Characterisation of interneurons in lamina II of the rat spinal cord

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Thesis submitted in fulfillment for the degree of Doctor of Philosophy

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February 2011

Summary

Lamina II of the dorsal horn contains numerous small neurons with varying morphologies, most of which have axons that remain within the spinal cord. It can be distinguished from the other laminae by its lack of myelinated fibres and its constituent interneurons that are densely packed. This region is the major termination site for unmyelinated (C) primary afferent fibres, which convey mostly nociceptive information. It also receives inputs from thinly myelinated (Aδ) fibres, some of which are nociceptive. In spite of its importance and several past attempts, little is known of its neuronal circuitry. This is mainly due to the great functional and morphological diversity of lamina II interneurons, which has made characterisation difficult. A comprehensive classification scheme is essential to identify discrete functional populations of lamina II interneurons, and to enable understanding of their roles in the local neuronal circuitry.

The present study aims to investigate the physiological, pharmacological and morphological properties of lamina II interneurons recorded in an *in vitro* slice preparation from adult rat spinal cord. These properties were correlated with the neurotransmitter content of each cell, which was identified by detection of vesicular transporters in axonal boutons, in order to distinguish discrete functional subpopulations of cells in this region.

Both inhibitory and excitatory interneurons were identified in lamina II, based on their expression of vesicular GABA transporter (VGAT) or vesicular glutamate transporter (VGLUT2), respectively. None of the cells that had VGAT-immunoreactive axons displayed staining for VGLUT2, and vice-versa. Injection of depolarising current evoked tonic-, transient-, delayed-, gap-, reluctant- and single spike-firing among these cells. Discharge pattern was strongly related to neurotransmitter phenotype, since most excitatory cells, but very few inhibitory cells had firing patterns that could be attributed to A-type potassium (I_A) currents (i.e. delayed, gap or reluctant-firing). This suggests that excitatory lamina II interneurons with I_A-type firing patterns are involved in plasticity that contributes to pain states. The majority of inhibitory cells displayed tonic-firing pattern in response to depolarisation. There was also an obvious difference in the response of lamina II neurons to hyperpolarisation, since the majority of

inhibitory cells showed inward currents while most excitatory cells displayed transient outward currents. Noradrenaline and serotonin hyperpolarised both inhibitory and excitatory neurons, while only inhibitory neurons responded to somatostatin. This is consistent with the findings of a previous study that had shown that the somatostatin 2 receptor (sst2a) is only expressed by inhibitory neurons in lamina II, and suggests that the pro-nociceptive effects of somatostatin are mediated by 'disinhibition'.

The somatodendritic morphology of 61 lamina II interneurons was reconstructed from projected confocal images of Neurobiotin labelling and assessed according to the morphological scheme developed by Grudt and Perl (2002). Although cells in the islet, central, vertical and radial class were identified, a substantial number of cells (19/61) had morphology that was atypical or intermediate between two classes and therefore could not be classified. Certain morphological types were consistently found in the inhibitory or excitatory population: all islet cells were GABAergic, while all radial cells and most vertical cells were glutamatergic. However, the correlation between these properties may be complex, since there was a considerable diversity in the remaining cells.

Some glutamatergic interneurons had axons that contained somatostatin and many of these also contained enkephalin. Somatostatin-expressing glutamatergic cells included various morphological types, while enkephalin was detected in the axons of vertical and radial cells. All cells with axons that were somatostatin- and enkephalin-immunoreactive had delayed-firing patterns. Taken together with the pharmacological data from the present study, this suggests that somatostatin released from these glutamatergic neurons would hyperpolarise subsets of inhibitory neurons and causes disinhibition. This could lead to alterations of pain thresholds.

The results from this study demonstrate that distinctive populations of inhibitory and excitatory interneurons can be recognised in lamina II, and these cells are most likely to correspond to discrete functional groups. Electrophysiological, neurochemical, morphological and pharmacological properties of neurons can be correlated but this is likely to be very complex. Future investigations that combine various approaches should allow further understanding of the specific roles of lamina II interneurons in nociceptive processing within the spinal cord.

Acknowledgements

This thesis is not a self accomplishment but one made possible by contributions and support from many. First of all, I would like to express my highest gratitude to my supervisor Professor Andrew Todd for his support and guidance from the initial to the final level in my course of study. His integrity, enthusiasm and optimism has encouraged me to always learn and do well. The writing up of this thesis would not be possible without his constructive criticisms and suggestions. I truthfully appreciate the opportunity to participate in this project.

It is my honour to work with Dr Toshiharu Yasaka, who has greatly contributed to this work. I am indebted to his generosity in teaching and sharing of his knowledge, particularly in electrophysiology. This work would not be attainable without the whole-cell patch-clamp recordings and electrophysiological analysis he carried out.

I would also like to thank Dr John Riddell who has assisted the study in numerous ways. My gratitude also extends to Dr David Hughes and Professor David Maxwell for their valuable advice and communication throughout the time I am here.

To Dr Anne Bannatyne and Dr Erika Polgár, thank you not only for the scientific knowledge that you have shared with me, but all the bits and pieces after work! You have truly made my experience here one to be remembered for life.

I also thank Mrs Christine Watt and Mr Robert Kerr for their technical support and their heartfelt humour. To the rest of the Spinal Cord Group, it is indeed my pleasure to work with all of you!

I am also grateful to Associate Professor Dr Durriyyah Sharifah Hasan Adli, University Malaya and the Ministry of Higher Education Malaysia for providing the opportunity, funds and support for this study.

Last but certainly not least, I owe my deepest gratitude to my parents, family and friends. Thank you for your unconditional love and faith!

All thanks be to God.

Declaration

I hereby declare that the work presented in this thesis is my own, except where explicit reference is made to the contribution of others. Dr Toshiharu Yasaka performed all electrophysiological recordings and the relevant analysis in this project. This thesis has not been submitted in any previous application for any other degree in the University of Glasgow or any other institution.

Tiong Yin Xin Sheena

February 2011

Dedication

To my most loving grandmother, parents and family

致最疼爱我的婆婆，父母与家人

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List of Abbreviations

CGRP	calcitonin gene-related peptide
CNS	central nervous system
Cy5	cyanine 5.18
DRG	dorsal root ganglion
DV	dorsoventral
Enk	enkephalin
ERK	extracellular signal-regulated kinases
GABA	gamma-aminobutyric acid
GAD	glutamic acid decarboxylase
GFP	green fluorescent protein
GFP	green fluorescent protein
GLYT2	glycine transporter 2
HeNe	helium neon
HRP	horseradish peroxidase
IB4	*Bandeiraea simplicifolia* isolectin B4
IgG	immunoglobulin G
LCN	lateral cervical nucleus
LII	lamina II

List of Abbreviations

LPb	lateral parabrachial area
nNOS	neuronal nitric oxide synthase
NPY	neuropeptide Y
NST	nucleus of the solitary tract
PAG	periaqueductal grey matter
PB	phosphate buffer
PBS	phosphate-buffered saline
PKC	protein kinase C
PSDC	postsynaptic dorsal column pathway
PV	parvalbumin
RC	rostroventral
SCT	spinocervical tract
SDH	superficial dorsal horn
SG	substantia gelatinosa
SST	somatostatin
VGAT	vesicular GABA transporter
VGLUT	vesicular glutamate transporter
WGA	wheatgerm agglutinin

Chapter 1

Introduction

1 Introduction

1.1 Central nervous system and the spinal cord

The mammalian central nervous system comprises the brain, which is situated rostrally in the cranial cavity, and the spinal cord that travels caudally and lies within the spinal canal of the vertebral column, more commonly known as the backbone or the spine. The spinal cord is continuous with the brain and together they act to communicate with the rest of the body and to generate appropriate outputs, such as limb movements, as responses to various sensory inputs.

The spinal cord is surrounded and protected by a cushion of cerebrospinal fluid and layers of spinal meninges: dura mater, arachnoid and pia mater (from external to internal). Intrinsically, the spinal cord is an elongated, unsegmented cylindrical structure; however, paired spinal nerves emerging along its entire length produce an appearance of external segmentation in correspondence to the vertebral column. A single spinal cord segment is defined as the cord region that connects the dorsal and ventral rootlets, which give rise to a single pair of spinal nerves. In human, there are altogether 31 pairs of spinal nerves: (in descending order) 8 cervical, 12 thoracic, 5 lumbar, 5 sacral and 1 coccygeal; each of which innervates a specific territory in the body. This applies to all mammals with slight alteration in the number of spinal nerves in different species, for example, in the rat the corresponding numbers are 8, 13, 6, 4 and 3 respectively. The vertebral column extends beyond the length of the spinal cord. Therefore, the length and obliquity of the spinal nerves increase progressively in the rostrocaudal direction as the distance between cord segments and the corresponding vertebral segments increases. The diameter of the spinal cord is not uniform. It appears to be enlarged at the cervical and lumbar segments.

The dorsal roots contain sensory (afferent) fibres that arise from ‘pseudo-unipolar’ neurons situated in dorsal root ganglia (DRG), from where an axon emerges and branches into two: central and peripheral processes. The peripherally directed axon innervates tissues and organs, and the central axon terminates within the spinal cord. The ventral root contains axons of preganglionic neurons entering the sympathetic trunk as part of the autonomic

nervous system; and also motor nerve fibres that originate from motor neurons in the spinal cord, are involved in muscular control and spinal reflexes in response to sensory inputs.

1.2 Organisation of spinal grey matter

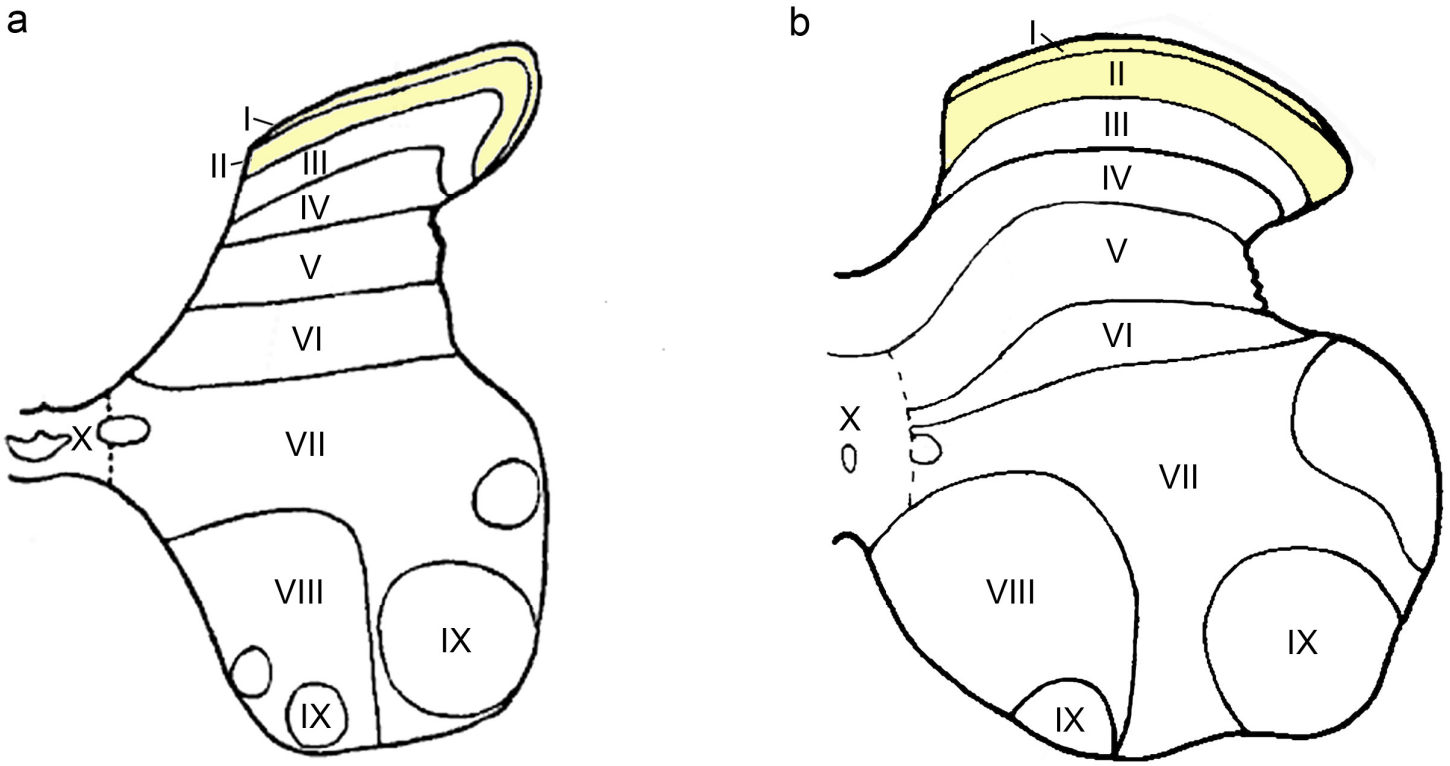
1.2.1 Overview of the spinal cord anatomy

Continuous with the fourth ventricle located in the hindbrain, the central canal extends longitudinally along the whole length of the spinal cord and is lined by ependymal epithelium. It is surrounded by white and grey matter. When viewed in a transverse section, the grey matter forms an H-shaped outline and the white matter lies on the outside. The white matter consists largely of myelinated nerve fibres, thus the white appearance. It also contains dendrites from neurons in the grey matter. It is anatomically divided into three main columns or funiculi: dorsal, lateral and ventral funiculus. The dorsal funiculus is made up of a medial fasciculus gracilis and the lateral fasciculus cuneatus. There is no distinct boundary between the remaining parts of the white matter, whereby nerve fibres decussate in the ventral white commissure while the dorsolateral tract of Lissauer intersects the area between the apex of the dorsal horn and the surface of the cord (Barr and Kiernan, 1983). The grey matter contains nerve cell bodies, the neuropil (which includes axonal and dendritic branches), glial cell processes and capillaries. The spinal grey matter is divided into dorsal and ventral horns.

1.2.2 Rexed's laminae

The laminar system introduced by Rexed (1952, Figure 1-1) remains the most widely accepted scheme for delineating regions in the spinal grey matter. Based on the cytological structure and arrangement of Nissl-stained neuronal cell bodies in cat spinal cord transverse sections, Rexed proposed a cytoarchitectonic organisation scheme which divides the grey matter into ten parallel regions or laminae: laminae I to VI in the dorsal horn, laminae VII-IX in the ventral horn and the tenth region, which surrounds the central canal (lamina X, substantia grisea centralis).

Figure 1-1 Laminar organisation of the grey matter. Rexed (1952) divided the grey matter in the cat into laminae I-X based on the size and packing density of neurons. This cytoarchitectonic organisation scheme has since been applied to several other mammalian species. Figure shows schematic drawings of the laminar subdivisions in the lumbar segment of the cat (**a**) and the rat (**b**). The superficial dorsal horn (laminae I and II) is highlighted in yellow. Images are modified from Figure 3 of Rexed (1952) and Figure 10 of Molander and Grant (1984).


a
I
II
III
IV
V
VI
X
VII
VIII
IX
IX
b
I
II
III
IV
V
VI
X
VII
VIII
IX
IX

The following description is based mainly on Rexed's observation in the cat (Rexed, 1952) and observations from more recent studies (Brown, 1982; Willis and Coggeshall, 2004). Lamina I was defined as the most marginal layer of the dorsal horn. It is the thinnest of all the laminae and runs around the most dorsal edge of the spinal grey matter, extending a short distance over the dorsal-lateral aspect. This layer is often penetrated by numerous small and large nerve fibre bundles, which give it a reticulated appearance and often makes its boundary with the white matter rather indistinct. Lamina II (also known as substantia gelatinosa, SG) is broader than lamina I and similarly, it extends around the lateral border of the dorsal horn to about half way down the lateral edge (Brown, 1982). It is distinguishable by its gelatinous appearance, which results from the lack of myelinated axons and is characterised by small-sized neurons with locally extended processes. Rexed observed the dorsal or outer part of this lamina to be more compact and to contain densely packed cells of smaller size than its ventral or inner region. Laminae III and IV are collectively known as the nucleus proprius. Lamina III is a broad band that runs just ventral and parallel to lamina II. It is thicker than the previous two layers with less tightly packed neurons. The cells are generally larger and more varied than those in lamina II and it also contains many myelinated fibres. Unlike laminae I and III, lamina IV does not show a ventral bend at its lateral part but extends across the dorsal horn with an almost uniform thickness. It contains cells of various size and these are loosely arranged. Some very large neurons can be found within this area and its neuronal population is rather heterogeneous. Lamina V extends as a thick band across the narrowest part of the dorsal horn. The neurons in this layer appear more varied in shape and size than those in lamina IV. The lateral region of lamina V is characterised by its reticulated appearance due to the many longitudinal nerve fibre bundles running through it. Lamina VI forms the base of the spinal dorsal horn. It exists only in the cervical and lumbosacral enlargements of the spinal cord. Lamina VI comprises small, regularly arranged neurons and has a darker appearance than its neighbouring laminae in Nissl stained sections.

The remainder of the spinal grey matter is further divided into lamina VII to lamina X. Lamina VII occupied a large area in the centre of the grey matter and has a homogeneous appearance. Lamina VIII and lamina IX are located at the

ventromedial and the ventrolateral edge of the ventral horn, respectively. Lamina VIII contains a mixture of small and large cells and lamina IX corresponds to the motor nuclei. Grey matter in the immediate vicinity around the central canal is considered as the tenth lamina and contains small sized interneurons.

Although this organisation method was first established using the cat spinal cord, it was found to be applicable to various other mammalian species in later studies. For example, Molander et al. (1984, 1989) had found a similar laminar arrangement in the rat and showed that the cytoarchitectonic organisation in rat spinal cord is very similar to that in the cat (Figure 1-1). In addition, lamina II was shown to have an outer zone with densely packed neurons and a less compact inner zone, as originally described by Rexed (1952). This laminar pattern has also been found in monkeys, especially in the dorsal horn (Beal and Cooper, 1978; Ralston, 1979). Ralston (1979) used light and electron microscopy to examine neuronal and synaptic organisation of laminae I to III, which he found to correspond closely with Rexed's description with few morphological differences. The substantia gelatinosa in the macaque monkey was characterised by its scarcity of myelinated axon and small neurons were abundant in this spinal region (Ralston, 1979). However, as in the cat, most studies found the distinction of borders between the deeper laminae to be ambiguous and not a sharp definitive line, but rather a transition zone. And more recently, Woodbury et al. (2000) pointed out that exact correspondence of SG may differ between species, as they found SG in mice to be relatively thicker than that in rat and cat.

A major advantage of Rexed's laminar organisation is that it avoids the problems of varied nomenclature that was used in classical anatomical descriptions and makes communication more efficient as far as neuronal location is concerned. Although it was widely adopted as the common ground for numerous studies, doubts were raised about its functional significance. Therefore, subsequent studies have examined the primary afferent inputs to different dorsal horn laminae and the physiological properties of neurons in each lamina to determine whether these varied between laminae. Development of microelectrode recording techniques had subsequently improved knowledge of these characteristics.

1.3 Overview of dorsal horn laminae

The spinal dorsal horn is the major site to receive inputs from primary afferent axons including nociceptive receptors, which terminate almost exclusively within this region. Among the series of six parallel laminae in dorsal dorn, as described by Rexed (1952), the superficial part of the dorsal horn (SDH), which referred to both laminae I and II has received considerable attention. It has long been suggested that inhibitory interneurons located within the superficial dorsal horn play a major role in controlling incoming sensory information before this information is transmitted to the brain (Melzack and Wall, 1965). It is therefore the first central synapse in the ascending pain-processing pathways where integration of sensory messages takes place and major somatosensory systems originate (Brown, 1982; Graham et al., 2007). It was reported to be the major target zone for small diameter Aδ and C-fibers primary afferents carrying noxious, thermal, itch and innocuous tactile information (Christensen and Perl 1970; Sugiura et al., 1986; Tuckett and Wei, 1987). Organisation of the SDH routinely includes neuronal components contained within this area and primary afferent inputs to the SDH. Many have postulated the existence of synaptic circuits involving intrinsic neurons and afferent fibers conveying distinct inputs in SDH, thus its roles in modulating pain (Cervero and Iggo, 1978; Willis and Coggeshall, 2004). Despite a vast number of studies and progress made to understand properties of the relevant neurons, knowledge of this spinal region is still far from complete. The complexity of neuronal organisation in the superficial dorsal horn makes understanding it in depth difficult but necessary. Several studies have attempted to characterise neurons present in this region by using different approaches. The highest density of neurons in the spinal cord is found in Rexed's laminae I-III of the dorsal horn (Rexed, 1952) and a great majority of those in each lamina are reported to be interneurons, although some in laminae I and III have projections to the brain.

1.3.1 Overview of the functions of dorsal horn laminae

Wall (1967) used extracellular recording and showed that there were physiological differences between cells in laminae IV to VI. For example, cells in all three of these laminae responded to cutaneous stimulation but only those in

lamina VI responded to movement. It was shown that lamina IV neurons had a small cutaneous receptive field. Electrical and natural stimulation of myelinated (A) and unmyelinated (C) primary afferent fibres provided evidence that fibres of varying diameter converged onto cells in the dorsal horn (Mendell and Wall, 1965; Mendell, 1966). In addition, Mendell (1966) observed that cells in the spinocervical tract have cell bodies situated in lamina IV and that this tract projects through the lateral cervical nucleus to the thalamus, S1 and S2 cortex. Christensen and Perl (1970) were able to record from neurons in lamina I, and found them to respond specifically to different stimuli that were related to primary afferents of varied conduction velocities. One group of neurons in lamina I was excited only by noxious mechanical stimulation, while others were activated by innocuous temperature change in the skin, in addition to intense mechanical stimulation. Electrical stimulation showed that these neurons responded to both Aδ- and C- primary afferents. In a subsequent study, Wall et al., (1979) found that lamina II/III neurons responded to a wide range of cutaneous stimulation intensity from light brushing to pinching. All of these neurons were excited by myelinated Aδ afferents, with some responding to both myelinated and unmyelinated fibres but none responded only to the latter. Price et al. (1979) investigated neurons in lamina I and II of primates. They found a significant proportion of wide dynamic range neurons, i.e. cells that responded to low threshold mechanical and noxious stimuli, in lamina I and dorsal part of lamina II. Similarly, nociceptive-specific neurons that did not respond to tactile stimulation were also located in these regions. However, neurons that responded to noxious heat were predominantly found in lamina II. Neurons that responded exclusively to innocuous mechanical stimuli were most abundant in lamina II ventral. They also found neurons that projected to the thalamus were confined to lamina I. This led to the suggestion that each lamina contains distinctive neuronal populations and receives specific primary afferent inputs.

1.3.1.1 Primary afferent inputs to the spinal dorsal horn

Primary afferents, which provide the main input to the spinal dorsal horn, enter the spinal cord through the dorsal roots and their subsequent course is dependent on the type of fibre involved. Primary afferent fibres vary in diameter. The smallest ones, C-fibres, are 0.2 to 1.5 μm in diameter and are unmyelinated. These fibres conduct at 0.5 to 2 m/second and are mainly

nociceptive and thermoreceptive, while some respond to tactile stimuli. The remaining fibres are myelinated (A-fibres) and can be divided into two main groups based on their functions and conduction velocity. The smaller fibres (Aδ) are 1 to 5 μm in diameter and have a conduction velocity of 6 to 30 m/second. Aδ afferents function as nociceptors or as low threshold mechanoreceptors. Aδ nociceptors have been further divided based on their responses to mechanical and thermal stimuli (Djouhri and Lawson, 2004). Non-nociceptive Aδ afferents can be either thermoreceptive or responsive to slow movement of hair (Aδ hair follicle afferents).

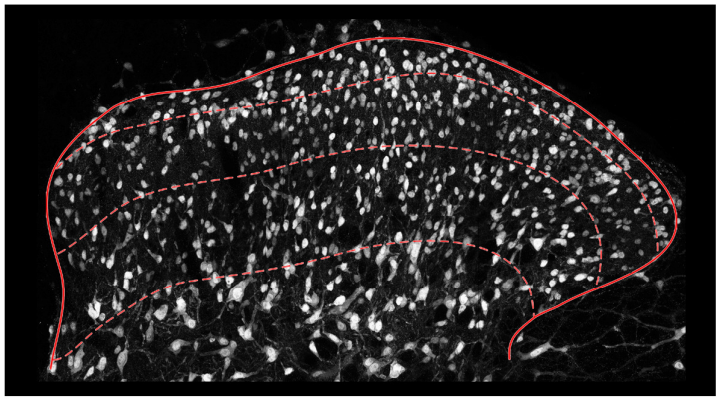
The largest cutaneous fibres (Aß), with diameters of 6 to 12 μm, are myelinated and conduct action potentials at a rate of up to 75 m/second. A great majority of Aß fibres are low threshold mechanoreceptors, although studies have suggested that a substantial number of these afferents could be nociceptive since nociceptors have been found to conduct in the Aß range (Djouhri and Lawson, 2004). Woodbury et al. (2008) also found myelinated cutaneous nociceptive fibres that exhibited morphology characteristic of Aß hair follicle afferents. This indicates that functions of primary afferents are closely related to their fibre size, but there may be some overlap in the sensory modalities and the intensity of stimulus required to activate these primary afferent axons.

After entering the spinal cord, large fibres send ascending axonal collaterals to the gracile or cuneate nuclei through the dorsal columns, and give local axonal branches in the spinal grey matter (Willis and Coggeshall, 2004). The small afferent axons (Aδ and C-fibres), however, bifurcate and enter the tract of Lissauer or dorsal columns, where they may extend for one or two segments as well as giving off local collaterals that arborise in the spinal dorsal horn (Szentágothai, 1964; Traub and Mendell, 1988).

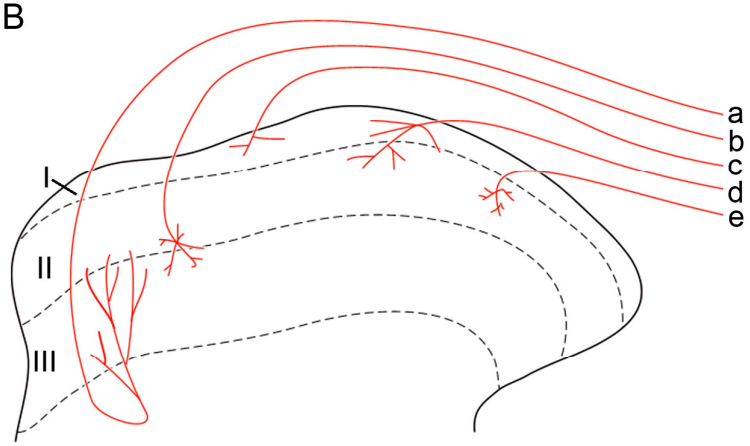
Primary afferents of different sensory modalities terminate within the spinal cord in a highly characteristic manner (Figure 1-2). Various methods have been used to determine the morphology and distribution of different-sized primary afferent fibres. A series of experiments was carried out by Brown et al. (1977, 1978 and 1980) to label different types of primary afferent by intra-axonal injection of horseradish peroxidase (HRP) in order to investigate their projection sites in the cat spinal cord. Axon collaterals originating from hair follicle

Figure 1-2 Laminae I-III and primary afferent inputs. (**A**): A transverse section from the mid-lumbar segment of the rat spinal cord that is immunostained with an antibody (NeuN) that specifically labels neurons. Laminar boundaries are marked by dashed lines. Lamina I and lamina II are characterised by a large number of densely packed small neurons. Lamina II can be divided into two zones: lamina II outer (IIo) and lamina II inner (IIi), with the former having a higher neuronal density than the latter. Neurons in lamina III are more scattered and varied in size. (**B**) Primary afferents terminate within the dorsal horn in a orderly, modality-specific manner. **a**: Large myelinated (Aß) hair follicle and tactile afferents are mainly terminated in the deep laminae (LIII-V) with some extension into lamina IIi; **b**: Thinly myelinated (Aδ) hair follicle afferents arborise on either side of the border between lamina II and lamina III; **c**: Aδ nociceptors terminate mainly in lamina I; **d**: Peptidergic primary afferents (C or Aδ nociceptors) terminate mainly in lamina I and lamina IIo; **e**: Most non-peptidergic C-fibres form a dense plexus in the central region of lamina II.

A



B



receptors descended into the deeper part of the dorsal laminae, before turning back to arborise mainly in lamina III, and very sparsely in deep lamina II. This distinctive arborisation pattern corresponded closely to the 'flame-shaped arbors' described in a previous study (Scheibel and Scheibel, 1968). Slowly and rapidly adapting mechanoreceptors innervated by myelinated afferent nerve fibres were found to terminate mainly in lamina III and IV (Brown et al., 1978; 1980). Collaterals from Pacinian corpuscle afferent fibres were most concentrated in laminae III and IV, but also innervated a small region in laminae V and VI (Brown et al, 1980). Group Ia and Ib muscle afferents, on the other hand, sent collaterals to deeper laminae, with some that entered the motor nuclei (Brown and Fyffe, 1978; 1979). With a similar approach, Light and Perl (1979b) reported a group of high threshold nociceptive Aδ afferents to end mainly in the lamina I (and lamina V) of the dorsal horn. They also found a population of low threshold nociceptive Aδ-fibres that innervate down hairs terminate at the most ventral of lamina II, often overlapping with the most dorsal portion of lamina III in both cats and monkeys. In additions, they also suggested that most fibres and terminals in lamina II originate from unmyelinated primary afferent C-fibres. Suguira et al. (1986) managed to label very fine unmyelinated cutaneous afferents of different sensory modalities in guinea pig. They found C fibres with somatic receptive fields to terminate primarily in lamina I and II outer.

However, innervation of neurons by the different classes of primary afferents is not constrained to the laminar boundaries. For example, although the main termination plexus of nociceptive C-fibres is in the superficial dorsal horn, subsets of laminae III-IV neurons frequently responded to inputs from these unmyelinated afferents (e.g. Woolf and Fitzgerald, 1980; Naim et al, 1997). One way for neurons in the deeper laminae to receive information carried by these nociceptive unmyelinated afferents is through synaptic contacts between the afferent fibres and the dorsally projected dendrites of lamina III-V neurons that reach the superficial layers (Todd, 1989; Brown et al., 1995; Naim et al, 1997). Primary afferent inputs may also be relayed by neurons in the superficial layers with axon collaterals that extend ventrally and terminate in the deep laminae (Light and Kavookjian, 1988). This explains the superficial dorsal horn consists mainly of nociceptive-specific neurons, whereas deep laminae contain rather

wide dynamic range neurons that respond to noxious and tactile inputs (Gauriau and Bernard, 2002).

Besides intra-axonal labelling, many studies have used 'bulk labelling' to identify primary afferents that innervate the dorsal horn. For example, Light and Perl (1979a) applied HRP to severed dorsal rootlets to reveal termination sites of primary afferent fibres in the lumbar, sacral and cervical segments of mammalian spinal cord. Selective lesions of dorsal roots allowed them to find that the superficial laminae are the major target site of thin afferent fibres originating from the lateral part of the dorsal root. Subsequently, HRP was used not only in its free form but conjugated to wheat germ agglutinin (WGA) to label nerve fibres (Swett and Woolf, 1985; Woolf and Fitzgerald, 1986). Conjugated forms of HRP not only enhance afferent tracing but different classes of primary afferents were observed to selectively transport different forms of HRP. HRP conjugated to WGA predominantly labels fine diameter primary afferents and their terminals in superficial dorsal horn, and thus reflecting concentration of presumably C-afferent fibres within this region (Swett and Woolf, 1985; Roberston and Arvidsson, 1985). Besides WGA, HRP conjugated to choleragenoid or beta-cholera toxin (B-HRP or CTb-HRP) has also been used to determine the pattern of primary afferent projections in the spinal cord. Unlike WGA-HRP, which labels mainly small primary afferents, B-HRP is primarily taken up by thick myelinated afferent fibres. B-HRP labelled axons are present in all laminae, most concentrated in lamina I, IV and V but minimally present in lamina II (Rivero-Melián and Grant, 1990; LaMotte et al., 1991).

Several types of primary afferents may innervate the same lamina and therefore convergent inputs from more than one type of sensory receptor are expected (Brown et al., 1992). The superficial dorsal horn, interestingly, receives inputs predominantly from unmyelinated (C) and thinly myelinated (Aδ) fibres that primarily function as nociceptors and thermoreceptors. Large number of fine primary afferents from the Lissauer's tract and the surrounding white matter enters lamina I to innervate the marginal plexus. Previous studies showed that primary afferent termination within lamina I is primarily of Aδ- and C-fibres origins, and there is no evidence of collaterals from large fibres entering the marginal layer (Brown, 1981; Fyffe, 1984; Mense and Prabhakar, 1986; Willis and Coggeshall, 2004). The primary afferent input into lamina II is generally

dominated by fine sensory fibres (Light and Perl, 1979a; Rethelyi et al., 1982; Ralston et al., 1984; Sugiura et al., 1986). Finely myelinated mechanical nociceptive afferent (Aδ) input was reported to be sparse or nonexistent in lamina II (Gobel and Binck, 1977; LaMotte, 1977; Gobel and Falls, 1979; Ralston and Ralston, 1979).

Earlier studies had identified collaterals from coarse primary afferents to innervate the substantia gelatinosa, which was then still regarded as both laminae II and III. Large myelinated fibres extend through the medial part of the dorsal horn in an angle and curve dorsally to end in large flame-shaped arbors (Figure 1-2 a) in the ventral region of lamina II (Ramon y Cajal, 1909; Szentágothai, 1964; Scheibel and Scheibel, 1968; Brown, 1981). This information was disputed since lamina III was excluded from the substantia gelatinosa later on and the flame-shaped arbors were thought to be largely restricted to lamina III (Brown, 1981). More recent investigations have, however, confirmed that a considerable input of these myelinated afferents is indeed present in the most ventral region of lamina II (Shortland et al., 1989; Woodbury et al., 2000; Hughes et al., 2003).

All primary afferents are thought to use glutamate as a neurotransmitter (de Biasi and Rustioni, 1988; Broman et al., 1993). Identification of vesicular glutamate transporters (VGLUTs), proteins that are responsible for vesicular storage of glutamate, made them suitable immunochemical markers of glutamatergic axons. VGLUT1, 2 or 3 label primary afferent terminals and their spatial distribution in the dorsal horn is highly distinctive. Most myelinated primary afferent fibres that end in lamina I expressed VGLUT2 while those with endings in the deeper laminae were all VGLUT1-immunoreactive (Todd et al., 2003). More recently, VGLUT3 was found to have a restricted distribution in lamina I and the inner part of lamina II. This novel vesicular glutamate transporter is related to umyelinated primary afferents of low threshold mechanoreceptive properties (Seal et al., 2009).

Unmyelinated primary afferent fibres can be differentiated into two major groups: those that contain neuropeptides and those without (Hunt and Rossi, 1985). Generally, all peptidergic C-fibres could be identified with calcitonin gene-related peptide or CGRP (Ju et al., 1987; Todd and Ribeiro-da-Silva, 2005)

and binding of *Bandeiraea simplicifolia* isolectin B4 (IB4) recognises those that lack peptides. The peptidergic and non-peptidergic C-fibre endings are distributed in different spinal dorsal horn regions. Those that contain peptides arborised mainly in lamina I and dorsal part of lamina II, with some terminating further ventrally. Non-peptidergic C-fibres, on the other hand, innervate the central region of lamina II (Ribeiro-da-Silva, 2002). The spatial difference of peptidergic and non-peptidergic C fibres in the dorsal horn suggests that they have different functions. Among them, subsets that express various neuropeptides are present. These neuropeptides colocalise in certain patterns that differ from species to species. In the rat but not cat, for example, primary afferents that contain substance P are exclusive from those that express somatostatin. Peptidergic primary afferent population that contains substance P can also express neurokinin A (Dalsgaard et al., 1985), galanin (Hökfelt et al., 1994) and the opiod peptide, endomorphin-2 (Martin-Schild et al., 1997). Functionally, all peptidergic C-fibres that express substance P were shown to be nociceptors (Lawson et al., 1997) but little is known about the other types of peptidergic C afferents. Some Aδ nociceptors also contain this peptide (Lawson et al., 1997). Vanilloid receptor protein that functions as a receptor for capsaicin and transduces noxious thermal stimuli is found to be present in the central terminals of unmyelinated primary afferents that are positively labelled with the binding of lectin IB4, but not in those of myelinated fibres (Guo et al., 1999). Snider and McMahon (1998), however, reported that both CGRP-expressing and IB4-binding neurons responded to capsaicin. This strongly suggests that there may be an overlap in the functions of the two groups of C-fibres, which involves nociception.

The specific termination patterns of primary afferents in the spinal dorsal horn, together with their complex neurochemical properties, strongly suggest that laminar organization of the spinal dorsal horn as well as the rest of the spinal grey matter may be functionally important.

1.3.1.2 Projection neurons

A major output from the spinal cord is constituted by a significant number of neurons in the dorsal horn with axons that extend beyond this region into various target nuclei in the brain. Anatomical and electrophysiological investigations

have been carried out to determine the termination sites of these projection neurons and their origins in the spinal cord. In the rat lumbar segment, the majority of projection neurons are located in lamina I and many are scattered throughout lamina III-VI, but virtually none is present in lamina II.

Many of these projection neurons send axon collaterals across the midline, passing through the contralateral ventrolateral white matter, then travel rostrally to terminate in specific brain site. Retrograde and anterograde transports of tracers allow individual neurons to be labelled from each brain region and their termination sites in the brain to be mapped, respectively. The main supraspinal target zones include the medullary reticular formation, the nucleus of the solitary tract (NTS), the lateral parabrachial area (LPb), the periaqueductal grey matter (PAG) and the thalamus (Gauriau and Bernard, 2004). Anatomical studies have found lamina I projection neurons that send axons to more than one target nuclei: LPb, PAG and thalamus (Al-Khater and Todd, 2009). Most projection neurons send their axons exclusively to the contralateral brain regions, but a significant proportion of these projects bilaterally (Spike et al., 2003). Anatomical approaches also enable distribution of projection neurons in different laminae to be quantitatively analysed (for example Al-Khater et al., 2008), as well as to identify distinct neurochemical populations among these neurons. For example, approximately 80% of lamina I projection neurons express the neurokinin 1 receptor, which is the main target of substance P (Todd et al., 2000; Spike et al., 2003; Al-Khater et al., 2008). This discrete population of neurons responds to noxious stimuli and is involved with the development of hyperalgesia (Salter and Henry, 1991; Mantyh et al., 1997; Nichols et al., 1999). Less is known of lamina I projection neurons that are not NK1 receptor-immunoreactive. However, a group of neurons with prominently large multipolar dendritic structures, although very sparse, is present in this region. These ‘giant’ cells were found to be enriched with glycine receptor-associated protein gephyrin (Puskár et al., 2001).

Origins and distribution of projection neurons can also be demonstrated by stimulating their axons antidromically in specific brain region. Descriptions of most electrophysiological studies are based on neurons that form the spinothalamic tract, considering its presumed importance in pain perception. Studies in rat, cat and monkey, have shown that a large number of back-fired

neurons after antidromic stimulation are located in lamina I and the deeper laminae (Trevino et al, 1972, 1973; Bester et al., 2000). This is in close agreement to the anatomical observations. Similarly, several studies found spinothalamic neurons to be activated from more than one site, indicating that the same neurons project to multiple targets (Willis and Coggeshall, 2004). Besides antidromic stimulation, intracellular and whole-cell patch-clamp recordings were also used to investigate physiological properties of lamina I projection neurons (for example Zhang et al., 1991; Han et al., 1998; Ikeda et al., 2003; Ruscheweyh et al., 2003). These studies also correlated morphology of the labelled neurons with their physiological characteristics. For example, Han et al. (1998) found modality-selective nociceptive and thermoreceptive neurons in lamina I are restricted to specific morphological type: pyramidal cells are thermoreceptive, fusiform cells are nociceptive, while multipolar cells are either polymodal or nociceptive-specific.

The anterolateral system is not the only output pathway from the spinal cord to the supraspinal regions. Axons of dorsal horn neurons that synapse in the dorsal column nuclei form the postsynaptic dorsal column pathway (PSDC). Retrograde labelling as well as electrophysiological techniques showed that these neurons were predominantly identified in lamina III and IV, and also deeper laminae V-VI of different mammalian species (Brown and Fyffe, 1981; Bennett et al., 1983; de Pommery, 1984; Giesler et al., 1984). In the rat, most neurons project their axons through the dorsal funiculus before terminating in the junction area between the spinal cord and the medulla, i.e. the dorsal column nuclei (Giesler et al., 1984). Interestingly, axons of these neurons extend to the dorsal column nuclei in a somatotopic manner: cells in the lumbosacral enlargement project to the nucleus gracilis while cervical cells terminate in the nucleus cuneatus, which lies lateral to the nucleus gracilis (de Pommery et al., 1984). These PSDC neurons respond to innocuous or to both innocuous and noxious cutaneous stimulation (Willis and Coggeshall, 2004).

The spinocervical tract (SCT) neurons also originate from the dorsal horn. Axons of these neurons project in the dorsal part of the ipsilateral lateral funiculus to terminate in the lateral cervical nucleus (LCN). Both anatomical and electrophysiological techniques were used to understand the characteristics of SCT neurons in the rat but relatively little is known. Baker and Giesler (1984)

found labelled neurons predominantly in lamina III and IV (or the nucleus proprius) after HRP injection in the rat LCN. These neurons were small and their axons ascend in the dorsal lateral funiculus. In the cat, SCT neurons were predominantly identified in lamina IV, with very few in lamina I and the deeper laminae (Craig, 1976). Brown et al. (1980) reported similar neuronal distribution and that the majority was found in the ipsilateral dorsal horn. However, they suggested that SCT neurons did not include those located in lamina I.

The large number of supraspinal target regions of SDH projection neurons suggests that these neurons are most likely to be functionally diverse (Todd, 2010). For example, the LPb (one of the major target regions of lamina I projection neurons) comprises many neurons that are activated by nociceptive inputs from the SDH and project to either the amygdala or the hypothalamus in the forebrain. The parabrachial-amygdala connection is most likely to contribute to the autonomic adaptations and emotional (including aversive) components of pain processing; whereas the parabrachial-hypothalamic tract could play a role in motivational-affective responses during pain states (Gauriau and Bernard, 2002). Projection neurons in the spinal dorsal horn also target thalamic nuclei including the ventral posterolateral nucleus (VPL), the posterior group and the posterior triangular nucleus. The VPL receives tactile inputs from the dorsal column neurons, which send axons to the primary somatosensory cortex. The interconnections between the superficial dorsal horn and these relay thalamic nuclei indicate a probable role in sensory discriminative aspects of pain (Gauriau and Bernard, 2004). Previous studies have also associated target areas in the caudal ventrolateral medulla and the NTS to nociceptive-cardiovascular and nociceptive-cardiorespiratory integration, respectively (Lima et al., 2002; Boscan et al., 2002). The PAG projects to the rostral ventromedial medulla, which in turn sends axons to the dorsal horn laminae. This system provides a critical means of descending modulation of activity in the dorsal horn, since the PAG is recognised as the central site of actions of analgesics and is involved in organising strategies for coping with stressors (Heinricher et al., 2009).

1.4 Lamina II (*substantia gelatinosa*)

For a long time there was debate as to whether the substantia gelatinosa, which was described as the darker and more gelatinous portion of the posterior grey matter, corresponded to Rexed's lamina II, lamina I and II, or lamina II and III (e.g., Wall, Merrill and Yaksh, 1979), However, this was clarified when significant differences in types of primary afferent input and distribution of neuronal markers were identified. One major difference is that lamina III is enriched with myelinated fibres while lamina II lacks them. Therefore, at present, the terms substantia gelatinosa and lamina II are used interchangeably to describe this well delineated band across the dorsal horn.

It is essential to identify discrete neuronal population in lamina II in order to understand their roles in the local circuitry. Despite numerous studies that have attempted to group these neurons into different classes based on their morphology, physiological properties and neurochemistry (see below), there is still no single universally acceptable classification scheme with a unified nomenclature that identifies lamina II neurons.

1.4.1 Morphological classification

Various techniques and approaches have been carried out to reveal morphological features of neurons, including their soma location, shape and sizes, dendritic arborisation and axonal trajectory.

1.4.1.1 Golgi studies

The Golgi stain reveals not only the cell bodies of neurons, but also their dendrites and axons. Generally, a cell body gives rise to a single axon and few primary dendrites that branch and taper along their lengths. The various shapes and sizes of dendritic trees are one of the main attributes used to classify neurons. Two major types of neurons are distinguished based on their axonal extents: Golgi type I neurons have long axons that extend beyond the vicinity of their cell bodies into adjacent laminae or further into the white matter and the brain; while Golgi type II neurons are cells with shorter and locally confined axons, otherwise known as local circuit neurons. The Golgi impregnation

technique has been used in a number of previous investigations to recognize neurons in the substantia gelatinosa, for example Gobel (1975, 1978), Beal and Cooper (1978) and Todd and Lewis (1986).

The Golgi studies by Gobel in 1975 identifies major neuronal cell types in cat's SG layer of the spinal trigeminal nucleus based on criteria such as dendritic and axonal branching patterns, laminar distribution of dendrites and axons, dendritic spine distribution and geometric shapes of dendritic trees. He found the axons and dendrites of SG neurons are highly branched, and a large number of fine dendritic shafts streamed through the layer in all directions (Gobel, 1975). Three morphologically distinctive cell types were identified in this study: islet cells, stalked cells and spiny cells.

The islet cells, which were most frequently encountered in small clusters, had fusiform or rounded cell bodies with dendrites and axons that run in the longitudinal axis. Their cell bodies were found throughout the thickness of layer II and had dendrites that extended rostrocaudally within the layer (Gobel, 1978). Dendrites of islet cells generally tapered in diameter and often had recurrent branches that traveled back in the direction of the soma. The axon emerged from the cell body or, more frequently, from one of the primary dendrites, arborized within the area of the dendritic tree. Like the dendrites, the axons of islet cells were confined within lamina II. Fine axonal branches were characterised by several small boutons en passant. Dendritic spines were widely scattered and sparse. Gobel also reported that this type of cell occupied a somewhat flattened cylinder within the SG, where its rostrocaudally extended dendrites correspond to the length of the cylinder and its cell body was located midway along this axis.

Stalked cells, on the other hand, were found individually in SG and had short, stalk-like dendritic branches that often extended beyond lamina II penetrating lamina III and IV. The cell bodies of these cells were usually situated at the apex of their dendritic trees, giving rise to a cone-shaped appearance (Gobel, 1978). The dendrites of stalked cells often emitted numerous short and long-necked spines evenly throughout their lengths. Their axons provide collaterals in the SG layer and entered the marginal layer (Gobel, 1975, 1978).

Gobel also described a few minor groups of lamina II neurons: spiny cells, arboreal cells and lamina II/III border cells based on their morphological features that were atypical from that of islet and stalked cells (Gobel, 1975, 1978). However, none of the three types was observed in subsequent studies.

With a similar approach, Beal and Cooper (1978) studied laminae I-III neurons in primates. They found lamina II had a higher density of neurons than lamina III. Generally, the laminar organization in adult monkey was comparable to that described by Rexed in the cat spinal cord. Similarly, neurons in laminae I-III were highly variable in size, shape and dendritic organisation. Cells with ventrally extended dendrites, which represent Gobel's stalked cells, were observed in outer region of lamina II. Also, neurons that displayed long dendrites running longitudinally through the ventral portion of lamina II corresponded to islet cells described in the previous studies (Gobel, 1975). Axons of neurons in primates mainly originated from the cell body or a primary dendrite, only occasionally a secondary dendrite. These, together with the presence of boutons en passant, once again support the comparability of dorsal horn neuronal organisation between different species.

Todd and Lewis (1986) identified two main types of Golgi-stained neurons in rat lamina II, which they divided into a dorsal and ventral part. Stalked cell, which typically had rounded soma located in the dorsal part of LII, made up half of the studied population. The main characteristic that distinguished stalked cells from other neurons was their ventrally extended dendrites with some branches spread along the rostrocaudal axis, which give a 'cone-shape' appearance with the soma located at the apex. The dendrites have stalk-like branches and are usually covered with spines. Axons of stalked cells were either limited within the lamina or traveled dorsally into lamina I. Another cell type found was islet cells, very similar to those described by Gobel, which were recognised by their distinctive rostrocaudal dendritic orientation. Typical islet cells were observed to have recurrent dendrite branches that were often restricted within the lamina and displayed few dendritic spines. Cell bodies of islet cells could lie dorsally to, ventrally to or within the same plane as the major part of the dendritic tree. However, over one third of the studied LII neuronal population consisted of unclassified, but morphologically varied, neurons. Cell bodies of these neurons were of various shapes: fusiform or spindle-shaped, round and polygonal. The

extent of their dendritic arborisation also varied. Some of these cells possessed features that resembled those of cells described in other studies, but many showed characteristics of more than one neuronal class.

1.4.1.2 Intracellular injection *in vivo*

As *in vivo* technology advanced, single cell electrophysiological analysis combined with intracellular labelling of neurons was made possible and this revealed the morphology of cells with greater clarity in correlation to their physiological properties.

In addition to information on neuronal receptive fields and their responses to natural or electrical stimulation, structural features of cells with varied functional attributes were investigated through *in vivo* electrophysiological techniques. Light et al. (1979), for example, combined analyses of unitary neuronal activity with intracellular staining to understand the relationship between functional and morphological characteristics of neurons found in the SG, which was divided into two regions: outer and inner. Cells that represent Gobel's stalked and islet cells were identified. Dendrites of most neurons displayed spines and these cells generally had a long rostrocaudal extent. Similarly, the morphologically distinctive islet and stalked cells of Gobel were also found to be present in Bennett et al. (1980)'s population of HRP-stained SG neurons. Cell bodies of islet cells were located throughout lamina II while most stalked cells were situated along the superficial border of this lamina. Both cell types resembled those described in previous studies (Gobel, 1975, 1978). Both islet and stalked cells were observed to have axons that arose from the soma or a primary dendrite, and had numerous boutons. In both studies, parameters such as soma location and size, dendritic extent and branching pattern as well as axonal arborisation were used to characterise neurons in lamina II. These neurons were also found to vary in their responses to natural and electrical afferent stimulation, which was found to correlate with the location and distribution of their principal dendritic arborisation (Bennett et al., 1980). This suggested that stalked and islet cells may represent functionally distinct components in the local neural circuitry.

Rèthelyi et al. (1989) observed no clear relationship between receptive fields and cell morphology but indicated that the laminar arrangement of dendrites could be associated to the nature of primary afferent excitation. Different terminologies were used in this study to describe lamina II cells. 'Limitroph' cells corresponded to the commonly found 'stalked' cells, while 'central' cells had a prominent rostrocaudal extended dendritic trees similar to islet cells. On top of that, they described a few 'central' cells that had dendrites protruding in all directions, which gave them a 'circular' appearance. In contrast to the observation that neuronal functions correlate closely with dendritic arborisation patterns, some have suggested that there is a relationship between the location of cell bodies and their responses to stimulation; hence neurons of same shape and location may serve different functions (Woolf and Fitzgerald, 1983). From such contradictory information, it is most reasonable to propose that morphology of neurons in lamina II is as, if not more, diverse than their functions, and that very little is known of the correlation between these features.

1.4.1.3 *In vitro* labelling

Grudt and Perl (2002) carried out tight-seal whole-cell recordings from individual neurons in spinal cord slices, which were filled with biocytin to reveal their morphology. This was first of several studies that investigated morphological characteristics of single neurons in relation to their electrophysiological properties from spinal cord slices instead of *in vivo*. Neurons that had their somata in lamina II were morphologically diverse and five categories were recognised: islet, central, vertical, radial and medial-lateral. Most lamina II neurons had dendrites that arborised primarily in the rostrocaudal dimension and relatively limited in the dorsoventral and mediolateral axes. Axon distribution, trajectory and branching pattern also varied between cell types. This study emphasized that the SDH is a highly complex region where no single morphological feature could distinguish one neuron from another. Subsequent investigations by the same group on connectivity between lamina II neurons confirmed the distinction between the major morphological types. Islet cells were recognized by their exceptionally extensive dendrites, typically over 400 µm, in the rostrocaudal dimension and central cells were of very similar shape but had considerably shorter dendrites (Lu and Perl, 2003). Vertical cells had

dendrites that travelled extensively in the ventral direction (Lu and Perl, 2005). However, they found about a quarter of lamina II cells in the sample that did not fit into any of the morphological classes.

Hantman and Perl (2004) investigated morphological and physiological properties of spinal dorsal horn neurons selectively labelled by green fluorescent protein (GFP) in a strain of transgenic mice, in which expression of GFP was driven by the mouse prion promoter. The majority of the GFP-expressing neurons had somata that were located in lamina II outer, and had dendrites that were most extensive in the rostrocaudal axis. Axons of these neurons projected within lamina II. Their dendritic and axonal arborisation patterns fitted closely to the morphological characteristics of central cells described in previous studies (Grudt and Perl, 2002; Lu and Perl, 2003). Besides recording from the GFP expressing neurons, they also recorded from non-GFP expressing cells located in the centre region of lamina II and found them to be morphologically varied and very similar to those recognised in hamsters by Grudt and Perl (2002). This study further confirms that characteristics of lamina II neurons highly correspond between species, namely hamster, rat and mouse.

With a similar method, Heinke et al. (2004) studied subsets of GABAergic neurons that expressed GFP under the control of the promoter for the GABA-synthesizing enzyme GAD67 in transgenic mice. They found these GAD67-GFP neurons contribute almost one third of the GABAergic neuron population in lamina II of the spinal dorsal horn. A proportion of GFP-expressing and non-GFP-expressing neurons in this region were morphologically analysed based mainly on the dendritic orientation and laminar location. Three major categories of cells were identified: islet, vertical and radial. Generally, characteristics of each neuronal group agreed with observations in previous studies (Gobel, 1978; Bicknell and Beal, 1984; Grudt and Perl, 2002). Islet cells were most abundant among GFP-expressing neuronal population while non-labelled neurons were more varied and fitted features of other previously classified morphological types. Islet cells had cell bodies located throughout the depth of lamina II but their dendrites were confined within the lamina. Vertical cells, conforming to descriptions from previous studies (Grudt and Perl, 2002), had dendrites that descended into lamina II or more ventrally. Cell bodies of vertical cells were mostly found in outer part of lamina II while majority of radial cells were

located in lamina II inner. Radial cells had dendrites that radiated in all directions and their dendritic trees could be either compact or loose.

Yasaka et al. (2007) carried out blind whole-cell voltage-clamp recording in horizontal slices from rat spinal cord and identified islet, central, radial and vertical cells in the SG. Morphology of the Neurobiotin-labelled neurons was defined based mainly on their dendritic arborisation patterns and the neurons closely resembled those described by Grudt and Perl (2002). To avoid subjective judgements and difficulties when classifying neurons by visual inspection, they combined several morphometric parameters to analyse the different morphological types. This method supported that each cell type could be quantitatively distinguished from others by using a combination of suitable geometric properties.

Maxwell et al. (2007) examined Neurobiotin-labelled neurons in rat spinal cord slices and included axonal projections as one of the criteria to characterise neurons in the superficial laminae. They found all lamina II neurons had axon collaterals that arborized locally. A quarter of the neurons resembled islet cells, and had cell bodies located in the inner part of lamina II. Axons of islet cells extended over a similar RC distance to their dendritic trees but were more extensive in the dorsal-ventral axis and also travelled in the mediolateral direction. Vertical cells appeared similar to those described by Yasaka et al. (2007). Locations of their axons varied: a few cells had axons confined to lamina II, while axons of others extended into both laminae I and II (Maxwell et al, 2007). They also described cells that resembled radial and central cells of previous studies (see above), as well as an antenna cell that was similar to those described by Rethelyi and Szentágothai (1969). Several concurrent studies have examined morphology of lamina II neurons as part of other investigations, and have found similar cell types to those described above (for example Melnick, 2008; Abe et al., 2009; Wang and Zylka, 2009).

These *in vitro* investigations were consistent in proving that distinctive morphologically varied neurons are present in lamina II and this suggests that each neuronal class is most likely to serve functions exclusive of each other.

1.4.1.4 Trans-synaptic labelling

Trans-synaptic labelling described by Cordero-Erausquin et al. (2009) is one of the most recent and novel approaches to label interneurons in the spinal dorsal horn. Retrogradely transported adenoviral vectors encoding GFP conjugated to TTC (nontoxic fragment C of the tetanus toxin) were use to label spinoparabrachial projection neurons in lamina I and the lamina II neurons that were presynaptically connected to them.

GFP expression allowed labelling of entire dendritic trees and made comparison of morphological properties between the projection neurons and neurons presynaptic to them possible. Since this approach allows only the fusion protein to be transported transneurally, labelling across multiple synapses was avoided. Although the fusion protein was expressed at a relatively low level in the second-order presynaptic neurons, morphology of these neurons could be revealed after amplification through immunostaining. Morphology of lamina II neurons was compared according to their dendritic extent and neurons with prominent ventrally extended dendrites were most frequently encountered. These neurons had features that closely resembled those of stalked cells or vertical cells described in previous studies (Gobel, 1978; Todd and Lewis, 1986; Grudt and Perl, 2002). Besides the vertical cells, neurons with pyramidal cell bodies located in lamina II outer and cells with dorsally projecting dendrites were also observed. Although not all neurons observed in lamina II could be morphologically classified, no cell resembled the typical islet cells defined in previous studies (e.g. Todd and Lewis, 1993, Grudt and Perl, 2002; Maxwell et al., 2007; Yasaka et al, 2007).

1.4.2 Functional and physiological properties

Neurons, like other living cells, are separated from the extracellular environment by a plasma membrane. This is characterised by transmembrane proteins embedded in its lipid bilayer that form ionic channels, which allow transportation of ions between the interior and the exterior of the cell. Ions are unevenly distributed across the neuronal plasma membrane, leading to a difference in electrical potential between the two sides of the membrane. This is maintained by a continuous active exchange of ions through the ion-specific

channels. Ions diffuse in a passive manner down their concentration gradient through open channels, with Na^+ moving from the extracellular fluid to the interior of the cell, while K^+ does the reverse. Ion pumps and transporters compensate for passive ionic diffusion by actively transporting ions in both directions through the membrane. The electrical potential difference between the interior of a neuron and its external environment gives rise to membrane potential. The presence and alteration of membrane potential in neurons, which are excitable, allows ionic currents to generate electrical signals from cell to cell and makes neuronal communication possible. Membrane potential changes as ion channels open and close, resulting in the interior voltage becoming more positive (depolarisation), or more negative (hyperpolarisation). When the flow of depolarising ionic currents across the membrane exceeds a certain threshold value, action potentials are generated. Action potentials vary in duration, amplitude, and involve different ions. Voltage-gated ion channels are responsible for generation of action potential in neurons, which lead to alteration in ion permeability and other electrotonic (passive) properties of neuronal membrane. Once initiated, action potentials propagate along the axon at high speed without attenuation as 'nerve impulses', thus allowing synaptic information to be conveyed from neuron to neuron. Firing patterns in response to depolarisation or hyperpolarisation are affected by the activity of subliminal voltage-currents in the somatic and/or dendritic membrane. These ionic currents are activated at voltages subthreshold to that of action potentials and have either a depolarising or hyperpolarising effect on the cell membrane Inward subliminal currents including persistent inward Na+ current (I_{NaP}), low-threshold transient Ca2+ current (I_{Ca}) and hyperpolarisation-activated cationic current (I_h) are responsible to depolarise, thus increase membrane excitability. On the other hand, outward currents carried by K^+ such as fast and slow inactivating K^+ currents (I_A, I_D), intracellular calcium-activated K^+ currents, muscarine-sensitive K^+ current (I_M) and inward rectifier K^+ current (I_{KIR}) contribute to membrane hyperpolarisation. These subliminal voltage-gated currents underlie intrinsic firing patterns and are involved in modulating neuronal excitability (Hammond, 2008). The different membrane properties and action potential discharge patterns are useful in characterizing neuronal functions and therefore included in numerous studies (e.g. Yoshimura and Jessell, 1989; Grudt and Perl, 2002;

Heinke et al., 2004; Hu et al., 2006; Graham et al., 2007; Santos et al., 2007; Davies and North, 2009; Wang and Zylka, 2009).

Yoshimura and Jessell (1989b) were among the first to investigate membrane properties of SG neurons in rats using an *in vitro* slice preparation approach. They investigated responses of neurons to depolarisation and hyperpolarisation, and found discharge patterns of individual neurons were affected by expression of various voltage-activated ionic conductances. Hyperpolarisation caused a proportion of SG neurons to exhibit transient outward current, while some showed a time-dependent inward current when the membrane was hyperpolarised from the resting potential. SG neurons that exhibited transient outward current displayed a delay in spike firing from the onset of depolarization, which was thought to be caused by an A-type potassium current (I_A). This may serve to decrease the probability of a neuron generating action potentials upon depolarisation. SG neurons that did not show a transient outward current upon hyperpolarisation fired in a repetitive manner when depolarised. These observations, among others, suggested that functional properties of SG neurons were heterogeneous and underlying currents were most likely to be involved in modification of firing patterns of SG neurons.

Action potential discharge patterns in response to injection of depolarising current pulses have often been used to identify neuronal classes in the dorsal horn. Investigations of lamina II neurons have generally been carried out *in vitro* (e.g. Grudt and Perl, 2002; Ruscheweyh and Sandkühler, 2002; Santos et al., 2007; Davies and North, 2009). The most frequently encountered types of discharge patterns of lamina II neurons include tonic firing, delayed firing, transient (or initial bursting) and single spiking (Graham et al., 2007). Another type of discharge pattern often observed is adapting-firing (also known as phasic firing), when neurons have irregular firings (Ruscheweyh and Sandkühler, 2002; Santos et al., 2007; Davies and North, 2009). Tonic-, transient-, delayed-firing and single-spiking neurons have also been observed in the deep dorsal horn (e.g. King et al., 1988; Morisset and Nagy, 1998).

Grudt and Perl (2002) described a constellation of electrophysiological properties besides firing patterns for superficial dorsal horn neurons. These include responses to electrical stimulation of primary afferents, current-voltage

relationships, the frequency of spontaneous miniature events and neuronal morphology. They observed that islet cells in lamina II responded to depolarising pulse stimulation with a sustained firing of action potentials (tonic firing). Radial cells, on the other hand, displayed spikes only after a significant delay from onset of the depolarising step. Among the central cell population, two subgroups of different action potential firing patterns were identified. One group discharged a few action potentials and went silent when depolarized, another fired in a tonic fashion. Vertical cells had tonic or delayed firing patterns. Functional features often overlapped between neurons of different geometrical forms. However when combined with morphological criteria, it was possible to make a coherent classification of most neurons identified in lamina II. For example, islet cells were found to have lower membrane potentials than neurons of other morphological types and most showed spontaneous inhibitory postsynaptic currents, while both radial and vertical cells exhibited excitatory postsynaptic currents.

Hantman et al. (2004) investigated a selectively GFP-labelled neuronal population in lamina II, which they found all to fire tonically upon depolarization and most of these cells displayed hyperpolarization-activated inward current (I_h). Most of these GFP-expressing neurons were located in the outer part of lamina II and were GABA-immunoreactive. Heinke et al. (2004) found initial bursting firing pattern to be typical of GAD67-GFP neurons, which accounted for about one-third of GABAergic neurons in lamina II, and most cells that did not express GAD67-GFP exhibited either delayed or gap firing pattern. However, no correlation was detected between morphology and firing patterns for this subset of GABAergic lamina II neurons. These two studies provided an insight to the possible presence of subgroups within the inhibitory neuronal population in lamina II (Heinke et al., 2004).

Santos et al. (2007) reported that discharge patterns of excitatory neurons in SG of rats included tonic firing, adapting firing and delayed firing. This study investigated the relationship between synaptically connected neurons and defined a neuron as excitatory when it evoked inward currents in a postsynaptic neuron. They found excitatory cells to dominate the neuronal population in lamina II. Unlike previous studies, Ruscheweyh and Sandkühler (2002) found no tonic firing neurons in lamina II but identified neurons that fired in a delayed or

transient pattern. Lamina II neurons had the highest absolute relative action potential thresholds among neurons in lamina I to III. Delayed firing only occurred when depolarisation was applied from holding potential more negative than -50 mV. Interestingly, they also found that depolarisation from different holding potentials evoked different discharge patterns among these neurons, which implies that action potential discharge patterns may be dependent on the initial holding potentials (Ruscheweyh and Sandkühler, 2002). Like Yoshimura and Jessell (1989b), they also concluded that I_A was responsible for the delayed firing pattern of lamina II neurons. Genetic elimination of Kv4.2 channel subunit, which mediates the majority of A-type current in dorsal horn neurons, increases the number of tonic firing neurons. These results suggest that Kv4.2-mediated I_A plays a major role in regulating the excitability of neurons in this region (Hu et al., 2006).

1.4.3 Neurochemical properties

Neurochemical properties have been extensively used to classify neurons in lamina II. Improvements in immunohistochemical techniques have greatly improved knowledge regarding the expression of various neuropeptides and proteins by individual neurons in this region. Colocalisation patterns of these components can be identified, and this approach can be used to provide information on the functional properties of neurons, thus enabling systematic classification of neurons in order to understand their roles in local somatosensory processing.

1.4.3.1 Classical neurotransmitter

Interneurons in lamina II are classically divided into two main functional groups according to their neurotransmitter content: inhibitory that uses GABA and/or glycine, and excitatory that is glutamatergic.

GABA-containing neurons were initially identified by immunocytochemistry with antiserum to the GABA synthesizing enzyme, glutamic acid decarboxylase (GAD) (McLaughlin et al., 1975; Barber et al., 1978; Hunt et al. 1981). GAD acts to catalyse decarboxylation of glutamate, which produces GABA. Low concentration of this enzyme in neuronal cell bodies makes detection difficult. To circumvent

this problem, colchicine was administered to block axoplasmic transport and to allow accumulation of GAD in cell bodies of neurons that express the enzyme (Barber et al., 1978; Hunt et al. 1981). Although many GABAergic neuronal cell bodies are present in lamina II, only a small number of these could be labelled with GAD since it was detected mostly in axonal boutons. Hunt et al. (1981) reported that GAD-containing neurons were large and predominantly found in the outer region of lamina II and close to the border between lamina II and III. However, later on GAD was found to exist in two isoforms of different molecular weights: GAD65 and GAD67 (Erlander and Tobin, 1991). Individual neurons are thought to express both forms of GAD but in different ratios (Mackie et al., 2003). Distribution of GAD65 and GAD67 immunoreactive neurons also varied between laminae in the spinal grey matter. Magoul et al. (1987) found GAD immunoreactive neurons to concentrate in the superficial part of the dorsal horn. Within lamina II, both GAD65 and GAD67 immunoreactivity were particularly strong in the inner part of lamina II compared with its dorsal portion (Mackie et al., 2003). Maxwell et al. (2007) used both GAD isoforms to identify inhibitory cells in the dorsal horn and found none of the positively-labelled axonal terminals was VGLUT-immunoreactive. In a recent study, it was suggested that GAD65- and GAD67-immunoreactive neurons had distinct responses to peripheral noxious stimulation, as evidenced by the expression of early gene c-fos mainly in GAD65-immunoreactive neurons in the dorsal horn but at a much reduced level in cells that expressed GAD67 (Nowak et al., 2011). This agrees with a previous report on the drastic reduction of GAD65 (but not GAD67) expression at dorsal horn level in post-neuropathic injury models (Moore et al., 2002).

GABA, the predominant inhibitory transmitter in the central nervous system, is present in certain neurons found in all dorsal horn laminae, but they are most abundant in lamina I-III (Willis and Coggeshall, 2004). GABA-immunoreactive cells were identically distributed at cervical, thoracic, lumbar and sacral levels in the rat (Magoul et al., 1987). Neurons that were immunoreactive for GABA were identified throughout laminae I to III in an even manner (Todd and McKenzie, 1989). Evidence from immunocytochemical studies showed that in these laminae, all neurons that contain glycine, another major inhibitory amino acid, were also GABAergic although some GABA-containing neurons in these

regions were not glycine-immunoreactive (Todd and Sullivan, 1990). More recently, stereological analysis showed that approximately 30% of neurons in lamina II are GABAergic, which is consistent with that observed in the previous study, and half of them were glycinergic (Polgár et al., 2003).

Like other neurotransmitters, GABA is stored in synaptic vesicles into which it is accumulated before being exocytotically released to the synaptic cleft. The vesicular GABA transporter (VGAT) is responsible for the uptake of both GABA and glycine into synaptic vesicles (Chaudhry et al., 1998) and is therefore a suitable marker for axons that use GABA and/or glycine. Glycinergic axons can also be revealed with antibody against glycine transporter 2 (GLYT2), a sodium/chloride-dependent transporter (Spike et al., 1997). The distribution of GLYT2 mRNA also suggests that this transporter may be largely restricted to neurons that express glycine throughout the central nervous system (Zafra et al., 1995). However, glycine-immunoreactive boutons are relatively less frequent in laminae I and II than in the deeper laminae (van den Pol and Gorcs, 1988; Todd and Sullivan, 1990).

Early information on morphology of GABA-immunoreactive neurons has been obtained in several studies that used immunocytochemistry with colchicine pretreatment or by combining Golgi method with postembedding immunocytochemistry. GABAergic cells were frequently found to include islet cells (e.g. Barber et al, 1982; Todd and McKenzie, 1989). Morphology of GABA immunoreactive neurons was more clearly delineated by Todd and McKenzie (1989) by incorporating immunocytochemical method with Golgi impregnation of lamina II neurons. More than a quarter of the Golgi-stained neurons were GABA immunoreactive and majority of these cells represented typical islet cells. These islet-like cells had fusiform cell bodies and dendrites that ran along the rostrocaudal axis, often giving out recurrent branches. The rest of the GABA-containing neurons had rostrocaudally extended dendrites that were more restricted in comparison to the typical islet cells but often with branches that extended for a short distance in the dorsal or ventral directions. Another type of GABAergic neuron had radiating dendrites. Interestingly, none of the neurons that had stalked-like dendritic arborisation was GABA immunoreactive. A few cells that resembled islet cells, with significantly smaller cell bodies, were also non-GABAergic. Observations from this particular study suggested that cells of

different morphological properties may be functionally different and cells that do not belong to the common islet or stalked classes may also contain GABA. This was confirmed in several recent studies, which reported GABA-containing cells to include islet, radial, central and vertical types (e.g. Hantman et al., 2004; Heinke et al., 2004; Maxwell et al., 2007).

Glutamate has long been known to be the major excitatory neurotransmitter in the central nervous system. Experimental destruction of interneurons greatly reduced glutamate concentration in the dorsal region of spinal grey matter (Davidoff et al., 1967), whereas severing of dorsal roots did not significantly altered the level of glutamate in the spinal cord (Roberts and Keen, 1974). In addition, the majority of neurons in the superficial dorsal horn do not contain GABA or glycine (Todd and Sullivan, 1990; Todd, 1991; Todd and Spike, 1993). Therefore, it is reasonable to assume that not only all primary afferents, but many intrinsic neurons in this area are excitatory and use glutamate. In early studies, identification of glutamatergic interneurons in the spinal dorsal horn was difficult largely due to lack of suitable immunocytochemical markers for glutamatergic axons (Todd and Spike, 1993). Glutamate immunoreactivity has been revealed in neuronal cell bodies in the rat superficial dorsal horn with glutamate antisera (Miller et al., 1988). However, later studies showed that it is not reliable to identify glutamatergic neuronal cell bodies because the antisera also labelled neurons that were GABAergic and glycinergic (Yingcharoen et al., 1989; Walberg et al., 1990). Lack of satisfactory markers for cell bodies of glutamatergic neurons and presence of primary afferent axons that are thought to also use glutamate make identification of glutamatergic neurons in the superficial dorsal horn more difficult (Todd and Spike, 1993). Like GABA and glycine, glutamate is also packaged and stored in specialized secretory vesicles. The discovery of the vesicular glutamate transporters (VGLUTs) has provided a useful tool to identify glutamatergic neurons. Three different transporters: VGLUT1, VGLUT2, and VGLUT3 have been identified. These VGLUTs accumulate glutamate into synaptic vesicles and therefore account for exocytotic release of glutamate at glutamatergic nerve endings. VGLUT1 and VGLUT2 are isoforms of Na^+-dependent inorganic phosphate co-transporter family which localise in largely exclusive glutamatergic CNS neuronal populations (Busch et al., 1996; Bellochio et al., 1998, 2000; Fujiyama et al., 2001; Kaneko et al., 2002; Varoqui

et al., 2002). Activity of both VGLUT1 and VGLUT2 are ATP-dependent and stimulated by physiologically relevant concentrations of chloride (Bai et al, 2001). VGLUT3 is highly homologous to the structures and functions of VGLUT1 and VGLUT2 (Gras et al., 2002). However, distribution of VGLUT3 is more restricted and it is expressed by not only excitatory neurons, but also subpopulations of inhibitory cells in the brain as well as cholinergic interneurons, monoamine neurons and glia (Fremeau et al., 2002; Gras et al; 2002; Somogyi et al., 2004). VGLUT1 and VGLUT2 are only present in nerve fibres and not cell bodies (Fremeau et al., 2002; Oliveira et al., 2003). VGLUT2 immunoreactivity is present throughout the spinal grey matter but it is particularly dense in lamina I, II and X (Todd et al., 2003). By using a combination of different neuropeptide markers (see below) together with VGLUT2, Todd et al. (2003) provided evidence that most of the VGLUT2 staining seen in the dorsal horn represented axons of local glutamatergic interneurons, whereas VGLUT1 and VGLUT3 were related to primary afferent axons. Previous studies have reported that glutamatergic neurons included vertical and radial cells (Todd and McKenzie, 1989; Maxwell et al., 2007). In addition, Todd and Spike (1992) found small islet cells that were not GABA-immunoreactive, suggesting that this group of cells were also likely to use glutamate.

1.4.3.2 Neuropeptides and proteins

Many peptides are present in high concentration within axons of neurons, particularly in the superficial region of the spinal dorsal horn. Some peptides are restricted to primary afferents; some are normally only present in local intrinsic neurons while others are found in both (Todd, 2006). The majority of peptide-containing neurons have axons that arborise locally, only with some exceptions that possess ascending axons that project to various targets in the brain (Todd and Spike, 1993). Peptides are stored in large dense-cored vesicles and released by a calcium-dependent mechanism. Neuropeptides are concentrated in axonal boutons but in some cases they are detectable in the perikaryal cytoplasm of the neurons that express them. They often coexist with the 'classical' neurotransmitters and more than one peptide may be present in an individual neuron. Therefore, distribution of neuropeptides and their colocalisation patterns with each other and with other amino acid transmitters provide means to systematically characterise neurons in the spinal dorsal horn.

Among the peptides that are commonly present in dorsal horn neurons, some are found mainly or exclusively in either GABAergic or glutamatergic neurons. **Enkephalin**, one of the opioid peptides that is present in the spinal dorsal horn, is most abundant in lamina II. There is evidence that enkephalin coexists with GABA in some neurons within the rat dorsal horn (Todd et al, 1992). However, more recent studies reported a great majority of enkephalin-immunoreactive axon terminals in the dorsal horn are glutamatergic (Todd et al., 2003; Marvizón et al., 2007, 2009).

Numerous **neurotensin**-immunoreactive cell bodies were present in the ventral part of lamina II and few were also seen in its dorsal region (Todd and Spike, 1993). Lamina II neurons that contained neurotensin were reported to have dendrites that were elongated along the rostrocaudal axis (Seybold and Elde, 1982). **Somatostatin** is present in all three superficial layers of the spinal dorsal horn (Ribeiro-da-Silva and Cuello, 1990; Alvarez and Priestley, 1990), but it is most concentrated in the ventral half of lamina II (Todd and Spike, 1993). Neurons that contained somatostatin or neurotensin were not GABA immunoreactive and their axons contained VGLUT2 (Todd et al., 1992; Todd and Spike, 1993; Simmons et al., 1995; Todd et al., 2003). However, these two types of peptides were never colocalised in non-GABAergic neurons (Proudlock et al., 1993).

Neuropeptide Y (NPY), a peptide of 36 amino acids, is normally only present in axons of local interneurons (Todd, 2006). Distribution of NPY differs between the superficial laminae, but many NPY-containing neurons were identified in lamina II (Hunt et al., 1981; Hökfelt et al., 1981; Sasek and Elde, 1985; Krukoff, 1987). Hunt and colleagues (1981) described the NPY-containing neurons in this region to be relatively large with a rostrocaudal or oblique dendritic arborisation. In the rat dorsal horn, NPY was reported to colocalise with GABA, but very infrequently with glycine (Rowan et al., 1993; Todd and Spike, 1993). By using double-labelling immunofluorescence method, NPY and Met-enkephalin were shown to be present in different axonal boutons in the dorsal horn (Rowan et al., 1993), and therefore it is likely that NPY and enkephalin exist in exclusive neuronal populations in the spinal dorsal horn (Todd and Spike, 1993). **Galanin** is a 19 amino-acid peptide observed in many neuronal cell bodies of interneurons in the superficial dorsal horn (Melander et al., 1986; Arvidsson et al., 1991;

Kordower et al., 1992). These galanin-immunoreactive neurons have small cell bodies (Todd and Spike, 1993) and which are GABA-immunoreactive (Simmons et al., 1995). Overall, results from previous studies suggest that certain neuropeptides (NPY and galanin) are expressed by GABAergic interneurons, while others (somatostatin, neurotensin) are restricted to neurons that contain glutamate. Enkephalin, however, is expressed by both GABAergic and glutamatergic interneurons (Todd, 2006; Todd and Spike, 1993; Fleming and Todd, 1994; Todd et al., 2003).

Certain proteins, besides the classical neurotransmitters and peptides, are also useful as neurochemical markers to distinguish neuronal populations in the superficial dorsal horn. However, proteins are present throughout the soma and dendrites and can thus reveal the morphological structures of neurons that express them. These proteins include calcium binding proteins, enzymes and some neuropeptides receptors (Todd, 2006). Three calcium-binding proteins have been found in superficial dorsal horn neurons: calbindin, calretinin and parvalbumin (Ren and Ruda, 1994). These were distributed throughout the cytoplasm of neurons that express them. All lamina II neurons that contain calbindin lack GABA and are therefore likely to be glutamatergic (Antal et al., 1991). Evidence showed that calbindin and calretinin are present in dorsal horn neuron populations that partially overlapped, and neither group is predominantly immunoreactive for GABA or glycine (Albuquerque et al., 1999). **Parvalbumin** (PV) is mostly expressed by inhibitory neurons that express both GABA and glycine (Antal et al., 1991; Laing et al., 1994). Neurons that contain parvalbumin are scattered in lamina II and III, but are most frequently found in the ventral part of lamina II (Laing et al., 1994). Nitric oxide is a gas that has diverse effects on neural signaling in the central nervous system (Vincent, 1994). The neuronal form of nitric oxide synthase (**nNOS**), a neuronal nicotinamide adenine dinucleotide phosphate (NADPH) diaphorase, is responsible for converting arginine to citrulline generating nitric oxide. Thus, histochemical reactions for NADPH diaphorase have been used interchangeably with nNOS antibody reactions to indicate NO production (Hama and Sagen, 1994). nNOS-labelled neurons are distributed throughout the spinal cord (Valtschanoff et al., 1992), but they are most abundant in the superficial laminae. Spike et al. (1993) reported that the majority of these neurons contained GABA and most were also glycine-

immunoreactive. nNOS is most likely to be restricted to inhibitory interneurons because nNOS is shown to not coexist with glutamate (Spike et al., 1993; Valtschanoff et al., 1993; Laing et al., 1994; Reuss and Reuss, 2001). Although both PV and nNOS were found in neurons that were GABA- and glycine-immunoreactive, these two populations were exclusive of each other (Laing et al., 1994). Protein kinase C is an enzyme that is essential in signal transduction. There has been much interest in its gamma-isoform (**PKCγ**) since the discovery of it as being involved in development of neuropathic pain (Malmberg et al., 1997). PKCγ is restricted to a population of excitatory interneurons in the dorsal horn that are concentrated in laminae II inner and III. These neurons include many of those that express neurotensin or somatostatin (Polgár et al., 1999). PKCγ labels both cell bodies and dendrites of immunoreactive cells, thus revealing their diverse morphology. In lamina II, however, many PKCγ neurons have dendrites that arborize along the rostrocaudal axis and resemble central cells (Todd and McKenzie, 1989; Polgár et al., 1999). The specific distribution of PKCγ makes it a useful marker to distinguish the ventral portion of lamina II from its dorsal part.

1.5 Aims and objectives

Despite numerous investigations on neurons in the superficial dorsal horn, with various techniques and approaches, there is still no universally accepted scheme that can be used to classify all neurons in this region. At present, very little is known of the distinct functions of neuronal types that populate this area or the relationship (if any) between their physiological, morphological and neurochemical characteristics. This study aimed to extend our knowledge by testing the following hypotheses:

1. That discrete populations of lamina II neurons can be distinguished by identification of firing pattern, morphology and neurotransmitter phenotype. This hypothesis was tested by carrying out blind whole-cell patch-clamp recordings on adult rat spinal cord slices from neurons in lamina II. Firing patterns of neurons were determined in response to depolarising current injections, and membrane properties were assessed. All neurons that had undergone whole-cell recording were identified histologically by the presence of

Neurobiotin labelling. Morphology of each cell was reconstructed after subsequent immunocytochemical processing and confocal microscopy. Expression of VGAT- or VGLUT2-immunoreactivity was examined in axon terminals belonging to each cell to identify inhibitory and excitatory interneurons, respectively.

2. That physiological, morphological and neurochemical properties of lamina II neurons are distinctively correlated. There have been numerous attempts to dissect the relationship between morphology and firing pattern of SDH neurons (Heinke at al., 2004; Melnick et al., 2004; Graham et al., 2007; Davies and North, 2009); as well as to identify correlations between firing pattern and neurotransmitter type (Heinke at al., 2004; Santos et al., 2007). However, the correlation pattern is likely to be complex, since certain firing patterns are found in overlapping morphological classes (Grudt and Perl, 2002; Melnick et al., 2004) and both GABAergic and glutamatergic cells have been identified among morphologically diverse groups (Hantman et al., 2004; Heinke et al., 2004; Lu and Perl, 2007; Maxwell et al., 2007). This study compared electrophysiological properties (in particular, firing pattern in response to current injection) and morphology of lamina II neurons, for which the neurotransmitter phenotype was identified, in order to distinguish specific functional subpopulations in lamina II.

3. That inhibitory and excitatory interneurons respond differently to neuromodulators. Noradrenaline, serotonin and somatostatin were applied to a sample of the recorded neurons to investigate whether responses to different neuromodulators were associated with particular cell types. This hypothesis is proposed since previous studies have shown that these neuromodulators act on the majority of lamina II cells (North and Yoshimura, 1984; Ji et al., 1999; Grudt et al., 1995; Kim et al., 2002; Lu and Perl, 2007; Abe et al., 2009) but information on responses of specific cell types to these drugs is very limited.

4. That only inhibitory neurons respond to somatostatin. Immunocytochemical evidence has shown that in the dorsal horn, only GABAergic neurons express the somatostatin receptor sst2a (Todd et al, 1998). Bath application of somatostatin was carried out to test the response of lamina II neurons to the drug.

5. That a proportion of excitatory lamina II interneurons contain somatostatin and/or enkephalin. Somatostatin is synthesised by glutamatergic cells (Proudlock

et al., 1993) and a substantial number of enkephalin-immunoreactive axon terminals in the dorsal horn also contained glutamate (Todd et al., 2003; Marvizón et al., 2007, 2009). This hypothesis was tested by reacting sections that contained NB-labelled axonal arborisation from lamina II glutamatergic cells with antibodies against somatostatin and enkephalin to determine expression of these neuropeptides.

Neuronal diversity in the superficial dorsal horn remains one of the biggest challenges in attempts to identify discrete functional populations in this region. This information is critical for an improved understanding of the neuronal circuitry involved in nociceptive processing within the spinal cord and for identifying mechanisms that underlie chronic pain states. The above hypotheses were proposed to examine various properties of lamina II neurons and to correlate these characteristics in order to classify neurons, which would allow refinement of neuronal classification and identification of their precise functions in the sensory processing pathways.

Chapter 2

Materials and Methods

2 Materials and Methods

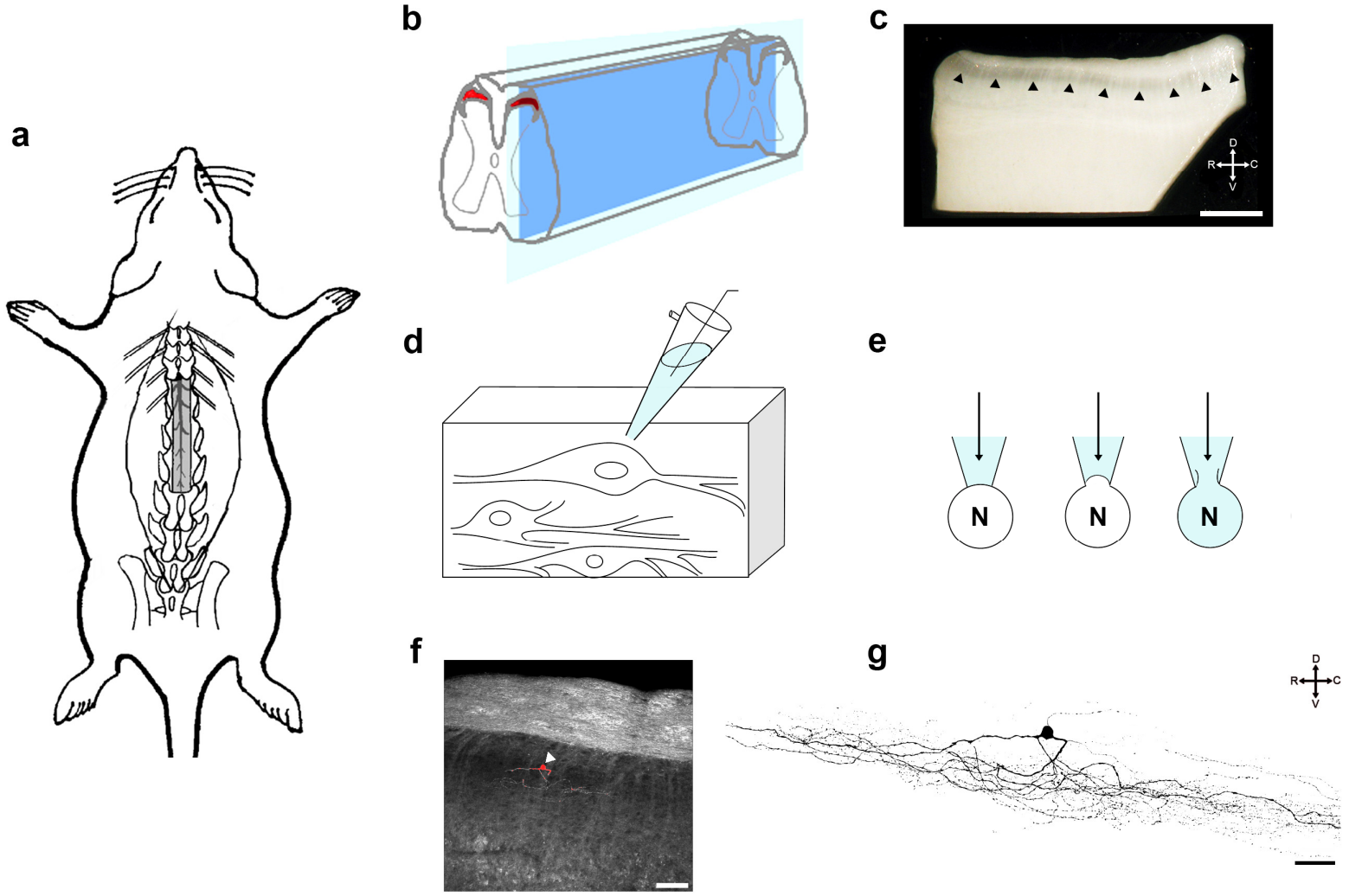
2.1 Animals and slice preparation

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

Adult male Wistar rats aged 6 to 10 weeks (162-324 g) were deeply anaesthetized with a volatile anaesthetic (halothane or isoflurane; 1.5-4%) in a mixture of oxygen and nitrous oxide. This was administered through a plastic tube attached to a mask covering the animal's mouth and nostrils. The level of anaesthesia was checked by applying pressure on the hindpaw and checking the withdrawal reflex before thoracolumbar laminectomy was carried out. The rat was placed prone on a heating blanket to maintain normal body temperature while its limbs were fixed on the blanket with cellophane tape. Hair on the animal's back was shaved and saline was injected subcutaneously to maintain the physiological condition during operation. An incision was made longitudinally along the rostral-caudal axis. Skin and connective tissues overlying the vertebral column were carefully cut away until the vertebral column was entirely visible. Occasional bleeding was stopped by cauterization or by applying a gentle pressure with cotton tips. Then, the dorsal part of the vertebral column was removed until the spinal cord was exposed. A two to 3 cm length of spinal cord from the L2-L5 region (Figure 2-1 a) was excised and immediately placed into a bath of preoxygenated, ice cold (1-3°C) dissection solution. The animal was then killed by anaesthetic overdose and decapitation.

Under magnification using a binocular dissecting microscope, dura mater and pia mater covering the spinal cord were removed. This was necessary to enable the vibrating blade microtome to properly cut the cord. In most cases, all dorsal and ventral roots were removed. Only in a few experiments, one or two dorsal roots were kept intact. The cord was trimmed at both ends and then laid flat, dorsal side facing downwards, in a Petri dish. Later, 3% low-melting-point agarose was prepared, cooled and slowly poured over the cord until it was fully embedded. This was kept at 4 °C for approximately 20 minutes until the agar was

Figure 2-1 Whole-cell patch-clamp recording. Schematic representation of slice preparation procedures for whole-cell patch-recording. **a**: Thoracolumbar laminectomy was carried out on anesthetized adult rats to excise a 2-3 cm length of spinal cord from the lumbar enlargement region (shaded); **b**: Parasagittal slices were cut using a vibrating blade microtome (blue pane shows plane of section, SDH is highlighted in red); **c:** the SDH appears as a distinctive dark band in dark field illumination due to the relative absence of myelinated fibres, which is most obvious at the border of laminae II and III (delineated by arrowheads). Scale bar = 1 mm; **d:** Glass micropipette electrode of 7 to 12 MΩ, filled with internal pipette solution that contains Neurobiotin, is slowly inserted into the spinal cord slice until it detects a neuron; **e:** tip of the micropipette is pressed against the neuronal membrane and suction is applied to assist formation of a tight gigaohm seal before the neuron (N) is ruptured to allow access to its intercellular space and to record from it; **f**: After completion of whole-cell patch-clamp recordings, a Neurobiotin-filled neuron is revealed with avidin-rhodamine (red). Scale bar = 100 μm; **g:** image of the recorded neuron obtained from projected confocal scans after morphology reconstruction. Crosshair shows the rostral (R), caudal (C), dorsal (D) and ventral (V) axes. Scale bar = 50 μm.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completely solidified. Blocks of agar-embedded spinal cord were trimmed and then cut using a VT1000S vibrating blade microtome (Leica Microsystems Ltd, Milton Keynes, UK) into 500 μm parasagittal or horizontal slices (Figure 2-1 b) while immersed in a continuously oxygenated cold dissection solution bath. The slices were then transferred to the recovery chamber where they were perfused by oxygenated (with 95% O_2, 5% CO_2) normal Krebs' solution at room temperature, for at least 30 minutes before they were ready for electrophysiology recording.

2.2 Whole-cell patch-clamp recording

All electrophysiological experiments and analysis were carried out by Dr Toshiharu Yasaka.

A single slice of tissue was transferred to a recording chamber and perfused with normal Krebs' solution at 10 ml min^{-1} at room temperature. Glass micropipettes of 7 to 12 MΩ were prepared by using a Flaming/Brown type micropipette puller (Model P-97, Sutter Instrument) and filled with internal pipette solution that contains Neurobiotin. In all cases, the micropipettes contained 0.2% Neurobiotin (catalogue number SP-1120, Vector Laboratories, Burlingame, CA).

Blind whole-cell current- and/or voltage-clamp recordings were made from neurons in lamina II, which was visualised using a dissecting microscope as a translucent band across the dorsal horn. After identifying the region to record from, using a stage manipulator, the micropipette was adjusted and lowered until its tip reached the surface of the tissue slice. The micropipette was slowly inserted into the slice until a neuron was detected. The aim was to achieve whole-cell configuration where a seal of high resistance was formed between the pipette tip and cell membrane before a neuron could be ruptured and recorded from (Figure 2-1 d, e). Signals were acquired with a patch-clamp amplifier (Axopatch 200B or MultiClamp 700B; Scientifica Ltd, Uckfield, UK) and pCLAMP 9 or 10 acquisition software (Scientifica; Uckfield, UK). Signals were low-pass filtered at 5 kHz, amplified 10-fold in voltage-clamp mode or 50-fold in current-clamp mode, sampled at 10 kHz and analysed offline using pCLAMP 9 or 10. No correction for the liquid junction potential was made during recording.

The resting membrane potential of the neuron was immediately determined after establishing the whole-cell configuration. In this study, only cells with resting membrane potentials of -40 mV and more negative were included in the sample. Passive membrane properties of neurons were monitored throughout recording using the built-in software of pCLAMP. However, Ruscheweyh et al (2003) had found this software to be rather unreliable in determining values of membrane properties, especially membrane resistance. Therefore, precise membrane resistance and capacitance were calculated from the currents in response to hyperpolarising voltage steps from -70 to -80 mV at duration of 200 ms.

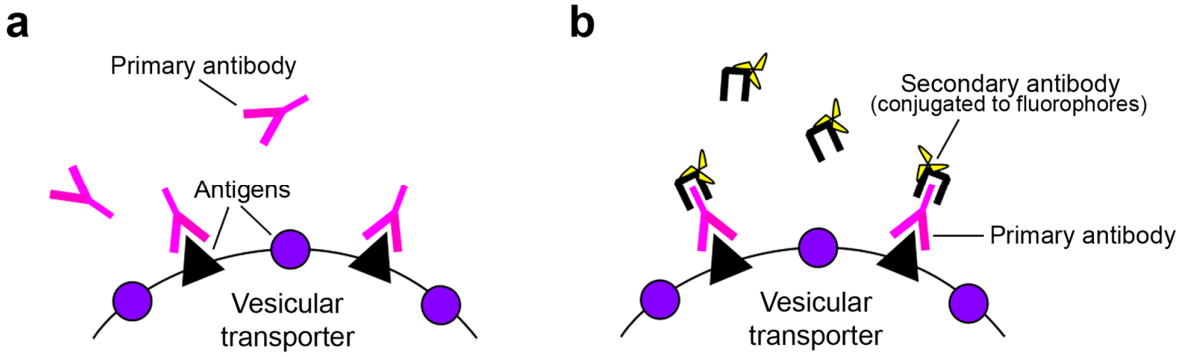
2.3 Drug application

The actions of neuromodulators noradrenaline (NA, bitartrate salt, 20-40 μM; Sigma-Aldrich, Gillingham, UK), serotonin (5HT, hydrochloride salt, 10-40 μM; Sigma-Aldrich, Gillingham, UK) and somatostatin (SST, 1-2 μM, Merck Chemicals Ltd, Nottingham, UK) were tested by bath application for 1 minute on a sample of the recorded neurons. The drugs were dissolved in Krebs' solution and were applied via three-way stopcocks without any change in either perfusion rate or temperature. The choice of doses for each drug was based on those found to be effective in previous studies, with noradrenaline at 20 or 40 μM, serotonin at 20 or 40 μM and somatostatin at 1 or 2 μM (Kim et al., 2002; Jiang et al., 2003; Lu and Perl, 2007). For each cell, only one concentration of each neuromodulator was tested.

2.4 Tissue processing and immunocytochemistry

The spinal cord slices were processed for confocal microscopy to reveal each intracellularly Neurobiotin-filled neuron. In addition, immunocytochemistry was used to assess the presence of specific antigens, in this case vesicular transporters, to allow visualisation of VGLUT2 and VGAT in each recorded neuron. In this study, the indirect immunocytochemistry method, where unlabelled primary antibodies that react to specific antigens in neurons and fluorescent-conjugated secondary antibodies that react with the primary antibodies, was used (Figure 2-2). For example, rabbit primary antibody raised

Figure 2-2 Indirect immunocytochemistry (ICC) method. Schema shows the basic mechanism of indirect ICC used to identify presence of specific neurotransmitters or neuropeptides in this study. **a**: Unlabelled primary antibodies identify and bind to specific antigens on the surface of vesicular transporters; **b:** Species-specific fluorescent-conjugated secondary antibodies are used to reveal the primary antibodies and enable examination with a confocal microscope.


a
Primary antibody
Antigens
Vesicular
transporter
b
Secondary antibody
(conjugated to fluorophores)
Primary antibody
Vesicular
transporter

against VGAT binds specifically to epitopes expressed by the vesicular transporter, if present, in neurons; which is then revealed by a fluorescent-conjugated secondary antibody raised against rabbit IgG in donkey that binds to the antigen sites on the primary antibody. Because the secondary antibody is covalently tagged to a fluorophore and it binds to the primary antibody, neurons that express the probed VGAT are visible when examined with a confocal microscope.

2.4.1 To reveal Neurobiotin-labelled neurons

Following completion of whole-cell patch-clamp recordings, the slices were fixed overnight in 4% formaldehyde in 0.1 M phosphate buffer (PB) at 4 °C. Slices were rinsed in 0.1 M PB, three times for 10 minutes each, then flat-embedded in 3% agarose. The tissue blocks were then cut into 60 µm-thick parasagittal sections with a VT1000S vibrating-blade microtome (Leica Microsystems Ltd, Milton Keynes, UK). Serial sections were collected and stored in 0.1 M PB. These sections were immersed free-floating in 50% ethanol for 30 minutes to enhance antibody penetration (Llewellyn-Smith and Minson, 1992) and then rinsed three times in phosphate-buffered saline that contains 0.3 M NaCl (PBS). After that, they were incubated free-floating at 4 ºC overnight in avidin-rhodamine (1:1000; Jackson ImmunoResearch, West Grove, PA, USA) to reveal the intracellularly Neurobiotin-labelled neurons (Figure 2-1 f).

All antibodies and avidin-rhodamine were diluted in PBS that contained 0.3 M NaCl and 0.3% Triton X-100. After 24 hours, all sections were rinsed three times with PBS, mounted on glass slides, covered with cover slips in anti-fade medium (Vectashield; Vector Laboratories, Burlingame, CA, USA) and stored at -20 ºC.

2.4.2 To reveal neurotransmitter phenotype of neurons

Details of the primary antibodies and secondary antibodies used in this study are shown in Table 2-1 and Table 2-2.

To determine the neurochemical phenotype of each of the labelled cells, selected sections containing axonal boutons (at least one section from each cell) were rinsed three times, for 10 minutes each, in PBS and were then reincubated

Table 2-1 Primary antibodies used in this study

Antibody	Species	Working dilution	Source	Catalogue no.
VGAT	Rabbit	1:1000	Synaptic Systems, Göttingen, Germany	131002
VGLUT2	Guinea pig	1:5000	Chemicon International (now Millipore), Temecula, CA, USA	AB5907 AB2251
Enkephalin	Mouse	1:1000	Millipore, Temecula, CA, USA	MAB350
Somatostatin	Rabbit	1:1000	Peninsula (now Bachem), San Carlos, CA, USA	T-4103

Table 2-2 Secondary antibodies used in this study

Antibody	Species	Working dilution	Source	Catalogue no.
Alexa 488	Rabbit	1:500	Molecular Probes, Eugene, OR, USA	A21206
Cyanine 5.18	Guinea pig	1:100	Jackson ImmunoResearch, West Grove, PA, USA	83927
Cyanine 5.18	Mouse	1:100	Jackson ImmunoResearch, West Grove, PA, USA	84536
Avidin rhodamine		1:1000	Jackson ImmunoResearch, West Grove, PA, USA	54078

for 3 days in a cocktail of guinea pig antibody raised against vesicular glutamate transporter 2 (VGLUT2; 1:5000; Millipore/Chemicon) and rabbit antibody raised against vesicular GABA transporter (VGAT; 1:1000; Synaptic Systems). This was followed by incubation in species-specific secondary antibodies that were raised in donkey and conjugated to cyanine 5.18 (Cy5; 1:100; Jackson ImmunoResearch) and Alexa 488 (1:500; Molecular Probes), respectively.

All antibodies and avidin-rhodamine were diluted in PBS that contained 0.3 M NaCl and 0.3% Triton X-100. On completion of immunocytochemical reactions, all sections were mounted in Vectashield and stored at -20°C.

2.4.3 To determine neurochemical subclasses of neurons

For 24 neurons that had VGLUT2-immunoreactive axons, additional sections that contained parts of the labelled axonal arborisation were reacted with antibodies against neuropeptides somatostatin and enkephalin. First, these sections were rinsed three times in PBS and were then incubated for 72 hours in a cocktail of rabbit antibody raised against somatostatin (SST; 1:1000; Peninsula/Bachem) and mouse antibody raised against enkephalin (EnK; 1:1000; Millipore). This was followed by overnight incubation in species-specific secondary antibodies that were raised in donkey and conjugated to Alexa 488 and Cy5, respectively.

All primary and secondary antibodies were diluted in PBS that contained 0.3 M NaCl and 0.3% Triton X-100. On completion of immunocytochemical reactions, all sections were mounted in anti-fade medium (Vectashield; Vector Laboratories) and stored at -20°C.

2.4.4 Antibody characterisation

Guinea pig anti-vesicular glutamate transporter 2 (VGLUT2) and rabbit anti-vesicular GABA transporter (VGAT) polyclonal antibodies were used in this study. Two VGLUT2 antibodies were used. Both were raised against a peptide from the C-terminus of the rat VGLUT2. Pre-absorption of the VGLUT2 antiserum with immunogen peptide eliminates all immunostaining (manufacturer's specification). Specificity of both VGLUT2 antibodies was confirmed by dual immunofluorescence staining with a well-characterised rabbit antibody against

VGLUT2 (Todd et al., 2003). In each case, identical structures were stained by the rabbit and guinea pig antibodies. The VGAT antibody was raised against a peptide corresponding to amino acids 75-87 of the rat VGAT in rabbit, and staining was abolished by pre-incubation with the immunising peptide at 10^{-6} M (manufacturer's specification).

The somatostatin antibody was raised against somatostatin-14, and shows 100% cross reaction with longer forms of the peptide (somatostatin -25 or -28), but none with substance P, neuropeptide-Y or vasoactive intestinal polypeptide after radioimmunoassay applications (manufacturer's specification). Absorption of the antibody with somatostatin has been shown to omit immunostaining with this antibody in the dorsal horn (Proudlock et al., 2003).

The monoclonal antibody against enkephalin used in this study was generated in mice by immunizing the animals with Leu-enkephalin conjugated to BSA. In radioimmunoassay, this antibody does not discriminate between Leu- and Met-enkephalin, and displayed 40% cross-reactivity with C-terminal extended Met-enkephalin hexapeptides (Cuello et al., 1984). It also does not recognise other endogenous peptides and shows no cross-reactivity to beta-endorphin or dynorphin by immunohistochemistry (manufacturer's specification).

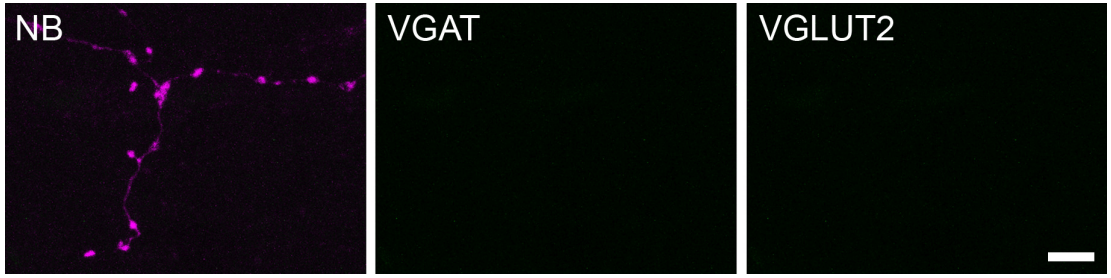
As a procedural control for antiserum specificity, primary antibodies were omitted and replaced with PBS. Under these conditions, no staining was observed (Figure 2-3).

2.5 Confocal microscopy and analysis

2.5.1 To determine morphology of neurons

All sections that contained the cell bodies, dendrites and axons of each Neurobiotin-labelled neuron were scanned with a confocal laser scanning microscope equipped with a Krypton-Argon laser (Bio-Rad MRC1024; Bio-Rad, Hemel Hempstead; UK). Overlapping fields from each section were scanned through an oil-immersion 40× lens with a zoom of 1 and a z-separation of 1 µm. Scans were saved in the format of raw images (Bio-Rad PIC files) and viewed

Figure 2-3 Primary antibody omission control. Neurobiotin-avidin rhodamine-labelled profiles (magenta) show parts of axon from a lamina II neuron. Primary antibodies (VGAT and VGLUT2) were omitted and replaced with PBS. No immunostaining was observed after incubation in species-specific secondary antibodies Alexa 488 and Cy5. The images are projections of 40 optical sections at 0.5 μm z-spacing. Scale bar = 5 μm.


NB
VGAT
VGLUT2

using Confocal Assistant Application software (version 4.02; Todd Clark Brelje, USA). Maximum intensity projections were made from these image stacks and saved in TIF format.

2.5.2 To determine neurotransmitter phenotype of neurons

Expression of VGLUT2 or VGAT by axons of each neuron was determined by scanning the sections with a Bio-Rad Radiance 2100 confocal laser scanning microscope equipped with Argon, HeNe and red diode lasers through an oil-immersion 60× lens with a zoom of 2 and a z-separation of 0.3 µm or 0.5 µm. Line-by-line sequential acquisition was performed and scans were saved in the format of raw images (Confocal Assistant image files) for each channel separately. These were later viewed with Metamorph Offline (version 6.1; Universal Imaging Corporation, Downingtown, PA, USA) to look for colocalisation of VGLUT2 or VGAT with Neurobiotin in axonal boutons of each cell.

2.5.3 To determine presence of somatostatin and enkephalin in the axons of excitatory neurons

Immunoreactivity for somatostatin and enkephalin in axons of the excitatory neurons tested was identified by scanning the sections with a Bio-Rad Radiance 2100 confocal laser scanning microscope equipped with Argon, HeNe and red diode lasers through an oil-immersion 60× lens with a zoom of 2 and a z-separation of 0.3 µm. Line-by-line sequential acquisition was performed and scans were saved in the format of raw images (Confocal Assistant image files) for each channel separately. These were later viewed with Metamorph Offline (version 6.1; Universal Imaging Corporation, Downingtown, PA, USA) to look for colocalisation of somatostatin and/or enkephalin with Neurobiotin in the axonal boutons of the each cell tested.

2.6 Discharge patterns and membrane properties analysis

The electrophysiological data was examined and obtained from analysis carried out by Dr Toshiharu Yasaka.

Action potential discharge patterns of neurons were determined in response to depolarizing current injections of 1s duration. Their firing patterns were observed and routinely elicited from a range of different holding potentials: at least one from -50 to -65 mV, -65 to -80 mV and one from a potential more negative than the previous, usually between -80 mV and -95 mV. The varied holding potentials were to detect whether these discharge patterns were voltage dependent. The protocol used in this study was based on reports by Sandkühler and co-workers (Ruscheweyh et al, 2002; Heinke et al, 2004; Ruscheweyh et al, 2004).

Active membrane properties of neurons were also observed during whole-cell patch-clamp recording. The presence of A-type potassium currents (A-currents, I_A), hyperpolarisation-activated currents (H-currents, I_H) and currents through low threshold calcium channels (I_{Ca}) was assessed. The membrane potential was held at -50 mV, and increasing negative voltage steps of 10 mV at 1 s duration were applied (usually over the range of -60 and -140 mV).

2.7 Neurotransmitter phenotype analysis

Axonal boutons from every cell were examined with confocal microscopy to determine their neurotransmitter content. This was confirmed by the presence of either VGLUT2 or VGAT in the terminals. The penetration of both VGAT and VGLUT2 immunostaining was limited, and therefore only boutons within the surface (within 15 µm from the surface in most cases) of sections were immunoreactive. In order for a neuron to be classified as VGAT- or VGLUT2-positive, at least six of its axonal boutons had to display immunoreactivity for either antibody, respectively, but not both.

2.8 Morphological reconstruction and analysis

Confocal image stacks obtained from scans (see above) that included the cell body, dendrites and axon of each labelled neuron were used to reveal morphology of every neuron. The entire somato-dendritic and axonal domains were reconstructed by orientating corresponding regions from serial Vibratome sections according to the cut surfaces of their dendrites and axons. Then, a

montage of each cell was created by aligning and stitching together the respective tiff images using Adobe Photoshop (Version CS3; Adobe Systems, San Jose, CA, USA). Laminar locations of the labelled cell bodies and neurites were determined by using dark field and epifluorescence microscopy. As described previously (Grudt and Perl, 2002; Yasaka *et al.*, 2007), the superficial dorsal horn (SDH), which includes both lamina I and II, was distinguishable as a relatively translucent band below Lissauer's tract when observed under a binocular microscope and appeared as a distinctive dark band in dark field illumination due to the relative absence of myelinated fibres in this region (Figure 2-1 c). However, the thickness of lamina I varies considerably, being thickest in the central part (Todd et al, 1998), while the border between lamina I and II is often difficult to determine. Therefore in this study, a region corresponding to 100 µm dorsal to the border between lamina II and lamina III was considered to be lamina II. This was then divided into three equal parts: lamina II outer (II_o, most superficial), II inner (II_i, deep third) and the middle third was defined as the border region between II_o and II_i. Dimensions of dendritic arbor in the rostrocaudal (RC) and dorsoventral (DV) plane were measured in parasagittal sections using the marquee tool in Adobe Photoshop. In addition, the dorsal (SD) and ventral extents (SV) of dendrites were measured from the centre of the soma. In some cases, mediolateral extent of the dendritic arbor was estimated from the number of 60 µm sagittal sections containing labelled profiles. Contrast and brightness of images were adjusted by using the level settings in Adobe Photoshop during preparation of images and this was done only for illustration purposes. Image of a neuron after morphological reconstruction is shown in Figure 2-1 g.

2.9 Neurochemical subclasses of excitatory cells

Axon terminals of the 24 VGLUT2-immunoreactive cells that were reacted with somatostatin and enkephalin antibodies were examined with confocal microscopy to look for the presence of these neuropeptides in the axons of each neuron. It is concluded that a neuron is positively-labelled with somatostatin and/or enkephalin if a minimum of 6 axonal boutons from each neuron displayed positive immunoreactivity for either or both neuropeptides.

2.10 Statistical tests

The unpaired t-test or *Chi*-squared test was used for statistical comparisons between groups, and a *p* value of <0.05 was taken as significant.

Chapter 3

Electrophysiological Data

3 Electrophysiological data

Information discussed in this chapter is based on the electrophysiological analysis carried out by Dr Toshiharu Yasaka.

Detailed electrophysiological data were obtained from 50 of the 61 lamina II cells revealed by the presence of Neurobiotin labelling, of which 45 had their action potential discharge patterns examined. The actions of noadrenaline (NA), serotonin (5HT) and somatostatin (SST) were tested on 30, 28 and 24 lamina II cells, respectively.

3.1 Electrophysiological analysis

Of the 50 lamina II cells that were analysed, firing patterns were assessed in 45 of them. The mean resting membrane potential for the whole sample was -62.6 ± 1.2 mV SEM. Membrane resistance (Rm) and capacitance (Cm) were calculated and the mean values were 607.9 ± 63.5 mΩ and 92.4 ± 8.1 pF, respectively.

3.1.1 Action potential discharge patterns

Actions potential discharge patterns in response to 1s injections of depolarising current were tested in 45 lamina II neurons. In each cell, the membrane potential was adjusted to three different levels: (1) between -50 and -65 mV, (2) between -65 and -80 mV, and (3) more negative than -80 mV by continuous current injection prior to the 1s depolarizing current pulses. In this study discharge patterns including tonic-firing, delayed-firing, gap-firing, transient-firing (or initial burst), single spiking and reluctant firing were observed. These discharge patterns were similar to those described in previous studies of lamina II neurons (Yoshimura and Jessell, 1989; Grudt and Perl, 2002; Ruscheweyh and Sandkühler, 2002; Heinke et al., 2004; Ruscheweyh et al., 2004; Graham et al., 2007). Examples of tonic-, delayed-, gap- and transient-firing at different holding potentials are shown in Figure 3-1, and a summary of the number of cells that displayed different discharge patterns is shown in Table 3-1. Twelve of the neurons had differing discharge patterns in response to current injection at the different holding potentials. Within this group, 9 neurons showed tonic or

Figure 3-1 Discharge patterns observed in lamina II neurons in response to depolarising current injection. For each cell, in current-clamp mode prior to application of pulses, the membrane potentials were adjusted to within three ranges: between -50 and -65 mV (in most cases -57 mV), between -65 and -80 mV (in most cases -72 mV) and more negative than -80 mV (in most cases -87mV). **a**: Tonic firing: sustained spiking of action potentials in response to electrical stimulation; **b**: Delayed firing: first action potential observed after a significant delay (arrowhead) from onset of the depolarising step; **c**: Gap firing: a long inter-spike interval (arrowhead) followed by regular firing; **d**: Transient firing: No spike was observed after an initial burst of firing (arrowhead). All current or voltage pulses are of 1 second duration and each column consists of data from a single cell.

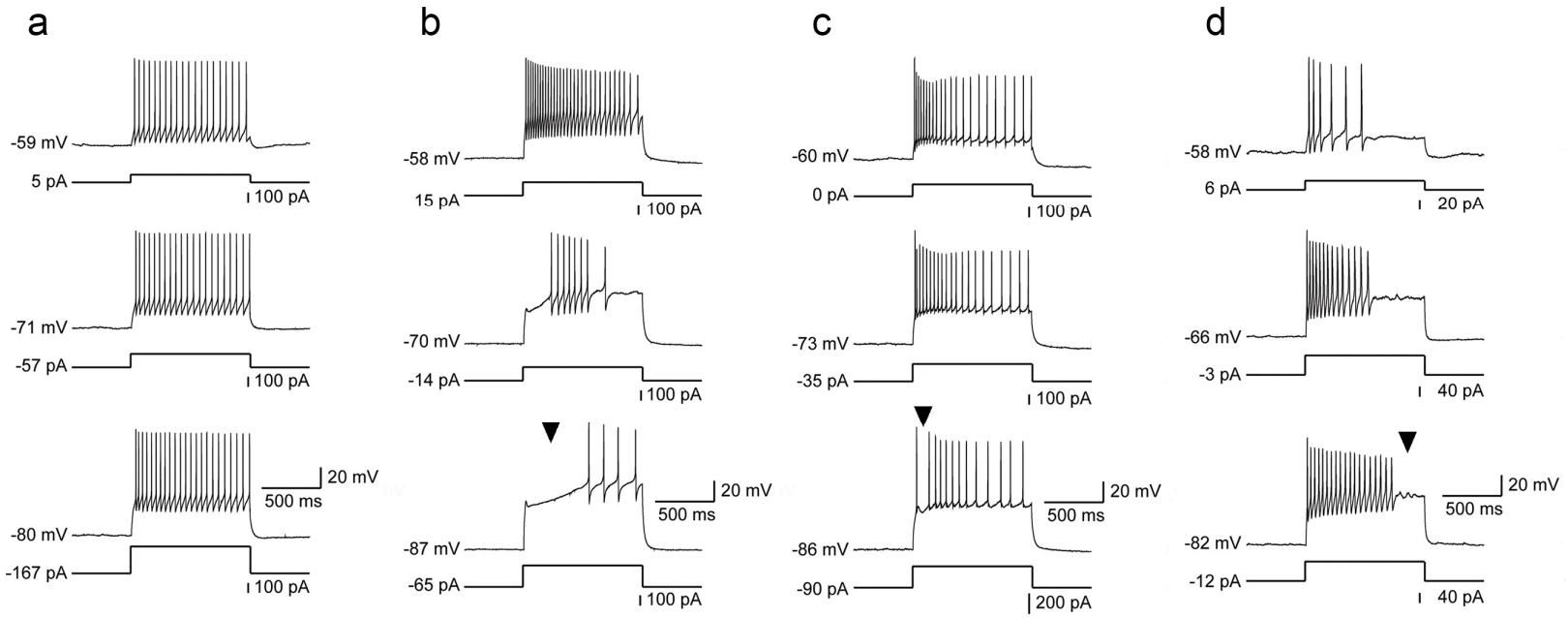

a
-59 mV
5 pA
100 pA
-71 mV
-57 pA
100 pA
20 mV
500 ms
-80 mV
-167 pA
100 pA
b
-58 mV
15 pA
100 pA
-70 mV
-14 pA
100 pA
20 mV
500 ms
-87 mV
-65 pA
100 pA
c
-60 mV
0 pA
100 pA
-73 mV
-35 pA
100 pA
20 mV
500 ms
-86 mV
-90 pA
200 pA
d
-58 mV
6 pA
20 pA
-66 mV
-3 pA
40 pA
20 mV
500 ms
-82 mV
-12 pA
40 pA

Table 3-1 Discharge patterns of 45 lamina II neurons tested.

No. of cells showing discharge pattern	Discharge pattern (from initial holding potential)		
	-50 to -65 mV	-65 to -80 mV	-80 to -95 mV
22	Tonic	Tonic	Tonic
8	Delayed	Delayed	Delayed
2	Transient	Transient	Transient
1	Reluctant	Reluctant	Reluctant
2	Tonic	Tonic	Gap
2	Tonic	Gap	Gap
1	Tonic	Delayed	Delayed
1	Transient	Transient	Reluctant
1	Transient	Transient/gap	Delayed
1	Transient	Transient/gap	Transient/delayed
1	Tonic	Transient/gap	Transient/delayed
1	Delayed	Delayed	Reluctant
1	Gap	Delayed	Delayed
1	Transient	Transient	Single

The table shows different discharge patterns displayed by lamina II neurons in response to 1s depolarising current injection. Note that firing patterns of some neurons differed at different holding potential range.

transient firing patterns at holding potentials less negative than -80 mV but delayed, gap or reluctant firing at more negative potentials. One neuron changed from delayed to reluctant firing and one from gap to delayed firing patterns under these conditions. This indicates that the discharge patterns of neurons are voltage-dependent and this is probably related to the properties of A-type potassium channels. In these cases the pattern observed at the most negative potential was used to classify the cell (Ruscheweyh et al., 2003; Heinke et al., 2004). One cell fired in a transient pattern at -50 to -80 mV and changed to single-spiking at more negative holding potentials. This cell was classified as transient-firing.

3.1.2 Responses to hyperpolarisation

Responses to hyperpolarising current injection were examined in most of the recorded neurons. In current-clamp mode, current pulses were applied to hold the membrane potential of each neuron at -70 ± 3 mV. Responses of neurons were then observed following release from hyperpolarisation and were classified with reference to the results of Graham et al. (2007). In general, four different types of responses were observed: rebound depolarisation with action potential discharge (Figure 3-2 a), rebound depolarisation without action potential discharge (Figure 3-2 b), long-lasting hyperpolarising response (Figure 3-2 c) and a passive response (Figure 3-2 d).

In voltage-clamp mode, voltage-activated currents observed in neurons following release from hyperpolarising voltage steps were examined. The holding potential of each neuron tested was maintained at -50 mV before application of the voltage pulses. A number of neurons responded by showing a transient fast outward current (Figure 3-3 a) at the end of the hyperpolarising voltage pulse, while some displayed a transient slow outward current instead (Figure 3-3 b). The difference in responses are most probably contributed by the different subclasses of A-type potassium channels involved, with the transient fast outward current being mediated by a subclass of I_A channel with fast kinetics, and another subclass with slow kinetics mediating those of the transient slow

Figure 3-2 Responses to hyperpolarising current injection observed in lamina II neurons. a-d: traces showing different voltage responses following releases from hyperpolarisation in current-clamp mode. Responses were classified in accordance with the results of Graham et al. (2007). **a**: rebound depolarisation with action potential discharge; **b**: rebound depolarisation without action potential discharge; **c**: long-lasting hyperpolarising response; **d**: passive response. Values at the left side of each trace indicate initial membrane voltage before application of current pulses to hold the membrane potential at -70 ± 3 mV. The time point of release from hyperpolarisation is shown by arrowheads at the bottom of each trace.

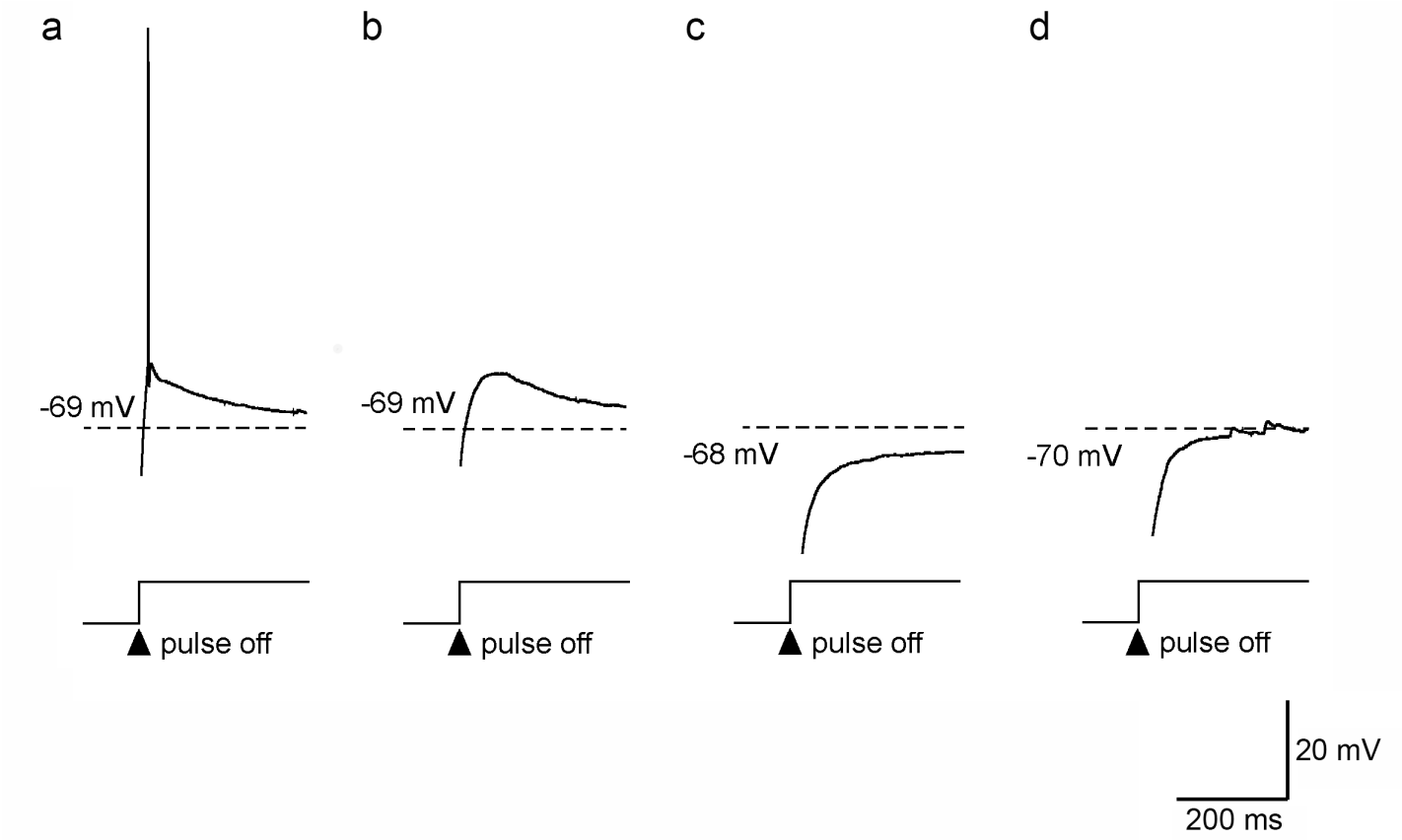
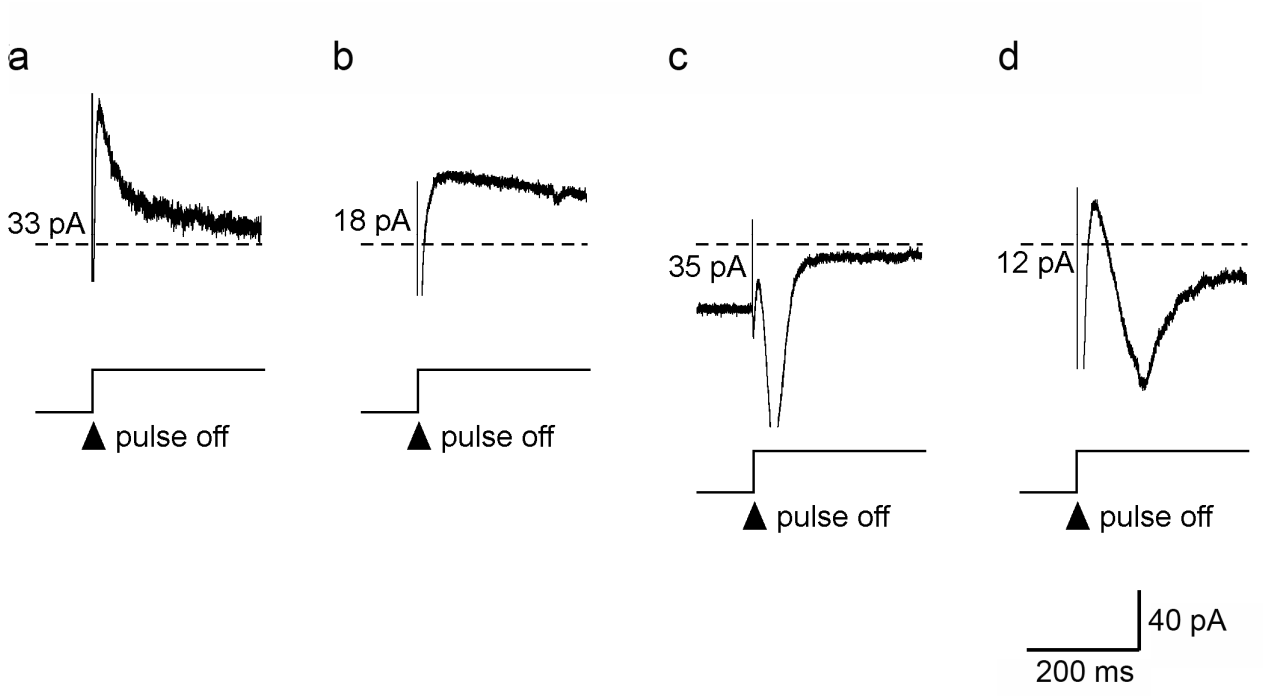

a
b
c
d
-69 mV
-69 mV
-68 mV
-70 mV
pulse off
pulse off
pulse off
pulse off
20 mV
200 ms

Figure 3-3 Voltage-activated currents observed in lamina II neurons after release from hyperpolarising voltage steps. a-d: traces showing different responses following release from hyperpolarisation in voltage-clamp mode. Response profiles were classified in accordance with the results of Graham et al. (2007) and Ruscheweyh et al. (2003). **a**: transient fast outward current observed at the end of the hyperpolarising voltage pulse; **b**: transient slow outward current induced by the same protocol; **c**: transient inward current; **d**: mixed response with both outward and inward currents. Values at the left side of each trace indicate initial membrane current to maintain holding potential at -50 mV before application of voltage pulses. The time point of release from hyperpolarisation is shown by arrowheads at the bottom of each trace.


a
b
c
d
33 pA
18 pA
35 pA
12 pA
pulse off
pulse off
pulse off
pulse off
40 pA
200 ms

outward current. There were also neurons that responded by showing a transient inward current, which is most likely to be mediated by a low threshold calcium channel (I_{Ca}, Figure 3-3 c). Among the neurons tested, four had a mixed response (Figure 3-3 d). These cells showed both outward and inward currents following release from hyperpolarisation.

Currents induced by hyperpolarising voltage steps (I_h) were measured by applying negative voltage steps at 1s duration increasing from -50 to -100 mV (at 10 mV steps). I_h was quantified at a holding potential of -100 mV to minimize the contribution from inward potassium currents below their reversal potentials. Amplitudes of I_h were calculated by subtracting values measured at the beginning of the hyperpolarising pulse from those at the end of the pulse. Within the population of cells that displayed I_h, five had pronounced I_h (> -40 pA), seven displayed I_h with amplitudes between -20 to -40 pA and the majority (33/45) showed small I_h (10-20 pA). Currents smaller than 10 pA were considered to be insignificant.

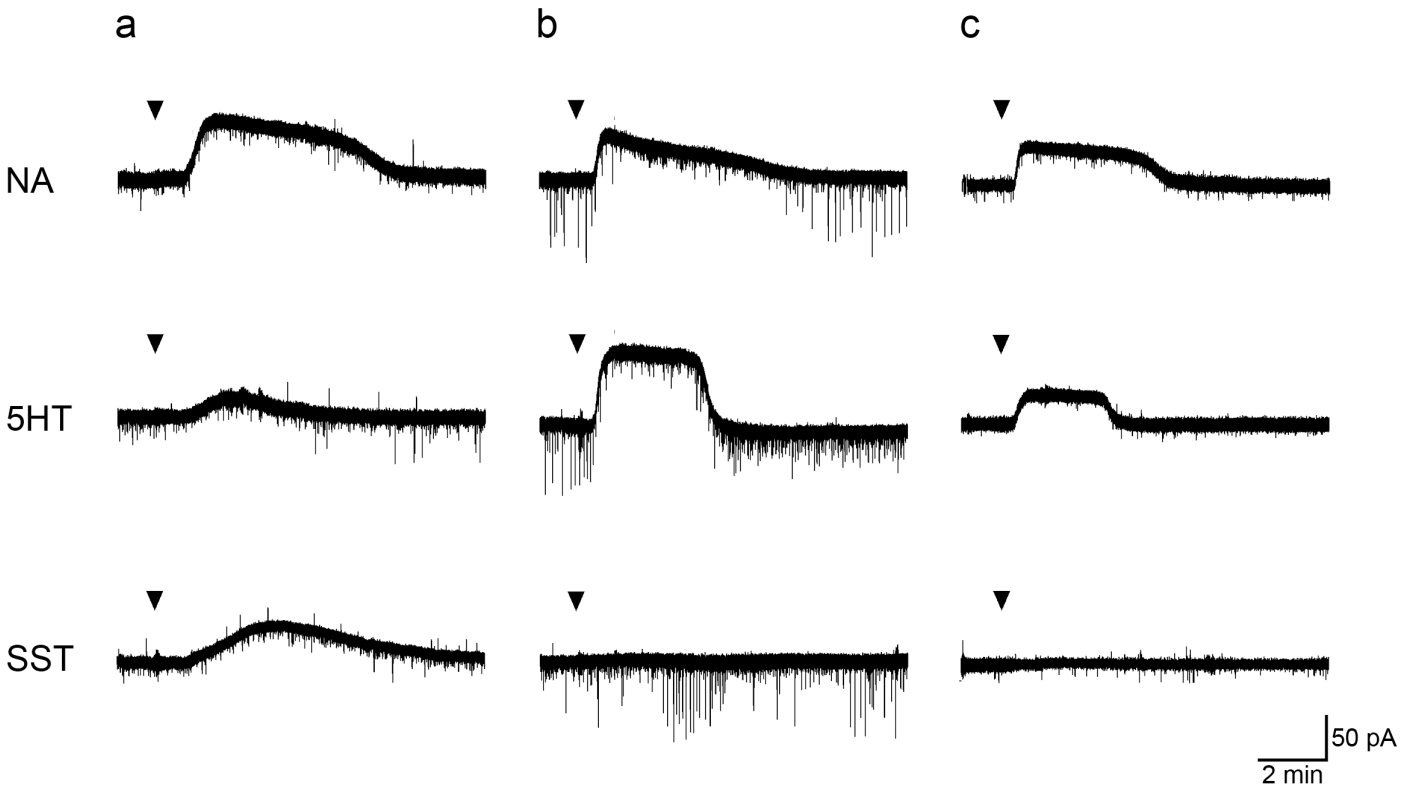
3.2 Responses to neuromodulators

Responses to application of NA, 5HT and/or somatostatin were tested on a sample of the recorded neurons. All three drugs caused an outward current in a proportion of the cells tested, while inward currents were not seen in response to application of any of these drugs in any of the cells (Figure 3-4). Responses were defined as currents that were larger than 10 pA. Spontaneous events were not analysed in this study.

Application of NA caused an outward current in 22 of the 30 (73%) neurons tested. Of the cells that responded to NA, only four cells displayed outward currents with amplitudes larger than 50 pA while the remainder had outward currents with smaller amplitudes.

Serotonin (10-40 μM) was applied to 28 neurons and induced an outward current in 12 (43%) of them. Most of the neurons that responded (9/12) showed outward currents with amplitudes smaller than 50 pA.

Figure 3-4 Responses of lamina II neurons to neuromodulators. Traces obtained from three lamina II neurons during a series of applications of noradrenaline (NA), serotonin (5HT) and somatostatin (SST). Each column (**a-c**) consists of response currents from a single cell during 1 minute bath application for each neuromodulator. Initial point of drug application is shown by arrowheads.


a
b
c
NA
5HT
SST
50 pA
2 min

Twenty-four lamina II neurons were tested with somatostatin and consistent with previous reports (Kim et al., 2002; Jiang et al., 2003), only a proportion (7/24, 29%) exhibited outward current in response to this drug. Application of somatostatin induced outward currents with amplitudes smaller than 50 pA in all of these neurons. The cells that responded to somatostatin were also tested with NA and 5HT. Within this sample, three exhibited response to NA but not 5HT, 1 to 5HT but not NA, and 3 responded to both monoamines.

Chapter 4

Results

4 Results

4.1 Overview of anatomical investigations

Altogether 74 neurons were histologically revealed by the presence of Neurobiotin labelling after whole-cell patch-clamp recording. In 67 cases it was possible to determine the neurotransmitter phenotype as GABA/glycinergic or glutamatergic, by the presence of VGAT or VGLUT2 in axonal boutons belonging to each cell. Since VGAT is found in axons of both GABAergic and glycinergic neurons (Chaudhry et al., 1998), it is not possible to distinguish between the two neurotransmitter types based on the presence of VGAT. However, previous evidence has shown that virtually all SDH neurons that contain glycine are GABAergic (Todd and Sullivan, 1990; Polgár et al., 2003). Therefore, neurons that had VGAT-immunoreactive boutons were defined as inhibitory neurons. Those with axons that contained VGLUT2 were defined as glutamatergic (excitatory). The cell bodies of 61 of these neurons were located within lamina II; two had somata located 15-18 μm below the dorsal white matter and were identified as being at the border between lamina I and II, while the cell bodies of the remaining 4 neurons were found in lamina III. Of the remaining 7 cells for which the transmitter type was not identified, three did not have visible axon, while axonal boutons of the other four were not immunoreactive for either VGAT or VGLUT2. The cells that showed no immunoreactivity for both VGAT and VGLUT2 had axons that were deep in the sections and had very small axonal boutons, which caused visualization of staining very difficult. These cells were excluded from further analysis, since the main purpose of this study is to compare morphological, physiological and neurochemical properties of inhibitory and excitatory interneurons.

4.2 VGAT- and VGLUT2-immunoreactivity

Distribution of VGAT- and VGLUT2-immunoreactivity observed in this study was consistent with that reported in previous studies (Varoqui et al., 2002; Todd et al., 2003). Staining for VGAT was present throughout the SDH in structures that resembled axonal varicosities, similar to that shown in perfusion-fixed tissues (Polgár and Todd, 2008). VGLUT2 is most abundant in laminae I-III but not in

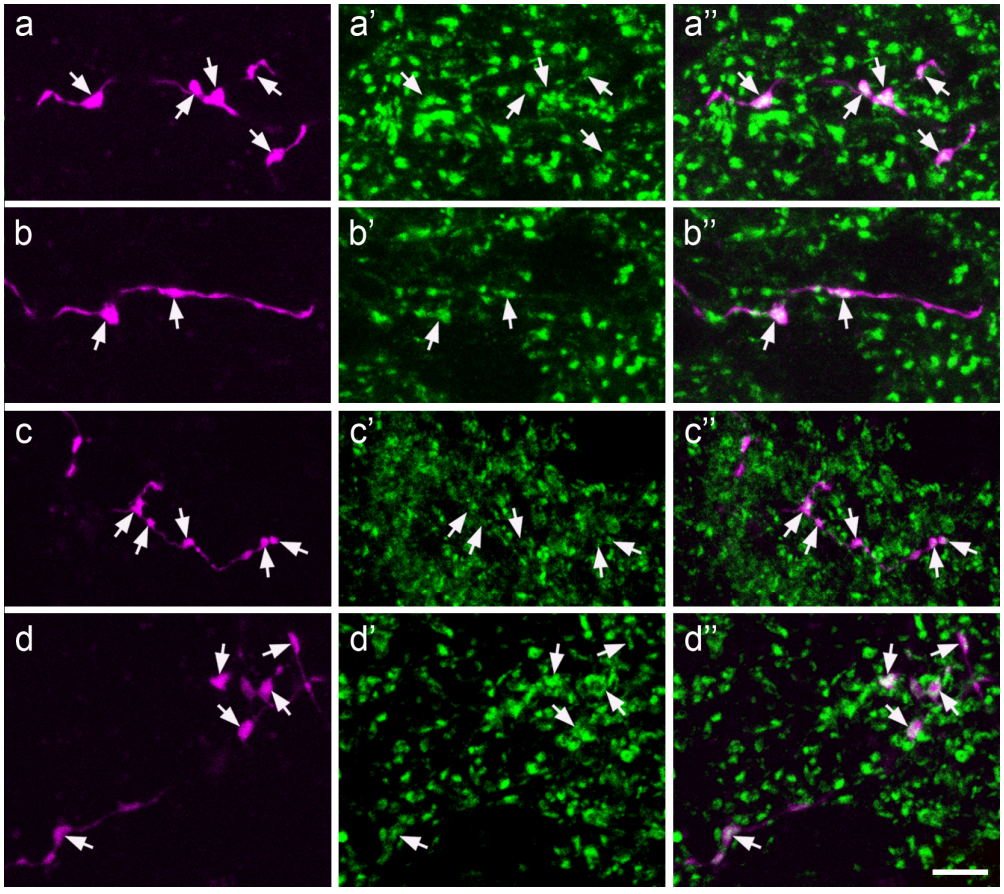
deeper dorsal horn. Both VGAT and VGLUT2 staining was observed in areas outside of the Neurobiotin-labelled profiles of each neuron. There was no incidence of VGAT and VGLUT2 colocalisation throughout the sections.

For each neuron, VGAT and VGLUT2 immunostaining was carried out on one or two selected sections that contained axonal boutons. VGAT and VGLUT2 staining were strongest within the first 5-15 μM from the surface of each section. Therefore, axonal boutons that were located deep in the sections were often not useful to determine the neurotransmitter phenotype of neuron due to poor antibody penetration. Cells of each neurotransmitter type were found in almost equal population: 33 neurons were inhibitory and the remaining 34 were excitatory. Every cell had to have at least 6 positively-labelled boutons in order to be concluded as VGAT- or VGLUT2-immunoreactive. None of the cells that had VGAT-immunoreactive axons displayed staining for VGLUT2, and vice-versa. Examples of VGAT and VGLUT2 immunostaining are shown in Figure 4-1 and Figure 4-2, respectively.

4.3 Cell morphology

Morphology of all 67 neurons was assessed according to the morphological classification scheme developed by Grudt and Perl in 2002. Every section that contained Neurobiotin-labelled profiles from each recorded neuron was included for morphology reconstruction and analysis. Dendrites of each cell were completely filled since intensity of NB labelling was consistent throughout the dendritic length and it did not fade even at the distal parts. Labelling of axons was also relatively complete since in all cases, the axonal arborisations could be followed up to considerable distances. Cell body location, branching patterns and dimension of dendritic trees, as well as extent and laminar distribution of axon were included to provide further morphological information for each cell. Dendritic extension in the rostrocaudal (RC) and dorsoventral (DV) axis, distance from the centre of the cell body to the dorsal limit of the dendritic arbor (SD) and distance from the centre of the cell body to the ventral limit of the dendritic arbor (SV) were also measured. Mediolateral dendritic span of each neuron was estimated from the number of parasagittal sections containing labelled dendritic profiles and generally dendrites of all cells extended for less

Figure 4-1 Examples of VGAT immunostaining. Projected images of immunostaining for VGAT in parts of the axons of 3 lamina II neurons. **a, b, c, d**: axon labelled with Neurobiotin and avidin-rhodamine (magenta). **a', b', c', d'**: VGAT immunostaining (green), while a merged image (**a''- d''**) appears on the right. Axonal boutons are indicated by arrows. Note that the boutons are labelled with antibody against VGAT. The images are projections of 4 (a, c), 5 (b, d) optical sections at 0.3 (a, b, d) or 0.5 μm (c) z-spacing. Scale bar = 5 μm.


a
a'
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b
b'
b''
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c'
c''
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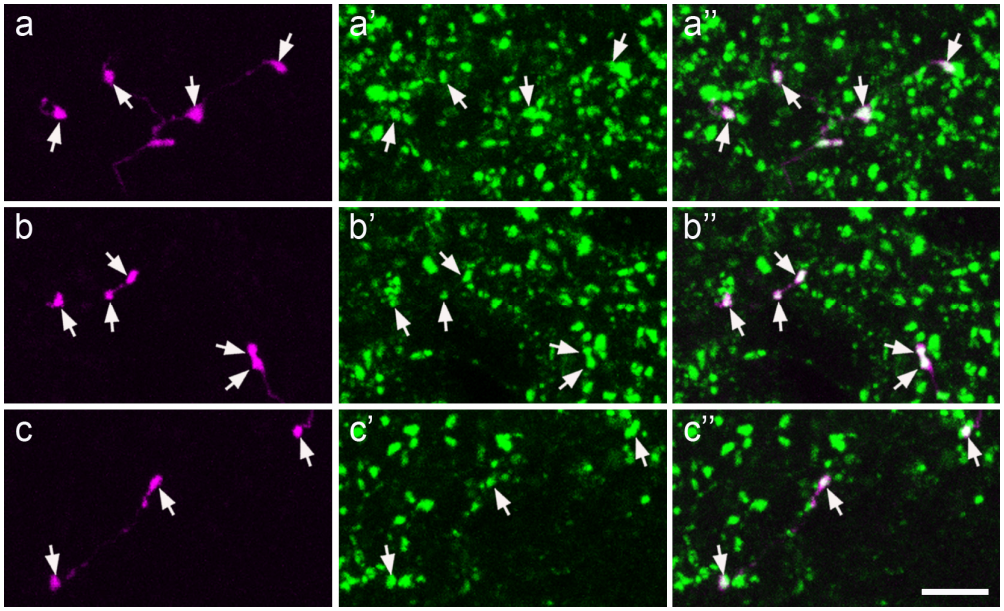
d'
d''

Fig 4-2 Examples of immunostaining for VGLUT2. Projected images of immunostaining for VGLUT2 in parts of the axons of a lamina II neuron. **a-c**: axon labelled with Neurobiotin and avidin-rhodamine (magenta); **a'-c'**: VGLUT2 immunostaining (green), while a merged image appears on the right (**a''-c''**). Axonal boutons are indicated by arrows. Note that these boutons are labelled with antibody against VGLUT2. The images are projections of 11 (a), 8 (b, c) optical sections at 0.5 μm z-spacing. Scale bar = 5 μm.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a''
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c''

than 300 μm in this plane. The axonal extent of each cell was also examined. Four distinctive morphological classes were identified among the 61 neurons that definitely had their cell bodies in lamina II: islet cells, central cells, vertical cells and radial cells. Forty-two neurons were assigned to one of these four types, while the remaining 19 cells could not be classified. Information on the cell body location and axonal extent, as well as dendritic dimensions of each morphological class identified in this sample is shown in Table 4-1 and Table 4-2, respectively.

4.3.1 Islet cells

Islet cells constituted almost a fifth (12/61) of the lamina II neuron population identified in this study. Examples of islet cell are shown in Figure 4-3. Cell bodies of 10 islet cells were located near the border between laminae II_o and II_i, and the remaining two had soma in lamina II_o and lamina II_i, respectively. The most characteristic feature that differentiated this group of cells from the other cell types was their distinctively extensive rostrocaudal dendritic arborisation. Dendrites of islet cells are remarkably elongated in the rostrocaudal axis reaching up to over 1000 μm, with a minimum extension of 466 μm. Generally, islet cells had relatively limited dorsoventral dendritic spread with some cells being more compact than the others. Density of dendritic branches varied among the islet cells but the major part of their dendritic trees usually arborised within the same plane as their cell bodies. Two islet cells had a very simple dendritic branching pattern with less than five primary dendrites that branched minimally (e.g. Figure 4-3 b). On the other hand, dendritic arborisations of a few islet cells were very complex with dendrites that curved back in the direction of the cell body after travelling for a certain distance (recurrent dendrites) (e.g. Figure 4-3 g).

Although all islet cells had rostrocaudally extensive dendrites, variations were observed among them. For example, distribution of dendrites was asymmetrical in a few cells, which have dendrites that arborised mainly on either the rostral or caudal side of the cell body (e.g. Figure 4-3 a). The dendritic extent from the cell body in either the dorsal or ventral direction also differed from cell to cell. Dendritic spines were often present on the whole dendritic length, but were

Table 4-1 Soma location and axonal arborisation of different morphological types of lamina II neurons.

		Axonal arbor			Soma location		
Morphology	**Number**	**I**	**II**	**III**	**IIo**	**IIo/i**	**IIi**
Islet	12	6	12	5	1	10	1
Central	8	4	8	3	2	3	3
Vertical	15	10	15	10	10	3	2
Radial	7	4	7	4	2	3	2
Unclassified	19	12	19	10	5	5	9

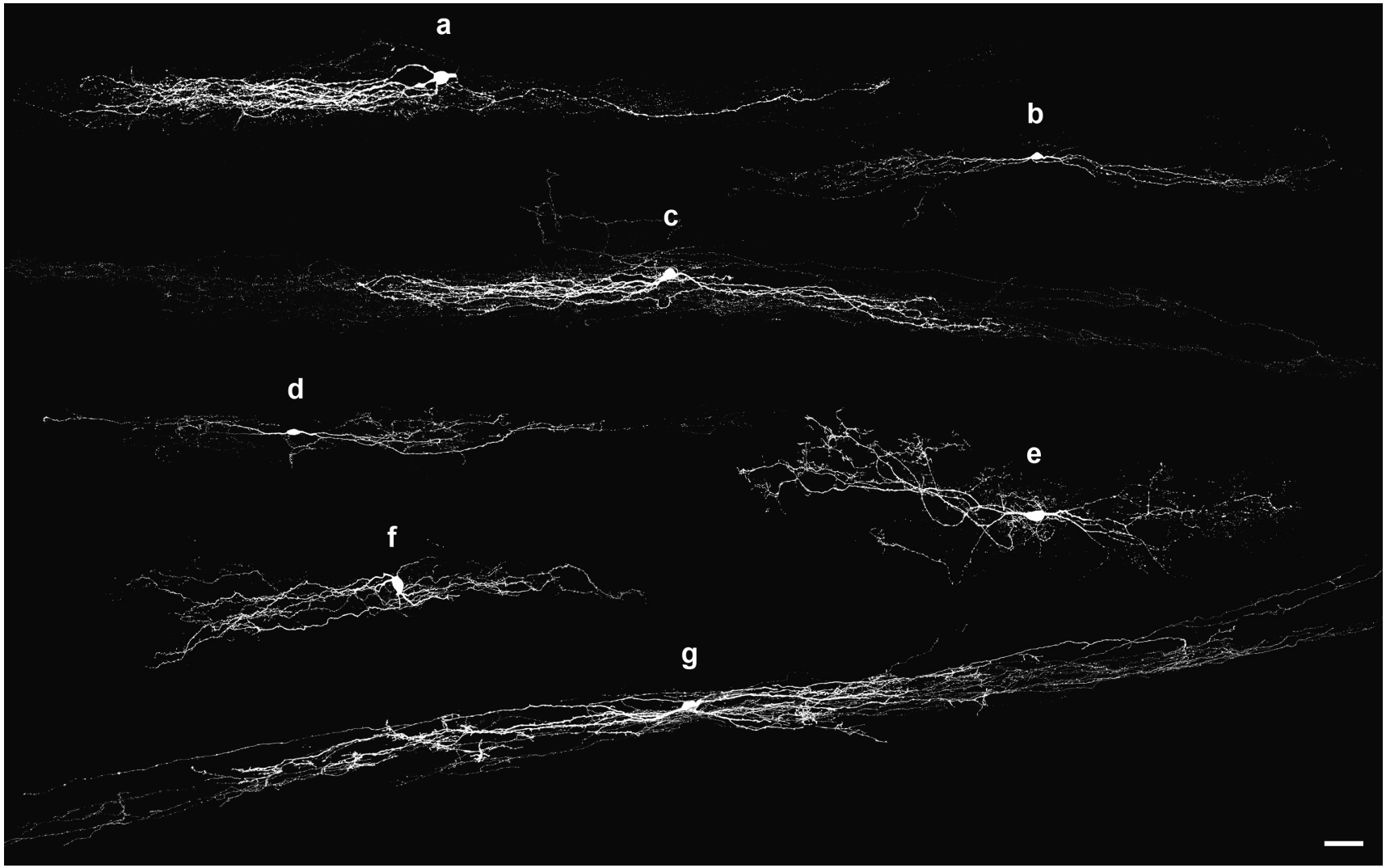
The table shows different morphological types together with the number of cells that had at least some of their axonal arbor in laminae I, II and/or III, and the number with their soma in each part of lamina II. Note that all cells had axons that arborised in lamina II.

Table 4-2 Dendritic dimensions of different morphological types of lamina II neurons.

Morphology	Dendritic dimensions (μm)			
	RC	DV	SD	SV
Islet	730.55 ± 202.99	108.00 ± 40.53	32.78 ± 37.75	75.55 ± 28.16
Central	271.70 ± 70.31	66.88 ± 13.22	31.83 ± 19.18	35.05 ± 22.49
Vertical	327.21 ± 138.41	264.19 ± 157.74	58.13 ± 46.23	206.07 ± 128.96
Radial	202.74 ± 47.16	117.77 ± 37.63	65.29 ± 33.01	52.49 ± 15.05
Unclassified	362.26 ± 342.94	123.38 ± 81.07	58.53 ± 37.25	73.85 ± 53.42

The table shows range of dendritic tree dimensions at different field spans for each morphological type (mean ± S.D.).
RC: rostrocaudal; DV: dorsoventral; SD: dendritic extent from center of soma to dorsal limit; SV: dendritic extent from center of soma to ventral limit

Figure 4-3 Examples of islet cells. Confocal images obtained from projected confocal scans showing cell bodies and dendrites of islet cells. All islet cells (**a-g**) were inhibitory. The dendrites of islet cells were distinctively extended in the rostrocaudal axis but were relatively limited in the dorsoventral direction. Note that most of the axon of each cell has been omitted. Scale bar = 50 μm.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more numerous on distal dendrites. However, the presence of spines on the dendrites was not a dominant feature among the islet cells. Axons of islet cells generally arborised within the volume occupied by the dendritic trees, which were limited to lamina II. However, in some cases they extended further rostrally or caudally than the dendrites, and axons of a few cells travelled into lamina I and/or lamina III (Table 4-1). Boutons were evenly distributed along the axon in high density and were relatively small in size.

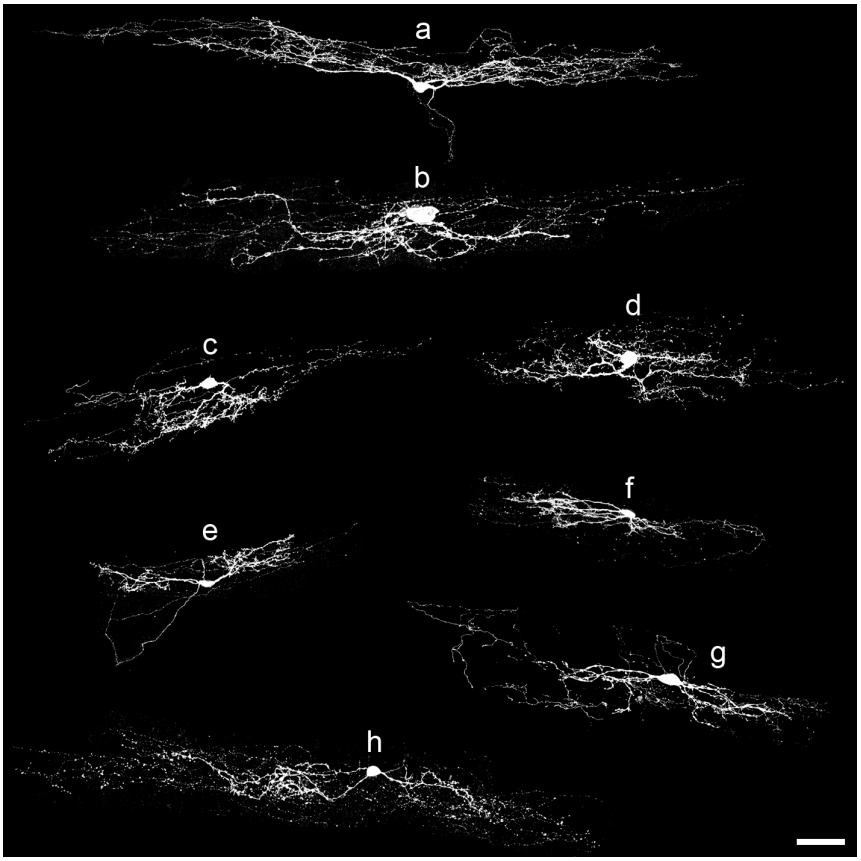
4.3.2 Central cells

Altogether eight neurons were classified as central cells, as shown in Figure 4-4. These cells resembled islet cells, but were much smaller in size. They had shorter dendritic extension in the rostrocaudal axis (mean = 271.7 ± 70.3 μm) and their dendritic arborisations were more compact. Cell bodies of central cells were generally spindle shaped and small in size. These neurons were found throughout the depth of lamina II. Central cells had relatively few primary dendrites, which arborised close to the cell body, were limited to lamina II and extended less than 100 μm dorsoventrally from the somata. Branches formed from these primary dendrites were often covered with short-necked spines. Generally, the central cells had dendrites that arborized in a simple pattern, matching descriptions of small islet cells by Todd and McKenzie (1989) and Grudt and Perl's (2002) central cells. Axons of central cells often arborised beyond the volume of the dendritic trees, especially in the rostrocaudal extent. Axons of central cells were contained within lamina II in most cases (Table 4-1). Two neurons with their cell bodies situated in the inner third of lamina II had axons that travelled far into the ventral laminae and axons of a few others entered laminae I or III. Axonal terminals are evenly distributed along the length of the axons.

4.3.3 Vertical cells

Vertical cells had dendrites that extended in both rostrocaudal and dorsoventral axes, but their ventrally-directed dendrites were particularly prominent. This distinctive characteristic gave the dendritic trees of vertical cells their triangular or cone-like appearance, with the cell bodies usually located dorsally

Figure 4-4 Examples of central cells. Confocal images obtained from projected confocal scans showing cell bodies and dendrites of central cells, which were inhibitory (**a-d**) and excitatory (**e-h**). Note that most of the axon of each cell has been omitted. Scale bar = 50 µm.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at the apex. Examples of vertical cell are shown in Figure 4-5. Consistent with past studies (Grudt and Perl, 2002; Maxwell et al., 2007; Yasaka et al., 2007), the majority of vertical cells had their cell bodies located in the outer region of lamina II. Two vertical cells were found in deep lamina II while the rest of the population was located at the border region between lamina II_i and IIo. Cell bodies of vertical cells were either rounded or pyramidal in shape. Dendrites of vertical cells extended throughout the whole depth of lamina II and in many cases extended into lamina III and further ventrally. Dendritic arbors of vertical cells could be loose or compact. Altogether 15 vertical cells were identified in this sample. Two of these had a dendrite that extended dorsally before making a curve and travelling back in the direction of the cell body and then continuing ventrally (e.g. Figure 4-5 b), which is uncommon among this neuronal population. The primary dendrites were considerably long and mostly branched at the distal parts. The occurrence of dendritic spines, both short- and long-necked, was frequent but the density varied from cell to cell. A few vertical cells that had spines-covered dendrites closely resembled the stalked cells identified by Gobel (1975, 1978). Axons of vertical cells were not confined within the vicinity of their dendrites and extended for considerable distances in all dimensions, especially in the rostrocaudal direction. The axons of vertical cells arborized mainly in lamina II. Of these vertical cells, five cells had axon collaterals in laminae I-III and the remainder had axons that extended into one of the adjacent laminae (Table 4-1). Axonal boutons were distributed along the length of the axons in a relatively even pattern.

4.3.4 Radial cells

Seven cells were included in the radial cell population based on their characteristic dendritic arbors, which appeared almost circular when viewed in the sagittal plane. Examples of radial cells are shown in Figure 4-6. Somata of radial cells were located in all parts of lamina II and most of them had a rounded shape. Dendrites of radial cells emerged from the cell bodies and extended in all directions, thus giving them their radiated or 'star-like' arborisation pattern. The number of primary dendrites varied, but at least three prominent processes emanated from the cell body. Generally, the dendritic span was more extended in the rostrocaudal direction than dorsoventrally, and relatively limited in the

Figure 4-5 Examples of vertical cells. Confocal images obtained from projected confocal scans showing cell bodies and dendrites of vertical cells, which were excitatory (**a-e**) and inhibitory (**f-g**). Note that most of the axon of each cell has been omitted. Scale bar = 50 μm.

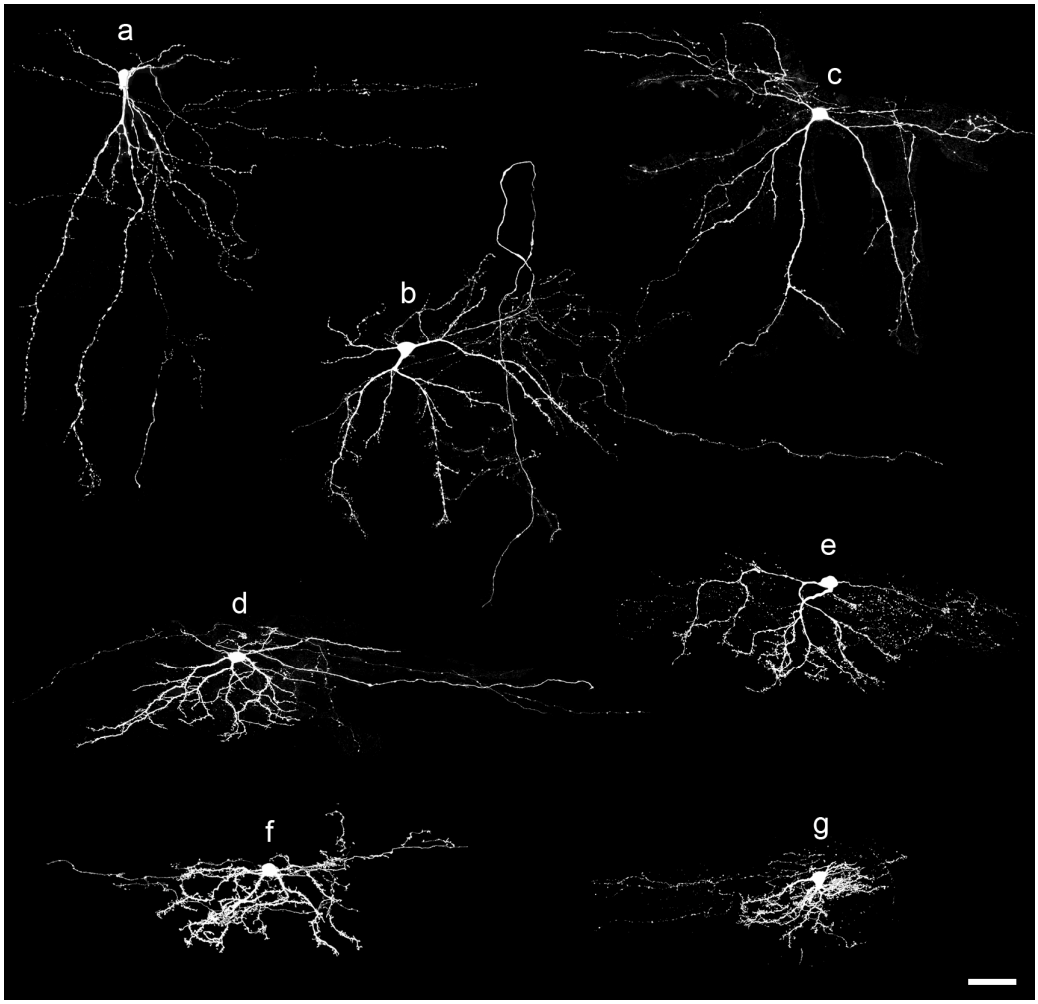
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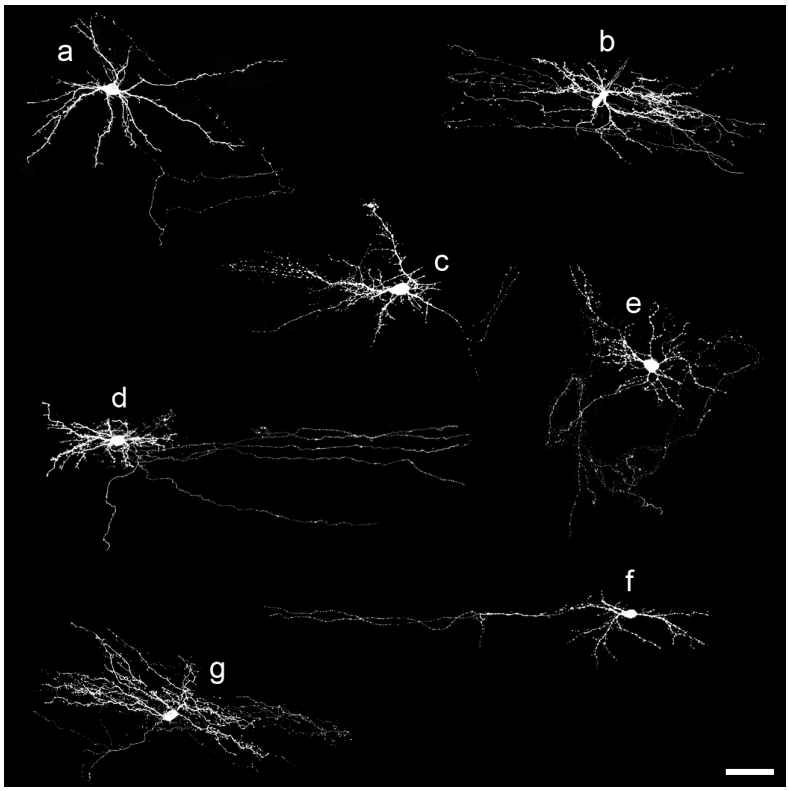
Figure 4-6 Examples of radial cells. Confocal images obtained from projected confocal scans showing cell bodies and dendrites of radial cells. All radial cells (**a-g**) were excitatory. Note that most of the axon of each cell has been omitted. Scale bar = 50 μm.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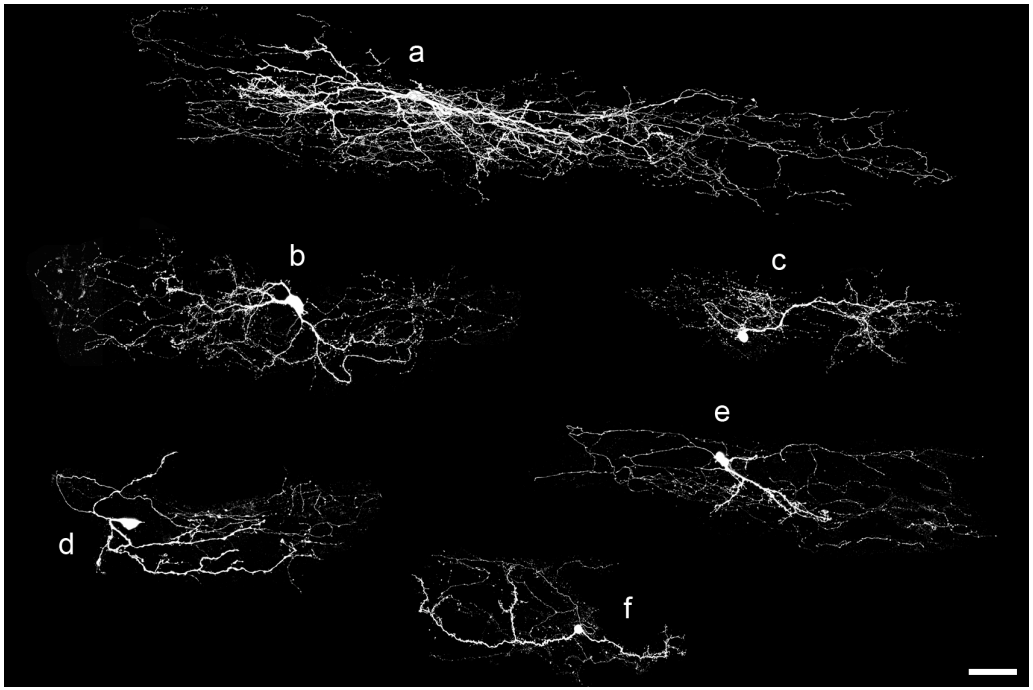
mediolateral axis. In most cases, the dendritic trees were compact and were limited to lamina II. This should be considered when comparing these cells to those of similar morphology, but had longer radiating dendrites described in previous studies (Todd and Mckenzie, 1989; Maxwell et al., 2007). Dendritic spines were present on dendrites of these cells at high densities. Axons of radial cells often travelled a considerable distance, exceeding 500 μm, either rostrally or caudally away from the cell bodies (e.g. Figure 4-6 d, f). A few radial cells had axons that were mainly located at one side of the cell body. Others had axon that filled the volume of their dendrites and beyond. In many cases, axon collaterals of radial cells entered laminae I and/or III (Table 4-1). Axon terminals were relatively sparse and more frequent at distal of axon.

4.3.5 Unclassified cells

A total of 19 neurons could not be classified and examples of these are shown in Figure 4-7. Cell bodies of these neurons also varied in location, appearance and size. These cells had morphology that was different from that of the other classes of neurons in lamina II, or characteristics that were intermediate between two classes; therefore they did not fit any of the described morphological types. For example, two neurons had dendrites that arborized in a highly complex manner, forming extensive dendritic trees with numerous branches emanating from their primary dendritic processes (e.g. Figure 4-7 a). Axons of both cells extended mostly within close proximity of their dendrites but exceeded the dendritic limits in the rostrocaudal axis. Spines were scarce on dendrites of these cells.

Dendrites of a few unclassified neurons (4/19) arborized extensively on one side of their cell bodies but had few primary dendrites on the other (e.g. Figure 4-7 d and e). Two of them displayed many dendritic spines but few were observed in the other two. These cells generally had simple dendritic arborisation with no more than five primary dendrites that branched infrequently. Axonal arborisations of these cells were more extensive than their dendrites. Most of their axons remained within lamina II but two cells had axons that extended dorsally into lamina I and/or ventrally into lamina III.

Figure 4-7 Examples of unclassified cells. Confocal images obtained from projected confocal scans showing cell bodies and dendrites of cells that were not able to be classified. These cells were found in both the inhibitory (**a-c, e**) and excitatory (**d, f**) populations. Dendritic arborisation patterns of these cells were atypical or resembled characteristics that were intermediate between two classes. Note that most of the axon of each cell has been omitted. Scale bar = 50 µm.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Morphology of some unclassified cells had features of more than a single known morphological type. For example, one neuron had dendrites that extended rostrocaudally, similar to those of islet cells, but its dorsoventral dendritic extension and appearance of dendritic spines were prominent, resembling a stalked-like vertical cell. Another similar case was cell b of Figure 4-7, which had a cell body located in outer lamina II and appeared to resemble a vertical cell, but lacked the distinctive 'cone-like' structure. The size of its dendritic and axonal arborisation were however, relatively similar to the central cell type.

There were unclassified cells with dendrites that followed a dorsal trajectory (Figure 4-7 c and f). These cells had dendrites that were sparsely distributed, with a single primary dendrite emanating from each side of the cell body and forming few branches. Dendritic spines were visible on dendrites of both cells. Their axons extended over a larger area in the sagittal plane than their dendritic trees. The axon of cell c was constrained to lamina II while cell f had an axon that travelled to lamina I and III.

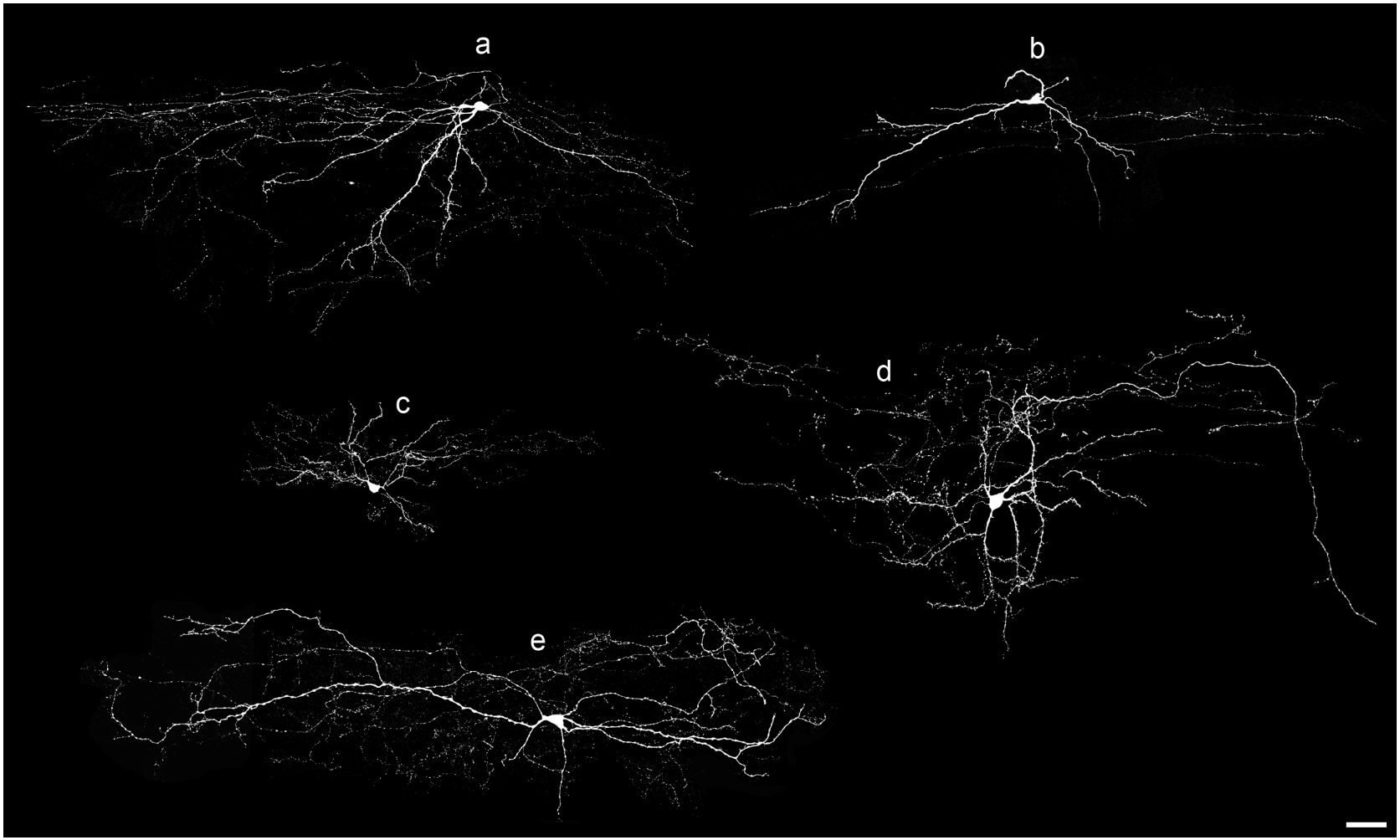
4.3.6 Cells at the border between lamina I and lamina II

Two neurons had cell bodies that were 15 to 18 μm below the dorsal white matter, therefore they were identified as being located at the border region of laminae I and II. Examples of these cells are shown in Figure 4-8 (a, b). Both cells had dendritic trees that extended in the rostrocaudal and dorsoventral planes, with the latter being more prominent. The main portions of their dendrites were distributed ventrally from the cell bodies. One of the two neurons displayed numerous spines on its dendrites, while the other had almost no dendritic spine at all. Axons of both neurons arborized extensively in the rostrocaudal axis and exceeded the volume of their dendritic trees. However, the axonal arborisation of one cell was highly complex and branched into more collaterals than the other. Axonal boutons were observed to be evenly distributed along the whole lengths of the axons.

4.3.7 Lamina III cells

The cell bodies of four neurons were located in lamina III and their morphological appearance differed from each other. Examples of these cells are

Figure 4-8 Examples of laminae I/II border cells and lamina III cells. Confocal images obtained from projected confocal scans showing cell bodies and dendrites of cells that were located at the border region of lamina I and II (**a-b**), and lamina III (**c-e**). All but cell **b** were inhibitory. Note that most of the axon of each cell has been omitted. Scale bar = 50 μm.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e

shown in Figure 4-8 (c-e). One neuron had dendrites that were directed dorsally into lamina II, resembling an 'inverted vertical cell', which were similar to that described of an antenna cell by Rethelyi and Szentágothai (1969) and more recently observed by Maxwell et al (2007) and Wang and Zylka (2009). The dendritic arborisation of another cell found in this lamina occupied an almost perfect circle when viewed in the sagittal plane. Its dendrites radiated from the cell body in all directions, very much the same as radial cells, but in a much greater span in both the rostrocaudal and dorsoventral axes. The remaining two cells had dendrites that extended primarily in the rostrocaudal direction, but unlike islet or central cells, their dendritic trees were not as compact and the dendrites were more extensive in the dorsoventral direction. Axonal arborisations of all four neurons exceeded the limits of their dendritic volume, mostly in the dorsoventral axis.

4.4 Cell morphology and neurotransmitter content

Figure 4-9 shows the proportions of cells in each morphological class found in the VGAT- and VGLUT2-immunoreactive populations. Although most morphological types were identified in both populations, consistent patterns were observed among certain cell types. All islet cells (n = 12) were VGAT-immunoreactive, while all radial cells (n = 7) were excitatory. Vertical (n = 15) and central (n = 8) cells included both inhibitory and excitatory neurons; although most (12/15) vertical cells were VGAT-immunoreactive. Morphological features, including soma location and axonal arborisation, of both excitatory and inhibitory neuronal populations are shown in Table 4-3.

Table 4-4 shows dendritic dimensions of the different morphological types of inhibitory and excitatory neurons identified in lamina II. When the rostrocaudal and dorsoventral dendritic extent of both excitatory (e.g. Figure 4-5 a-e) and inhibitory vertical cells (e.g. Figure 4-5 f-g) were analysed, it was found that the 3 inhibitory cells were among the smallest of the vertical cell population. Maxwell et al (Maxwell et al., 2007) classified six lamina II neurons in rat as vertical cells, of which 4 were identified as glutamatergic and two are GABAergic, based on the presence of GAD in their axons. In order to compare a larger sample of excitatory and inhibitory vertical cells, measurements of the

Figure 4-9 Morphological classes identified in lamina II neurons. This histogram shows proportions of each morphological type found in the inhibitory (blue bars) and the excitatory (red bars) populations. Note that all islet cells were inhibitory, all radial cells were excitatory and the majority of vertical cells were excitatory.

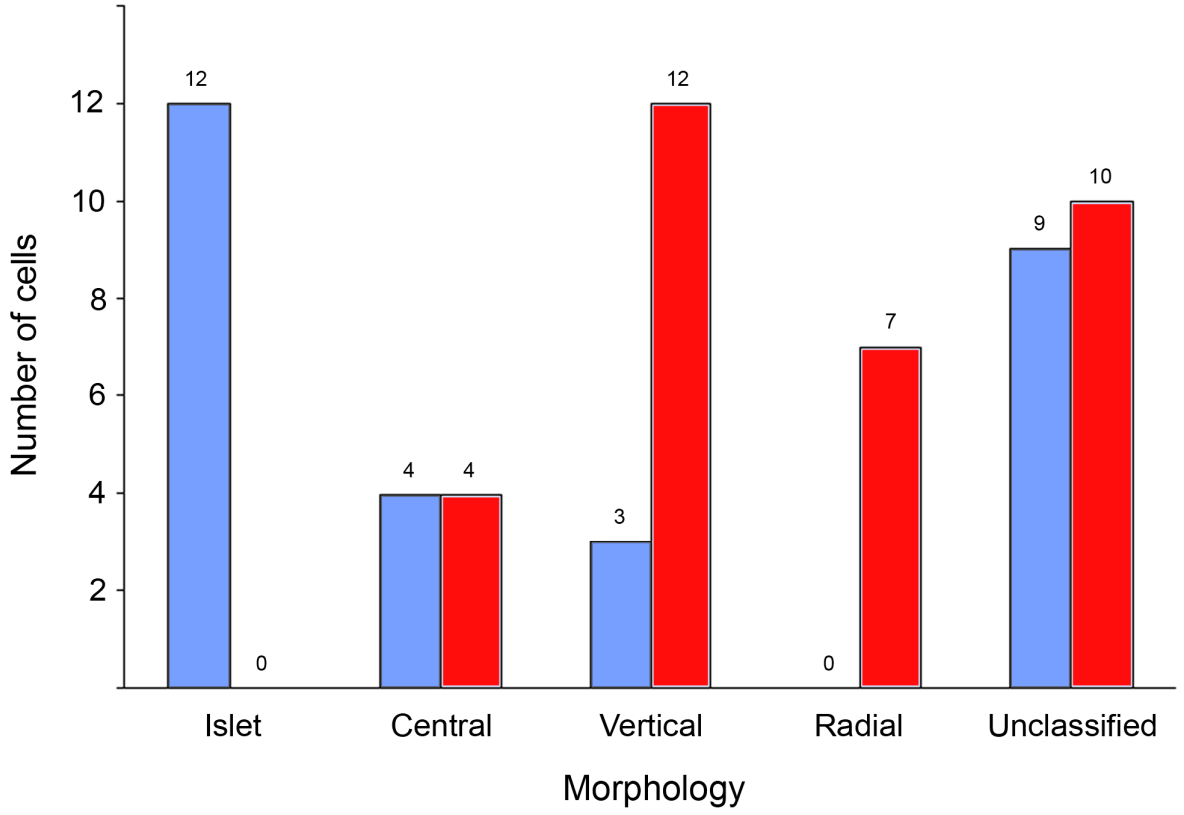

Number of cells
12
10
8
6
4
2
12
0
4
4
3
12
0
7
9
10
Islet
Central
Vertical
Radial
Unclassified
Morphology

Table 4-3 Soma location and axonal arborisation of different morphological types of inhibitory and excitatory lamina II neurons.

			Axonal arbor			Soma location		
Neurotransmitter phenotype	**Morphology**	**Number**	**I**	**II**	**III**	**IIo**	**IIo/i**	**IIi**
Inhibitory, VGAT (n = 28)	Islet	12	6	12	5	1	10	1
	Central	4	1	4	1	1	2	1
	Vertical	3	1	3	0	3	0	0
	Unclassified	9	4	9	3	3	2	4
Excitatory, VGLUT2 (n = 33)	Central	4	3	4	2	1	1	2
	Vertical	12	9	12	10	7	3	2
	Radial	7	4	7	4	2	3	2
	Unclassified	10	8	10	7	2	3	5

The table shows different morphological cell types of inhibitory and excitatory neurons together with the number of cells that had at least some of their axonal arbor in laminae I, II and/or III, and the number with their soma in each part of lamina II. Note that all cells had axons that arborised in lamina II.

Table 4-4 Dendritic dimensions of different morphological types of inhibitory and excitatory lamina II neurons.

Neurotransmitter phenotype	Morphology	Dendritic dimensions (μm)			
		RC	DV	SD	SV
Inhibitory, VGAT (n = 28)	Islet	730.55 ± 202.99	108.00 ± 40.53	32.78 ± 37.75	75.55 ± 28.16
	Vertical	156.77 ± 56.70	103.40 ± 11.30	24.63 ± 10.90	78.77 ± 10.83
	Central	294.30 ± 81.39	76.43 ± 8.54	35.28 ± 23.99	41.15 ± 28.03
	Unclassified	518.86 ± 444.26	135.00 ± 79.28	55.30 ± 46.00	79.73 ± 42.01
Excitatory, VGLUT2 (n = 33)	Vertical	369.83 ± 117.87	304.38 ± 151.10	66.51 ± 48.12	237.90 ± 124.98
	Radial	202.74 ± 47.16	117.77 ± 37.63	65.29 ± 33.01	52.49 ± 15.05
	Central	249.10 ± 59.58	57.33 ± 9.56	28.38 ± 15.86	28.95 ± 17.19
	Unclassified	221.32 ± 114.82	129.99 ± 86.79	61.44 ± 29.58	68.55 ± 63.82

The table shows range of dendritic tree dimensions at different field spans for each morphological type (mean ± S.D.).

RC: rostrocaudal; DV: dorsoventral; SD: dendritic extent from center of soma to dorsal limit; SV: dendritic extent from center of soma to ventral limit

rostrocaudal and dorsoventral dendritic extent of the 15 vertical cells described in the present study and of the 6 vertical cells from Maxwell et al. (2007) were plotted and compared (Figure 4-10). The dendritic trees of the excitatory vertical cells were generally larger than those of the inhibitory ones. Axonal projections of excitatory and inhibitory vertical cells in the present sample also differed. The majority of excitatory vertical cells (10/12) had axons that entered lamina III, while those belonging to the 3 inhibitory vertical cells were confined within lamina II with the axon of one cell going into lamina I. The cell bodies of all 3 inhibitory vertical cells were located in the outer region of lamina II, whereas cell bodies of the excitatory cells were found throughout lamina II with the majority of them located in lamina IIo. VGLUT2 immunostaining in parts of the axon of a vertical cell (Figure 4-5 b) is shown in Figure 4-11.

Of the 8 central cells identified in the present study, half of them had axonal boutons that were VGAT-immunoreactive (Figure 4-4 a-d) and the other half had axons that were VGLUT2-immunoreactive (Figure 4-4 e-h). Measurements of the dendritic tree dimensions showed that the dendritic extent of the four excitatory central cells were generally more restricted than those of their inhibitory counterparts. However, the limited sample size made it difficult to determine whether this represented a genuine size difference among the central cell population. However, cell bodies of both excitatory and inhibitory central cells, as well as their axonal arborisation patterns did not show distinct differences.

The unclassified group included 9 inhibitory (Figure 4-7 a-c, e) and 10 excitatory neurons (Figure 4-7 d, f) of diverse morphology. Dendritic tree dimensions of the two groups overlapped, with the size of their dendritic trees varying from cell to cell. Both groups contained cells with axons that entered lamina I and/or lamina III. Cell bodies of unclassified cells, either inhibitory or excitatory, were not restricted to any of the three regions in lamina II.

4.5 Neurotransmitter content and electrophysiology

Of the 50 lamina II neurons for which detailed electrophysiology data and neurotransmitter content were obtained, the mean resting membrane potential

Figure 4-10 Dendritic tree extents of inhibitory and excitatory vertical cells. Scatter plot of rostrocaudal and dorsoventral extent of the dendritic trees of inhibitory (blue open circles) and excitatory (red open cirles) vertical cells. Fifteen of the cells included in this scatter plot are from the present study and the other six are those illustrated in Figure 1 of Maxwell et al. (2007). Note that excitatory vertical cells generally have larger dendritic trees.

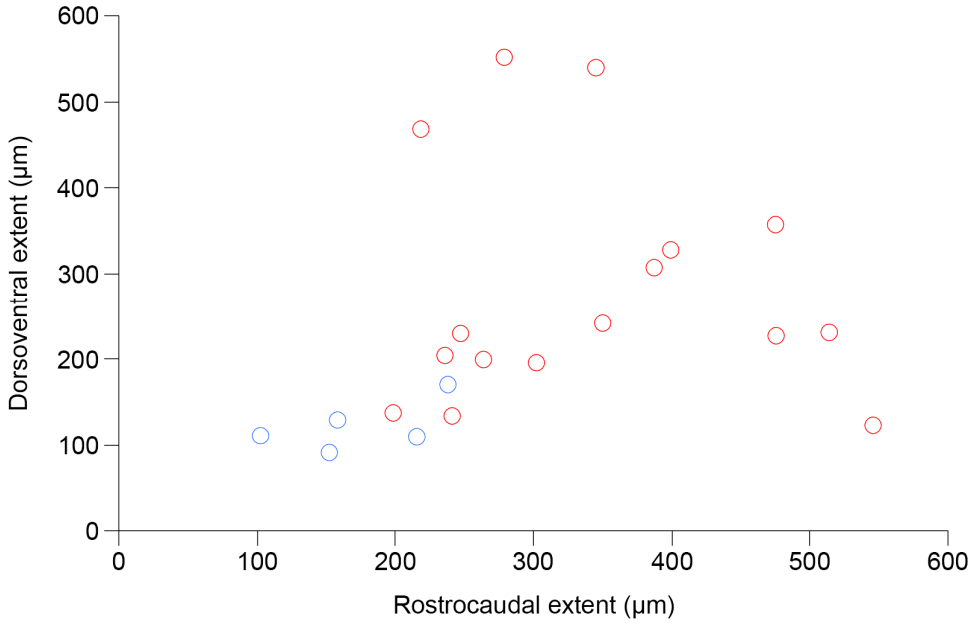
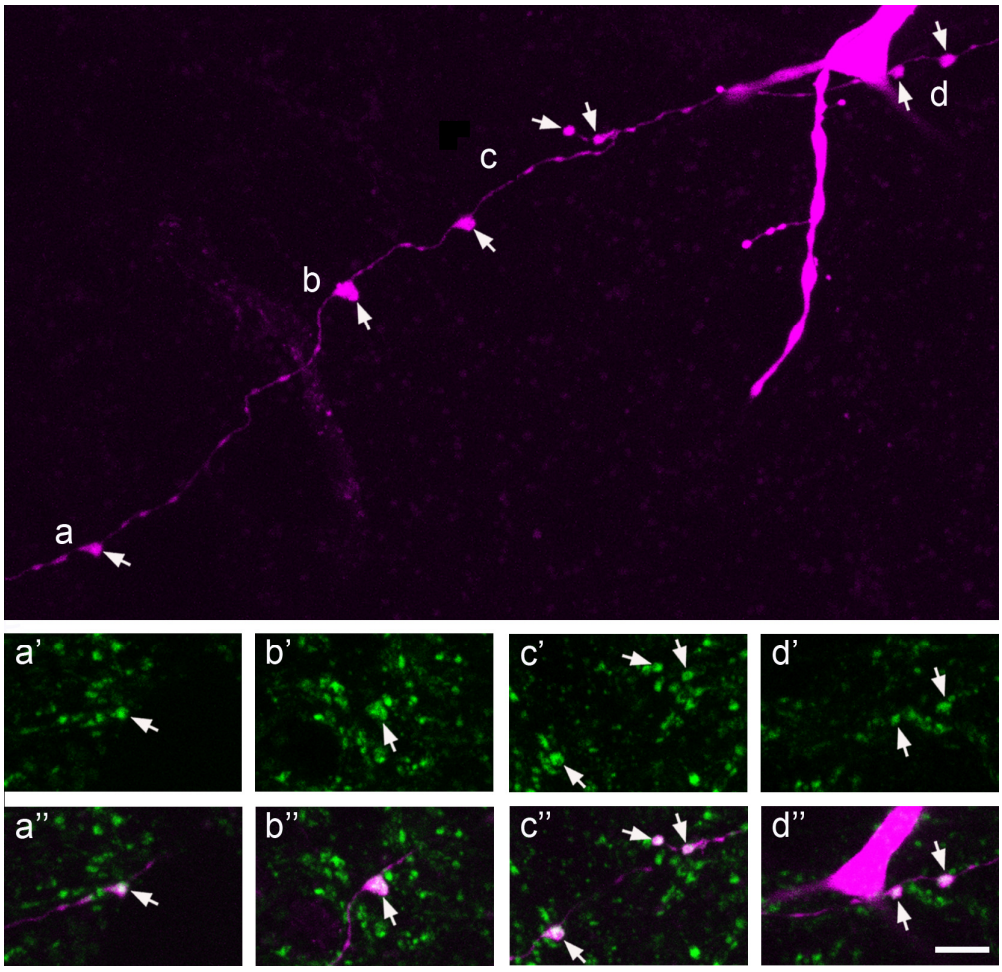

600
500
400
300
200
100
0
Dorsoventral extent (µm)
0
100
200
300
400
500
600
Rostrocaudal extent (µm)

Figure 4-11 VGLUT2-immunoreactive axon of a vertical cell. Projected images of immunostaining for VGLUT2 in parts of the axon of a vertical cell (cell b in Fig. 3-8). The top panel shows a projection of 30 optical sections through part of the axon of this cell labelled with Neurobiotin and avidin-rhodamine (**a-d**, magenta). The larger Neurobiotin-labelled profiles in this field are regions of dendrites that belong to this cell. Several axonal boutons (arrows) are present on the axon. **a'- d'**: VGLUT2 immunostaining (green), while a merged image (**a''-d''**) appears on the bottom row; The images are projections of 6 (**a, b**), 8 (**c**) and 4 (**d**) optical sections at 0.3 µm z-spacing. Scale bar = 5 µm.


a
b
c
d
a'
b'
c'
d'
a''
b''
c''
d''

of the inhibitory group (n = 23) was -60.7 ± 1.6 mV SEM and this did not significantly differ from that of the 27 excitatory neurons (-64.1 ± 1.7 mV) (t-test, $p > 0.05$). Passive membrane properties of the neurons tested are shown in Table 4-5. Action potential discharge patterns in response to 1s current injection were tested in 23 inhibitory neurons and 22 that were VGLUT2-immunoreactive. All major classes of firing patterns were seen in both inhibitory and excitatory neuronal groups. Delayed, gap and reluctant firing were considered to represent an A-current-related discharge pattern (Yoshimura and Jessell, 1989; Ruscheweyh et al., 2003; Heinke et al., 2004; Graham et al., 2007). Therefore, cells that displayed these discharge patterns were grouped together for descriptive purposes. In total, 20/45 (44.4%) of the tested neurons belong to this category. However, the proportion of excitatory (18/22, 81.8%) and inhibitory (2/23, 8.7%) neurons that showed these types of discharge patterns was significantly different ($p < 0.0005$, Chi-square test).

Figure 4-12 shows discharge patterns identified among the different morphological classes. All radial cells had an A-current related pattern, and within the vertical group the presence of this pattern was closely related to neurotransmitter type, being found in all of the glutamatergic, but none of the GABAergic cells. Figure 4-13 shows examples of delayed-firing from a radial cell (A) and a vertical cell (B).

Among the eleven islet cells tested for their firing patterns in response to 1s depolarizing currents, the majority (8/11, 72.7 %) showed tonic firing pattern, while two showed transient-firing pattern and the remainder was gap-firing. All inhibitory central (n = 3) and vertical (n = 2) cells displayed tonic firing pattern. The cells that were morphologically unclassified displayed all major types of firing patterns. Among the 7 inhibitory unclassified cells tested, only one showed transient/gap firing pattern, which differed from the rest of the group (n = 6) that fired in a tonic pattern. Figure 4-14 shows tonic-firing from an islet cell (A) and a central cell (B).

Five out of 21 inhibitory neurons displayed large inward currents (I_h, larger than -40 pA) in response to hyperpolarising voltage steps, but this was not seen in any of the 24 VGLUT2-immunoreactive cells tested. Most of the excitatory cells responded with inward currents of small amplitudes (10-20 pA). A summary of

Table 4-5 Passive membrane properties (mean ± S.D.) of lamina II neurons.

Neurotransmitter phenotype	Morphology	Number of cells tested	RMP (mV)	Cm (pF)	Rm (MΩ)
VGAT	Islet	11	-62.27 ± 8.59	160.45 ± 56.53	444.45 ± 578.88
	Central	3	-56.33 ± 8.50	110.89 ± 19.99	724.73 ± 275.08
	Vertical	2	-59.00 ± 1.41	71.61 ± 25.24	1650.00 ± 636.40
	Unclassified	7	-60.71 ± 7.39	101.45 ± 65.11	538.43 ± 505.87
VGLUT2	Central	3	-64.33 ± 8.08	47.86 ± 8.66	584.83 ± 267.78
	Vertical	9	-65.07 ± 9.04	78.23 ± 24.79	500.00 ± 135.02
	Radial	6	-71.50 ± 3.73	48.88 ± 8.49	648.93 ± 227.08
	Unclassified	9	-58.22 ± 8.87	58.50 ± 41.11	679.43 ± 383.59

Figure 4-12 Discharge patterns displayed by different morphological classes of inhibitory and excitatory lamina II neurons. The histograms show the different discharge patterns (tonic, transient, delayed, gap, reluctant, transient/delayed) displayed by VGAT-immunoreactive (histogram on the left) and VGLUT2-immunoreactive (histogram on the right) cells of different morphological classes (I: islet; C: central; V: vertical; R: radial; U: unclassified) in response to 1 second injections of depolarising current. Note that most inhibitory cells were tonic-firing and the majority of excitatory cells had I_A-currents related firing patterns (delayed, gap, reluctant).

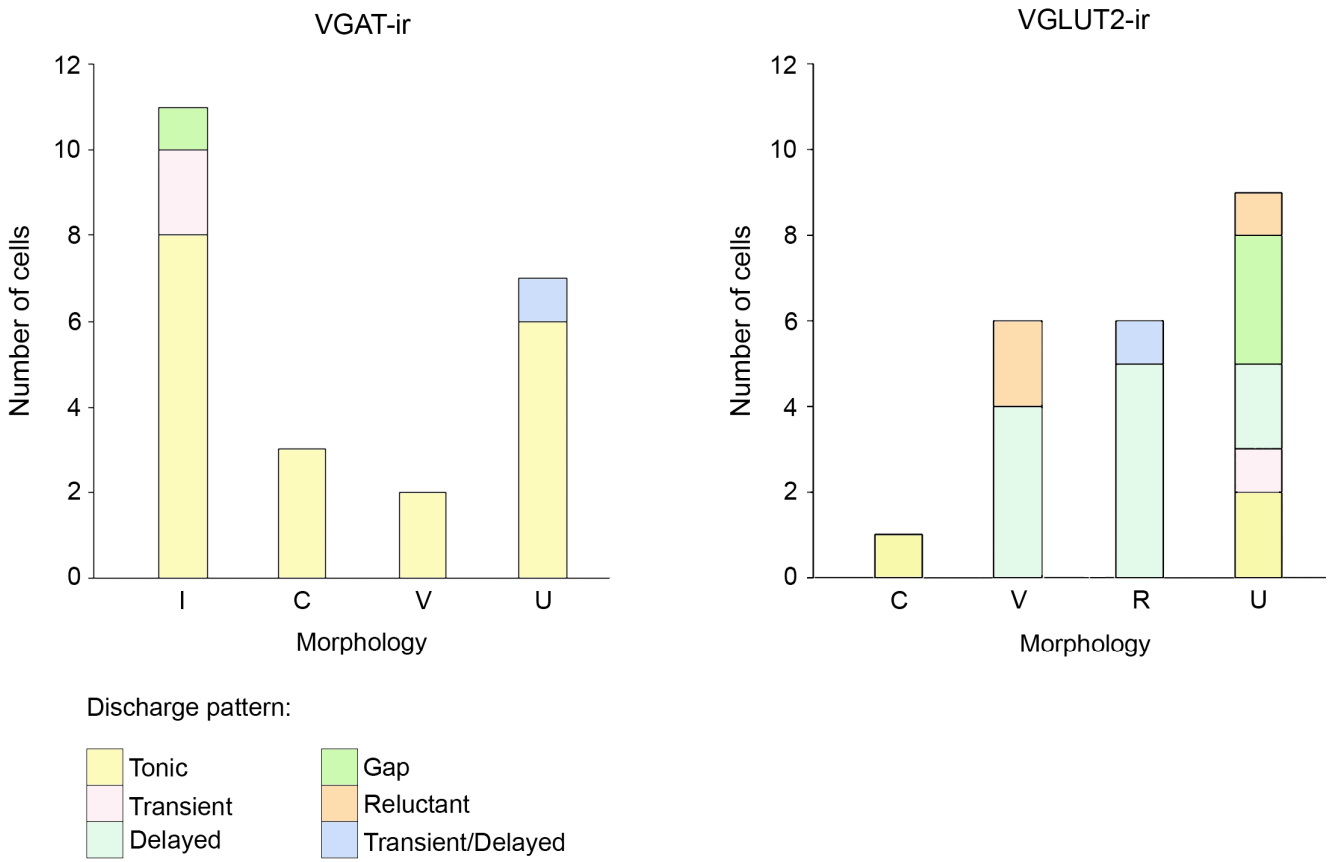

VGAT-ir
VGLUT2-ir
Number of cells
0
2
4
6
8
10
12
I
C
V
U
R
Morphology
Discharge pattern:
Tonic
Transient
Delayed
Gap
Reluctant
Transient/Delayed

Figure 4-13 Delayed-firing displayed by excitatory neurons. Delayed discharge pattern displayed by a radial cell **(A)** and a vertical cell **(B)** at three different holding membrane potentials in current-clamp mode (**a**: -50 and -65 mV; **b**: -65 and -80 mV; **c**: more negative than -80 mV). Values at the left side of each trace indicate the initial membrane voltage or current before application of current or voltage pulses to hold the membrane potential at a certain level. **d**: For the test of voltage responses to hyperpolarising current injection, membrane potentials were initially adjusted to -70 ± 3 mV. **e**: Ionic currents were tested by applying voltage steps in voltage-clamp mode from an initial holding potential of -50 mV (or in some cases, -40 mV). Note that in **e** of (**A**), transient outward currents with slow kinetics were observed at the end of hyperpolarising voltage pulses (arrowhead), which are most probably mediated by a subclass of I_A with slow kinetics. Both cells were excitatory. All current or voltage pulses are of 1 second duration.

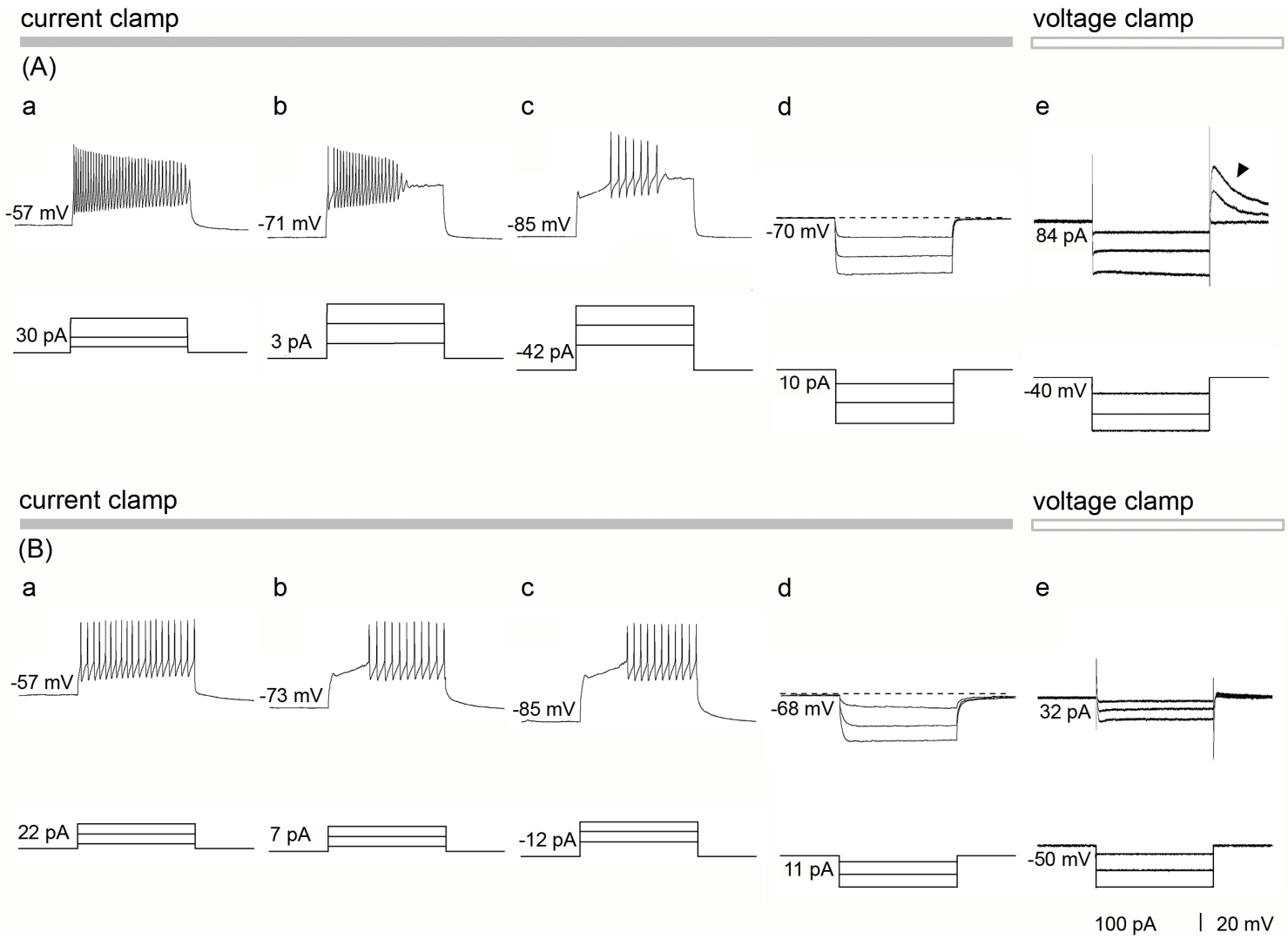
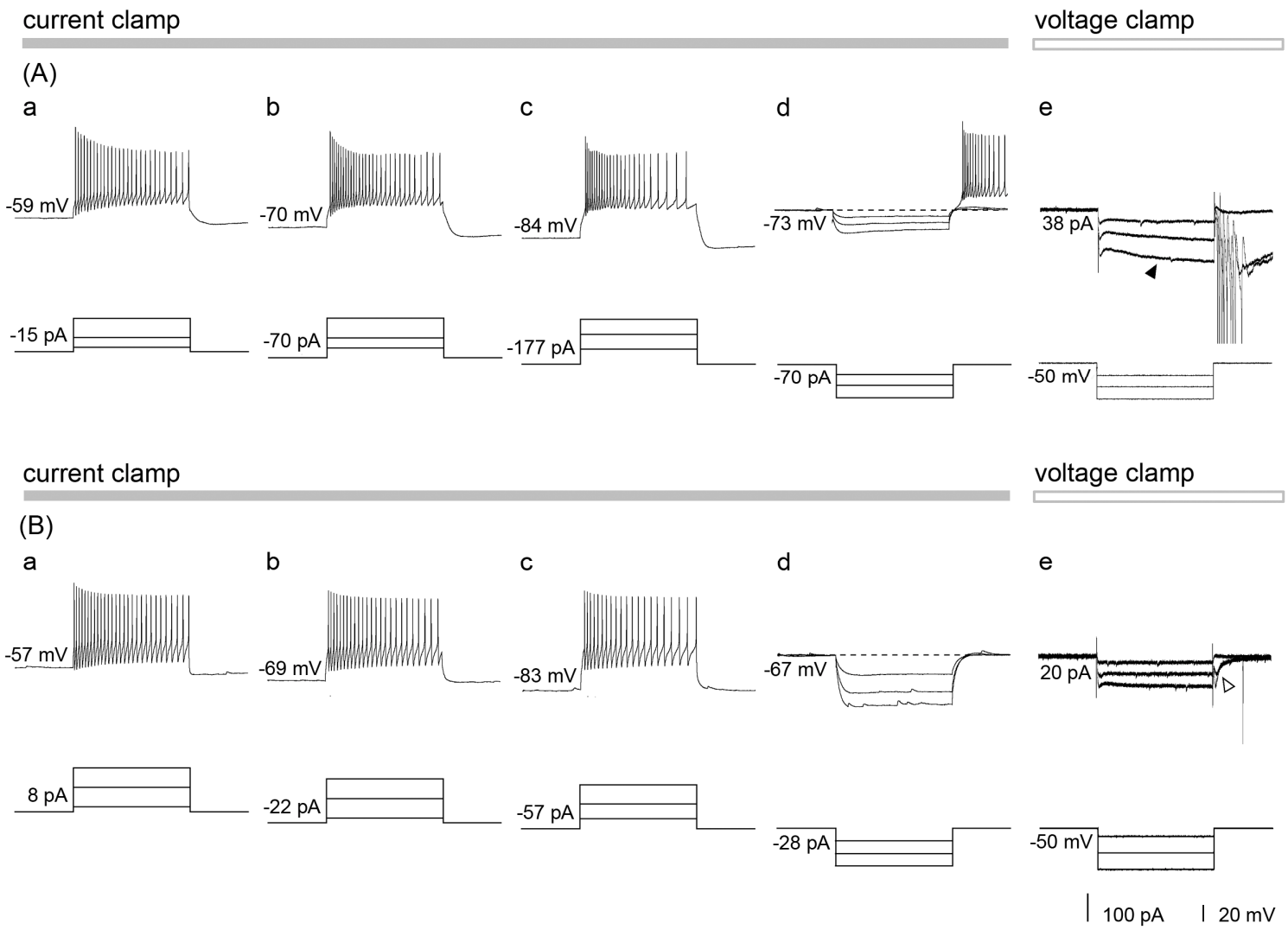

current clamp
voltage clamp
(A)
a
b
c
d
e
-57 mV
-71 mV
-85 mV
-70 mV
84 pA
30 pA
3 pA
-42 pA
10 pA
-40 mV
current clamp
voltage clamp
(B)
a
b
c
d
e
-57 mV
-73 mV
-85 mV
-68 mV
32 pA
22 pA
7 pA
-12 pA
11 pA
-50 mV
100 pA
20 mV

Figure 4-14 Tonic-firing displayed by neurons. Tonic discharge pattern from an islet cell **(A)** and a central cell (B) observed consistently at three different holding membrane potentials in current-clamp mode (**a**: -50 and -65 mV; **b**: -65 and -80 mV; **c**: more negative than -80 mV). Values at the left side of each trace indicate the initial membrane voltage or current before application of current or voltage pulses to hold the membrane potential at a certain level. **d**: For the test of voltage responses to hyperpolarising current injection, membrane potentials were initially adjusted to -70 ± 3 mV. **(A) e**: I_h, current induced by hyperpolarisation (filled arrowhead). **(B) e**: Transient inward current were observed at the end of hyperpolarising voltage pulses, which are probably mediated by I_{Ca} (open arrowhead). Both cells were inhibitory. All current or voltage pulses are of 1 second duration.


current clamp
voltage clamp
(A)
a
b
c
d
e
-59 mV
-70 mV
-84 mV
-73 mV
38 pA
-15 pA
-70 pA
-177 pA
-70 pA
-50 mV
current clamp
voltage clamp
(B)
a
b
c
d
e
-57 mV
-69 mV
-83 mV
-67 mV
20 pA
8 pA
-22 pA
-57 pA
-28 pA
-50 mV
100 pA
20 mV

the incidence of I_h currents in inhibitory and excitatory neurons is shown in Figure 4-15.

4.6 Cell morphology, neurotransmitter content and pharmacology

The amplitudes of outward currents in response to application of neuromodulators for different types of lamina II neurons that were observed in the present study are shown in Figure 4-16. Traces in Figure 3-4 were obtained from an islet cell (a), an excitatory vertical cell (b) and a radial cell (c). Note that somatostatin only evoked outward current in the islet cell (a).

Noradrenaline was applied to 13 inhibitory and 17 excitatory neurons. The proportion of inhibitory (10/13, 77%) and excitatory (12/17, 71%) neurons showing outward currents in response to NA was very similar. When the effects of NA on different morphological types were compared, it was found that all islet cells (6/6), all radial cells (4/4) and most excitatory vertical cells (4/5) responded (Figure 4-16). Among the morphologically unclassified neurons, 2 of 5 inhibitory cells and 4 of 7 excitatory cells responded to NA.

The proportion of inhibitory neurons (4/12, 33%) that exhibited outward currents in response to 5HT was smaller than that of excitatory ones (8/16, 50%), although the difference was not significant ($p = 0.6$, Chi-squared test). The neurons that responded to 5HT included islet, vertical, radial and unclassified types but none of the central cells tested showed response to 5HT. One third of the islet cells (2/6), most radial cells (3/4) and majority of excitatory vertical cells (4/5) responded to 5HT.

However, neurons that responded to somatostatin were restricted to the inhibitory population (7/8, 88%) and none of the 16 excitatory cells that were tested responded. Within the limited sample of cells tested with somatostatin, 5 out of 6 islet cells were found to respond to somatostatin.

Figure 4-15 The incidence of I_h currents in inhibitory and excitatory lamina II neurons. The amplitude of I_h in inhibitory (blue open circles) and excitatory neurons (red open circles) was measured during 1 second hyperpolarizing voltage step from -50 to -100 mV. Values measured immediately after application of the hyperpolarizing pulse were subtracted from those at the end of the pulse to calculate I_h. Currents < 10 Pa are considered to be insignificant. Dashed lines indicate currents range from small (10-20 pA), through medium (20-40 pA) to large (>40 pA). Note that while I_h currents are seen in both types of cell, the large currents are restricted to the inhibitory population. **I**: islet; **C**: central; **V**: vertical; **R**: radial; **U**: unclassified.

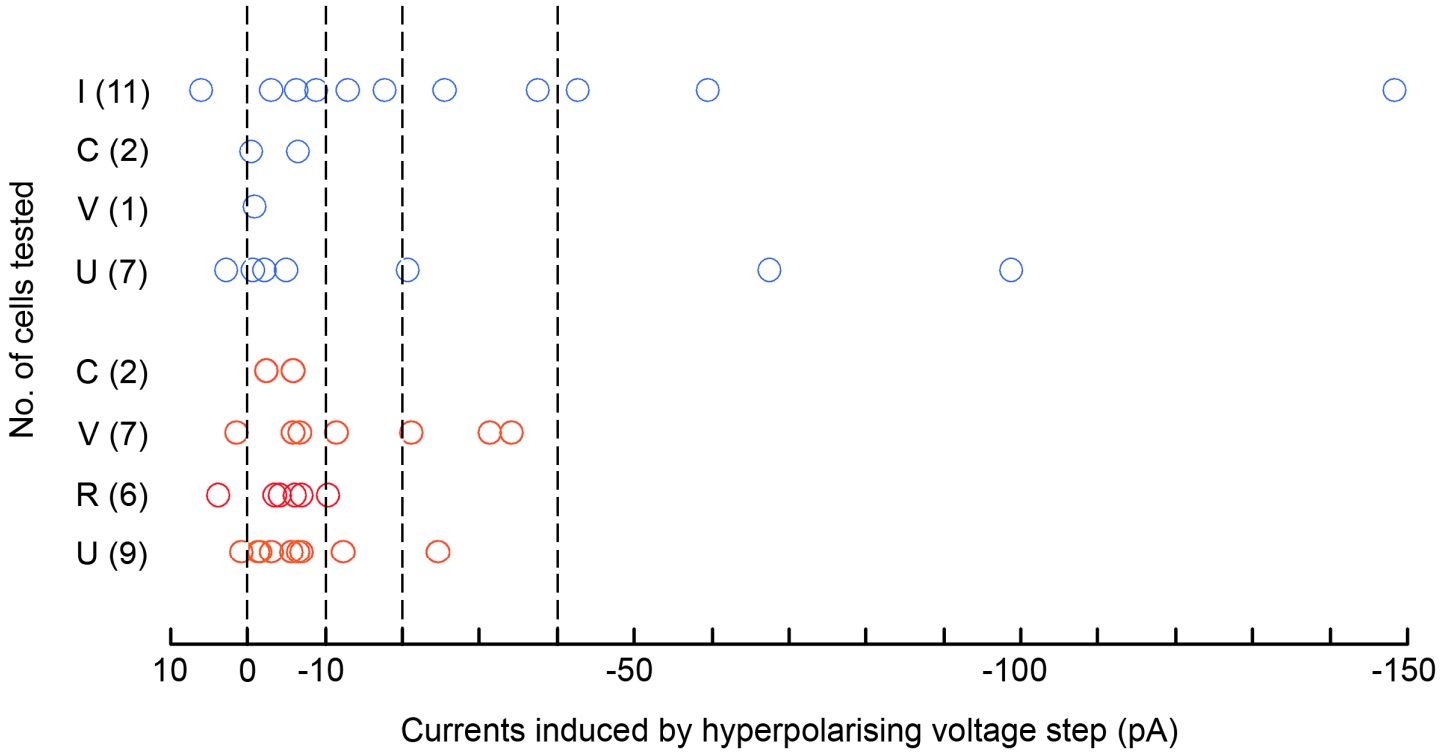
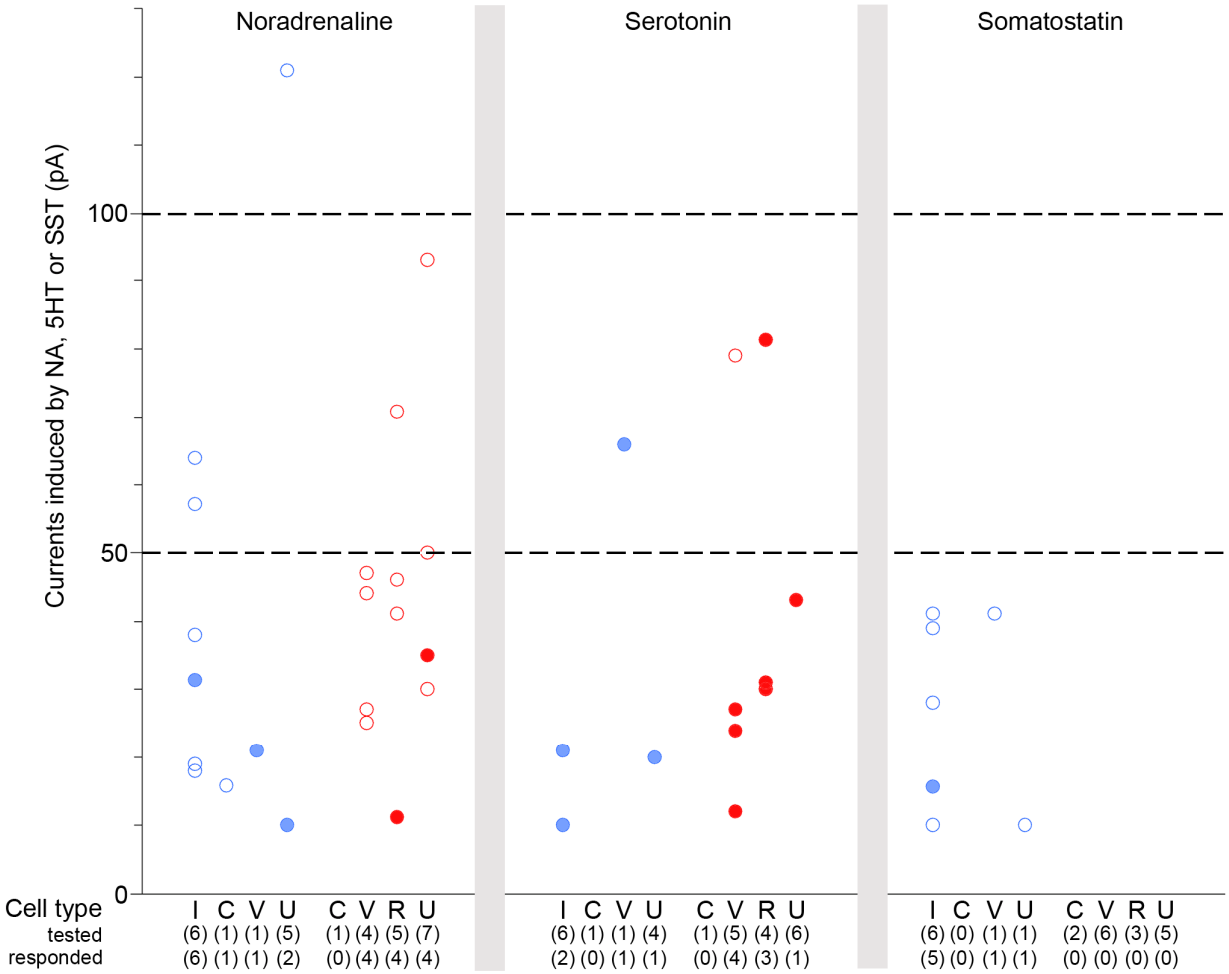

I (11)
C (2)
V (1)
U (7)
C (2)
V (7)
R (6)
U (9)
No. of cells tested
10
0
-10
-50
-100
-150
Currents induced by hyperpolarising voltage step (pA)

Figure 4-16 Responses of inhibitory and excitatory lamina II neurons to noadrenaline, serotonin and somatostatin. The amplitudes of outward currents in response to drug application for each of the inhibitory (blue open or filled circles) and excitatory (red open or filled circles) cells tested, grouped according to morphological class. Open circles show responses to 40 μM NA, 40 μM 5HT or 2 μM somatostatin, while filled circles show responses to 20 μM NA, 20 μM 5HT or 1 μM SST. For each cell, only one concentration of each neuromodulator was tested. Note that most (7/8) of the inhibitory cells, but none of the excitatory cells, responded to somatostatin. **I**: islet; **C**: central; **V**: vertical; **R**: radial; **U**: unclassified.


Noradrenaline
Serotonin
Somatostatin
Currents induced by NA, 5HT or SST (pA)
100
50
0
Cell type
tested
responded
I C V U C V R U
(6) (1) (1) (5) (1) (4) (5) (7)
(6) (1) (1) (2) (0) (4) (4) (4)
I C V U C V R U
(6) (1) (1) (4) (1) (5) (4) (6)
(2) (0) (1) (1) (0) (4) (3) (1)
I C V U C V R U
(6) (0) (1) (1) (2) (6) (3) (5)
(5) (0) (1) (1) (0) (0) (0) (0)

In the sample tested with both NA and 5HT, most radial cells (3/4) and all excitatory vertical cells (4/4) showed responses to both drugs while only some islet cells (2/6) did.

4.7 Neurochemical subclasses

Previous studies have shown that somatostatin and enkephalin are present in glutamatergic neurons (Todd et al., 1992; Todd and Spike, 1993; Simmons et al., 1995; Todd et al., 2003; Marvizón et al., 2007, 2009). Therefore, for 24 of the VGLUT2-immunoreactive neurons, additional sections that contained parts of the axonal arborisation were reacted with antibodies against somatostatin and enkephalin. The morphology and discharge patterns of the neurons that were tested for somatostatin- and enkephalin-immunoreactivity are shown in Table 4-6 and Table 4-7.

4.7.1 Somatostatin

In the sections reacted with somatostatin antibody, immunoreactivity was seen to concentrate in the superficial dorsal horn, where it was found in numerous small structures that resembled axonal boutons, as well as in some cell bodies. Of the twenty-four cells tested, 14 were somatostatin-immunoreactive. Immunostaining appeared in individual terminals, and also appeared in small clumps that probably correspond to clusters of dense-cored vesicles (Todd et al., 2003). Example of somatostatin-immunoreactivity is shown in Figure 4-17.

All five of the radial cells tested expressed somatostatin, as were half of the central (1/2) and vertical (5/10) cells, and 3 of 7 unclassified cells. No obvious morphological differences was observed between vertical or unclassified cells that contained somatostatin and those that did not.

Fifteen of the twenty-four cells tested for somatostatin immunoreactivity had their firing patterns investigated. Seven of ten delayed-firing cells were somatostatin-immunoreactive but none of the cells with other firing patterns contained somatostatin. Although the sample of cells with tonic, gap, transient and reluctant firing patterns was too small to allow specific interpretation, this

Table 4-6 Morphology of lamina II neurons tested for somatostatin- and enkephalin-immunoreactivity.

	Morphology			
	Central	**Vertical**	**Radial**	**Unclassified**
SST+ / EnK+	0	5	4	1
SST+ / EnK-	1	0	1	2
SST- / EnK-	1	5	0	4
Total	2	10	5	7

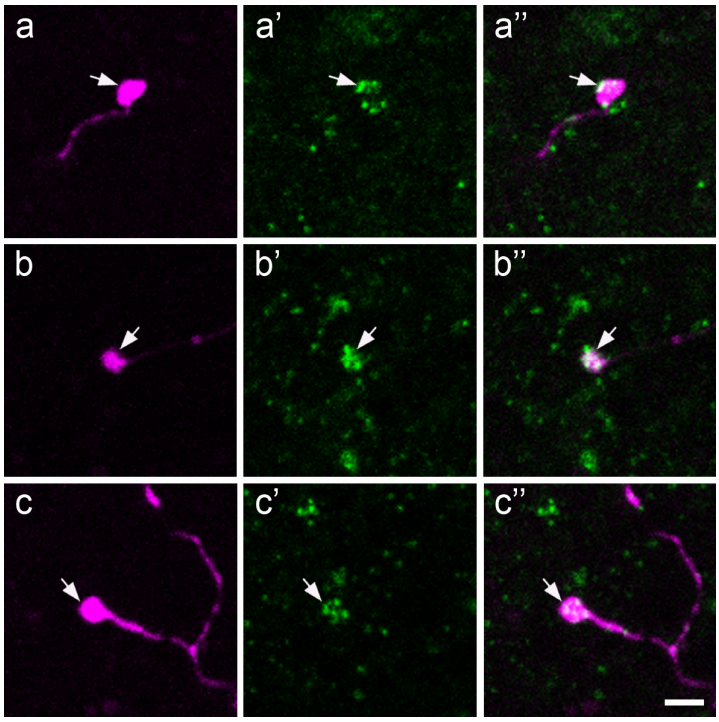
The table shows discharge patterns displayed by excitatory lamina II neurons that were immunoreactive for somatostatin (SST+) and/or enkephalin (EnK+). Some cells contained neither neuropeptide (SST-/EnK-). Note that most (7/10) somatostatin-containing cells had delayed firing.

Table 4-7 Discharge patterns of lamina II neurons tested for somatostatin- and enkephalin-immunoreactivity.

	Discharge patterns					
	Delayed	**Gap**	**Transient**	**Tonic**	**Reluctant**	**Not tested**
SST+ / EnK+	5	0	0	0	0	5
SST+ / EnK-	2	0	0	0	0	2
SST- / EnK-	3	2	1	1	1	2
Total	10	2	1	1	1	9

The table shows discharge patterns displayed by excitatory lamina II neurons that were immunoreactive for somatostatin (SST+) and/or enkephalin (EnK+). Some cells contained neither neuropeptide (SST-/EnK-). Note that most (7/10) somatostatin-containing cells had delayed firing.

Figure 4-17 Somatostatin immunoreactivity in excitatory neuron. Figure shows three axonal boutons from an excitatory radial cell (cell **e** in Fig. 3-10) in a section that were reacted to reveal Neurobiotin (magenta, **a-c**) and somatostatin (green). In each field, several small patches of somatostatin (**a'**, **b'**, **c'**) immunoreactivity are visible, and some of these are contained within the boutons (arrows). Somatostatin immunoreactivity outside the labelled boutons represents expression of the peptide by nearby axons belonging to other neurons. Note that each bouton is labelled with antibody against somatostatin (**a''**, **b'', c''**). The images are obtained from two 0.3 µm optical sections. Scale bar = 2 µm.


a
a'
a''
b
b'
b''
c
c'
c''

result suggests that many somatostatin-containing cells will show delayed-firing patterns.

4.7.2 Enkephalin

Enkephalin immunoreactivity was distributed throughout the superficial dorsal horn in structures resembling axon terminals, which varied in size and were occasionally clustered. Ten of twenty-four cells tested with antibody against enkephalin had axonal boutons that were positively-labelled. Example of enkephalin-immunoreactivity is shown in Figure 4-18.

The majority of the radial cells (4/5) and half of the vertical cells tested were enkephalin-immunoreactive. Two central cells were tested but both did not contain enkephalin, and only 1 of 7 unclassified neurons had axons that were enkephalin-immunoreactive.

Discharge patterns were investigated for 15 of the 24 neurons that were tested for enkephalin immunoreactivity. Half of the cells (5/10) with delayed firing pattern contained enkephalin, but none of the cells with other firing patterns: tonic, delayed, gap, transient, and reluctant, was enkephalin-immunoreactive. Enkephalin was present in axonal terminals of five other cells but their discharge properties were not examined.

4.7.3 Somatostatin and enkephalin colocalisation

Interestingly, all neurons that had axonal boutons that were immunoreactive for enkephalin (n = 10) also contained somatostatin. Examples of somatostatin and enkephalin colocalisation are shown in Figure 4-19. Result from this study suggests that somatostatin and enkephalin colocalise in axonal boutons of neurons that express glutamate.

Figure 4-18 Enkephalin immunoreactivity in excitatory neuron. Figure shows axonal boutons from an excitatory vertical cell in a section that were reacted to reveal Neurobiotin (magenta, **a-c**) and enkephalin (green**, a'**- **c'**). In each field, several small patches of enkephalin immunoreactivity are visible, and some of these are contained within the boutons (arrows). Enkephalin immunoreactivity outside the labelled boutons represents expression of the peptide by nearby axons belonging to other neurons. Note that each bouton is labelled with antibody against enkephalin (**a''-c''**). The images are obtained from two (top and middle rows) or three (bottom row) 0.3 µm optical sections. Scale bar = 2 µm.

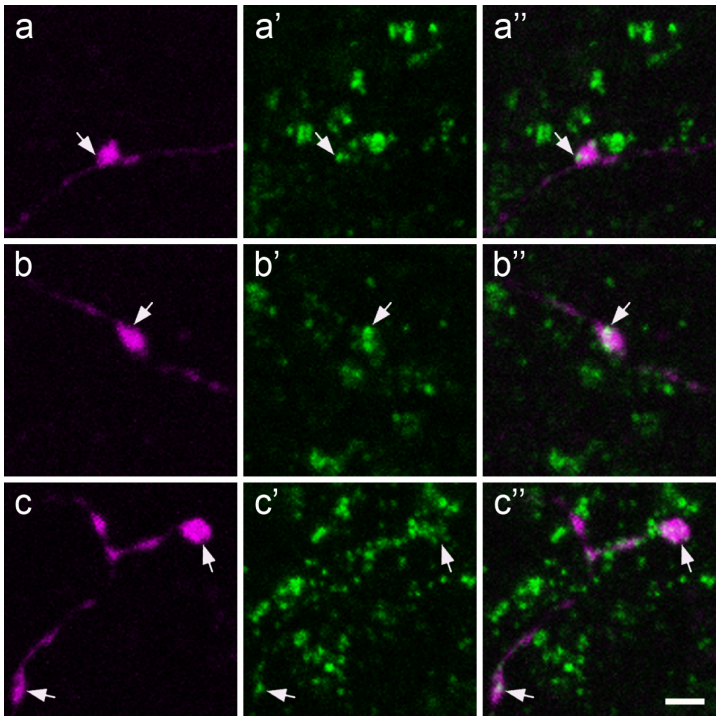
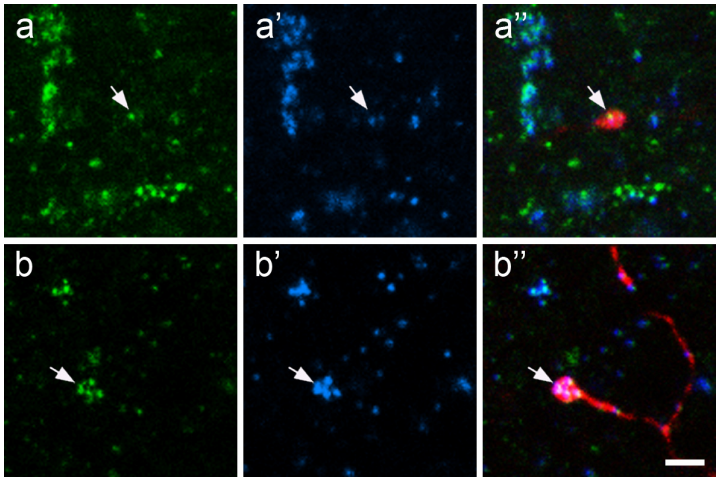

a
a'
a''
b
b'
b''
c
c'
c''

Figure 4-19 Somatostatin and enkephalin colocalisation in excitatory neurons. Figure shows an axonal bouton each from two lamina II neurons (top row: radial cell, cell **e** in Fig. 3-10; bottow row: vertical cell) in sections that were reacted to reveal Neurobiotin (red), somatostatin (green, **a-b**) and enkephalin (blue, **a'-b'**). In each field, several small patches of somatostatin (**a, b**) or enkephalin (**a'**, **b'**) immunoreactivity are visible, and some of these are contained within the boutons (arrows). Note that each bouton is labelled with antibodies against both somatostatin and enkephalin (**a''**, **b''**). The images are obtained from two (top row) and three 0.3 µm optical sections. Scale bar = 2 µm.


a
a'
a''
b
b'
b''

Chapter 5

Discussion

5 Discussion

5.1 Major findings

The major findings of this study are as follows: (1) Both inhibitory and excitatory interneurons were identified in lamina II, by the presence of VGAT or VGLUT2 in axonal boutons; (2) Although certain morphological types were consistently found in each group: all islet cells were inhibitory, while all radial cells and most vertical cells were excitatory; a considerable morphological heterogeneity is present in the rest of the population. (3) A substantial proportion of inhibitory (32.1%) and excitatory (30.3%) interneurons could not be assigned to any of the main morphological classes, since their morphology was atypical or intermediate between two classes; (3) Injection of depolarising current evoked tonic-, transient-, delayed-, gap-, reluctant- and single spike-firing in lamina II interneurons; (4) I_A-related firing patterns (i.e. delayed-, gap- and reluctant-firing) were predominantly displayed by excitatory neurons, while tonic-firing was most frequently observed in the inhibitory population; (5) Noradrenaline and serotonin hyperpolarised both inhibitory and excitatory neurons, but the hyperpolarising action of somatostatin was restricted to the inhibitory group; (6) Some glutamatergic interneurons contained somatostatin and many of these also had enkephalin. Somatostatin was present in the axons of various morphological types of glutamatergic interneurons, while enkephalin was detected in vertical and radial cells. All cells with axons that were somatostatin- and enkephalin-immunoreactive had delayed-firing pattern.

5.2 Technical considerations

5.2.1 Slice preparation and maintenance

Spinal cord slices were placed in a bath of preoxygenated, ice cold (1-3ºC) dissection solution immediately after being excised from the animal. Tissue slices are prone to deteriorate as a result of oxygen depletion and loss of nutrient supply during dissection, therefore the aim of the cold bath was to reduce the cellular metabolic rate and thus to maintain the slices in the best physiological condition possible.

The composition of the dissection solution is important to preserve the physiological stability of the tissue slices *in vitro*. The low sodium content of the dissection solution was designed to keep the ionic concentration in the extracellular environment in a state where Na^+ influx could be minimised, and thus prevent neurons from firing. This helps to conserve energy in cells so that they could easily repolarise when being transferred to the normal Krebs' solution during recording. The dissection solution also had a high magnesium concentration, which reduced synaptic transmission by blocking high threshold calcium channels that are present at the axon terminals of neurons. The high level of sucrose was necessary to compensate for the osmolarity difference between the intracellular compartment and the extracellular buffer solution. The composition of the normal Krebs' solution used to immerse the spinal cord slices during recording mimics that of the cerebrospinal fluid.

Graham et al. (2008) have reported that recording temperature may affect neuronal excitability of some SDH cells and elevated temperature increased the number of reluctant-firing cells. In this study, slices were maintained at room temperature during patch-clamp recording since this is most commonly practised and it avoids the possibility of altered observations as a result of temperature differences.

5.2.2 Membrane holding potentials

The firing patterns of lamina II neurons were examined following application of depolarising current pulses from three different membrane holding potential ranges: -50 to -65 mV, -65 to -80 mV and -80 to -95 mV. This was done in order to avoid certain firing patterns being 'masked' at an insufficient hyperpolarisation state. Sandkühler and co-workers (Ruscheweyh and Sandkühler, 2002; Ruscheweyh et al., 2003; Heinke et al., 2004) have reported that gap-firing could only be evoked from holding potentials more negative than -75mV and this type of firing pattern could easily be mistaken for the tonic-firing pattern when the neuron is not recorded from a sufficiently-hyperpolarised holding potential. Graham et al. (2008) reported that some neurons were reluctant to fire when held at a particular holding potential, but it was not clear whether this was caused by the difference in the holding potential itself or

intrinsic membrane properties of the individual neuron. Consistent with these observations, discharge patterns of 12/45 neurons tested differed when depolarised from different holding potentials and only one cell was reluctant to fire at all three holding potential ranges. The protocol used in this study may explain the differences in the firing patterns of certain cell of specific morphology in comparison to those observed in other studies. For example, central cells tested in this study displayed only tonic firing but they were reported to include a population of transient firing cells, which was further divided by the presence and absence of I_A currents (Grudt and Perl, 2002). However, the relatively small number of central cells for which firing patterns were examined in the present study may also have contributed to this discrepancy. The present study, together with these previous findings, emphasizes the importance of testing firing patterns from different holding potentials.

5.2.3 Drug application

Noradrenaline and serotonin were applied to a sample of the recorded neurons, and both drugs caused an outward current in a proportion of the cells tested. Neither drug evoked depolarisation in any of these cells. Lu and Perl (2007) found that these drugs induced both outward and inward currents on subpopulations of lamina II neurons. However, other conventional whole-cell patch-clamp experiments reported that NA application caused only outward currents (Grudt et al., 1995; Sonohata et al., 2004). Using the perforated patch-clamp technique, Gassner et al. (2009) detected NA-evoked depolarisations in lamina II neurons. North and Yoshimura (1984) reported similar observations in cell recording experiments using sharp electrodes. NA-induced depolarisation and hyperpolarisation are dependent on two types of metabotropic adrenergic receptors: α_1- and α_2- adrenoceptors, respectively (Piascik and Perez, 2001; Hague et al., 2003; Gassner et al., 2009). One possible explanation of the variable NA actions in different experiments is that the intracellular pathways that are triggered by α_2- adrenoceptors are membrane delimited, and are therefore less susceptible to be lost during whole-cell recording and thus leads to hyperpolarisation being preferably induced (Gassner et al., 2009). In addition, elusion of diffusible signals caused by dialysis of postsynaptic neurons are

required for depolarisation (but not for hyperpolarisation) and these are more easily detectable with sharp electrodes and perforated patch clamp (Trussell and Jackson, 1987; Gassner et al., 2009). Similarly, the role of serotonin seems to be controversial since in spite of the monoamine being predominantly inhibitory (e.g. Grudt et al, 1995; Millan, 2002), there were reports on 5HT causing pronociceptive effects (e.g. Green et al., 2000; Oatway et al., 2004). The various serotonergic receptor subtypes are most likely to contribute to the differences in action. Among the known 5HT receptors, subclasses of $5HT_1$ and $5HT_3$ are abundant in the spinal cord (Yoshimura and Furue, 2006). Grudt et al. (1995) reported that $5HT_{1A}$ was accounted for hyperpolarisation in 70% of lamina II neurons. Activation of $5HT_{1A}$ causes the opening of K+ and closing of Ca2+ channels conductance through coupling negatively to adenylate cyclase which produces an inhibitory effect on the sensory transmission (Yoshimura and Furue, 2006). The excitatory role of serotonin has been associated with selective activation of receptor subtype $5HT_3$ (Green et al., 2000; Oatway et al., 2004; Abe et al., 2009). Unlike other known serotonergic receptors that are metabotropic, $5HT_3$ is a ligand-gated ionotropic receptor that has been related to inhibitory interneurons in the SDH as well as small primary afferent fibres (Kidd et al., 1993; Maxwell et al, 2003; Abe et al., 2009). Abe et al. (2009) suggested that large $5HT_{1A}$- mediated outward currents may mask the responses induced by $5HT_3$, since 5HT-induced inward currents were revealed only after application of $5HT_{1A}$ antagonist and $5HT_3$ agonist. This could possibly explain why only outward currents were observed among the lamina II neurons tested in this study in response to serotonin. Selective agonists and/or antagonists can be utilised to refine the present pharmarcological analysis, thus to further understand the distinct roles of these monoamines at the spinal level.

5.2.4 Immunocytochemistry

The best available markers for distinguishing inhibitory (GABAergic/glycinergic) and excitatory (glutamatergic) neurons are proteins that are present in axon terminals. Antibodies against GAD65 and GAD67 have been used to identify GABAergic neurons in previous studies (Schneider and Lopez, 2002; Maxwell et al., 2007). However, these were not used in the present study because the intensity of immunostaining of each antibody has been found to vary in

individual boutons and can be very weak in some (Mackie et al., 2003). This would make the identification of GABAergic neurons difficult and less reliable.

Chaudhry et al. (1998) showed that VGAT localised to synaptic vesicles and is present in both GABAergic and glycinergic nerve endings in the CNS. It is therefore thought to transport both inhibitory amino acids into vesicles. Other studies have provided evidence that virtually all glycinergic neurons in lamina II are GABAergic (Todd and Sullivan, 1990; Polgár et al., 2003). Although it is not possible to know whether the VGAT-immunoreactive boutons identified in this study use glycine as a co-transmitter, they still effectively define a group of inhibitory (and GABAergic) lamina II interneurons. There are, however, reports on subpopulations of GABAergic or glycinergic nerve endings in the CNS that do not express VGAT (Chaudhry et al., 1998). Although this may suggest alternative modes of GABA/glycine transport and the possibility that some GABAergic boutons lack VGAT, this is unlikely to be a common occurrence in the superficial dorsal horn since over 96% of gephyrin puncta in this region were associated with VGAT-containing boutons (AJ Todd and E Polgár, unpublished data) and 94% of $GABA_A$ receptor ß3 subunit-immunoreactive puncta were in contact with a VGAT-positive bouton (Polgár and Todd, 2008). Furthermore, all but four cells for which the neurotransmitter phenotype was tested in the present study had boutons that were either VGAT- or VGLUT2-immunoreactive (but never both). The remaining four cells were excluded from the final analysis due to insufficient amount of axon available for testing with immunocytochemistry.

Three vesicular glutamate transporter proteins have been characterised: VGLUT1, VGLUT2 and VGLUT3. Within the spinal cord, VGLUT1 is largely found in the central terminals of myelinated primary afferents that occupy the inner part of lamina II, while VGLUT2 is present at high density throughout the dorsal horn, including lamina II (Varoqui et al., 2002; Todd et al., 2003). It is thought to be contained in axon terminals of excitatory interneurons, based on its colocalisation with peptides such as somatostatin and neurotensin that are found in non-GABAergic interneurons in the superficial laminae (Todd et al., 2003). In addition, mRNA for VGLUT2 (but not for VGLUT1 or VGLUT3) is present throughout the dorsal horn (Kullander et al., 2003; Oliveira et al., 2003). VGLUT3 has recently been identified in the central terminals of primary afferents that are thought to be low threshold mechanoreceptive C-fibres (Seal

et al., 2009). VGLUT2 is a proton gradient-dependent glutamate transporter that regulates glutamate uptake in internal membranes. Therefore it not only identifies glutamatergic neurons but is sufficient to make neurons store glutamate in synaptic vesicles and release it by exocytosis (Takamori et al., 2001). Therefore, VGLUT2 is generally accepted as a marker for glutamatergic interneurons.

5.2.5 Cell morphology

Morphological classification of neurons can be complicated and not always straightforward due to the subjectivity of this method. Cell morphology is generally assessed by visual inspection based on different criteria, which could vary depending on the experimental design and purpose. In general, morphology of individual cells is described according to the appearance of the cell body, dendritic tree and axonal arborisation. However, these basic criteria could be expanded to meet the needs of different experiments. Two previous studies have attempted to avoid the need for subjective judgement by using a limited morphometric analysis based on extent of dendritic arbors to investigate the morphological properties of SDH neurons (Prescott and De Koninck, 2002; Yasaka et al., 2007). Although this morphometric approach provides a more objective way of classifying neurons, it could also be problematic. Since the analysis is based mainly on the dendritic extent of neurons, any inconsistent branching of dendrites can have a dramatic effect on the geometrical measurements. For example, some of the neurons in the present study had a single dendrite that extended for a great distance in one direction, without any nearby branches, thus distorting the measurement in that particular dimension.

Only one cell was recorded from each spinal cord slice to ensure that all labelled dendritic and axonal profiles originated from the recorded neuron. The morphology of every neuron was reconstructed by aligning and stitching together scanned images obtained from serial Vibratome sections that contained labelled profiles by matching the cut surfaces of the corresponding dendrites and axons. Unlike previous studies that obtained images of intracellularly labelled neurons from scans of intact spinal cord slices (e.g. Hantman et al., 2004; Heinke et al., 2004; Maxwell et al., 2007; Melnick, 2008), the cells in the present study were

scanned after re-sectioning of the slices. This is because cells were recorded from a substantial depth in the slice (usually more than 100 μm) and therefore could not be scanned in the intact slice due to the limited working distance of the 40× oil-immersion objective lens. In addition, the slices had to be cut into 60 μm sections to allow immunocytochemical processing.

An alternative to the approach used in this study would be to reconstruct the cells using software such as Neurolucida for Confocal. This would allow detailed reconstruction of the dendritic and axonal arbors of each cell. However, it would be extremely time consuming and labour intensive when applied to neurons with very complex dendritic arborisation, such as some of those identified in this study. Reconstruction based on scanned images from sections, instead of intact whole slices, makes reconstruction even more complicated particularly in the cases where dendrites or axon are slightly distorted as a result of tissue sectioning. This would contribute to problems when aligning sections and matching them together in Neurolucida. Taken together, these factors would limit the number of cells being sampled, despite providing a significant amount of details for each neuron. Therefore, numerous issues ought to be considered in order to produce a sufficient sample size that includes adequate information from each neuron and more importantly allows the morphology of cells to be represented as accurately as possible.

A more comprehensive approach for classifying SDH neurons would be to carry out cluster analysis (e.g. McGarry et al., 2010). This would require combining various quantified anatomical and physiological parameters to distinguish neuronal populations in an unbiased way. The anatomical measures could include soma size and shape, laminar distribution of dendrites and axon, dendritic thickness and dimension, the number of dendrite branches and the presence of dendritic spines. Cluster analysis was not used in this study firstly because Neurolucida reconstructions were not performed for reasons discussed above. In addition, the diversity of neurons within lamina II suggests that there will be numerous functional populations. This would therefore require a very large sample size in order to allow identification of discrete groups with this approach.

5.2.6 Sample size

Altogether 61 lamina II interneurons were analysed in this study to compare their neurotransmitter content, electrophysiological, morphological and pharmacological properties. This relatively large sample was sufficient to show the heterogeneity of neuronal populations in this region, and it demonstrated the different morphological types among inhibitory and excitatory neurons. It also showed the discharge patterns and responses to neuromodulators among these neurons. However, the numbers of cells identified in this study should not be regarded as representing the real proportions of each cell type in lamina II. This is largely due to the inevitable possibility that certain cell types, or neurons of certain functional properties, are more likely than others to be recorded from in such experiments. Although a sample size that is too small is not reliable for providing an accurate representation in this sort of analysis, a very large sample size would be too time-consuming to obtain. A very large sample would ideally overcome the problem of certain neurons being more frequently encountered than others and thus gives a more realistic representation, but the bias that occurs towards certain cells due to the nature of the experiment still cannot be ruled out.

5.2.7 Sampling bias

In this study, sampling bias that occurs towards certain types of neurons may be caused by a few issues. Parasagittal and horizontal slices were used in this study since dendritic trees of lamina II neurons usually arborized in the rostrocaudal axis and such section planes would preserve these cells better. This, however, could not eliminate the problems caused by neurons with dendrites that branch extensively in all directions. Dendrites of these cells are more likely to be damaged during tissue sectioning and therefore the physiological condition of these cells would deteriorate and make them more difficult to detect by the electrode during recording. The size of neurons varied considerably, and the ones with larger cell bodies were more likely to be patched onto by the non-visually guided recording electrode. Sampling bias is present in both targeted and blind patch-clamp recording. While larger neurons are more likely to be selected in blind recording (as described above), visually targeted recording can

only be performed on neurons that are located superficially within the slice and therefore favours neurons with compact dendritic arborisations. These technical preferences could account for the higher than expected proportion of inhibitory neurons (46%), instead of 31% as previously reported (Polgár et al., 2003). For example, islet cells, which are all inhibitory, were among the largest cells identified in this study, and therefore this cell type could be over-represented for the reasons discussed above. The proportions of radial and vertical cells found in this study were very similar to those reported by Grudt and Perl (2002), but central cells only constituted 13% of the sample in this study compared to 29% by Grudt and Perl (2002). This variation could be due to species difference, since rats were used in this study and hamsters were used in the previous study. However, technical issues such as plane of section and whether neurons were visually targeted are more likely to contribute to this difference. Despite the inevitable technical bias, neurons of each major type were identified in this study.

Neurons that had a resting membrane potential less negative than -40 mV were excluded from the electrophysiological recording. This relatively low value was used because among the major cell types, islet cells were reported to have a mean resting potential of -47.7 mV (Grudt and Perl, 2002). Therefore there could be a tendency for certain cell types to be excluded from the sample if their membrane potentials were less negative than those of other types.

Since the main purpose of this study was to compare morphological, physiological and pharmacological properties of inhibitory and excitatory neurons, those for which the transmitter was not identified were excluded from further analysis. Neurons differed in the amount of axon available for testing with immunocytochemistry. Some cells have very limited axonal arbor, and thus have fewer boutons located superficially in the section where immunostaining was successful. In certain cases, neurons had boutons that were very small and they were difficult to visualise immunostaining for either vesicular transporter. Although these issues may have contributed to the likelihood of a neuron being eliminated from further analysis, only four cells were excluded on this basis.

5.3 Classification of lamina II interneurons

5.3.1 Why is neuronal classification important?

The dorsal horn contains a diverse array of interneurons with a relatively small number of projection neurons. Cells from each population have been extensively investigated and evidence has been found to show that they are not only functionally diverse, but they also vary widely in their structural characteristics and neurochemical properties. This complex diversity has therefore made classification of either the interneurons or the projection neurons difficult, and thus complicates the understanding of the roles of these cells in the local neuronal circuitry. However, distinct classes of morphologically discrete neurons that correspond to functional populations have been identified in other areas of the central nervous system. For example, five morphologically distinctive cell types are present in the cerebellar cortex: stellate, basket, Golgi, Purkinje and granule cells. These cells are organised in three layers and they form specific synaptic connectivity. The inhibitory stellate and basket cells synapse selectively onto different parts of the Purkinje cells, while the GABAergic terminals of Golgi cells form axodendritic synapses on the dendrites of excitatory granule cells. Axons of granule cells form the parallel fibres, which provide an excitatory (glutamatergic) input to the Purkinje cells, which provide the only output of the cerebellar cortex. The highly specific functional organisation of these neurons suggests that cells of particular morphological appearance may represent discrete functional classes and form specific synaptic connections.

Dendritic geometry has been used to define neuronal classes in the SDH. Electrophysiological recording from pairs of neurons in this region has suggested that connectivity between neurons is likely to be associated with the different morphological classes. For example, GABAergic islet cells were presynaptic to transient-firing central cells (Lu and Perl, 2003), while vertical cells send excitatory synaptic projections to lamina I neurons (Lu and Perl, 2005). Zheng et al. (2010) have recently found a group of functionally and morphologically homogeneous inhibitory neurons in lamina II that are presynaptic to islet and vertical cells. These cells were morphologically equivalent to central cells.

The improved understanding of connectivity among SDH neurons that are structurally distinctive provide important information to fill in the gaps on neuronal circuitry. The two studies by Lu and Perl (2003, 2005) have shown unidirectional excitatory and inhibitory synaptic connections between neurons (see above), which strongly suggested that discrete groups of neurons are linked to each other in a highly specific manner. SDH is densely innervated by nociceptive primary afferents, and its constituent neurons play the major role in somatosensory information processing before the signals are conveyed to the brain. Therefore, the balance between excitation and inhibition of SDH neurons that form the complex processing circuits is very important to maintain normal sensory function (Todd, 2010). Depending on whether inhibitory or excitatory neurons are involved, changes in neuronal excitability would result in different overall output from the SDH (Graham et al., 2007).

Neuronal responses to depolarising and/or hyperpolarising currents (e.g. Yoshimura and Jessell, 1989b; Lopez-Garcia and King, 1994; Lu and Perl, 2003) as well as peripheral cutaneous stimulation (e.g. Christensen and Perl, 1970; Woolf and Fitzgerald, 1983; Réthelyi et al., 1989; Furue et al., 1999) are regularly used to characterise neurons in the SDH. Both approaches showed that SDH neurons displayed a variety of responses to current injection and different modalities of cutaneous stimulation (Graham et al., 2004). However, the direct functional relationship between action potential discharge patterns and cell responses to peripheral cutaneous afferent stimulation remains unclear. Lopez-Garcia and King (1994) reported that biophysical properties of dorsal horn neurons are significantly associated with their response to peripheral afferent stimulation, since cells that responded to both low- and high-threshold mechanical stimuli largely displayed tonic firing. Graham et al., (2004), however, found no distinct relationship between current-evoked discharge patterns and peripherally-evoked responses. This suggested that firing patterns do not necessarily identify neuronal responses to physiologically relevant stimuli in all cases.

A comprehensive classification scheme that compares and combines physiological, morphological and neurochemical properties may shed light on the heterogeneity of SDH neurons, thus establish the functions of different neurons in the highly complex sensory processing circuitry.

5.3.2 How has the present study extended previous findings?

Islet cells were first described by Gobel (1975, 1978) to have dendrites that were elongated to a great extent in the rostrocaudal axis and often had recurrent dendritic branches. These features were consistently found in subsequent studies (Todd and Lewis, 1986; Grudt and Perl, 2002). Gobel et al., (1980) suggested that they were inhibitory and this was later confirmed by immunocytochemical studies showing that they were GABAergic (Todd and McKenzie, 1989; Todd and Sullivan, 1990). Similarly, it was found that axons of islet cells contained GAD (Maxwell et al., 2007) and 62% of EGFP-GAD67 expressing neurons were classified as islet cells by Heinke et al. (2004). In addition, physiological studies have demonstrated that islet cells form GABAergic synapses (Lu and Perl, 2003; Zheng et al., 2010). This information, together with the finding that all twelve islet cells recognised in this study had axons that were VGAT-immunoreactive, strongly suggests that islet cells constitute a morphologically and functionally discrete population of lamina II inhibitory interneurons.

Gobel (1978) grouped neurons with ventrally directed spine-covered dendrites in lamina II as stalked cells, and suggested that these neurons were excitatory. Consistent with this, none of the stalked cells examined by Todd and McKenzie, (1989) showed immunoreactivity when tested with antibody against GABA. Grudt and Perl (2002) identified neurons of similar morphology, but noted that some these had few dendritic spines and/or axons that did not enter lamina I, and included these cells in a larger population of 'vertical' cells. As noted above, Lu and Perl (2005) obtained physiological evidence that vertical cells were glutamatergic. Maxwell et al., (2007) also found that axonal boutons of the majority of vertical cells expressed VGLUT2-immunoreactivity. However, they also found two vertical cells that were GAD-immunoreactive. Consistent with this, the present study identified many vertical cells that were glutamatergic (12/15), but also identified three cells that had the morphological appearance of vertical cells with axons that were GABAergic. These inhibitory vertical cells, however, had less extensive dendritic tree than the glutamatergic vertical cells and displayed tonic firing pattern instead of the delayed firing that is predominantly observed in the excitatory group. The cell bodies of vertical cells

identified in this sample, consistent with previous studies, were principally located in outer lamina II (Grudt and Perl, 2002; Maxwell et al., 2007; Yasaka et al., 2007). When viewed in the sagittal plane, radial cells seen in this study had compact and almost-circular dendritic trees similar to those described in previous studies (Grudt and Perl, 2002; Yasaka et al., 2007). All of them were glutamatergic and displayed I_A-type firing patterns. However, these cells differed from those with much longer radiating dendrites identified in previous studies, some of which contained GABA (Todd and McKenzie, 1989; Maxwell et al., 2007). These observations suggest that there are possibly populations of lamina II neurons that are morphologically very similar but differ in size and functions, and therefore require careful characterisation to avoid inaccurate interpretation.

Rèthelyi et al. (1989) were the first to describe a group of lamina II neurons with morphology that closely resembled islet cells as 'central cells'. Todd and Mckenzie (1989) identified a group of 'small islet cells' that were not GABA-immunoreactive. Grudt and Perl (2002) identified cells of similar morphology and classified them as central cells, which they further divided into tonic, transient-I_A, and transient non-I_A firing types based on their discharge properties. Tonic and transient non-I_A central cells can form glutamatergic synapses onto vertical cells (Lu and Perl, 2003, 2005). However, others have identified a population of GABAergic tonic central cells that are presynaptic to vertical and islet cells (Hantman et al., 2004; Zheng et al., 2010; see below). Maxwell et al. (2007) only found one central cell, which was inhibitory. Consistent with the previous reports, both GABAergic and glutamatergic central cells were identified in this study. However, all four central cells (3 inhibitory and 1 excitatory) tested for their discharge patterns in the present study were tonic firing. The variety that exists among the central cell group suggests that there may be more subclasses yet to be identified. A subset of lamina II GABAergic neurons that expresses GFP under the control of mouse prion promoter was recently discovered and interestingly, these cells had characteristics that fit those of central cells and are functionally homogeneous (Hantman et al., 2004, 2005; Zheng et al., 2010). This further suggests that the central cell category may be more diverse than what is presently characterised.

Results from the present study and those from previous reports showed that the islet cells invariably represent a morphologically distinctive inhibitory neuronal group in lamina II. All radial cells and most vertical cells are excitatory and therefore these are also likely to constitute discrete populations. However, the remaining neurons of lamina II consists of inhibitory and excitatory cells that are morphologically and functionally diverse. This suggests that although the relationship between morphology and neurotransmitter content of LII neurons is evident for certain cell types, it is not as straightforward for others.

To make this yet more complicated, previous reports as well as results from this study consistently observed a relatively high proportion of lamina II neurons that do not belong to any of the known morphological classes (Todd and Lewis, 1986; Todd and McKenzie, 1989; Grudt and Perl, 2002; Heinke et al., 2004; Maxwell et al., 2007; Yasaka et al., 2007). These cells have morphology that do not conform to any of the major cell types or have geometrical features that represent more than one cell type. These neurons vary not only in their structural characteristics, but also displayed different neurotransmitter content and firing patterns. The central cell population that included both inhibitory and excitatory neurons, together with those currently unclassified, demonstrates the complexity of lamina II neuronal populations and suggests that there are several further classes still to be recognised.

Axonal arborisation of lamina II neurons provides important information on the potential connectivity between neurons in this region and those located in adjacent laminae. For a long time it was believed that the gelatinosal complex, which includes both laminae II-III, was a 'closed system' (Szentágothai, 1964). It was suggested that axons of lamina II the local interneurons remained within these laminae, with no direct pathways for further conduction apart from connections with neurons in laminae IV and V. However, this is unlikely to be entirely true since it was previously shown that some neurons in lamina II had axons that extended through laminae III-V and terminated within the deeper laminae (Light and Kavookjian, 1988). Among the 61 lamina II neurons identified in this study, 36 had axons that extended into lamina I and axons of 32 travelled ventrally into lamina III (and often beyond). This suggests that lamina II neurons do not only interact with neurons that are located within the lamina, but also

form synapses onto cells in neighbouring laminae as part of the complex local neuronal circuitry.

Inhibitory neurons in the superficial dorsal horn have been extensively investigated because they are presumed to be involved in the processing of somatosensory information and possibly play important roles in synaptic regulation that underlies pain mechanisms (Melzack and Wall, 1965; Sandkühler et al., 2009). Intrathecal application of GABA or glycine receptor antagonists has been shown to elicit pain following innocuous stimuli, which further suggests that a decrease in the efficacy of spinal inhibitory circuits would lead to hypersensitive pain states (Yaksh, 1989; Sivilotti and Woolf, 1994). Immunocytochemical studies have attempted to distinguish subpopulations present in lamina II based on the expression of various neuropeptides and proteins, aiming to eventually identify discrete functional classes among them. For example, NPY and galanin are found exclusively in GABAergic neurons, but these neurons very infrequently contain glycine (Rowan et al., 1993; Todd and Spike, 1993; Simmons et al., 1995). In contrast, parvalbumin and nNOS are identified in neurons that contain both GABA and glycine, but they constitute separate populations (Antal et al., 1991; Laing et al., 1994). Similarly, nNOS and NPY are found in non-overlapping populations of GABAergic neurons in laminae I-III (Laing et al., 1994). GABAergic NPY-containing neurons are found to synapse onto lamina III projection neurons that express neurokinin 1 receptor, while some of those that contain nNOS innervate the giant projection neuron in lamina I (Puskár et al., 2001; Polgár et al., 2008). This suggests that these neurochemically defined classes are most likely to be functionally distinctive populations. In addition, parvalbumin-expressing inhibitory neurons with fast-spiking firing pattern have been identified as a distinctive population, which contributes to gamma frequency oscillations that are thought to provide a robust temporal network structure for information processing in the brain (Bartos et al., 2007). Parvalbumin is present in 70% of GABAergic neurons in lamina II (Antal et al., 1991) and the majority (19/23) of inhibitory lamina II neurons tested for their firing pattern in the present study exhibited tonic firing. In transgenic mice, parvalbumin-expressing neurons that were selectively labelled by GFP were also fast-spiking (B Graham and D Hughes, personal communication). These observations suggest that neurons in different spinal level may be related in

their roles and it is likely that discrete neuronal groups in the CNS that express specific neuropeptides may be homogeneous in their functions.

Less is known about the glutamatergic neurons present in lamina II, since immunocytochemical markers for these neurons are still limited to VGLUTs, and these only label glutamatergic axon terminals, but not the cell bodies. Previous studies have consistently assumed that neurons that are not GABAergic/glycinergic use glutamate and this is confirmed by the results of the present study. A large number of glutamatergic interneurons in the SDH are responsible to synthesise peptides, (including somatostatin, enkephalin, substance P, neurokinin B and neurotensin) since many nonprimary axonal terminals that contain one of these peptides also express VGLUT2 (Todd and Spike, 1993; Todd et al., 2003).

By obtaining a larger sample or combining neurochemical identification with various markers may shed light on these morphologically heterogeneous populations of inhibitory and excitatory interneurons, and thus refine the neuronal classification in this region. However, this needs to be carried out with caution since not all neuropeptides are expressed exclusively by either inhibitory or excitatory interneurons; others (e.g. enkephalin) are present in both glutamatergic and GABAergic neurons and this could make the definition of discrete classes difficult.

5.4 Firing patterns associated with I_A currents

Most excitatory neurons but very few inhibitory cells have delayed-, gap- and reluctant-firing patterns in response to depolarisation. These firing patterns are attributed to I_A currents as reported in several studies (e.g. Yoshimura and Jessell, 1989b; Ruscheweyh and Sandkühler, 2002). I_A is a voltage-dependent K^+ current that activates and inactivates rapidly in response to depolarisation from holding potentials more negative than the resting membrane potentials. This is supported by the observation that discharge patterns of several cells in this sample changed from tonic or transient firing patterns to I_A-related firing patterns at more negative holding potentials. I_A act to regulate neuronal excitability by delaying the first action potential and reducing discharge

frequency, thus generating delayed-, gap- or reluctant-firing. These firing patterns have been associated to particular cell types in the SDH (Grudt and Perl, 2002; Prescott and De Koninck, 2002; Ruscheweyh et al., 2004; Graham et al., 2008). There is also an obvious difference in the response of lamina II neurons to hyperpolarisation, since the majority of inhibitory cells showed inward currents while most excitatory cells (including radial and vertical types) displayed transient outward currents, with either fast or slow kinetics, which is associated to delayed- or gap-firing, respectively.

This A-type potassium current is mediated by voltage-gated K^+ channel subunits, with the major component being Kv4.2 since genetic elimination of Kv4.2 drastically reduces I_A in the SDH (Hu et al., 2006). Kv4.2 modulates transmission of nociceptive signals from the periphery to the brain. Heinke et al. (2004) found that delayed- and gap-firing patterns were mainly displayed by GFP-negative cells in mice of which GFP is expressed by some GABAergic lamina II neurons. Expression of Kv4.2 and Kv4.3 were also observed in calretinin-containing and μ-opiod receptor-immunoreactive lamina II neurons (Huang et al., 2005), which are thought to be glutamatergic (Kemp et al., 1996; Albuquerque et al., 1999). The results from this study confirm and extend these previous observations by directly demonstrating that I_A-type firing patterns are predominantly restricted to excitatory cells and associated with most of these cells.

I_A currents have been related with plasticity, and loss of I_A in Kv4.2 knockout animals has been shown to enhance neuronal excitability and firing frequency, resulting in increased sensitivity to tactile and thermal stimuli (Hu et al., 2006). Kv4.2, which produces I_A is directly phosphorylated by ERKs, which are specifically activated by noxious stimuli (Ji et al., 1999) and have been implicated in the development of spinal central sensitisation that underlies chronic pain. Nociceptive plasticity could involve pERK-mediated phosphorylation of Kv4.2, resulting in the reduction of I_A currents (Hu et al., 2006). Therefore, the results from this study strongly suggest that excitatory lamina II interneurons with I_A –type firing patterns are involved in synaptic plasticity in inflammatory pain states.

5.5 Responses to neuromodulators

5.5.1 Actions of NA and 5HT on lamina II neurons

The SDH receives adrenergic and serotoninergic inputs from the brain stem (Satoh et al., 1982; Miletic et al., 1984), and these descending pathways are thought to produce analgesic effects upon stimulation (Reynolds, 1969). However, specific actions of these monoamines on individual neurons, and how they interact to modulate sensory transmission in the SDH, are still not very well understood. Effects of exogenous application of NA and 5HT onto individual neurons were investigated in this study and both drugs evoked outward currents in those cells that responded. It was previously suggested that NA and 5HT actions on SDH neurons may vary according to neuronal type (Lu and Perl, 2007). In the present study, all of the major morphological types responded to NA and 5HT, although the proportions varied. Very few central cells responded to both drugs, but this may be due to the relatively small number of central cells tested. Incidence of lamina II interneurons that respond to NA and 5HT in the present sample is very similar to that reported by other groups (North and Yoshimura, 1984; Grudt et al., 1995; Lu and Perl, 2007; Abe et al., 2009). When the numbers of inhibitory and excitatory cells that responded to NA were compared, no major difference was found between the two populations. The same was observed of the cells that responded to 5HT. This suggests that it is unlikely that these two monoamines act selectively on either inhibitory or excitatory cells.

5.5.2 Somatostatin hyperpolarises inhibitory lamina II neurons

A proportion (29%) of lamina II neurons responded to somatostatin but unlike NA and 5HT, effect of this neuromodulator was restricted to the inhibitory population. This proved the proposed hypothesis and agrees with the finding that the somatostatin receptor sst_{2a}, the main receptor for somatostatin in the spinal cord, is highly expressed in the dorsal horn (Schindler et al., 1997) and is restricted to GABA-immunoreactive neurons (Todd et al., 1998). When morphological types of inhibitory cells that responded to somatostatin were compared, islet cells constituted the majority (5/8). However it is difficult to determine whether somatostatin acts on cells of a particular morphological

type, since it was only applied to a small sample of vertical and unclassified cells. Somatostatin induced outward currents in all the neurons that responded. Somatostatin was reported to activate K^+ channels, resulting in postsynaptic hyperpolarisation of LII neurons, thus reduces neuronal excitability and it has been suggested to contribute to analgesia (Kim et al., 2002; Jiang et al., 2003). Nakatsuka et al (2008) also showed a similar hyperpolarising action of somatostatin has on a subset of SG neurons. These previous observations, together with the findings in the present study, suggest that somatostatin has an inhibitory effect on its target neurons. However, since these cells are inhibitory, the overall effect of somatostatin is likely to be pro-nociceptive. Although analgesic effects of intrathecal somatostatin have been reported, this may be the result of neurotoxicity on nociceptive neurons (Gaumann and Yaksh, 1988). Others have reported that the anti-nociceptive effects of somatostatin are dose-dependent, with high doses of somatostatin applied intrathecally causing neuronal damage (Mollenholt et al., 1988), while at physiological levels, intrathecal somatostatin appears to have a pro-nociceptive effect (Seybold et al., 1982; Wiesenfeld-Hallin, 1985, 1986). This indicates that the role of somatostatin in neuromodulation can be complicated and should be interpreted with caution.

5.6 Somatostatin and enkephalin immunoreactivity in excitatory lamina II neurons

Somatostatin is widely distributed in the CNS and it is synthesised by primary afferents and many glutamatergic interneurons in lamina I-II (Proudlock et al., 1993; Todd at al., 2003). Consistent with previous reports (Mizukawa et al., 1988; Chung et al., 1989), somatostatin immunoreactivity is seen in both cell bodies and nerve terminals in lamina II in the present study. Somatostatin was present in the axons of 14/24 excitatory neurons tested with antibody against the neuropeptide, and ten of these were enkephalin-immunoreactive. Enkephalin is one of the two opioid peptides that are present most abundantly in lamina II and it is known that the majority of enkephalin-expressing neurons are glutamatergic and that some contained somatostatin (Todd and Spike, 1992; Todd et al., 2003; Marvizón et al., 2007, 2009). However, not as many glutamatergic terminals contain enkephalin (Marvizón et al., 2009).

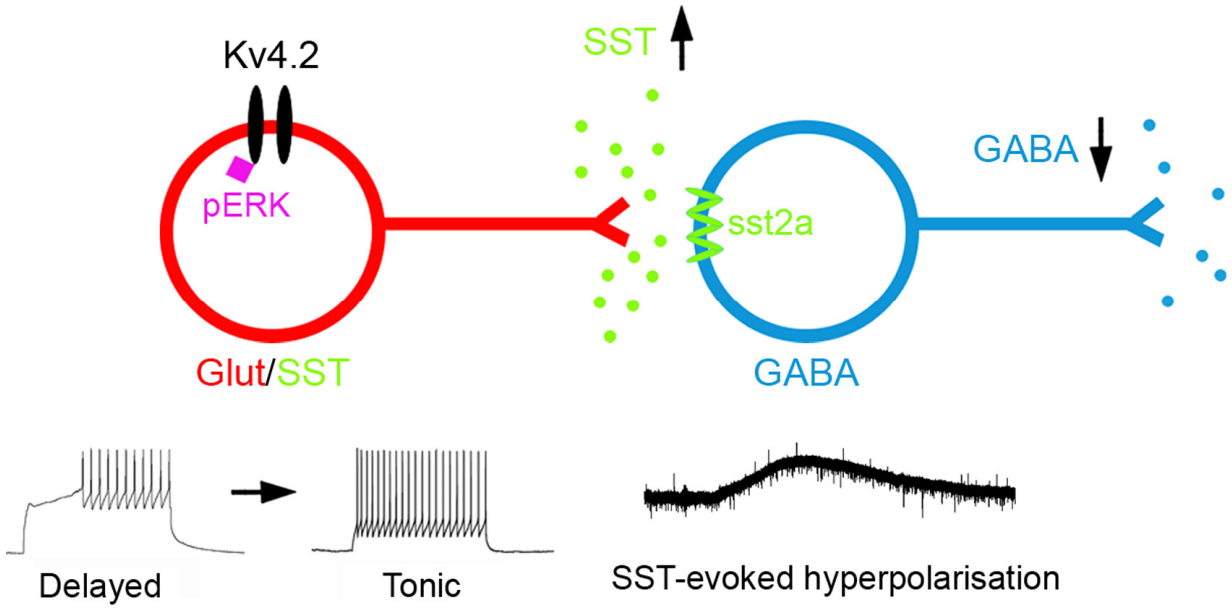
All radial cells that were tested contained somatostatin in their axons, while five out of the ten vertical cells tested were somatostatin-immunoreactive. Although it is very tempting to assume that these neuropeptides are always present in the same terminals of a discrete excitatory neuronal group in lamina II, a larger sample needs to be investigated to specify the frequency of somatostatin and enkephalin colocalisation. The present observation confirms that some excitatory cells in lamina II contain somatostatin with or without enkephalin, but this does not represent the definite number of cells of each subtype in this spinal region because of the limited numbers of cells tested. Quantitative analysis is necessary to determine the precise proportion of glutamatergic cells that express these neuropeptides in lamina II.

5.6.1 Proposed disinhibitory mechanism involving somatostatin

Interestingly, all of the somatostatin-immunoreactive neurons that had their firing patterns tested displayed delayed-firing pattern. Although the sample of cells that were tested for somatostatin-immunoreactivity and discharge patterns is limited, it is most likely that many somatostatin-containing neurons will show delayed-firing when depolarised. Noxious stimulation is likely to activate ERKs in these neurons and thus phosphorylate Kv4.2 (Hu et al., 2006), which mediates I_A. This would lead to an elevation in neuronal excitability and firing frequency, thus increasing release of somatostatin following peripheral stimulation. Somatostatin released from these neurons would hyperpolarise neighbouring inhibitory neurons through volume transmission and cause an inhibitory effect. The resulting reduction in GABA release from these inhibitory neurons corresponds to a 'disinhibition' mechanism, which may contribute to plasticity in pain states (Hu et al., 2006). A schematic representation of this proposed mechanism is shown in Figure 5-1.

Inhibitory control is essential to maintain sensory information processing and for normal perception of pain (Sandkühler, 2009). Disruption or alteration of the inhibitory neurotransmission system is commonly associated with the development and maintenance of chronic pain states (e.g. Yaksh 1989; Sivilotti and Woolf, 1994; Hwang and Yaksh, 1997; Moore et al., 2002; Coull et al., 2003; Drew et al., 2004; Harvey et al., 2004). Disinhibition leads to abnormal pain

Figure 5-1 Proposed mechanism for involvement of somatostatin-evoked disinhibition in Kv4.2-mediated synaptic plasticity in the superficial dorsal horn. Noxious stimulation is likely to activate ERKs in somatostatin-expressing glutamatergic neurons (Glut/SST, red) and thus phosphorylate Kv4.2, which mediates I_A. This would lead to an elevation in neuronal excitability, which may be associated with a change from a delayed- to a tonic-firing pattern, and thus increasing release of somatostatin (green, SST) in response to peripheral stimulation. The somatostatin acts through volume transmission on sst2a receptors expressed by nearby inhibitory interneurons (GABA, blue), causing hyperpolarisation and a decrease of their excitability. The consequent reduction in GABA release from these inhibitory neurons corresponds to a 'disinhibition' mechanism.


Kv4.2
pERK
Glut/SST
SST
sst2a
GABA
GABA
Delayed
Tonic
SST-evoked hyperpolarisation

perception including allodynia, hyperalgesia and spontaneous pain (Sandkühler, 2009). Several mechanisms that could contribute to disinhibition have been proposed. Previous studies have reported loss of GABAergic and/or glycinergic neurons after peripheral nerve injury in rats that developed neuropathic pain (Ibuki et al., 1997; Eaton et al., 1998). However, others have found no significant difference in the number of SDH neurons and no alteration in the proportion of neurons that were GABA-immunoreactive in animals that developed clear signs of neuropathic pain (Polgár et al, 2004, 2005; Polgár and Todd, 2008). Reduction of presynaptic GABA release and downregulation of GAD in different types of neuropathic pain models could also lead to disinhibition (Moore et al., 2002). Coull et al. (2003) have shown that a shift in transmembrane anion gradient, resulting from downregulation of potassium-chloride exporter KCC2, could diminish (or reverse) the inhibitory actions of GABA and glycine (Coull et al., 2003). Therefore, the specific mechanisms that underlie disinhibition are most likely to be complex and knowledge on this is still far from complete. However, the proposed mechanism for involvement of somatostatin-evoked disinhibition in Kv4.2-mediated synaptic plasticity in the superficial dorsal horn (as discussed above) may provide a novel approach in understanding the specific changes that underlie chronic pain.

Treatment for pain condition is highly dependent on modulations of excitability of neurons that are involved in the sensory processing circuits. Manipulation of specific neurotransmitters, receptors and ion channels expressed by certain types of neurons remains the main interest of current drug development to treat neuropathic as well as chronic pain (Graham et al, 2007). If taking the proposed disinhibition mechanism into consideration, a probable approach would be to apply sst2a antagonist, which would act on inhibitory neurons that express this receptor, and thus prevent hyperpolarisation of GABAergic neurons by somatostatin released from glutamatergic neurons. However, the precise distribution of the relevant molecular targets on different types of dorsal horn neurons has to be determined in order to manipulate excitability and transmission of pain signals, and hence to treat pain efficiently (Graham et al., 2007). Lack of a definitive classification scheme has made it very difficult to establish roles of dorsal horn neurons in the neuronal circuits. Results from this study have identified neuronal properties that not only allow further

clarification of sensory processing mechanisms in the SDH, but also greatly contribute to identify potential targets for development of novel analgesics.

Chapter 6

Conclusions and Future Directions

6 Conclusions and future directions

The present study has confirmed and extended previous findings by identifying discrete populations of inhibitory and excitatory lamina II interneurons. Although the neuronal organisation of lamina II is highly complex, a considerable correlation was observed between the electrophysiological, morphological, neurochemical and pharmacological properties of these cells. All islet cells were GABAergic, while all radial cells and the majority of vertical cells were glutamatergic. However, there was a substantial proportion (31.1%) of cells that could not be morphologically classified. Firing pattern is strongly related to neurotransmitter phenotype, since the firing patterns associated with I_A currents were largely restricted to excitatory interneurons, while the tonic-firing pattern was most frequently observed in the inhibitory population. These observations strongly suggest that lamina II interneurons comprise a large number of functional groups, which are likely to have specific roles in the neuronal circuitry in this region.

The availability of transgenic mice that selectively express GFP in neurochemically defined subpopulations of SDH neurons would be useful to refine the present findings. This approach would allow targeted recording from a particular set of GFP-expressing neurons, while detailed analysis of their physiological properties together with structural characteristics would provide sufficient measures that could be quantified and further interpreted with cluster analysis (e.g. McGarry et al., 2010). This approach provides an objective and unbiased way of selectively characterising specific neuronal groups identified in this area. For example, recent studies have used mice in which a subset of lamina II GABAergic neurons express GFP under the control of prion promoter to show synaptic connections between cells that are morphologically discrete (Hantman et al., 2004; Zheng et al., 2010). Furthermore, electrophysiological recordings that include dorsal root afferent stimulation would provide the means to establish the modular organisation of these targeted neurons by revealing their responses to different primary afferent inputs. *In vivo* patch-clamp recordings from targeted neurons would allow their responses to various somatosensory stimuli to be assessed. This would lead to a better understanding

of the functional significance of these lamina II interneurons, many of which are known to be nociceptive.

The SDH is the major termination site for fine myelinated and unmyelinated primary afferent fibres, which are distributed differentially, based on the fibre size and sensory modalities (Figure 1-2). Nociceptive and thermoreceptive Aδ and C afferents innervate lamina I and most of lamina II, whereas axons of low threshold mechanoreceptive (Aβ) and some Aδ hair follicle afferents arborise in the most ventral part of lamina II. Lamina II interneurons may constitute a substantial postsynaptic target of these primary afferent fibres. Several studies have shown selective patterns of synaptic inputs from Aδ and C fibres to morphologically identified cells in lamina II. Islet cells and central cells are principally innervated by C-fibres, while radial and vertical cells receive inputs from both Aδ and C afferents (Grudt and Perl, 2002; Hantman et al., 2004; Lu and Perl, 2003, 2005; Yasaka et al., 2007). It is shown in the present study that all islet cells are GABAergic and that all radial cells are glutamatergic. This indicates that there is likely to be a difference in the functional groups of primary afferents that innervate the inhibitory and excitatory populations. Several neurochemical markers can be used to identify the type of primary afferent inputs to the different classes of interneurons in anatomical studies. Unmyelinated fibres that contain neuropeptides and those that lack neuropeptides can be recognised with antibodies against CGRP and binding to IB4, respectively. The difference in the central distribution and peripheral targets suggest that the two classes of unmyelinated fibres vary in functions, and therefore identifying the types of input to the interneurons would advance the understanding of the specific functional roles of these cells in the local neuronal circuits. In addition, VGLUT1 can be used to identify terminals of low threshold myelinated afferents in the dorsal horn, while VGLUT3 is present in a group of low threshold C-fibres that are non-peptidergic. These neurochemical markers are useful to determine whether primary afferents terminate differentially in lamina II, and thus refine the functional characterisation of its neuronal populations. Besides primary afferent inputs, interneurons also receive synapses from other interneurons. Recent electrophysiological studies have identified specific synaptic connectivity between different types of lamina II interneurons (e.g. Lu and Perl, 2003, 2005). Intrinsic neurochemical markers can be used to

identify contacts between these cells. For example, expression of NPY and galanin are restricted to GABAergic cells, and therefore the different types of lamina II neurons can be tested with antibodies against these neuropeptides and a neurochemical marker for inhibitory neuron to determine if they receive GABAergic synapses from cells that contain these neuropeptides.

The incoming information from primary afferents is processed by a complex circuitry of inhibitory and excitatory interneurons before they are transmitted to the projection neurons, which form the major output from the SDH to the brain. Some of the lamina II interneurons identified in the present study had axons that arborised extensively into lamina I and/or lamina III. This indicates that these cells may form synapses on projection neurons, which are mostly concentrated in these adjacent laminae. One marker that is widely used to identify projection neurons is the neurokinin 1 receptor (NK1R). NK1R is the main target for substance P and is expressed by around 80% of lamina 1 projection neurons (Todd et al., 2000; Spike et al., 2003; Al-Khater et al., 2009), which are activated by noxious stimuli (Salter and Henry, 1991). NK1R-immunoreactive projection cells are also present in lamina III, but in a much smaller proportion. However, synaptic inputs onto the NK1R-expressing projection neurons in lamina I and lamina III originate from different sources. Those in lamina I receive excitatory input from glutamatergic vertical cells in lamina II outer (Lu and Perl, 2005), whereas the NK1R-expressing lamina III projection neurons are densely innervated by inhibitory boutons that contain NPY. Among the lamina I projection neurons that lack NK1R, a population of very large multipolar cells have been identified with the glycine receptor-associated protein gephyrin (Puskár et al., 2001; Polgár et al., 2008). The cell bodies and dendrites of these giant cells are densely coated with gephyrin-containing inhibitory synapses, and they also receive a very high density of glutamatergic inputs from presumably axons of excitatory interneurons (Polgár et al., 2008). These lamina I giant cells are also innervated by inhibitory cells that express nNOS (Puskár et al., 2001). It is reasonable to hypothesise that a substantial proportion of the inhibitory and excitatory contacts on these projection cells are derived from populations of GABAergic or glutamatergic interneurons in lamina II. By combining various neurochemical markers (e.g. NPY, nNOS) with NK1R or gephyrin, it is possible to determine whether specific groups of SDH cells form contacts onto particular

types of projection neurons. This information would reveal the synaptic connectivity between intrinsic neurons and projection neurons, and thus clarify the links between these neurons and the incoming primary afferents. Future studies can also be advanced by combining these approaches (as above) with investigation at the ultrastructural level using electron microscopy. In addition, simultaneous recording from pairs of pre-labelled neurons, would allow detailed characterisation of specific interactions between these major components of the neuronal circuitry.

It is not a simple task to unravel the extremely complex neuronal organisation and circuitry in the SDH. The present findings together with those of previous studies, however, provide an important tool to distinguish lamina II interneurons according to various characteristics. One of the prerequisites to improve the limited knowledge of SDH circuitry would be to have a comprehensive classification scheme for its diverse constituent neuronal populations. Therefore, identification of distinct neuronal classes allows further investigation of these neuronal components in order to understand their specific roles within the neuronal circuits. This information is essential to understand nociceptive processing within the spinal cord, and the changes that contribute to the development of pain states. Although neuronal functions are clearly related to certain structural or neurochemical features, it is not always straightforward. To add to the complication, a substantial proportion of inhibitory and excitatory neurons in lamina II remain morphologically unclassified. Further studies should take these issues into consideration and combine various techniques in order to obtain a clearer picture of the neuronal circuitry in the spinal dorsal horn.

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Appendix

Appendix

Dissection solution

NaCl	0.0 mM
$CaCl_2$	0.5 mM
KCl	1.8 mM
$MgCl_2$	7.0 mM
KH_2PO_4	1.2 mM
$NaHCO_3$	26.0 mM
Glucose	15.0 mM
Sucrose	254.0 mM

Appendix

Krebs' solution

NaCl	127 mM
$CaCl_2$	2.4 mM
KCl	1.8 mM
$MgCl_2$	1.3 mM
KH_2PO_4	1.2 mM
$NaHCO_3$	26.0 mM
Glucose	15.0 mM

Appendix

Internal pipette solution

D-gluconic acid potassium	130.0 mM
KCl	10.0 mM
$MgCl_2$	2.0 mM
NA 2ATP	2.0 mM
NA GTP	0.5 mM
HEPES	5.0 mM
Phosphocreatin	5.0 mM
EGTA	0.5 mM
Neurobiotin	6.2 mM
pH	7.28

Appendix

0.1 M phosphate buffer (PB)

Solution A: $NaH_2PO(2H_2O)$	37.44 g in 1200 ml H_2O
Solution B: Na_2HPO_4	84.90 g in 3000 ml H_2O

Add 1120 ml of solution A to 2880 ml of solution B and mix well. Adjust pH to 7.4 with either HCL or NaOH. Add 3000 ml distilled water to the final solution.

Phosphate buffer with 0.3 M saline (PBS)

0.2 M phosphate buffer	100 ml
Distilled water	1900 ml
NaCl	36 g

PBS with Triton X-100 (PBST)

PBS	1000 ml
Triton® X-100	3 ml

Appendix

4% paraformaldehyde

Paraformaldehyde	40 g
0.2 M phosphate buffer	500 ml
Distilled water	400 ml
1N NaOH	a few drops

In a fume hood, heat distilled water to 68 °C and add paraformaldehyde.

Add NaOH, a few drops at a time, until paraformaldehyde is completely dissolved.

Add 0.2 M phosphate buffer and top up with distilled water to make one litre of final solution.

Then, filter final solution.

Publication

Pain. 2010 Nov;151(2):475-88.

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http://dx.doi.org/10.1016/j.pain.2010.08.008