Populations of spinal cord dorsal horn neurons and their role in nociception

Kholoud Saad Al Ghamdi (MBBS, MSc)

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Institute of Neuroscience and Psychology

College of Medical, Veterinary and Life Sciences

University of Glasgow

Glasgow, Scotland

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Summary

Nociception involves detection of tissue damage by specialized receptors; nociceptors. These convey information to the first synaptic relays in the dorsal horn of the spinal cord. Within the dorsal horn itself are the dorsal horn neurons which can be divided into two broad classes, based on their axonal projections: projection neurons and interneurons.

The neurokinin 1 receptor (NK1r), the main target for substance P, is expressed by most projection neurons and many interneurons in the dorsal horn. These NK1r-expressing neurons show a bimodal size distribution in lamina I. The 1st part of the project tested the hypothesis that large NK1r-immunoreactive cells in this lamina are projection neurons, while the small cells are interneurons. Rats were anaesthetised and received injections of tracers into two supraspinal areas that are likely to label all contralateral lamina I projection neurons. The rats were re-anaesthetized and perfused 3 days later and 1341 NK1r-positive cells were analysed, of which 441 were retrogradely labelled. Cross-sectional soma areas of projection neurons were larger than those of cells that were not retrogradely labelled. This difference was highly significant. Nearly all (99.4%) of the NK1r-expressing cells that were not retrogradely labelled had soma areas <200 μm^2, while only 9.8% of the retrogradely labelled NK1r-expressing cells had somata <200 μm^2. These results provide a means of distinguishing lamina I NK1r-expressing projection neurons from interneurons based on their soma sizes without the need of retrograde tracing surgeries.

Lamina I contains another population of projection neurons that lack or weakly express the NK1 receptor and consists of very large cells: giant cells, which are coated with the glycine and gamma-aminobutyric acid (GABA) receptor associated protein, gephyrin. There is also a group of large NK1r-expressing projection neurons with cell bodies in laminae III-IV and dendrites that pass dorsally to enter lamina I. Extracellular signal-regulated kinase (ERK) is expressed in dorsal horn neurons and is activated (phosphorylated) by noxious stimuli. In the 2nd part of the project, ERK phosphorylation in NK1r-expressing neurons as well as in lamina I giant cells was investigated following different type of noxious stimuli. Anaesthetised rats received noxious cutaneous, deep or

visceral stimuli. They remained anaesthetized for 5 min after the end of the stimulus, and were then fixed by perfusion. Spinal cord sections were immunoreacted to reveal NK1r, gephyrin and phosphorylated ERK (pERK). Among the NK1r-expressing lamina I neurons, pERK was detected in both projection (somata >200 μm^2) neurons and interneurons, with a significantly higher proportion in the larger cells, after all types of noxious stimulation. There was no significant difference in the frequency of pERK expression between the three morphological classes (fusiform, pyramidal and multipolar) of lamina I NK1r-expressing projection neurons after these stimuli. Most of the giant cells contained pERK after noxious cutaneous stimuli, but few did so following noxious deep stimulation. Only a few of laminae III-IV NK1r-expressing projection cells contained pERK after noxious deep or visceral stimulation, and the labelling in these was very weak. Results from the present study indicate that different types of neurons have different roles in conveying nociceptive information.

The superficial dorsal horn (SDH) is also a vital area for modulating nociception and contains large number of excitatory and inhibitory interneurons. Glutamate, released by primary afferents and local excitatory neurons, acts on G-protein-coupled metabotropic glutamate receptors (mGlus). Group I mGlus ($mGlu_1$ and $mGlu_5$) are strongly expressed in the SDH. It has been reported that intrathecal administration of the $mGlu_{1/5}$ agonist 3,5-dihydroxyphenylglycine (DHPG) induces spontaneous nociceptive behaviours, which are ERK-dependent. In the 3rd part of the project, ERK phosphorylation in $mGlu_5$-expressing neurons following the administration of DHPG was investigated. Anaesthetized rats underwent a laminectomy procedure. DHPG or saline was applied to their exposed lumbar cord for 8 minutes after which they were perfused. Sections from the lumbar spinal cord were immunoreacted to reveal $mGlu_5$, pERK and one of various markers for excitatory or inhibitory interneurons. Following DHPG (but not saline), numerous pERK-positive cells were seen in the SDH, particularly lamina II, and the great majority of these were $mGlu_5$-positive. ERK phosphorylation was detected in both inhibitory and excitatory $mGlu_5$-expressing cells, suggesting that type I mGlus have a complex role in nociceptive processing.

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"وماتوفيقي إلا بالله عليه توكلت وإليه أنيب".

“My success can only come from Allah: in Him I trust and unto Him I turn”.

Author's declaration

I declare that the work presented in this thesis is my own work, with the exception of part of the data that are presented in Figure 3-7, which was pooled, with permission, from work published by Al-Khater and Todd, (2009).

Prof. Andrew Todd performed the retrograde tracing surgery, and Dr John Riddell performed the laminectomy procedure. This thesis has not been submitted in any previous application for any other degree in the University of Glasgow or any other institution.

Dedication

This thesis is dedicated to you Layla ☺, you always said: ‘Mama, Be Brave!’

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List of abbreviations

AMPA	α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
BDNF	brain-derived neurotrophic factor
BNPI	brain-specific Na^+-dependent inorganic phosphate transporter I
CFA	complete Freund's adjuvant
CGRP	calcitonin gene-related peptide
CNS	central nervous system
CREB	cAMP response element binding protein
CRD	colorectal distension
CTb	cholera toxin subunit B
CVLM	caudal ventrolateral medulla
DHPG	3,5-dihydroxyphenylglycine
DRG	dorsal root ganglion
DRt	dorsal reticular nucleus
ERK	extracellular signal-related kinase
GABA	gamma-aminobutyric acid
LPb	lateral parabrachial nucleus
LTMs	low-threshold mechanoreceptors
MAPK	mitogen activated protein kinase

mGlu	metabotropic glutamate receptor
mRNA	messenger ribonucleic acid
NADPH-d	nicotineamide adenine dinucleotide phosphate diaphorase
NGF	nerve growth factor
NK1r	neurokinin 1 receptor
NMDA	N-methyl-D-aspartic acid
nNOS	neuronal nitric oxide synthase
NPY	neuropeptide Y
NTS	nucleus of the solitary tract
PAG	periaqueductal grey matter
PB	phosphate buffer
PBS	phosphate-buffered saline
pERK	phosphorylated form of extracellular signal-related kinase
PKC	protein kinase C
PoT	posterior triangular nucleus
SDH	superficial dorsal horn
SP-SAP	substance P conjugated to the cytotoxin saporin
STT	spinothalamic tract
TrkB	tropomyosin-related kinase B

TRPs	transient receptor potential receptors
TSA	tyramide signal amplification
VGAT	vesicular GABA transporter
VGLUT	vesicular glutamate transporter

1. Introduction

Pain is among the most complex perceptions, and its research brings together elements of many disciplines. It is defined by the International Association for the Study of Pain as “An unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage”. Nociception on the other hand is defined as “The processes of encoding and processing of noxious stimuli, which is an actual or potential tissue damaging event, by the nervous system” (Treede, 2009). The SDH of the spinal cord represents the first area of the central nervous system (CNS) where transmission and modulation of pain-related information occurs.

1.1 Nociception and the spinal cord

Nociception involves detection of tissue damage by specialized receptors. Both somatic and visceral nociceptors can be activated by noxious chemical, mechanical or thermal stimulation in addition to stimuli that only threaten damage. Nociceptors convey this information to the first synaptic relays in the dorsal horn of the spinal cord. In order to understand the mechanisms of nociception and to produce more selective analgesics for use in the treatment of both animal and human pain, it is essential to develop various nociception eliciting models. These include models of acute and chronic pain such as applying noxious or innocuous stimuli to the hindpaw of a rodent, or using peripheral nerve injury induced models of pain (Garry *et al.*, 2004).

The dorsal horn of the spinal cord is a major relay region for nociceptive inputs and in the 1950s there was a need to develop a widely accepted scheme that divided its grey matter into distinct regions. In 1952, Rexed divided the dorsal horn of the cat spinal cord into a series of six parallel laminae based on certain cytoarchitectonic characteristics. This scheme was then adapted to other species including the rat (Steiner and Turner, 1972; Clark, 1984; Molander *et al.*, 1984). Lamina I, which is sometimes referred to as the marginal zone, is located at the most dorsal aspect of the dorsal horn, and it represents an important nociceptive area. Lamina II, which is also known as ‘Substantia Gelatinosa of Rolando’ after Luigi Rolando (Caputi *et al.*, 1995) has a very low concentration of myelinated fibres, thus it appears translucent under the microscope in thick wet sections. Lamina II is further divided into two layers: lamina II outer (IIo) with densely packed small cells and predominant primary afferent input from C-

fibres, and lamina II inner (IIi) with slightly larger neurons and a significant input from myelinated fibres. Laminae III and IV are known as the 'Nucleus Proprius'. Most cells in lamina III are small and densely packed with some scattered large neurons compared to the more heterogenous lamina IV cells. Laminae V and VI are referred to as the neck and base of the dorsal horn, respectively.

The SDH, which includes laminae I and II, is the main target of nociceptive primary afferents. Some nociceptive primary afferents also terminate in deeper laminae (III-VI), indicating that this region has an important role in both physiological and pathological pain states (Light and Perl, 1979a, 1979b; Cervero and Iggo, 1980; Sugiura *et al.*, 1986).

In addition to the primary afferent input to the dorsal horn, it receives pain modulating descending axons from various parts of the brain (Fields *et al.*, 2006; Heinricher *et al.*, 2009). The dorsal horn contains neurons which, based on their axonal projections, can be divided into two broad classes: interneurons, with axons that remain in the spinal cord and projection neurons with axons travelling to supraspinal targets such as the brainstem and thalamus. Projection neurons are less numerous than interneurons and are known to be excitatory while interneurons can be either excitatory or inhibitory (Todd, 2006). In order for us to understand the neural circuits that link incoming primary afferents to projection neurons, these two broad classes have to be further divided into distinct functional populations (Todd, 2010).

1.2 Primary afferents fibres

Primary afferent fibres have a remarkable capacity of detecting and transmitting sensory information from the periphery to the spinal cord, thus linking external and internal environments to the CNS. The somata of primary afferent neurons are located in the trigeminal ganglion and the dorsal root ganglia (DRG). A central branch from the axons of these neurons travels through a dorsal root to enter the spinal cord where it forms axodendritic or axosomatic synapses with second-order neurons. Primary sensory fibres are most commonly classified based on the conduction velocity of their axons, which is directly related to the axonal diameter and whether or not the axon is myelinated. Sensory fibres in cutaneous and visceral nerves are routinely divided into Aα/β-, Aδ- and C-fibres.

The Aα/β group consists of large myelinated axons that have the fastest conduction velocities (25-100 m/s) and most of these are classified as low-threshold mechanoreceptors (LTM), responding to touch, pressure, stretch or hair movement. Aδ-fibres are thinly myelinated and conduct at an intermediate velocity (5-25 m/s). C-fibres are the smallest, unmyelinated and most slowly conducting fibres (<2.5 m/s). The majority of Aδ [excluding the down (D) hair afferents] and most C-fibres are classified as nociceptors or thermoreceptors (Todd and Koerber, 2006). Different terminology is used for sensory fibres in muscles and joint nerves. Their myelinated afferents are subdivided into groups I, II and III, while group IV corresponds to the unmyelinated afferents (Willis and Coggeshall, 2004). Figure 1-1 shows Rexed's laminae and the laminar termination pattern of primary afferents in the rat dorsal horn.

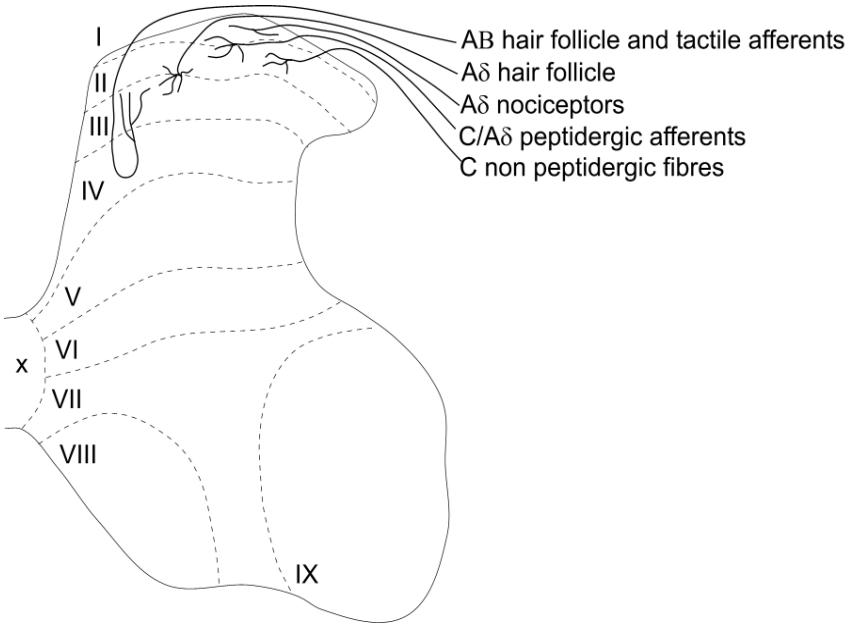


Figure 1-1 Rexed's laminae and the laminar termination pattern of primary afferents. The image shows the central terminations of the major primary afferent neurons, the dashed lines show laminar boundaries. Modified from Todd (2010).

Primary afferent fibres are either nociceptive or non-nociceptive and can be divided into somatic (cutaneous or deep) or visceral depending on their peripheral targets. When a stimulus is applied to a sensory receptor, it elicits a response through stimulus transduction, which is the process of converting a physical stimulus into a generated potential leading to an action potential. Some fibres have specialized response properties since they only respond to one type of stimulus, while most nociceptors respond to different sensory modalities

including mechanical, thermal and chemical stimuli, and are considered polymodal. In most early studies of nociceptors, primary afferents that responded to both mechanical and thermal stimulation were referred to as mechano-heat-sensitive fibres. These were also considered polymodal nociceptors because if an afferent responded to mechanical and heat stimuli, it would, in most cases, respond to chemical stimuli as well (Davis *et al.*, 1993).

The term 'nociceptive nerves' was first introduced by Sir Charles Sherrington in 1906 (Burke, 2007). Nociceptors are specialized primary sensory neurons that detect damaging or potentially damaging stimuli, which may result in the perception of pain and subsequently initiate protective responses. Nociceptors play an important role in this protective function of the pain pathway. They are able to: (1) detect various physical and chemical stimuli, (2) set specific response thresholds for each stimulus, thus being able to distinguish between noxious and innocuous events, and even (3) reset these thresholds in order to protect against further damage, a phenomenon known as sensitization (Julius and McCleskey, 2006).

1.2.1 Nociceptive unmyelinated primary afferents

The majority of cutaneous C-fibres are classified as nociceptors since they can be activated exclusively by intense mechanical, thermal or chemical stimuli and are typically polymodal (Lynn and Carpenter, 1982; Fleischer *et al.*, 1983). It was also reported that some of the small diameter afferents from muscles and tendons (Mense and Meyer, 1985) as well as from joints (Schaible and Schmidt, 1985) are considered nociceptors, while others fall under the low-threshold receptor category.

C-fibres are divided into two major neurochemical groups: peptidergic, which innervate various tissues including the epidermis and deeper regions of the skin, and non-peptidergic, which innervate mainly the epidermis (Rice *et al.*, 1997; Zylka *et al.*, 2005). Peptidergic C-fibres express either one or a combination of the following peptides: calcitonin gene-related peptide (CGRP), substance P, somatostatin, galanin, cholecystokinin, and vasoactive intestinal polypeptide /peptide histidine isoleucine (Ju *et al.*, 1987; Bennett *et al.*, 1996). CGRP is the most useful marker for detecting peptidergic afferents for two reasons: first, it

appears to be present in all of these, including those that contain substance P or somatostatin (Ju *et al.*, 1987), and second, within the dorsal horn, CGRP is exclusively present in primary afferents (Chung *et al.*, 1988). Lawson *et al.* (2002) reported that CGRP was detected in less than half of the nociceptive DRG neurons of the guinea-pig including those with C-fibres. The majority of peptidergic C nociceptors terminate in lamina I and lamina IIo (Figure 1-1).

Most non-peptidergic C-fibres bind the plant lectin IB4 from Bandeiraea simplicifolia (Plenderleith and Snow, 1993). These can be recognized by the presence of IB4-binding and the lack of CGRP-immunoreactivity (Sakamoto *et al.*, 1999). However, Dhaka *et al.* (2008) identified a subpopulation of non-peptidergic C-fibres in the mice that lack peptides and do not bind IB4. Zylka *et al.* (2005) identified another population of IB4-positive non-peptidergic C nociceptors that was revealed by axonal tracers targeted to Mrgprd, a Mas-related gene which is expressed in specific subsets of nociceptive sensory neurons in mice (Zylka *et al.*, 2003). These $Mrgprd^{+}$ fibres exclusively innervate the epidermis and project centrally to lamina II of the spinal cord suggesting a central topographic segregation of the projection of peptidergic and non-peptidergic nociceptive sensory neurons to the spinal cord (Figure 1-1).

The central arborisations of peptidergic primary afferents are concentrated in lamina I and the outer half of lamina II (IIo), and also extend to laminae III-IV. These peptidergic fibres form simple (non-glomerular) endings that rarely receive axo-axonic synapses (Ribeiro-da-Silva *et al.*, 1989). In contrast, the IB4-binding non-peptidergic C-fibres that correspond to the subpopulation identified by Zylka *et al.* (2005) terminate in a narrow band that occupies the central part of lamina II and form central axons of type I synaptic glomeruli (Ribeiro-da-Silva and Coimbra, 1982). These axons are postsynaptic to GABAergic boutons at axo-axonic synapses and therefore are targets to classical presynaptic inhibition (Todd, 1996).

1.2.2 Nociceptive myelinated primary afferents

Myelinated nociceptors include both the slow Aδ-fibres, which were first identified by Burgess and Perl (1967), and a propotion of the fast Aα/β-fibres (Djouhri and Lawson, 2004). A-fibre nociceptors respond to external stimulation

with higher discharge frequencies, compared to the responses of C-fibre nociceptors. This suggests that A-fibre nociceptors provides more discriminable information regarding stimulus intensity and they are thought to signal fast pricking or sharp pain (Slugg *et al.*, 2000; Lumpkin and Caterina, 2007).

Although the neurochemistry of myelinated nociceptors is not yet fully known, it has been reported that substance P and CGRP are among the neuropeptides that are expressed by these nociceptors (Lawson *et al.*, 1997; Lawson *et al.*, 2002).

The central projections of myelinated nociceptors were initially described in the cat and monkey by Light and Perl (1979b). Their study reported that lamina I, the most dorsal part of lamina II as well as lamina V of the spinal cord received projections from relatively slow conducting myelinated primary afferent nociceptors; the Aδ type. More recently, a second type of A-fibre nociceptors was identified, which resembled myelinated LTMs in its central projections, since these fibres projected to deeper laminae. However, they also projected to the SDH, suggesting a more diffuse pattern of central projection (Woodbury and Koerber, 2003; Woodbury *et al.*, 2008).

One of the methods for identifying the central terminals of myelinated primary afferents is by their ability to transport peripherally injected cholera toxin B subunit (CTb). CTb is a protein that binds specifically to ganglioside GM1, which is expressed by most myelinated afferents but not by intact C-fibres in somatic nerves of rodents. However, the transganglionic labelling with CTb seen in lamina I (which is thought to be contained in terminals of Aδ nociceptors) rarely colocalised with CGRP or substance P, suggesting that peptidergic Aδ afferents do not normally express the GM1 ganglioside and therefore could not be identified with CTb (Shehab and Hughes, 2011).

1.2.3 Non-nociceptive primary afferents

Non-nociceptive primary afferents are mostly low-threshold thermoreceptors and/or LTMs. Most fibres projecting to the skin and skeletal muscles from the DRG are LTMs (Djouhri and Lawson, 2004). Cutaneous LTMs respond to innocuous mechanical stimulation such as light brushing of the limb fur, light pressure with blunt objects or distortion.

LTMs of the fast conducting myelinated type (Aα/β) make up a large proportion of cutaneous and muscle fibres. A smaller proportion of fibres in skin and muscles are of the slowly conducting Aδ group (Perl, 1992). Cutaneous LTMs of the Aα/β type can be divided into rapidly adapting and slowly adapting fibres (Willis and Coggeshall, 2004).

Brown (1981) summarized the findings of numerous studies of LTMs in the cat, and reported that these fibres migrate medially within the dorsal columns as they move away from their point of entry in the spinal cord. Most of them bifurcate to give rostral and caudal branches within the dorsal horn. Collaterals from these fibres terminate in dense arborisations that lie within a region extending from lamina III to lamina V, with fibres innervating hair follicles terminating more superficially than those innervating slowly adapting receptors. Shortland *et al.* (1989) studied the central projections of hair follicle afferents in the rat, and reported that these afferents formed narrow sagittal sheets of arbors extending from lamina IIi to lamina IV. In addition, Hughes *et al.* (2003) found that in the rat, the termination zone of afferents with hair follicle-like morphology extended dorsally into the ventral half of lamina II (Figure 1-1).

LTMs of the Aδ type include D-hair afferents and are found in hairy skin. They are sensitive to the slow movements of hairs (Rethelyi *et al.*, 1982). These fibres were found to project extensively into laminae IIi and III-IV of the dorsal spinal cord (Light *et al.*, 1979; Woodbury and Koerber, 2003). They gave rise to large varicosities that formed the central terminals of type II glumeruli (Rethelyi *et al.*, 1982).

LaMotte *et al.* (1982) reported that the threshold of warm receptors in the monkey is below the pain threshold (41-49°C), and these receptors responded better to painless heat than to painful heat. The conduction velocity of these receptors in the glabrous skin of the monkey is of the Aδ range. The low-threshold cold receptors, which discharged statically at 20-30°C skin temperature, also had a homogenous distribution of conduction velocities of the Aδ range in the glabrous skin of the monkey (LaMotte and Thalhammer, 1982).

It was reported that in the cat, a fraction of C-fibres responded to both low-threshold mechanical stimulation and thermal stimulation, and did not respond uniquely to noxious stimulation (Bessou and Perl, 1969; Bessou *et al.*, 1971). A subclass of these low-threshold C-fibres was recently found to respond vigorously to slow and light stroking of the hairy skin in humans (Loken *et al.*, 2009). Additionally, selective stimulation of these fibres activated the insular cortex; an area known to process positive emotions (Olausson *et al.*, 2002).

Recently, certain molecular and histological markers of subtypes of unmyelinated LTMs (Seal *et al.*, 2009) and rapidly adapting myelinated mechanoreceptors (Bourane *et al.*, 2009; Luo *et al.*, 2009) have been identified in the mice. Seal *et al.* (2009) reported that a subset of glutamatergic C-fibre LTMs that terminated in laminae I and IIi of the spinal cord of the mice appear to be important for the onset of hypersensitivity caused by spared nerve injury.

1.2.4 Neurochemistry of primary afferents

Glutamate is the principal excitatory neurotransmitter in the CNS and it is used by all primary afferents. Glutamate and substance P, a neuropeptide involved in nociception transmission, coexist in many primary afferent terminals in the superficial laminae of the spinal cord (De Biasi and Rustioni, 1988) and these will be discussed in more detail.

1.2.4.1 Glutamate

Glutamate was discovered in 1907 by the Japanese chemist Kikunae Ikeda (Kurihara, 2009). After its synthesis in the presynaptic cytoplasm, glutamate is transported into the synaptic vesicles for its exocytotic release. Glutamate transport requires a specific energy generating system that uses the H^+-ATPase pump. Certain proteins have been shown to function as vesicular glutamate transporters (VGLUTs) and were named VGLUT1, VGLUT2 and VGLUT3 (Takamori, 2006). VGLUT1 was originally characterized as a brain-specific Na^+-dependent inorganic phosphate transporter I (BNPI) (Ni *et al.*, 1994), while VGLUT2 was known originally as differentiation-associated Na^+-dependent inorganic phosphate transporter I, a protein that highly resembled BNPI (Aihara *et al.*, 2000). Subsequently, VGLUT3 was identified and found to be expressed in

a more restricted pattern in the CNS in addition to its expression in the DRG (Gras *et al.*, 2002). Antibodies against VGLUTs helped in identifying glutamatergic axons in anatomical studies. VGLUT1 has been found to be expressed by all low-threshold myelinated fibres, while VGLUT2 is expressed by lamina I Aδ-fibres as well as by axons of most glutamatergic spinal neurons in the rat (Oliveira *et al.*, 2003; Todd *et al.*, 2003; Alvarez *et al.*, 2004). The expression of VGLUT2 by unmyelinated primary afferents is found to be weak in the rat and the mouse, and these fibres do not express VGLUT1 (Todd *et al.*, 2003; Brumovsky *et al.*, 2007). VGLUT3, however, is found to be expressed by a population of unmyelinated primary sensory neurons projecting to lamina I and the inner part of lamina II of the mice dorsal horn (Seal *et al.*, 2009).

Glutamate acts on both ionotropic and metabotropic receptors. These are the most widespread excitatory neurotransmitter receptors in the CNS, thus they contribute to both normal and pathological neural activities. Ionotropic receptors are subdivided into three groups: AMPA (α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate), Kainate and NMDA (N-methyl-D-aspartic acid). AMPA receptors are made up from 4 different subunits: GluA1-4 (GluR1-4), with receptors lacking the GluA2 (GluR2) subunit being permeable to Ca^{2+} (Burnashev *et al.*, 1992). They are tetramers that exist either as homomeric, containing single receptor subtype, or heteromeric receptors. Kainate receptor subtypes include GluK1-5 (GluR5-7, KA1 and KA2) (Pinheiro and Mulle, 2006). NMDA receptor subunits include GluN1-3 (NR1-3), and GluN2 is further divided into 4 subunits (GluN2A-D). NMDA receptors are heteromeric complexes, which are composed of the GluN1 subunit and one or more of the GluN2 subunits (Matsuda *et al.*, 2002). There are 8 cloned metabotropic glutamate receptors (mGlus) that are classified into 3 groups. Group I: $mGlu_1$ and $mGlu_5$ (mGluR1 and mGluR5), group II: $mGlu_2$ and $mGlu_3$ (mGluR2 and mGluR3) and group III: $mGlu_{4,\ 6\text{-}8}$ (mGluR4, 6-8) (Conn and Pin, 1997). mGlus will be discussed in Chapter 5.

1.2.4.2 Substance P

Substance P is an undecapeptide that is involved in nociception; it acts on the NK1 receptor. Substance P is a member of the mammalian tachykinin group which include two other peptides: neurokinin A and neurokinin B, which act on the neurokinin 2 and 3 receptors (NK2R and NK3R), respectively (Nakanishi,

1991). Substance P and neurokinin A are both derived from the same gene (preprotachykinin I), while neurokinin B is derived from a different gene, preprotachykinin II (Helke *et al.*, 1990). Substance P and neurokinin A are expressed by both primary afferents and dorsal horn neurons, while neurokinin B is found in the SDH interneurons of the rat (Ogawa *et al.*, 1985; Moussaoui *et al.*, 1992). Substance P was discovered by von Euler and Gaddum (1931) and its sequence was identified by Chang and Leeman (1970). In the rat, substance P is colocalised with CGRP in many of the small DRG neurons. It is packed into the large dense-core vesicles at the trans-Golgi network in the cell bodies. It is then transported to the peripheral terminals of C-fibres in tissues, and to the central terminals in laminae I-II of the spinal cord (Todd, 2002, 2006). Substance P is stored in these terminals and can be released into the dorsal horn of the spinal cord following intense peripheral stimulation (Duggan *et al.*, 1987; Duggan *et al.*, 1988; Liu *et al.*, 1994; Mantyh *et al.*, 1995). Substance P plays a role in nociceptive signal transmission by promoting central hyperexcitability and increasing sensitivity to pain (Salter and Henry, 1991; Ma and Woolf, 1995). Traub (1996) also reported that substance P, released upon peripheral tissue insult, contributes mainly to generation but not maintenance of hyperalgesia, by facilitating the activation of NMDA receptors, leading to SDH hyperexcitabilty.

1.2.5 Receptors on primary afferents

Most studies of receptors on primary afferents have been performed in the DRG, since locating these receptors on terminals of primary fibres in the spinal cord requires the development of specific antibodies against them (Ribeiro-da-Silva and De Koninck, 2009). A number of primary afferent receptors have been reported to play a role in peripheral modulation of nociception. These include glutamate receptors, opioid receptors, cholinergic receptors, α_2-adrenergic receptors, somatostatin receptors and transient receptor potential receptors (TRPs) (Meyer *et al.*, 2006; Todd and Koerber, 2006). The TRPs, particularly TRPV1, will be discussed in more detail since capsaicin, which acts on TRPV1, is used in this study as a peripheral noxious stimulus.

1.2.5.1 Transient receptor potential receptors (TRPs)

TRPs are members of a family of transducer protein molecules which are present in the surface membranes of nociceptors, thermoreceptors and thermoreceptive neurons. These receptors are essential for nociceptive excitation after chemical or thermal stimuli. There are four vanillin TRPs that form a group of thermosensitive channels in mammals: TRPV1, TRPV2, TRPV3 and TRPV4. Non-vanilloid TRPs include TRPM8 and TRPA1 (also called ANKTM1).

In mammals, each of the thermally sensitive TRPs responds to a distinct temperature range from cold to noxious heat, and most are expressed in cutaneous sensory neurons (Patapoutian *et al.*, 2003). TRPV1, formerly known as the vanilloid receptor, is among the earliest proteins that were known to play a major role in heat transduction. It is highly expressed on many nociceptive Aδ and C-fibres (Caterina and Julius, 2001). In addition to its activation by noxious heat (temperatures ≥42°C), TRPV1 is also activated by chemicals such as capsaicin [the active substance in chilli peppers (8-methyl-*N*-vanillyl-6-nonenamide)] and its agonist resiniferatoxin (Caterina *et al.*, 1997) as well as by some endogenous lipids (Vennekens *et al.*, 2008). The function of TRPV1 in the peripheral nervous system has been extensively studied. It is specifically activated by capsaicin, a lipophillic vanilloid compound found in a wide variety of red peppers of the genus capsicum. Binding of capsaicin to TRPV1 activates nonselective cation channels, leading to Na^+ and Ca^{2+} influx, which triggers action potential firing and the consequent burning sensation associated with spicy food (Holzer, 1991). Caterina *et al.* (2000) reported that $TRPV1^{-/-}$ (knock-out) mice exhibited no capsaicin/vanilloid evoked pain behaviour and an impaired response to noxious thermal stimuli, but responded normally to noxious mechanical stimuli. In the spinal cord dorsal horn, the role of TRPV1 in pain transmission and modulation is not yet fully understood (Spicarova and Palecek, 2008). Recently, Cavanaugh *et al.* (2011) found that during development, a substantial percentage of IB4-binding, non-peptidergic neurons lose the expression of functional TRPV1 and that in adult mice, TRPV1 expression is restricted to a subset of peptidergic primary afferent neurons in the DRG.

In the periphery, TRPV2 is strongly expressed on a subset of Aδ nociceptors as well as in other neuronal and non-neuronal cells (Caterina and Julius, 2001).

TRPV2 is only activated by intense noxious heat (temperatures ≥52°C), while TRPV3 and TRPV4 respond to lower temperatures. However, TRPV3 responses continue to increase with increasing temperatures that reach the noxious range, suggesting that it may play a role in nociception (Patapoutian *et al.*, 2003). TRPM8 is known to play a role in cold transduction (McKemy *et al.*, 2002; Peier *et al.*, 2002). The TRPM8 channel, which is expressed almost exclusively in a subpopulation of C-fibres, can be activated by low temperatures (15-30°C) and by other chemicals that produce a cooling sensation such as menthol (Lumpkin and Caterina, 2007). Further, the exposure to temperatures below 18°C activates another member of the TRP family, TRPA1, suggesting that TRPA1 may contribute to TRPM8-independent cold transduction of the noxious cold temperature range (Story *et al.*, 2003; Lumpkin and Caterina, 2007).

Thermal or chemical activation of the TRP receptors, which are expressed on C- and Aδ-fibres, subsequently results in the release of glutamate and peptides including substance P, neurokinin A and CGRP into the dorsal horn, contributing to the excitation of second order sensory neurons (Sorkin and McAdoo, 1993; Sun *et al.*, 2004; Yan *et al.*, 2006).

1.3 Dorsal horn projection neurons

Dorsal horn projection neurons have axons that ascend to various areas of the brain. These neurons are not uniformly distributed throughout the grey matter of the spinal cord, and this has been shown by injecting retrograde tracers into all known supraspinal targets. In the rat, neurons with axons projecting to the brain are present in relatively large numbers in lamina I, are largely absent from lamina II and are scattered throughout the remainder of the dorsal horn (Todd, 2010).

1.3.1 Dorsal horn projection neurons in lamina I

It has been estimated that projection neurons make up around 5-10% of the neuronal population in lamina I (Bice and Beal, 1997a, 1997b; Spike *et al.*, 2003; Al-Khater *et al.*, 2008). Many lamina I neurons have axons that remain in the spinal cord, and these interneurons are thought to play a major role in modulating the process of nociception.

Projection neurons in lamina I have axons that cross the midline ascending to a variety of supraspinal targets. These include: (1) The caudal ventrolateral medulla (CVLM), an area that may play a significant role in modulating cardiovascular responses to nociceptive stimulation (Lima *et al.*, 1991; Lima *et al.*, 2002; Spike *et al.*, 2003). (2) The dorsal reticular nucleus (DRt) and the nucleus of the solitary tract (NTS), which partially but significantly mediates cardiorespiratory reflex responses to noxious stimulation (Lima, 1990; Boscan and Paton, 2001; Gamboa-Esteves *et al.*, 2004). (3) The lateral parabrachial area of the pons (LPb), which in turn projects to areas in the brain involved in emotional aspects and autonomic components of pain such as the amygdala and hypothalamus (Cechetto *et al.*, 1985; Hylden *et al.*, 1989; Bernard and Besson, 1990; Bester *et al.*, 1995; Ding *et al.*, 1995; Gauriau and Bernard, 2002). (4) The midbrain periaqueductal grey matter (PAG), which forms part of a descending analgesic pathway that projects via the rostral ventromedial medulla, modulating nociceptive transmission within the spinal cord dorsal horn (Todd *et al.*, 2000; Spike *et al.*, 2003; Al-Khater and Todd, 2009; Drew *et al.*, 2009). (5) Certain nuclei in the thalamus, which in the rat include the ventral posterolateral nucleus that projects to the primary somatosensory cortex and the posterior triangular nucleus (PoT) projecting to the 2nd somatosensory area. The ventral posterolateral nucleus contributes to the sensory-discriminative aspect of pain, while PoT contributes to the affective-motivational aspect of pain (Burstein *et al.*, 1990; Gauriau and Bernard, 2004; Al-Khater *et al.*, 2008; Todd, 2010).

Todd (2010) summarized the quantitative data collected from a series of studies of projection neurons in the L4 segment of the rat spinal cord. Most of lamina I projection neurons can be retrogradely labelled from the CVLM and/or the LPb. Almost all lamina I cells projecting to the thalamus, PAG or NTS also project to LPb. However, since the region of CVLM is close to the main bundle of ascending axons, the tracer injected into this area may be up taken by the fibres of passage rather than projection neurons axon terminals.

Almost a century ago in 1909, Ramon Cajal described the morphology of neurons in the nervous system (Garcia-Lopez *et al.*, 2011). Cells in lamina I of the spinal cord were known traditionally as the marginal cells of Waldeyer. Lamina I contains neurons of different shapes and sizes, and the morphology of these

neurons has been examined in various species. Lima and Coimbra (1983, 1986) distinguished 4 morphological classes of lamina I neurons in the rat based on their dendroarchitecture and cell body shape: (1) Fusiform spiny cells having longitudinally-orientated perikarya with bipolar dendrtic trees. (2) Pyramidal prismatic wedge shaped cells with 3 to 5 dendritic trunks arising from the apical and basal angles of the cell body. (3) Flattened aspiny neurons with a polygonal flattened body and horizontal dendritic arbor confined to lamina I. (4) Multipolar cells with a cuboidal cell body and dense dendritic trees originating from numerous primary trunks. Subsequent studies of projection neurons in lamina I of the rat, cat and monkey confirmed the findings of Lima and Coimbra (1983, 1986) fusiform, pyramidal and flattened cells. However, flattened cells were named as multipolar and Lima and Coimbra's multipolar cells have not been further described in the literature (Zhang *et al.*, 1996; Zhang and Craig, 1997; Prescott and De Koninck, 2002; Spike *et al.*, 2003; Almarestani *et al.*, 2007).

Lamina I neurons were also classified based on their soma sizes, and this means of classification will be further discussed in Chapter 3. In addition, a substantial number of lamina I neurons responded specifically to various forms of noxious stimuli, and their responses will be discussed in detail in Chapter 4.

Lamina I projection neurons have been classified based on the expression of the NK1 receptor into NK1r-expressing projection neurons and projection neurons that either lacked or weakly expressed the receptor. The NK1 receptor, on which substance P acts, is a G protein-coupled receptor consisting of seven membrane spanning domains (Yokota *et al.*, 1989).

1.3.1.1 Lamina I NK1r-expressing projection neurons

Cells possessing the NK1 receptor are present in all dorsal horn laminae but are most numerous in lamina I (Todd *et al.*, 2002). They make up around 80% of lamina I projection neurons, and project to the CVLM, NTS, PAG, LPb and thalamus (Todd *et al.*, 2000; Spike *et al.*, 2003; Al-Khater *et al.*, 2008).

Lamina I NK1r-expressing projection neurons selectively receive input from peptidergic primary afferents most of which contain substance P (Todd *et al.*, 2002). These cells also receive glutamatergic input from lamina II vertical

interneurons (Lu and Perl, 2005). These glutamatergic vertical cells receive peripheral inputs from TRPA1- and TRPV1-expressing C-fibres (Uta *et al.*, 2010), as well as from Aδ-fibres, in addition to a central input from neighbouring lamina II glutamatergic central interneurons (Lu and Perl, 2005).

Lamina I NK1r-expressing neurons have been studied extensively in the past few years. These cells have attracted attention since they responded specifically to noxious stimulation (Salter and Henry, 1991). They also internalized the NK1 receptor into their cytoplasm following acute noxious stimulation (Mantyh *et al.*, 1995; Abbadie *et al.*, 1997; Allen *et al.*, 1997). Further, the number of neurons with internalized NK1 receptors was proportional to the severity of the noxious stimuli (Wang and Marvizon, 2002). These cells also up-regulated fos (the protein product of the immediate-early gene *c-fos*), and phosphorylated ERK after noxious stimulation (Doyle and Hunt, 1999; Ji *et al.*, 2002; Todd *et al.*, 2002; Polgar *et al.*, 2007). Activation of NK1r-expressing neurons following noxious stimulation will be further discussed in Chapter 4.

It was reported that intrathecal injection of substance P conjugated to the cytotoxin saporin (SP-SAP) selectively destroyed NK1r expressing neurons in the dorsal horn (Mantyh *et al.*, 1997; Nichols *et al.*, 1999). SP-SAP injected-rats showed signs of dramatically reduced hyperalgesia in both inflammatory and neuropathic pain models, while their response to acute painful stimuli was normal. However, mice that lacked either the NK1 receptor (De Felipe *et al.*, 1998) or the gene coding for substance P formation (Cao *et al.*, 1998), showed little reduction in signs of hyperalgesia in inflammatory models, yet significantly reduced responses to acute painful stimulation. These findings suggest that although substance P acting on the NK1r-expressing neurons plays a role in nociception, there are other substances, such as glutamate released from primary afferents (De Biasi and Rustioni, 1988) that are more important in the development of hyperalgesia.

1.3.1.2 Lamina I giant cells

Lamina I giant cells have been identified by Puskar *et al.* (2001) as a population of very large multipolar lamina I projection neurons that either lacked or weakly expressed the NK1 receptor. They are very sparse since there are only around 10

of these cells on each side of the L4 segment of the rat spinal cord. They therefore make up ~2.5% of lamina I projection neurons. Most of the giant cells were retrogradely labelled from the parabrachial area (Puskar *et al.*, 2001; Polgar *et al.*, 2008).

These cells are identified by the presence of gephyrin; the glycine and GABA receptor associated protein, on their cell bodies and proximal dendrites. They receive extensive inputs from neuronal nitric oxide synthase (nNOS)-containing GABAergic internurons (Puskar *et al.*, 2001). They also receive a high density input from VGLUT2-containing excitatory interneurons (Polgar *et al.*, 2008). However, these cells appear to receive little direct synaptic input from primary afferents, suggesting that their responses to noxious stimuli are mainly mediated by excitatory interneurons (Polgar *et al.*, 2008; Todd, 2010).

It was reported that after the injection of formalin to the rat hindpaw, 26 out of 30 giant cells up-regulated fos (Puskar *et al.*, 2001). Polgar *et al.* (2008) also reported that 38% of the giant cells contained fos after noxious heat. The above studies suggest that giant cells, which lack or weakly express the NK1 receptor, may contribute to the normal responses to acute noxious stimuli seen after the injection of SP-SAP, which selectively destroys NK1r-expressing neurons.

1.3.2 Dorsal horn projection neurons in laminae III-IV

Rexed (1952) observed that lamina III of the spinal cord contained numerous small cells and scattered larger ones, while lamina IV cells were more variable in size. Further, early Golgi studies in the cat identified large laminae III-IV neurons with dorsal dendrites extending to superficial laminae (Szentagothai, 1964; Rethelyi and Szentagothai, 1969). Schoenen (1982) also reported that in humans, laminae III-IV contained a mixed population of antenna-like neurons with dorsally oriented dendritic domains. Thereafter, these cells were identified in the rat (Todd, 1989) and some were found to express the neurokinin 1 receptor (Bleazard *et al.*, 1994; Brown *et al.*, 1995; Littlewood *et al.*, 1995).

Todd *et al.* (1998) reported that around 11% of lamina III cells were NK1r-immunoreactive, while the percentage of NK1r-expressing neurons in lamina IV was approximately 28%. However, the population of large NK1r-immunoreactive

projection neurons with long dorsal dendrites represents only approximately 0.1% of all neurons in lamina III (Polgar *et al.*, 2004). These cells have similar supraspinal targets to the lamina I projection cells, since they project to the CVLM, NTS, LPb, PAG and the thalamus (Marshall *et al.*, 1996; Naim *et al.*, 1997; Todd *et al.*, 2000; Al-Khater *et al.*, 2008; Al-Khater and Todd, 2009).

Laminae III-IV NK1r-immunoreactive cells are densely innervated by substance P-containing primary afferents, which appear to run ventrally along their dorsal dendrites (Naim *et al.*, 1997). In addition, these cells receive a moderate input from myelinated LTMs (Naim *et al.*, 1998), but they do not seem to receive any significant input from non-peptidergic IB4-binding C-fibres (Sakamoto *et al.*, 1999). These cells are also densely innervated by GABAergic interneurons that contain neuropeptide Y (Rowan *et al.*, 1993; Polgar *et al.*, 1999b). It has been recently reported that these neuropeptide Y-positive interneurons constitute around 30% of laminae III-IV NK1r-immunoreactive cells' inhibitory input (Polgar *et al.*, 2011).

Both laminae I and III-IV NK1r-expressing neurons are innervated by descending serotoninergic axons from the raphe nuclei that selectively depress the transmission of nociceptive information (Stewart and Maxwell, 2000; Polgar *et al.*, 2002). This is evidenced by the high density contacts of serotonin-containing axons forming synapses with the NK1r-expressing neurons, suggesting that a significant part of the descending raphe-spinal inhibitory system is mediated directly by serotoninergic axons (Polgar *et al.*, 2002). These cells also receive some noradrenergic input from the locus coeruleus and other pontine regions (Stewart, 2001; Todd, 2010).

1.4 Dorsal horn interneurons

Interneurons have axons that remain in the spinal cord and arborize locally. They form the vast majority of neurons in laminae I-III. They include virtually all neurons in lamina II and most of laminae I and III neurons. Interneurons can be divided into two main functional types: inhibitory cells, using GABA and/or glycine as their principal transmitter, and excitatory cells with glutamate being their main neurotransmitter (Todd and Spike, 1993). Interneurons are thought to play an important role in pain processing. They modify incoming somatosensory

information before it is relayed to higher centres. For instance, inhibitory interneurons exert a powerful control on spinal nociceptive neurons. A review by Sandkuhler (2009) summarized the role of inhibitory interneurons in 4 distinct functions. (1) They attenuate the responses of nociceptive neurons during nociception. (2) They prevent spontaneous pain by muting nociceptive neurons during normal states. (3) They separate nociceptive from non-nociceptive information in order to prevent cross-talk between different sensory modalities. (4) They also limit the spread of excitation by inhibiting excitatory interneurons that cross somatotopic borders.

Attempts have been made to classify interneurons morphologically, electrophysiologically, neurochemically and sometimes by combining two methods of classification together. However, there is not a generally accepted scheme that applies to all of these cells. Neurochemical classification of laminae I, II and III-IV interneruons will be discussed separately, since the expression of most dorsal horn neurotransmitters is not confined to one lamina.

1.4.1 Interneurons in lamina I

The organisation of lamina I interneurons is not yet fully understood. This may be due to the fact that lamina I contains projection neurons and inteneurons that are not easily distinguished (Todd, 2010). Three-quarter of lamina I interneurons are thought to be glutamatergic since the remaining 25-30% of these interneurons contain GABA (Todd and Sullivan, 1990; Polgar *et al.*, 2003).

1.4.1.1 Morphological and electrophysiological classification

Grudt and Perl (2002) studied a sample of 13 lamina I neurons, in spinal cord slices prepared from hamsters, 8 of which did not project to supraspinal targets and were therefore presumed to be interneurons. These cells had ipsilateral directed axons in contrast to the axons of the projection neurons that ran ventrally and medially toward the contralateral cord. The axonal arborisation of lamina I interneurons was mainly limited to lamina I with few cells having axons that arborized in adjacent lamina IIo. These cells had relatively sparse dendrites, which were confined mainly to laminae I and IIo. The study also reported that all lamina I interneurons produced a sustained discharge in

response to injecting a depolarizing current. In addition, Ruscheweyh and Sandkuhler (2002) reported a laminar difference in membrane and discharge properties of neurons of the rat dorsal horn *in vitro*. Although the study did not differentiate between lamina I projection neurons and interneurons, it showed that most neurons in lamina I, but not in lamina II, discharged action potentials at regular intervals throughout the current pulse (tonic discharge). However, delayed firing neurons, which showed a delayed onset of firing in response to current injection, were encountered in both laminae I and II. Prescott and De Koninck (2002) also identified 4 physiological cellular classes of lamina I neurons in the rat *in vitro*: tonic, delayed, phasic (firing a high frequency burst of variable duration) and single spike (firing only one spike in response to strong depolarisation). The study also reported the existence of at least three morphological cellular classes in lamina I, which correlated with the electrophysiological properties of these cells. Tonic cells were typically fusiform, phasic cells were pyramidal and multipolar cells fired in a delayed and/or single spike patterns.

1.4.2 Interneurons in lamina II

Most of the studies regarding dorsal horn interneurons were conducted in lamina II, since the majority of neurons in this lamina are interneurons. In lamina II of the rat, it was found that approximately 30% of the neurons were inhibitory, containing GABA and/or glycine (Polgar *et al.*, 2003). The remaining neurons are thought to be glutamatergic (Todd *et al.*, 2003; Maxwell *et al.*, 2007).

1.4.2.1 Morphological classification

Lamina II interneurons can be classified morphologically using different techniques. Gobel (1975, 1978) studied the cat substantia gelatinosa and identified certain morphological types of cells that have distinct characteristics using the Golgi technique. Cells were identified based on certain criteria: dendritic and axonal branching patterns, dendritic spine distribution, geometric shape of the dendritic tree and the laminar distribution of the dendrites and axons. Three morphological types were identified in this study: islet, stalked and spiny cells. Islet cells, mostly found in clusters, had fusiform or rounded cell bodies with axons and dendrites arborising longitudinally. Stalked cells on the

other hand were found individually with cone-shaped cell bodies and dendrites emitting numerous fine stalked-like branches and dendritic spines. Spiny cells were found singly between the islet cell clusters with extensive dendritic trees spanning rostrocaudally and mediolaterally. These spiny cells have not been further mentioned in the literature that described interneuron morphology. In the rat, Todd and Lewis (1986) recognised two main types of Golgi stained neurons in lamina II: islet and stalked cells with a description that almost resembled the cells identified by Gobel (1975). Todd and Lewis (1986) also identified a group of 'unclassified' cells that represented over one-third of lamina II neuronal population.

Cells can also be classified morphologically after being revealed by intracellular injection *in vivo*. Bennett *et al*. (1980) studied the responses of a population of 22 lamina II neurons of the cat to both natural and electrical stimulation of their receptive fields. These neurons were then filled intracellularly with horseradish peroxidase and their morphology was studied. Twenty of them were recognised as being either islet or stalked cells. There was also a clear correlation between the location of the neurons' dendritic arborisations and their responses to natural and electrical stimulation. For instance, all stalked cells and those islet cells with dendritic trees located superficially (lamina IIo) responded either specifically to noxious stimulation or to both gentle mechanical and tissue-damaging stimulations (wide dynamic range neurons). On the other hand, islet cells with deeply located dendritic trees responded to low-threshold mechanical stimulation, suggesting that different layers have different functional roles. In agreement with Bennett *et al*. (1980), Rethelyi *et al*. (1989) reported a relation between the laminar location of neuronal dendritic trees and the nature of primary afferent excitation in the cat. However, there was no clear relation between the morphology of these cells and their responses to various types of noxious and innocuous stimuli. Additionally, in the rat SDH, there was no correlation seen between function and either the cellular configuration or the distribution of the dendritic trees (Woolf and Fitzgerald, 1983).

A third morphological means of classification was developed in the hamster by combining whole cell recording in slices obtained from *in vitro* preparations with neuronal labelling using biocytin from the patch pipette (Grudt and Perl, 2002). Four main classes were identified based on different dendritic morphology: islet,

central, radial and vertical cells. Islet cells had elongated (>400 μm) dendritic trees in the rostrocaudal axis. Central cells resembled islet cells but with shorter (<400 μm) dendritic trees. Unlike the islet and central cells, radial cell dendrites were compact and extended in several directions in the parasagittal plane. Vertical cell dendrites fanned out ventrally from a dorsally placed soma located in lamina IIo. Some of the vertical cells were morphologically similar to Gobel's stalked cells but others did not show all the features of stalked cells. Similar results were seen in the rat dorsal horn (Maxwell *et al.*, 2007; Yasaka *et al.*, 2007; Yasaka *et al.*, 2010). Despite the identification of the above 4 cellular classes, approximately one-third of the studied neuronal populations did not fall under any of them, and were considered as 'unclassified' (Grudt and Perl, 2002; Maxwell *et al.*, 2007; Yasaka *et al.*, 2007; Yasaka *et al.*, 2010).

Using the Golgi method, Todd and McKenzie (1989) reported that islet cells were GABAergic while none of the stalked cells were immunoreactive to GABA. More recently, Maxwell *et al*. (2007) and Yasaka *et al*. (2010) immunocytochemically stained axons of populations of lamina II neurons that were obtained in *vitro* preparations (whole cell patch clamp) with VGLUT2 and the vesicular GABA transporter (VGAT). The two studies concluded that all islet cells were GABAergic while radial and most vertical cells were glutamatergic, and central cells could be either excitatory or inhibitory. In addition, all islet cells received monosynaptic excitatory inputs exclusively from C-fibres and primary afferent-evoked inhibitory inputs only from Aδ-fibres, while vertical and radial cells received excitatory and inhibitory primary afferent-evoked inputs from both C- and Aδ-fibres (Grudt and Perl, 2002; Yasaka *et al.*, 2007). This suggests that morphology may correlate with different patterns of excitatory and inhibitory inputs to lamina II interneurons. Moreover, within the dorsal horn, certain specific circuits between the interneurons have been identified. Lu and Perl (2003) performed simultaneous whole cell recordings from pairs of cells *in vitro* preparation and identified a specific inhibitory pathway between islet and central cells in which islet cell axons formed GABAergic synapses onto central cells. Lu and Perl (2005) also reported that lamina II central cells excited vertical cells monosynaptically through glutamatergic synapses. Vertical cells in turn excited neurons in lamina I that were presumed to be projection neurons.

1.4.2.2 Electrophysiological classification

Dorsal horn neurons can be classified electrophysiologically based on their action potential discharge during the injection of a depolarizing current. During the injection of a current, most lamina II neurons either discharged tonically (repetitive action potential discharges), fired transiently (initial bursting after which adaptation occurs), displayed a prominent single spike or exhibited a delayed action potential discharge (Grudt and Perl, 2002; Graham *et al.*, 2007).

Yoshimura and Jessell (1989) studied the membrane properties of the substantia gelatinosa neurons *in vitro*. Their study found that the discharge properties of these neurons are modified by the expression of various types of voltage-gated currents. It has been suggested that a tonic firing pattern is mainly generated by a combination of a voltage gated Na^+ current with a pronounced delayed inward rectifier K^+ (K_{DR}) current (Ruscheweyh and Sandkuhler, 2002; Melnick *et al.*, 2004). In addition, it was suggested that the response of delayed firing neurons to sudden depolarisation was caused by the transient outward rectifying current (I_A) that served to delay the onset of the first action potential. This A current is a transient voltage-dependant outward potassium current that counteracts fast depolarisations leading to the delay to the appearance of the first action potential (Yoshimura and Jessell, 1989; Ruscheweyh and Sandkuhler, 2002). Furthermore, Hu *et al.* (2006) reported that in the SDH, the I_A current is mediated by the Kv4-containing potassium channel particularly Kv4.2, and that genetically eliminating the Kv4.2 channel subunit increased the number of tonically firing neurons.

Grudt and Perl (2002) studied the responses of certain morphological populations of lamina II neurons to depolarising current injections. It was observed that all islet cells studied responded with a sustained repetition of action potentials (tonically), while the action potentials of the radial neurons were delayed from the onset of the depolarising step. Central cells acted in two different ways, one group went silent when depolarised while the other group responded to the depolarising step in a sustained (tonic) fashion. The vertical cells had either tonic or delayed response patterns.

In a recent study, Yasaka *et al*. (2010) reported that the morphology and neurotransmitter type of lamina II interneurons were related to the firing patterns of these neurons. Most excitatory neurons including vertical and radial cells but few inhibitory cells including islet cells had delayed, gap or reluctant pattern of firings, which was associated with A-type potassium (I_A) currents.

1.4.3 Interneurons in lamina III-IV

Laminae III-IV contains variety of cells of different sizes and shapes including both projection neurons and interneurons. A limited number of studies have focused on laminae III-IV interneurons, for instance, Schneider (2008) studied the local circuit connections between neurons in lamina III and IV of the hamster. Whole cell recordings between synaptically connected pairs of neurons in laminae III and IV showed that these connections were mostly unidirectional inhibitory connections. Subsequentely, Schneider (2008) processed the biocytin filled-cells histochemically and described their morphology. Intracellular labelling suggested that the recording was made from local axon interneurons, since the neurons were relatively small in size and located within laminae III-IV. Each interneuron had 2 to 4 tapered dendrites and a thin cylindrical axon that terminated within the dorsal horn. Polgar *et al*. (2003) also reported that around 40% of lamina III interneurons were GABAergic in the rat. Further, Ruscheweyh and Sandkuhler (2002) obtained whole cell patch-clamp recordings from 27 lamina III-IV neurons (projection neurons and/or interneurons). The study reported that some of these deeper neurons generated tonic firing after the injection of a depolarizing current, and none showed delayed firing.

1.4.4 Neurochemical classification of the SDH interneurons

Interneurons can be classified alternatively based on their expression of neurochemical markers, including various proteins and neuropeptides (Todd, 2010). Despite the complexity of the SDH in term of its neurochemistry, specific populations of excitatory and inhibitory interneurons can be identified when using combinations of neurochemical markers (Sardella *et al.*, 2011b).

Certain proteins are expressed in the dorsal horn of the spinal cord including the calcium-biding proteins: calbindin, calretinin, parvalbumin (Ren and Ruda, 1994)

and the γ-isoform of protein kinase C (PKCγ) (Mori *et al.*, 1990). Apart from parvalbumin, the above proteins are largely restricted to glutamatergic cells (Todd, 2010). ***Calbindin*** and ***calretinin*** expression patterns in the SDH are known to partially overlap and most of the calbindin- calretinin-containing neurons were not reactive for either GABA or glycine (Albuquerque *et al.*, 1999). Moreover, Antal *et al.* (1991) reported that the majority of the calbindin-immunoreactive cells in the SDH were negative for GABA-immunostaining, suggesting that these neurons are more likely to be glutamatergic. The calcium binding protein ***Parvalbumin*** is expressed by a subpopulation of neurons that are found mainly in laminae IIi and III and ~70% of these showed both GABA and glycine immunoreactivity, while ~30% were non-GABAergic (Antal *et al.*, 1991; Laing *et al.*, 1994).

Several protein kinase C isoenzymes have been identified in the SDH including α, βI, βII and γ (Mori *et al.*, 1990; Polgar *et al.*, 1999a). Among these, ***PKCγ*** is thought to play an important role in nociceptive processing, evidenced by the reduction of signs of neuropathic pain in mice lacking the PKCγ isoform (Malmberg *et al.*, 1997). Polgar *et al.* (1999a) reported that the great majority of PKCγ-containing neurons, which are concentrated mainly in lamina IIi (Mori *et al.*, 1990), were likely to be excitatory, since they were neither GABA- nor glycine-immunoreactive. Polgar *et al.* (2011) recently reported that lamina IIi PKCγ-expressing interneurons receive input from a subset of NPY-expressing inhibitory interneurons.

Furst (1999) reported that nitric oxide, which is synthesized by nitric oxide synthase in pre- and post-synaptic sites, is involved in certain spinal functions such as mediating nociception. Nitric oxide activates the enzyme guanylyl cyclase which in turn increases the production of the second messenger cGMP (Schmidt *et al.*, 1992). Nitric oxide synthase possesses a nicotineamide adenine dinucleotide phosphate (NADPH) reduction capacity. This reduction capacity is called NADPH diaphorase (NADPH-d) activity. NADPH-d is known to colocalise with nitric oxide synthase in some areas in the CNS including the spinal cord (Bredt *et al.*, 1991; Reuss and Reuss, 2001). ***neuronal nitric oxide synthase*** (nNOS) is one of three nitric oxide synthase isoforms which is found in neurons that synthesise nitric oxide and is thought to immunocytochemically correspond

to NADPH-d (Valtschanoff *et al.*, 1992a). NADPH-d was used in early studies to identify nNOS-containing neurons. NADPH-d/nNOS-positive neurons are concentrated mainly in lamina II of the spinal cord and they were most likely GABAergic and glycinergic (Valtschanoff *et al.*, 1992b; Spike *et al.*, 1993). Puskar *et al.* (2001) also reported that GABAergic axons containing nNOS selectively innervate lamina I giant cells of the rat dorsal horn.

The dorsal horn also contains certain neuropeptides that are found exclusively in glutamatergic neurons such as substance P, neurokinin B, somatostatin and neurotensin (Todd, 2010). ***Somatostatin***-expressing interneurons are found throughout lamina II (Kiyama and Emson, 1990; Proudlock *et al.*, 1993; Todd and Spike, 1993). Most of the somatostatin-containing axons (in laminae I and II) are thought to be derived from somatostatin interneurons along with a minor contribution from primary afferents (Ribeiro-da-Silva and Cuello, 1990). ***Neurotensin*** is thought to be present mainly in cells in the ventral part of lamina II (Seybold and Elde, 1982; Todd and Spike, 1993) and these neurotensin-containing neurons were found to be GABA-negative (Todd *et al.*, 1992a). Further, Todd *et al.* (2003) reported that the great majority of axons that are likely to be derived from neurotensin- and somatostatin-containing interneurons were VGLUT2-immunoreactive.

It has been shown that certain neuropeptides are restricted to GABAergic neurons, such as neuropeptide Y (NPY) and galanin (Todd, 2010). ***Neuropeptide Y*** is found exclusively in GABAergic neurons in laminae I-III (Rowan *et al.*, 1993). It has been reported that lamina III NK1r- expressing projection neurons receive a substantial inhibitory input from a set of NPY-containing GABAergic interneurons (Polgar *et al.*, 1999b). In a recent study, Polgar *et al.* (2011) reported that lamina IIi PKCγ-containing interneurons also receive input from a presumably different subset of NPY-expressing neurons. ***Galanin*** is another neuropeptide which is found mainly in laminae I and IIo (Todd and Spike, 1993). It has been reported that all of laminae I and IIo galanin-containing cells are GABAergic (Simmons *et al.*, 1995; Zhang *et al.*, 1995) and recently, Tiong *et al.* (2011) reported that these cells represent a distinct population of inhibitory interneurons.

Among the opioid peptides that are expressed by both excitatory and inhibitory interneurons are dynorphin and enkephalin. ***Dynorphin***-immunoreactive neurons are concentrated mainly in laminae I and IIo (Todd and Spike, 1993). They can be clearly revealed immunocyochemically with antibodies against dynorphin precursor proteins: prodynorphin and preprodynorphin. Preprodynorphin is also present in dynorphin-containing axon terminals (Li *et al.*, 1999). Prodynorphin and preprodynorphin are expressed in glutamatergic (Marvizon *et al.*, 2009) and galanin-containing GABAergic neurons (Sardella *et al.*, 2011a), respectively. ***Enkephalin***-containing neurons are present throughout the dorsal horn but are concentrated in lamina II (Todd and Spike, 1993). They are found in both excitatory (Todd *et al.*, 2003; Marvizon *et al.*, 2009) and inhibitory (Todd *et al.*, 1992b) interneurons.

1.5 Markers of neuronal activity

Markers of neuronal activity provide an approach for studying the functions of the spinal cord neurons. Neuronal markers such as fos and ERK are up-regulated/phosphorylated by variety of noxious stimuli, and have been used widely in basic research. Fos, a protein encoded by the cellular immediate-early gene *c-fos* (Hunt *et al.*, 1987) and more recently, ERK (a mitogen activated protein kinase, MAPK) (Ji *et al.*, 1999), are recognised as useful markers of neuronal activation. Immunocytochemical labelling of these markers has enabled researchers to investigate their colocalisation with other cellular markers, making it possible to study the characteristics of positively-labelled neurons.

1.5.1 Fos

Almost two decades ago, Hunt *et al.* (1987) reported that fos is up-regulated in nuclei of neurons in the dorsal horn of the rat after noxious stimulation. Since then, this neuronal marker of activity has been extensively used in pain studies. The *c-fos* messenger ribonucleic acid (mRNA) is typically identified using either northern blot analysis (Draisci and Iadarola, 1989) or in situ hybridization (Elliott *et al.*, 1995), while its protein product fos is identified immunocytochemically (Hunt *et al.*, 1987). It has been shown that noxious stimulation increased *c-fos* mRNA levels in the spinal dorsal horn within 30 minutes of stimulation (Draisci and Iadarola, 1989). The nuclear protein fos is transported from the cytoplasm,

where it is synthesized, and it accumulates in the nuclei of the spinal neurons, reaching its maximal detectible levels approximately 2 (1-3) hours after the application of the stimulus (Presley *et al.*, 1990). Following noxious thermal and chemical stimulation, Williams *et al.* (1990) reported that fos expression reached its peak 2 hours later, mainly in laminae I and II of lumbar spinal segments ipsilaterally, with more fos-positive neurons in laminae III-IV after formalin injection than after noxious heat. The study also showed that 8 hours after formalin injection, there was no fos-immunoreactivity in the spinal cord, while numerous fos-positive cells appeared, mainly in the contralateral side of the deep dorsal horn, 8 hours following noxious heat. Fos-immunoreactivity lasted for 24 hours and became more diffuse and symmetrical, suggesting a complex role of fos in nociception transmission. Bullitt (1991) also reported that during the first few hours after the peripheral stimulation of one side of the body, fos-positive neurons appeared in the appropriate spinal segments.

Fos expression after somatic noxious stimulation is mostly localised to laminae I and IIo of the spinal cord (Harris, 1998). This neuronal distribution differs if the stimulus was innocuous, since fos-immunoreactive neurons were seen mainly in deeper laminae (IIi-IV) in response to hair brushing, gentle joint manipulation (Hunt *et al.*, 1987) and tactile stimulation (Jasmin *et al.*, 1994). It was also reported that itch, which was induced either by topical application of histamine to the skin of the rat (Nakano *et al.*, 2008) or by the injection of histamine-independent itch mediators to the mouse hindpaw (Akiyama *et al.*, 2009), led to the expression of fos in the superficial laminae (I and II). Thus, it could therefore be suggested that spinal transmission of both itch and pain sensations may have a lot in common (Ross, 2011).

1.5.2 pERK

In this study, phosphorylation of ERK was used as the marker of neuronal activity, and it will therefore be discussed in more detail. In the past few years, the MAPK family of signalling cascades has received a great deal of attention. This family includes 3 major members: extracellular signal-regulated kinases (ERK, including ERK1/2), p38 (including p38α, p38β, p38γ and p38δ) and c-Jun N-terminal kinases (JNK, including JNK1, JNK2, and JNK3) (Ji *et al.*, 2009).

ERK1 (also known as p44 MAPK) and ERK2 (p42 MAPK) belong to a group of serine/threonine protein kinases. They are both activated by dual phosphorylation on their regulatory tyrosine and threonine residues by the upstream MAPK kinases: MEK1 and MEK2 (Boulton *et al.*, 1991; Seger and Krebs, 1995). Both ERK1 and ERK2 are usually referred to as ERK since they resemble each other structurally and are often activated together, as they have similar sensitivities to activation by MEKs (Zheng and Guan, 1993). In addition, neither MEK inhibitors nor pERK antibodies distinguish between ERK1 and ERK2 (Ji *et al.*, 2009). However, ERK1 knock-out mice are viable, fertile and of normal size (Pages *et al.*, 1999), while ERK2 knock-out mice die before embryonic day 8.5, indicating that ERK2 can compensate for ERK1 loss but not *vice versa* (Hatano *et al.*, 2003).

Both p38 and JNK are stress-regulated protein kinases that play a role in inflammatory responses and neuronal survival, while the ERK/MAPK cascade is known to regulate cellular proliferation and differentiation (Xia *et al.*, 1995). In addition to its role in regulating a variety of cellular functions, the ERK/MAPK cascade is also involved in neuronal plasticity such as that involved in learning and memory (Impey *et al.*, 1999) and in pain hypersensitivity (Ji *et al.*, 2009). Ruan *et al.* (2010) suggested the involvement of all three members of the MAPK family in nociception. Their study reported that activation of ephrin receptor tyrosine kinases in the spinal cord induced hyperalgesia in mice, which was accompanied by phosphorylation of p38 and JNK in both spinal neurons and astrocytes, whereas pERK appeared in spinal neurons only.

1.5.2.1 The role of ERK in nociception

In the nervous system, ERK pathway activation occurs in a variety of situations, including painful conditions. Activation of ERK produces short-term functional changes by phosphorylating kinases, receptors and ion channels as well as long-term transcriptional changes (Ji *et al.*, 1999). After exposure to a noxious stimulus, the ERK pathway is activated at different neuraxis levels, including DRG, spinal and supraspinal centres, leading to modulatory changes in the nervous system (Cruz and Cruz, 2007). Ji *et al.* (1999) provided the first indication of pERK involvement in nociception in the spinal cord. Their study showed that ERK phosphorylation was specific to noxious stimulation and that

noxious stimuli of different types led to the appearance of many pERK-positive neurons in the SDH of the spinal cord of the rat.

The ERK/MAPK cascade is activated by various pathways, including for instance those that involve activation of certain receptors or neurotransmitters. NMDA receptor-dependent phosphorylation of ERK occurs after the removal of the Mg^{2+} blockade of the NMDA channels, thus opening these channels and allowing the entry of Ca^{2+}. On the other hand, NMDA receptor-independent mechanisms involve the GluA2-lacking AMPA receptors that allow the influx of calcium, which produces lasting facilitation of synaptic transmission (Woolf and Salter, 2000). It also involves group I mGlus, for instance, Karim *et al.* (2001) reported that $mGlu_1$ and $mGlu_5$ are required for phosphorylation of ERK in the SDH. It was also suggested that group I mGlu-induced ERK activation are of importance in maintaining longer lasting forms of inflammatory pain. Adwanikar *et al.* (2004) showed that group I mGlus activation led to nociceptive behaviours as well as pERK expression in the SDH, both of which were enhanced after the injection of complete Freund's adjuvant (CFA) into the mouse hindpaw.

Substance P also plays an important role in the MAPK/ERK cascade, as evidenced by the induction of pERK mainly in lamina I neurons of the SDH of the rat after both intrathecal injection of substance P *in vivo* and application of substance P to a spinal cord slice preparation *in vitro* (Kawasaki *et al.*, 2004). Wei *et al.* (Wei *et al.*, 2006) reported that the bath application of substance P into spinal cord slices activated ERK in the SDH in both rat and mouse. Further, both capsaicin- and substance P-induced ERK activation were inhibited after the application of a selective NK1r antagonist (Kawasaki *et al.*, 2004; Wei *et al.*, 2006). However, it was reported by Lever *et al.* (2003) that after stimulating C-fibres in the mouse dorsal horn *in vitro*, ERK was phosphorylated mainly in the SDH, and this phosphorylation was NMDA and $mGlu_1$ but not AMPA or NK1 receptor dependent. Their study also reported that the brain-derived neurotrophic factor (BDNF) signalling through the tropomyosin-related kinase B (TrkB) receptor, but not substance P signalling through the NK1 receptor, may contribute to ERK activation in the dorsal horn neurons in this model. This discrepancy may be attributed to different nature of stimuli applied in each study as well as to different pharmacological degrees of NK1r inhibition (Ji *et al.*, 2009).

It has been also shown that noxious stimulation led to the release of BDNF in the dorsal horn after its anterograde transport from the DRG (Zhou and Rush, 1996). It was suggested that following nociceptive stimulation, BDNF is released at the synaptic cleft in the dorsal horn and binds to its high affinity postsynaptic receptor: TrkB (Pezet *et al.*, 2002), activating the MAPK/ERK cascade (Slack *et al.*, 2004). Subsequently, Slack *et al.* (2005) reported that dorsal horn neurons that projected to the thalamus (spinothalamic tract, STT neurons) expressed the TrkB receptor, and that both exogenous and induced-endogenous release of BDNF led to ERK phosphorylation in TrkB-containing STT neurons.

Activation of the MAPK/ERK cascade leads to short term changes in the dorsal horn, such as phosphorylating receptors or channels, as well as long term changes, altering the expression of neuronal genes (Ji *et al.*, 2003). It was hypothesized that the activating ERK phosphorylates the GluN1 subunit of the NMDA receptor, thus increasing dorsal horn neuronal excitability (Slack and Thompson, 2002; Slack *et al.*, 2004). Recent studies have also demonstrated the involvement of activated ERK in phosphorylating the pore-forming subunit of the potassium channel Kv4.2, which is a major contributor to A-type K^+ currents that modulate nociceptive behaviours and pain plasticity and in the dorsal horn (Adams *et al.*, 2000; Hu *et al.*, 2003; Ji *et al.*, 2003; Hu *et al.*, 2006).

The MAPK/ERK cascade is also involved in regulating neuronal gene expression, which is involved in induction and maintenance of inflammatory pain. Ji *et al.* (2002) reported that two days following noxious stimulation, which was induced by the injection of CFA into the hindpaw of the rat, pERK-positive neurons were concentrated in the SDH. Both prodynorphin and NK1r were up-regulated in these SDH neurons and their up-regulation was prevented by blocking ERK activation. Recently, Matsuoka and Yang (2012) reported that selectively activating ERK1/2 led to nerve growth factor (NGF)-induced up-regulation of BDNF mRNA in cultured DRG neurons. The study also showed that the application of AZD6244, a MEK1/2 specific inhibitor, to the rat spinal cord attenuated pathological pain, which was induced by either chronic constrictive injury or spared nerve injury. AZD6244 also decreased the expression of pERK in the DRG and BDNF in the SDH. In addition to the above study, several studies have used inhibitors of ERK phosphorylation, which block its upstream kinase MEK, in order to reverse pERK-induced pain state. For instance, intrathecal application of the

MEK inhibitor PD98059 reduced the 2nd phase of formalin-induced pain behaviour in mice (Choi *et al.*, 2006). This 2nd phase is thought to result from central sensitization, a nociceptor-mediated activity-dependent increase in the excitability of spinal neurons (Woolf and Costigan, 1999).

It is often assumed that spinal ERK phosphorylation is exclusively dependent on sensory afferent input from the periphery. However, supraspinal input may contribute to either facilitating or inhibiting spinal ERK activation (Cruz *et al.*, 2006; Svensson *et al.*, 2006). For example, Svensson *et al.* (2006) reported that formalin injection to the hindpaw of the rat evoked the activation of spinal ERK. This ERK expression was facilitated by input from an excitatory serotonergic descending pathway, which arose from the brainstem medullary 5-hydroxytrytamine-containing nuclei of the raphe. It was shown that the intrathecal administration of a spinal 5-hydroxytrytamine 3 receptor antagonist decreased ERK activation in the spinal cord. However, Cruz *et al.* (2006) reported that in animals with spinal cord injury, which was induced by spinal transection at the thoracic level, spinal ERK phosphorylation was enhanced at the L6 spinal cord segment, and this enhancement was more intense after stimulating the bladder. This suggests that supraspinal input through different mediators may play a complex role in both inhibiting and stimulating spinal ERK activation (Cruz and Cruz, 2007).

Phosphorylation of ERK in the dorsal horn following various types of noxious stimuli will be discussed in more detail in Chapter 4. Figure 1-2 shows examples of mechanisms of ERK activation in the spinal cord after noxious stimulation.

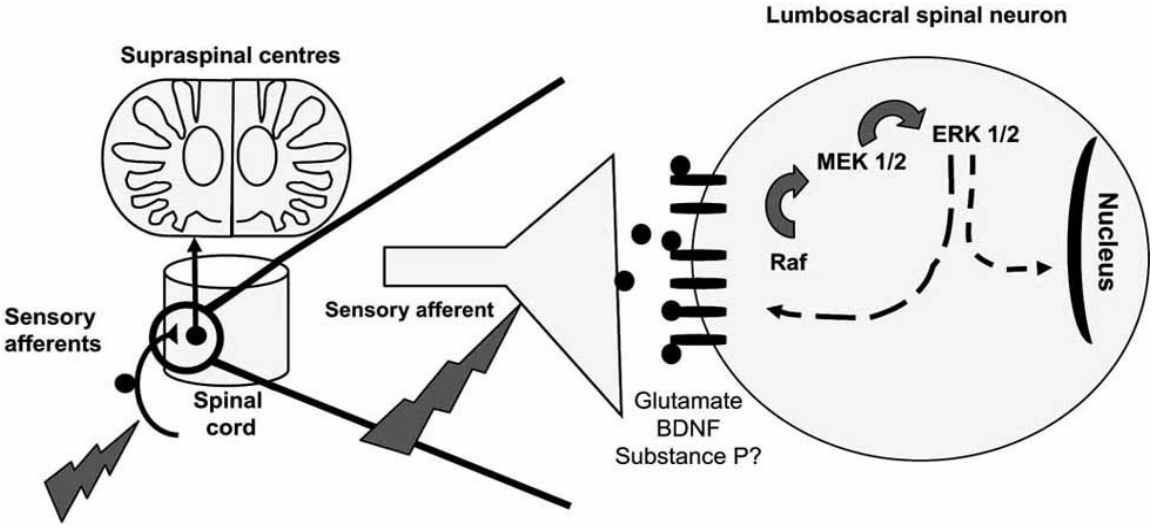


Figure 1-2 Examples of mechanisms of ERK activation in the spinal cord.
Following noxious stimulation, glutamate, BDNF and substance P are released onto the spinal cord and bound to their receptors activating ERK in the cytoplasm. Activated ERK then phosphorylates specific subunits or subsequently leads to gene expression, modulating different cellular functions. From Cruz and Cruz, (2007).

1.5.3 Fos and pERK: pros and cons

In 1987, fos was first used as a marker of neuronal activity in the SDH (Hunt *et al.*, 1987), yet 12 years later, it was shown that pERK is specifically induced by noxious stimulation (Ji *et al.*, 1999). Since then, both fos and pERK have been extensively used as markers of neuronal activation following noxious stimulation.

There are several advantages to using fos as a marker of neuronal activity, such as the fact that its expression in the SDH is very robust and requires high threshold stimuli. In addition, it can be easily revealed and quantified immunocytochemically. However, the direct role of fos in nociception is not yet fully understood. Fos expression is thought to be due to monosynaptic activation of spinal cord neurons following small-diameter high-threshold primary afferents stimulation (Hunt *et al.*, 1987). It is usually assumed that fos is related to nociceptive processes, since its expression leads to long term intracellular changes in spinal nociceptive neurons, producing either hyperalgesia or allodynia (Morgan and Curran, 1991; Harris, 1998). This link between nociception and fos expression in the dorsal horn is further strengthened by the finding that fos expression is decreased after treating animals with variety of analgesics, such as kappa1-opioid receptor agonists and non steroidal anti-inflammatory drugs (Catheline *et al.*, 1999; Buritova and Besson, 2000). However, dissociation

between the sensation of pain and fos expression has also been reported. For instance, morphine abolished pain-related behaviours in rats injected with formalin, yet failed to significantly decrease the expression of fos in the SDH (Presley *et al.*, 1990). This suggests that in addition to the direct relation between fos expression in the dorsal horn and nociception, spinal fos expression may reflect processes that contribute indirectly to pain transmission. It is also essential to consider that the absence of fos expression does not always indicate the absence of neuronal activity, since it is expressed only in certain neurons following peripheral stimulation. For instance, pinching the hindpaw of lightly anesthetised rats led to positive withdrawal reflexes, indicating the activity of ventral horn motor neurons. However, there was no fos-immunoreactivity in the ventral horn (Bullitt, 1990). Additionally, there is a risk of false positive results, for instance Bullit (1990) reported that at least few supraspinal neurons expressed fos in the absence of noxious stimulation, such as following prolonged inhalational anaesthesia. This may be due to the time lag between the induction and expression of fos, making it difficult to determine which precise events were directly responsible for the gene induction (Cruz and Cruz, 2007). It was also reported that walking for an hour on a rotating rod induced fos expression in spinal and supraspinal areas in the rat. However, the pattern of this expression differed from fos up-regulation following noxious stimulation, since fos was expressed in laminae IIi, V-VII as well as in motor neurons in the ventral horn following this innocuous stimulus (Jasmin *et al.*, 1994).

Like fos expression, ERK activation requires high-threshold stimuli. pERK expression is also topographically specific, and predominantly induced in somatotopically appropriate neurons (Ji *et al.*, 1999). Unlike fos, the expression of pERK is more rapid and occurs in various cellular and subcellular regions. In addition, ERK phosphorylation is more likely to be only associated with noxious stimulation. Importantly, the availability of specific inhibitors of the MAPK/ERK pathway has led to a clear demonstration of the role of pERK in central sensitization (Karim *et al.*, 2001).

Although it was reported that fos expression in supraspinal neurons may occur without the participation of ERK (Johnson *et al.*, 1997), it was shown that the situation may differ in the spinal cord (Cruz and Cruz, 2007). It was reported that spinal fos expression, induced by both noxious somatic and visceral

stimulation, was almost completely suppressed upon ERK blockade (Kawasaki *et al.*, 2004; Cruz *et al.*, 2007). In addition, Kominato *et al.* (2003) reported that after the injection of a MEK inhibitor in a rat model of neuritis, the increase in fos expression seen in the SDH neurons was significantly suppressed, while fos expression in deep dorsal horn neurons was ERK independent. This suggests that fos expression in the SDH may have been the result of activation of the MAPK/ERK cascade. It was also shown that formalin-induced fos expression in the SDH of the rat was almost blocked after intrathecal injection of a p38 inhibitor (Svensson *et al.*, 2005). Ruan *et al.* (2010) also reported that p38, JNK, ERK and fos were phosphorylated/up-regulated in the mouse SDH following the activation of ephrin receptor tyrosine kinases, which also led to hyperalgesia. Their study also showed that inhibition of any of the three MAPKs pathways almost prevented fos expression and reversed pain behaviour. This therefore suggests that blocking any of the interactions in this signalling molecules network will result in a decreased activation of the whole network and pain behaviour relief as a result.

The specificity, distribution and time course of fos and pERK are compared in Table 1-1, which is partially adapted from a review by Gao and Ji (2009).

Table 1-1 Comparison of the specificity, distribution and time course of fos and pERK

	Fos	**pERK**
Mechanism of action	Gene expression	phosphorylation
Induction by noxious stimuli	Yes	Yes
Induction by innocuous stimuli	Sometimes	No
Stimulus intensity-dependent	Yes	Yes
Subcellular distribution in neurons	Nucleus	Nucleus, cytoplasm, dendrites, and axons
Lamina localization in the SDH		
I-II	Strong	Strong
III-IV	Weak	Weak
V-VI	Medium	Weak
Time course after formalin injection		
Onset	>30 minutes	1-3 minutes
Peak induction	1-2 hours	2-10 minutes
Return to base	8-24 hours	1-2 hours
Correlation to pain behaviour	Partially	Closely
Involvement in central sensitization	Unknown	Yes
Specific inhibitors	No	Yes

1.6 Aims of the project

The overall aim of the project is to investigate the role of different populations of spinal cord projection neurons and interneurons in nociception.

The project initially starts with examining whether NK1r-expressing projection neurons can be distinguished from NK1r-expressing interneurons, in lamina I of the spinal cord, based on their soma sizes. The results from the first part of the project are then used to assess the responses of 'putative' lamina I NK1r-expressing interneurons and projection neurons (categorized based on their soma sizes) to various noxious stimuli. The 2nd part of the project also investigates the responses of other populations of projection neurons in the SDH to various noxious stimuli. These include laminae III-IV NK1r-expressing neurons and lamina I giant cells (both are previously known, from retrograde studies, to be projection neurons). Finally, and since it was reported that spinal activation of group I mGlus induced hyperalgesia, the role of $mGlu_5$-expressing neurons in nociceptive modulation is further investigated.

The individual aims of each part of the project will be discussed in further detail in the relevant Chapters.

2. General Methods

This Chapter deals with the general methods that are used in order to pursue the three principal aims of the project. Specific experimental procedures that are applied in each part of the project are explained in detail in the corresponding Chapters. Overall, the following procedures are similar in every Chapter: animal perfusion, tissue processing and immunocytochemistry in addition to confocal microscopy and analysis. All experiments were approved by the Ethical Review Process Applications Panel of the University of Glasgow, and were performed in accordance with the UK Animals (Scientific Procedures) Act 1986.

2.1 Perfusion of the animals

Following the appropriate survival time for each experimental protocol (retrograde tracing, noxious or pharmacological stimulation), pentobarbitone- or urethane-anesthetized rats were perfused intracardially with a fixative at 4°C, preceded by a wash with ***Ringer's solution**** for couple of seconds. The fixative that was used contained 4% freshly de-polymerised ***formaldehyde**** in ***0.1 M phosphate buffer**** (PB). After completion of the perfusion process, the appropriate spinal segments were removed and put into the same fixative solution and stored at 4°C overnight. In those experiments that involved retrograde tracing, in order to identify the injection sites, the brain tissue was removed and put in 30% sucrose overnight. Detail of handling the brain tissue is mentioned in the next Chapter.

2.2 Tissue processing and immunocytochemistry

On the following day after perfusing the animals, appropriate thoracic, lumbar or sacral spinal segments were cut into 60 μm thick horizontal, parasagittal or transverse sections with a Vibratome. The choice of the plane of cutting the

***Ringer's solution** typically contains sodium chloride, potassium chloride, calcium chloride and sodium bicarbonate (to balance the pH). Other additions can include chemical fuel sources for cells, including ATP and dextrose, as well as antibiotics and antifungals.

***4% formaldehyde** is made in a fume hood by heating distilled water to 68°C and adding 40 g of paraformaldehyde. Few drops of 1 N NaOH are added at a time till the paraformaldehyde is completely dissolved. 0.2 M phosphate buffer is then added and topped up with distilled water to make one litre of the solution, which is then filtered.

***0.1 M phosphate buffer** is made by adding 1120 ml of solution A to 2880 ml of solution B and mixing well after adjusting the pH to 7.4. 3000 ml distilled water is then added to the solution.
Solution A: NaH2PO ($2H_2O$) 37.4 g in 1200 ml H_2O
Solution B: Na2HPO4 84.9 g in 3000 ml H_2O

segments depended on the orientation of the cells of interest as well as on the purpose of processing these cells. Horizontal sections are ideal for viewing and assessing the morphology of lamina I neurons, since the branches of their dendritic trees are oriented mainly rostrocaudally and/or mediolaterally with very limited ventral spread (Cheunsuang and Morris, 2000). On the other hand laminae III-IV neurons posses dorsally-directed dendrites that arborize toward the superficial laminae (Todd, 1989) making them more visible in parasagittal sections. Transverse sections are useful for showing the laminar distribution of cells of interest. Prior to cutting the segments, some blocks were notched at one side in order to orientate the free-floating sections. Spinal blocks from animals that were unilaterally injected with a tracer to the brain were notched ipsilateral to the injection site. Blocks from the stimulated animals were notched contralateral to the side of stimulation. After cutting the segments, sections were immersed in 50% ethanol for 30 minutes in order to enhance the penetration of the antibodies (Llewellyn-Smith and Minson, 1992). These sections were then rinsed in ***phosphate buffer saline*** (PBS) ***that contained 0.3 M NaCl**** three times over a period of half an hour, unless otherwise stated. They were then incubated at 4°C for three days in a cocktail of primary antibodies. Antibodies that were used in this project are listed in Table 2-1. The sections were then rinsed again in PBS three times over a period of half an hour followed by their reaction with species-specific secondary antibodies for 24 hours. These secondary antibodies (anti-IgG) were raised in donkey and conjugated to Alexa 488 (Invitrogen, Paisley, UK), Rhodamine Red, Cy5 or DyLight 649 (Jackson ImmunoResearch, West Grove, PA, USA) and were used at 1:500 (Alexa 488 and DyLight 649 conjugates) or 1:100 (Rhodamine Red and Cy5 conjugates).

All antibodies were made in ***PBS that contained 0.3% Triton® X-100**** (PBST) unless otherwise stated. Sections were then rinsed three times with PBS, mounted on glass slides, covered-slipped with anti-fade medium (Vectashield; Vector Laboratories, Burlingame, CA, USA) and stored at -20°C.

phosphate buffer saline that contained 0.3 M NaCl is composed of 100 ml of 0.2 M PB in 1900 ml of distilled water with 36 g of NaCl. It is found that this concentration of NaCl reduces the non-specific binding of antibodies and avoids need for the addition of other proteins, such as blocking sera.

PBS that contained 0.3% Triton® X-100 is made up with 3 ml of Triton® X-100 in 1000 ml of PBS.

Tyramide signal amplification (TSA) was used in some reactions where two of the primary antibodies were raised in the same species (Shindler and Roth, 1996). The amplification was performed with a TSA kit (PerkinElmer Life Sciences, Boston, MA) according to the instructions of the manufacturer and will be explained further in the appropriate Chapter.

2.3 Antibody characterisation

Specificity of the ***CTb*** and ***Fluorogold*** antibodies is shown by the lack of staining in regions of the CNS that did not contain neurons that had transported the tracer, and by the presence of immunostaining in populations of neurons that are known to project to the injection sites.

The ***NK1r*** antibody is raised in rabbit against amino acids 393-407 of the rat NK1 receptor conjugated to keyhole limpet haemocyanin. It has been shown that there is no immunostaining with this antibody in sections of medulla and cervical spinal cord from knock-out mice compared to wild-type mice (Ptak *et al.*, 2002).

The mouse monoclonal antibody against gephyrin (***mAb 7a***) was generated against an extract of rat spinal cord synaptic membranes (Pfeiffer *et al.*, 1984), and has been shown with Western blots to bind to a 93-kDa peripheral membrane protein (gephyrin) in extracts of rat brain membranes (Becker *et al.*, 1989; Kirsch and Betz, 1993).

The monoclonal ***pERK*** antibody detects both ERK1 and ERK2 that are dually phosphorylated at Thr202 and Tyr204 sites, and does not cross-react with JNK or p38 MAP kinase that are phosphorylated at the corresponding residues.

Specificity of the ***mGlu$_5$*** antibody is shown by the lack of staining in regions of the brain in knock-out mice, as well as the lack of cross-reactivity with the mGlu$_1$ antibody (manufacturer's specification).

The ***calbindin*** antibody (CB-$_{28kD}$) detects a single protein band at 28-kDa (manufacturer's specification). The specificity of the ***calretinin*** antibody is shown by the absence of staining in the cerebral cortex of knock-out mice.

The ***GABA*** antibody was raised against GABA conjugated with glutaraldehyde to porcine thyroglobulin, and shows negligible cross-reactivity against other amino acids, including glutamate, aspartate, glycine or taurine (Pow and Crook, 1993).

2.4 Confocal microscopy and analysis

Two confocal laser scanning microscopes were used in this project. For the first two parts of the study, sections were scanned with a ***Bio-Rad Radiance 2100*** confocal system equipped with blue diode (405 nm) argon multi-line, green HeNe (543 nm) and red diode (637 nm) lasers (Bio-Rad, Hemel Hampstead, UK), and connected to a Nikon Eclipse E600 microscope (Nikon UK Ltd, Surry, UK). The sections were scanned through dry (10×, 20×) objective lenses and a 40× oil-immersion lens (numerical aperture [NA] 1.3). Z-series of various numbers of optical sections at 2 μm z-separation were produced.

For the 3rd part of the study, a ***Zeiss LSM710*** confocal microscope equipped with argon multi-line, 405 nm diode, 561 nm solid-state and 633 nm HeNe lasers was used (Carl Zeiss MicroImaging, Germany). Sections were scanned through a 40× oil-immersion objective lens (NA 1.3) to produce z-series consisting of optical sections at 1 μm z-separation.

Scans that were produced using both the Bio-Rad Radiance 2100 confocal system and the Zeiss LSM710 confocal microscope were saved in the form of raw images (Bio-Rad pic and Zeiss lsm), and viewed using Neurolucida for Confocal software (MicroBrightField Inc., Colchester, VT, USA). Figures were composed with Adobe Photoshop (CS) software and in some cases, the brightness and contrast of the images was adjusted.

2.5 Statistical analysis

For statistical comparison between different groups of data, the following tests were used: Kruskal–Wallis test, Post-hoc Mann–Whitney *U*-tests with sequential Bonferroni correction and Chi-square test. A *P* value of less than 0.05 was considered significant.

Table 2-1 Primary antibodies used in this study

Antibody	Species	Working Dilution	Supplier
CTb	Goat	1:50,000 (brain sections)	List Biological Laboratories, Campbell, CA, USA
	Goat	1:5000 (spinal cord sections)	Sigma-Aldrich, Poole, UK
Fluorogold	Guinea pig	1:500 (spinal cord sections)	Protos Biotech Corp., New York, USA
NK1r	Rabbit	1:10,000	Sigma-Aldrich, Poole, UK
Gephyrin, mAb 7a	Mouse	1:100,000 (TSA)	Synaptic Systems, Göttingen, Germany
pERK	Mouse	1:1000	Cell Signalling, Beverley, MA, USA
$mGlu_5$	Rabbit	1:500	Gift from Prof. M. Watanabe, Hokkaido, Japan
Calretinin	Goat	1:1000	Swant, Bellizona, Switzerland
Calbindin	Goat	1:500	Gift from Prof. M. Watanabe, Hokkaido, Japan
GABA	Guinea pig	1:1000	Gift from Prof. D.V. Pow, Brisbane, Australia

3. Soma size distinguishes lamina I NK1r-expressing projection neurons from interneurons

3.1 Introduction

Lamina I is supplied mainly by Aδ- and nociceptive peptidergic C-fibres as well as by thermoreceptive fibres (Light and Perl, 1979b; Todd and Koerber, 2006; Todd, 2010). It contains neurons that are activated by noxious and/or thermal stimuli (Christensen and Perl, 1970; Han *et al.*, 1998). These can be either projection neurons, which convey nociceptive, thermoreceptive and itch information to higher brain centres (Craig, 1995; Willis and Coggeshall, 2004) or interneurons. Lamina I interneurons play a major role in modulating nociception, yet they are not easily distinguished from projection neurons in the same lamina. As described previously, projection neurons make up around 5-10% of the neuronal population in lamina I, and these neurons project to various supraspinal targets.

Lamina I neurons of the rat that project to the LPb respond to noxious stimulation, as evidenced by immunocytochemical (fos up-regulation) (Lanteri-Minet *et al.*, 1994; Bester *et al.*, 1997) and electrophysiological (Bester *et al.*, 2000; Andrew, 2009) studies, suggesting the importance of this area in processing nociception. Craig (1995) reported that the CVLM; specifically the area between the lateral reticular and spinal trigeminal nuclei, is a major target for axons of lamina I neurons in the cat and the monkey. It was also reported that large numbers of lamina I neurons in the rat were retrogradely labelled from the CVLM (Lima *et al.*, 1991; Spike *et al.*, 2003). It was shown that in the rat, the thalamus (including its PoT) received fewer projections from lamina I neurons that were located in the lumbar region, compared to neurons in the cervical area (Burstein *et al.*, 1990; Al-Khater *et al.*, 2008). It was also found that in the lumbar cord of the rat, the largest numbers of labelled lamina I neurons were seen following tracer injections into the CVLM or LPb, compared to the smaller number of cells observed after the injection of a tracer into the PAG. In each case, most labelled cells were located in the contralateral dorsal horn with some cells having bilateral projections (Todd *et al.*, 2000; Spike *et al.*, 2003; Polgar *et al.*, 2010). In addition, Al-Khater and Todd (2009) reported that almost all (>95%) lamina I spinothalamic neurons in both lumbar and cervical enlargements were labelled from the LPb.

Many lamina I neurons express the NK1 receptor, on which substance P acts. It was estimated that in the rat, around 45% of lamina I neurons express the NK1 receptor (Todd *et al.*, 1998), and a large number of these do not project to supraspinal areas. Approximately 80% of lamina I neurons that were retrogradely labelled from various supraspinal regions, expressed the NK1 receptor in the rat. NK1r-expressing neurons are of particular interest, since they are activated by noxious stimuli (Doyle and Hunt, 1999) and their ablation leads to a dramatic reduction of hyperalgesia in inflammatory and neuropathic pain models (Mantyh *et al.*, 1997; Nichols *et al.*, 1999).

Attempts have been made to classify lamina I NK1r-expressing neurons. For instance, Cheunsuang and Morris (2000) identified two distinct populations of NK1r-expressing neurons in lamina I. These included small cells, which stained weakly for the receptor and had fusiform somata, with a mean value of their cross-sectional area of 81 μm^2 (in a horizontal plane), and large multipolar cells that were described as strongly immunoreactive (mean of the soma cross sectional area was 288 μm^2). Cheunsuang and Morris (2000) also suggested that many of the larger neurons may project to the thalamus and have polymodal nociceptive characteristics, thus being a part of a system that evaluates the severity of an injury, and then initiates the appropriate response for protection and endogenous suppression of this pain. In contrast, the study showed that smaller neurons had a very distinctive fusiform morphology, which has been suggested to be characteristic of nociceptive specific neurons (Han *et al.*, 1998). Subsequently, Polgar *et al.* (2002) reported that a sample of 45 NK1r-immunoreactive lamina I neurons that were labelled from the CVLM had soma sizes that were similar to those of the large cells identified by Cheunsuang and Morris (2000). However, the study could not determine whether all of the large cells were projection neurons, since not all projection neurons in this lamina would have been labelled from the CVLM (Spike *et al.*, 2003).

It was reported that morphology of lamina I neurons correlated with functional properties in the rat, cat and monkey. For instance, pyramidal cells that lacked the NK1 receptor responded to innocuous cooling only, while multipolar and fusiform cells were activated by noxious stimuli (Han *et al.*, 1998; Yu *et al.*, 1999; Almarestani *et al.*, 2007). Almarestani *et al.* (2007) also reported that

most of the spinoparabrachial NK1r-expressing neurons in the rat were either multipolar or fusiform, while the pyramidal projection cells were seldom NK1r-immunoreactive. However, it was shown that in the rat, there was no difference between the morphological classes of NK1r-expressing projection neurons in either the density of contacts from substance P-containing afferents or the percentage of cells that up-regulated fos after noxious chemical stimulation (Todd *et al.*, 2002; 2005).

The main aim of this part of the study was to test the hypothesis that all of the large lamina I NK1r-immunoreactive cells identified by Cheunsuang and Morris (2000) are projection neurons, while the small cells are interneurons. To achieve this, tracers were injected into both the LPb and the CVLM, since it has been shown that virtually all lamina I projection neurons can be labelled from these sites (Spike *et al.*, 2003). It has been reported that a large number of lamina I pyramidal projection neurons lacked the NK1 receptor, and these cells tended to be smaller than the NK1r-expressing projection neurons (Almarestani *et al.*, 2007). Therefore, an additional aim of this study was to further investigate the retrogradely labelled pyramidal cells that were NK1r-negative, and compare them with the NK1r-expressing projection neurons of the pyramidal class.

3.2 Experimental procedures

3.2.1 Animals and operative procedures

Three adult male Wistar rats (250–280 g; Harlan, Loughborough, UK) were used for this part of the study. The rats were anaesthetized with Isofluorane (1.5-4%). They were then placed on a heating pad in order to maintain their body temperature. Their heads were fixed in a stereotaxic frame, after which the anaesthetic was administered through a mask attached to the frame. The level of anaesthesia was monitored throughout the procedure by occasionally pinching the hindpaw and observing the appearance of any withdrawal reflexes. In order to prevent the rat corneas from drying during the surgery, their eyes were covered with Vaseline petroleum jelly. The points in the skull that corresponded to the LPb and the CVLM were determined using a standard rat brain atlas (Paxinos and Watson, 2005). Under aseptic conditions, the two areas of interest: the LPb and the CVLM were made accessible by performing a craniotomy with a

fine drill after separating the underlying fascia. Each rat received two injections: (1) 50 nl of 4% Fluorogold (Fluorochrome Inc, Englewood, CO, USA) targeted on the left LPb, and (2) 200 nl of 1% CTb (Sigma-Aldrich, Poole, UK) into the left CVLM. Injections to the CVLM were aimed to regions between the lateral reticular nucleus and the adjacent part of the spinal trigeminal nucleus (Spike *et al.*, 2003). The injections were made under pressure using two different glass micropipettes, one for each area. In order to prevent the backflow of the tracers after injecting them, the micropipettes were left in place for 5 minutes. At the end of the procedure, the incision was sutured and the rats were allowed to recover from anaesthesia after which they were subcutaneously injected with 5 mg/kg carprofen; an analgesic. Following a 3 day survival period, the rats were re-anaesthetized with pentobarbitone (40 mg/kg ip) and perfused with 4% formaldehyde as described in the previous Chapter.

3.2.2 Tissue processing and immunocytochemistry

The brain and the lumbar spinal cord were dissected out and post-fixed at 4°C for 24 hours. The brain fixative contained 30% sucrose as a cryoprotectant, preventing large ice crystal formation. The regions of the brainstem that contained the injection sites were cut into 100 μm thick coronal sections with a freezing microtome. The brain sections were cut serially into 5 bottles and the series in one of the bottles was used for further analysis. Sections through the Fluorogold injection were mounted on slides with a glycerol-based antifade medium (Vectashield, Vector laboratories, Peterborough, UK) and viewed with a fluorescent microscope. For detection of CTb, sections from one of the bottles were incubated for 3 days in goat antiserum to CTb (1:50,000). On the 3rd day, the sections were rinsed 3 times in PBS over a period of half an hour, and then they were incubated overnight in biotinylated anti-goat IgG (Jackson ImmunoResearch, West Grove, PA; 1:500). On the 4th day, and after the sections had been rinsed 3 times in PBS for 30 minutes, they were reacted for 3-4 hours with streptavidin-horseradish peroxidase (Sigma-Adrich, Poole, UK; diluted in PBS, 1:1000). Peroxidase activity was revealed with 0.025 M diaminobenzidine in the presence of 0.001% hydrogen peroxide. The sections were then rinsed 3 times in PBS over a period of half an hour and mounted on gelatinized slides and air dried. On the following day, sections were rinsed in water, dehydrated in

ascending concentrations of alcohol, cleared in Histo-Clear and mounted with Histomount.

The spread of the tracers was plotted onto drawings of the LPb and the CVLM (Paxinos and Watson, 2005) and representative examples were photographed.

The spinal L4 segment from each animal was initially notched on the left side (ipsilateral to the injection side), and cut into 60 μm horizontal sections with a Vibratome. The sections were then treated for 30 minutes with 50% ethanol. These were rinsed 3 times with PBS for half an hour, and then incubated free-floating at 4°C for 3 days in a cocktail of antibodies. The primary antibodies consisted of guinea-pig anti-Fluorogold (1:500), goat anti-CTb (1:5000) and rabbit anti-NK1r (1:10,000). The sections were rinsed 3 times with PBS for 30 minutes, reacted with fluorescent labelled species-specific secondary antibodies that were raised in donkey and conjugated to either Alexa 488, Rhodamine Red or Cy5. The sections were then rinsed 3 times with PBS, mounted and stored at -20°C. Characteristics and suppliers of antibodies used in this part of the study are described in the previous Chapter.

3.2.3 Confocal microscopy and analysis

Horizontal sections from the L4 spinal segments were used to analyze the soma sizes of lamina I NK1r-expressing neurons that projected to the LPb and/or the CVLM, as well as the soma sizes of the NK1r-expressing neurons that were not retrogradely labelled.

Sections were initially examined with a 20× objective lens using a fluorescent microscope. All of those on the contralateral (right, un-notched) side that contained lamina I were identified by the presence of numerous retrogradely labelled cells, as well as a relatively high density of NK1r-immunoreactivity compared to lamina II. In this way, between one and three sections from each animal were selected for further analysis. These sections were scanned with a Bio-Rad Radiance 2100 confocal system through a 40× oil-immersion lens to produce image stacks with a 2 μm z-separation. Since the area covered by this lens was approximately 300×300 μm^2, several overlapping fields (between 5 and 7 from each section) were scanned in order to include most of the medial two-

thirds of lamina I. The lateral part of the dorsal horn was not analysed, as the orientation of the lamina is different in this region. Scans were performed sequentially with each laser line to avoid fluorescent bleedthrough.

Confocal image stacks were analysed with Neurolucida for Confocal software. Initially, and to avoid selection bias, only the channel corresponding to NK1r-immunoreactivity was viewed and this was used to identify lamina I. NK1r-immunoreactive cells in lamina I were then selected and the outlines of their cell bodies were drawn by examining all of the optical sections through each cell. Preliminary observation confirmed that there was a large population of small cells (<200 μm^2 soma cross-sectional area) that showed weak NK1r-immunoreactivity, and these were found to be far more numerous than the larger NK1r-immunoreactive cells. For this reason, all of the larger cells (>~200 μm^2 soma cross-sectional area) together with a sample of small cells were selected for analysis. For each of the selected cells, the maximum cross-sectional area of the soma was measured from projected confocal images of NK1 receptor staining with Neurolucida for Confocal (Puskar *et al.*, 2001). In addition, the intensity of NK1r-immunostaining in the plasma membrane was assigned a score ranging from 4 (strong) to 1 (very weak), as described previously (Al-Khater and Todd, 2009). This scoring system was used because variation in immunofluorescence intensity at different depths of the Vibratome sections makes it difficult to use a more objective measure (Spike *et al.*, 2003). When all of the selected cells in a field had been analysed, the channels corresponding to Fluorogold and CTb were examined, and the presence or absence of these tracers in each of the selected cells was determined. In addition, any retrogradely labelled cells that had not been included in the selected sample were identified and analysed in the same way. These additional cells included those that were NK1r-negative as well as some that showed very weak immunostaining and had not been recognized in the initial survey. Cells were excluded from the analysis if part of the soma had been removed from the Vibratome section in such a way that the cross-sectional area would be underestimated.

As described previously, Almarestani *et al.* (2007) reported the presence of a distinctive population of pyramidal spinoparabrachial neurons that lacked the

NK1 receptor. In order to further investigate this, the morphology of all retrogradely labelled cells that were not NK1r-immunoreactive was analysed by examining confocal image stacks with Neurolucida software. Cells were classified into multipolar, pyramidal or fusiform based on the criteria described by Zhang *et al.* (1996) and Zhang and Craig (1997). The soma size of these neurons and the number and orientation of their primary dendrites were clearly defined, since it has been suggested that these features are particularly important for classification (Almarestani *et al.*, 2009).

3.3 Results

3.3.1 Injection sites

The extent of spread of Fluorogold from the LPb and of CTb from the CVLM was plotted for each experiment. Drawings, which were based on those of Paxinos and Watson (2005), are illustrated in Figure 3-1. Photomicrographs of representative injection sites are shown in Figure 3-2.

The Fluorogold injection, which targeted the LPb covered almost all of it in each case, with variable spread into surrounding structures, such as the superior cerebellar peduncle, medial parabrachial area, cuneiform nucleus, and inferior colliculus.

The CTb injection, which was targeted on the CVLM filled the lateral part of the lateral reticular nucleus in all three cases, with spread into surrounding regions, including the area between this nucleus and the spinal trigeminal nucleus and the ventrolateral white matter.

3.3.2 NK1r-immunoreactivity in lamina I

The highest densities of NK1r-immunostaining, for both cell bodies and dendrites, were seen in lamina I (Figure 3-3). This finding is in agreement with previous studies (Bleazard *et al.*, 1994; Nakaya *et al.*, 1994; Littlewood *et al.*, 1995; Todd *et al.*, 1998). Lamina I NK1r-immunoreactive cells appeared to have a bimodal size distribution, with one group consisting of large neurons of various shapes, and the other group consisted of many smaller fusiform cells that resembled what was described by Cheunsuang and Morris (2000). The large cells

showed different somatodendritic morphology (including fusiform, pyramidal and multipolar types), and were also much more variable in regard to their strength of NK1r-immunoreactivity. This ranged from strong to very weak in contrast to the small cells, which were generally weakly immunoreactive. In regard to the smaller NK1r-expressing cells, it was observed, but not systematically analysed, that these cells were particularly numerous in the ventral part of lamina I (as reported by Cheunsuang and Morris, 2000).

Altogether, 1341 NK1r-immunoreactive cells were identified in lamina I in sections from the three experiments. Of these, 441 neurons were retrogradely labelled from the CVLM, LPb, or both. The 900 remaining cells were not labelled from either the CVLM or the LPb.

3.3.3 Retrograde labelling

In addition to the 441 NK1-immunoreactive projection neurons, 101 NK1r-negative retrogradely labelled cells were identified. The total number of lamina I projection neurons that were selected for analysis from the 3 experiments was therefore 542, (156–206 per experiment). Of these, 83–88% contained both tracers, while 5–6% was labelled only with CTb injected into the CVLM and 5–12% was labelled only with Fluorogold injected into the LPb (Table 3-1, Figure 3-4a).

From the three experiments, between 76 and 86% (mean 81%) of the retrogradely labelled lamina I cells were NK1r-immunoreactive. The strength of NK1r expression on these cells varied from very weak to strong (Table 3-2). Examples of retrogradely labelled neurons that were NK1r-immunoreactive and those that lacked the NK1 receptor are shown in Figures 3-3 and 3-4.

Of the 441 NK1r-immunoreactive retrogradely labelled neurons, 420 had been identified in the initial surveys of NK1r immunoreactivity in the confocal image stacks, as described in the experimental procedures section. The remaining 21 (most of which showed weak NK1r-immunoreactivity) were identified only after the channels corresponding to CTb and Fluorogold were observed.

3.3.4 Soma cross-sectional areas

As described previously, the total number of lamina I NK1r-immunoreactive neurons that had been selected in this study is 1341. Of these, 900 cells were not retrogradely labelled (214–390 from each experiment). The remaining 441 NK1r-immunoreactive neurons were retrogradely labelled. Regarding the strength of the NK1r immunoreactivity, the great majority of un-labelled cells (784, 87%) were assigned a score of 1, while 106 (12%) were given a score of 2, and 10 (1%) a score of 3.

Figure 3-5 shows the frequency distributions of soma cross-sectional areas for the different groups of neurons based on both their NK1r expression and retrograde labelling. The histogram for all NK1r-immunoreactive neurons shows a clear bimodal distribution with two peaks. The first one corresponds to soma cross-sectional area values between 50 and 200 μm^2, and the second broader peak extends from 200 to 600 μm^2 with fewer cells having larger soma cross-sectional areas (up to 1200 μm^2).

Figure 3-5 also shows the cross-sectional areas of cell bodies for the NK1r-immunoreactive cells that were not retrogradely labelled. These ranged from 61 to 568 μm^2, with a median value of 124 μm^2 (n=900), and they corresponded to the first peak of the combined NK1r-immunoreactive group. The cross-sectional area values for the retrogradely labelled NK1r-immunoreactive cells were 128–1198 μm^2, with a median of 298 μm^2 (n=441), and these corresponded to the second peak of the combined group. The retrogradely labelled cells that lacked the NK1 receptor had soma cross-sectional area values that ranged between 137 and 1129 μm^2 with a median of 272 μm^2 (n=101).

Differences between these three groups ($NK1r^+$ non-retrograde, $NK1r^+$ retrograde and $NK1r^-$ retrograde) were found to be significant (Kruskal–Wallis test, P<0.001). Post-hoc Mann–Whitney U-tests with sequential Bonferroni correction showed a highly significant difference between the soma sizes of the NK1r-immunoreactive neurons that were not retrogradely labelled and those of both groups of retrogradely labelled neurons (P<0.001). However, there was no significant difference between the retrogradely labelled neurons that were NK1r-immunoreactive and those that were not immunoreactive (P=0.16).

Nearly all (895/900, 99.4%) of the NK1r-immunoreactive cells that were not retrogradely labelled had soma cross-sectional areas that were less than 200 μm^2. In contrast, only 43/441 (9.8%) of the retrogradely labelled NK1r-positive cells had cell bodies smaller than 200 μm^2 (Figure 3-5 and Table 3-3).

3.3.5 Morphology of NK1r-negative projection neurons

In order to fulfill one of the aims of this part of the study regarding investigating lamina I pyramidal projection neurons that lacked the NK1 receptor, the morphology of all the projection cells that were NK1r-negative was examined. Within this population (*n*=101), 15 were classified as pyramidal, 36 as multipolar and 24 as fusiform. Of the remainder 26, 13 fell under the 'unclassified' group, while 13 could not be classified because of incomplete filling of primary dendrites. An example of a retrogradely labelled pyramidal cell that lacked NK1r-immunoreactivity is illustrated in Figure 3-4 (cell 3). Figure 3-6 shows drawings of the cell bodies and proximal dendrites of five of these cells. The pyramidal cells were characterized by a triangular cell body, and in most cases (11/15) this gave rise to three primary dendrites (one from each pole of the soma). Each of the remaining four pyramidal cells had four primary dendrites. In one of these cases, two of the dendrites arose from one pole, while in the other three cases an additional thin dendrite was given off from the soma (Figure 3-6). One of these travelled dorsally towards the dorsal columns. Although the morphology of the retrogradely labelled NK1r-immunoreactive neurons was not analysed systematically, many of these cells were also pyramidal (e.g. cells 1 and 2 in Figure 3-4). In order to compare the soma sizes of NK1r-immunoreactive and non-immunoreactive pyramidal projection neurons, the results from the 15 NK1r-negative pyramidal projection cells seen in this part of the project were pooled with data from a study of spinoparabrachial neurons in the L3 segment of the rat reported by Al-Khater and Todd (2009). This latter group consisted of 55 retrogradely labelled pyramidal cells, of which 37 were NK1r-immunoreactive and 18 were non-immunoreactive. Soma sizes of the NK1r-immunoreactive pyramidal cells varied from 196 to 616 μm^2 (median 333, *n*=37) while those of the non-immunoreactive cells ranged from 171 to 533 μm^2 (median 285, *n*=33). Although the NK1r-immunoreactive cells tended to be larger than the non-immunoreactive ones (Figure 3-7), this difference did not reach significance (*P*=0.07, Mann–Whitney *U*-test).

Figure 3-1 Drawings of Fluorogold and CTb injection sites in the three experiments.

Injection sites in the three experiments (Fluorogold injected into LPb and CTb into CVLM). The drawings show the spread of tracer in each of these experiments. Each vertical column represents a single experiment, and the experiment number is shown at the bottom of the column. Numbers on the left correspond to the position of the section anterior or posterior (-) to the interaural plane. Drawings are based on those of Paxinos and Watson (2005). The upper four outlines in each column represent the Fluorogold injection, while the lower five show the spread of CTb.

CnF, cuneiform nucleus; Cu, cuneate nucleus; IC, inferior colliculus; KF, Kölliker-Fuse nucleus; LPb, lateral parabrachial nucleus; LRt, lateral reticular nucleus; MPB, medial parabrachial nucleus; PAG, periaqueductal grey matter; py, pyramidal tract; scp, superior cerebellar peduncle; Sol, nucleus of the solitary tract; Sp5, spinal trigeminal nucleus.

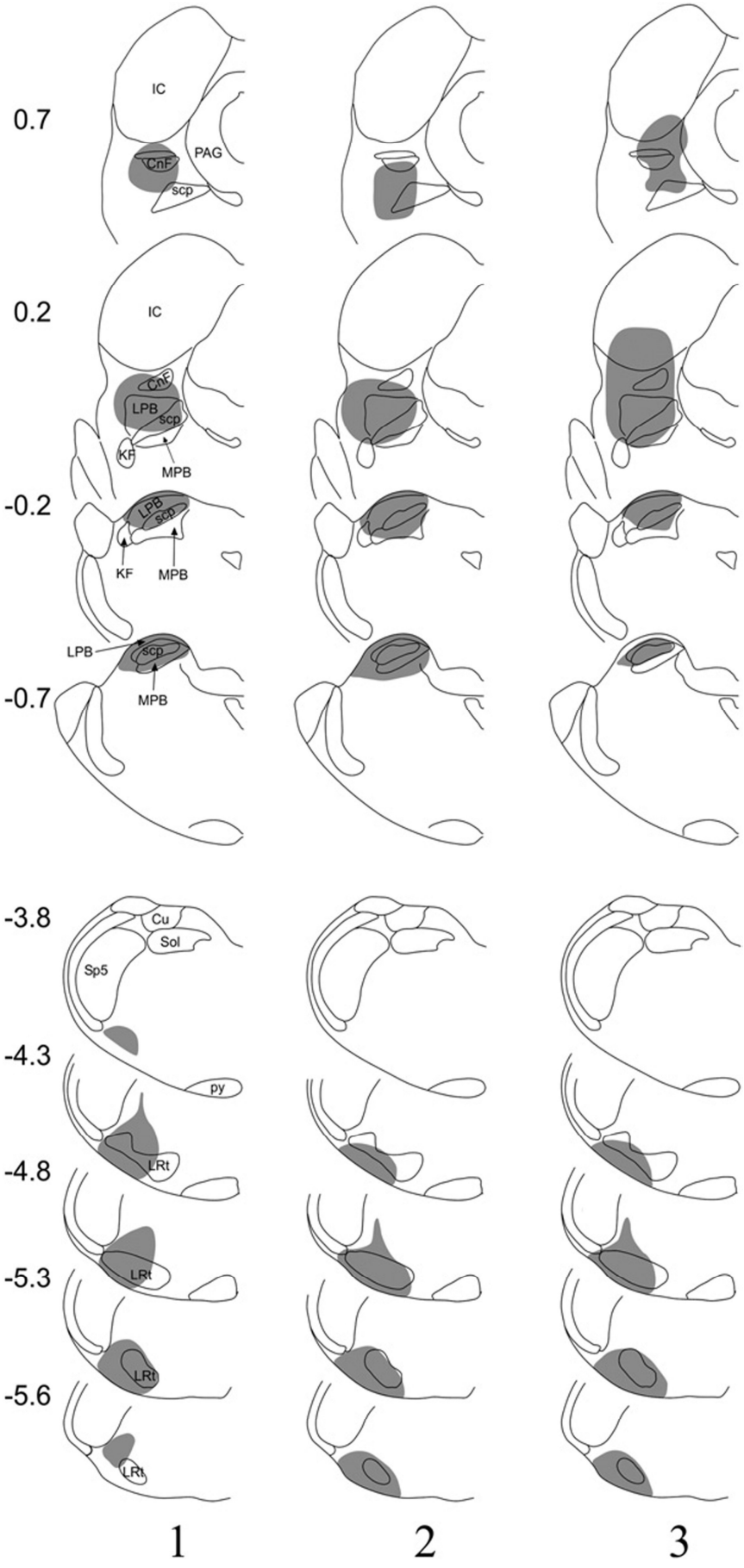

0.7
IC
PAG
CnF
scp
0.2
IC
CnF
LPB
scp
KF
MPB
-0.2
LPB
scp
KF
MPB
LPB
scp
-0.7
MPB
-3.8
Cu
Sol
Sp5
-4.3
py
-4.8
LRt
-5.3
LRt
-5.6
LRt
LRt
1
2
3

Figure 3-2 Examples of CTb and Fluorogold injection sites.

Photomicrographs of representative sites for the CVLM and LPb. Transverse sections through the brain are shown for injection into (a) the CVLM (experiment 2, interaural ~-5.3 mm) and (b, c) the LPb (experiment 3, interaural ~-0.2 mm). The section shown in (a) had been reacted with an immunoperoxidase method to reveal CTb, while (b, c) are fluorescent and bright field photomicrographs of a section through the Fluorogold injection. The spread of tracer is indicated by arrowheads. Scale bar=1 μm.

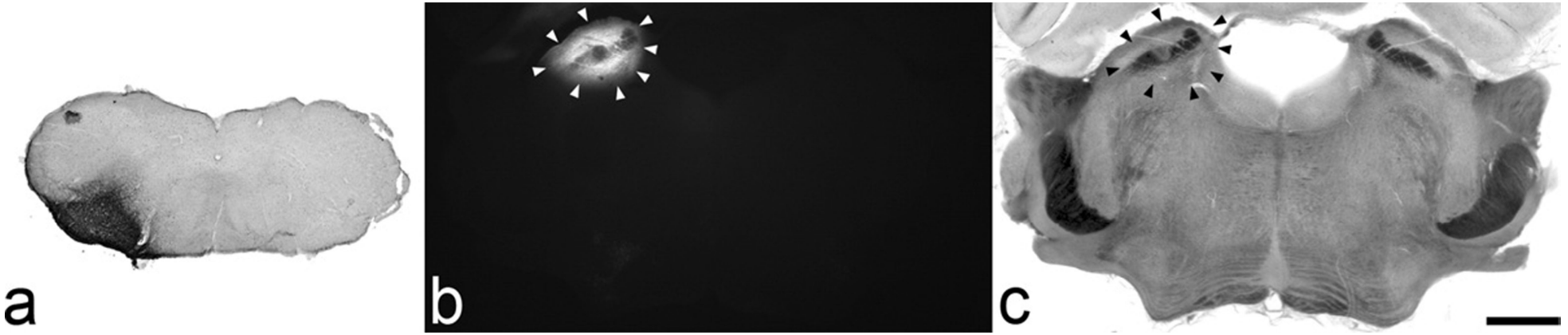

a
b
c

Figure 3-3 Immunoreactivity of the NK1 receptor, CTb and Fluorogold.

A horizontal section from the L4 segment of experiment 2. (a) shows a field scanned to reveal NK1r-immunoreactivity (green), (b) has been scanned for CTb (red) and Fluorogold (FG, blue), while (c) is a merged image. The NK1r-immunoreactivity is associated with thin elongated profiles, which are dendrites, as well as with larger structures, which are cell bodies.

This field contains the cell bodies of five NK1r-immunoreactive projection neurons (asterisks) that were retrogradely labelled from both CVLM and LPb, and therefore contain both tracers. In addition, several smaller NK1r-immunoreactive cell bodies are visible, and these are not retrogradely labelled. Six of these cells are indicated with arrowheads. The images are projections of two optical sections at 2 μm z-separation. Scale bar=20 μm.

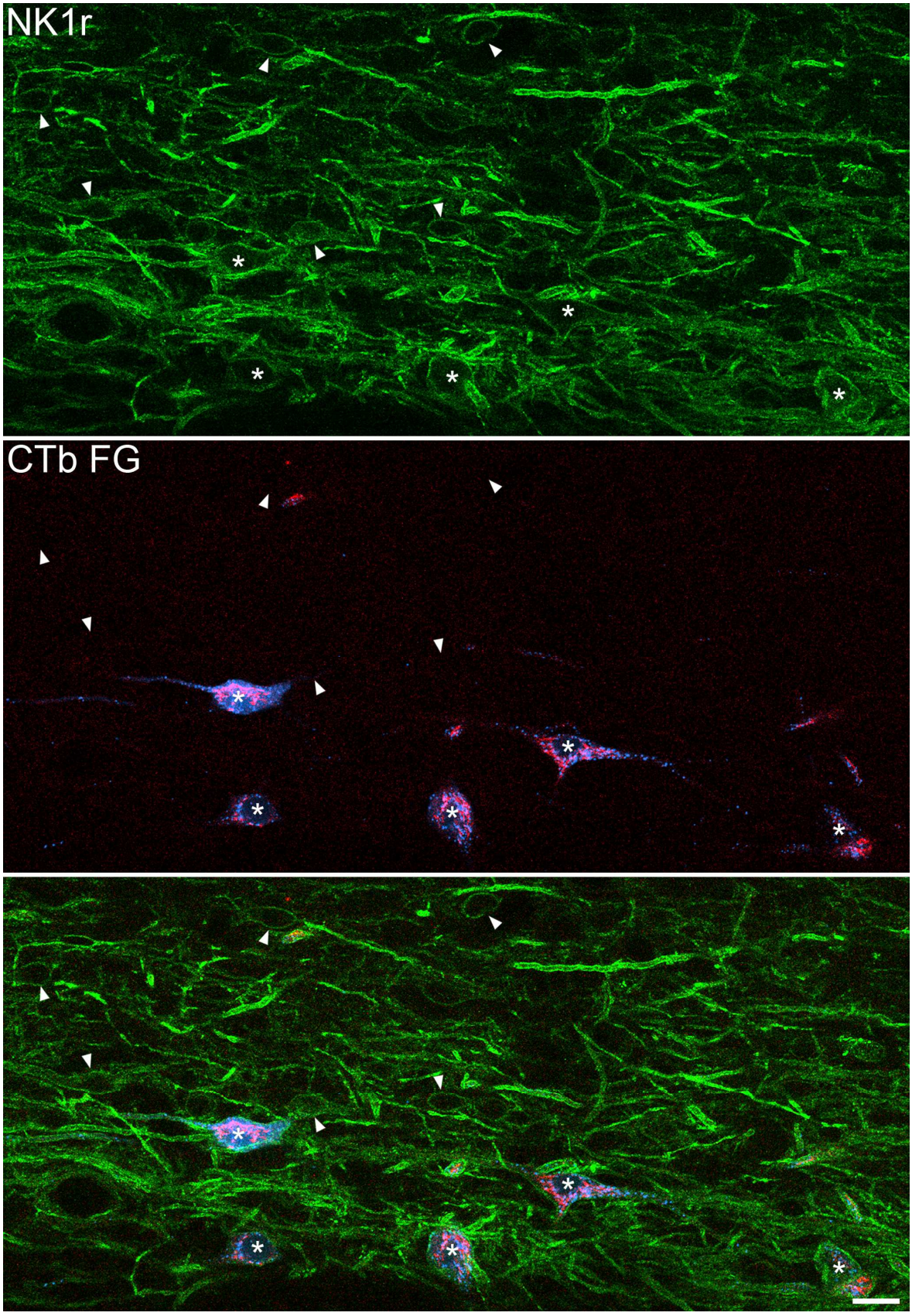

NK1r
CTb FG

Table 3-1 Quantitative retrograde labelling data

Experiment	Rertrogradely labelled	Double labelled	CVLM only	LPb only
1	206	182 (88%)	13 (6%)	11 (5%)
2	180	150 (83%)	10 (6%)	20 (11%)
3	156	130 (83%)	8 (5%)	18 (12%)
Total	**542**	**462 (85%)**	**31 (6%)**	**49 (9%)**

The table shows the total number of retrogradely labelled neurons analysed in each experiment, as well as the proportions (and percentages) that were double-labelled or labelled only with CTb or Fluorogold.

Table 3-2 NK1r-immunoreactivity in projection neurons

	NK1r-immunoreactivity score					
	0	1	2	3	4	**Total**
Experiment 1	29 (14%)	56 (27%)	56 (27%)	39 (19%)	26 (13%)	**206**
Experiment 2	34 (19%)	46 (26%)	35 (19%)	29 (16%)	36 (20%)	**180**
Experiment 3	38 (24%)	53 (34%)	18 (12%)	24 (15%)	23 (15%)	**156**
Total	**101 (19%)**	**155 (29%)**	**109 (20%)**	**92 (17%)**	**85 (16%)**	**542**

The table shows proportions (and percentages) of retrogradely labelled cells that were defined as having strong (4), medium (3), weak (2) or very weak (1) NK1r-immunoreactivity, or as being non-immunoreactive (0).

Figure 3-4 Examples of retrogradely labelled neurons.

A horizontal section from L4 in experiment 1. (a) A projected image through the cell bodies of several retrogradely labelled cells, with CTb shown in red and Fluorogold in blue. Most cells have taken up both tracers and therefore appear pink, while the cell numbered 1 is labelled only with Fluorogold, and the one marked with an asterisk is labelled only with CTb. The three numbered cells are pyramidal in shape and single optical sections through the cell bodies of each of these are shown in (b–g).

From these images it can be seen that cells 1 and 2 are NK1r-immunoreactive, while cell 3 is not. The image in (a) is a projection of eight optical sections at 2 μm z-spacing. Scale bar=20 μm.

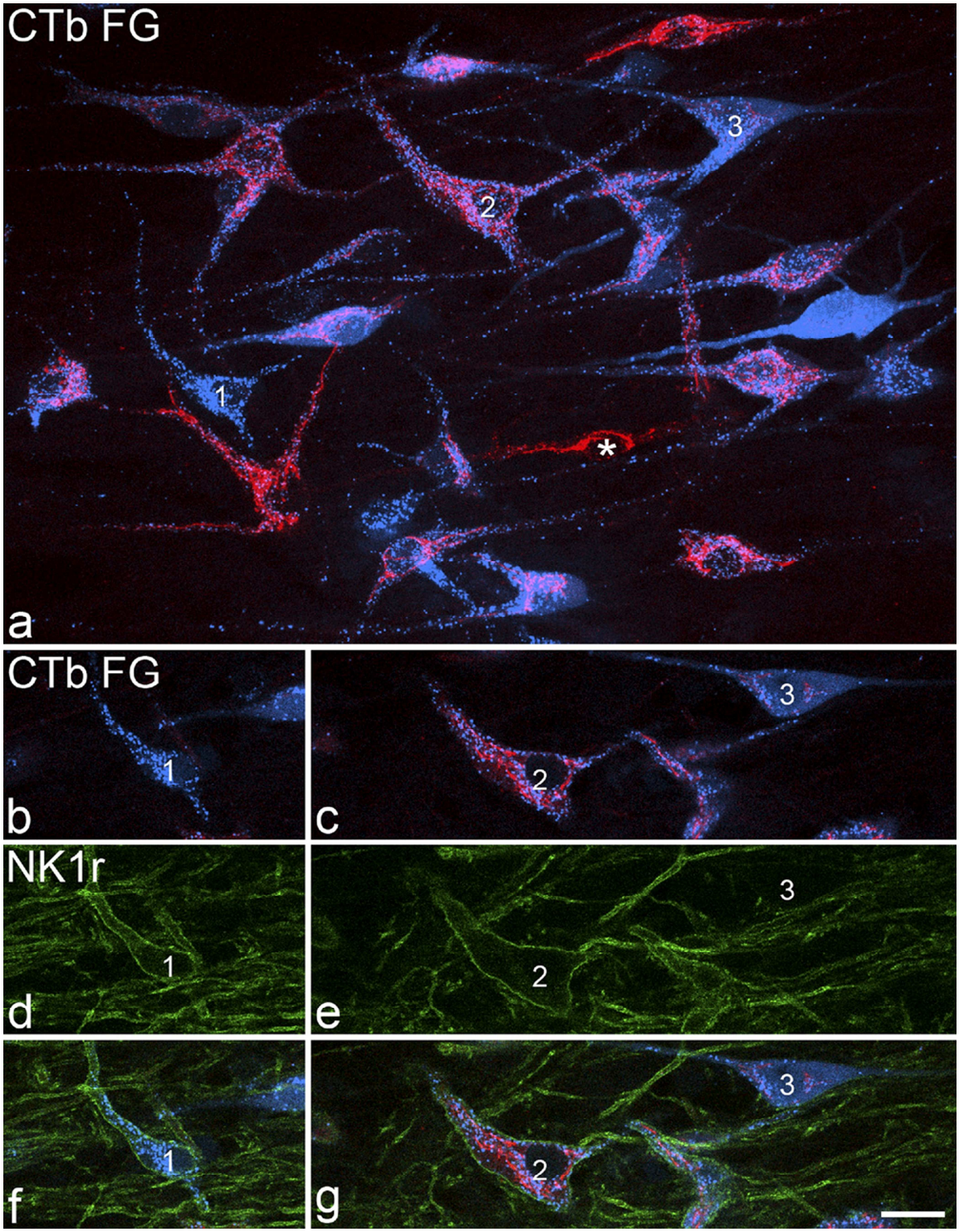

CTb FG
3
2
1
*
a
CTb FG
1
b
3
2
c
NK1r
1
d
3
2
e
1
f
3
2
g

Figure 3-5 Frequency histogram showing the soma cross-sectional areas for different groups of lamina I neurons.

The histogram shows all of the NK1r-immunoreactive cells (All $NK1r^+$, *n*=1341), those that were NK1r-immunoreactive but not retrogradely labelled ($NK1r^+$ non-retrograde, *n*=900), those that were NK1r-immunoreactive and retrogradely labelled ($NK1r^+$ retrograde, *n*=441) and those that were retrogradely labelled and not NK1r-immunoreactive ($NK1r^-$ retrograde, *n*=101).

In each case, the y-axis represents percentage. The dashed line corresponds to a cross-sectional area of 200 μm^2. The NK1r-immunoreactive neurons show a clear bimodal distribution, with the first and second peaks corresponding to the non-retrogradely labelled and the retrogradely labelled populations, respectively.

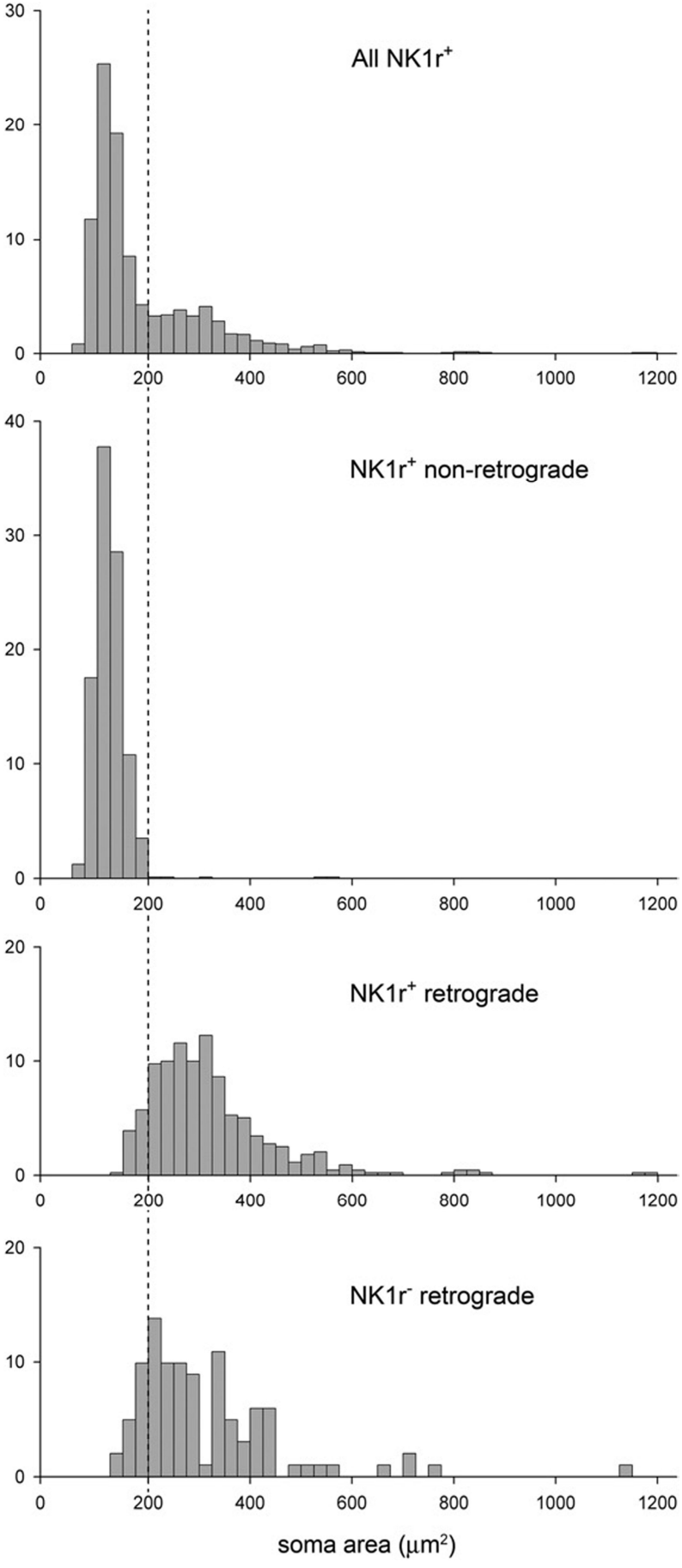

All NK1r+
NK1r+ non-retrograde
NK1r+ retrograde
NK1r- retrograde
soma area (μm2)

Table 3-3 Proportions of lamina I NK1r$^+$ interneurons and NK1r$^+$ projection neurons in regard to their soma sizes

All NK1r$^+$ cells	Number of cells with somata <200 μm^2	Number of cells with somata >200 μm^2	Total
NK1r$^+$ non-retrograde	895	5	900
NK1r$^+$ retrograde	43	398	441
Total	938	403	1341

The table shows the total number of NK1r-expressing neurons (from the three experiments) that were either retrogradely labelled or not, in regard to their soma sizes.

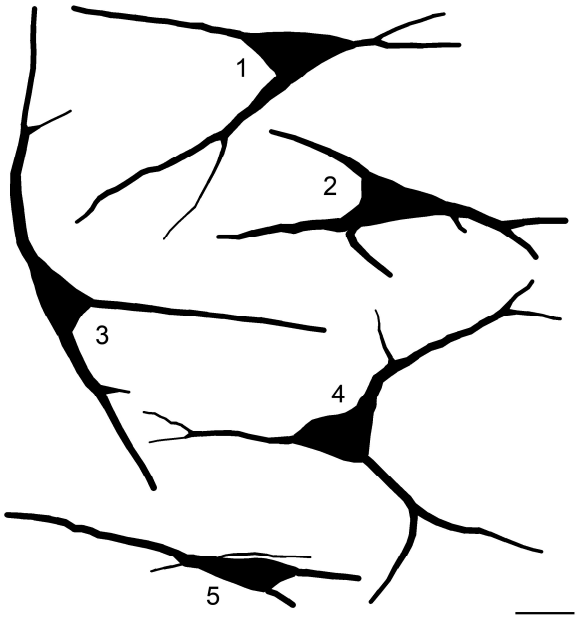


Figure 3-6 Drawings of NK1r-negative retrogradely labelled pyramidal cells.

Drawings showing five of the 15 retrogradely labelled pyramidal cells that were not NK1r-immunoreactive. Cell 1 corresponds to cell 3 in Figure 3-4. Three of these cells (1, 3 and 4) each give rise to three primary dendrites. Cell 2 has two primary dendrites originating from one pole of the soma, while cell 5 has an additional very fine dendrite originating from the soma. Scale bar=20 μm.

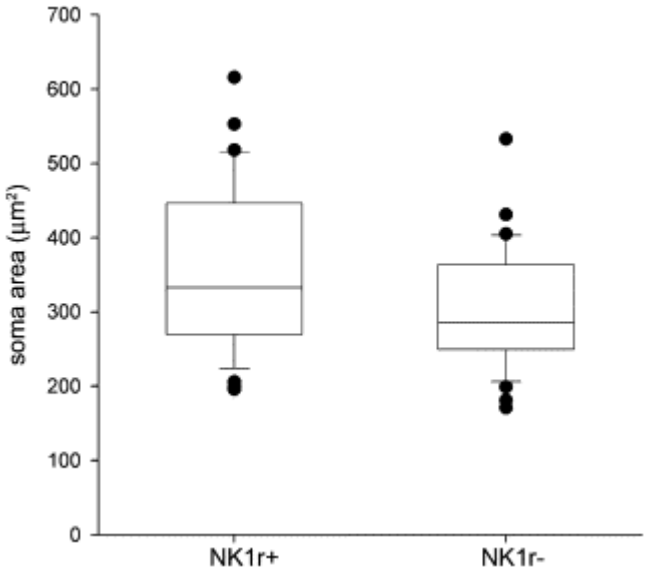


Figure 3-7 Box and whisker plot of the soma sizes of retrogradely labelled pyramidal cells.

The plot shows the soma sizes of retrogradely labelled pyramidal cells that were NK1r-immunoreactive ($NK1r^+$, *n*=37) or non-immunoreactive ($NK1r^-$, *n*=33). The boxes represent the median and interquartile range, while the upper and lower error bars show the 90th and 10th percentiles and the filled symbols are values outside these ranges.

3.4 Discussion

The main finding of this part of the study is that NK1r-expressing projection neurons can be distinguished from NK1r-expressing interneurons, in lamina I, based on their cell body sizes. The great majority of NK1r-expressing cells that were not retrogradely labelled from CVLM and/or LPb have soma cross-sectional areas of less than 200 μm^2, while most of the retrogradely labelled cells are larger than this.

3.4.1 Choice of the retrograde tracers and injection targets

In this part of the study, a retrograde labelling technique was used in order to identify lamina I projection neurons. It was reported that this technique allows both quantitative and qualitative assessments of connections between various CNS areas (Vercelli *et al.*, 2000). A combination of two retrograde tracers (CTb and Fluorogold), which were injected into two different supraspinal targets on one side, CVLM and LPb, enabled targeting almost all lamina I projection neurons on the contralateral side, for reasons that will be explained shortly.

It has been shown from previous studies that both CTb and Fluorogold are sensitive markers, as evidenced by their ability to detect large numbers of retrogradely labelled cells, as well as their extensive filling of labelled neurons (Spike *et al.*, 2003; Al-Khater and Todd, 2009; Polgar *et al.*, 2010). They are also equally efficient, since Spike *et al.* (2003) reported that the injection of either Fluorogold or CTb into the LPb resulted in labelling almost the same number of cells. It was observed that Fluorogold may cause necrosis of the tissues to which it is injected, this is why it is preferable, that if used with another marker, for it to be injected to the more rostral target, which is the LPb in this case (Akintunde and Buxton, 1992; Bice and Beal, 1997b).

It has been concluded from previous studies in our laboratory that the injection of tracers into both CVLM and LPb labels almost all lamina I projection neurons. Ikeda *et al.* (2006) suggested that lamina I spino-PAG neurons represent a distinct population, since they showed a functional difference compared to lamina I neurons that projected to the LPb. Their study provided evidence for a difference in the type of long-term potentiation between lamina I neurons that

were retrogradely labelled from the PAG and those that were labelled from the LPb. However, Spike *et al*. (2003) have shown that nearly all of the cells that projected to the contralateral PAG can also be labelled from contralateral LPb. This suggests that although spino-PAG neurons may represent a distinct functional population, they still can be labelled from the LPb. It was also reported that axons from the SDH that travel to the PAG pass through the LPb (Bernard *et al*., 1995; Feil and Herbert, 1995). Therefore, the tracers used in both studies (Spike *et al*., 2003; Ikeda *et al*., 2006) would have been taken not only by axon terminals, but also by fibres of passage. Regarding the thalamus as a potential distinct projection target of lamina I neurons, Al-Khater *et al*. (2008) reported that lamina I spinothalamic tract neurons make up only ~3–5% of the total number of projection cells in the mid-lumbar segments of the rat. Almost all of these cells send axon collaterals to the LPb (Al-Khater and Todd, 2009). It was also found that lamina I neurons can be labelled following injection of tracer into the dorsal part of the caudal medulla, which is an area that includes the DRt and NTS (Menetrey and Basbaum, 1987; Lima, 1990; Todd *et al*., 2000). However, it was recently shown that cells labelled from the dorsal part of the caudal medulla are also retrogradely labelled from the LPb (Polgar *et al*., 2010).

Regarding the percentages of labelled cells, the present study showed that between 83 and 88% of the retrogradely labelled lamina I neurons contained Fluorogold and CTb, indicating that they had been labelled from both LPb and CVLM respectively. However, Spike *et al*. (2003), who used similar injection and immunostaining protocols, reported a lower percentage of these labelled cells. Their study found that 63–78% of labelled cells contained both tracers. This difference is mainly due to an increase in the number of cells labelled from the LPb (94–95% of all retrogradely labelled cells in the present study, compared to 81–91% in the study of Spike *et al*. (2003). This may be attributed to a technical difference between those two studies, since the method of detecting Fluorogold-positive cells in the present study seems to be more sensitive. It involved using a highly sensitive gallium arsenide phosphide photomultiplier tube to detect Fluorogold, which was conjugated to Cy5.

One issue in this part of the study is that some of the retrograde labelling with CTb that was seen resulted from uptake of the tracer by fibres passing through the CVLM before reaching their targets (Spike *et al*., 2003). This might be

attributed to the fact that the CTb injections extended into the ventrolateral white matter, which contains many axons that ascend from the spinal cord (Zemlan *et al.*, 1978). However, this did not affect the overall outcome of the study, since the purpose of it was to label all projection neurons regardless of their supraspinal projection targets.

3.4.2 NK1r-mmunoreactive neurons

The present findings strongly support what was hypothesized by Cheunsuang and Morris (2000) that lamina I NK1r-expressing neurons are divided into two populations based on their soma sizes. The present study has identified a population of large NK1r-immunoreactive neurons that are considered projection neurons, since virtually all (398/403, 99%) of those with soma areas >200 μm^2 were retrogradely labelled. In contrast, the size distribution for NK1r-immunoreactive cells that were not retrogradely labelled indicates that these correspond to the population of small neurons. Neurons that were located in the most lateral part of lamina I were not analysed, since this part curves around the lateral margin of the dorsal horn leading to a different orientation of the neurons in this region, which makes it difficult to compare their soma sizes. However, it was observed that retrogradely labelled lamina I neurons in this region have a similar appearance to those elsewhere in the lamina. It is therefore unlikely that the lack of data from the lateral part of lamina I affects the validity of this conclusion.

Lamina I contains a very large number of the small NK1r-immunoreactive neurons, and it was not possible therefore to include all of these in the sample that was used for analysis. These cells also had weak immunoreactivity, which would have made it very time-consuming to identify them all. However, it is most probably that the present study did not underestimate the extent of retrograde labelling within this group, since in all cases a search for retrogradely labelled cells was carried out after the sample of NK1r-immunoreactive neurons had been collected. Another possible explanation for the absence of retrograde labelling in the small cells may be the lack of time for transport of the tracers, since the animals were perfused three days after the tracer injection surgery. However, it was found (in our laboratory) that increasing the post-operative survival time does not result in any increase in the number of labelled cells or in

the appearance of smaller labelled cells (Todd and Polgar, unpublished observations). In addition, this absence of labelling is unlikely to be due to a tracer sensitivity issue, since there was a clear distinction between cells that were positive and negative for CTb and Fluorogold. For the reasons stated above, it is unlikely that the present study underestimated the extent of retrograde labelling in the small NK1r-immunoreactive neurons. Although Cheunsuang and Morris (2000) speculated that the small NK1r-immunoreactive cells might be projection cells, it is very unlikely. However, the possibility that they project to some other brain region, other than the CVLM, LPb, PAG, thalamus, DRt or NTS can not be ruled out, but it is more likely that these cells are intrinsic spinal interneurons.

Of the 5 large NK1r-immunoreactive cells that were not retrogradely labelled, 3 had cell bodies >300 μm^2, and clearly these do not belong to the population of small neurons. Although it is possible that these cells are interneurons, it seems more likely that they are projection cells that were not retrogradely labelled from either of the injection sites used in these studies. The very low number of large (>200 μm^2) NK1r-immunoreactive cells that were not retrogradely labelled (5/403, 1%) provides additional support for the suggestion that the two injections used in this study labelled the great majority of lamina I projection cells on the contralateral side.

Although the small NK1r-immunoreactive cells were not quantified systematically, these appeared to be much more numerous than the large ones. This presumably accounts for the fact that ~45% of lamina I cells are NK1r-immunoreactive (Todd *et al.*, 1998) and that projection neurons (~80% of which express the NK1r) are thought to make up less than 10% of the total neuronal population in this lamina (Bice and Beal, 1997a, 1997b; Spike *et al.*, 2003; Al-Khater *et al.*, 2008). Although these small cells outnumber the larger projection neurons, more research has been done on projection neurons, and even the studies that investigated lamina I interneurons did not distinguish the NK1r-positive from the NK1r-negative smaller cells.

Little is known about the function of the small NK1r-expressing cells in lamina I. However, since NK1r-immunoreactive cells in this lamina are not GABA-immunoreactive (Littlewood *et al.*, 1995), it is likely that they are glutamatergic

excitatory interneurons. In the next part of this project, it will be shown that some of these cells phosphorylate ERK following noxious stimulation. Since they only express low levels of the NK1 receptor, it is likely that these cells would not have been destroyed by intrathecal administration of SP-SAP, and this presumably explains why there is no detectable reduction in the number of neurons in lamina I following this treatment (Nichols *et al.*, 1999). Additionally, A recent study by Choi *et al.* (2012) reported that after the pretreatment of the rats with SP-SAP, ~30% of the NK1r immunoreactivity, determined by pixel count, remained in the SDH, which may be attributed to the retained small NK1r-expressing interneurons.

An important practical outcome from the above results is that soma size can be used to identify putative lamina I NK1r-expressing projection neurons in studies that have not used retrograde tracing. Although NK1r-immunoreactive cells that have soma areas between 150 and 200 μm^2 cannot be classified with certainty, those with areas above 200 μm^2 are very likely to be projection cells, while those below 150 μm^2 are probably interneurons. A similar approach (without the need of retrograde labelling) can be used to identify the large laminae III-IV NK1r-immunoreactive cells as projection neurons, since virtually all of these were shown to be labelled from the CVLM, with some projecting to LPb and/or PoT (Todd *et al.*, 2000; Al-Khater and Todd, 2009). This approach will be used in the next Chapter as a means of distinguishing responses of lamina I projection neurons from those of interneurons to various types of noxious stimulation.

3.4.3 Projection neurons that lack the NK1r

The present study shows that of the 542 retrogradely labelled cells from the CVLM and/or LPb, 101 lacked the expression of the NK1 receptor. Although some of these cells will correspond to lamina I giant cells that were described previously, there are only ~10 cells of this type on each side in the L4 segment in the rat, and they therefore constitute only ~3% of lamina I projection neurons (Polgar *et al.*, 2008).

Little is known about the morphology and function of the remaining lamina I NK1r-negative projection neurons. Although Almarestani *et al.* (2007) have reported that ~90% of these neurons that projected to the LPb were of the

pyramidal class, with some having a 4th dorsally-directed dendrite, the present study shows that this number may be over-representative. Out of the 88 lamina I NK1r-negative projection neurons that could be classified morphologically in the present study (13 cells had incomplete filling of their dendrites) only 15 (17%) were classified as pyramidal. The present study also shows that from the 15 identified pyramidal cells only one had a 4th dendrite that travelled dorsally towards the white matter. Almarestani *et al*. (2009) provided evidence that these pyramidal cells can display novel expression of the NK1r (and also an increase in innervation by substance P-containing primary afferents) following a chronic inflammatory stimulus. However, there is also a population of large pyramidal NK1r-immunoreactive neurons (Cheunsuang and Morris, 2000) that are included in the spinoparabrachial population (Spike *et al.*, 2003; Almarestani *et al.*, 2007; Al-Khater and Todd, 2009; Almarestani *et al.*, 2009). It may therefore be difficult to distinguish pyramidal cells that are normally NK1r-negative and up-regulate the receptor after inflammation from those that constitutively express the receptor. Almarestani *et al*. (2009) reported that the pyramidal cells that normally lack NK1r are smaller than the NK1r-immunoreactive type, and typically have a fourth dendrite that emerges from the dorsal aspect of the soma and travels towards the overlying white matter. However, the present study shows that there is a considerable overlap in the distribution of soma sizes of NK1r-immunoreactive and non-immunoreactive pyramidal neurons, and that the difference between the two populations is not significant. In addition, the fourth, dorsally-directed dendrite described by Almarestani *et al*. (2009) is only observed on one of the 15 non-NK1r-immunoreactive pyramidal neurons.

Further experiments are needed to identify the function of the remaining projection neurons that lacked the NK1 receptor in lamina I.

4. ERK phosphorylation in NK1r-expressing dorsal horn neurons and lamina I giant cells after noxious stimulation

4.1 Introduction

Superficial dorsal horn neurons respond specifically or preferentially to various forms of noxious stimuli. These neurons may receive a direct input from primary afferents (monosynaptic), and/or are indirectly activated through excitatory interneurons (polysynaptic). Lamina I is a major termination site for Aδ- and C-fibre primary afferents. Neurons in this lamina receive input from cutaneous, visceral, muscle and joint primary afferents (Cervero and Connell, 1984; Mense and Prabhakar, 1986). The primary afferent input to lamina II is mainly from unmyelinated fibres innervating cutaneous tissues as well as from Aδ hair follicle afferents (Light and Perl, 1979b; Shortland *et al.*, 1989). It was also revealed recently that many large afferent fibre terminals enter the inner part of lamina II (Woodbury *et al.*, 2000). Much of the input to laminae III-IV is from large primary afferents that enter the cord and curve into the medial part of the dorsal horn (Hamano *et al.*, 1978). Cells in lamina IV have been also reported to receive visceral (Pomeranz *et al.*, 1968) and joint (Schaible *et al.*, 1986) inputs. In addition, these cells commonly have an input from cutaneous and muscle receptors (Schaible *et al.*, 1987).

Christensen and Perl (1970) were the first to record the responses of lamina I neurons to noxious stimulation in the cat. The study divided lamina I neurons into 3 groups according to their excitatory input. Group I, which responded only to intense mechanical stimulation of high threshold myelinated primary afferents. The 2nd group, which received afferent input from both Aδ- and C-fibres, was activated by both intense mechanical and noxious heat stimulation. The 3rd group responded to the above 2 stimuli as well as to innocuous temperature changes. Similar findings were seen in the monkey dorsal horn (Kumazawa and Perl, 1978). It was also reported that in the rat, the marginal zone (lamina I) contained cells that were only activated by noxious pinching or pin-pricking stimuli (Menetrey *et al.*, 1977). Light and Willcockson (1999) recorded *in vivo* from a sample of 73 neurons in laminae I-II of the lumbar spinal cord of the rat. Of these, 39 were classified as nociceptive, responding to heat and/or mechanical noxious stimuli, and these were located mainly in laminae I and IIo. Most cells in lamina II, in both primates and cat, were activated by hair movement (single brush stimulus) or innocuous mechanical stimulation, but some

cells required more intense stimulation (Kumazawa and Perl, 1978; Wall *et al.*, 1979; Cervero and Iggo, 1980). Laminae III-IV was found to contain narrow- and wide-dynamic-range cells in primates and cats. Neurons in these laminae responded to both cutaneous stimulation and joint movements (Wall, 1967). Some of these neurons responded only to noxious stimuli (Kolmodin and Skoglund, 1960), while others were activated just by mechanical stimuli such as hair movement, touch, pressure and pinch (Wall, 1967; Brown and Franz, 1969).

Receptive fields of SDH neurons usually vary in shape and size, and can also be modifiable. Excitatory neurons' receptive fields are often ovoid and larger than the receptive fields of primary afferents. The central part of these receptive fields is usually the most sensitive, and the application of a strong stimulus to the skin adjacent to an excitatory receptive field could produce inhibition (Wall, 1960). Receptive fields often vary from 1-1000 mm^2 depending on the body part encoded. The smallest excitatory receptive field for lamina I neurons can be as small as that of a C-fibre nociceptor (<1 mm^2). In contrast, lamina I neurons with A-fibre nociceptive inputs have more uniform, continuous receptive fields rather than spot-like ones, which indicate considerable convergence from primary afferent onto the lamina I neurons (Light and Lee, 2009). Further, noxious mechanical stimulation of the region that surrounded an excitatory receptive field caused inhibitory postsynaptic potentials in the recorded neuron, and this inhibitory field faded out gradually as the distance from the excitatory field increased (Light and Lee, 2009). Wall (1967) initially described a somatotopic organisation of the dorsal horn, with the toes being represented medially, while the lateral aspect of the foot and more proximal regions are represented laterally in the dorsal horn of the cat. It was also found that the receptive fields of some dorsal horn neurons are changeable in primates, cats and rats. For instance, it was reported that noxious stimulation caused an expansion of the receptive fields of dorsal horn neurons, mostly of the wide-dynamic-range class, to both innocuous and noxious mechanical stimulation as well as to noxious heat (Kenshalo *et al.*, 1979, 1982; Laird and Cervero, 1989; Simone *et al.*, 1989; Simone *et al.*, 1991).

The responses of dorsal horn projection neurons, which were identified by antidromic activation from contralateral supraspinal targets, to various noxious stimuli have been investigated. Many studies were done on STT neurons in the

cat and monkey. It has been shown that lamina I STT cells play an important role in transmitting both nociceptive and thermoreceptive information to the thalamus (Willis *et al.*, 1974). It has also been reported that the receptive fields of STT cells in lamina I are generally smaller than those of STT cells in deeper laminae (IV-IV) (Apple Baum *et al.*, 1975). Andrew and Craig (2002) studied the responses of lamina I spinothalamic neurons in the cat to various stimuli. Of the 125 lamina I spinothalamic neurons recorded, 33 were classified as nociceptive specific, 43 were considered polymodal-nociceptive, since they responded to heat, pressure and cold and the remaining 49 neurons were classified as thermoreceptive specific, responding to cooling and innocuous warming. As described previously, there are not many lamina I STT neurons in the rat, compared their number in the cat and monkey (Al-Khater *et al.*, 2008). Zhang *et al*. (2006) investigated the responses of 33 lamina I STT neurons in the rat. Of these, 30 neurons were activated by noxious, but not innocuous, mechanical stimuli applied to their cutaneous receptive fields. Half of the 17 thermally tested STT cells were activated by both innocuous cooling as well as noxious heat and cold, while the other half were nociceptive-specific. The study also reported a difference in the thalamic terminations of axons of cells that responded to innocuous cooling, compared to cells that responded to noxious heat and cold.

The parabrachial area, which surrounds the superior cerebellar peduncle at the ponto-mesencephalic junction, has been reported to be one of the major targets of lamina I dorsal horn neurons in primates, cats and rats (Hylden *et al.*, 1989; Craig, 1995; Spike *et al.*, 2003). Bester *et al*. (2000) recorded from 53 lamina I spinoparabrachial neurons in anesthetized rats. Most of these neurons (75%) were nociceptive-specific, while the remainder responded to both noxious and innocuous thermal and mechanical stimulation. The majority of these neurons (92%) responded to two modalities of noxious stimuli (mechanical and thermal), and about a third of the neurons in this group also responded to noxious cold stimuli. Andrew (2009) also studied the responses of lamina I spinoparabrachial neurons of the rat to different stimuli. Of the 40 neurons studied, 28 were nociceptive specific, 6 were polymodal-nociceptive, 4 were wide-dynamic-range neurons and 2 were thermoreceptive-specific.

In addition to their responses to noxious and thermal stimuli, Andrew and Craig (2001) found that a class of lamina I spinothalamic neurons in the cat responded to pruritic stimulation, since they were selectively excited by iontophoretic histamine; an itch producing agent. However, recent studies in primates recorded from a group of spinothalamic neurons and reported that these were polymodal with respect to their excitation by pruritic and painful stimuli (Simone *et al.*, 2004; Davidson *et al.*, 2007).

The function of substance P has been studied at both cellular and systemic levels. Henry *et al.* (1975) reported that substance P, when applied by iontophoresis into the vicinity of dorsal horn neurons, led to a prolonged excitation (lasting for several minutes) of almost half of the neurons tested in cats. Subsequently, Salter and Henry (1991) found that iontophoretic application of substance P in the cat activated most of nociceptive dorsal horn neurons, but not non-nociceptive ones. Substance P also led to a slow depolarisation of dorsal horn neurons, as shown by intracellular recordings in spinal cord slice preparation in the rat (Murase *et al.*, 1982). The mechanism of action of substance P has been investigated by voltage clamp experiments on dorsal horn neurons in slice preparations. Substance P's action was attributed to its augmentation of an inward Ca^{2+} current and its inhibition of the M-current, which is a voltage-sensitive K^{+} current, causing depolarisation accompanied by an increase in the membrane resistance (Murase *et al.*, 1986). Substance P has also been reported to potentiate the action of glutamate on its NMDA receptor (Dougherty and Willis, 1991). This long lasting effect of substance P has been shown to play an important role in the generation of hyperalgesia and allodynia (Woolf, 1983; Dougherty *et al.*, 1995).

Intrathecal injection of substance P has been reported to induce nociceptive behaviours that were prevented by NK1 receptor antagonists. Substance P increased the gain of nociceptive flexion withdrawal reflexes (Ma and Woolf, 1995), and decreased the latency of paw withdrawal following noxious heat (Malmberg and Yaksh, 1992). It also led to biting and scratching behaviours, which were similar to those induced by the peripheral injection of noxious chemicals (Wilcox, 1988; Bjorkman *et al.*, 1994). NK1 receptor antagonists also prevented behavioural responses that were associated with noxious visceral stimulation and those related to the 2^{nd} phase of formalin injection (Garret *et*

al., 1991; Seguin *et al.*, 1995). This prevention of nociception-related behaviours was successful only if the NK1 receptor antagonist was given either before or during the process of stimulation. It is therefore assumed that substance P-induced activation of the NK1 receptor contributes mainly to the generation, but not the maintenance, of hyperalgesia.

A number of studies focused on the role of NK1r-expressing neurons in nociception. It was reported that following the injection of formalin into the rat hindpaw, the expression of the NK1 receptor gene was increased in the SDH, and this increase was blocked following the administration of a specific NK1 receptor antagonist (McCarson and Krause, 1996). It has been also shown that natural and electrical noxious stimulation, as well as the administration of substance P or NMDA led to internalisation of the NK1 receptor. This internalisation was stimulus intensity-dependent, occurred within 5-10 minutes, and the receptor returned to the cell membrane within 1 hour (Mantyh *et al.*, 1995; Abbadie *et al.*, 1997). This NK1 receptor internalisation can be prevented by NK1 receptor antagonists as well as by NMDA antagonists, but not by AMPA or kainite antagonists (Liu *et al.*, 1997; Marvizon *et al.*, 1997). This therefore suggests a role of NMDA in controlling internalisation of the NK1 receptor, which is initiated by substance P. Afrah *et al.* (2001) also reported that capsaicin-induced substance P release (capsaicin in the perfusion fluid for 30 min, a microdialysis study) into the dorsal horn of the rat spinal cord was partially dependent on an action of NMDA receptors. Their study suggest that capsaicin may activate the release of glutamate from primary afferents, which in turn acts on presynaptic NMDA receptors located on substance P-containing primary afferents leading to the release of substance P. Although it was reported that $GABA_B$ agonists reduced the release of substance P from primary afferents, these failed to reduce NMDA-induced NK1 receptor internalisation (Marvizon *et al.*, 1999). Trafton *et al.* (1999) reported that morphine alone produced a modest decrease in noxious-pinch induced internalisation of the NK1 receptor in the rat, but when combined with an NK1 antagonist, internalisation was almost prevented. Their study suggests that opioid analgesia may involve controlling non-substance P primary afferents. However, Kondo *et al.* (2005) showed that the administration of spinal μ- and δ-opioid agonists prevented afferent-evoked NK1 internalisation both *in vivo* and *ex vivo*. More recently Beaudry *et al.* (2011) reported that

capsaicin- and formalin-induced NK1 receptor internalisation, which can be used as an indication of substance P release, as well as fos up-regulation were inhibited by selective spinal activation of the μ- and δ-opioid receptors in the mice SDH.

The involvement of NK1r-expressing neurons in nociception has been also studied by using markers of neuronal activity, such as fos and pERK. For instance, it was reported that the number of chemical- or electrical-induced dorsal horn neurons that up-regulated fos was significantly reduced after the pretreatment of rats with selective NK1r antagonists (Chapman *et al.*, 1996; Tao *et al.*, 1997; Redburn and Leah, 1999). Similarly, De Felipe *et al.* (1998) reported that in NK1 receptor-knock-out mice, which were injected with formalin, the number of fos-positive neurons in the SDH was reduced by 30% compared to the wild type. Lu *et al.* (1995) also found that 2 hours after the injection of formalin into the urinary bladder of the rat, fos was up-regulated in NK1r-expressing neurons, which were found mainly in the medial aspects of lamina I and in the dorsal commissural nucleus and sacral parasympathetic nucleus of the lumbosacral cord. This suggests that these neurons receive nociceptive input from the urinary bladder through substance P-containing afferents. Subsequently, Seki *et al.* (2005) reported that instillation of capsaicin into the urinary bladder of the rat up-regulated fos in the SDH of the lumbosacral cord. The study also reported that this capsaicin-induced fos expression was reduced after the pretreatment of the rats with SP-SAP, indicating an important role of the NK1r-expressing neurons in conveying nociceptive afferent information from the bladder to supraspinal relay centres.

In contrast to the several studies that showed fos up-regulation in NK1r-expressing neurons after noxious stimulation, little is known about the types of neurons that contained pERK. For instance, Ji *et al.* (2002) reported that 24 hours after the injection of CFA into the rat hindpaw, most neurons in lamina I that contained prodynorphin or expressed the NK1 receptor were pERK-immunoreactive. Ji *et al.* (2002) suggested that the contribution of pERK in pain hypersensitivity may be attributed to activation of the MAPK/ERK pathway, which then regulates the expression of the NK1 receptor and prodynorphin, as well as other target genes. It was also reported that pERK expression in the SDH

of adult rat spinal slice preparations was increased by 8 to 10 fold after C-fibres activation, and that this expression was decreased by blocking the NK1 receptor (Kawasaki *et al.*, 2004). Choi *et al.* (2005) also found that intrathecal injection of substance P led to pERK expression in laminae I and II of the spinal cord of the mice. This expression was attenuated after the pretreatment with a MEK1/2 inhibitor in a dose dependent manner, suggesting that ERK plays a role in substance P-induced nociception. Moreover, and in agreement with studies that reported fos up-regulation after the stimulation of the urinary bladder, Yoo and Hwang (2007) reported that pERK was expressed in lamina I neurons of the rat after both mechanical (distension) and chemical (mustard oil) noxious stimulation of the bladder. Their study also showed that the majority of pERK-positive cells were NK1r-immunoreactive, and received contacts from TRPV1-positive primary afferents, suggesting that these fibres may mediate ERK phosphorylation in the SDH.

It was described above that some of lamina I giant cells up-regulated fos after either formalin injection into the rat hindpaw (Puskar *et al.*, 2001), or exposing it to noxious heat (Polgar *et al.*, 2008). Laminae III-IV NK1r-expressing neurons also showed NK1 receptor internalisation as well as ERK phosphorylation after mechanical, thermal and chemical stimulation in the rat (Polgar *et al.*, 2007). Polgar *et al.* (2007) reported that almost all laminae III-IV NK1r-expressing neurons contained pERK 5 minutes after repeated pinching of the skin of the hindpaw, immersion of the hindpaw in hot water or subcutaneous injection of formalin into the hindpaw. The majority of these cells also showed internalisation of the NK1 receptor following noxious stimulation, particularly following thermal and chemical stimulation.

Work done in the previous Chapter showed that lamina I NK1r-expressing projection neurons can be distinguished from interneurons, as they were generally larger. In this part, the responses of lamina I NK1r-expressing presumed projection neurons and interneurons to cutaneous, deep and visceral noxious stimuli were investigated, using pERK as the marker of neuronal activity. Responses of lamina I giant cells to cutaneous and deep noxious stimuli were also studied. The responses of laminae III-IV NK1r-expressing neurons to deep and visceral noxious stimuli were investigated, since their responses to cutaneous stimulation have already been demonstrated.

4.2 Experimental procedures

The first part of the study (Chapter 3) showed that NK1r-expressing projection neurons in lamina I could be distinguished from interneurons based on their soma sizes. Specifically, it showed that 99% of the cells with soma cross-sectional areas >200 μm^2 are projection neurons, while the great majority (~95%) of those below this size are interneurons. Therefore, the investigation regarding responses of lamina I NK1r-expressing neurons to different noxious stimuli was carried out on rats that had not received injections of retrograde tracer, and soma size was used to identify NK1r-immunoreactive cells as putative projection neurons or interneurons.

4.2.1 Animals and noxious stimulation

Thirty adult male Wistar rats (250–280 g; Harlan, Loughborough, UK) were used for this part of the study. The rats were deeply anaesthetized with 10% urethane (1.3 g/kg ip) and the depth of anesthesia was sufficient that the animals did not respond to gentle touching of the cornea (negative corneal reflex). Body temperature was maintained at 38.0 ± 0.5°C with an electric blanket and was controlled by a body thermometer. They then received one of the following noxious stimuli:

(1) Pinching of folds of skin at 12 points on the left hindpaw (six each on the dorsal and ventral surfaces, applied with forceps for 5 s at each point; *n*=3).
(2) Injection of 25 μl of **1% capsaicin*** (Sigma-Aldrich, Poole, UK) into the plantar surface of the left hindpaw (*n*=3).
(3) Immersion of the left hindpaw in water at 52°C for 20s (*n*=3) or 45s (*n*=3).
(4) Injection of 20 μl of **10% mustard oil*** (95% Allyl isothiocyanate, Sigma-Aldrich, Poole, UK) into the left knee joint (*n*=6), followed by flexion and extension of the knee joint (3-5 times).
(5) Injection of 30 μl of 10% mustard oil to the left gastrocnemius muscle (*n*=6).

* **1% capsaicin** is made by adding 800 μl of solution A to solution B and mixing well,
Solution A: 700 μl Tween80 added to 9.3 ml saline and stirred on a magnetic stirrer
Solution B: 10 mg capsaicin dissolved in 200 μl ethanol
The dissolved solution is heated for 1hr at 50°C with the lid off to evaporate the alcohol, optional.

* **10% mustard oil** is made by dissolving 10.5 μl of 95% Allyl isothiocyanate in 89.5 μl paraffin oil.

(6) Distension of the rectum and colon (colorectal distension, CRD) with a 6-7 cm latex balloon at 80 mmHg for 20s (*n*=3), since CRD at 80 mmHg for 20s has been demonstrated behaviourally to be noxious (Ness *et al.*, 1991). The balloon was made by connecting the finger of a surgical glove with tygon tubing, using a tight ligature. The pressure was monitored and maintained with a handheld manometer attached to the balloon. Three additional rats were considered controls as a balloon was inserted into their colon but not inflated. The rats that underwent visceral stimulation were fasted with unlimited access to water for 24 hours prior to distension.

In each case, the animals were maintained under general anaesthesia for 5 minutes after the end of the stimulus, and were then perfused through the heart with 4% formaldehyde. The first three stimuli were considered cutaneous, while mustard oil into joint and muscle was a means of deep stimulation, and CRD was the visceral stimulus.

4.2.2 Tissue processing and immunocytochemistry

Thoracic, lumbar and sacral spinal segments (T13, L1-L6, S1-S2) were removed, post-fixed at 4°C overnight. Apart from the spinal segments from rats that were exposed to visceral stimulation, all other segments were notched on the right side, in order to distinguish it from the left (stimulated) side.

4.2.2.1 Lamina I NK1r-expressing neurons and giant cells

To investigate the responses of lamina I NK1r-expressing neurons and giant cells to various noxious stimuli, the appropriate spinal segments (Table 4-1) were cut into 60 μm thick horizontal sections with a Vibratome. They were then treated for 30 minutes in 50% ethanol and processed immunocytochemically. The sections were incubated in mouse monoclonal antibody (mAb) against gephyrin (mAb 7a, 1:100,000), which was detected with a TSA kit (PerkinElmer Life Sciences, Boston, MA), see below. The mouse mAb 7a was combined with rabbit anti-NK1r (1:10,000) followed by incubation in mouse monoclonal antibody against pERK (1:1000). Sections were incubated in species-specific secondary antibodies raised in donkey and conjugated to either Cy5 or Alexa 488.

The TSA method was performed by applying one primary antibody, which is the mAb 7a in this case, at a level that is too low to be detected with a fluorescent labelled secondary antibody, yet sufficient for detection with the tyramide system. This first primary antibody will then be effectively neglected during application of a second primary antibody that will be detected by conventional fluorescently labelled secondary antibodies (Shindler and Roth, 1996).

4.2.2.2 Laminae III-IV NK1r-expressing neurons

To investigate the responses of laminae III-IV NK1r-expressing neurons to various noxious stimuli, deep horizontal 60 μm thick sections as well as parasagittal 60 μm thick sections from the left side of the appropriate segments (Table 4-1) were treated with 50% ethanol for 30 minutes. The sections were then incubated for 72 hours in a mixture of primary antibodies: rabbit anti-NK1r (1:10,000) and mouse monoclonal antibody against pERK (1:1000). Sections were incubated for 24 hours in species-specific secondary antibodies conjugated to either Rhodamine Red or Alexa 488.

For reactions that did not involve TSA, antibodies were diluted in PBS containing 0.3 M NaCl and 0.3% Triton® X-100. For those involving TSA, antibodies were diluted in PBS that contained 0.15 M NaCl, together with a blocking serum, which was supplied by the manufacturer. Sections were mounted in anti-fade medium and stored at -20°C.The primary antibodies that were used in this part of the study, and their characterisations are described in Chapter 2.

4.2.3 Confocal microscopy and analysis

4.2.3.1 Lamina I NK1r-expressing projection neurons and interneurons

To investigate the responses of lamina I NK1r-expressing projection neurons and interneurons to noxious cutaneous, deep and visceral stimuli, horizontal sections from the ipsilateral side of the appropriate segments were analysed. Sections were initially scanned through the confocal microscope (Bio-Rad Radiance 2100) at 20× objective lens, and the regions of lamina I that contained numerous pERK-immunoreactive cells were identified.

These regions were then scanned through a 40× oil-immersion lens to produce overlapping fields of image stacks, with a z-separation of 2 μm. Confocal image stacks were were analysed with Neurolucida for Confocal software. A sample of NK1r-immunoreactive cells was selected, and the outlines of their cell bodies and proximal dendrites were drawn by examining all of the optical sections through each cell. For each of the selected cells, cross-sectional area of its cell body was measured and its morphology was assessed. When all of the selected cells in a field had been analysed, the files containing pERK-immunostaining were then viewed and the presence or absence of pERK in each of the selected cells was determined. Based on the results from Chapter 3, NK1r-immunoreactive lamina I neurons with soma areas >200 μm^2 were assumed to be projection cells, and those with somata <200 μm^2 were putative interneurons.

4.2.3.2 Lamina I giant cells

To investigate the responses of lamina I giant cells to noxious cutaneous and deep stimuli, horizontal sections from the ipsilateral side of the appropriate segments were analysed. Initially, the region of lamina I that contained numerous pERK-positive cells was determined using 20× objective lens, and then all of the giant cells that were present within this region were identified by the presence of numerous gephyrin puncta on their cell bodies and dendrites. These cells were then scanned through a 40× lens to reveal gephyrin, NK1r, and pERK immunoreactivity. They were also examined for their NK1 receptor expression. The presence or absence of pERK immunoreactivity in the selected cells was determined.

4.2.3.3 Laminae III-IV NK1r-expressing neurons

To investigate the responses of laminae III-IV NK1r-expressing neurons to noxious deep and visceral stimuli, parasagittal and deep horizontal sections from the ipsilateral side of the appropriate segments were analysed.

Parasagittal sections were initially scanned through a 10× lens to reveal pERK-immunoreactivity and the sections from each animal that showed maximal staining in the superficial laminae were selected for further analysis. At this magnification it was possible to identify the band of pERK-immunoreactivity in

the superficial dorsal horn, but individual pERK-positive neurons in deeper laminae were not well seen. This was not applicable for the deep horizontal sections from the rats that had received noxious visceral stimulation, since lamina I was absent in these sections. However, unlike joint and muscle stimulation, CRD produced diffuse, bilateral pERK immunoreactivity in lamina I. It is therefore unlikely to select deep horizontal sections from an area that had minimal pERK immuoreactivity in lamina I.

Selected sections were then examined through a 20× lens and all of the NK1r-immunoreactive neurons with cell bodies in laminae III or IV and dendrites that could be traced dorsally into superficial laminae (either in the same section or by following the dendrites through serial sections) were identified. Care was taken to avoid double-counting neurons with cell bodies that appeared on two adjacent sections. For all of the selected cells, the presence or absence of pERK staining in the soma and dendrites was recorded. In this way, the proportion of laminae III-IV NK1r-immunoreactive cells in each animal that showed pERK-immunostaining was determined.

Table 4-1 Spinal cord segments studied in regard to different types of noxious stimuli used

Type of the stimulus	Laminar distribution	Cells of interest	Plane of cutting	Spinal segments
Pinch, *n*=3	Lamina I	NK1r-expressing neurons + giant cells	Horizontal	L4-L5
Capsaicin, *n*=3	Lamina I	NK1r-expressing neurons + giant cells	Horizontal	L4-L5
Heat, *n*=6	Lamina I	NK1r-expressing neurons + giant cells	Horizontal	L4-L5
Mustard oil into joint, *n*=6	Lamina I	NK1r-expressing neurons + giant cells	Horizontal	L2
	Laminae III-IV	NK1r-expressing neurons	Parasagittal	L2
Mustard oil into muscle, *n*=6	Lamina I	NK1r-expressing neurons + giant cells	Horizontal	L5
	Laminae III-IV	NK1r-expressing neurons	Parasagittal	L5
Colorectal distension, *n*=3	Lamina I	NK1r-expressing neurons	Superficial horizontal	L6-S1
	Laminae III-IV	NK1r-expressing neurons	Deep horizontal	L6-S1

4.3 Results

4.3.1 pERK in lamina I NK1r-expressing neurons

After the application of cutaneous and deep noxious stimuli, numerous pERK-positive cells were seen in lamina I, in the medial part of the dorsal horn on the left side (ipsilateral to the stimulus) in the lumbar cord, while very few were present on the contralateral side. Following CRD, pERK-positive cells were seen in both sides of lamina I of the lumbosacral spinal cord. Table 4-1 shows the appropriate spinal segments that were analysed in regard to the stimuli applied.

Most of the NK1r-immunoreactive cells with soma cross-sectional areas >200 μm^2 were pERK-positive following the ***pinch*** stimulus. When data from the three rats were pooled, pERK was found in 68% (228 out of 335 cells) of the NK1r-positive cells >200 μm^2, and only 28% (109/393) of the cells that were smaller than 200 μm^2 were pERK-positive. In the three rats that had received the ***capsaicin*** injection, 81% (229/283) of NK1r-positive cells with cell bodies >200 μm^2, and 39% (135/344) of those with somata <200 μm^2 contained pERK. In rats that were stimulated with noxious ***heat*** (immersion of the hindpaw in 52°C for 20s), almost all (97%, 289/299) of the NK1r-immunoreactive cells >200 μm^2 were pERK-positive, as were 72% (236/327) of the cells <200 μm^2. After injection of mustard oil into the ***joints*** and ***muscles***, 77% (61/79) and 74% (75/101) of the NK1r-immunoreactive cells with soma cross-sectional areas >200 μm^2 were pERK-positive, respectively. However, only 34% (28/83) and 33% (30/82) of the smaller cells, <200 μm^2, were pERK-positive following deep noxious stimulation of the joints and muscles, respectively. Following ***CRD***, but not tubal insertion, pERK was found in 75% (80/106) of the large NK1r-positive cells (>200 μm^2) while only 16% (29/180) of the NK1r-immunoreactive cells <200 μm^2 phosphorylated ERK. Chi-square tests revealed that the expression of pERK differed significantly between cells with somata >200 μm^2 and cells smaller than 200 μm^2 following all 6 noxious stimuli (P<0.05). Figure 4-1 shows the percentages of $pERK^+$ NK1r-immunoreactive small and large neurons after the exposure to different types of noxious stimuli.

There was no significant difference in the frequency of pERK expression between the three morphological classes (fusiform, multipolar and pyramidal) of lamina I NK1r-presumed projection neurons after the exposure to different types of noxious stimuli (P>0.05, Chi-square tests, Table 4-2).

After each type of noxious stimulation, many of the NK1r-immunoreactive cells showed extensive internalisation of the receptor, which gave rise to numerous endosomes in their cells bodies and dendrites, and the great majority of these cells were pERK-positive. In some cases, NK1r-expressing neurons that did not appear to have significant internalisation showed pERK immunoreactivity, and most of these were presumed to be interneurons (soma size <200 μm^2). In rare occasions, NK1r-expressing cells that showed clear signs of internalisation did not appear to be pERK-immunoreactive. Examples of pERK and lamina I NK1r-immunostaining are shown in Figures 4-2 and 4-3.

4.3.2 pERK in lamina I giant cells

After application of cutaneous and deep noxious stimuli, a clear band of pERK-positive cells was observed in the medial part of lamina I on the ipsilateral sides of the appropriate segments (Table 4-1). Giant cells lateral to this band were also often pERK-immunoreactive, suggesting that they may have large receptive fields. For this reason, in addition to the fact that these cells are scarce, all giant cells identified were included in the analysis.

In the rats that had received the pinch stimulus, the percentages of giant cells that were pERK-positive varied from 80 to 92% (mean of 85%). After the injection of capsaicin, the mean percentage of giant cells that contained pERK was 84% (77-88%). Noxious heat (immersion of the hindpaw in 52°C for 45s) led to the phosphorylation of ERK in 74% of the giant cells (67-88%). After the injection of mustard oil into the knee joint, 44% of the giant cells were pERK-positive (33-60%) and only 13% contained pERK after the injection of mustard oil into the gastrocnemius muscle (0-33%). Table 4-3 shows the proportions of giant cells that expressed pERK after the exposure to the different noxious stimuli. Examples pERK-positive giant cells are shown in Figures 4-4, 4-5 and 4-6. The intensity of pERK-immunostaining in the giant cells varied but mostly was moderately strong.

4.3.3 pERK in laminae III-IV NK1r-expressing neurons

The number of large laminae III-IV NK1r-immunoreactive cells that were identified in parasagittal sections from the ipsilateral side of the appropriate segments (Table 4-1) in each of the 6 rats that received mustard oil injections into their joints and muscles varied from 6 to 15. In each of the 3 rats that underwent CRD, the number of large laminae III-IV NK1r-expressing neurons that were identified in deep horizontal sections (both sides) of the lumbosacral cord varied from 38 to 41. The dorsal dendrites of all identified cells could be followed to at least as far as lamina II.

After the injection of mustard oil into the left knee joint, 8 of the 35 large laminae III-IV NK1r-expressing neurons identified contained pERK (mean of 23%). Following the injection of mustard oil into the left gastrocnemius muscle, 8 of the 27 identified large NK1r-expressing cells in laminae III-IV were pERK-positive (mean of 30%). Of the 118 large laminae III-IV NK1r-expressing neurons identified in the 3 rats that underwent CRD, but not tubal insertion, only 13 were pERK-immunoreactive (mean of 11%). Table 4-4 shows the proportions of pERK-positive laminae III-IV NK1r-expressing cells after the exposure to deep and visceral noxious stimuli. Figure 4-7 shows 2 pERK-positive laminae III-IV NK1r-expressing cells after the injection of mustard oil into the knee joint. Figure 4-8 shows a laminae III-IV NK1r-expressing cell that did not contain pERK after the injection of mustard oil into the knee joint.

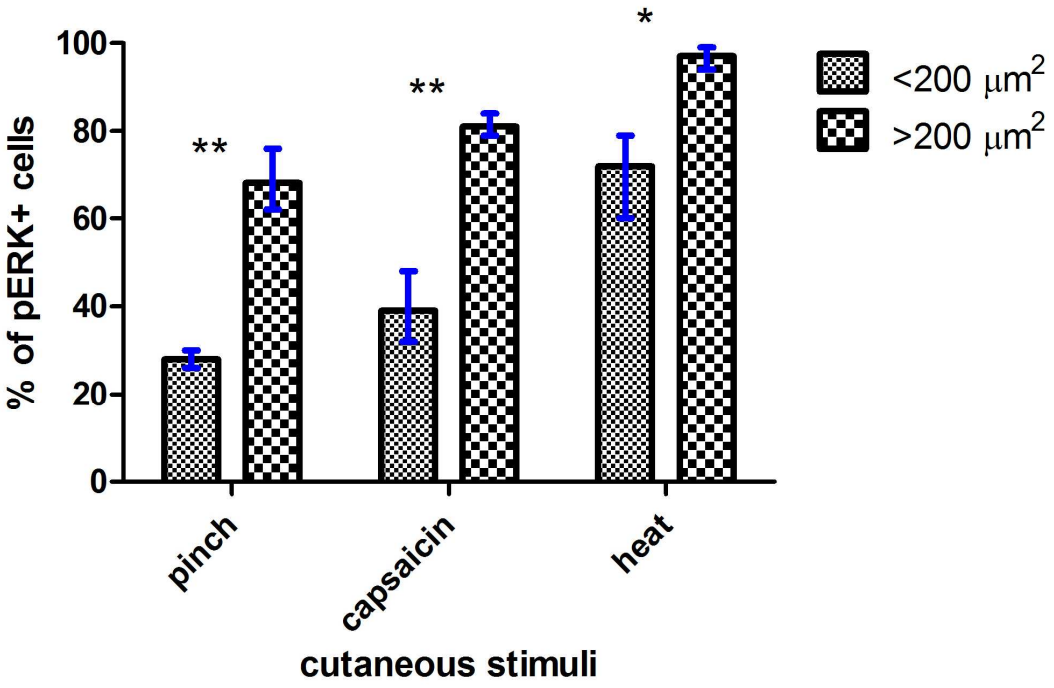
The intensity of pERK-immunostaining in these cells varied from very weak to moderately strong. In most of the pERK-positive laminae III-IV NK1r-expressing neurons, pERK-immunoreactivity was detected in both cell body and dendrites. However, in few of these cells, pERK staining was seen clearly only in their dorsal dendrites. pERK staining was often considerably stronger in the distal parts of the dorsal dendrites compared to the cell bodies of these neurons.

Most of the laminae III-IV NK1r-expressing neurons showed internalisation of the NK1 receptor, mainly of their dorsal dendrites. However, pERK-immunoreactivity was not restricted to dendrites that showed internalisation.

Figure 4-1 Histogram showing the percentages of pERK$^+$ NK1r-expressing small and large cells in lamina I after noxious stimulation.

The histogram shows the percentages of pERK$^+$ NK1r-immunoreactive cells that had soma cross-sectional areas <200 μm^2 and those that had somata >200 μm^2 in lamina I following various noxious stimuli.

The percentages of pERK$^+$ NK1r-immunoreactive presumed projection neurons were significantly higher than those of pERK$^+$ NK1r-immunoreactive interneurons following noxious cutaneous, deep and visceral stimuli (*****P*<0.05, ******P*<0.001, Chi-square tests). In each case, the y-axis represents the total percentage of pERK$^+$ NK1r-immunoreactive cells from the three experiments, and the upper and lower error bars show the higher and lower percentages after pooling the results from the three animals.


100
80
60
40
20
0
% of pERK+ cells
**
**
*
pinch
capsaicin
heat
cutaneous stimuli
<200 μm2
>200 μm2

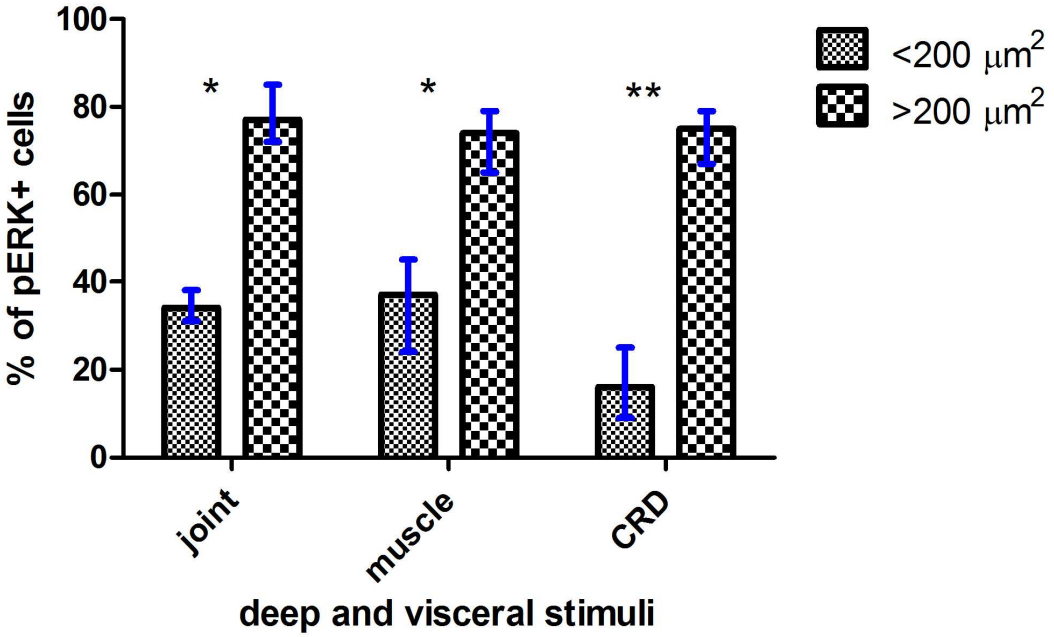

100
80
60
40
20
0
% of pERK+ cells
*
*
**
joint
muscle
CRD
deep and visceral stimuli
<200 μm2
>200 μm2

Table 4-2 Proportions of pERK$^+$ NK1r-expressing projection neurons of various morphological classes after noxious stimulation

Stimulus	Proportions (percentages %)			*P* value
	Fusiform	Multipolar	Pyramidal	
Pinch (*n*=3)	59/95 (62)	89/131 (68)	60/86 (70)	*P*=0.873
Capsaicin (*n*=3)	90/119 (76)	83/97 (86)	50/60 (83)	*P*=0.818
Heat (*n*=3)	101/106 (95)	100/103 (97)	65/67 (97)	*P*=0.995
Joint (*n*=3)	29/40 (73)	20/29 (69)	10/16 (63)	*P*=0.951
Muscle (*n*=3)	30/42 (71)	28/30 (93)	17/27 (63)	*P*=0.591
CRD (*n*=3)	39/45 (87)	18/28 (64)	15/25 (60)	*P*=0.562

In each case, proportions from the 3 animals are shown, with percentages in brackets. The table also shows no significant difference between the percentages of pERK$^+$ NK1r-expressing projection neurons of the 3 morphological classes in lamina I after the exposure to cutaneous, deep and visceral stimuli ($P>0.05$, Chi-square tests).

Figure 4-2 Examples of pERK-immunoreactivity in lamina I NK1r-expressing cells following noxious pinch.

This Figure shows a projected confocal image stack from a horizontal section through the ipsilateral side of the L5 segment. Two large NK1r–immunoreactive cells with somata >200 μm^2 cross-sectional area are marked with asterisks. These two cells were positive for pERK and showed internalisation of the receptor. Five small NK1r–immunoreactive cells with somata <200 μm^2 are marked with arrowheads, and these were not immunoreactive for pERK. Images are projections of 2 optical sections at 2 μm z-spacing. Scale bar=20 μm.

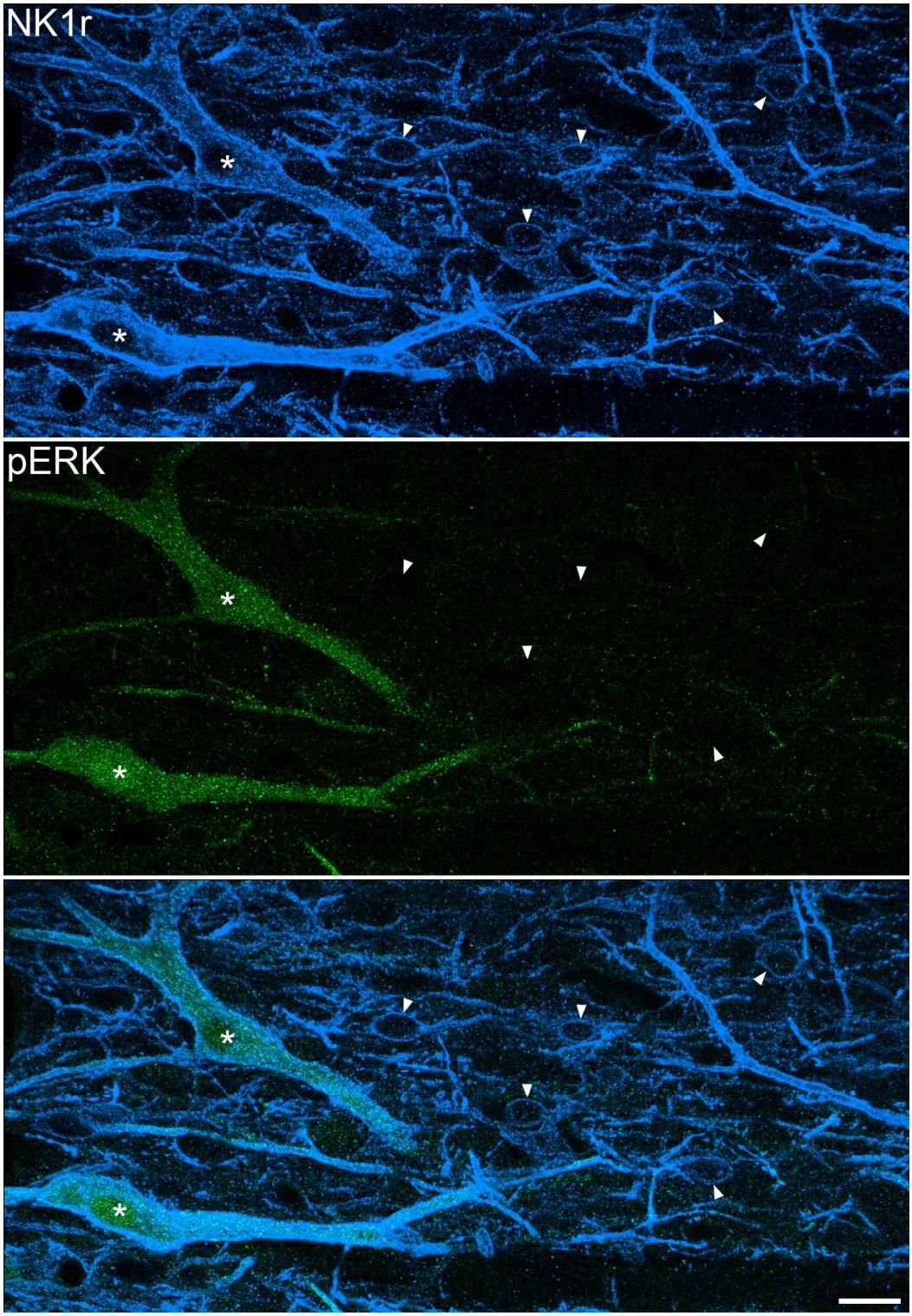

NK1r
pERK

Figure 4-3 Examples of pERK-immunoreactivity in lamina I NK1r-expressing cells following capsaicin injection.

The upper part shows a projected confocal image stack of 9 optical horizontal sections at 2 μm z-spacing through the ipsilateral side of the L5 segment. Several pERK-immunoreactive neurons can be seen and five of these are numbered. The lower part shows more limited projections of 3-4 optical sections through cells 1, 2, 3, 4 and 5. Each of these cells is immunostained for both NK1r and pERK, and there is internalisation of the receptor. Cells 1 to 5 had somata >200 μm^2 cross-sectional area and all of these cells contained pERK. A small NK1r-immunoreactive cell (soma area of 149 μm^2) that was negative for pERK is marked with an arrowhead. Scale bar=20 μm.

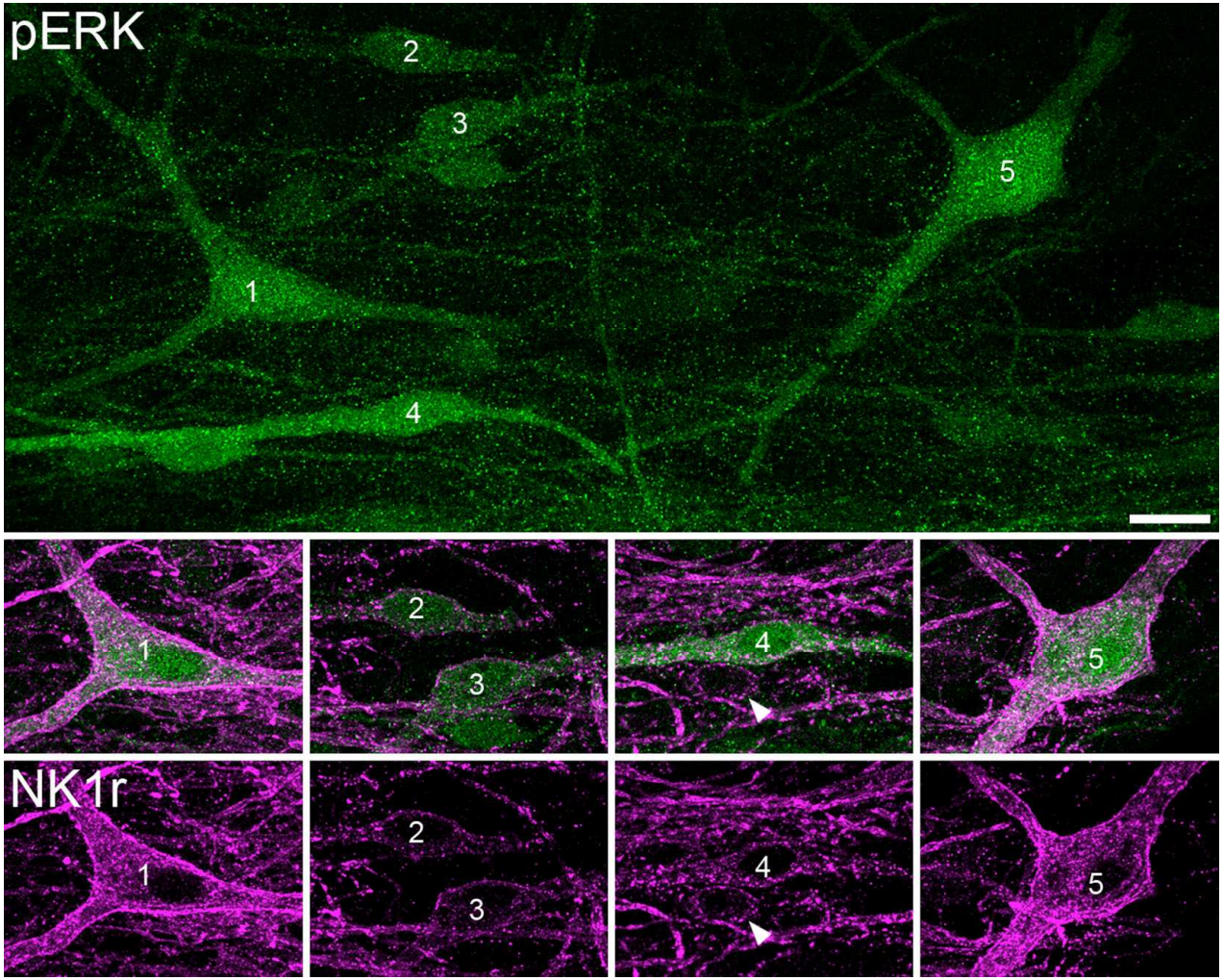

pERK
2
3
5
1
4
1
2
3
4
5
NK1r
1
2
3
4
5

Table 4-3 Proportions of pERK$^+$ giant cells after noxious stimulation

Stimulus	Proportions (percentages %)			
	Experiment 1	Experiment 2	Experiment 3	Total
Pinch (*n*=3)	11/12 (92)	12/15 (80)	10/12 (83)	33/39 (85)
Capsaicin (*n*=3)	7/8 (88)	10/13 (77)	14/16 (88)	31/37 (84)
Heat (*n*=3)	7/8 (88)	8/11 (73)	8/12 (67)	23/31 (74)
Joint (*n*=3)	2/6 (33)	3/5 (60)	2/5 (40)	7/16 (44)
Muscle (*n*=3)	0/5 (0)	0/4 (0)	2/6 (33)	2/15 (13)

In each case, proportions from the 3 animals are shown, with percentages in brackets.

Figure 4-4 Example of pERK-immunoreactivity in a lamina I giant cell following noxious pinch.

The upper part shows a projected confocal image stack of 15 optical horizontal sections at 2 μm z-spacing through the ipsilateral side of the L4 segment. A large giant cell (gephyrin coated) is visible, scale bar=50 μm. The lower part shows more limited projections of 5 optical sections through the giant cell that is positive for pERK, scale bar=30 μm.

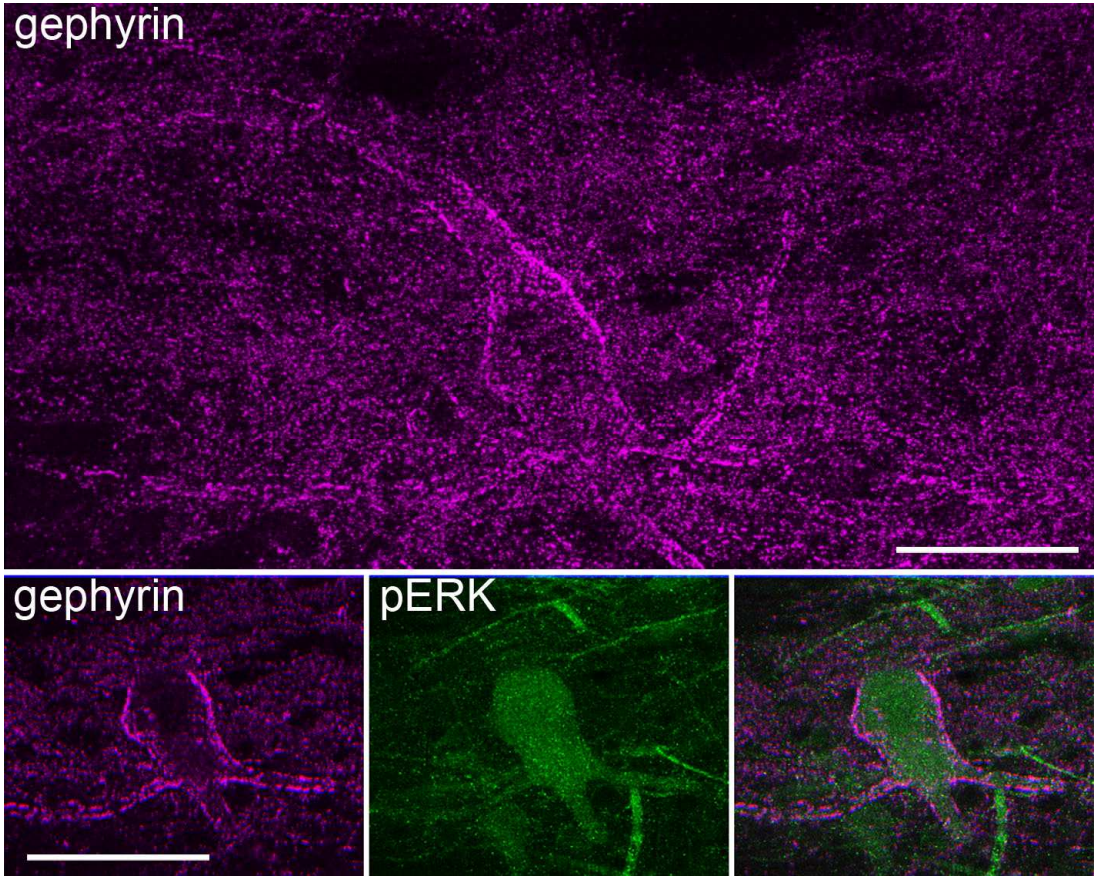

gephyrin
gephyrin
pERK

Figure 4-5 Example of pERK-immunoreactivity in a lamina I giant cell after the injection of mustard oil into the knee joint.

This figure shows a projected confocal image stack from a horizontal section through the ipsilateral side of the L2 segment. A giant cell (gephyrin coated) is visible in the left side of the image. The middle part shows pERK immunoreactivity only and the right side shows a merged image of the pERK-positive giant cell. Images are projections of 8 optical horizontal sections at 2 μm z-spacing. Scale bar=50 μm

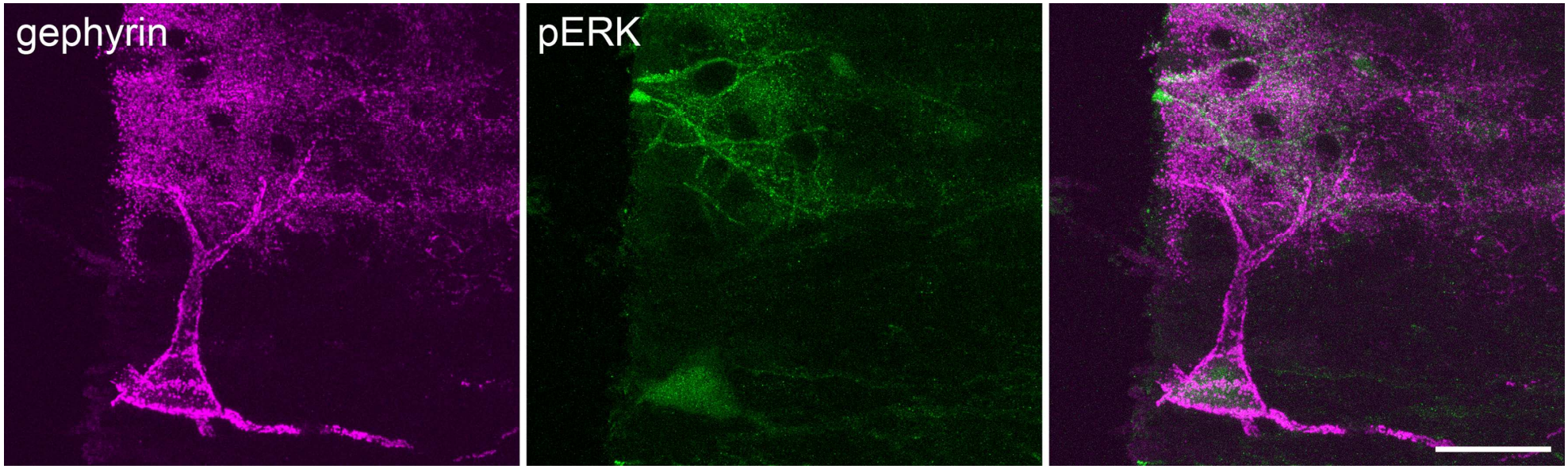

gephyrin
pERK

Figure 4-6 Examples of pERK-immunoreactivity in a lamina I giant cell and in NK1r-expressing cells after noxious pinch.

This figure shows a projected confocal image stack from a horizontal section through the ipsilateral side of the L4 segment. A giant cell (gephyrin coated, arrow) is seen in the 1st image, which is negative for the NK1 receptor as seen in the 2nd image and is positive for pERK as seen in the 3rd image. The 2nd image also shows 4 large NK1r-immunoreactive cells (asterisks) with somata >200 μm^2 cross-sectional area and all these cells contained pERK as seen in the 3rd image. The 4th image is merged showing the giant cell and the 4 NK1r-expressing neurons. Images are projections of 8 optical horizontal sections at 2 μm z-spacing. Scale bar=20 μm.

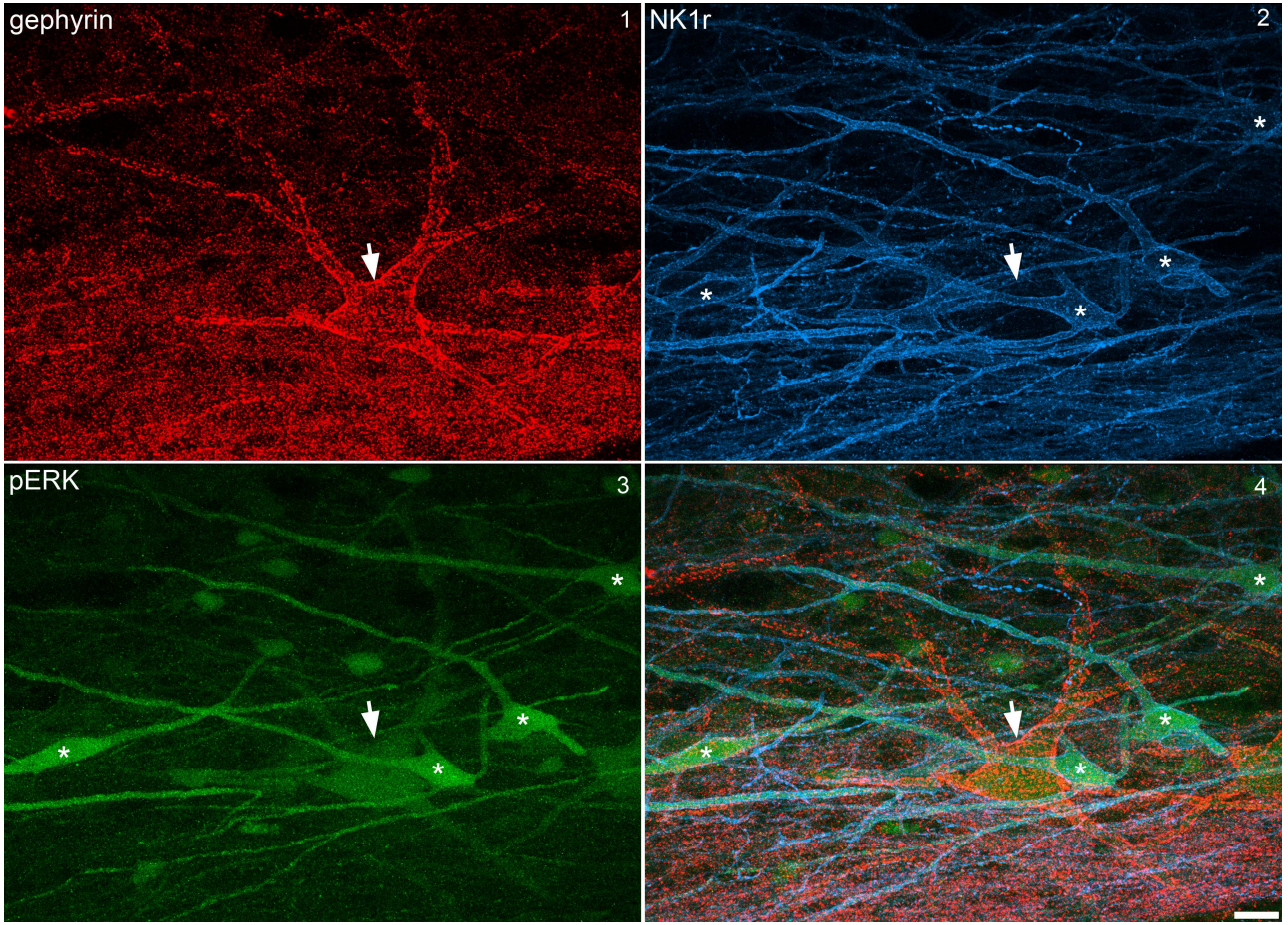

gephyrin
1
NK1r
2
pERK
3
4

Table 4-4 Proportions of pERK$^+$ laminae III-IV NK1r-expressing cells after noxious stimulation

Stimulus	Proportions (percentages %)			
	Experiment 1	**Experiment 2**	**Experiment 3**	**Total**
Joint (*n*=3)	0/6 (0)	2/14 (14)	6/15 (40)	8/35 (23)
Muscle (*n*=3)	5/13 (38)	1/8 (13)	2/6 (33)	8/27 (30)
CRD (*n*=3)	3/41 (7)	5/38 (13)	5/39 (13)	13/118 (11)

In each case, proportions from the 3 animals are shown, with percentages in brackets.

Figure 4-7 Examples of pERK-immunoreactivity in lamina III NK1r-expressing cells after the injection of mustard oil into the knee joint.

This figure shows a projected confocal image stack from a parasagittal section through the ipsilateral side of the L2 segment. It shows 2 large lamina III NK1r-immunoreactive neurons that are also weakly pERK-positive. The Image is a projection of 12 optical sections at 2 μm z-spacing. Scale bar=20 μm.

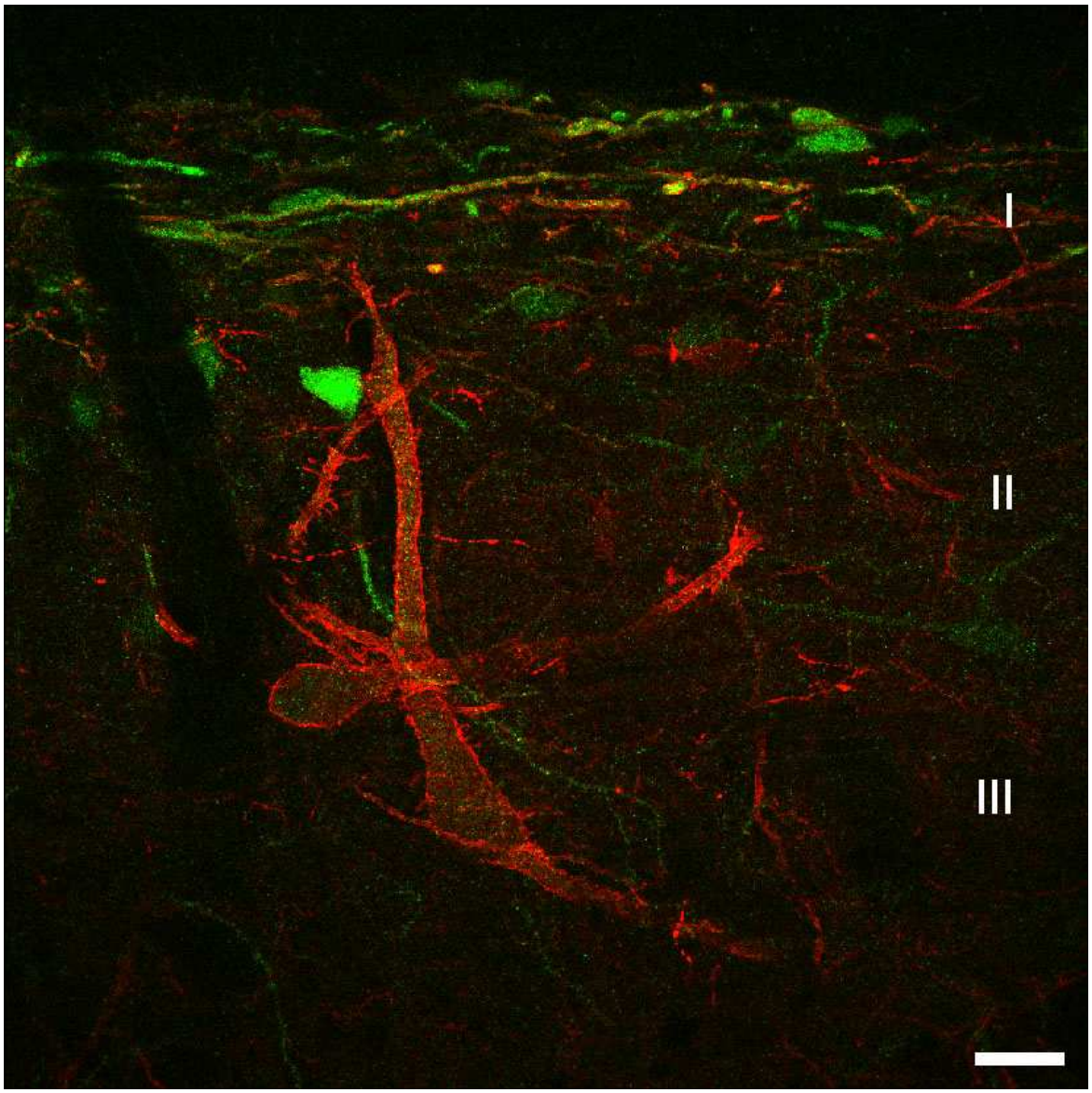
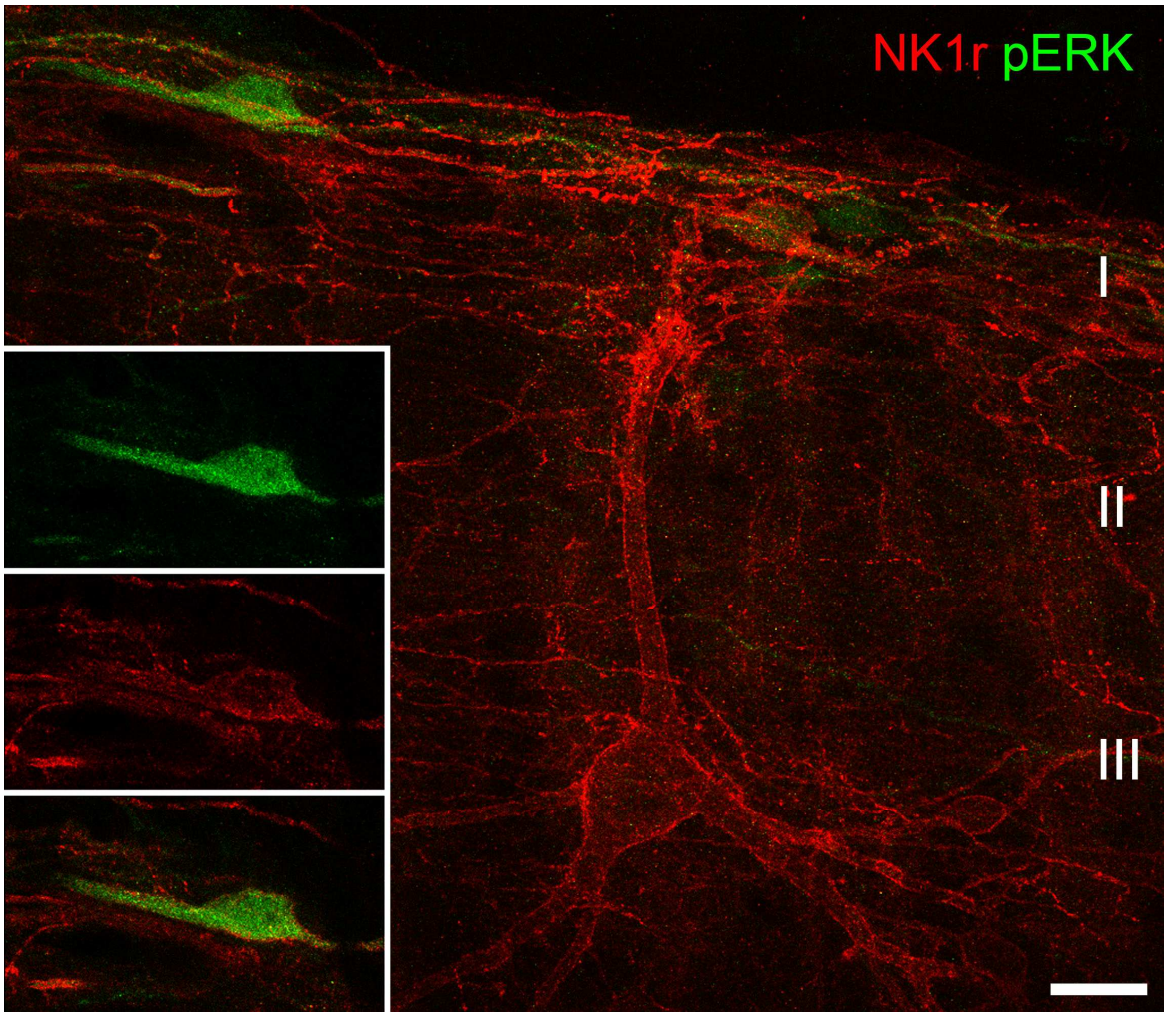

I
II
III

Figure 4-8 Example of the lack of pERK-immunoreactivity in a lamina III NK1r-expressing cell after the injection of mustard oil into the knee joint.

This figure shows a projected confocal image stack of 15 optical parasagittal sections at 2 μm z-spacing through the ipsilateral side of the L2 segment. It shows a large lamina III NK1r-immunoreactive cell that is negative for pERK. The inset shows a more limited projection of 5 optical sections through a lamina I NK1r-expressing cells that is pERK-positive. Scale bar=25 μm.


NK1r pERK
I
II
III

4.4 Discussion

Several studies have used immunocytochemistry or electrophysiology to investigate the responses of dorsal horn neurons to various noxious stimuli. However, few of these studies attempted to identify the specific population of neurons that responded to these noxious stimuli. This part of the project shows that a proportion of lamina I NK1r-expressing neurons, both putative projection neurons and interneurons, phosphorylated ERK within 5 minutes of noxious cutaneous, deep and visceral stimulation. The study also reports that the responses of different morphological classes of lamina I NK1r-expressing projection neurons did not differ significantly following noxious stimulation. These projection cells were more likely to phosphorylate ERK than the presumed NK1r-expressing interneurons after the exposure to noxious stimuli. Additionally, following noxious deep and visceral stimulation, few of the large laminae III-IV NK1r-expressing projection neurons contained pERK. Regarding the giant cells, a varying proportion of them developed pERK-immunorectivity after noxious cutaneous and deep stimulation.

4.4.1 pERK as a marker of neuronal activity

As described previously, stimuli of different nature have been used to induce the phosphorylation of ERK in the spinal cord, and to further study the causes and effects of this activation. pERK expression in the SDH was first studied by Ji *et al*. (1999) after the exposure of the rat to different noxious stimuli (thermal, mechanical, chemical and electrical). Ji *et al*. (1999) tested the time course of capsaicin-induced ERK phosphorylation, and for how long this phosphorylation lasted. The study reported that ERK was initially activated at 1 minute after stimulation and this activation peaked at 2 minutes, was maintained for 5 minutes, but decreased at 10 minutes, and finally reached its basal level at 2 hours. The study also showed that pERK induction is intensity dependent, since the number of pERK expressing neurons increased as the noxious temperature increased from 45 to 55°C. Fukui *et al*. (2007) reported that electrically stimulating C-fibres at different frequencies induced pERK expression in the SDH of the rat in a frequency dependent manner.

In this part of the project, and based on the findings of Ji *et al*. (1999) as well as on the findings from our laboratory (Polgar *et al.*, 2007), the animals were perfused 5 minutes after the exposure to noxious stimulation. The short time course of ERK phosphorylation, compared to that of fos up-regulation, is one of the advantages of using pERK as the marker of neuronal activity. The time course of fos up-regulation after noxious stimulation was studied by Herdegen *et al*. (1991). Their study investigated the expression of different proto-oncogene proteins, including fos, after electrically stimulating the sciatic nerve of the rat at Aδ/C-fibre intensity. Fos expression reached its maximal level in both superficial and deep spinal dorsal horn 1 hour after the electrical stimulus. Fos immunoreactivity decreased to basal levels between 8 and 16 hours after applying the stimulus, with its expression persisting longer in the deeper laminae. Another technical reason, for preferring pERK over fos, is that pERK immunoreactivity occurs in the cell bodies and dendrites of the cells, while fos is up-regulated in the nucleus only. The cellular morphology of pERK-positive neurons can therefore be easily identified.

4.4.1.1 Spinal ERK phosphorylation following noxious stimulation

Several studies have reported the effect of various types of noxious stimuli (cutaneous, deep and visceral) on spinal ERK activation. Ji *et al*. (1999) were the first to show that phosphorylation of ERK in the spinal cord is stimulus-specific and is also activity-dependent. ERK was activated by noxious cutaneous stimuli as well as by electrical stimulation of primary afferents at the C- and Aδ-fibre range, but not after Aβ-fibre stimulation. The study reported that 2 minutes after the application of an intense punctate mechanical stimulus to the hindpaw of the rat, pERK was expressed in the SDH. The injection of either capsaicin or formalin into the hindpaw of anesthetised or awake rats respectively, also induced pERK mainly in the medial half of laminae I and IIo of the L4-L5 spinal segments. In addition, Ji *et al*. (1999) examined the effect of noxious heat on pERK expression in the SDH, and reported that ERK phosphorylation was stimulus intensity dependent, since it varied with the water bath temperature. Few pERK-immunoreactive cells appeared at the threshold of activating heat-sensitive nociceptors (45°C), and the number increased with increasing temperature from 48°C to 55°C. This pERK expression was only partially inhibited by an NMDA antagonist, suggesting that the activation of ERK may be initiated

through the action of glutamate on non-NMDA (AMPA, mGlu) glutamate receptors, or through the action of neuropeptides or growth factors such as substance P, CGRP or BDNF. Galan *et al.* (2002) showed that intraplantar injection of carrageenan into both hindpaws of the rat evoked a peak five-fold activation of ERK in the SDH of the lumbar spinal cord at 30 minutes, compared to saline injection, which resulted in a two-fold activation. Saline-treated animals showed a higher level of ERK activation compared to control animals, suggesting that the process of injection itself may be sufficient to activate ERK.

The involvement of the MAPK/ERK cascade in central (spinal cord) and peripheral (DRG) processing of chronic inflammatory articular pain has been reported (Cruz *et al.*, 2005; Seino *et al.*, 2006). The above studies showed that passive movement of the inflamed ankle or knee joints (induced by the injection of CFA) of anesthetized rats increased pERK-immunoreactivity in both superficial and deep dorsal horn of the L3-L5 spinal segments, as well as in the DRG. Additionally, intrathecal and intra-articular injection of a MEK inhibitor reduced the joint movement-induced pain behaviour in monoarthritic rats, and decreased pERK labelling in the DRG neurons. This suggests that ERK phosphorylation in the spinal cord or in the DRG may play a role in decreasing mechanical pain threshold. Recently, Tsujimura *et al.* (2011) reported that the MAPK/ERK cascade was activated following noxious muscle stimulation in the rat. It was shown that following the injection of capsaicin into the muscles of mastication, a large number of pERK-positive cells were seen in the SDH of the upper cervical cord. The study also showed a significant reduction in the number of pERK-positive cells following the intrathecal administration of a MEK1/2 inhibitor, further supporting the involvement of this cascade in muscle pain.

Regarding visceral pain, Zhang *et al.* (2009) reported that noxious CRD evoked pERK expression in the SDH of the lumbosacral cord of rats. This suggests the involvement of ERK in CRD-induced neuronal responses in the spinal cord. Lai *et al.* (2011) also studied the effects of noxious distention of the urinary bladder of anaesthetized mice with or without a 10-day treatment with cyclophosphamide to pre-sensitize the bladder. The study reported that after distending the bladder, pERK was expressed in both superficial and deep laminae of the L6-S1 cord, and this expression was more profound in the cyclophosphamide-treated animals. Intrathecal injection of a MEK inhibitor attenuated distention-induced

bladder pain, suggesting that ERK activation may contribute to the development of inflammatory bladder painful conditions.

4.4.2 Lamina I NK1r-expressing projection neurons

The present study shows that following noxious cutaneous mechanical, chemical, or thermal stimulation, the majority of lamina I NK1r-expressing presumed projection neurons (with somata >200 μm^2) phosphorylated ERK. It also shows that after the injection of mustard oil into the knee joint or the gastrocnemius muscle, as well as after noxious visceral stimulation, three-quarter of these presumed projection neurons contained pERK. Noxious CRD, compared to tubal insertion without inflation, led to a large number of bilateral pERK-immunostaining in the SDH of the lumbosacral cord mainly, with some labelling in the thoracolumbar area.

4.4.2.1 Input to lamina I NK1r-expressing neurons

Lamina I NK1r-expressing projection neurons represent 80% of the projection neurons in this lamina (Todd *et al.*, 2000; Spike *et al.*, 2003; Al-Khater *et al.*, 2008). These NK1r-expressing projection neurons receive selective monosynaptic input from substance P-containing primary afferents (Todd *et al.*, 2002). They also receive glutamatergic input (Todd, 2010), presumably from Aδ primary afferents (Andrew, 2010), as well as from lamina II vertical interneurons (Lu and Perl, 2005). Uta *et al.* (2010) showed that these vertical interneurons receive peripheral inputs from TRPA1-expressing C-fibres, which can be activated by formalin (McNamara *et al.*, 2007), and from C-fibres that express TRPV1, on which capsaicin acts (Pingle *et al.*, 2007). Labrakakis and MacDermott (2003) used fluorescence-conjugated substance P to label NK1r-expressing neurons in lamina I of the rat spinal cord in a slice preparation. They reported that capsaicin increased the frequency of excitatory postsynaptic currents in 73% of these neurons, suggesting that these cells also receive a direct input from capsaicin and heat sensitive nociceptors. Consistent with the above finding, the present study shows that 81% of lamina I NK1-expressing neurons contained pERK after the injection of capsaicin. Torsney and MacDermott (2006) also reported that lamina I NK1r-expressing neurons receive monosynaptic input mainly from high-threshold C- (52%) and Aδ- (29%) fibres.

4.4.2.2 Responses of lamina I projection neurons to noxious stimuli; electrophysiological studies

It has been shown in electrophysiological studies that many lamina I projection neurons in the rat responded to one or more noxious stimuli. These studies did not distinguish between neurons that expressed the NK1 receptor from those that did not. However, since the NK1 receptor is expressed on 80% of lamina I projection neurons, the majority of neurons in these studies would presumably have been NK1r-positive. As described previously, Bester *et al*. (2000) showed that almost all of the spinoparabrachial neurons were activated by noxious stimuli; of these 75% were nociceptive specific (responding only to stimuli of the noxious range). The study also showed that 92% of these projection neurons responded to both noxious thermal and mechanical stimuli. Further, Andrew (2009) showed that 70% of the spinoparabrachial neurons were activated by noxious thermal and/or mechanical stimulation (nociceptive specific). Zhang *et al*. (2006) also reported that almost all lamina I STT neurons were activated by noxious mechanical stimuli, and half of those neurons that were activated by thermal stimulation were nociceptive specific (activated only by noxious heat). Consistent with the above findings of physiological studies, which suggested that almost all STT and spinoparabrachial neurons were excited by noxious mechanical and/or thermal stimuli, the present study shows that ERK was phosphorylated in almost all NK1r-expressing projection neurons after noxious heat, and in the majority of these cells following noxious pinch or capsaicin injection. This higher proportion of cells that responded to noxious heat, compared to the other noxious modalities, may be attributed to the fact that in case of thermal stimulation, the whole hindpaw is immersed in hot water covering a larger skin area. In contrast, pinching is a punctate stimulus that is not applied to the entire hindpaw and therefore, cells with receptive fields outside this area would not be activated.

The present study shows the responses of NK1r-expressing neurons to individual noxious stimuli, and therefore avoids the phenomenon of sensitisation, by which one stimulus may affect the neuronal responses to a 2^{nd} stimulus (Campbell and Meyer, 1983). It also shows that the majority of the NK1r-expressing projection cells are heat or capsaicin or pinch activated, therefore, they are presumed to respond to all three stimuli.

4.4.2.3 Responses of lamina I NK1r-expressing neurons to noxious stimuli; activation of fos and ERK

Doyle and Hunt (1999) studied the effects of noxious stimuli on fos expression, specifically in NK1r-immunorective neurons in both superficial (laminae I, III-IV) and deep (laminae V-X) dorsal horn of the rat. It was found that in the SDH, but not in deeper laminae, the number of fos$^+$ NK1r-expressing neurons positively correlated with the intensity of the noxious stimulus. Their study therefore suggests the involvement of laminae I and III-IV NK1r-expressing neurons in intensity discriminative aspects of pain. Regarding lamina I NK1r-expressing neurons, their study showed that 70-80% of these neurons up-regulated fos following topical application of mustard oil onto the hindpaw, injection of formalin and immersion of the hindpaw in water at 52°C for 10s. Results from the present study show that almost all lamina I NK1r-expressing projection neurons activated ERK following noxious heat (immersion of the hindpaw in water at 52°C for 20s). The higher proportion of pERK$^+$ NK1r-expressing neurons following noxious heat in this study, compared to the proportion of fos$^+$ NK1r-expressing cells seen by Doyle and Hunt (1999), may be attributed to more than one reason. First of all, Doyle and Hunt (1999) did not distinguish between the responses of small and large NK1r-expressing neurons, therefore their sample could have included a proportion of small NK1r-expressing cells that did not up-regulate fos. The number of fos$^+$ large NK1r-expressing (projection) neurons may be therefore underestimated. Secondly, in the present study, a longer duration of the stimulus was used: 20s compared to 10s, and Ji *et al*. (1999) reported that pERK expression is intensity-dependent. It has also been found that the number of lamina I NK1r-expressing neurons that internalized the receptor, as well as the number of endosomes within each NK1r-expressing neuron, was increased as the intensity of noxious heat increased (from 43 to 55°C) (Allen *et al*., 1997). Although Todd *et al*. (2005) used a similar method to what has been used in the present study (immersion of the rat hindpaw in water at 52°C for 20s), 57-69% of lamina I spinoparabrachial NK1r-expressing neurons up-regulated fos, compared to 97% pERK$^+$ NK1-expressing neurons in the present study. The overall higher number of NK1r-expressing neurons that responded to noxious heat in the present study, compared to Doyle and Hunt (1999) and Todd *et al*. (2005) studies may be attributed to the difference in the markers of neuronal activity that were used in these studies; fos versus pERK. It therefore seems that ERK

phosphorylation is more sensitive, at least in detecting the activity of NK1r-expressing neurons after noxious heat. The present study also shows that following noxious pinch and capsaicin injection, 68 and 81% of lamina I NK1r-expressing projection neurons contained pERK, respectively. In support of the above finding, Mantyh *et al.* (1995) found that 5 minutes after the exposure of rats to similar noxious mechanical and chemical stimuli, 67-70% of lamina I NK1r-expressing cells internalised the NK1 receptor.

Doyle and Hunt (1999) also reported that after the injection of mustard oil into either the gastrocnemius muscle or the knee joint, 20% or 45% of lamina I NK1r-expressing neurons up-regulated fos, respectively. The present study shows that ~75% of the large cells contained pERK after noxious deep stimulation, which was of similar quantity and quality to what was used by Doyle and Hunt (1999). However, for the reasons described above, the comparison between the present study and that of Doyle and Hunt (1999) in regard to the responses of lamina I NK1r-expressing neurons to noxious stimuli is not straightforward, because of the many variables between the two studies. In support of the present study, it has been reported that the gastrocnemius muscle afferents [mostly of the unmyelinated class, (Langford, 1983)] distribute collaterals to lamina I and deeper laminae (IV-IV) in cats and rats (Cervero *et al.*, 1976; McMahon and Wall, 1985; Mense and Craig, 1988). It was also reported that 50% of the gastrocnemius muscle afferents in the L5 DRG contained substance P in the rat, suggesting the importance of the NK1 receptor in muscle pain transmission (Perry and Lawson, 1998). Further, Craig and Kniffki (1985) recorded from lamina I STT neurons in the cat and reported that some of these neurons responded either specifically to deep noxious stimulation of the gastrocnemius muscle (algesic chemical and noxious mechanical stimulation), or to cutaneous noxious and innocuous stimuli (polymodal). Responses to noxious knee joint stimulation have also been recorded from lamina I neurons in the cat (Schaible *et al.*, 1986), consistent with the termination pattern of nociceptive knee joint afferents in the spinal cord; laminae I and VI-VI (Craig *et al.*, 1988). Langford (1983) also reported that ~50% of the cat knee joint afferents are group IV axons (unmyelinated).

Previous studies have shown that lamina I neurons can also be activated by visceral stimulation. Traub *et al.* (1992) showed that after repetitive distension of the colon of anesthetised rats to 80 mmHg, more fos-immunoreactive neurons

were observed in laminae I-II, V-VII and X of the lumbosacral cord, compared to what was seen after the distension of the colon to 20 mmHg only. In a subsequent behavioural study, Traub *et al*. (2002) confirmed that CRD at 80 mmHg is a noxious stimulus, since it caused avoidance behaviours in rats. In contrast, distending the colon at 20 mmHg was considered innocuous, since there was no difference in the behaviour of these animals compared to the non-distended controls. Honore *et al*. (2002) reported that internalisation of the NK1 receptor was seen in lamina I neurons in the T13 and S1 spinal segments after noxious CRD in awake rats. Further, Gamboa-Esteves *et al*. (2004) specifically reported that ~70% of lamina I NK1r-expressing neurons that projected to the NTS up-regulated fos after the exposure to noxious visceral stimulation (formalin injection into the urinary bladder). The present study shows that 75% of lamina I NK1r-expressing neurons phosphorylated ERK following noxious CRD, and that a large number of these cells showed internalisation of the receptor. Fewer pERK-positive cells were seen following tubal insertion, compared to CRD, and these were scattered in laminae I and II of the lumbosacral cord.

It has been suggested that the spinal phosphorylation of ERK may be due to a direct action of substance P on the NK1 receptor, as well as the co-release of glutamate from primary afferents and excitatory interneurons. For instance, Choi *et al*. (2005) reported that the intrathecal injection of substance P, presumably acting on the NK1 receptor, induced pain behaviour in the mouse and led to the expression of pERK in laminae I and II of the spinal cord at 10 minutes. This substance P-induced pain behaviour was attenuated after the blockade of ERK activity by the pretreament of the mice with PD98059. Anderson and Seybold (2000) reported that the phosphorylation of cAMP response element binding protein (CREB), which is required for the transcription of many neuronal genes and long-term synaptic plasticity (Ji and Rupp, 1997), was increased in NK1r-expressing neurons in the SDH 30 minutes after the injection of formalin into the rat hindpaw. Their study speculated that phospho CREB (pCREB) may regulate the expression of the NK1 receptor gene as well as other genes such as *c-fos* (Ji and Rupp, 1997), suggesting an indirect role of pCREB in regulating the expression of the NK1 receptor mRNA through the up-regulation of fos. Kawasaki *et al*. (2004) reported an additional indirect evidence of the involvement of NK1r-expressing neurons in the activation of ERK. They showed that the

activation of C-fibre primary afferents, in adult rat spinal slice preparation, by brief exposure to capsaicin led to an 8 to 10 fold increase in pERK in the SDH neurons. This pERK immunoreactivity was reduced by blocking the NK1 receptor. Their study also showed that C-fibre stimulation led to ERK-dependent phosphorylation of CREB, suggesting that the MAPK/ERK cascade may contribute to the production of long-lasting changes in sensory processing. Recently, Choi *et al*. (2012) reported that the carrageenan-induced phosphorylation of Akt, which is a serine/threonine kinase that plays an important role in various cellular processes including nociception, in the deep dorsal horn of the rat is dependent on the prior activation of the NK1r-expressing neurons in the SDH. It is therefore likely that NK1r-expressing projection neurons, most of which phosphorylated ERK following noxious stimulation, play an important role in conveying nociceptive information to higher brain centres through pathways that involve activation of ERK.

4.4.2.4 Morphology of lamina I NK1r-expressing projection neurons

Previous attempts to classify lamina I projection neurons based on their morphology have been controversial. It was suggested that morphology of these cells is related to their function; since Han *et al*. (1998) reported that in the cat, pyramidal cells specifically responded to innocuous cooling only, in comparison to the other two morphological classes, which were mostly nociceptive-specific. Yu *et al*. (2005) and Almarestani *et al*. (2007) also reported that lamina I pyramidal neurons that projected to the thalamus or to the parabrachial area were seldom NK1r-immunoreactive in the rat. However, it is unlikely that all lamina I pyramidal projection neurons in the rat respond to innocuous cooling for the reasons stated below. Although it has been reported that spinoparabrachial neurons of the pyramidal class make up 23-30% of this population (Almarestani *et al*., 2007; Al-Khater and Todd, 2009), Bester *et al*. (2000) did not find cells that responded to innocuous cooling in a sample of 53 lumbar lamina I spinoparabrachial neurons in the rat. In addition, out of the 40 spinoparabrachial cells that were studied by Andrew (2009), only 2 were identified as cooling-specific, while the majority of those (70%) were nociceptive specific. Further, although Zhang *et al*. (2006) identified cervical spinothalamic lamina I cells that were activated by cooling, these cells also responded to noxious stimuli. Todd *et al*. (2002, 2005) also showed that lamina I NK1r-expressing projection neurons of

all three morphological classes up-regulated fos after the exposure to noxious chemical (formalin) and thermal (noxious heat and cold) stimulation, with the responses of these neurons to noxious cold being more common in the multipolar class. The present study shows further evidence that morphology of lamina I NK1r-expressing projection neurons does not correlate with their function. There is also no significant difference between the responses of fusiform, multipolar or pyramidal lamina I NK1r-expressing cells to various noxious cutaneous (pinch, capsaicin or heat), deep or visceral stimuli.

4.4.3 Lamina III-IV NK1r-expressing projection neurons

Immunocytochemical studies have revealed a population of large laminae III-IV NK1r-expressing neurons of the rat spinal cord that have prominent dorsal dendrites entering the superficial lamina (Bleazard *et al.*, 1994; Brown *et al.*, 1995; Littlewood *et al.*, 1995; Mantyh *et al.*, 1995). These are also known to be projection neurons, since virtually all of them projected to supraspinal targets as described previously (Al-Khater and Todd, 2009). There are approximately 20 cells of this type on each side of the L4 segment in the rat (Todd *et al.*, 2000). These cells receive a large input from substance P-containing primary afferents, which form numerous synapses on their cell bodies and dendrites (Naim *et al.*, 1997). They therefore provide a route through which information transmitted by nociceptive primary afferents can be conveyed to deeper laminae and to supraspinal areas in the CNS. Torsney and MacDermott (2006) recorded from laminae III-IV NK1r-exressing neurons in spinal cord slices, and were unable to demonstrate monosynaptic input from Aδ- or C-fibres on the majority of these cells. However, they attributed this to the possibility of recording from neurons that did not posses long dorsal dendrites, or to not being able to retain the afferent input to these cells during the slice preparation.

The involvement of laminae III-IV NK1r-expressing neurons in transmitting nociceptive information from cutaneous tissues has been demonstrated by internalisation of the NK1 receptor on these cells after the injection of capsaicin (Mantyh *et al.*, 1995) or formalin (Honor *et al.*, 1999) into the rat hindpaw. Doyle and Hunt (1999) also reported that 40% of laminae III-IV NK1r-expressing cells up-regulated fos following the injection of formalin into the rat hindpaw. In contrast, a lower proportion of these cells were fos-positive following the topical

application of mustard oil onto the skin or noxious heat (immersion of the hindpaw in water at 52°C for 10s). However, Polgar *et al*. (2007) showed that almost all laminae III-IV NK1r-expressing neurons phosphorylated ERK after more intense noxious heat (immersion of the hindpaw in water at 52°C for 60s), noxious pinch or the injection of formalin.

The involvement of substance P and laminae III-IV NK1r-expressing cells in the spinal processing of nociceptive information from the knee joint of the rat has been suggested. Neugebauer *et al*. (1994) recorded from a sample of deep dorsal horn (laminae IV-VI) neurons, which may include some of lamina IV NK1r-expressing cells, that responded to pressure applied to the knee joint. Although the SDH contained neurons with inputs from the knee, the above study was done on deeper laminae neurons, since it was not easy to distinguish neurons with joint input from those with input from the skin in the SDH. Their study reported that the administration of substance P and neurokinin A led to a transient increase in the responses of neurons to both innocuous and noxious joint stimulation. However, the application of a specific NK1r inhibitor blocked the excitatory effects of substance P on neurons that were exposed to noxious, but not innocuous, stimuli.

The present study shows that after the injection of mustard oil into the knee joint or the gastrocnemius muscle, less than one-third of the large laminae III-IV NK1r-expressing projection neurons contained pERK. Doyle and Hunt (1999) also reported that less than 5% of all laminae III-IV NK1r-expressing neurons up-regulated fos following deep noxious stimuli of the joint and muscle. This lower proportion of fos-positive cells that was seen in Doyle and Hunt (1999) study, compared to the present study, following noxious deep stimulation may be due to the difference in the criteria of selection of laminae III-IV NK1r-expressing neurons. Doyle and Hunt (1999) included in their sample both the large neurons that had dorsal dendrites reaching the superficial laminae as well as the smaller non-projection ones, which were not included in the present study. This may have led to an increase in the sample size of Doyle and Hunt (1999), and a subsequent decrease in the proportion fos^+ NK1r-expressing projection cells in laminae III-IV.

The present study also reports a lower proportion of laminae III-IV NK1r-expressing neurons that phosphorylated ERK after noxious deep and visceral stimulation, compared to what was seen following noxious cutaneous stimulation (Polgar *et al.*, 2007). In addition, the responses of laminae III-IV NK1-expressing neurons to noxious deep and visceral stimulation differed from the responses of lamina I NK1r-expressing neurons to the same noxious stimuli. This suggests a difference in the type of information (cutaneous versus deep and visceral) that is transmitted to supraspinal areas through populations of NK1r-expressing projection neurons in laminae III-IV versus those in lamina I. Hu and Zhao (2001) also reported that the injection of turpentine oil into either the hindpaw or the gastrocnemius muscle of anesthetised rats led to fos expression in the superficial (laminae I-IIo) and deep (laminae V-VI) dorsal horn of the of the L4-L5 spinal segments. Their study showed that the number of fos-positive cells, induced by the intramuscular injection, was significantly decreased after the pretreatment of the animals with an AMPA and kainate receptor antagonist. In contrast, an NMDA receptor antagonist decreased the number of fos-positive cells that were induced only by intraplantar injection into the hindpaw. This suggests that the transmission of nociceptive information from various tissues (deep versus cutaneous) may be mediated by different glutamate receptors in the spinal cord.

4.4.4 Lamina I giant cells

The giant cells represent a distinct population of very large lamina I projection neurons that either lack or weakly express the NK1 receptor. These cells can be identified by the presence of high density of gephyrin (the glycine and GABA receptor-associated protein) puncta on their cell bodies and dendrites (Puskar *et al.*, 2001). These giant cells receive inhibitory input from lamina II nNOS-containing GABAergic neurons, which provide around one quarter of their GABAergic input (Puskar *et al.*, 2001; Todd, 2010). They are also densely innervated by VGLUT2-immunoreactive boutons that are associated with GluA4-containing puncta. This glutamatergic input is thought to be derived from excitatory interneurons, since the giant cells receive little direct synaptic input from peptidergic or Aδ primary afferents, compared to the NK1r-expressing neurons, which are densely innervated by peptidergic primary afferents as described earlier (Polgar *et al.*, 2008).

The giant cells have extensive, radiating dendritic trees, as seen in the present study and also reported by Puskar *et al*. (2001). It is therefore more likely that these cells have large peripheral receptive fields. In agreement with the above finding, results from the present study shows that following noxious pinch, 85% of the giant cells phosphorylated ERK, and even those giant cells lateral to the band of pERK immunolabelling were often pERK-positive. The present study also shows that a large number of the giant cells (74-85%) contained pERK following all three noxious cutaneous stimuli (pinch, capsaicin, heat). This, together with the fact that the giant cells form a small population that project mostly to the parabrachial area (Polgar *et al.*, 2008), suggests that these cells are involved in transmitting nociceptive information that alerts about the presence of a noxious stimulus, rather than providing accurate (discriminative) information about the noxious stimulus itself. These cells, in relation to their projection target, may also play a role in the affective and autonomic responses to pain. The fact that the giant cells lack, or weakly express, the NK1 receptor makes them resistant to being ablated by SP-SAP (Mantyh *et al.*, 1997). Although Mantyh *et al*. (1997) reported a dramatic reduction in hyperalgesia following SP-SAP treatment, responses to acute noxious stimuli remained intact. It is therefore likely that the giant cells are involved in transmitting nociceptive signals that led to the responses observed by Mantyh *et al*. (Mantyh *et al.*, 1997).

The involvement of giant cells in nociception was first shown by Puskar *et al*. (2001). Their study reported that that 87% of the giant cells up-regulated fos after the injection formalin into the rat hindpaw. The present study shows a similar percentage of giant cells (84%) that contained pERK after the injection of capsaicin. Regarding the responses of the giant cells to noxious heat, Polgar *et al*. (2008) reported that 38% of these cells up-regulated fos following thermal stimulation (immersing the hindpaw in water at 52°C for 45s). However, the present study shows that following noxious heat, of similar intensity and duration, 74% of the giant cells phosphorylated ERK. This discrepancy between the sensitivity of pERK and fos may be due to the possibility that in order for fos to be up-regulated, it requires a certain threshold of pERK activity to be reached (if it is assumed that spinal fos activation is ERK-dependent). It is likely therefore that the occasional lower levels of pERK expression, which are manifested as weak pERK immunolabelling, in this study, are insufficient to

subsequently up-regulate fos. In agreement with this, noxious heat also led to a higher proportion of $pERK^+$- compared to fos^+-NK1r-expressing neurons.

Results from the present study show that a lower proportion of the giant cells were activated by noxious stimulation of the joint or muscle, in comparison to the responses of lamina I NK1r-expressing neurons to noxious deep stimulation. This may be attributed to the observation that these cells receive less direct input from primary afferents, including those innervating joints and muscles. This also suggests that different populations of projection neurons in lamina I convey different messages to higher brain centres.

Puskar *et al.* (2001) found that most of the giant cells were located in the mid-lumbar spinal cord in the rat (~10 cells on each side of the L4 segment). In the present study, the pERK-immunostaining that was seen after noxious CRD was concentrated mainly in L6-S1 spinal segments, with some staining seen in T13-L1 segments. It was not applicable therefore to assess the responses of giant cells to visceral stimulation due to the rarity of these cells in the area that showed the highest pERK labelling after noxious CRD.

4.4.5 Lamina I NK1r-expressing interneurons

It was described previously that projection neurons in lamina I make up ~5% of the neuronal population in this lamina. It is therefore assumed that most lamina I cells (~95%) are interneurons. Twenty-five percent of these interneurons are found to be GABAergic (Polgar *et al.*, 2003), suggesting that the majority of lamina I neurons are excitatory interneurons. Todd *et al.* (1998) reported that 45% of neurons in lamina I express the NK1 receptor. From the above data, it could be calculated that 60% of all excitatory interneurons express the NK1 receptor. Despite the large number of interneurons in lamina I, knowledge of their organisation and function is very limited. Prescott and De Koninck (2002) reported a clear correlation between morphology and firing pattern for a sample of small neurons (presumably interneurons) in lamina I. However, little is known about the relation between morphology, function and the neurotransmitter phenotype for lamina I interneurons.

Although Doyle and Hunt (1999) reported that lamina I NK1r-expressing neurons up-regulated fos after exposure to various noxious stimuli, the interpretation of their findings is complicated, since they did not distinguish between the responses of NK1r-expressing projection neurons from those of NK1r-expressing interneurons. However, the present study shows the responses of both the large and small (with somata <200 μm^2) NK1r-expressing neurons to various noxious cutaneous, deep and visceral stimuli. Following pinch, capsaicin or noxious heat, 28%, 39% and 72% of these putative interneurons contained pERK, respectively. One-third of these cells were pERK-positive after the injection of mustard oil into the joint and muscle, and only 16% phosphorylated ERK after noxious CRD.

The first part of the project showed that the NK1 receptor was weakly expressed on this interneuronal population, while most of the larger NK1r-positive projection neurons expressed the NK1 receptor more strongly. This suggests that these smaller cells may not have been killed by SP-SAP, and may contribute to the normal behavioural responses to noxious heat that were seen in rats after the treatment with SP-SAP (Mantyh *et al.*, 1997). They may provide excitatory input to neighboring giant cells or even to other NK1r-expressing projection neurons that had survived SP-SAP treatment. Overall, the results from the present study suggest that this population of excitatory interneurons in lamina I plays a role in modulating and fine tuning nociceptive information before it is transmitted to higher brain centres.

4.4.6 Technical considerations and future directions

4.4.6.1 Noxious deep stimulation

One of the technical issues in the present study is the possibility that introducing a needle into the skin, rather than the noxious deep stimulus itself (mustard oil), may be the cause of activating the large numbers of lamina I NK1r-exressing neurons that were seen after deep noxious stimulation. Panfil *et al*. (2006) investigated the expression of fos in the SDH of the mice after the injection of NGF into the semispinal neck muscle, which was exposed surgically. Following the injection, fos-immunoreactivity was increased significantly in laminae I and II of the cervical cord, compared to sham-operated and saline injected controls. Although NGF was injected into a surgically exposed neck muscle, the number of

fos-positive cells was significantly higher in those operated animals compared to the sham group, on which a cannula needle was only inserted without any injection. In the present study, mustard oil was injected into the gastrocnemius muscle without the need to surgically expose it and risk the activation of additional cutaneous afferents.

4.4.6.2 Use of anaesthetics

The validity of the use of anaesthetics in experiments that investigates the responses of neurons to various noxious stimuli has been questioned. Banks *et al.* (1988) reported that although anaesthesia may influence the responses of neurons, it succeeded in minimizing the effects of intra-individual variability, which occurred while measuring these responses in awake rats. The study also showed a difference in the effects of pentobarbital, urethane and ketamine on the responses of mice to noxious thermal stimulation, assessed by measuring their tail flick latency. Although pentobarbital had no effect on latencies, in comparison with awake mice that received saline, if used in higher doses, which resulted in very deep level of anaesthesia, it led to an increase in the latencies. Urethane increased the mice tail flick latencies (decreased their levels of nociception), while ketamine decreased the latencies leading to more positive responses to noxious heat.

Anaesthesia may also affect the number of fos/pERK-positive neurons that are seen after noxious stimulation, since it was shown that it may dampen the responses of these neurons; resulting in false negative data. For instance, Willcockson *et al.* (1995) reported that the use of anaesthetics (a combination of ketamine-xylazine) increased the threshold of fos expression in the SDH as evidenced by the requirement of higher intensities of electrical stimulations in order to up-regulate fos in a pattern similar to the pattern of fos expression in un-anesthetised rats. It was also reported that the number of formalin-induced fos-positive neurons in the SDH of the lumbar cord was decreased in rats that were anaesthetised with halothane or isofluorane, compared to awake ones (Fukada *et al.*, 1999; Sommers *et al.*, 2008; Liu *et al.*, 2011). However, a number of studies reported that inhalational anaesthesia had failed to decrease the number of formalin-induced fos-positive cells in the SDH in rats (Sun *et al.*, 1996; Hagihira *et al.*, 1997).

In the present study, urethane was used as the anaesthetic, and a large number of pERK-positive neurons were seen in the SDH after the exposure of urethane-anesthetized rats to various noxious stimuli. Specifically, noxious heat induced the expression of pERK in almost all large lamina I NK1r-expressing neurons. This suggests that urethane is less likely to lead to under-estimation of the number of neurons that are activated following noxious stimulation, at least at spinal levels. Abbadie *et al.* (1994) also showed that under urethane anaesthesia, the temperature threshold that induced a consistent fos expression in the SDH of the rat was ~46°C. This threshold is similar to the threshold that led to heat-induced nociceptive reactions in awake rats, and that also activated C nociceptors.

Anaesthesia has also been reported to cause unexpected false positive results. However, these fos-positive neurons were found in supraspinal areas rather than in the spinal cord. Bullitt (1990) showed that prolonged inhalational anaesthesia led to the appearance of fos-positive cells in unexpected areas such as the olfactory cortex in the rat. Additionally, it was reported that after urethane anaesthesia without stimulation, numerous fos-positive cells were seen in the NTS and in the intermediate reticular formation in the rat (Strassman and Vos, 1993). Lanteri-Minet *et al.* (1993) also reported that controlled volatile anaesthesia alone did not cause fos labelling in the spinal cord of the rat, while it was effective in evoking fos in discrete hindbrain regions such as the rostral ventrolateral medulla, nuclei of the parabrachial area and the NTS. In the present study, pERK-positive cells were found mostly in the dorsal horn, ipsilateral to the stimulus, while the contralateral side showed minimal pERK labelling, suggesting that urethane alone has no effect in, at least, evoking spinal false positive cells.

Although carrying out experiments on awake rats may seem to prevent unwanted effects of anaesthesia, exposing the animals to noxious stimuli without the use of anaesthetics raises ethical issues. It has also been reported that experimenting on awake rats may indirectly lead to false negative results. For instance, Fukuda *et al.* (2009) showed that, in awake rats, the number of pERK-positive cells after the injection of formalin was decreased by the 'analgesic' act of licking, compared to the number of these cells in restrained awake rats, which were unable to lick their hindpaws.

Work from the present study sheds light on specific populations of dorsal horn neurons, as important contributors in conveying (projection neurons) or modulating (interneurons) nociceptive information, received from cutaneous, deep and visceral tissues. Future work to further investigate the effects inhibiting specific cascades that activated these neurons is recommended.

5. Responses of $mGlu_5$-expressing neurons to spinal application of DHPG

5.1 Introduction

Glutamate, which is released by primary afferents and local excitatory neurons, is the principal excitatory neurotransmitter in the CNS. It acts on both ionotropic and metabotropic receptors. Metabotropic glutamate receptors (mGlus) are G protein-coupled receptors. Their binding to glutamate regulates a series of cellular activities including phosphorylation of ion channels and/or gene transcription. There are 8 cloned mGlus, which are broadly classified into 3 groups based on their sequence homology, pharmacology and association with intracellular effector systems (Conn and Pin, 1997). Group I mGlus ($mGlu_1$ and $mGlu_5$) are linked to phospholipase C, an enzyme which hydrolyzes phosphoinositide leading to calcium release from intracellular stores. Groups II ($mGlu_2$ and $mGlu_3$) and III ($mGlu_4$, $mGlu_6$, $mGlu_7$, and $mGlu_8$) mGlus are linked to adenylate cyclase, an enzyme which catalyses the production of cyclic adenosine monophosphate (Simeone *et al.*, 2004). Each mGlu is composed of seven transmembrane-spanning domains with a large N-terminal domain.

mGlus are expressed on spinal dorsal horn neurons as well as on primary afferents. For instance, Ohishi *et al.* (1995) found that some axon terminals of primary afferent fibres, which terminated in the SDH, expressed $mGlu_7$. Although primary afferent terminals may contain mGlus, Jia *et al.* (1999) suggested that most of these receptors were typically in dendrites and cell bodies of neurons within the dorsal horn. These mGlus work synergistically and/or antagonistically with ionotropic receptors to control the release of glutamate from primary afferents and interneurons.

Localization of mGlus subtypes in the spinal cord has been investigated. Regarding group I mGlus, it was reported that SDH neurons strongly express $mGlu_{5a}$ and $mGlu_{5b}$ (two alternative splice variants), while deep dorsal horn neurons predominantly express the $mGlu_{1a}$ subtype (Valerio *et al.*, 1997; Boxall *et al.*, 1998; Berthele *et al.*, 1999; Alvarez *et al.*, 2000). Additionally, in lamina II of the rat dorsal horn, $mGlu_5$ was found either at the postsynaptic densities of asymmetrical synapses, which have thickened postsynaptic densities and are thought to be excitatory (Gray, 1959), or localized extrasynaptically at dendritic and somatic membranes (Vidnyanszky *et al.*, 1994; Jia *et al.*, 1999; Alvarez *et al.*, 2000). Tao *et al.* (2000) also identified a synaptic relationship of $mGlu_5$-

containing neurons in the SDH of the rat with nociceptive primary afferents and GABAergic terminals of presumed interneurons. Regarding group II mGlus, it was reported that the antibody against $mGlu_{2/3}$ was found mainly in laminae IIi, III and IV (Young *et al.*, 1998; Jia *et al.*, 1999). Additionally, Boxall *et al.* (1998) and Berthele *et al.* (1999) reported the absence of $mGlu_2$ mRNA from the spinal cord of rat. In regard to group III mGlus, it was reported that $mGlu_4$ mRNA was expressed in a disperse manner over the grey matter, while $mGlu_6$ mRNA was not expressed in the spinal cord (Boxall *et al.*, 1998; Berthele *et al.*, 1999). Their studies also showed that mGlu7 mRNA was localized with a high concentration in the SDH, in comparison to its lesser expression in deeper laminae.

Fisher and Coderre (1996a) reported that the application of group I mGlus agonist DHPG to the rat spinal cord induced spontaneous nociceptive behaviours. These behaviours were attenuated after the treatment of rats with antagonists acting on groups I and II mGlus, as well as on NMDA. Mechanical and thermal thresholds were also increased after the application of group I mGlu antagonists to the rat spinal cord (Young *et al.*, 1997). It was also reported that activation of groups I and II mGlus enhanced the excitatory responses of spinal neurons to ionotropic glutamate receptor (AMPA, kainite and NMDA) agonists both *in vivo* and *in vitro* (Bleakman *et al.*, 1992; Bond and Lodge, 1995; Budai and Larson, 1998). The excitability of dorsal horn neurons of the rat was also increased after the activation of group I mGlus (Zhong *et al.*, 2000; Park *et al.*, 2004). Park *et al.* (2010) recently reported that the bath application of DHPG enhanced the number of fos-immunoreactive neurons in the spinal trigeminal subnucleus caudalis *in vitro* (brainstem slices). The study also showed that the neuronal expression of fos was significantly decreased after the application of an NMDA receptor antagonist, as well as protein kinase C (PKC) and ERK inhibitors. This facilitatory effect of group I mGlus is either attributed to their interaction with ionotropic receptors (Fisher and Coderre, 1996b), or to their direct effect of increasing neuronal excitability (Jones and Headley, 1995). However, the role of group I mGlus in modulation of nociception is still poorly understood.

ERK is expressed in dorsal horn neurons and is phosphorylated by noxious stimuli, as described in the previous Chapter. It has been reported that nociceptive behaviour, which resulted from the activation of spinal group I mGlus, was ERK-dependent (Karim *et al.*, 2001). Karim *et al.* (2001) also showed that DHPG-

induced activation of group I mGlus led to ERK phosphorylation in mice dorsal horn neurons, identifying the ERK/MAPK cascade as a potential mediator of mGlu-induced enhancement of nociception. Hu *et al*. (2007) also reported that ERK activation was induced pharmacologically by activating group I mGlus in mouse spinal cord.

The role of $mGlu_5$ in modulating nociception is not yet clear. Karim *et al*. (2001) reported that nociceptive behaviours resulting from the activation of spinal group I mGlus is ERK-dependent. In this part of the study, phosphorylation of ERK in $mGlu_5$-expressing neurons after the administration of DHPG into the lumbar spinal cord was investigated. The study also examined the phenotype of these $pERK^+$ $mGlu_5$-expressing neurons by investigating the colocalisation of pERK and $mGlu_5$ with excitatory and/or inhibitory neurochemical markers.

5.2 Experimental procedures

5.2.1 Animals and DHPG application

Eight adult male Wistar rats (250–280 g; Harlan, Loughborough, UK) were used for this part of the study. The rats were deeply anaesthetized with 10% urethane (1.3 g/kg ip). They were then placed on a heating pad in order to maintain their body temperature. The depth of anaesthesia was monitored throughout the procedure by occasionally pinching the forepaw and observing the appearance of any withdrawal reflexes. A midline incision was made over the back, the paraspinous muscle was dissected, and a dorsal laminectomy was performed at T13 and L1 to expose the mid-lumbar spinal cord. Connective tissue and muscle were dissected from the dorsolateral surfaces of T12 and L2 to enable placement of vertebral clamps. DHPG [165 nmol (3.3 mM, 30 μg/50 μl), Tocris Cookson, Ballwin, MO, (Karim *et al*., 2001)] was applied to the exposed mid-lumbar spinal cord and maintained for 8 minutes (n=3). 100 μl of normal saline was applied to the lumbar cord of the remaining 5 rats for 8 minutes. The rats were then perfused with 4% formaldehyde.

5.2.2 Tissue processing and immunocytochemistry

Lumbar spinal cord was dissected out and post-fixed at 4°C for 24 hours. Mid-lumbar (L3-L5) spinal segments were cut into 60 μm thick transverse sections with a Vibratome. The transverse sections were cut sequentially into 5 bottles and were then treated for 30 minutes with 50% ethanol. Spinal sections from one of the bottles were reacted for 3 days in rabbit anti-$mGlu_5$ (1:500), and mouse monoclonal antibody against pERK (1:1000). Sections from the 2nd, 3rd and 4th bottles were reacted in both rabbit anti-$mGlu_5$ and mouse anti-pERK, as well as in one of the following primary antibodies: (1) goat anti-calretinin (1:1000) (2) goat anti-calbindin (1:500) or (3) guinea pig anti-GABA (1:1000), respectively. Sections were then incubated overnight in appropriate species-specific secondary antibodies raised in donkey and conjugated to either Alexa 488, Rhodamine Red, or DyLight 649. They were then rinsed 3 times with PBS, mounted and stored at -20°C. Characteristics and suppliers of the antibodies used in this part of the study are described in Chapter 2.

5.2.3 Confocal microscopy and analysis

5.2.3.1 ERK phosphorylation in $mGlu_5$-expressing neurons

To investigate the proportion of $pERK^+$ $mGlu_5$-expressing neurons after the application of DHPG, transverse sections from the mid-lumbar (L3-L5) spinal cord were analysed. For this part of the study, Zeiss LSM710 confocal microscope was used. Sections from the L3, L4 and L5 spinal segments were initially examined with a 20× lens using fluorescence microscopy for the presence of pERK. Three sections from each of the 2 segments that showed the most numerous pERK immunoreactive neurons were selected for further analysis. These were then scanned throughout the depth of the section using a 40× oil-immersion objective lens to produce z-series consisting of 24 to 26 optical sections at 2 μm z-separation. Between 4 and 6 contiguous fields were scanned for each section in order to cover the entire cross-sectional area of lamina I and II on one side. Files corresponding to pERK-immunostaining were opened with Neurolucida for Confocal software and every pERK-immunoreactive cell in lamina II was selected. The files containing $mGlu_5$-immunostaining were then viewed and the

presence or absence of $mGlu_5$ in each of the selected pERK–positive cells was determined.

5.2.3.2 $mGlu_5$-expressing excitatory and inhibitory neurons

The proportions of lamina II $mGlu_5$-expressing neurons that were positive for calretinin, calbindin or GABA were investigated. Three sections from each segment were scanned with the confocal microscope through a 40× oil-immersion objective lens to produce z-series at 2 μm z-separation that covered the entire area of lamina II on one side. Sections were then analysed with Neurolucida. The $mGlu_5$ channel was initially viewed, and $mGlu_5$-immunoreactive cells were selected from 4 optical sections at the start of the z-series. The selection was performed by applying a 5 μm × 5 μm grid and choosing the cell nearest the lower right hand corner of each grid square. It started at the most dorsal part of the lamina working from dorsal to ventral (1st 4 optical sections) and then from left to right. Once the cells had been selected, the calretinin, calbindin or GABA channel was viewed, and the presence or absence of calretinin-, calbindin- or GABA-immunostaining in each of the selected $mGlu_5$-positive cell was recorded.

5.2.3.3 Colocalisation of pERK with calretinin, calbindin and GABA in $mGlu_5$-expressing neurons

To investigate the proportion $pERK^+$ $mGlu_5$-expressing neurons that were assumed to be excitatory, sections from the L3-L4 or L4-L5 spinal segments that were incubated to reveal pERK, $mGlu_5$ and calretinin or calbindin were analysed. Initially, colocalisation of calretinin or calbindin with $mGlu_5$ in pERK-positive neurons was investigated. All the $pERK^+$ $mGlu_5$-expressing neurons were selected throughout the depth of the section. Files containing calretinin- or calbindin-immunostaining were then viewed and the presence or absence of calretinin or calbindin in each of the selected $pERK^+$ $mGlu_5$-expressing cells was determined.

To investigate the proportion $pERK^+$ $mGlu_5$-expressing neurons that were GABA-immunoreactive, sections from the L3-L4 and L4-L5 spinal segments that were incubated to reveal pERK, $mGlu_5$ and GABA were analysed. Three sections from each segment were scanned with the confocal microscope through a 40× oil-immersion objective lens to produce z-series at 2 μm z-separation that covered

the entire area of lamina II on one side. Since penetration of the GABA-immunostaining was extremely limited, only the superficial parts of each section were scanned. Sections were then analysed with Neurolucida. The pERK-positive neurons that were at the surface of the section were selected and the files containing $mGlu_5$- immunostaining were then viewed and the presence or absence of $mGlu_5$ in each of the selected pERK–positive cells was determined. The GABA channel was then revealed, and the presence or absence of GABA immunoreactivity in each of the selected $pERK^+$ $mGlu_5$-expressing cells was recorded.

In order to determine whether differences in the sizes of GABA+ and GABA- neurons could have led to a bias in selection, the distance in the z-axis between the upper surface of the Vibratome section and the bottom of the nucleus for a sample of pERK-positive neurons was estimated, by counting the number of optical sections between these levels. The presence or absence of GABA-immunostaining was then recorded.

5.3 Results

5.3.1 $pERK^+$ $mGlu_5$-expressing neurons

$mGlu_5$ staining was localized in the SDH, mostly in lamina II, consistent with previous studies that showed that $mGlu_5$ expression was concentrated in the superficial laminae of rats and mouse. $mGlu_5$ immunoreactivity was present as numerous granules forming distinct annuli that occupied cellular membranes.

Following DHPG (but not saline) application, numerous pERK-positive cells were seen in the SDH, particularly in lamina II (Figure 5-1). Of the 606 lamina II pERK-positive cells analysed, 531 (88%, range 81-94%) were $mGlu_5$-immunoreactive.

Animals that received saline showed variable results. In the first two rats, scattered pERK-positive cells were seen in both laminae I and II, with more of these cells in lamina I. In addition to the difference in the laminar distribution of these cells, compared to cells that activated ERK after DHPG, their rostrocaudal distribution differed too. They were mainly concentrated at one end of the exposed lumbar cord: L3 or L5 spinal segment and this segment corresponded to

an external swelling of the cord, which was induced by the laminectomy itself. These pERK-positive cells were also larger than those seen after DHPG, and were seldom $mGlu_5$-immunoreactive. The other three animals that received saline showed minimal pERK expression, and this may be attributed to the laminectomy procedure after which less swelling of the cord was seen.

Results of the quantitative analysis of pERK in $mGlu_5$-expressing neurons in lamina II, after DHPG application, are shown in Table 5-1, and an example of pERK and $mGlu_5$-immunostaining is seen in Figure 5-2.

5.3.2 Neurochemistry of $mGlu_5$-expressing cells

5.3.2.1 Calretinin and calbindin

In the sections reacted to reveal calretinin, which is expressed by excitatory neurons, calretinin-immunostaining was present in the superficial dorsal horn, mostly in lamina II. The percentage of calretinin$^+$ $mGlu_5$-immunoreactive neurons in lamina II was 38% (37-39%, Table 5-2). Additionally, of the 578 pERK$^+$ $mGlu_5$-immunoreactive cells analysed, only 58 cells (10%, 6-15%) contained calretinin (Table 5-2 and Figure 5-3). Table 5-2 also shows a significant difference between the expression of calretinin in $mGlu_5$-immnuoreactive cells compared to its expression in pERK$^+$ $mGlu_5$-expressing cells (P<0.001, Chi-square test).

In the sections reacted to reveal calbindin, a marker for excitatory neurons, calbindin-positive cells were seen in the SDH, mostly in lamina II. Fifty-seven percent (51-64%) of the $mGlu_5$-expressing neurons in lamina II contained calbindin (Table 5-3). Additionally, of the 674 pERK$^+$ $mGlu_5$-immunoreactive cells analysed, 138 cells (20%, 13-30%) were calbindin-positive (Table 5-3 and Figure 5-4). Chi-square test shows that there is a significant difference between the expression of calbindin in $mGlu_5$-immnuoreactive cells and its expression in pERK$^+$ $mGlu_5$-expressing cells (P<0.001, Table 5-3).

5.3.2.2 Gamma-aminobutyric acid (GABA)

In sections reacted to reveal GABA, which is expressed exclusively by inhibitory interneurons, the distribution of GABA immunostaining was very similar to that reported previously in the rat dorsal horn. Staining was particularly dense in

laminae I-III, and this region contained numerous immunoreactive cell bodies. Non-immunoreactive cells were seen as dark areas, surrounded by strongly immunoreactive structures in the neuropil, which presumably corresponded to dendrites and axonal boutons of GABAergic neurons. However, as reported previously, the penetration of GABA-immunostaining was extremely limited in formaldehyde-fixed tissues compared to glutaraldehyde-fixed ones (Somogyi *et al.*, 1985; Sardella *et al.*, 2011a), and only cells for which the nucleus appeared on the top surface of the section were examined.

The percentage of $GABA^+$ $mGlu_5$-expressing neurons in lamina II was 35% (31-43%, Table 5-4). However, GABA-immunoreactivity was seen in 79% (74-84%) of the $pERK^+$ $mGlu_5$-expressing cells. Results of the quantitative analysis of GABA expression in both $mGlu_5$-expressing neurons and $pERK^+$ $mGlu_5$-immunoreactive neurons in laminae II are shown in Table 5-4. Table 5-4 also shows a significant difference between GABA expression in $pERK^+$ $mGlu_5$-immnuoreactive cells compared to its expression in $mGlu_5$-expressing cells (P<0.001, Chi-square test). An example of GABA, pERK and $mGlu_5$-immunostaining is seen in Figure 5-5.

Since only pERK-positive neurons that were located in the surface of the section were selected for further analysis, and both $GABA^+$ and $GABA^-$ pERK-immunoreactive neurons were found in laminae II, it was unlikely to avoid a selection bias towards the larger pERK-positive cells. However, the present study did not show any significant difference between the sizes of the pERK-positive neurons that expressed GABA (8.97 μm, range 6-12 μm, median 9 μm, n=114) and those that were GABA-negative (9.01 μm, range 6-13 μm, median 9 μm, n=74) (P=0.984, Mann-Whitney U-test). It is therefore unlikely that the estimate of the proportion of pERK-positive neurons that were GABA-immunoreactive was influenced by this selection bias.

Figure 5-1 Examples of pERK-immunoreactivity in lamina II after DHPG application.

This figure shows a projected confocal image stack from a transverse section through the L4 segment. Eight minutes after DHPG application, numerous pERK-positive cells are seen, mostly in lamina II. Images are projections of 5 optical horizontal sections at 2 μm z-spacing. Scale bar=20 μm.

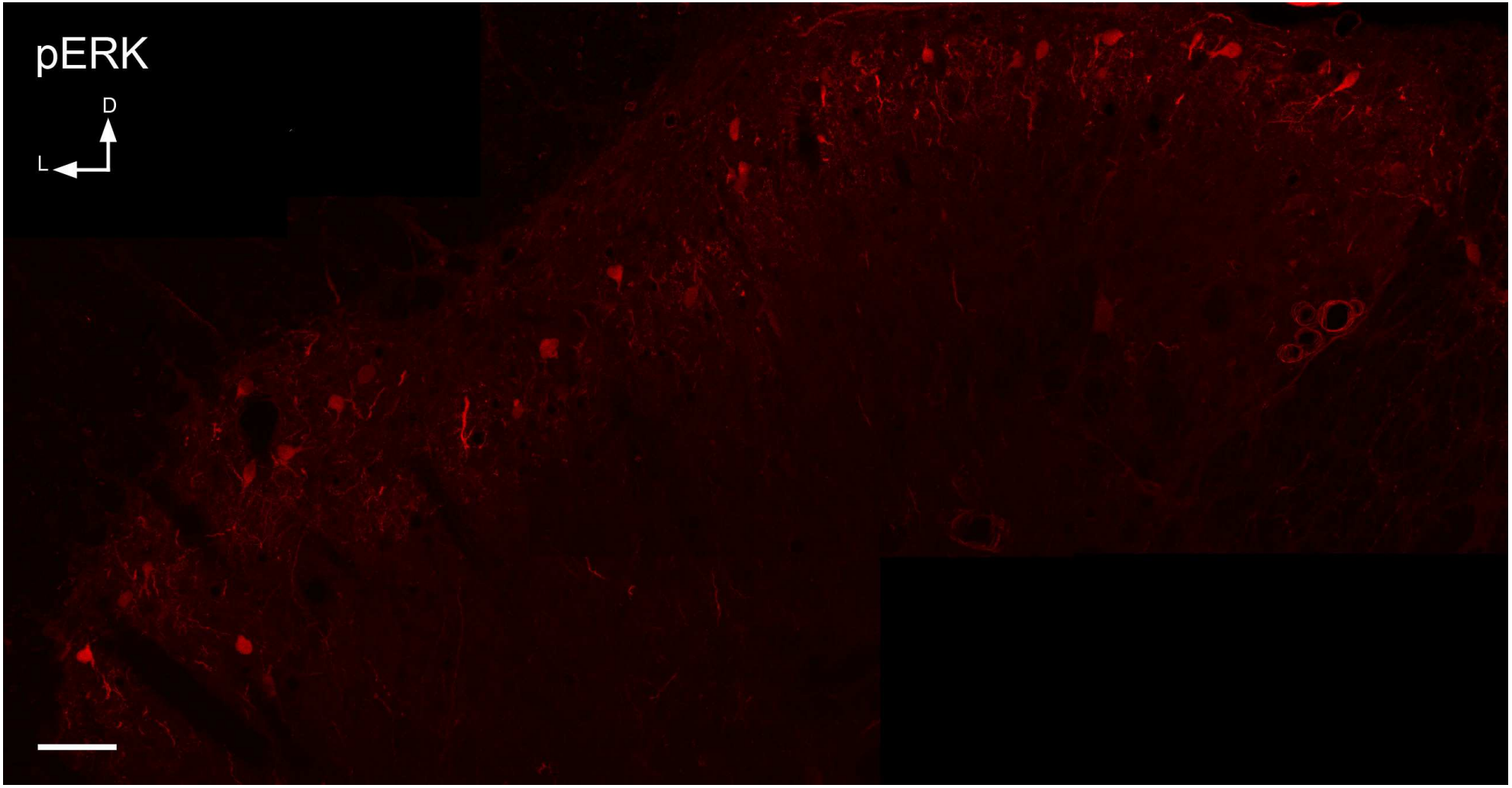

pERK
D
L

Table 5-1 Proportions of pERK$^+$ mGlu$_5$-expressing cells in lamina II after DHPG application

Experiment	Spinal segments	Number of pERK$^+$ cells	Proportions (and percentages) of pERK$^+$ mGlu$_5$-expressing cells
1	L4-L5	226	212 (94)
2	L4-L5	166	146 (88)
3	L3-L4	214	173 (81)
Total		**606**	**531 (88)**

In each case, proportions from the 3 animals are shown, with percentages in brackets.

Figure 5-2 Examples of pERK-immunoreactivity in $mGlu_5$-expressing cells following DHPG application.

This figure shows a projected confocal image stack from a transverse section through the L4 segment. Eight minutes after DHPG application, numerous pERK-positive cells are seen, of which 88% are $mGlu_5$-immunoreactive. The arrows show 2 pERK-positive cells, which are also $mGlu_5$-immunoreactive. The insets show enlarged images of the two $pERK^+$ $mGlu_5$-expressing neurons. Images are projections of 5 optical horizontal sections at 2 μm z-spacing. Scale bar=20 μm.

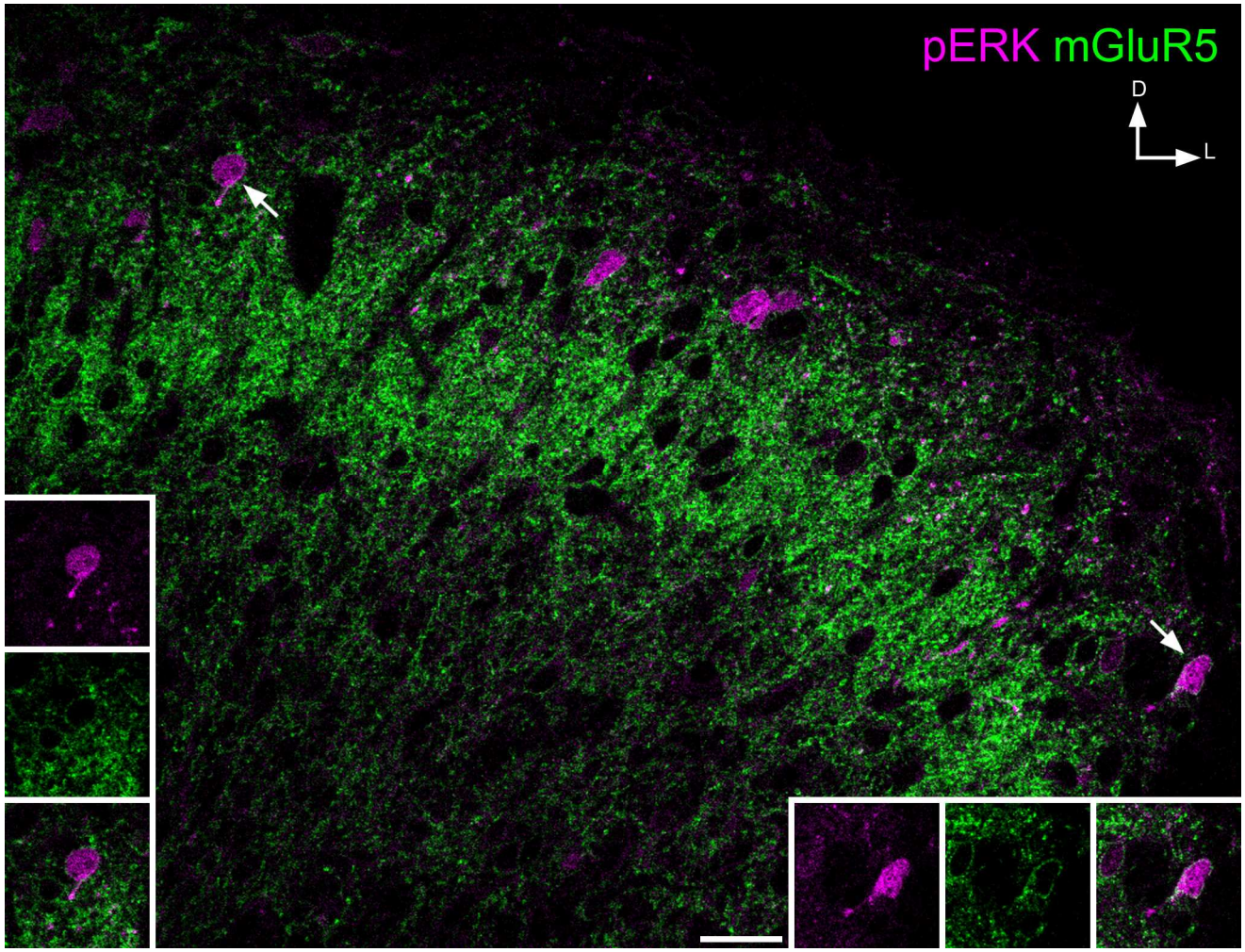

pERK mGluR5
D
L

Table 5-2 Calretinin-immunostaning in $mGlu_5$-expressing cells and in $pERK^+$ $mGlu_5$-expressing neurons after DHPG application

Experiment	Spinal segments	Number of $mGlu_5$-expressing cells that are positive for calretinin	Number of $pERK^+$ $mGlu_5$-expressing cells that are positive for calretinin	*P* value
1	L4-L5	82/220 (37)	26/268 (7)	
2	L4-L5	79/210 (38)	22/143 (15)	
3	L3-L4	68/176 (39)	10/167 (6)	
Total		**229/606 (38)**	**58/578 (10)**	***P*<0.001**

In each case, proportions from the 3 animals are shown, with percentages in brackets. The table also shows a significant difference between the expression of calretinin in $mGlu_5$-immnuoreactive cells and in pERK+ $mGlu_5$-expressing cells (*P*<0.001, Chi-square test).

Figure 5-3 Examples of calretinin-immunoreactivity in pERK$^+$ mGlu$_5$-expressing cells following DHPG application.

This figure shows a projected confocal image stack from a transverse section through the L4 segment. Numerous pERK-positive mGlu$_5$-expressing neurons cells are seen 8 minutes after DHPG application. The arrows show 2 pERK$^+$ mGlu$_5$-expressing cells, which are also calretinin-immunoreactive. The inset shows an enlarged image of the two calretinin-containing pERK$^+$ mGlu$_5$-expressing neurons. Images are projections of 7 optical horizontal sections at 2 μm z-spacing. Scale bar=20 μm.

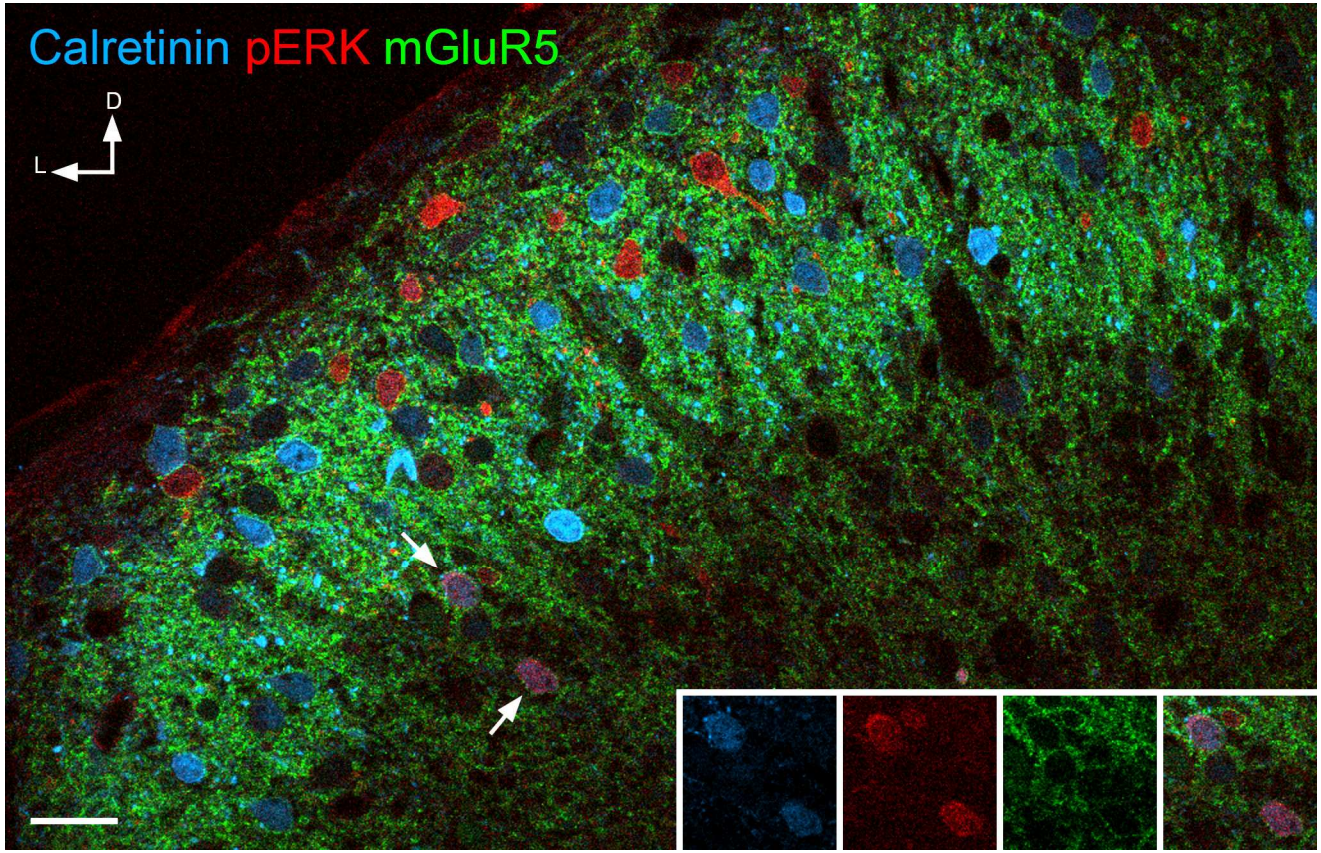

Calretinin pERK mGluR5
D
L

Table 5-3 Calbindin-immunostaning in $mGlu_5$-expressing cells and in $pERK^+$ $mGlu_5$-expressing neurons after DHPG application

Experiment	Spinal segments	Number of $mGlu_5$-expressing cells that are positive for calbindin	Number of $pERK^+$ $mGlu_5$-expressing cells that are positive for calbindin	*P* value
1	L4-L5	84/164 (51)	31/234 (13)	
2	L4-L5	138/217 (64)	61/205 (30)	
3	L3-L4	103/192 (54)	46/235 (20)	
Total		**325/573 (57)**	**138/674 (20)**	***P*<0.001**

In each case, proportions from the 3 animals are shown, with percentages in brackets. The table also shows a significant difference between the expression of calbindin in $mGlu_5$-immnuoreactive cells and in pERK+ $mGlu_5$-expressing cells (*P*<0.001, Chi-square test).

Figure 5-4 Examples of calbindin-immunoreactivity in pERK$^+$ mGlu$_5$-expressing cells following DHPG application.

This figure shows a projected confocal image stack from a transverse section through the L4 segment. Numerous pERK-positive mGlu$_5$-expressing neurons cells are seen 8 minutes after DHPG application. The arrows show 2 pERK$^+$ mGlu$_5$-expressing cells, which are also calbindin-immunoreactive. The inset shows an enlarged image of the two calbindin-containing pERK$^+$ mGlu$_5$-expressing neurons. Images are projections of 5 optical horizontal sections at 2 μm z-spacing. Scale bar=20 μm.

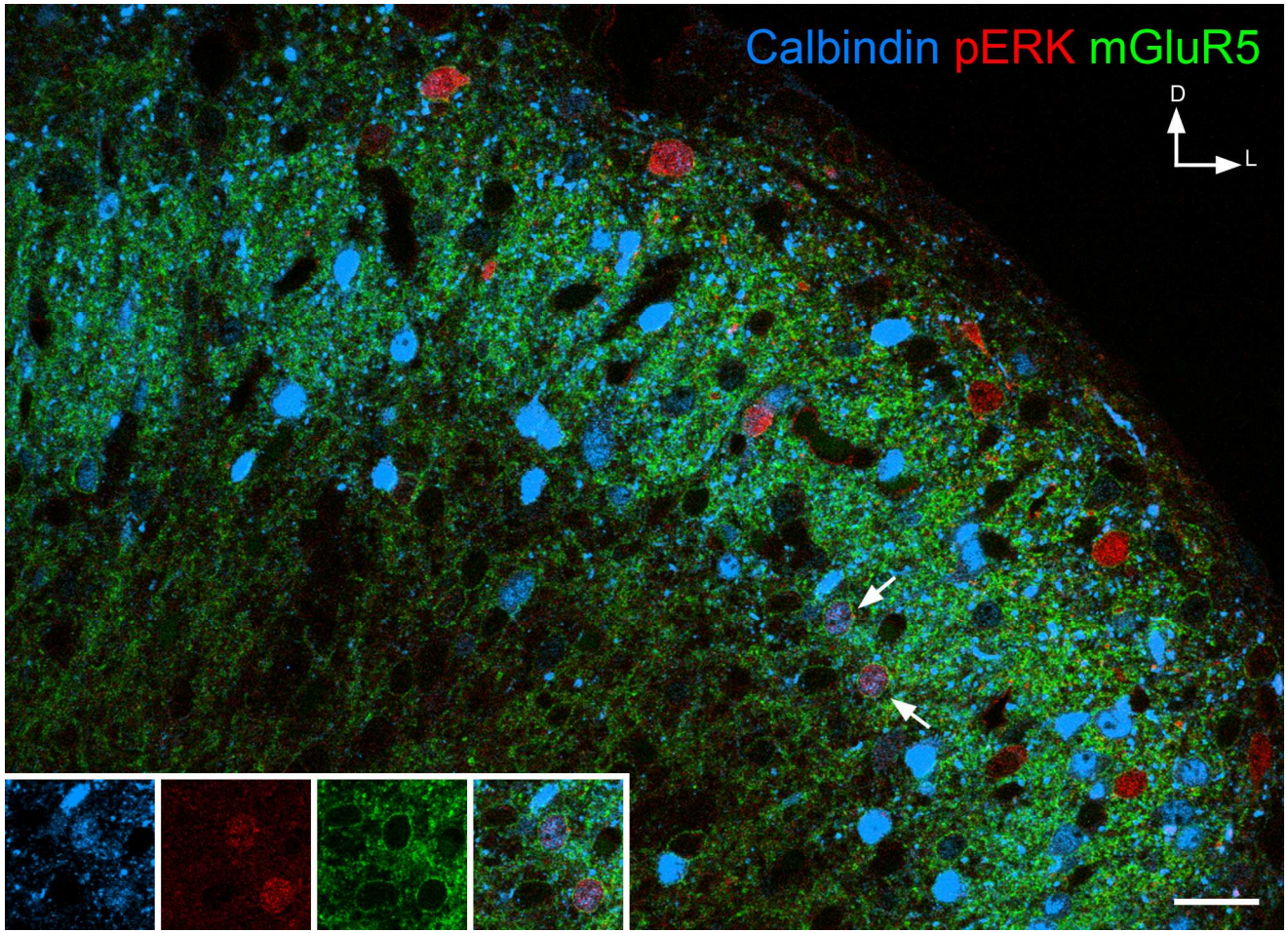

Calbindin pERK mGluR5
D
L

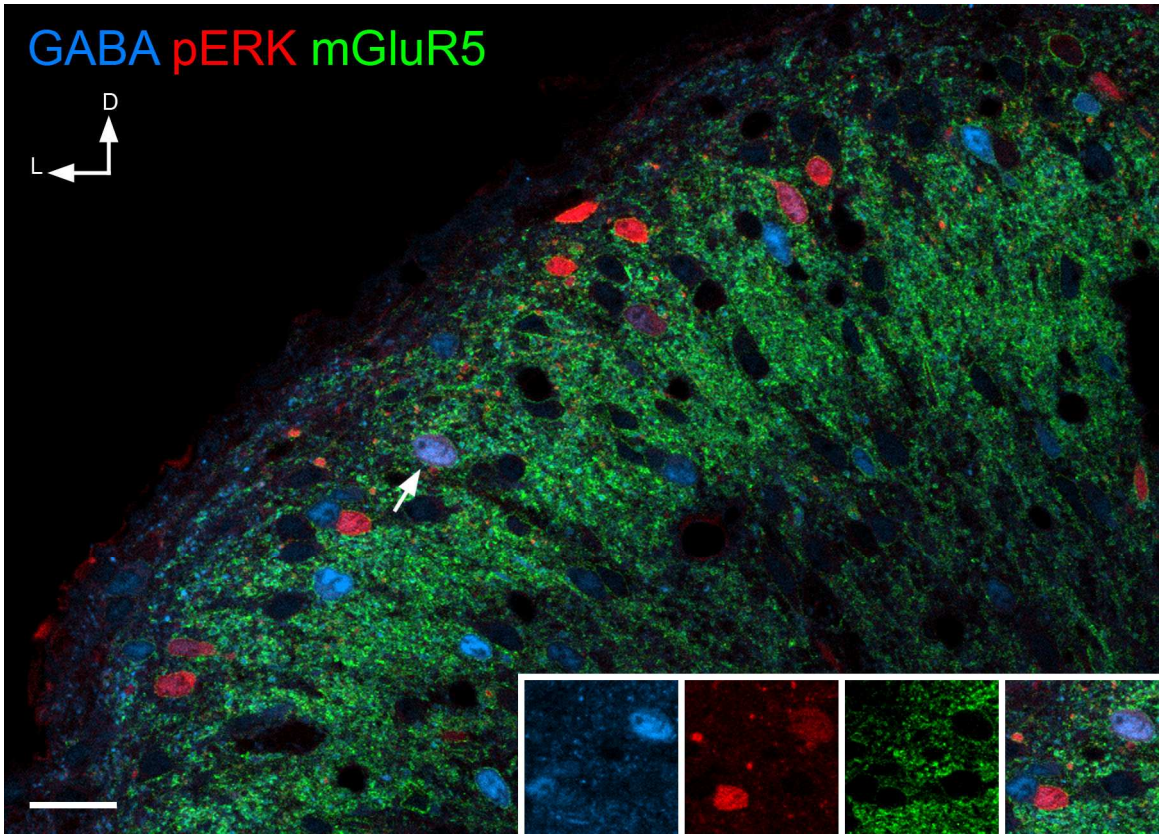
Table 5-4 GABA-immunostaning in $mGlu_5$-expressing cells and in pERK+ $mGlu_5$-expressing neurons after DHPG application

Experiment	Spinal segments	Number of $mGlu_5$-expressing cells that are positive for GABA	Number of $pERK^+$ $mGlu_5$-expressing cells that are positive for GABA	*P* value
1	L4-L5	58/180 (32)	109/130 (84)	
2	L4-L5	75/175 (43)	73/93 (78)	
3	L3-L4	46/159 (31)	85/115 (74)	
Total		**179/505 (35)**	**267/338 (79)**	***P*<0.001**

In each case, proportions from the 3 animals are shown, with percentages in brackets. The table also shows a significant difference between the expression of GABA in $mGlu_5$-immnuoreactive cells and in pERK+ $mGlu_5$-expressing cells (*P*<0.001, Chi-square test).

Figure 5-5 Examples of GABA-immunoreactivity in pERK$^+$ mGlu$_5$-expressing cells following DHPG application.

This figure shows a projected confocal image stack from a transverse section through the L4 segment. Numerous pERK-positive mGlu$_5$-expressing neurons cells are seen 8 minutes after DHPG application. The arrow shows a pERK$^+$ mGlu$_5$-expressing cell, which is also positive for GABA. The inset shows an enlarged image of the GABA-containing pERK$^+$ mGlu$_5$-expressing neuron. Images are projections of 10 optical horizontal sections at 2 μm z-spacing. Scale bar=20 μm.


GABA pERK mGluR5
D
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5.4 Discussion

This part of the study reports that following spinal activation of group I mGlus, numerous pERK-positive cells were seen in lamina II of the spinal cord, and the great majority of these (88%) were $mGlu_5$-positive. It also shows that while 38% of lamina II $mGlu_5$-positive neurons contained calretinin, only 10% of these cells phosphorylated ERK after DHPG. Additionally, more than half of the $mGlu_5$-positive cells (57%) in lamina II are double-labelled with calbindin, but only 20% were also positive for pERK following DHPG. Despite the finding that 35% of the $mGlu_5$-expressing neurons were GABA-immunorective, a high proportion (79%) of the $pERK^+$ $mGlu_5$-expressing neurons was positive for GABA after DHPG. Overall, lamina II $mGlu_5$-expressing neurons contain various proportions of calretinin, calbindin and GABA. However, activating these cells phosphorylated ERK in a lower proportion of $mGlu_5$-expressing excitatory interneurons ($calretinin^+$ and/or $calbindin^+$), and in a higher proportion of $mGlu_5$-expressing inhibitory interneurons ($GABA^+$). This suggests that $mGlu_5$-expressing cells, when activated, play a complex role in processing nociceptive information.

5.4.1 Spinal $mGlu_5$-expressing neurons

Although it has been well established that ionotropic glutamate receptors are involved in the transmission and mediation of spinal nociception (Yamamoto and Yaksh, 1992; Kristensen *et al.*, 1994; Meller *et al.*, 1996), the role of spinal mGlus in processing nociceptive information is not yet fully understood.

The involvement of spinal mGlus in nociception was initially supported by pharmacological studies that demonstrated the effects of applying non specific AMPA/mGlu agonists such as quisqualic acid into the lumbar spinal cord (Aanonsen *et al.*, 1990; Sun and Larson, 1991). Subsequently, selective mGlu agonists and antagonists were available, and these helped in exploring the specific roles of mGlu groups, as well as the roles of each member of these groups. For instance, it has been reported that intrathecal application of DHPG ($mGlu_{1/5}$ receptor agonist) in rats induced spontaneous nociceptive behaviours and enhanced formalin-evoked nociception in the 2^{nd} phase of the formalin test (Fisher and Coderre, 1996a, 1996b). DHPG also led to thermal hyperalgesia (persistent decrease in noxious heat response latency), mechanical hyperalgesia

(vigorous nociceptive responses to tail pinch) and mechanical allodynia (a reduction in the 50% response threshold to von Frey hairs) in rats (Fisher and Coderre, 1998). In addition, it was reported that the application of relatively selective group I mGlu antagonists [(S)-4-carboxyphenylglycine or (S)-4-carboxy-3-hydroxyphenylglycine] reduced nociceptive responses in the 2nd phase of the formalin test (Fisher and Coderre, 1996b). Furthermore, antisense knockdown of spinal $mGlu_1$ in rats decreased neuronal responses to topical mustard oil application (Young *et al.*, 1998), and also attenuated thermal hyperalgesia and mechanical allodynia in a rat model of chronic inflammation (Fundytus *et al.*, 2002). Together, the above findings suggest a role of group I mGlus in spinal modulation of nociception.

As described previously, one of the established cellular mechanisms of action of group I mGlus are through their activation of phospholipase C, which leads to the release of intracellular calcium and subsequently the activation of PKC (Masu *et al.*, 1991; Abe *et al.*, 1992). Recent studies also showed that group I mGlus can activate other pathways such as the ERK/MAPK cascade, which has been shown to be involved in nociception (Ji *et al.*, 1999; Karim *et al.*, 2001; Adwanikar *et al.*, 2004). Karim *et al.* (2001) reported that intrathecal application of DHPG into the mouse spinal cord led to spontaneous nociceptive behaviours, which were attenuated by the administration of selective $mGlu_1$ or $mGlu_5$ antagonists. Immunoblot analysis of the mouse spinal cord after DHPG administration showed a dose-dependent increase in phosphorylation of ERK1 and ERK2, with stronger activation of ERK2. The doses of DHPG that were required to activate ERK were similar to those that induced nociceptive behaviours, suggesting that group I mGlu activation leads to an ERK-dependent nociceptive behaviour in mice. This suggestion was further confirmed in a subsequent study by Adwanikar *et al.* (2004) that showed that DHPG-induced nociceptive behaviours were attenuated after the pretreatment with a MEK inhibitor, which blocks the activation of ERK.

Karim *et al.* (2001) also reported that $mGlu_5$ colocalised with pERK in the SDH after DHPG administration, however, they did not attempt to quantify these $pERK^+$ $mGlu_5$–expressing neurons. The present study, on the other hand, demonstrates the proportion of these cells and further confirms the involvement of group I mGlus as an upstream activator for ERK in rats. Results from this study show that the spinal application of DHPG led to the expression of high levels of

pERK, mostly in lamina II. The majority (88%) of these pERK-positive cells were $mGlu_5$-immunoreactive, suggesting a direct involvement of $mGlu_5$ in activating ERK, rather than $mGlu_5$ indirectly acting on other dorsal horn receptors and subsequently activating ERK.

The present study also reports that a low proportion (12%) of the pERK-positive cells did not express $mGlu_5$ after DHPG. One possible technical reason for this is that the $mGlu_5$ antibody used in this study detects only one splice variant of $mGlu_5$: $mGlu_{5a}$ or $mGlu_{5b}$, suggesting that some $mGlu_5$-expressing cells were not detected. However, this possibility is ruled out since it is found that the $mGlu_5$ antibody used detects the last 28 amino acids from the C terminal, which are the similar in both splice variants: $mGlu_{5a}$ and $mGlu_{5b}$ (Joly *et al.*, 1995). Another explanation for the presence of these pERK-positive cells that lacked $mGlu_5$ is that they could be activated by nearby $pERK^+$ $mGlu_5$–expressing interneurons. It can also be attributed to the fact that DHPG is a non-specific group I agonist, which activates both $mGlu_1$ and $mGlu_5$, and therefore, suggests that these $pERK^+$ $mGlu_5$-negative cells may express $mGlu_1$. This suggestion is further supported by the finding of Karim *et al.* (2001), who showed that both $mGlu_1$ and $mGlu_5$ antagonists attenuated DHPG-induced pain behaviours. However, the distribution of $mGlu_{1a}$ and $mGlu_{5a/b}$ has been described above, and $mGlu_{1a}$ is found mainly in deep dorsal horn, compared to the expression of $mGlu_{5a/b}$ which is concentrated in the SDH (Valerio *et al.*, 1997; Alvarez *et al.*, 2000). It is therefore unlikely that those $mGlu_5$ lacking pERK-positive cells express $mGlu_{1a}$. In support of the above finding, Karim *et al.* (2001) reacted mouse spinal cord sections with a selective antibody against $mGlu_{1a}$ and reported no detectable $mGlu_{1a}$-immunostaining in the SDH. Another explanation for these pERK-positive cells that neither expressed $mGlu_{5a/b}$ nor $mGlu_{1a}$ is that they express other known splice variants of $mGlu_1$ such as $mGlu_{1b}$ and $mGlu_{1d}$. Alvarez *et al.* (2000) also reported the expression of low levels of $mGlu_{1b}$ in lamina II in the rat. Additionally, Karim *et al.* (2001) reported the presence of 2 RNA splice variants of $mGlu_1$ in the spinal cord dorsal horn of the mouse: $mGlu_{1b}$ and $mGlu_{1d}$.

Recent studies have demonstrated the involvement of activated ERK in phosphorylating the pore-forming subunit of the potassium channel Kv4.2, which is a major contributor to A-type K^+ currents (Adams *et al.*, 2000; Hu *et al.*, 2003; Ji *et al.*, 2003; Hu *et al.*, 2006). The down regulation of these A-type currents is

known to enhance neuronal activity (Hu and Gereau, 2003; Hu *et al.*, 2003). Subsequently, Hu *et al.* (2007) showed that ERK-mediated phosphorylation of Kv4.2 is downstream of $mGlu_5$ activation. Their study examined the effects of DHPG on action potentials evoked by depolarising current injection in spinal cord dorsal horn neurons in culture and in acute slices from mice. DHPG application decreased first spike latency and increased spike frequency. Their study also showed that DHPG decreased A-type currents in dorsal horn neurons from wild-type mice, but not in those from Kv4.2 knock-out mice. This DHPG-induced inhibition of A-type currents was blocked by ERK inhibitors. Additionally Hu *et al.* (2007) reported that 71% of DHPG-induced $pERK^+$ neurons from three different cultures were also positive for Kv4.2, and all Kv4.2-expressing neurons contained pERK. It was also found that the blockade of $mGlu_5$, but not $mGlu_1$, activation completely abolished the modulation of A-type K^+ currents and neuronal excitability that were seen following DHPG application. Their study concluded that $mGlu_5$, but not $mGlu_1$, modulates A-type currents in the SDH, while the mechanism that explains the contribution of $mGlu_1$ to nociceptive behaviour remains unclear.

It has been reported (Ji *et al.*, 1999; Polgar *et al.*, 2007) and also shown in the previous Chapter that ERK is phosphorylated in the SDH following noxious stimulation. The present study shows that many of the $mGlu_5$-expressing neurons in the SDH did not phosphorylate ERK following DHPG. The reason for the presence of these $pERK^-$ $mGlu_5$-expressing neurons is not clear. It may be duo to the possibility that these cells express non-functioning $mGlu_5$, or that DHPG activates these $pERK^-$ $mGlu_5$-expressing cells through different pathways other than the MAPK/ERK cascade.

5.4.2 pERK in $mGlu_5$-expressing excitatory and inhibitory neurons

Lamina II contains both inhibitory and excitatory interneurons, and these play an important role in modulating nociception, by their action on neighbouring interneurons or on projection neurons in neighbouring laminae (I and III-IV). Calcium binding proteins have been used as markers for excitatory (calretinin, calbindin) or inhibitory (parvalbumin) neurons, as described in the first Chapter.

The present study is the first to report that one-third of lamina II $mGlu_5$-expressing neurons contain calretinin, and more than half of the $mGlu_5$-positive cells in lamina II are double-labelled with calbindin. Both calretinin and calbindin are expressed by excitatory neurons, and Albuquerque *et al*. (1999) reported that the majority of calbindin- calretinin-containing neurons were not reactive for either GABA or glycine. Antal *et al*. (1991) also showed that most of the calbindin-positive cells in the SDH were not GABAergic, which further confirms that these calretinin- calbindin-expressing $mGlu_5^+$ neurons are more likely to be glutamatergic.

Polgar *et al*. (2003) reported that one-third of lamina II interneurons of the rat were inhibitory, containing GABA. The present study shows that 35% of the $mGlu_5$-expressing neurons are also GABA-immunoreactive, suggesting that $mGlu_5$ is expressed on both excitatory and inhibitory neurons in lamina II. Jia *et al*. (1999) used electron microscopy to investigate colocalisation of GABA and $mGlu_5$ in SDH neurons of the rat. They reported that about half of the vesicle-containing profiles immunostained for $mGlu_5$ were stained for GABA.

Hu and Gereau (2011) recently showed that only 16% of $mGlu_5$-expressing cells also expressed GAD67-GFP (inhibitory cells marker) in cultured neurons. This discrepancy between the present study and that of Hu and Gereau (2011) may be attributed to the different inhibitory markers used, since an unknown proportion (estimated between 35% and 67%) of GAD67-GFP neurons represent GABAergic neurons (Heinke *et al.*, 2004; Dougherty *et al.*, 2009). However, GABA, which is the marker of choice in this study, presumably labels all inhibitory neurons. Another reason of this difference is that Hu and Gereau (2011) results were based on a study done in cultured neurons, which may differ in their responses compared to the responses of dorsal horn neurons of rats (*in vivo*).

Regarding the phenotype of these DHPG-induced $pERK^+$ $mGlu_5$-expressing cells, results from the present study show that 10 and 20% of these cells contained calretinin and calbindin, respectively. The majority (79%) of $pERK^+$ $mGlu_5$-expressing neurons were GABAergic. The above percentages add up to more than a 100%, however, it has been shown that calretinin and calbindin expression patterns in the SDH partially overlap (Albuquerque *et al.*, 1999). The finding from the present study that a proportion of $pERK^+$ $mGlu_5$-expressing cells were

excitatory is in agreement with the results by Huang *et al*. (2005) and Hu and Gereau (2011). Huang *et al*. (2005) reported that almost all Kv4.3-positive cells (which also express Kv4.2) in lamina II of the rat expressed $mGlu_5$, and Kv4.3 was expressed in a subset of lamina II calretinin$^+$ (calbindin$^-$ and PKCγ^-) excitatory neurons. Hu and Gereau (2011) recently showed in cultured dorsal horn neuron that Kv4.2-containing potassium channels, which were modulated by $mGlu_5$-ERK signalling as described above, were found primarily in excitatory cells, since these cells expressed VGLUT1/2, PKCγ and NK1r.

Results from the present study suggest that the pronociceptive effect of activating $mGlu_5$ in the spinal cord is likely to involve a direct action of a subset of $mGlu_5$-expressing excitatory interneurons on projection neurons. This effect may also involve an indirect inhibition of local inhibitory interneurons by $mGlu_5$-expressing inhibitory cells, a process of disinhibition. However, the finding that DHPG induced pERK expression in a high proportion of $mGlu_5$-expressing inhibitory cells speculates that this group of cells may be involved in inhibiting other spinal pathways such as itch, rather than their indirect involvement in nociception (Ross *et al.*, 2010; Ross, 2011). This part of the project helps in understanding the various mechanisms that underlie the role of $mGlu_5$ in the spinal cord. It also shows that there is a balance in the dorsal horn between inhibition and excitation, resulting in accurately modulating nociceptive information. Further studies investigating other mechanisms of actions of group I mGlus in the spinal cord are required.

6. Concluding remarks

This thesis highlighted some important aspects regarding different populations of spinal cord dorsal horn neurons and their role in nociception. Initially it aimed to assess whether NK1r-expressing projection neurons can be distinguished from NK1r-expressing interneurons, in lamina I of the spinal cord, based on their soma sizes. It showed that cell bodies of NK1r-immunoreactive neurons in lamina I had a clear bimodal size distribution that is related to the presence or absence of retrograde labelling from CVLM and/or LPb. It found that nearly all of the NK1r-immunoreactive cells that were not retrogradely labelled had soma cross-sectional areas that were less than 200 μm^2. In contrast, less than 10% of the retrogradely labelled NK1r-positive cells had cell bodies smaller than 200 μm^2. An important practical outcome from the above results is that soma size can be used to identify putative lamina I NK1r-expressing projection neurons in studies that have not used retrograde tracing.

Results from the 1st part were then used to assess the responses of putative lamina I NK1r-expressing interneurons and projection neurons (cell bodies >200 μm^2) to various noxious stimuli. It reported that both lamina I NK1r-expressing projection cells and interneurons phosphorylated ERK after noxious cutaneous, deep and visceral stimulation with significantly more of the projection neurons were pERK-positive compared to the interneurons. It also showed that there was no significant difference in the responses of various morphological classes of lamina I NK1r-expressing projection cells to different types of noxious stimuli. The 2nd part of the project also showed that other populations of projection neurons (laminae III-IV NK1r-expressing neurons and lamina I giant cells) in the SDH phosphorylated ERK following noxious stimulation. Few laminae III-IV NK1r-expressing cells were pERK-positive after noxious deep and visceral stimulation and these were very weakly labelled. Most of lamina I giant cells contained pERK after noxious cutaneous stimulation, and a lower proportion of these cells phosphorylated ERK after noxious deep stimulation. These results indicate that different types of projection neurons have different roles in conveying nociceptive messages.

The 3rd and final part investigated ERK phosphorylation in $mGlu_5$-expressing cells following spinal DHPG application. It showed that following spinal activation of group I mGlus, numerous pERK-positive cells were seen in lamina II of the spinal

cord, and the great majority of these were $mGlu_5$-positive. It also reported that lamina II $mGlu_5$-expressing neurons contained various proportions of calretinin, calbindin and GABA. However, activating these cells phosphorylated ERK in a lower proportion of $mGlu_5$-expressing excitatory neurons (calretinin$^+$ and/or calbindin$^+$), and in a higher proportion of $mGlu_5$-expressing inhibitory cells (GABA$^+$). This suggests that $mGlu_5$-expressing cells, when activated, play a complex role in processing nociceptive information.

List of references

Aanonsen LM, Lei S & Wilcox GL. (1990). Excitatory amino acid receptors and nociceptive neurotransmission in rat spinal cord. *Pain* **41,** 309-321.

Abbadie C, Honore P, Fournie-Zaluski MC, Roques BP & Besson JM. (1994). Effects of opioids and non-opioids on c-Fos-like immunoreactivity induced in rat lumbar spinal cord neurons by noxious heat stimulation. *Eur J Pharmacol* **258,** 215-227.

Abbadie C, Trafton J, Liu H, Mantyh PW & Basbaum AI. (1997). Inflammation increases the distribution of dorsal horn neurons that internalize the neurokinin-1 receptor in response to noxious and non-noxious stimulation. *J Neurosci* **17,** 8049-8060.

Abe T, Sugihara H, Nawa H, Shigemoto R, Mizuno N & Nakanishi S. (1992). Molecular characterization of a novel metabotropic glutamate receptor mGluR5 coupled to inositol phosphate/Ca2+ signal transduction. *J Biol Chem* **267,** 13361-13368.

Adams JP, Anderson AE, Varga AW, Dineley KT, Cook RG, Pfaffinger PJ & Sweatt JD. (2000). The A-type potassium channel Kv4.2 is a substrate for the mitogen-activated protein kinase ERK. *J Neurochem* **75,** 2277-2287.

Adwanikar H, Karim F & Gereau RWt. (2004). Inflammation persistently enhances nocifensive behaviors mediated by spinal group I mGluRs through sustained ERK activation. *Pain* **111,** 125-135.

Afrah AW, Stiller CO, Olgart L, Brodin E & Gustafsson H. (2001). Involvement of spinal N-methyl-D-aspartate receptors in capsaicin-induced in vivo release of substance P in the rat dorsal horn. *Neurosci Lett* **316,** 83-86.

Aihara Y, Mashima H, Onda H, Hisano S, Kasuya H, Hori T, Yamada S, Tomura H, Yamada Y, Inoue I, Kojima I & Takeda J. (2000). Molecular cloning of a novel brain-type Na(+)-dependent inorganic phosphate cotransporter. *J Neurochem* **74,** 2622-2625.

Akintunde A & Buxton DF. (1992). Quadruple labeling of brain-stem neurons: a multiple retrograde fluorescent tracer study of axonal collateralization. *J Neurosci Methods* **45,** 15-22.

Akiyama T, Merrill AW, Carstens MI & Carstens E. (2009). Activation of superficial dorsal horn neurons in the mouse by a PAR-2 agonist and 5-HT: potential role in itch. *J Neurosci* **29,** 6691-6699.

Al-Khater KM, Kerr R & Todd AJ. (2008). A quantitative study of spinothalamic neurons in laminae I, III, and IV in lumbar and cervical segments of the rat spinal cord. *J Comp Neurol* **511,** 1-18.

Al-Khater KM & Todd AJ. (2009). Collateral projections of neurons in laminae I, III, and IV of rat spinal cord to thalamus, periaqueductal gray matter, and lateral parabrachial area. *J Comp Neurol* **515,** 629-646.

Albuquerque C, Lee CJ, Jackson AC & MacDermott AB. (1999). Subpopulations of GABAergic and non-GABAergic rat dorsal horn neurons express Ca2+-permeable AMPA receptors. *Eur J Neurosci* **11,** 2758-2766.

Allen BJ, Rogers SD, Ghilardi JR, Menning PM, Kuskowski MA, Basbaum AI, Simone DA & Mantyh PW. (1997). Noxious cutaneous thermal stimuli induce a graded release of endogenous substance P in the spinal cord: imaging peptide action in vivo. *J Neurosci* **17,** 5921-5927.

Almarestani L, Waters SM, Krause JE, Bennett GJ & Ribeiro-da-Silva A. (2007). Morphological characterization of spinal cord dorsal horn lamina I neurons projecting to the parabrachial nucleus in the rat. *J Comp Neurol* **504,** 287-297.

Almarestani L, Waters SM, Krause JE, Bennett GJ & Ribeiro-da-Silva A. (2009). De novo expression of the neurokinin 1 receptor in spinal lamina I pyramidal neurons in polyarthritis. *J Comp Neurol* **514,** 284-295.

Alvarez FJ, Villalba RM, Carr PA, Grandes P & Somohano PM. (2000). Differential distribution of metabotropic glutamate receptors 1a, 1b, and 5 in the rat spinal cord. *J Comp Neurol* **422,** 464-487.

Alvarez FJ, Villalba RM, Zerda R & Schneider SP. (2004). Vesicular glutamate transporters in the spinal cord, with special reference to sensory primary afferent synapses. *J Comp Neurol* **472,** 257-280.

Anderson LE & Seybold VS. (2000). Phosphorylated cAMP response element binding protein increases in neurokinin-1 receptor-immunoreactive neurons in rat spinal cord in response to formalin-induced nociception. *Neurosci Lett* **283,** 29-32.

Andrew D. (2009). Sensitization of lamina I spinoparabrachial neurons parallels heat hyperalgesia in the chronic constriction injury model of neuropathic pain. *J Physiol* **587,** 2005-2017.

Andrew D. (2010). Quantitative characterization of low-threshold mechanoreceptor inputs to lamina I spinoparabrachial neurons in the rat. *J Physiol* **588,** 117-124.

Andrew D & Craig AD. (2001). Spinothalamic lamina I neurons selectively sensitive to histamine: a central neural pathway for itch. *Nat Neurosci* **4,** 72-77.

Andrew D & Craig AD. (2002). Quantitative responses of spinothalamic lamina I neurones to graded mechanical stimulation in the cat. *J Physiol* **545,** 913-931.

Antal M, Polgar E, Chalmers J, Minson JB, Llewellyn-Smith I, Heizmann CW & Somogyi P. (1991). Different populations of parvalbumin- and calbindin-D28k-immunoreactive neurons contain GABA and accumulate 3H-D-aspartate in the dorsal horn of the rat spinal cord. *J Comp Neurol* **314,** 114-124.

Applebaum AE, Beall JE, Foreman RD & Willis WD. (1975). Organization and receptive fields of primate spinothalamic tract neurons. *J Neurophysiol* **38,** 572-586.

Banks WA, Trentman TL, Kastin AJ & Galina ZH. (1988). The general anesthesia induced by various drugs differentially affects analgesia and its variability. *Pharmacol Biochem Behav* **31,** 397-403.

Beaudry H, Dubois D & Gendron L. (2011). Activation of Spinal {micro}- and {delta}-Opioid Receptors Potently Inhibits Substance P Release Induced by Peripheral Noxious Stimuli. *J Neurosci* **31,** 13068-13077.

Becker CM, Hoch W & Betz H. (1989). Sensitive immunoassay shows selective association of peripheral and integral membrane proteins of the inhibitory glycine receptor complex. *J Neurochem* **53,** 124-131.

Bennett DL, Dmietrieva N, Priestley JV, Clary D & McMahon SB. (1996). trkA, CGRP and IB4 expression in retrogradely labelled cutaneous and visceral primary sensory neurones in the rat. *Neurosci Lett* **206,** 33-36.

Bennett GJ, Abdelmoumene M, Hayashi H & Dubner R. (1980). Physiology and morphology of substantia gelatinosa neurons intracellularly stained with horseradish peroxidase. *J Comp Neurol* **194,** 809-827.

Bernard JF & Besson JM. (1990). The spino(trigemino)pontoamygdaloid pathway: electrophysiological evidence for an involvement in pain processes. *J Neurophysiol* **63,** 473-490.

Bernard JF, Dallel R, Raboisson P, Villanueva L & Le Bars D. (1995). Organization of the efferent projections from the spinal cervical enlargement to the parabrachial area and periaqueductal gray: a PHA-L study in the rat. *J Comp Neurol* **353,** 480-505.

Berthele A, Boxall SJ, Urban A, Anneser JM, Zieglgansberger W, Urban L & Tolle TR. (1999). Distribution and developmental changes in metabotropic glutamate receptor messenger RNA expression in the rat lumbar spinal cord. *Brain Res Dev Brain Res* **112,** 39-53.

Bessou P, Burgess PR, Perl ER & Taylor CB. (1971). Dynamic properties of mechanoreceptors with unmyelinated (C) fibers. *J Neurophysiol* **34,** 116-131.

Bessou P & Perl ER. (1969). Response of cutaneous sensory units with unmyelinated fibers to noxious stimuli. *J Neurophysiol* **32,** 1025-1043.

Bester H, Chapman V, Besson JM & Bernard JF. (2000). Physiological properties of the lamina I spinoparabrachial neurons in the rat. *J Neurophysiol* **83,** 2239-2259.

Bester H, Matsumoto N, Besson JM & Bernard JF. (1997). Further evidence for the involvement of the spinoparabrachial pathway in nociceptive processes: a c-Fos study in the rat. *J Comp Neurol* **383,** 439-458.

Bester H, Menendez L, Besson JM & Bernard JF. (1995). Spino (trigemino) parabrachiohypothalamic pathway: electrophysiological evidence for an involvement in pain processes. *J Neurophysiol* **73,** 568-585.

Bice TN & Beal JA. (1997a). Quantitative and neurogenic analysis of the total population and subpopulations of neurons defined by axon projection in the superficial dorsal horn of the rat lumbar spinal cord. *J Comp Neurol* **388,** 550-564.

Bice TN & Beal JA. (1997b). Quantitative and neurogenic analysis of neurons with supraspinal projections in the superficial dorsal horn of the rat lumbar spinal cord. *J Comp Neurol* **388,** 565-574.

Bjorkman R, Hallman KM, Hedner J, Hedner T & Henning M. (1994). Acetaminophen blocks spinal hyperalgesia induced by NMDA and substance P. *Pain* **57,** 259-264.

Bleakman D, Rusin KI, Chard PS, Glaum SR & Miller RJ. (1992). Metabotropic glutamate receptors potentiate ionotropic glutamate responses in the rat dorsal horn. *Mol Pharmacol* **42,** 192-196.

Bleazard L, Hill RG & Morris R. (1994). The correlation between the distribution of the NK1 receptor and the actions of tachykinin agonists in the dorsal horn of the rat indicates that substance P does not have a functional role on substantia gelatinosa (lamina II) neurons. *J Neurosci* **14,** 7655-7664.

Bond A & Lodge D. (1995). Pharmacology of metabotropic glutamate receptor-mediated enhancement of responses to excitatory and inhibitory amino acids on rat spinal neurones in vivo. *Neuropharmacology* **34,** 1015-1023.

Boscan P & Paton JF. (2001). Role of the solitary tract nucleus in mediating nociceptive evoked cardiorespiratory responses. *Auton Neurosci* **86,** 170-182.

Boulton TG, Nye SH, Robbins DJ, Ip NY, Radziejewska E, Morgenbesser SD, DePinho RA, Panayotatos N, Cobb MH & Yancopoulos GD. (1991). ERKs: a family of protein-serine/threonine kinases that are activated and tyrosine phosphorylated in response to insulin and NGF. *Cell* **65,** 663-675.

Bourane S, Garces A, Venteo S, Pattyn A, Hubert T, Fichard A, Puech S, Boukhaddaoui H, Baudet C, Takahashi S, Valmier J & Carroll P. (2009). Low-threshold mechanoreceptor subtypes selectively express MafA and are specified by Ret signaling. *Neuron* **64,** 857-870.

Boxall SJ, Berthele A, Laurie DJ, Sommer B, Zieglgansberger W, Urban L & Tolle TR. (1998). Enhanced expression of metabotropic glutamate receptor 3 messenger RNA in the rat spinal cord during ultraviolet irradiation induced peripheral inflammation. *Neuroscience* **82,** 591-602.

Bredt DS, Glatt CE, Hwang PM, Fotuhi M, Dawson TM & Snyder SH. (1991). Nitric oxide synthase protein and mRNA are discretely localized in neuronal

populations of the mammalian CNS together with NADPH diaphorase. *Neuron* **7,** 615-624.

Brown AG. (1981). *Organization in the Spinal Cord: the Anatomy and Physiology of Identified Neurons*. New York, Springer Verlag.

Brown AG & Franz DN. (1969). Responses of spinocervical tract neurones to natural stimulation of identified cutaneous receptors. *Exp Brain Res* **7,** 231-249.

Brown JL, Liu H, Maggio JE, Vigna SR, Mantyh PW & Basbaum AI. (1995). Morphological characterization of substance P receptor-immunoreactive neurons in the rat spinal cord and trigeminal nucleus caudalis. *J Comp Neurol* **356,** 327-344.

Brumovsky P, Watanabe M & Hokfelt T. (2007). Expression of the vesicular glutamate transporters-1 and -2 in adult mouse dorsal root ganglia and spinal cord and their regulation by nerve injury. *Neuroscience* **147,** 469-490.

Budai D & Larson AA. (1998). The involvement of metabotropic glutamate receptors in sensory transmission in dorsal horn of the rat spinal cord. *Neuroscience* **83,** 571-580.

Bullitt E. (1990). Expression of c-fos-like protein as a marker for neuronal activity following noxious stimulation in the rat. *J Comp Neurol* **296,** 517-530.

Bullitt E. (1991). Somatotopy of spinal nociceptive processing. *J Comp Neurol* **312,** 279-290.

Burgess PR & Perl ER. (1967). Myelinated afferent fibres responding specifically to noxious stimulation of the skin. *J Physiol* **190,** 541-562.

Buritova J & Besson JM. (2000). Effects of flurbiprofen and its enantiomers on the spinal c-Fos protein expression induced by noxious heat stimuli in the anaesthetized rat. *Eur J Pharmacol* **406,** 59-67.

Burke RE. (2007). Sir Charles Sherrington's the integrative action of the nervous system: a centenary appreciation. *Brain* **130,** 887-894.

Burnashev N, Monyer H, Seeburg PH & Sakmann B. (1992). Divalent ion permeability of AMPA receptor channels is dominated by the edited form of a single subunit. *Neuron* **8,** 189-198.

Burstein R, Dado RJ & Giesler GJ, Jr. (1990). The cells of origin of the spinothalamic tract of the rat: a quantitative reexamination. *Brain Res* **511,** 329-337.

Campbell JN & Meyer RA. (1983). Sensitization of unmyelinated nociceptive afferents in monkey varies with skin type. *J Neurophysiol* **49,** 98-110.

Cao YQ, Mantyh PW, Carlson EJ, Gillespie AM, Epstein CJ & Basbaum AI. (1998). Primary afferent tachykinins are required to experience moderate to intense pain. *Nature* **392,** 390-394.

Caputi F, Spaziante R, de Divitiis E & Nashold BS, Jr. (1995). Luigi Rolando and his pioneering efforts to relate structure to function in the nervous system. *J Neurosurg* **83,** 933-937.

Caterina MJ & Julius D. (2001). The vanilloid receptor: a molecular gateway to the pain pathway. *Annu Rev Neurosci* **24,** 487-517.

Caterina MJ, Leffler A, Malmberg AB, Martin WJ, Trafton J, Petersen-Zeitz KR, Koltzenburg M, Basbaum AI & Julius D. (2000). Impaired nociception and pain sensation in mice lacking the capsaicin receptor. *Science* **288,** 306-313.

Caterina MJ, Schumacher MA, Tominaga M, Rosen TA, Levine JD & Julius D. (1997). The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature* **389,** 816-824.

Catheline G, Le Guen S & Besson JM. (1999). Effects of U-69,593, a kappa-opioid receptor agonist, on carrageenin-induced peripheral oedema and Fos expression in the rat spinal cord. *Eur J Pharmacol* **370,** 287-296.

Cavanaugh DJ, Chesler AT, Braz JM, Shah NM, Julius D & Basbaum AI. (2011). Restriction of transient receptor potential vanilloid-1 to the peptidergic subset of primary afferent neurons follows its developmental downregulation in nonpeptidergic neurons. *J Neurosci* **31,** 10119-10127.

Cechetto DF, Standaert DG & Saper CB. (1985). Spinal and trigeminal dorsal horn projections to the parabrachial nucleus in the rat. *J Comp Neurol* **240,** 153-160.

Cervero F & Connell LA. (1984). Distribution of somatic and visceral primary afferent fibres within the thoracic spinal cord of the cat. *J Comp Neurol* **230,** 88-98.

Cervero F & Iggo A. (1980). The substantia gelatinosa of the spinal cord: a critical review. *Brain* **103,** 717-772.

Cervero F, Iggo A & Ogawa H. (1976). Nociceptor-driven dorsal horn neurones in the lumbar spinal cord of the cat. *Pain* **2,** 5-24.

Chang MM & Leeman SE. (1970). Isolation of a sialogogic peptide from bovine hypothalamic tissue and its characterization as substance P. *J Biol Chem* **245,** 4784-4790.

Chapman V, Buritova J, Honore P & Besson JM. (1996). Physiological contributions of neurokinin 1 receptor activation, and interactions with NMDA receptors, to inflammatory-evoked spinal c-Fos expression. *J Neurophysiol* **76,** 1817-1827.

Cheunsuang O & Morris R. (2000). Spinal lamina I neurons that express neurokinin 1 receptors: morphological analysis. *Neuroscience* **97,** 335-345.

Choi JI, Koehrn FJ & Sorkin LS. (2012). Carrageenan induced phosphorylation of Akt is dependent on neurokinin-1 expressing neurons in the superficial dorsal horn. *Mol Pain* **8,** 4.

Choi SS, Seo YJ, Kwon MS, Shim EJ, Lee JY, Ham YO, Park SH & Suh HW. (2005). Involvement of phosphorylated extracellular signal-regulated kinase in the mouse substance P pain model. *Brain Res Mol Brain Res* **137,** 152-158.

Choi SS, Seo YJ, Shim EJ, Kwon MS, Lee JY, Ham YO & Suh HW. (2006). Involvement of phosphorylated Ca2+/calmodulin-dependent protein kinase II and phosphorylated extracellular signal-regulated protein in the mouse formalin pain model. *Brain Res* **1108,** 28-38.

Christensen BN & Perl ER. (1970). Spinal neurons specifically excited by noxious or thermal stimuli: marginal zone of the dorsal horn. *J Neurophysiol* **33,** 293-307.

Chung K, Lee WT & Carlton SM. (1988). The effects of dorsal rhizotomy and spinal cord isolation on calcitonin gene-related peptide-labeled terminals in the rat lumbar dorsal horn. *Neurosci Lett* **90,** 27-32.

Clark RG. (1984). Anatomy of the mammalian cord. In *Handbook of the spinal cord*, ed. A. DR, pp. 1-45.

Conn PJ & Pin JP. (1997). Pharmacology and functions of metabotropic glutamate receptors. *Annu Rev Pharmacol Toxicol* **37,** 205-237.

Craig AD. (1995). Distribution of brainstem projections from spinal lamina I neurons in the cat and the monkey. *J Comp Neurol* **361,** 225-248.

Craig AD, Heppelmann B & Schaible HG. (1988). The projection of the medial and posterior articular nerves of the cat's knee to the spinal cord. *J Comp Neurol* **276,** 279-288.

Craig AD & Kniffki KD. (1985). Spinothalamic lumbosacral lamina I cells responsive to skin and muscle stimulation in the cat. *J Physiol* **365,** 197-221.

Cruz CD & Cruz F. (2007). The ERK 1 and 2 pathway in the nervous system: from basic aspects to possible clinical applications in pain and visceral dysfunction. *Curr Neuropharmacol* **5,** 244-252.

Cruz CD, Ferreira D, McMahon SB & Cruz F. (2007). The activation of the ERK pathway contributes to the spinal c-fos expression observed after noxious bladder stimulation. *Somatosens Mot Res* **24,** 15-20.

Cruz CD, McMahon SB & Cruz F. (2006). Spinal ERK activation contributes to the regulation of bladder function in spinal cord injured rats. *Exp Neurol* **200,** 66-73.

Cruz CD, Neto FL, Castro-Lopes J, McMahon SB & Cruz F. (2005). Inhibition of ERK phosphorylation decreases nociceptive behaviour in monoarthritic rats. *Pain* **116,** 411-419.

Davidson S, Zhang X, Yoon CH, Khasabov SG, Simone DA & Giesler GJ, Jr. (2007). The itch-producing agents histamine and cowhage activate separate populations of primate spinothalamic tract neurons. *J Neurosci* **27,** 10007-10014.

Davis KD, Meyer RA & Campbell JN. (1993). Chemosensitivity and sensitization of nociceptive afferents that innervate the hairy skin of monkey. *J Neurophysiol* **69,** 1071-1081.

De Biasi S & Rustioni A. (1988). Glutamate and substance P coexist in primary afferent terminals in the superficial laminae of spinal cord. *Proc Natl Acad Sci U S A* **85,** 7820-7824.

De Felipe C, Herrero JF, O'Brien JA, Palmer JA, Doyle CA, Smith AJ, Laird JM, Belmonte C, Cervero F & Hunt SP. (1998). Altered nociception, analgesia and aggression in mice lacking the receptor for substance P. *Nature* **392,** 394-397.

Dhaka A, Earley TJ, Watson J & Patapoutian A. (2008). Visualizing cold spots: TRPM8-expressing sensory neurons and their projections. *J Neurosci* **28,** 566-575.

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

Djouhri L & Lawson SN. (2004). Abeta-fiber nociceptive primary afferent neurons: a review of incidence and properties in relation to other afferent A-fiber neurons in mammals. *Brain Res Brain Res Rev* **46,** 131-145.

Dougherty KJ, Sawchuk MA & Hochman S. (2009). Phenotypic diversity and expression of GABAergic inhibitory interneurons during postnatal development in lumbar spinal cord of glutamic acid decarboxylase 67-green fluorescent protein mice. *Neuroscience* **163,** 909-919.

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

Dougherty PM & Willis WD. (1991). Enhancement of spinothalamic neuron responses to chemical and mechanical stimuli following combined micro-iontophoretic application of N-methyl-D-aspartic acid and substance P. *Pain* **47,** 85-93.

Doyle CA & Hunt SP. (1999). Substance P receptor (neurokinin-1)-expressing neurons in lamina I of the spinal cord encode for the intensity of noxious stimulation: a c-Fos study in rat. *Neuroscience* **89,** 17-28.

Draisci G & Iadarola MJ. (1989). Temporal analysis of increases in c-fos, preprodynorphin and preproenkephalin mRNAs in rat spinal cord. *Brain Res Mol Brain Res* **6,** 31-37.

Drew GM, Lau BK & Vaughan CW. (2009). Substance P drives endocannabinoid-mediated disinhibition in a midbrain descending analgesic pathway. *J Neurosci* **29,** 7220-7229.

Duggan AW, Hendry IA, Morton CR, Hutchison WD & Zhao ZQ. (1988). Cutaneous stimuli releasing immunoreactive substance P in the dorsal horn of the cat. *Brain Res* **451,** 261-273.

Duggan AW, Morton CR, Zhao ZQ & Hendry IA. (1987). Noxious heating of the skin releases immunoreactive substance P in the substantia gelatinosa of the cat: a study with antibody microprobes. *Brain Res* **403,** 345-349.

Elliott KJ, Brodsky M, Hynansky AD, Foley KM & Inturrisi CE. (1995). Dextromethorphan suppresses both formalin-induced nociceptive behavior and the formalin-induced increase in spinal cord c-fos mRNA. *Pain* **61,** 401-409.

Feil K & Herbert H. (1995). Topographic organization of spinal and trigeminal somatosensory pathways to the rat parabrachial and Kolliker-Fuse nuclei. *J Comp Neurol* **353,** 506-528.

Fields HL, Basbaum AI & Heinricher MM. (2006). Central nervous system mechanisms of pain modulation. In *Wall and Melzack's Textbook of Pain*, ed. McMahon SB & Koltzenburg M.

Fisher K & Coderre TJ. (1996a). Comparison of nociceptive effects produced by intrathecal administration of mGluR agonists. *Neuroreport* **7,** 2743-2747.

Fisher K & Coderre TJ. (1996b). The contribution of metabotropic glutamate receptors (mGluRs) to formalin-induced nociception. *Pain* **68,** 255-263.

Fisher K & Coderre TJ. (1998). Hyperalgesia and allodynia induced by intrathecal (RS)-dihydroxyphenylglycine in rats. *Neuroreport* **9,** 1169-1172.

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

Fukada Y, Otsuki M & Tase C. (1999). [The study of the anesthetic action of halothane on the rat spinal cord by fos immunoreactivity]. *Masui* **48,** 966-976.

Fukuda T, Hisano S & Tanaka M. (2009). Licking decreases phosphorylation of extracellular signal-regulated kinase in the dorsal horn of the spinal cord after a formalin test. *Anesth Analg* **109,** 1318-1322.

Fukui T, Dai Y, Iwata K, Kamo H, Yamanaka H, Obata K, Kobayashi K, Wang S, Cui X, Yoshiya S & Noguchi K. (2007). Frequency-dependent ERK phosphorylation in spinal neurons by electric stimulation of the sciatic nerve and the role in electrophysiological activity. *Mol Pain* **3,** 18.

Fundytus ME, Osborne MG, Henry JL, Coderre TJ & Dray A. (2002). Antisense oligonucleotide knockdown of mGluR1 alleviates hyperalgesia and allodynia associated with chronic inflammation. *Pharmacol Biochem Behav* **73,** 401-410.

Furst S. (1999). Transmitters involved in antinociception in the spinal cord. *Brain Res Bull* **48,** 129-141.

Galan A, Lopez-Garcia JA, Cervero F & Laird JM. (2002). Activation of spinal extracellular signaling-regulated kinase-1 and -2 by intraplantar carrageenan in rodents. *Neurosci Lett* **322,** 37-40.

Gamboa-Esteves FO, McWilliam PN & Batten TF. (2004). Substance P (NK1) and somatostatin (sst2A) receptor immunoreactivity in NTS-projecting rat dorsal horn neurones activated by nociceptive afferent input. *J Chem Neuroanat* **27,** 251-266.

Gao YJ & Ji RR. (2009). c-Fos and pERK, which is a better marker for neuronal activation and central sensitization after noxious stimulation and tissue injury? *Open Pain J* **2,** 11-17.

Garcia-Lopez P, Garcia-Marin V, Martinez-Murillo R & Freire M. (2011). Cajal's achievements in the field of the development of dendritic arbors. *Int J Dev Biol* **54,** 1405-1417.

Garret C, Carruette A, Fardin V, Moussaoui S, Peyronel JF, Blanchard JC & Laduron PM. (1991). Pharmacological properties of a potent and selective nonpeptide substance P antagonist. *Proc Natl Acad Sci U S A* **88,** 10208-10212.

Garry EM, Jones E & Fleetwood-Walker SM. (2004). Nociception in vertebrates: key receptors participating in spinal mechanisms of chronic pain in animals. *Brain Res Brain Res Rev* **46,** 216-224.

Gauriau C & Bernard JF. (2002). Pain pathways and parabrachial circuits in the rat. *Exp Physiol* **87,** 251-258.

Gauriau C & Bernard JF. (2004). Posterior triangular thalamic neurons convey nociceptive messages to the secondary somatosensory and insular cortices in the rat. *J Neurosci* **24,** 752-761.

Gobel S. (1975). Golgi studies in the substantia gelatinosa neurons in the spinal trigeminal nucleus. *J Comp Neurol* **162,** 397-415.

Gobel S. (1978). Golgi studies of the neurons in layer II of the dorsal horn of the medulla (trigeminal nucleus caudalis). *J Comp Neurol* **180,** 395-413.

Graham BA, Brichta AM & Callister RJ. (2007). Moving from an averaged to specific view of spinal cord pain processing circuits. *J Neurophysiol* **98,** 1057-1063.

Gras C, Herzog E, Bellenchi GC, Bernard V, Ravassard P, Pohl M, Gasnier B, Giros B & El Mestikawy S. (2002). A third vesicular glutamate transporter expressed by cholinergic and serotoninergic neurons. *J Neurosci* **22,** 5442-5451.

Gray EG. (1959). Axo-somatic and axo-dendritic synapses of the cerebral cortex: an electron microscope study. *J Anat* **93,** 420-433.

Grudt TJ & Perl ER. (2002). Correlations between neuronal morphology and electrophysiological features in the rodent superficial dorsal horn. *J Physiol* **540,** 189-207.

Hagihira S, Taenaka N & Yoshiya I. (1997). Inhalation anesthetics suppress the expression of c-Fos protein evoked by noxious somatic stimulation in the deeper layer of the spinal cord in the rat. *Brain Res* **751,** 124-130.

Hamano K, Mannen H & Ishizuka N. (1978). Reconstruction of trajectory of primary afferent collaterals in the dorsal horn of the cat spinal cord, using Golgi-stained serial sections. *J Comp Neurol* **181,** 1-15.

Han ZS, Zhang ET & Craig AD. (1998). Nociceptive and thermoreceptive lamina I neurons are anatomically distinct. *Nat Neurosci* **1,** 218-225.

Harris JA. (1998). Using c-fos as a neural marker of pain. *Brain Res Bull* **45,** 1-8.

Hatano N, Mori Y, Oh-hora M, Kosugi A, Fujikawa T, Nakai N, Niwa H, Miyazaki J, Hamaoka T & Ogata M. (2003). Essential role for ERK2 mitogen-activated protein kinase in placental development. *Genes Cells* **8,** 847-856.

Heinke B, Ruscheweyh R, Forsthuber L, Wunderbaldinger G & Sandkuhler J. (2004). Physiological, neurochemical and morphological properties of a subgroup of GABAergic spinal lamina II neurones identified by expression of green fluorescent protein in mice. *J Physiol* **560,** 249-266.

Heinricher MM, Tavares I, Leith JL & Lumb BM. (2009). Descending control of nociception: Specificity, recruitment and plasticity. *Brain Res Rev* **60,** 214-225.

Helke CJ, Krause JE, Mantyh PW, Couture R & Bannon MJ. (1990). Diversity in mammalian tachykinin peptidergic neurons: multiple peptides, receptors, and regulatory mechanisms. *FASEB J* **4,** 1606-1615.

Henry JL, Krnjevic K & Morris ME. (1975). Substance P and spinal neurones. *Can J Physiol Pharmacol* **53,** 423-432.

Herdegen T, Kovary K, Leah J & Bravo R. (1991). Specific temporal and spatial distribution of JUN, FOS, and KROX-24 proteins in spinal neurons following noxious transsynaptic stimulation. *J Comp Neurol* **313,** 178-191.

Holzer P. (1991). Capsaicin: cellular targets, mechanisms of action, and selectivity for thin sensory neurons. *Pharmacol Rev* **43,** 143-201.

Honor P, Menning PM, Rogers SD, Nichols ML, Basbaum AI, Besson JM & Mantyh PW. (1999). Spinal substance P receptor expression and internalization in acute, short-term, and long-term inflammatory pain states. *J Neurosci* **19,** 7670-7678.

Honore P, Kamp EH, Rogers SD, Gebhart GF & Mantyh PW. (2002). Activation of lamina I spinal cord neurons that express the substance P receptor in visceral nociception and hyperalgesia. *J Pain* **3,** 3-11.

Hu H & Gereau Iv RW. (2011). Metabotropic Glutamate Receptor 5 Regulates Excitability and Kv4.2-Containing K+ channels Primarily in Excitatory Neurons of the Spinal Dorsal Horn. *J Neurophysiol*.

Hu HJ, Alter BJ, Carrasquillo Y, Qiu CS & Gereau RWt. (2007). Metabotropic glutamate receptor 5 modulates nociceptive plasticity via extracellular signal-regulated kinase-Kv4.2 signaling in spinal cord dorsal horn neurons. *J Neurosci* **27,** 13181-13191.

Hu HJ, Carrasquillo Y, Karim F, Jung WE, Nerbonne JM, Schwarz TL & Gereau RWt. (2006). The kv4.2 potassium channel subunit is required for pain plasticity. *Neuron* **50,** 89-100.

Hu HJ & Gereau RWt. (2003). ERK integrates PKA and PKC signaling in superficial dorsal horn neurons. II. Modulation of neuronal excitability. *J Neurophysiol* **90,** 1680-1688.

Hu HJ, Glauner KS & Gereau RWt. (2003). ERK integrates PKA and PKC signaling in superficial dorsal horn neurons. I. Modulation of A-type K+ currents. *J Neurophysiol* **90,** 1671-1679.

Hu JY & Zhao ZQ. (2001). Differential contributions of NMDA and non-NMDA receptors to spinal Fos expression evoked by superficial tissue and muscle inflammation in the rat. *Neuroscience* **106,** 823-831.

Huang HY, Cheng JK, Shih YH, Chen PH, Wang CL & Tsaur ML. (2005). Expression of A-type K channel alpha subunits Kv 4.2 and Kv 4.3 in rat spinal lamina II excitatory interneurons and colocalization with pain-modulating molecules. *Eur J Neurosci* **22,** 1149-1157.

Hughes DI, Scott DT, Todd AJ & Riddell JS. (2003). Lack of evidence for sprouting of Abeta afferents into the superficial laminas of the spinal cord dorsal horn after nerve section. *J Neurosci* **23,** 9491-9499.

Hunt SP, Pini A & Evan G. (1987). Induction of c-fos-like protein in spinal cord neurons following sensory stimulation. *Nature* **328,** 632-634.

Hylden JL, Anton F & Nahin RL. (1989). Spinal lamina I projection neurons in the rat: collateral innervation of parabrachial area and thalamus. *Neuroscience* **28,** 27-37.

Ikeda H, Stark J, Fischer H, Wagner M, Drdla R, Jager T & Sandkuhler J. (2006). Synaptic amplifier of inflammatory pain in the spinal dorsal horn. *Science* **312,** 1659-1662.

Impey S, Obrietan K & Storm DR. (1999). Making new connections: role of ERK/MAP kinase signaling in neuronal plasticity. *Neuron* **23,** 11-14.

Jasmin L, Gogas KR, Ahlgren SC, Levine JD & Basbaum AI. (1994). Walking evokes a distinctive pattern of Fos-like immunoreactivity in the caudal brainstem and spinal cord of the rat. *Neuroscience* **58,** 275-286.

Ji RR, Baba H, Brenner GJ & Woolf CJ. (1999). Nociceptive-specific activation of ERK in spinal neurons contributes to pain hypersensitivity. *Nat Neurosci* **2,** 1114-1119.

Ji RR, Befort K, Brenner GJ & Woolf CJ. (2002). ERK MAP kinase activation in superficial spinal cord neurons induces prodynorphin and NK-1 upregulation and contributes to persistent inflammatory pain hypersensitivity. *J Neurosci* **22,** 478-485.

Ji RR, Gereau RWt, Malcangio M & Strichartz GR. (2009). MAP kinase and pain. *Brain Res Rev* **60,** 135-148.

Ji RR, Kohno T, Moore KA & Woolf CJ. (2003). Central sensitization and LTP: do pain and memory share similar mechanisms? *Trends Neurosci* **26,** 696-705.

Ji RR & Rupp F. (1997). Phosphorylation of transcription factor CREB in rat spinal cord after formalin-induced hyperalgesia: relationship to c-fos induction. *J Neurosci* **17,** 1776-1785.

Jia H, Rustioni A & Valtschanoff JG. (1999). Metabotropic glutamate receptors in superficial laminae of the rat dorsal horn. *J Comp Neurol* **410,** 627-642.

Johnson CM, Hill CS, Chawla S, Treisman R & Bading H. (1997). Calcium controls gene expression via three distinct pathways that can function independently of the Ras/mitogen-activated protein kinases (ERKs) signaling cascade. *J Neurosci* **17,** 6189-6202.

Joly C, Gomeza J, Brabet I, Curry K, Bockaert J & Pin JP. (1995). Molecular, functional, and pharmacological characterization of the metabotropic glutamate receptor type 5 splice variants: comparison with mGluR1. *J Neurosci* **15,** 3970-3981.

Jones MW & Headley PM. (1995). Interactions between metabotropic and ionotropic glutamate receptor agonists in the rat spinal cord in vivo. *Neuropharmacology* **34,** 1025-1031.

Ju G, Hokfelt T, Brodin E, Fahrenkrug J, Fischer JA, Frey P, Elde RP & Brown JC. (1987). Primary sensory neurons of the rat showing calcitonin gene-related peptide immunoreactivity and their relation to substance P-, somatostatin-, galanin-, vasoactive intestinal polypeptide- and cholecystokinin-immunoreactive ganglion cells. *Cell Tissue Res* **247,** 417-431.

Julius D & McCleskey EW. (2006). Cellular and molecular properties of primary afferent neurons In *Wall and Melzack Textbook of Pain*, ed. McMahon SB & Koltzenburg M.

Karim F, Wang CC & Gereau RWt. (2001). Metabotropic glutamate receptor subtypes 1 and 5 are activators of extracellular signal-regulated kinase signaling required for inflammatory pain in mice. *J Neurosci* **21,** 3771-3779.

Kawasaki Y, Kohno T, Zhuang ZY, Brenner GJ, Wang H, Van Der Meer C, Befort K, Woolf CJ & Ji RR. (2004). Ionotropic and metabotropic receptors, protein kinase A, protein kinase C, and Src contribute to C-fiber-induced ERK activation and cAMP response element-binding protein phosphorylation in dorsal horn neurons, leading to central sensitization. *J Neurosci* **24,** 8310-8321.

Kenshalo DR, Jr., Leonard RB, Chung JM & Willis WD. (1979). Responses of primate spinothalamic neurons to graded and to repeated noxious heat stimuli. *J Neurophysiol* **42,** 1370-1389.

Kenshalo DR, Jr., Leonard RB, Chung JM & Willis WD. (1982). Facilitation of the response of primate spinothalamic cells to cold and to tactile stimuli by noxious heating of the skin. *Pain* **12,** 141-152.

Kirsch J & Betz H. (1993). Widespread expression of gephyrin, a putative glycine receptor-tubulin linker protein, in rat brain. *Brain Res* **621,** 301-310.

Kiyama H & Emson PC. (1990). Distribution of somatostatin mRNA in the rat nervous system as visualized by a novel non-radioactive in situ hybridization histochemistry procedure. *Neuroscience* **38,** 223-244.

Kolmodin GM & Skoglund CR. (1960). Analysis of spinal interneurons activated by tactile and nociceptive stimulation. *Acta Physiol Scand* **50,** 337-355.

Kominato Y, Tachibana T, Dai Y, Tsujino H, Maruo S & Noguchi K. (2003). Changes in phosphorylation of ERK and Fos expression in dorsal horn neurons following noxious stimulation in a rat model of neuritis of the nerve root. *Brain Res* **967,** 89-97.

Kondo I, Marvizon JC, Song B, Salgado F, Codeluppi S, Hua XY & Yaksh TL. (2005). Inhibition by spinal mu- and delta-opioid agonists of afferent-evoked substance P release. *J Neurosci* **25,** 3651-3660.

Kristensen JD, Karlsten R, Gordh T & Berge OG. (1994). The NMDA antagonist 3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid (CPP) has antinociceptive effect after intrathecal injection in the rat. *Pain* **56,** 59-67.

Kumazawa T & Perl ER. (1978). Excitation of marginal and substantia gelatinosa neurons in the primate spinal cord: indications of their place in dorsal horn functional organization. *J Comp Neurol* **177,** 417-434.

Kurihara K. (2009). Glutamate: from discovery as a food flavor to role as a basic taste (umami). *Am J Clin Nutr* **90,** 719S-722S.

Labrakakis C & MacDermott AB. (2003). Neurokinin receptor 1-expressing spinal cord neurons in lamina I and III/IV of postnatal rats receive inputs from capsaicin sensitive fibers. *Neurosci Lett* **352,** 121-124.

Lai HH, Qiu CS, Crock LW, Morales ME, Ness TJ & Gereau RWt. (2011). Activation of spinal extracellular signal-regulated kinases (ERK) 1/2 is associated with the development of visceral hyperalgesia of the bladder. *Pain* **152,** 2117-2124.

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

Laird JM & Cervero F. (1989). A comparative study of the changes in receptive-field properties of multireceptive and nociceptive rat dorsal horn neurons following noxious mechanical stimulation. *J Neurophysiol* **62,** 854-863.

LaMotte RH & Thalhammer JG. (1982). Response properties of high-threshold cutaneous cold receptors in the primate. *Brain Res* **244,** 279-287.

LaMotte RH, Thalhammer JG, Torebjork HE & Robinson CJ. (1982). Peripheral neural mechanisms of cutaneous hyperalgesia following mild injury by heat. *J Neurosci* **2,** 765-781.

Langford LA. (1983). Unmyelinated axon ratios in cat motor, cutaneous and articular nerves. *Neurosci Lett* **40,** 19-22.

Lanteri-Minet M, Isnardon P, de Pommery J & Menetrey D. (1993). Spinal and hindbrain structures involved in viscероception and visceronociception as revealed by the expression of Fos, Jun and Krox-24 proteins. *Neuroscience* **55,** 737-753.

Lanteri-Minet M, Weil-Fugazza J, de Pommery J & Menetrey D. (1994). Hindbrain structures involved in pain processing as revealed by the expression of c-Fos and other immediate early gene proteins. *Neuroscience* **58,** 287-298.

Lawson SN, Crepps B & Perl ER. (2002). Calcitonin gene-related peptide immunoreactivity and afferent receptive properties of dorsal root ganglion neurones in guinea-pigs. *J Physiol* **540,** 989-1002.

Lawson SN, Crepps BA & Perl ER. (1997). Relationship of substance P to afferent characteristics of dorsal root ganglion neurones in guinea-pig. *J Physiol* **505 (Pt 1),** 177-191.

Lever IJ, Pezet S, McMahon SB & Malcangio M. (2003). The signaling components of sensory fiber transmission involved in the activation of ERK MAP kinase in the mouse dorsal horn. *Mol Cell Neurosci* **24,** 259-270.

Li JL, Li YQ, Kaneko T & Mizuno N. (1999). Preprodynorphin-like immunoreactivity in medullary dorsal horn neurons projecting to the thalamic regions in the rat. *Neurosci Lett* **264,** 13-16.

Light AR & Lee S. (2009). Spinal Cord Physiology of Nociception. In *Science of Pain*, ed. Basbaum AI & Bushnell MC. Elsevier.

Light AR & Perl ER. (1979a). Reexamination of the dorsal root projection to the spinal dorsal horn including observations on the differential termination of coarse and fine fibers. *J Comp Neurol* **186,** 117-131.

Light AR & Perl ER. (1979b). Spinal termination of functionally identified primary afferent neurons with slowly conducting myelinated fibers. *J Comp Neurol* **186,** 133-150.

Light AR, Trevino DL & Perl ER. (1979). Morphological features of functionally defined neurons in the marginal zone and substantia gelatinosa of the spinal dorsal horn. *J Comp Neurol* **186,** 151-171.

Light AR & Willcockson HH. (1999). Spinal laminae I-II neurons in rat recorded in vivo in whole cell, tight seal configuration: properties and opioid responses. *J Neurophysiol* **82,** 3316-3326.

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

Lima D, Albino-Teixeira A & Tavares I. (2002). The caudal medullary ventrolateral reticular formation in nociceptive-cardiovascular integration. An experimental study in the rat. *Exp Physiol* **87,** 267-274.

Lima D & Coimbra A. (1983). The neuronal population of the marginal zone (lamina I) of the rat spinal cord. A study based on reconstructions of serially sectioned cells. *Anat Embryol (Berl)* **167,** 273-288.

Lima D & Coimbra A. (1986). A Golgi study of the neuronal population of the marginal zone (lamina I) of the rat spinal cord. *J Comp Neurol* **244,** 53-71.

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

Littlewood NK, Todd AJ, Spike RC, Watt C & Shehab SA. (1995). The types of neuron in spinal dorsal horn which possess neurokinin-1 receptors. *Neuroscience* **66,** 597-608.

Liu CR, Duan QZ, Wang W, Wei YY, Zhang H, Li YQ, Wu SX & Xu LX. (2011). Effects of intrathecal isoflurane administration on nociception and Fos expression in the rat spinal cord. *Eur J Anaesthesiol* **28,** 112-119.

Liu H, Brown JL, Jasmin L, Maggio JE, Vigna SR, Mantyh PW & Basbaum AI. (1994). Synaptic relationship between substance P and the substance P receptor: light and electron microscopic characterization of the mismatch

between neuropeptides and their receptors. *Proc Natl Acad Sci U S A* **91,** 1009-1013.

Liu H, Mantyh PW & Basbaum AI. (1997). NMDA-receptor regulation of substance P release from primary afferent nociceptors. *Nature* **386,** 721-724.

Llewellyn-Smith IJ & Minson JB. (1992). Complete penetration of antibodies into vibratome sections after glutaraldehyde fixation and ethanol treatment: light and electron microscopy for neuropeptides. *J Histochem Cytochem* **40,** 1741-1749.

Loken LS, Wessberg J, Morrison I, McGlone F & Olausson H. (2009). Coding of pleasant touch by unmyelinated afferents in humans. *Nat Neurosci* **12,** 547-548.

Lu Y, Jin SX, Xu TL, Qin BZ, Li JS, Ding YQ, Shigemoto R & Mizuno N. (1995). Expression of c-fos protein in substance P receptor-like immunoreactive neurons in response to noxious stimuli on the urinary bladder: an observation in the lumbosacral cord segments of the rat. *Neurosci Lett* **198,** 139-142.

Lu Y & Perl ER. (2003). A specific inhibitory pathway between substantia gelatinosa neurons receiving direct C-fiber input. *J Neurosci* **23,** 8752-8758.

Lu Y & Perl ER. (2005). Modular organization of excitatory circuits between neurons of the spinal superficial dorsal horn (laminae I and II). *J Neurosci* **25,** 3900-3907.

Lumpkin EA & Caterina MJ. (2007). Mechanisms of sensory transduction in the skin. *Nature* **445,** 858-865.

Luo W, Enomoto H, Rice FL, Milbrandt J & Ginty DD. (2009). Molecular identification of rapidly adapting mechanoreceptors and their developmental dependence on ret signaling. *Neuron* **64,** 841-856.

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

Ma QP & Woolf CJ. (1995). Involvement of neurokinin receptors in the induction but not the maintenance of mechanical allodynia in rat flexor motoneurones. *J Physiol* **486 (Pt 3),** 769-777.

Malmberg AB, Chen C, Tonegawa S & Basbaum AI. (1997). Preserved acute pain and reduced neuropathic pain in mice lacking PKCgamma. *Science* **278,** 279-283.

Malmberg AB & Yaksh TL. (1992). Hyperalgesia mediated by spinal glutamate or substance P receptor blocked by spinal cyclooxygenase inhibition. *Science* **257,** 1276-1279.

Mantyh PW, DeMaster E, Malhotra A, Ghilardi JR, Rogers SD, Mantyh CR, Liu H, Basbaum AI, Vigna SR, Maggio JE & et al. (1995). Receptor endocytosis

and dendrite reshaping in spinal neurons after somatosensory stimulation. *Science* **268,** 1629-1632.

Mantyh PW, Rogers SD, Honore P, Allen BJ, Ghilardi JR, Li J, Daughters RS, Lappi DA, Wiley RG & Simone DA. (1997). Inhibition of hyperalgesia by ablation of lamina I spinal neurons expressing the substance P receptor. *Science* **278,** 275-279.

Marshall GE, Shehab SA, Spike RC & Todd AJ. (1996). Neurokinin-1 receptors on lumbar spinothalamic neurons in the rat. *Neuroscience* **72,** 255-263.

Marvizon JC, Chen W & Murphy N. (2009). Enkephalins, dynorphins, and beta-endorphin in the rat dorsal horn: an immunofluorescence colocalization study. *J Comp Neurol* **517,** 51-68.

Marvizon JC, Grady EF, Stefani E, Bunnett NW & Mayer EA. (1999). Substance P release in the dorsal horn assessed by receptor internalization: NMDA receptors counteract a tonic inhibition by GABA(B) receptors. *Eur J Neurosci* **11,** 417-426.

Marvizon JC, Martinez V, Grady EF, Bunnett NW & Mayer EA. (1997). Neurokinin 1 receptor internalization in spinal cord slices induced by dorsal root stimulation is mediated by NMDA receptors. *J Neurosci* **17,** 8129-8136.

Masu M, Tanabe Y, Tsuchida K, Shigemoto R & Nakanishi S. (1991). Sequence and expression of a metabotropic glutamate receptor. *Nature* **349,** 760-765.

Matsuda K, Kamiya Y, Matsuda S & Yuzaki M. (2002). Cloning and characterization of a novel NMDA receptor subunit NR3B: a dominant subunit that reduces calcium permeability. *Brain Res Mol Brain Res* **100,** 43-52.

Matsuoka Y & Yang J. (2012). Selective inhibition of extracellular signal-regulated kinases 1/2 blocks nerve growth factor to brain-derived neurotrophic factor signaling and suppresses the development of and reverses already established pain behavior in rats. *Neuroscience*.

Maxwell DJ, Belle MD, Cheunsuang O, Stewart A & Morris R. (2007). Morphology of inhibitory and excitatory interneurons in superficial laminae of the rat dorsal horn. *J Physiol* **584,** 521-533.

McCarson KE & Krause JE. (1996). The neurokinin-1 receptor antagonist LY306,740 blocks nociception-induced increases in dorsal horn neurokinin-1 receptor gene expression. *Mol Pharmacol* **50,** 1189-1199.

McKemy DD, Neuhausser WM & Julius D. (2002). Identification of a cold receptor reveals a general role for TRP channels in thermosensation. *Nature* **416,** 52-58.

McMahon SB & Wall PD. (1985). The distribution and central termination of single cutaneous and muscle unmyelinated fibres in rat spinal cord. *Brain Res* **359,** 39-48.

McNamara CR, Mandel-Brehm J, Bautista DM, Siemens J, Deranian KL, Zhao M, Hayward NJ, Chong JA, Julius D, Moran MM & Fanger CM. (2007). TRPA1 mediates formalin-induced pain. *Proc Natl Acad Sci U S A* **104,** 13525-13530.

Meller ST, Dykstra C & Gebhart GF. (1996). Acute thermal hyperalgesia in the rat is produced by activation of N-methyl-D-aspartate receptors and protein kinase C and production of nitric oxide. *Neuroscience* **71,** 327-335.

Melnick IV, Santos SF, Szokol K, Szucs P & Safronov BV. (2004). Ionic basis of tonic firing in spinal substantia gelatinosa neurons of rat. *J Neurophysiol* **91,** 646-655.

Menetrey D & Basbaum AI. (1987). Spinal and trigeminal projections to the nucleus of the solitary tract: a possible substrate for somatovisceral and viscerovisceral reflex activation. *J Comp Neurol* **255,** 439-450.

Menetrey D, Giesler GJ, Jr. & Besson JM. (1977). An analysis of response properties of spinal cord dorsal horn neurones to nonnoxious and noxious stimuli in the spinal rat. *Exp Brain Res* **27,** 15-33.

Mense S & Craig AD, Jr. (1988). Spinal and supraspinal terminations of primary afferent fibers from the gastrocnemius-soleus muscle in the cat. *Neuroscience* **26,** 1023-1035.

Mense S & Meyer H. (1985). Different types of slowly conducting afferent units in cat skeletal muscle and tendon. *J Physiol* **363,** 403-417.

Mense S & Prabhakar NR. (1986). Spinal termination of nociceptive afferent fibres from deep tissues in the cat. *Neurosci Lett* **66,** 169-174.

Meyer RA, Ringkamp M, Campbell JN & Raja SN. (2006). Peripheral mechanisms of cutaneous nociception In *Wall and Melzack Textbook of Pain*, 5th edn, ed. McMahon SB & Koltzenburg M. Elsevier Limited.

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

Morgan JI & Curran T. (1991). Stimulus-transcription coupling in the nervous system: involvement of the inducible proto-oncogenes fos and jun. *Annu Rev Neurosci* **14,** 421-451.

Mori M, Kose A, Tsujino T & Tanaka C. (1990). Immunocytochemical localization of protein kinase C subspecies in the rat spinal cord: light and electron microscopic study. *J Comp Neurol* **299,** 167-177.

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

Murase K, Nedeljkov V & Randic M. (1982). The actions of neuropeptides on dorsal horn neurons in the rat spinal cord slice preparation: an intracellular study. *Brain Res* **234,** 170-176.

Murase K, Ryu PD & Randic M. (1986). Substance P augments a persistent slow inward calcium-sensitive current in voltage-clamped spinal dorsal horn neurons of the rat. *Brain Res* **365,** 369-376.

Naim M, Spike RC, Watt C, Shehab SA & Todd AJ. (1997). Cells in laminae III and IV of the rat spinal cord that possess the neurokinin-1 receptor and have dorsally directed dendrites receive a major synaptic input from tachykinin-containing primary afferents. *J Neurosci* **17,** 5536-5548.

Naim MM, Shehab SA & Todd AJ. (1998). Cells in laminae III and IV of the rat spinal cord which possess the neurokinin-1 receptor receive monosynaptic input from myelinated primary afferents. *Eur J Neurosci* **10,** 3012-3019.

Nakanishi S. (1991). Mammalian tachykinin receptors. *Annu Rev Neurosci* **14,** 123-136.

Nakano T, Andoh T, Lee JB & Kuraishi Y. (2008). Different dorsal horn neurons responding to histamine and allergic itch stimuli. *Neuroreport* **19,** 723-726.

Nakaya Y, Kaneko T, Shigemoto R, Nakanishi S & Mizuno N. (1994). Immunohistochemical localization of substance P receptor in the central nervous system of the adult rat. *J Comp Neurol* **347,** 249-274.

Ness TJ, Randich A & Gebhart GF. (1991). Further behavioral evidence that colorectal distension is a 'noxious' visceral stimulus in rats. *Neurosci Lett* **131,** 113-116.

Neugebauer V, Schaible HG, Weiretter F & Freudenberger U. (1994). The involvement of substance P and neurokinin-1 receptors in the responses of rat dorsal horn neurons to noxious but not to innocuous mechanical stimuli applied to the knee joint. *Brain Res* **666,** 207-215.

Ni B, Rosteck PR, Jr., Nadi NS & Paul SM. (1994). Cloning and expression of a cDNA encoding a brain-specific Na(+)-dependent inorganic phosphate cotransporter. *Proc Natl Acad Sci U S A* **91,** 5607-5611.

Nichols ML, Allen BJ, Rogers SD, Ghilardi JR, Honore P, Luger NM, Finke MP, Li J, Lappi DA, Simone DA & Mantyh PW. (1999). Transmission of chronic nociception by spinal neurons expressing the substance P receptor. *Science* **286,** 1558-1561.

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

Ohishi H, Nomura S, Ding YQ, Shigemoto R, Wada E, Kinoshita A, Li JL, Neki A, Nakanishi S & Mizuno N. (1995). Presynaptic localization of a

metabotropic glutamate receptor, mGluR7, in the primary afferent neurons: an immunohistochemical study in the rat. *Neurosci Lett* **202,** 85-88.

Olausson H, Lamarre Y, Backlund H, Morin C, Wallin BG, Starck G, Ekholm S, Strigo I, Worsley K, Vallbo AB & Bushnell MC. (2002). Unmyelinated tactile afferents signal touch and project to insular cortex. *Nat Neurosci* **5,** 900-904.

Oliveira AL, Hydling F, Olsson E, Shi T, Edwards RH, Fujiyama F, Kaneko T, Hokfelt T, Cullheim S & Meister B. (2003). Cellular localization of three vesicular glutamate transporter mRNAs and proteins in rat spinal cord and dorsal root ganglia. *Synapse* **50,** 117-129.

Pages G, Guerin S, Grall D, Bonino F, Smith A, Anjuere F, Auberger P & Pouyssegur J. (1999). Defective thymocyte maturation in p44 MAP kinase (Erk 1) knockout mice. *Science* **286,** 1374-1377.

Panfil C, Makowska A & Ellrich J. (2006). Brainstem and cervical spinal cord Fos immunoreactivity evoked by nerve growth factor injection into neck muscles in mice. *Cephalalgia* **26,** 128-135.

Park ES, Kim SY & Youn DH. (2010). NMDA receptor, PKC and ERK prevent fos expression induced by the activation of group I metabotropic glutamate receptors in the spinal trigeminal subnucleus oralis. *Mol Cells* **30,** 461-466.

Park YK, Galik J, Ryu PD & Randic M. (2004). Activation of presynaptic group I metabotropic glutamate receptors enhances glutamate release in the rat spinal cord substantia gelatinosa. *Neurosci Lett* **361,** 220-224.

Patapoutian A, Peier AM, Story GM & Viswanath V. (2003). ThermoTRP channels and beyond: mechanisms of temperature sensation. *Nat Rev Neurosci* **4,** 529-539.

Paxinos G & Watson C. (2005). *The rat brain in stereotaxic coordinates*. Elsevier Academic Press New York.

Peier AM, Moqrich A, Hergarden AC, Reeve AJ, Andersson DA, Story GM, Earley TJ, Dragoni I, McIntyre P, Bevan S & Patapoutian A. (2002). A TRP channel that senses cold stimuli and menthol. *Cell* **108,** 705-715.

Perl ER. (1992). Function of dorsal root ganglion neurons: An overview. In *Sensory neurons: Diversity, Development and Plasticity*, ed. Scott SA, pp. 3-23. Oxford University Press, New York.

Perry MJ & Lawson SN. (1998). Differences in expression of oligosaccharides, neuropeptides, carbonic anhydrase and neurofilament in rat primary afferent neurons retrogradely labelled via skin, muscle or visceral nerves. *Neuroscience* **85,** 293-310.

Pezet S, Malcangio M, Lever IJ, Perkinton MS, Thompson SW, Williams RJ & McMahon SB. (2002). Noxious stimulation induces Trk receptor and

downstream ERK phosphorylation in spinal dorsal horn. *Mol Cell Neurosci* **21,** 684-695.

Pfeiffer F, Simler R, Grenningloh G & Betz H. (1984). Monoclonal antibodies and peptide mapping reveal structural similarities between the subunits of the glycine receptor of rat spinal cord. *Proc Natl Acad Sci U S A* **81,** 7224-7227.

Pingle SC, Matta JA & Ahern GP. (2007). Capsaicin receptor: TRPV1 a promiscuous TRP channel. *Handb Exp Pharmacol***,** 155-171.

Pinheiro P & Mulle C. (2006). Kainate receptors. *Cell Tissue Res* **326,** 457-482.

Plenderleith MB & Snow PJ. (1993). The plant lectin Bandeiraea simplicifolia I-B4 identifies a subpopulation of small diameter primary sensory neurones which innervate the skin in the rat. *Neurosci Lett* **159,** 17-20.

Polgar E, Al-Khater KM, Shehab S, Watanabe M & Todd AJ. (2008). Large projection neurons in lamina I of the rat spinal cord that lack the neurokinin 1 receptor are densely innervated by VGLUT2-containing axons and possess GluR4-containing AMPA receptors. *J Neurosci* **28,** 13150-13160.

Polgar E, Campbell AD, MacIntyre LM, Watanabe M & Todd AJ. (2007). Phosphorylation of ERK in neurokinin 1 receptor-expressing neurons in laminae III and IV of the rat spinal dorsal horn following noxious stimulation. *Mol Pain* **3,** 4.

Polgar E, Fowler JH, McGill MM & Todd AJ. (1999a). The types of neuron which contain protein kinase C gamma in rat spinal cord. *Brain Res* **833,** 71-80.

Polgar E, Gray S, Riddell JS & Todd AJ. (2004). Lack of evidence for significant neuronal loss in laminae I-III of the spinal dorsal horn of the rat in the chronic constriction injury model. *Pain* **111,** 144-150.

Polgar E, Hughes DI, Riddell JS, Maxwell DJ, Puskar Z & Todd AJ. (2003). Selective loss of spinal GABAergic or glycinergic neurons is not necessary for development of thermal hyperalgesia in the chronic constriction injury model of neuropathic pain. *Pain* **104,** 229-239.

Polgar E, Puskar Z, Watt C, Matesz C & Todd AJ. (2002). Selective innervation of lamina I projection neurones that possess the neurokinin 1 receptor by serotonin-containing axons in the rat spinal cord. *Neuroscience* **109,** 799-809.

Polgar E, Sardella TC, Watanabe M & Todd AJ. (2011). Quantitative study of NPY-expressing GABAergic neurons and axons in rat spinal dorsal horn. *J Comp Neurol* **519,** 1007-1023.

Polgar E, Shehab SA, Watt C & Todd AJ. (1999b). GABAergic neurons that contain neuropeptide Y selectively target cells with the neurokinin 1 receptor in laminae III and IV of the rat spinal cord. *J Neurosci* **19,** 2637-2646.

Polgar E, Wright LL & Todd AJ. (2010). A quantitative study of brainstem projections from lamina I neurons in the cervical and lumbar enlargement of the rat. *Brain Res* **1308,** 58-67.

Pomeranz B, Wall PD & Weber WV. (1968). Cord cells responding to fine myelinated afferents from viscera, muscle and skin. *J Physiol* **199,** 511-532.

Pow DV & Crook DK. (1993). Extremely high titre polyclonal antisera against small neurotransmitter molecules: rapid production, characterisation and use in light- and electron-microscopic immunocytochemistry. *J Neurosci Methods* **48,** 51-63.

Prescott SA & De Koninck Y. (2002). Four cell types with distinctive membrane properties and morphologies in lamina I of the spinal dorsal horn of the adult rat. *J Physiol* **539,** 817-836.

Presley RW, Menetrey D, Levine JD & Basbaum AI. (1990). Systemic morphine suppresses noxious stimulus-evoked Fos protein-like immunoreactivity in the rat spinal cord. *J Neurosci* **10,** 323-335.

Proudlock F, Spike RC & Todd AJ. (1993). Immunocytochemical study of somatostatin, neurotensin, GABA, and glycine in rat spinal dorsal horn. *J Comp Neurol* **327,** 289-297.

Ptak K, Burnet H, Blanchi B, Sieweke M, De Felipe C, Hunt SP, Monteau R & Hilaire G. (2002). The murine neurokinin NK1 receptor gene contributes to the adult hypoxic facilitation of ventilation. *Eur J Neurosci* **16,** 2245-2252.

Puskar Z, Polgar E & Todd AJ. (2001). A population of large lamina I projection neurons with selective inhibitory input in rat spinal cord. *Neuroscience* **102,** 167-176.

Redburn JL & Leah JD. (1999). The induction and potentiation of c-Jun and c-Fos expression in spinal neurons mediated by NK1 and NK2 receptors. *Neuropeptides* **33,** 115-119.

Ren K & Ruda MA. (1994). A comparative study of the calcium-binding proteins calbindin-D28K, calretinin, calmodulin and parvalbumin in the rat spinal cord. *Brain Res Brain Res Rev* **19,** 163-179.

Rethelyi M, Light AR & Perl ER. (1982). Synaptic complexes formed by functionally defined primary afferent units with fine myelinated fibers. *J Comp Neurol* **207,** 381-393.

Rethelyi M, Light AR & Perl ER. (1989). Synaptic ultrastructure of functionally and morphologically characterized neurons of the superficial spinal dorsal horn of cat. *J Neurosci* **9,** 1846-1863.

Rethelyi M & Szentagothai J. (1969). The large synaptic complexes of the substantia gelatinosa. *Exp Brain Res* **7,** 258-274.

Reuss MH & Reuss S. (2001). Nitric oxide synthase neurons in the rodent spinal cord: distribution, relation to Substance P fibers, and effects of dorsal rhizotomy. *J Chem Neuroanat* **21,** 181-196.

Rexed B. (1952). The cytoarchitectonic organization of the spinal cord in the cat. *J Comp Neurol* **96,** 414-495.

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

Ribeiro-da-Silva A & Cuello AC. (1990). Ultrastructural evidence for the occurrence of two distinct somatostatin-containing systems in the substantia gelatinosa of rat spinal cord. *J Chem Neuroanat* **3,** 141-153.

Ribeiro-da-Silva A & De Koninck Y. (2009). Morphological and Neurochemical Organization of the Spinal Dorsal Horn In *Science of Pain*, ed. Basbaum AI & Bushnell MC. Elsevier.

Ribeiro-da-Silva A, Tagari P & Cuello AC. (1989). Morphological characterization of substance P-like immunoreactive glomeruli in the superficial dorsal horn of the rat spinal cord and trigeminal subnucleus caudalis: a quantitative study. *J Comp Neurol* **281,** 497-415.

Rice FL, Fundin BT, Arvidsson J, Aldskogius H & Johansson O. (1997). Comprehensive immunofluorescence and lectin binding analysis of vibrissal follicle sinus complex innervation in the mystacial pad of the rat. *J Comp Neurol* **385,** 149-184.

Ross SE. (2011). Pain and itch: insights into the neural circuits of aversive somatosensation in health and disease. *Curr Opin Neurobiol* **21,** 880-887.

Ross SE, Mardinly AR, McCord AE, Zurawski J, Cohen S, Jung C, Hu L, Mok SI, Shah A, Savner EM, Tolias C, Corfas R, Chen S, Inquimbert P, Xu Y, McInnes RR, Rice FL, Corfas G, Ma Q, Woolf CJ & Greenberg ME. (2010). Loss of inhibitory interneurons in the dorsal spinal cord and elevated itch in Bhlhb5 mutant mice. *Neuron* **65,** 886-898.

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

Ruan JP, Zhang HX, Lu XF, Liu YP & Cao JL. (2010). EphrinBs/EphBs signaling is involved in modulation of spinal nociceptive processing through a mitogen-activated protein kinases-dependent mechanism. *Anesthesiology* **112,** 1234-1249.

Ruscheweyh R & Sandkuhler J. (2002). Lamina-specific membrane and discharge properties of rat spinal dorsal horn neurones in vitro. *J Physiol* **541,** 231-244.

Sakamoto H, Spike RC & Todd AJ. (1999). Neurons in laminae III and IV of the rat spinal cord with the neurokinin-1 receptor receive few contacts from

unmyelinated primary afferents which do not contain substance P. *Neuroscience* **94,** 903-908.

Salter MW & Henry JL. (1991). Responses of functionally identified neurones in the dorsal horn of the cat spinal cord to substance P, neurokinin A and physalaemin. *Neuroscience* **43,** 601-610.

Sandkuhler J. (2009). Models and mechanisms of hyperalgesia and allodynia. *Physiol Rev* **89,** 707-758.

Sardella TC, Polgar E, Garzillo F, Furuta T, Kaneko T, Watanabe M & Todd AJ. (2011a). Dynorphin is expressed primarily by GABAergic neurons that contain galanin in the rat dorsal horn. *Mol Pain* **7,** 76.

Sardella TC, Polgar E, Watanabe M & Todd AJ. (2011b). A quantitative study of neuronal nitric oxide synthase expression in laminae I-III of the rat spinal dorsal horn. *Neuroscience* **192,** 708-720.

Schaible HG & Schmidt RF. (1985). Effects of an experimental arthritis on the sensory properties of fine articular afferent units. *J Neurophysiol* **54,** 1109-1122.

Schaible HG, Schmidt RF & Willis WD. (1986). Responses of spinal cord neurones to stimulation of articular afferent fibres in the cat. *J Physiol* **372,** 575-593.

Schaible HG, Schmidt RF & Willis WD. (1987). Convergent inputs from articular, cutaneous and muscle receptors onto ascending tract cells in the cat spinal cord. *Exp Brain Res* **66,** 479-488.

Schmidt HH, Gagne GD, Nakane M, Pollock JS, Miller MF & Murad F. (1992). Mapping of neural nitric oxide synthase in the rat suggests frequent co-localization with NADPH diaphorase but not with soluble guanylyl cyclase, and novel paraneural functions for nitrinergic signal transduction. *J Histochem Cytochem* **40,** 1439-1456.

Schneider SP. (2008). Local circuit connections between hamster laminae III and IV dorsal horn neurons. *J Neurophysiol* **99,** 1306-1318.

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

Seal RP, Wang X, Guan Y, Raja SN, Woodbury CJ, Basbaum AI & Edwards RH. (2009). Injury-induced mechanical hypersensitivity requires C-low threshold mechanoreceptors. *Nature* **462,** 651-655.

Seger R & Krebs EG. (1995). The MAPK signaling cascade. *FASEB J* **9,** 726-735.

Seguin L, Le Marouille-Girardon S & Millan MJ. (1995). Antinociceptive profiles of non-peptidergic neurokinin1 and neurokinin2 receptor antagonists: a comparison to other classes of antinociceptive agent. *Pain* **61,** 325-343.

Seino D, Tokunaga A, Tachibana T, Yoshiya S, Dai Y, Obata K, Yamanaka H, Kobayashi K & Noguchi K. (2006). The role of ERK signaling and the P2X receptor on mechanical pain evoked by movement of inflamed knee joint. *Pain* **123,** 193-203.

Seki S, Erickson KA, Seki M, Nishizawa O, Igawa Y, Ogawa T, de Groat WC, Chancellor MB & Yoshimura N. (2005). Elimination of rat spinal neurons expressing neurokinin 1 receptors reduces bladder overactivity and spinal c-fos expression induced by bladder irritation. *Am J Physiol Renal Physiol* **288,** F466-473.

Seybold VS & Elde RP. (1982). Neurotensin immunoreactivity in the superficial laminae of the dorsal horn of the rat: I. Light microscopic studies of cell bodies and proximal dendrites. *J Comp Neurol* **205,** 89-100.

Shehab SA & Hughes DI. Simultaneous identification of unmyelinated and myelinated primary somatic afferents by co-injection of isolectin B4 and Cholera toxin subunit B into the sciatic nerve of the rat. *J Neurosci Methods* **198,** 213-221.

Shindler KS & Roth KA. (1996). Double immunofluorescent staining using two unconjugated primary antisera raised in the same species. *J Histochem Cytochem* **44,** 1331-1335.

Shortland P, Woolf CJ & Fitzgerald M. (1989). Morphology and somatotopic organization of the central terminals of hindlimb hair follicle afferents in the rat lumbar spinal cord. *J Comp Neurol* **289,** 416-433.

Simeone TA, Sanchez RM & Rho JM. (2004). Molecular biology and ontogeny of glutamate receptors in the mammalian central nervous system. *J Child Neurol* **19,** 343-360; discussion 361.

Simmons DR, Spike RC & Todd AJ. (1995). Galanin is contained in GABAergic neurons in the rat spinal dorsal horn. *Neurosci Lett* **187,** 119-122.

Simone DA, Baumann TK, Collins JG & LaMotte RH. (1989). Sensitization of cat dorsal horn neurons to innocuous mechanical stimulation after intradermal injection of capsaicin. *Brain Res* **486,** 185-189.

Simone DA, Sorkin LS, Oh U, Chung JM, Owens C, LaMotte RH & Willis WD. (1991). Neurogenic hyperalgesia: central neural correlates in responses of spinothalamic tract neurons. *J Neurophysiol* **66,** 228-246.

Simone DA, Zhang X, Li J, Zhang JM, Honda CN, LaMotte RH & Giesler GJ, Jr. (2004). Comparison of responses of primate spinothalamic tract neurons to pruritic and algogenic stimuli. *J Neurophysiol* **91,** 213-222.

Slack SE, Grist J, Mac Q, McMahon SB & Pezet S. (2005). TrkB expression and phospho-ERK activation by brain-derived neurotrophic factor in rat spinothalamic tract neurons. *J Comp Neurol* **489,** 59-68.

Slack SE, Pezet S, McMahon SB, Thompson SW & Malcangio M. (2004). Brain-derived neurotrophic factor induces NMDA receptor subunit one

phosphorylation via ERK and PKC in the rat spinal cord. *Eur J Neurosci* **20,** 1769-1778.

Slack SE & Thompson SW. (2002). Brain-derived neurotrophic factor induces NMDA receptor 1 phosphorylation in rat spinal cord. *Neuroreport* **13,** 1967-1970.

Slugg RM, Meyer RA & Campbell JN. (2000). Response of cutaneous A- and C-fiber nociceptors in the monkey to controlled-force stimuli. *J Neurophysiol* **83,** 2179-2191.

Sommers MG, Nguyen NK, Veening JG, Vissers KC, Ritskes-Hoitinga M & van Egmond J. (2008). Suppression of noxious-induced c-fos expression in the rat lumbar spinal cord by isoflurane alone or combined with fentanyl. *Anesth Analg* **106,** 1303-1308, table of contents.

Somogyi P, Hodgson AJ, Chubb IW, Penke B & Erdei A. (1985). Antisera to gamma-aminobutyric acid. II. Immunocytochemical application to the central nervous system. *J Histochem Cytochem* **33,** 240-248.

Sorkin LS & McAdoo DJ. (1993). Amino acids and serotonin are released into the lumbar spinal cord of the anesthetized cat following intradermal capsaicin injections. *Brain Res* **607,** 89-98.

Spicarova D & Palecek J. (2008). The role of spinal cord vanilloid (TRPV1) receptors in pain modulation. *Physiol Res* **57 Suppl 3,** S69-77.

Spike RC, Puskar Z, Andrew D & Todd AJ. (2003). A quantitative and morphological study of projection neurons in lamina I of the rat lumbar spinal cord. *Eur J Neurosci* **18,** 2433-2448.

Spike RC, Todd AJ & Johnston HM. (1993). Coexistence of NADPH diaphorase with GABA, glycine, and acetylcholine in rat spinal cord. *J Comp Neurol* **335,** 320-333.

Steiner TJ & Turner LM. (1972). Cytoarchitecture of the rat spinal cord. *J Physiol* **222,** 123P-125P.

Stewart W. (2001). The organization of the monoaminergic and cholinergic systems in the spinal cord. University of Glasgow Glasgow.

Stewart W & Maxwell DJ. (2000). Morphological evidence for selective modulation by serotonin of a subpopulation of dorsal horn cells which possess the neurokinin-1 receptor. *Eur J Neurosci* **12,** 4583-4588.

Story GM, Peier AM, Reeve AJ, Eid SR, Mosbacher J, Hricik TR, Earley TJ, Hergarden AC, Andersson DA, Hwang SW, McIntyre P, Jegla T, Bevan S & Patapoutian A. (2003). ANKTM1, a TRP-like channel expressed in nociceptive neurons, is activated by cold temperatures. *Cell* **112,** 819-829.

Strassman AM & Vos BP. (1993). Somatotopic and laminar organization of fos-like immunoreactivity in the medullary and upper cervical dorsal horn induced by noxious facial stimulation in the rat. *J Comp Neurol* **331,** 495-516.

Sugiura Y, Lee CL & Perl ER. (1986). Central projections of identified, unmyelinated (C) afferent fibers innervating mammalian skin. *Science* **234,** 358-361.

Sun RQ, Lawand NB, Lin Q & Willis WD. (2004). Role of calcitonin gene-related peptide in the sensitization of dorsal horn neurons to mechanical stimulation after intradermal injection of capsaicin. *J Neurophysiol* **92,** 320-326.

Sun WZ, Shyu BC & Shieh JY. (1996). Nitrous oxide or halothane, or both, fail to suppress c-fos expression in rat spinal cord dorsal horn neurones after subcutaneous formalin. *Br J Anaesth* **76,** 99-105.

Sun XF & Larson AA. (1991). Behavioral sensitization to kainic acid and quisqualic acid in mice: comparison to NMDA and substance P responses. *J Neurosci* **11,** 3111-3123.

Svensson CI, Fitzsimmons B, Azizi S, Powell HC, Hua XY & Yaksh TL. (2005). Spinal p38beta isoform mediates tissue injury-induced hyperalgesia and spinal sensitization. *J Neurochem* **92,** 1508-1520.

Svensson CI, Tran TK, Fitzsimmons B, Yaksh TL & Hua XY. (2006). Descending serotonergic facilitation of spinal ERK activation and pain behavior. *FEBS Lett* **580,** 6629-6634.

Szentagothai J. (1964). Neuronal and Synaptic Arrangement in the Substantia Gelatinosa Rolandi. *J Comp Neurol* **122,** 219-239.

Takamori S. (2006). VGLUTs: 'exciting' times for glutamatergic research? *Neurosci Res* **55,** 343-351.

Tao YX, Li YQ, Zhao ZQ & Johns RA. (2000). Synaptic relationship of the neurons containing a metabotropic glutamate receptor, MGluR5, with nociceptive primary afferent and GABAergic terminals in rat spinal superficial laminae. *Brain Res* **875,** 138-143.

Tao YX, Wei F & Zhao ZQ. (1997). A contribution of neurokinin-1 receptor to formalin-induced c-fos expression in the rat spinal dorsal horn. *Neurosci Lett* **221,** 105-108.

Tiong SY, Polgar E, van Kralingen JC, Watanabe M & Todd AJ. (2011). Galanin-immunoreactivity identifies a distinct population of inhibitory interneurons in laminae I-III of the rat spinal cord. *Mol Pain* **7,** 36.

Todd AJ. (1989). Cells in laminae III and IV of rat spinal dorsal horn receive monosynaptic primary afferent input in lamina II. *J Comp Neurol* **289,** 676-686.

Todd AJ. (1996). GABA and glycine in synaptic glomeruli of the rat spinal dorsal horn. *Eur J Neurosci* **8,** 2492-2498.

Todd AJ. (2002). Anatomy of primary afferents and projection neurones in the rat spinal dorsal horn with particular emphasis on substance P and the neurokinin 1 receptor. *Exp Physiol* **87,** 245-249.

Todd AJ. (2006). Anatomy and neurochemistry of the dorsal horn. In *Handbook of Clinical Neurology* ed. Cervero F & Jensen TS, pp. 61-76. Elsevier, B. V.

Todd AJ. (2010). Neuronal circuitry for pain processing in the dorsal horn. *Nat Rev Neurosci* **11,** 823-836.

Todd AJ, Hughes DI, Polgar E, Nagy GG, Mackie M, Ottersen OP & Maxwell DJ. (2003). The expression of vesicular glutamate transporters VGLUT1 and VGLUT2 in neurochemically defined axonal populations in the rat spinal cord with emphasis on the dorsal horn. *Eur J Neurosci* **17,** 13-27.

Todd AJ & Koerber HR. (2006). Neuroanatomical substrates of spinal nociception. In *Wall and Melzack's Textbook of Pain*, ed. McMahon SB & Koltzenburg M, pp. 73-90. Elsevier.

Todd AJ & Lewis SG. (1986). The morphology of Golgi-stained neurons in lamina II of the rat spinal cord. *J Anat* **149,** 113-119.

Todd AJ, McGill MM & Shehab SA. (2000). Neurokinin 1 receptor expression by neurons in laminae I, III and IV of the rat spinal dorsal horn that project to the brainstem. *Eur J Neurosci* **12,** 689-700.

Todd AJ & McKenzie J. (1989). GABA-immunoreactive neurons in the dorsal horn of the rat spinal cord. *Neuroscience* **31,** 799-806.

Todd AJ, Puskar Z, Spike RC, Hughes C, Watt C & Forrest L. (2002). Projection neurons in lamina I of rat spinal cord with the neurokinin 1 receptor are selectively innervated by substance p-containing afferents and respond to noxious stimulation. *J Neurosci* **22,** 4103-4113.

Todd AJ, Russell G & Spike RC. (1992a). Immunocytochemical evidence that GABA and neurotensin exist in different neurons in laminae II and III of rat spinal dorsal horn. *Neuroscience* **47,** 685-691.

Todd AJ & Spike RC. (1993). The localization of classical transmitters and neuropeptides within neurons in laminae I-III of the mammalian spinal dorsal horn. *Prog Neurobiol* **41,** 609-645.

Todd AJ, Spike RC & Polgar E. (1998). A quantitative study of neurons which express neurokinin-1 or somatostatin sst2a receptor in rat spinal dorsal horn. *Neuroscience* **85,** 459-473.

Todd AJ, Spike RC, Russell G & Johnston HM. (1992b). Immunohistochemical evidence that Met-enkephalin and GABA coexist in some neurones in rat dorsal horn. *Brain Res* **584,** 149-156.

Todd AJ, Spike RC, Young S & Puskar Z. (2005). Fos induction in lamina I projection neurons in response to noxious thermal stimuli. *Neuroscience* **131,** 209-217.

Todd AJ & Sullivan AC. (1990). Light microscope study of the coexistence of GABA-like and glycine-like immunoreactivities in the spinal cord of the rat. *J Comp Neurol* **296,** 496-505.

Torsney C & MacDermott AB. (2006). Disinhibition opens the gate to pathological pain signaling in superficial neurokinin 1 receptor-expressing neurons in rat spinal cord. *J Neurosci* **26,** 1833-1843.

Trafton JA, Abbadie C, Marchand S, Mantyh PW & Basbaum AI. (1999). Spinal opioid analgesia: how critical is the regulation of substance P signaling? *J Neurosci* **19,** 9642-9653.

Traub RJ. (1996). The spinal contribution of substance P to the generation and maintenance of inflammatory hyperalgesia in the rat. *Pain* **67,** 151-161.

Traub RJ, Pechman P, Iadarola MJ & Gebhart GF. (1992). Fos-like proteins in the lumbosacral spinal cord following noxious and non-noxious colorectal distention in the rat. *Pain* **49,** 393-403.

Traub RJ, Zhai Q, Ji Y & Kovalenko M. (2002). NMDA receptor antagonists attenuate noxious and nonnoxious colorectal distention-induced Fos expression in the spinal cord and the visceromotor reflex. *Neuroscience* **113,** 205-211.

Treede RD. (2009). The Adequate Stimulus. In *Science of Pain*, ed. Basbaum AI & Bushnell MC. Elsevier.

Tsujimura T, Shinoda M, Honda K, Hitomi S, Kiyomoto M, Matsuura S, Katagiri A, Tsuji K, Inoue M, Shiga Y & Iwata K. (2011). Organization of pERK-immunoreactive cells in trigeminal spinal nucleus caudalis, upper cervical cord, NTS and Pa5 following capsaicin injection into masticatory and swallowing-related muscles in rats. *Brain Res* **1417,** 45-54.

Uta D, Furue H, Pickering AE, Rashid MH, Mizuguchi-Takase H, Katafuchi T, Imoto K & Yoshimura M. (2010). TRPA1-expressing primary afferents synapse with a morphologically identified subclass of substantia gelatinosa neurons in the adult rat spinal cord. *Eur J Neurosci* **31,** 1960-1973.

Valerio A, Rizzonelli P, Paterlini M, Moretto G, Knopfel T, Kuhn R, Memo M & Spano P. (1997). mGluR5 metabotropic glutamate receptor distribution in rat and human spinal cord: a developmental study. *Neurosci Res* **28,** 49-57.

Valtschanoff JG, Weinberg RJ & Rustioni A. (1992a). NADPH diaphorase in the spinal cord of rats. *J Comp Neurol* **321,** 209-222.

Valtschanoff JG, Weinberg RJ, Rustioni A & Schmidt HH. (1992b). Nitric oxide synthase and GABA colocalize in lamina II of rat spinal cord. *Neurosci Lett* **148,** 6-10.

Vennekens R, Owsianik G & Nilius B. (2008). Vanilloid transient receptor potential cation channels: an overview. *Curr Pharm Des* **14,** 18-31.

Vercelli A, Repici M, Garbossa D & Grimaldi A. (2000). Recent techniques for tracing pathways in the central nervous system of developing and adult mammals. *Brain Res Bull* **51,** 11-28.

Vidnyanszky Z, Hamori J, Negyessy L, Ruegg D, Knopfel T, Kuhn R & Gorcs TJ. (1994). Cellular and subcellular localization of the mGluR5a metabotropic glutamate receptor in rat spinal cord. *Neuroreport* **6,** 209-213.

von Euler US & Gaddum JH. (1931). An unidentified depressor substance in certain tissue extracts. *J Physiol* **72,** 74-87.

Wall PD. (1960). Cord cells responding to touch, damage, and temperature of skin. *J Neurophysiol* **23,** 197-210.

Wall PD. (1967). The laminar organization of dorsal horn and effects of descending impulses. *J Physiol* **188,** 403-423.

Wall PD, Merrill EG & Yaksh TL. (1979). Responses of single units in laminae 2 and 3 of cat spinal cord. *Brain Res* **160,** 245-260.

Wang X & Marvizon JC. (2002). Time-course of the internalization and recycling of neurokinin 1 receptors in rat dorsal horn neurons. *Brain Res* **944,** 239-247.

Wei F, Vadakkan KI, Toyoda H, Wu LJ, Zhao MG, Xu H, Shum FW, Jia YH & Zhuo M. (2006). Calcium calmodulin-stimulated adenylyl cyclases contribute to activation of extracellular signal-regulated kinase in spinal dorsal horn neurons in adult rats and mice. *J Neurosci* **26,** 851-861.

Wilcox GL. (1988). Pharmacological studies of grooming and scratching behavior elicited by spinal substance P and excitatory amino acids. *Ann N Y Acad Sci* **525,** 228-236.

Willcockson HH, Taylor-Blake B & Light AR. (1995). Induction of fos-like immunoreactivity by electrocutaneous stimulation of the rat hindpaw. *Somatosens Mot Res* **12,** 151-161.

Williams S, Evan GI & Hunt SP. (1990). Changing patterns of c-fos induction in spinal neurons following thermal cutaneous stimulation in the rat. *Neuroscience* **36,** 73-81.

Willis WD & Coggeshall RE. (2004). *Sensory Mechanisms of The Spinal Cord* Kluwer Academic, New York.

Willis WD, Trevino DL, Coulter JD & Maunz RA. (1974). Responses of primate spinothalamic tract neurons to natural stimulation of hindlimb. *J Neurophysiol* **37,** 358-372.

Woodbury CJ & Koerber HR. (2003). Widespread projections from myelinated nociceptors throughout the substantia gelatinosa provide novel insights into neonatal hypersensitivity. *J Neurosci* **23,** 601-610.

Woodbury CJ, Kullmann FA, McIlwrath SL & Koerber HR. (2008). Identity of myelinated cutaneous sensory neurons projecting to nocireceptive laminae following nerve injury in adult mice. *J Comp Neurol* **508,** 500-509.

Woodbury CJ, Ritter AM & Koerber HR. (2000). On the problem of lamination in the superficial dorsal horn of mammals: a reappraisal of the substantia gelatinosa in postnatal life. *J Comp Neurol* **417,** 88-102.

Woolf CJ. (1983). Evidence for a central component of post-injury pain hypersensitivity. *Nature* **306,** 686-688.

Woolf CJ & Costigan M. (1999). Transcriptional and posttranslational plasticity and the generation of inflammatory pain. *Proc Natl Acad Sci U S A* **96,** 7723-7730.

Woolf CJ & Fitzgerald M. (1983). The properties of neurones recorded in the superficial dorsal horn of the rat spinal cord. *J Comp Neurol* **221,** 313-328.

Woolf CJ & Salter MW. (2000). Neuronal plasticity: increasing the gain in pain. *Science* **288,** 1765-1769.

Xia Z, Dickens M, Raingeaud J, Davis RJ & Greenberg ME. (1995). Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. *Science* **270,** 1326-1331.

Yamamoto T & Yaksh TL. (1992). Comparison of the antinociceptive effects of pre- and posttreatment with intrathecal morphine and MK801, an NMDA antagonist, on the formalin test in the rat. *Anesthesiology* **77,** 757-763.

Yan JY, Sun RQ, Hughes MG, McAdoo DJ & Willis WD. (2006). Intradermal injection of capsaicin induces acute substance P release from rat spinal cord dorsal horn. *Neurosci Lett* **410,** 183-186.

Yasaka T, Kato G, Furue H, Rashid MH, Sonohata M, Tamae A, Murata Y, Masuko S & Yoshimura M. (2007). Cell-type-specific excitatory and inhibitory circuits involving primary afferents in the substantia gelatinosa of the rat spinal dorsal horn in vitro. *J Physiol* **581,** 603-618.

Yasaka T, Tiong SY, Hughes DI, Riddell JS & Todd AJ. (2010). Populations of inhibitory and excitatory interneurons in lamina II of the adult rat spinal dorsal horn revealed by a combined electrophysiological and anatomical approach. *Pain* **151,** 475-488.

Yokota Y, Sasai Y, Tanaka K, Fujiwara T, Tsuchida K, Shigemoto R, Kakizuka A, Ohkubo H & Nakanishi S. (1989). Molecular characterization of a functional cDNA for rat substance P receptor. *J Biol Chem* **264,** 17649-17652.

Yoo CJ & Hwang SJ. (2007). The VR1-Positive Primary Afferent-Mediated Expression of pERK in the Lumbosacral Neurons in Response to Mechanical and Chemical Stimulation of the Urinary Bladder in Rats. *J Korean Neurosurg Soc* **42,** 462-469.

Yoshimura M & Jessell TM. (1989). Membrane properties of rat substantia gelatinosa neurons in vitro. *J Neurophysiol* **62,** 109-118.

Young MR, Blackburn-Munro G, Dickinson T, Johnson MJ, Anderson H, Nakalembe I & Fleetwood-Walker SM. (1998). Antisense ablation of type I metabotropic glutamate receptor mGluR1 inhibits spinal nociceptive transmission. *J Neurosci* **18,** 10180-10188.

Young MR, Fleetwood-Walker SM, Dickinson T, Blackburn-Munro G, Sparrow H, Birch PJ & Bountra C. (1997). Behavioural and electrophysiological evidence supporting a role for group I metabotropic glutamate receptors in the mediation of nociceptive inputs to the rat spinal cord. *Brain Res* **777,** 161-169.

Yu XH, Ribeiro-da-Silva A & De Koninck Y. (2005). Morphology and neurokinin 1 receptor expression of spinothalamic lamina I neurons in the rat spinal cord. *J Comp Neurol* **491,** 56-68.

Yu XH, Zhang ET, Craig AD, Shigemoto R, Ribeiro-da-Silva A & De Koninck Y. (1999). NK-1 receptor immunoreactivity in distinct morphological types of lamina I neurons of the primate spinal cord. *J Neurosci* **19,** 3545-3555.

Zemlan FP, Leonard CM, Kow LM & Pfaff DW. (1978). Ascending tracts of the lateral columns of the rat spinal cord: a study using the silver impregnation and horseradish peroxidase techniques. *Exp Neurol* **62,** 298-334.

Zhang ET & Craig AD. (1997). Morphology and distribution of spinothalamic lamina I neurons in the monkey. *J Neurosci* **17,** 3274-3284.

Zhang ET, Han ZS & Craig AD. (1996). Morphological classes of spinothalamic lamina I neurons in the cat. *J Comp Neurol* **367,** 537-549.

Zhang X, Davidson S & Giesler GJ, Jr. (2006). Thermally identified subgroups of marginal zone neurons project to distinct regions of the ventral posterior lateral nucleus in rats. *J Neurosci* **26,** 5215-5223.

Zhang X, Nicholas AP & Hokfelt T. (1995). Ultrastructural studies on peptides in the dorsal horn of the rat spinal cord--II. Co-existence of galanin with other peptides in local neurons. *Neuroscience* **64,** 875-891.

Zhang XJ, Li Z, Chung EK, Zhang HQ, Xu HX, Sung JJ & Bian ZX. (2009). Activation of extracellular signal-regulated protein kinase is associated with colorectal distension-induced spinal and supraspinal neuronal response and neonatal maternal separation-induced visceral hyperalgesia in rats. *J Mol Neurosci* **37,** 274-287.

Zheng CF & Guan KL. (1993). Properties of MEKs, the kinases that phosphorylate and activate the extracellular signal-regulated kinases. *J Biol Chem* **268,** 23933-23939.

Zhong J, Gerber G, Kojic L & Randic M. (2000). Dual modulation of excitatory synaptic transmission by agonists at group I metabotropic glutamate receptors in the rat spinal dorsal horn. *Brain Res* **887,** 359-377.

Zhou XF & Rush RA. (1996). Endogenous brain-derived neurotrophic factor is anterogradely transported in primary sensory neurons. *Neuroscience* **74,** 945-953.

Zylka MJ, Dong X, Southwell AL & Anderson DJ. (2003). Atypical expansion in mice of the sensory neuron-specific Mrg G protein-coupled receptor family. *Proc Natl Acad Sci U S A* **100,** 10043-10048.

Zylka MJ, Rice FL & Anderson DJ. (2005). Topographically distinct epidermal nociceptive circuits revealed by axonal tracers targeted to Mrgprd. *Neuron* **45,** 17-25.

List of publications

Al Ghamdi KS, Polgar E & Todd AJ. (2009). Soma size distinguishes projection neurons from neurokinin 1 receptor-expressing interneurons in lamina I of the rat lumbar spinal dorsal horn. *Neuroscience* **164,** 1794-1804.

Polgar E, Al Ghamdi KS & Todd AJ. (2010). Two populations of neurokinin 1 receptor-expressing projection neurons in lamina I of the rat spinal cord that differ in AMPA receptor subunit composition and density of excitatory synaptic input. *Neuroscience* **167,** 1192-1204.