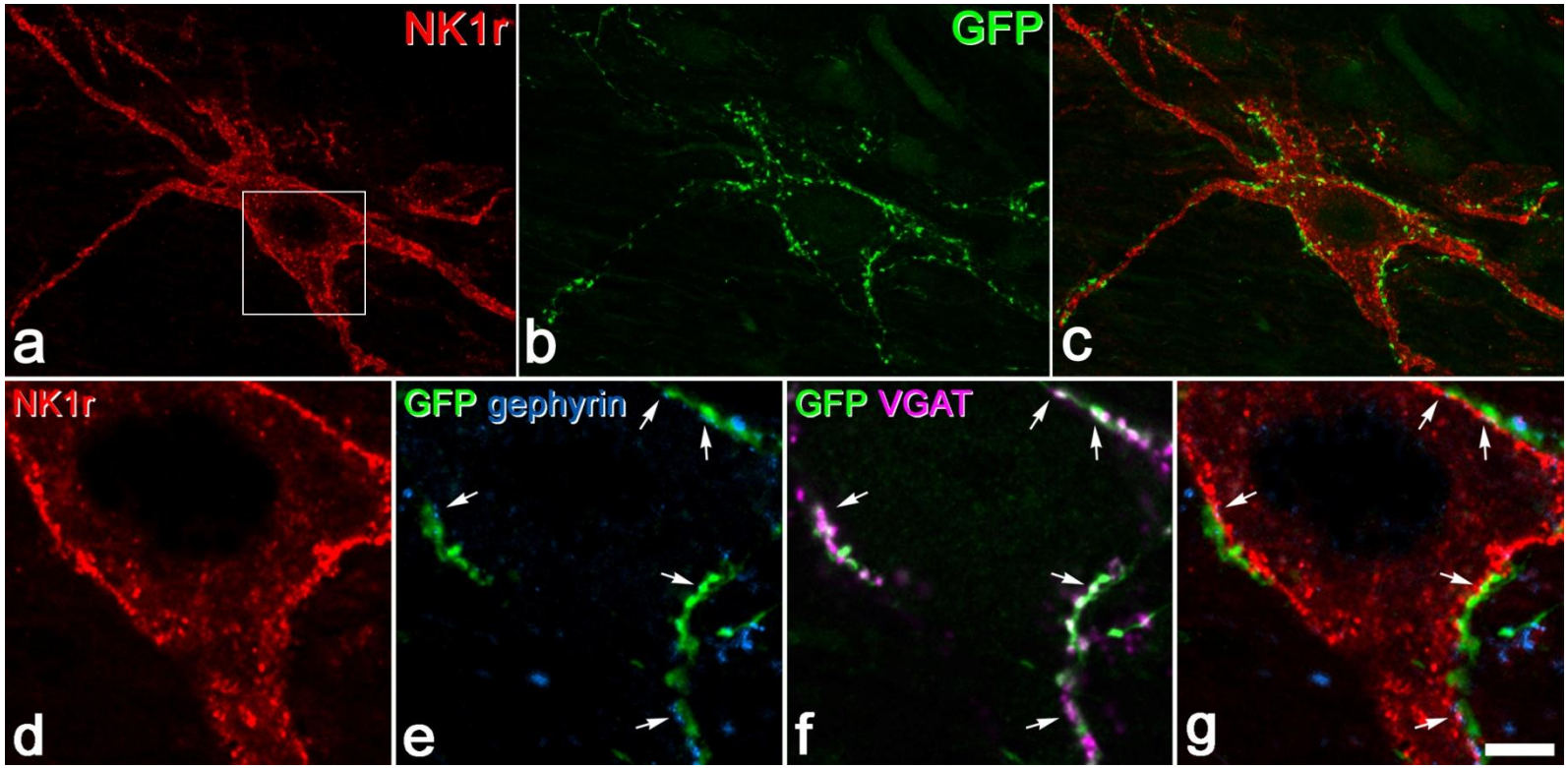

NK1r
n
g
a
GFP gephyrin
b
NK1r GFP gephyrin
n
g
c

Figure 5-6 Synapses between VGAT labelled PrP-GFP axons and a large lamina I NK1r-expressing neuron.

a-c, low magnification image through the soma and proximal dendrites of the lamina I NK1r cell, showing association with GFP axons that almost completely outline it. This cell illustrates an example of large lamina I NK1r-expressing cell that receives dense innervation from PrP-GFP labelled inhibitory boutons. The area in the box is shown under higher magnification in **d-g**. This part of the cell receives several synapses from axons that are labelled with GFP (green) and lie adjacent to inhibitory synaptic marker gephyrin (blue) (arrows).**f** GFP boutons are also immunoreactive for VGAT (magenta) and therefore appear white (some marked with arrows). Images are from projection of 8 **(a-c)** and 2 **(d-g)** optical sections at 0.3 µm z-spacing. Scale bar: 10 µm.


NK1r
GFP
a
b
c
NK1r
GFP gephyrin
GFP VGAT
d
e
f
g

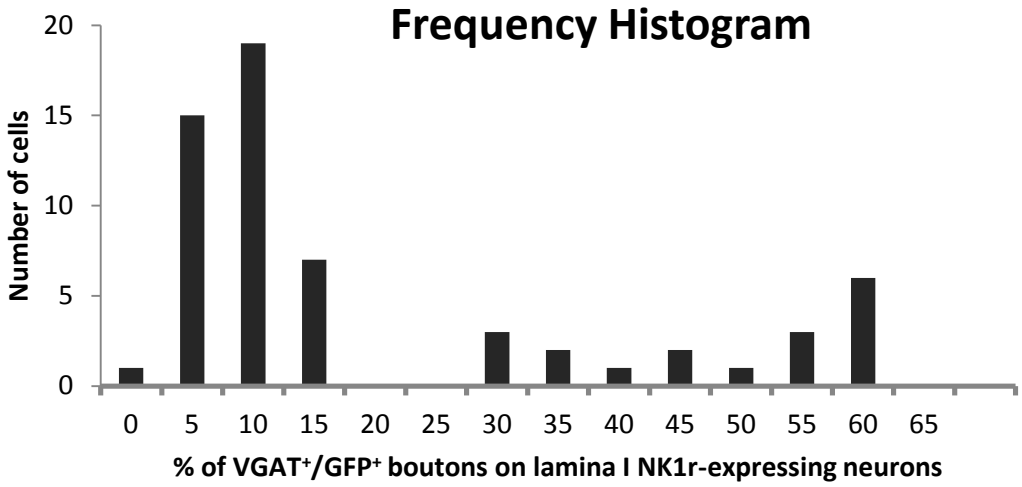


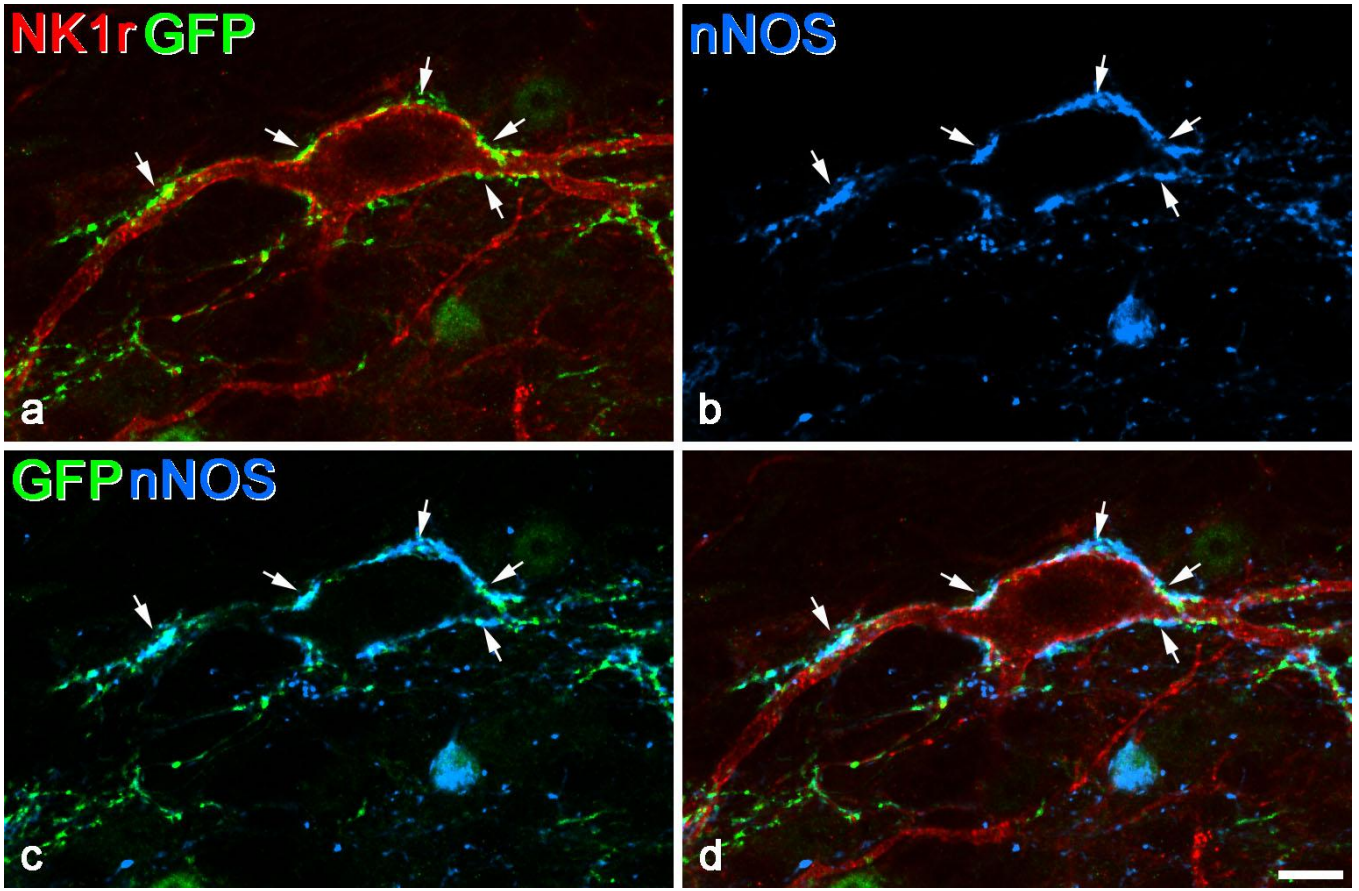
Figure 5-7 Frequency histogram showing PrP-GFP cells input to lamina I NK1r cells
A subset of lamina I NK1r projection cells (18/60) receives substantial inhibitory input (45.4%) from GFP labelled axons in lamina I of PrP-GFP mice. The cells receiving sparse GFP input correspond to the values between 5% and 15% and a second broader peak extending between 30% and 60% constitutes the large NK1r-expressing cells with substantial GFP synaptic input.

5.3.4 Selective innervation of nNOS-containing PrP-GFP axons on lamina I NK1r cells

This part of the analysis was carried out on 11 large lamina I NK1r-expressing cells that received considerable number of contacts from PrP-GFP boutons. The total dendritic lengths of these cells ranged from 222.8-2242.8 µm (mean 502.8 µm). Here again, it was possible to determine the number of PrP-GFP boutons synapsing on these cells due to the presence of gephyrin puncta adjacent to GFP boutons. The number of PrP-GFP boutons that contacted these cells varied from 36-220 boutons (mean 71.2) and the great majority (~92%) of them also expressed nNOS-immunoreactivity (Figure 5-8). The overall contact density of GFP boutons on these cells was consistent with what was observed in 18/60 highly innervated large lamina I NK1r-expressing cells (16.8 ± 5.2/100 µm).

Figure 5-8 nNOS labelled PrP-GFP input to lamina I NK1r cells

a-d Confocal scan through soma and proximal dendrite of large lamina I NK1r cell showing contacts from nNOS-containing GFP axons. **a** NK1r cell (red) receives dense innervation from GFP boutons (green). **b** nNOS boutons (blue) also appear to contact NK1r cell. This innervation is so dense that both GFP and nNOS boutons outline the soma and proximal dendrites of NK1r cell **(c)** and the great majority of GFP boutons contacting this cell are nNOS-immunoreactive **(c and d)**. Images are from projection of 5 optical sections at 0.3 µm z-spacing. Scale bar: 10 µm.


NK1rGFP
nNOS
GFPnNOS
a
b
c
d

5.3.5 Selective loss of nNOS inhibitory synaptic input to lamina I giant cells in the *Bhlhb5*$^{-/-}$ mice

Observations made previously and in this study suggest that lamina I giant cells receive selective innervation from inhibitory nNOS-containing boutons. Recently it is reported that the inhibitory interneurons that normally express nNOS are lost in the *Bhlhb5*$^{-/-}$ mice (Kardon *et al.*, 2014). Therefore, the aim of this part of the study was to determine whether the nNOS boutons synapsing with lamina I giant cells are derived from this selective population and this input is altered in the *Bhlhb5*$^{-/-}$. Giant lamina I cells in *Bhlhb5*$^{-/-}$ mice and wild type littermates were identified and analysis was carried out on a total of 59 cells (29 from *Bhlhb5*$^{-/-}$ and 30 from WT mice; ranging from 2-8 cells from each animal). Unlike the analysis of lamina I giant cells in PrP-GFP mice, here in this part of the study, NF200-immunoreactivity was used to reveal the dendritic morphology of giant cells and it was therefore possible to determine the density of contacts that these cells received from nNOS-immunoreactive boutons (Polgár *et al*., 2008b). Although, NF200 immunoreactivity was found to be faint in the soma, it was readily visible in the dendrites and the dendritic tree of these giant cells could be followed to a considerable distance in WT and *Bhlhb5*$^{-/-}$ mice (range: 138-1394.5µm) (Figure 5-9). These lamina I giant cells received numerous contacts from VGLUT2 and VGAT boutons that almost completely outlined the soma and dendritic tree of these cells (Figure 5-10 a, d).The contact densities of excitatory boutons on the giant cells did not vary much between wild type and their mutant littermates (mean: 81.3 and 86.8/100 µm in WT and *Bhlhb5*$^{-/-}$, respectively) ($p = 0.2$). However, the contact density of VGAT boutons differed significantly between the two groups (67.3 and 44.6/100 µm in WT and *Bhlhb5*$^{-/-}$, respectively) ($p < 0.05$; t-test). These results indicate that in the *Bhlhb5*$^{-/-}$, the overall density of inhibitory synapses on these cells was reduced to ~65% of that seen in the wild type (Figure 5-11).

Consistent with results of this study and Puskar *et al.* (2001), the quantitative analysis in the wild type mice showed that ~73% of the VGAT boutons expressed nNOS. The contact density of these nNOS-labelled VGAT boutons was 49.2 ± 9/100 µm (Figure 5-10). In most cases, this input was so dense that it almost outlined the soma and dendrites of these cells (Figure 5-10 b,c and g-i). In contrast, the giant cells from the *Bhlhb5*$^{-/-}$ showed a substantial reduction (~80%) in their nNOS input (Figure 5-10 d-f, j-l). Only ~16% of the VGAT boutons expressed nNOS-immunoreactivity and the contact density of nNOS boutons differed significantly from the wild type cells (6.8 ± 3.4/100 µm) ($p < 0.05$).

Figure 5-9 Immunostaining for NF200, VGLUT2 and VGAT to identify lamina I giant cell
a-c Confocal images showing a lamina I giant cell with soma and proximal dendrites, scanned to reveal NF200 (blue), VGLUT2 (green) and VGAT(red) together with a merged image. This cell contains NF200 immunoreactivity, which is relatively weak in the soma and strong in the dendrites. **b** Soma and dendrites of this giant cell are coated with VGLUT2 and VGAT boutons. Due to the presence of NF200, it was possible to identify the contacts from different neurochemical types of axonal boutons on these cells. Scale bar: 10 μm.

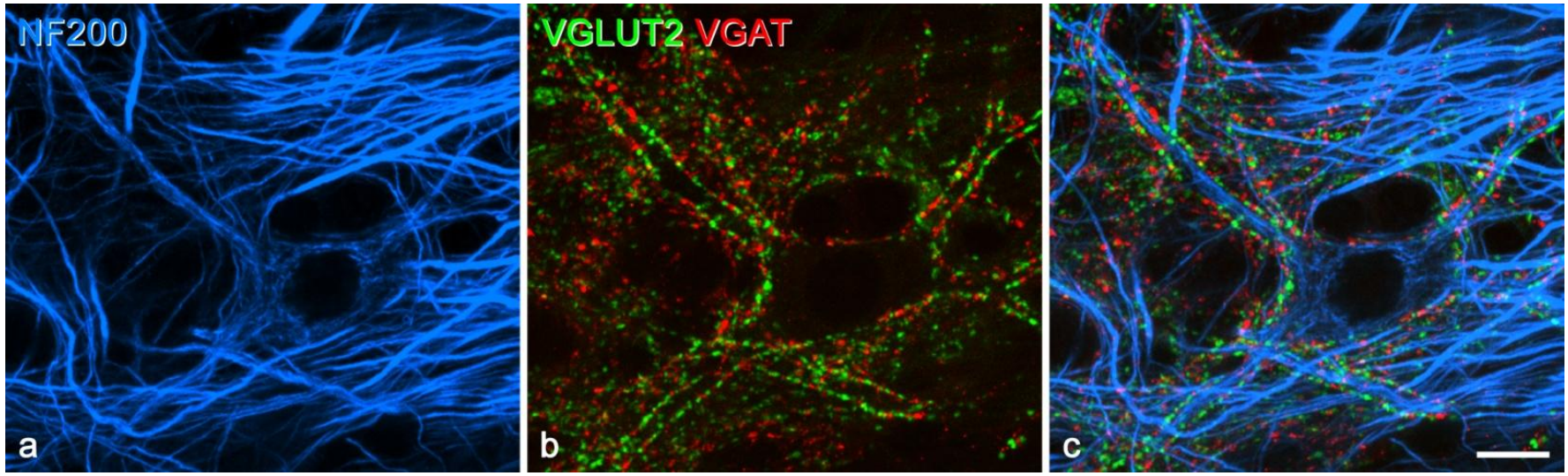
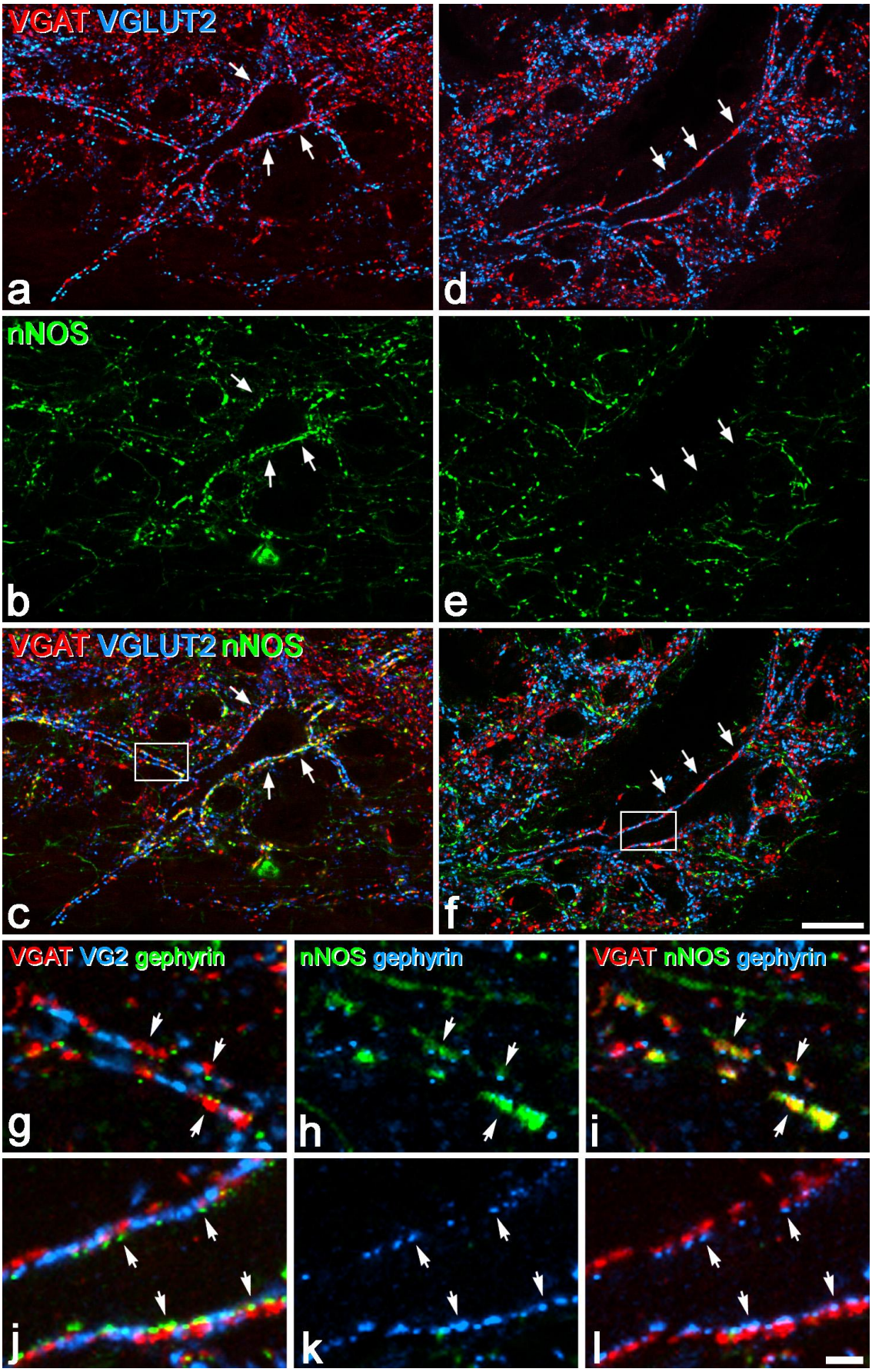

NF200
a
VGLUT2 VGAT
b
c

Figure 5-10 Comparison between giant lamina I cell in a wild type and *Bhlhb5*$^{-/-}$

a, A giant lamina I spinoparabrachial cell from a wild type mouse. The soma and proximal dendrites of the cell are densely innervated by VGLUT2 (blue) and VGAT (red) boutons. **b** The same field showing a very heavy nNOS input (green), almost outlining the respective cell (arrows). **d** Another giant lamina I cell from a *Bhlhb5*$^{-/-}$ mouse. The cell again receives a dense excitatory input and somewhat moderate inhibitory input. **e** The same field scanned for nNOS immunoreactivity, showing a virtual absence of nNOS input to this cell (arrows).**c and f** showing the merged images from WT and KO mouse, respectively. **g-i** The region in the box in **c** from WT type mouse. **g** Giant cell dendrite showing dense innervation from VGLUT2 (blue) and VGAT (red) boutons that lie adjacent to gephyrin puncta (green). **h-i** show heavy nNOS input (green) to this dendrite. **j-l** The region in the box in **f** from KO mouse. Here again giant cell receives substantial excitatory and inhibitory input **j** with virtual absence of input from nNOS axons **k-l**. Images are from projection of 2 optical sections at 0.3 µm z-spacing and scale bar; 20µm **(a-f)** 10 µm **(g-l).**


VGAT VGLUT2
a
d
nNOS
b
e
VGAT VGLUT2 nNOS
c
f
VGAT VG2 gephyrin
nNOS gephyrin
VGAT nNOS gephyrin
g
h
i
j
k
l

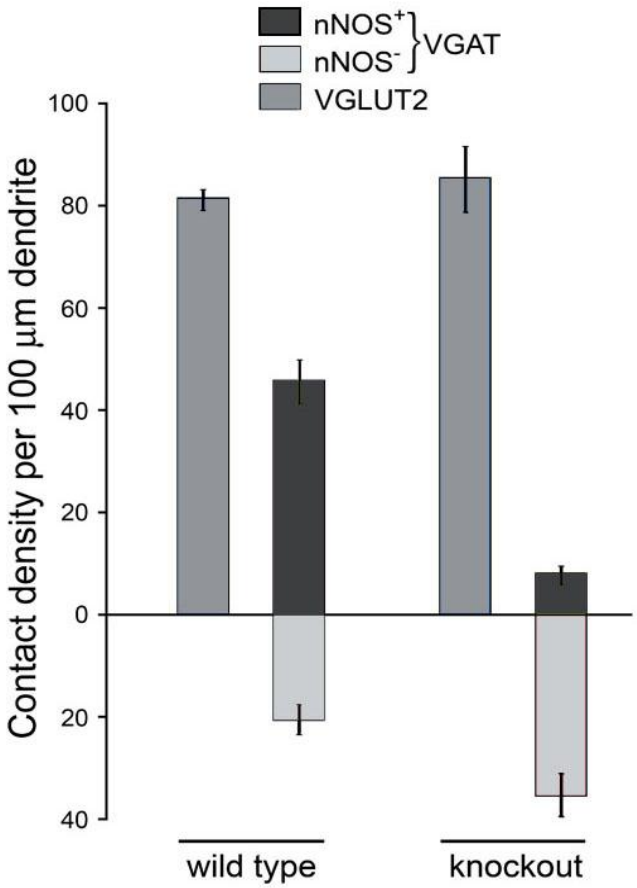


Figure 5-11 Comparison between excitatory and inhibitory input density/100 µm dendrite of lamina I giant cell dendrites in the WT and *Bhlhb5*$^{-/-}$ mice

The graph shows that the difference between the excitatory inputs to lamina I giant cells in WT and *Bhlhb5*$^{-/-}$ mice do not differ significantly. In the WT animals, inhibitory nNOS boutons constitute ~70% of the overall inhibitory input, while the giant cells in *Bhlhb5*$^{-/-}$ mice show moderate reduction in their overall inhibitory input with a significant loss of inhibitory nNOS input. There was a significant increase in the number of nNOS^{-}/VGAT^{+} boutons in the *Bhlhb5*$^{-/-}$ mouse, suggesting some degree of compensation from other inhibitory interneurons populations, the source of which is yet to be determined. This partial compensation was indicated by a significant increase in nNOS^{-}/VGAT^{+} boutons in the mutant mice (18 ± 4.6 and 37.7 ± 8.2/100 µm of the dendrite in WT and KO, respectively) ($p < 0.05$; t-test).

5.4 Discussion

The main findings of this part of the study are; (1) In the PrP-GFP mouse, the majority of the inhibitory synapses on lamina I giant cells are from GFP/nNOS-immunoreactive boutons, even though only a small proportion of all the inhibitory boutons in superficial dorsal horn express GFP. (2) PrP-GFP axons also target a subset of large lamina I NK1r-expressing neurons. Thus, these observations suggest 2 potential targets for GFP^{+}/$nNOS^{+}$-immunoreactive inhibitory interneurons in the superficial dorsal horn of PrP-GFP mouse. (3) Giant cells in the *Bhlhb5*$^{-/-}$ mice show a substantial reduction in their input from nNOS-containing inhibitory boutons and a moderate reduction in their overall inhibitory synapses with some compensation from other inhibitory cells.

5.4.1 PrP-GFP cells and innervation of giant lamina I neurons

The results suggest that while GFP is expressed in only 7-9% of VGAT (inhibitory) boutons in superficial dorsal horn, it is present in ~70% of those innervating lamina I giant cells and in ~45% of those that contacted a subset of NK1r-expressing cells. This indicates a dramatic enrichment of input (~10 and ~4 fold) from GFP^{+}/$nNOS^{+}$-immunoreactive cells on lamina I giant and subset NK1r-expressing cells, respectively. PrP-GFP cells constitute 3.4% of all neurons in laminae I-II (Iwagaki *et al.*, 2013), and GABA-immunoreactive interneurons make up ~26% of the total neuronal population in this region in the mouse (Polgár *et al*., 2013a), which is almost similar to that in the rat (~30%) (Polgár *et al.*, 2013). Based on these observations, PrP-GFP cells would constitute ~13% of inhibitory interneurons in the superficial laminae.

Hantman *et al.* (2004) reported that the axons of all PrP-GFP cells are limited to lamina II. However, the findings of the present study together with the recent data from Iwagaki, Ganley and Todd (unpublished observations) suggest that the axons of approximately 1/3rd of PrP-GFP cells (22 out of 60 cells in their sample) enter lamina I, where they selectively target lamina I giant cells and a subset of NK1r-expressing cells. Nonetheless, the axons of these PrP-GFP cells may also target lamina II cells, presumably interneurons, since lamina II cells do not project supraspinally. However the targets of these cells in lamina II of the spinal dorsal horn are not yet known.

It has previously been reported that nNOS-containing GABAergic axons innervate giant cells in the rat, where they constitute ~30% of the inhibitory synapses. However, their

source could not be determined (Puskár *et al.*, 2001). Most of the nNOS-immunoreactive boutons on giant cells in the PrP-GFP mice were also GFP-immunoreactive, and this strongly suggests that they originated from nNOS-containing inhibitory interneurons in lamina II. Since nNOS was found to be present in 70% of the GFP cells (Iwagaki *et al.*, 2013), neurons containing both GFP and nNOS presumably account for less than 10% of the inhibitory interneurons in the superficial dorsal horn. Their dense inhibitory input to lamina I giant neurons indicates a highly selective innervation. However, it is not yet known whether this innervation is derived from a restricted subset of $GFP^+/nNOS^+$ cells or from many of these cells, each providing a small number of synaptic inputs. The finding that some giant cells (4/25) received a far lower proportion of their input from $GFP^+/nNOS^+$ boutons may indicate that the inhibitory innervation of each giant cell arises from a relatively small number of neurons, and that in those cases with low density of GFP input, several of these neurons lacked GFP and nNOS. This interpretation is also consistent with the observation by Hantman *et al.* (2004) that axons of the GFP^+ neurons did not appear to project outside lamina II, as their sample may have included only those neurons that did not innervate the giant cells.

Functionally, PrP-GFP cells have been shown to express c-*fos* after formalin injection into the hind paw (Hantman *et al.*, 2004). These also receive a monosynaptic input from TRPV1-expressing primary afferents (Zheng *et al.*, 2010). These findings together with the fact that both lamina I giant and NK1r-expressing projection cells are also activated in response to noxious stimulation (Puskár *et al*., 2001, Todd *et al*., 2002, Polgár *et al*., 2008b) and that they receive substantial input from PrP-GFP boutons, suggest that PrP-GFP cells might be involved in attenuating nociceptive signals to lamina I projection cells. According to Sandkuhler (2009), one of the proposed principal functions of inhibitory interneurons includes pre- and post-synaptic inhibition of nociceptive dorsal horn cells. The subsequent failure of this inhibition results in the generation of hyperalgesia. This suggests that under physiological conditions, noxious stimuli would activate both lamina I projection cells and PrP-GFP cells. The latter would in turn inactivate (inhibit) projection cells by a mechanism involving feed forward inhibition and thus limit the intensity of pain felt after a noxious stimulus.

Recent studies have shown that a great majority of $nNOS^+/GABA^+$ neurons in lamina I-II of both rat and mouse are sst_{2A}-immunoreactive and this receptor is not expressed by excitatory neurons in this region. (Polgár *et al*., 2013a, Iwagaki *et al*., 2013). Therefore,

sst_{2A}-receptor expression can be used to distinguish inhibitory cells from the excitatory ones (Polgár *et al*., 2013a, Iwagaki *et al*., 2013). It has been shown that low doses of somatostatin when administered intrathecally, have a pro-nociceptive effect (Seybold *et al.*, 1982) and cause hyperpolarisation of a subset of inhibitory interneurons (disinhibition) (Yasaka *et al.*, 2010, Iwagaki *et al.*, 2013). In the rat, lamina I projection neurons respond to noxious stimuli by up-regulating c-*fos* and phosphorylation of extracellular regulatory kinase (ERK) (Puskár *et al*., 2001, Todd *et al*., 2002, Polgár *et al*., 2008b). It is therefore possible that all PrP-GFP cells in laminae I-II, many of which are activated by noxious stimuli (Hantman *et al.*, 2004, Polgár *et al.*, 2013) are likely to contribute to the pro-nociceptive effect of somatostain, presumably through their input to nociceptive projection neurons in lamina I.

It has been reported that 98% of PrP-GFP cells in lamina II are nNOS-and/or galanin-immunoreactive (Iwagaki *et al.*, 2013). The great majority of GFP boutons that synapse with lamina I giant cells (~70%) and large lamina I NK1r expressing cells (~90%) are nNOS-immunoreactive (as mentioned before). However, in this study ~20% of GFP boutons in lamina I and ~23% of those in lamina II did not express either nNOS and/or galanin. This discrepancy between somatic and axonal staining of nNOS and galanin can be attributed to variable intensity of nNOS and galanin staining among GFP cells (Iwagaki *et al.*, 2013). It is suggested that axonal nNOS is detectable only in those cells that have a strong somatic labelling for nNOS (Sardella *et al.*, 2011b). Furthermore, excitatory nNOS cells in lamina I-III have relatively weak nNOS staining as compared to the inhibitory cells and very few nNOS boutons in this region are VGLUT2-immunoreactive. It is therefore possible that the cells that express weak somatic labelling of nNOS are unable express detectable levels of nNOS in their axons.

5.4.2 Selective loss of nNOS-labelled inhibitory synaptic input to giant lamina I cells in *Bhlhb5*$^{-/-}$ mouse

Different theories on the neural mechanism of itch have already been discussed with the introductory chapter. Several studies have supported the labelled line theory of itch. The role of itch-specific unmyelinated C-afferents in humans (Schmelz *et al.*, 1997) and MrgprA3-expressing sensory neurons in the perception of itch (Han *et al.*, 2013) have been proposed. Some distinct populations of dorsal horn cells such as lamina I spinothalamic cells (Andrew and Craig, 2001) and gastrin-releasing peptide receptor (GRPR)-expressing cells (Sun and Chen, 2007, Sun *et al.*, 2009) have also been considered as itch specific.

Furthermore, the selective ablation of GRPR-expressing cells eradicates itch but not pain (Sun *et al.*, 2009). However, a precise circuitry underlying the perception of itch is not yet known.

Recently, Ross *et al.* (2010) reported that loss of inhibitory interneurons in mice lacking the transcription factor *Bhlhb5* leads to increased itching in these animals. In addition to that, an increased response to second phase of formalin test was also observed while the responses to all other noxious stimuli were found to be normal. Another observation made in the same study suggested that the number of GRPR-expressing cells and the input of GRP-expressing cells remained unchanged in the dorsal horn of *Bhlhb5*$^{-/-}$ mice. Furthermore, the great majority (~90%) of *Bhlhb5*-expressing cells co-expressed sst_{2A} receptor and many showed hyperpolarization in response to somatostatin. *Bhlhb5*$^{-/-}$ mouse show a substantial depletion of both galanin- and nNOS-containing inhibitory interneurons, but not any other type of inhibitory cells (Kardon *et al.*, 2014). Although, the number of sst_{2A}-receptor expressing cells was reduced to 2/3rd in the knockout mice as compared to what was observed in the WT animals, there was no substantial change in the number of nNOS or galanin-negative sst_{2A} receptor cells. As many nNOS- and galanin-immunoreactive cells are activated by noxious stimuli (Polgár *et al.*, 2013), it is possible that one or both of these populations may be responsible for scratch-evoked inhibition of itch (also termed as counter stimulation) (Kardon *et al.*, 2014). Moreover, the loss of this subset of inhibitory cells may underlie the exaggerated itch response seen in the *Bhlhb5*$^{-/-}$ mice. Kardon *et al.* (2014) further showed that *Bhlhb5*-expressing cells were innervated by those primary afferents that respond to counter stimuli such as heat and cool (capsaicin-, mustard oil- and menthol-responsive primary afferents). In addition, they reported the responsiveness of *Bhlhb5*-expressing inhibitory interneurons to menthol in the WT mice while this response was lost in the *Bhlhb5*$^{-/-}$ mice. All these findings suggest the role of *Bhlhb5*-expressing inhibitory cells in the chemical counter stimulation.

It has already been shown that both giant cells and nNOS-containing inhibitory cells also respond to formalin (Puskár *et al.*, 2001, Polgár *et al.*, 2013). A dramatic reduction of this feed forward inhibitory circuit in the *Bhlhb5*$^{-/-}$ mouse may lead to increased activation of the giant cells. This increased central sensitization would have resulted in an exaggerated response in the second phase of formalin test seen in *Bhlhb5*$^{-/-}$ mice (Ross *et al.*, 2010) (Figure 5-11).

The results from this part of the study establish the selective innervation of lamina I giant cells by nNOS-containing inhibitory interneurons (a subset of *Bhlhb5*-expressing inhibitory interneurons), and that this input is lost in *Bhlhb5*$^{-/-}$ mice. This raises the possibility that the giant cells are involved in the perception of itch, which can be further evaluated by determining the expression of c-*fos* by lamina I giant cells in response to various pruritogens such as histamine and chloroquine. Apart from the loss of nNOS input, there was a significant increase in the number of nNOS^{-}/VGAT^{+} boutons in the *Bhlhb5*$^{-/-}$ mouse, suggesting some degree of compensation from other inhibitory interneurons populations, the source of which is yet to be determined. This partial compensation was indicated by a significant increase in nNOS^{-}/VGAT^{+} boutons in the mutant mice (18 ± 4.6 and 37.7 ± 8.2/100 µm of the dendrite in WT and KO, respectively) (p<0.05; t-test) (Fig 5-11).

5.4.3 Possible role of Lamina I NK1r cells in itch perception

The question whether PrP-GFP axons target lamina I NK1r-expressing neurons is important for several reasons. Firstly, it determines whether lamina I giant cells are the only target for this population of inhibitory cells. Secondly, it is not known whether a subset of lamina I NK1r cells also lose some of their nNOS-containing inhibitory input in *Bhlhb5*$^{-/-}$ mouse, and may therefore contribute to the exaggerated itch response seen in these mice. Finally, it aids in providing further information about the circuitry of dorsal horn. It has been suggested that approximately 5% of lamina I cells in the mouse are projection neurons (Wang *et al*., 2013, Polgár *et al*., 2013a). Although, not much is known about the NK1r-expressing projection cells in lamina I of the mouse dorsal horn, in a separate set of experiments, it was observed that ~90% of lamina I cells that projected to contralateral LPb were NK1r-expressing cells. Furthermore, almost all NK1r-expressing projection cells showed strong NK1r immunoreactivity and had soma size greater than 200 µm^2 (Baseer, Mcintyre and Todd unpublished observations). Although, in the current study, retrograde tracer was not injected into LPb to label lamina I NK1r projection cells, only large NK1r cells that expressed strong NK1r immunoreactivity were selected. Therefore, it is likely all lamina I NK1r cells that were included in this study's sample were projection neurons.

There are several lines of evidence to suggest a role of lamina I NK1r-expressing projection cells in the perception of itch. Andrew and Craig (2001) reported a small subset

of histamine responsive lamina I spinothalamic cells in the cat. These projection cells did not appear to respond to mustard oil or mechanical and thermal stimuli. Later, Simone *et al.* (2004) showed that in the primates, all lamina I STT cells were polymodal in nature. They were not only histamine sensitive, but were also activated in response to noxious mechanical and chemical stimuli such as capsaicin. Although, these studies were performed on spinothalamic cells in lamina I of the primates, it is already known that there are few STT cells in lamina I of the rodent dorsal horn. Since most spinoparabrachial cells in this region also project to thalamus, these cells probably correspond to STT component of the anterolateral tract in the primates (Todd *et al.*, 2000, Spike *et al.*, 2003, Al-Khater and Todd, 2009).

An observation made by Carstens *et al* (2010) showed that rats, which were treated with substance P conjugated to saporin (SP-saporin), did not apparently itch. SP-saporin resulted in the neurotoxic ablation of lamina I NK1r-expressing cells, thereby suggesting the role of NK1r-expressing superficial dorsal horn cells in the transmission of itch. In addition to that, a distinct subset of primate polymodal spinothalamic tract (STT) neurons is also thought to be involved in the itch perception (Davidson *et al.*, 2012). These pruriceptive projection neurons are polymodal in nature and respond to pruritogens as well as to multiple noxious stimuli. They are situated mostly in superficial dorsal horn and constitute approximately 1/3rd of all the STT neurons while the remaining 2/3rd are either nociceptive in nature or respond to tactile sensation. There is a possibility that the subset of lamina I NK1r-expressing neurons, which received substantial input from GFP axons, constitute the pruriceptive cells identified by Davidson *et al.* (2012). Since 80% of lamina I projection neurons express NK1 receptor (Marshall *et al.*, 1996), the loss of nNOS input to a subset of these cells could also underlie the exaggerated itch response seen in *Bhlhb5*$^{-/-}$ mouse (Figure 5-12).

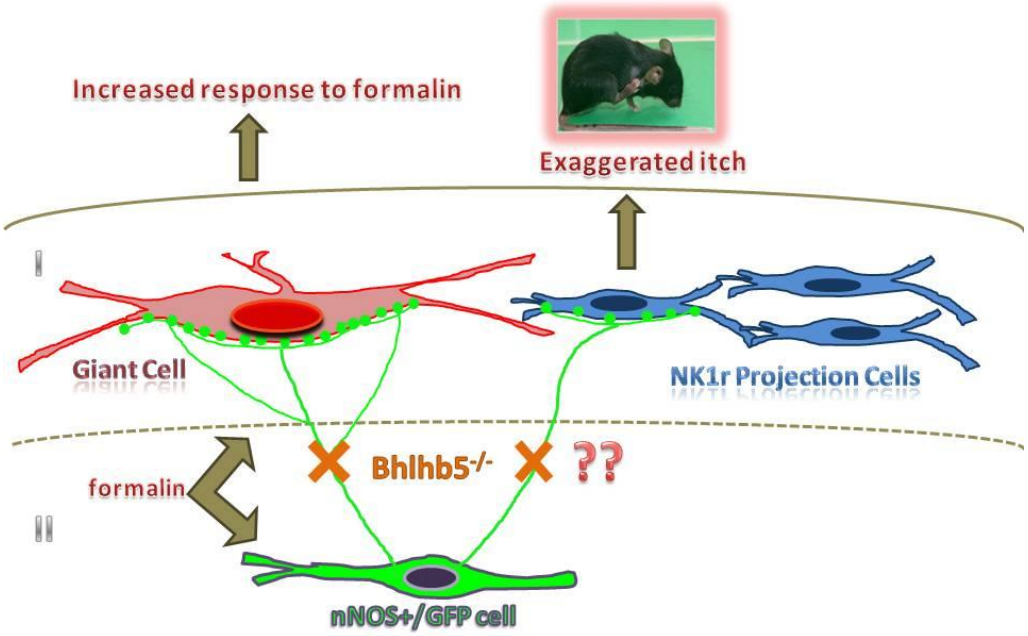


Figure 5-12 Diagrammatic representation of nNOS input to lamina I projection cells and the effects of loss of this input in the *Bhlhb5*$^{-/-}$ mouse.

Axons from nNOS-containing PrP-GFP cells in lamina II of the spinal dorsal horn of PrP-GFP mouse target both lamina I giant cells and a subset of lamina I NK1r expressing projection cells. In the *Bhlhb5*$^{-/-}$ mice, the loss of this input to giant and large lamina I NK1r cells may contribute to an increased response to formalin and exaggerated itch, respectively. Grey and white matter boundary as well as lamina I-II border is drawn using plain and dotted lines, respectively.

6 Concluding remarks

Primary afferents carry sensory information to the spinal dorsal horn, which is then processed by a complex circuitry of excitatory and inhibitory interneurons before being transmitted to the brain via projection cells (Todd, 2010). This study emphasized the importance of selective innervation of dorsal horn projection cells by different populations of nociceptive primary afferents and spinal neurons (excitatory and inhibitory). The initial aim of this project was to test whether lamina III NK1r-expressing projection cells received innervation from PPD-expressing spinal excitatory neurons. The results of this part of the study suggest that, apart from nociceptive peptidergic afferents (Naim *et al.*, 1997) and NPY-containing inhibitory interneurons (Polgár *et al*., 1999b), lamina III NK1r-expressing projection cells also receive selective innervation from PPD-expressing glutamatergic cells. Furthermore, combined confocal and electron microscopy established the presence of excitatory synapses at the sites of contacts between PPD boutons and lamina III NK1r projection cells. This approach is important since the contacts seen with the confocal microscopy do not always make corresponding synapses (Kemp *et al.*, 1996).

Although this study demonstrates selective targeting of lamina III NK1r projection cells by excitatory spinal neurons, the exact source of this input is not known. The excitatory PPD cells are distributed in deep as well as superficial dorsal horn (Sardella *et al.*, 2011a); however it is not known whether this input is provided by a discrete subset of PPD excitatory cells, and if so, whether these cells are scattered throughout the dorsal horn or located in deep or superficial laminae. This can be further investigated using transgenic mouse in which GFP is expressed under the control of promoter preprodynoprhin (Pdyn) (Anderson *et al.*, 2013). In these transgenic mice, PPD cells can be readily identified by whole cell patch clamp recordings. These cells can be filled using neuroanatomical tracers such as neurobiotin and their axons can be followed to determine whether they innervate lamina III projection cells. Although in the mouse, lamina III projection cells do not express NK1 receptor (A. J. Todd unpublished observation), they can be identified using antibodies against CGRP and NPY-containing axons that provide dense innervation to lamina III projection cells (Naim *et al*., 1997, Polgár *et al*., 1999b).

It has already been mentioned that dynorphin acts on both kappa-opioid (KOR) (James *et al.*, 1982) and glutamate specific NMDA-receptors (Shukla and Lemaire, 1992, Laughlin *et al.*, 1997, Drake *et al.*, 2007). The presence of glutamatergic synapses at the site of contacts between PPD-containing VGLUT2 boutons and lamina III NK1r cells suggest that dynorphin's action on these cells is mediated through NMDA receptors. Although KORs

are present in the dorsal horn (Arvidsson *et al.*, 1995), it is not known whether these receptors are expressed by lamina III NK1r cells. This can be addressed by using a reliable antibody against KOR to determine its expression and co-localization with PPD-containing VGLUT2 contacts on lamina III NK1r cells.

Since many PPD-expressing dorsal horn neurons respond to noxious stimulation (Noguchi *et al.*, 1991), this suggests that lamina III projection cells receive powerful mono- and polysynaptic nociceptive input. Excitatory interneurons in the dorsal horn have been shown to possess I_A currents (Yasaka *et al.*, 2010), which limit their excitability and can underlie a form of activity-dependent intrinsic plasticity (Sandkühler, 2009). It is therefore likely that during the development of chronic pain states, polysynaptic inputs to lamina III projection neurons are recruited due to the suppression of I_A currents (Hu *et al.*, 2006) in the excitatory PPD cells. These changes could contribute to the chronic hyperalgesia seen in the inflammatory pain states. Based on these observations, this study is first to provide evidence that a neurochemically defined distinct population of excitatory cells selectively innervate lamina III projection neurons, thus expanding our knowledge about the role of excitatory spinal cells in the neuronal circuitry of spinal dorsal horn.

Studies in the past have reported selective targeting of dorsal horn cells by Aδ and C afferents. It has already been mentioned that lamina I and III NK1r-expressing projection cells receive substantial input from peptidergic primary afferents (Naim *et al.*, 1997, Todd *et al.*, 2002, Torsney and MacDermott, 2006). Moreover, lamina II interneurons such as islet and central cells are principally targeted by C-fibres while radial and vertical cells receive input from both Aδ and C afferents (Grudt and Perl, 2002, Lu and Perl, 2003, 2005, Hantman *et al.*, 2004, Yasaka *et al.*, 2007). However, not much is known about the inputs to lamina I projection cells that do not express NK1r. This study provides first evidence of preferential targeting of lamina I $NK1r^-$ projection cells by CTb-labelled non-peptidergic Aδ nociceptors. However, whether this sub group of lamina I $NK1r^-$ projection cells represents a homogenous population, is not known. Further studies are needed to investigate features that are independent to these projection cells such as morphology, electrophysiology, response properties and synaptic inputs.

Torsney (2011) carried out whole-cell-patch clamp recordings of lamina I NK1r cells in response to electrical stimulation of dorsal root and reported that ~30% of lamina I NK1r cells received monosynaptic Aδ input. Furthermore, the proportion of lamina I NK1r cells

that received this input increased to 60% in the presence of hind paw inflammation. It was suggested that the increase in the proportion of NK1r cells resulted from the activation of previously silent receptors. The results from this current study show that Aδ afferents that provide synaptic input to lamina I NK1r-expressing projection cells are mostly non-peptidergic and therefore are likely to correspond to type I Aδ nociceptors. Furthermore these afferents can be related to superficial AHTM identified by Lawson and colleagues (1997). However, a considerably low proportion of CTb-labelled peptidergic Aδ boutons observed in this study is probably due to the lack of GM1 ganglioside on peptidergic afferents that is required to transport CTb injected into sciatic nerve (Robertson and Grant, 1989).

It has already been mentioned that type I Aδ afferents under lie fast pricking pain (Ringkamp *et al.*, 2013). Since, NK1r ablation results in the loss of hyperalgesia but acute noxious responses remain intact (Mantyh *et al.*, 1997, Nichols *et al.*, 1999), there is a possibility that the $NK1^-$ cells carry acute noxious information perceived as acute pain and therefore contribute to the normal responses seen in these studies. These results confirm the fact that the synaptic inputs to dorsal horn projection neurons are arranged in a considerably specialized way and the functional differences between different types of nociceptors are retained at the level of projection neurons. This type of arrangement has been described by the specificity theory of pain that suggests that pain is a specific modality that is transmitted to the brain in a highly specialized manner (Malzack and Wall, 1965). This observation is further supported by the presence of specialized receptors (nociceptors) and pain pathways such as spinothalamic tract, for the transmission of nociception (Perl, 1998).

Different lines of transgenic mice have made valuable contributions to the better understanding of dorsal horn circuitry (Heinke *et al.*, 2004, Hantman *et al.*, 2004, 2005, Labrakakis *et al.*, 2009, Punnakkal *et al.*, 2014). One such example is the PrP-GFP mouse that selectively expresses GFP under the control of prion-promoter in neurochemically defined subpopulations of SDH neurons (Hantman *et al.*, 2004, Iwagaki *et al.*, 2013). It is therefore possible to perform targeted recordings from a distinct set of GFP-expressing cells to determine their physiological and morphological properties as well as synaptic connections. The third and final part of this study investigated whether lamina I projection cells are innervated by GFP/NOS cells in lamina II of the spinal dorsal horn of PrP-GFP mouse. Preliminary observations suggested that PrP-GFP cells extend their axons to lamina

I. This observation was quite surprising since Hantmann and colleagues (2004) reported that the axons of PrP-GFP cells are limited to lamina II. The probable explanation is that their sample would have included only those cells that have locally arborizing axons. It is now known that approximately 1/3rd of PrP-GFP cells in lamina II do extend their axons to lamina I of the dorsal horn (Iwagaki, Ganley and Todd unpublished observation). This information is interesting since it would assist in the identification of polysynaptic pathways that convey sensory information to the brain via spinal dorsal horn.

In the rat, the selective innervation of lamina III NK1r-expressing projection cells is derived from a specific subset of NPY-containing inhibitory neurons (Polgár *et al*., 1999b). Based on this observation, the input from nNOS^{+}/GFP^{+} boutons to lamina I giant cells would correspond to a subset of nNOS-containing PrP-GFP cells. Taken together, these findings suggest that neurochemically defined populations of dorsal horn cells are not homogenous but they are likely to contain sub populations that may vary in their post synaptic targets and functions.

In the rat, lamina I giant cells express c-fos in response to noxious stimuli such as formalin (Puskár *et al*., 2001, Polgár *et al*., 2008b). However, it is not known whether these cells are also activated in response to pruritogens such as histamine (Andrew and Craig, 2001) or chloroquine (CQ) (Liu *et al.*, 2009). It would be interesting to see whether lamina I giant cells respond to such pruritogens by up regulating fos. If so, then the major nNOS input to these cells could be important in itch prevention. Furthermore, the loss of this input could explain the abnormal itch behaviour seen in *Bhlhb5*$^{-/-}$ mice. The results of this current study suggest that a small subset of lamina I NK1r cells are also innervated by inhibitory nNOS-containing axons, but it is not known whether this input is also lost in the *Bhlhb5*$^{-/-}$ mice. If these cells are also activated in response to pruritogens, then the loss of nNOS input to a subset of these cells could also underlie the exaggerated itch response seen in the *Bhlhb5*$^{-/-}$ mice.

The results from the experiments based on *Bhlhb5*$^{-/-}$ mice provide an insight to the changes taking place at dorsal horn circuitry level in certain pathological conditions such as chronic refractory itch. In these mice, self inflicted skin lesions are observed while the responses to painful stimuli usually remain intact (Ross *et al.*, 2010). These findings as well as the observations made in the many other studies (Schmelz *et al.*, 1997, Andrew and Craig,

2001, Sun *et al.*, 2009, Han *et al.*, 2013) support labelled line theory of itch, indicating that different sensory modalities are probably transmitted through distinct pathways.

The findings of the present study have confirmed and extended previous observations by suggesting that projection cells play an important role not only in the transmission of sensory information to their supraspinal targets but their inputs from dorsal horn excitatory and inhibitory cells are important in processing sensory information at the dorsal horn level. However, many aspects of this circuitry are still unknown and require further investigations.

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8 Publications

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