# Chronic neuropathic pain and spinal dorsal horn plasticity

Abeer Wael Saeed

Department of Pharmacology and Therapeutics
McGill University, Montreal

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To my family, your devotion to the
pursuit of knowledge is my inspiration

# Abstract

Chronic pain is a debilitating disease with a very important socio-economic burden. The objective of this thesis was to contribute to our understanding of the normal organization of the dorsal horn of the spinal cord and its changes in chronic neuropathic pain, a form of chronic pain that sometimes follows lesions of the nervous system. Our studies focused on two important components of spinal cord pain-related circuitry, the projection neurons and their innervation by the small diameter nociceptive afferents. Spinal lamina I projection neurons have been classified, based on their morphology into fusiform, multipolar and pyramidal neurons. The two former types have been shown to respond to noxious stimuli and express the substance P receptor (NK-1r), while pyramidal neurons seldom express the NK-1r and respond to innocuous cooling only. The two main populations of small diameter nociceptive afferents are the peptidergic, which expresses the neuropeptides substance P (SP) and calcitonin gene-related peptide (CGRP), and the non-peptidergic, which is mostly devoid of neuropeptides but binds the plan lectin IB4.

In the first experimental chapter, we investigated the changes that occur at the level of the spinal dorsal horn in an animal model of chronic neuropathic pain. We demonstrated a de novo expression of NK-1r by pyramidal neurons, similar to that previously observed in our laboratory in a chronic arthritis model. This phenotypic switch was associated with a dramatic increase in the peptidergic (SP-immunoreactive) innervation of this cell population, which normally is sparsely innervated by these fibers. To assess whether pyramidal neurons responded to noxious stimuli in neuropathic animals, we injected capsaicin in the hind paw, which induced a massive internalization of NK-1r on these neurons, an indication of cell activation.

To assess whether a chronic pain state was needed to trigger the expression of NK-1r by pyramidal neurons, in the second experimental chapter of this thesis we used a model in which there is no chronic pain but in which

previous work from our laboratory had revealed a marked increase in NK-1r in the dorsal horn. In this model, the non-peptidergic population of nociceptive afferents is specifically ablated by the injection of the neurotoxin saporin conjugated to the lectin IB4 (IB4-SAP) into the sciatic nerve. The animals did not display any pain-related behavioral changes. However, we observed a significant upregulation of NK-1r in lamina I, in neuronal types that normally expressed it (i.e. fusiform and multipolar cells), with no de novo expression by pyramidal neurons.

In the third experimental chapter, we addressed the issue of whether lamina I projection neurons which express the NK-1r are innervated by non-peptidergic nociceptive afferents, as a study in a transgenic mouse model had provided data suggesting that non-peptidergic afferents had connections with deep dorsal horn neurons but not with lamina I NK-1r-expressing cells. We performed a systematic study aimed at identifying the normal connections of the non-peptidergic nociceptive fibers with lamina I neurons using both confocal and electron microscopy and we found a considerable innervation by non-peptidergic afferents on all three types of lamina I projection neurons.

The results of this thesis, taken together with previous data from our lab, suggest that a chronic pain state, such as neuropathic pain, seems necessary to trigger a de novo expression of NK-1r in pyramidal neurons and their increased innervation by peptidergic afferents. Further studies are required to clarify the role, in normal nociception and chronic pain states, of the significant direct innervation of lamina I projection neurons by non-peptidergic afferents which we revealed for the first time.

# Résumé

La douleur chronique est une condition débilitante ayant de sérieuses répercussions socio-économiques. L'objectif de cette thèse était de mieux comprendre l'organisation de la corne dorsale de la moelle épinière et les changements qui s'y produisent dans les cas de douleurs chronique neuropathique suite à une lésion du système nerveux. Nos études se sont concentrées sur deux composantes importantes des circuits de la douleur: les neurones de projection et leur innervation par les afférents nociceptifs de petit diamètre. Les neurones de projection de la couche 1 de la moelle épinière sont classées selon leur morphologie en 3 types: les neurones fusiformes, multipolaires et pyramidaux. Les deux premiers répondent aux stimuli douloureux et expriment le récepteur de la substance P (NK-1r), alors que les neurones pyramidaux n'expriment ce récepteur qu'occasionellent et répondent au froid non-douloureux. Les deux populations d'afférents principales sont les fibres de petit diamètre peptidergiques, qui expriment la substance P et le "calcitonin gene-related peptide" (CGRP), et les non-peptidergiques, qui sont dépourvues de neuropeptides et qui s'associent avec la lectine IB4.

Lors du premier chapitre expérimental, nous avons étudié les changement qui se produisent dans la corne dorsale de la moelle épinière dans un modèle de douleur neuropathique chronique. Nous avons démontré une expression de novo du NK-1r sur les neurones pyramidales, un changement similaire à celui se produisant dans un modèle d'arthrite chronique. Ce changement de phénotype était associé à une augmentation significative du nombre d'appositions peptidergiques faites sur cette population neuronale, qui reçoit habituellement très peu de ces entrées. Afin de vérifier si ces récepteurs sont fonctionnels et répondent aux stimuli douloureux, nous avons injecté de la capsaicine dans la patte arrière, ce qui a mené à une internalisation du récepteur, marquant l'activation de celui-ci.

Le deuxième chapitre de cette thèse vérifie si un état de douleur chronique est nécéssaire pour ce changement phénotypique, utilisant une lésion non douloureuse qui cause une augmentation signnificative du NK-1r dans la corne dorsale. Dans ce modèle, une population de nocicepteurs non peptidergiques est excise par une injection dans le nerf sciatique de la toxine saporine conjuguée à la lectine IB4 (IB4-SAP). En absence de symptômes douloureux, la couche 1 de la corne dorsale des animaux lésés a subi une augmentation générale du NK-1r mais sa distribution cellulaire est restée normale, sans expression de novo sur les cellules pyramidales.

Lors du troisième chapitre de cette thèse, nous avons vérifié si les neurones de projection de la couche 1 exprimant le NK-1r recevaient des entrées des fibres nociceptives non peptidergiques, comme ce sujet était controversé suite à une publication utilisant des souris transgéniques démontrant une absence de connections de la sorte. Nous avons fait une étude systématique utilisant la microscopie confocale et électronique et avons démontré que les 3 types morphologiques de cellules de projection reçoivent des entrées non peptidergiques directes.

Pris ensembles, les résultats de cette thèse suggèrent qu'une condition de douleur chronique est nécessaire pour l'expression du NK-1r sur les neurones pyramidaux et l'augmentation des entrées peptidergiques faites sur celles-ci. D'autres études seront nécessaires pour clarifier l'implication des entrées non peptidergiques faites sur les neurones de projection dans la nociception normale et dans la douleur chronique.

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## Contributions of Authors

This thesis is based on data obtained from the generation of the following three manuscripts:

**Manuscript 1:**
*De novo* expression of neurokinin-1 receptors by spinoparabrachial lamina I pyramidal neurons following a neuropathic lesion. Abeer W. Saeed and Alfredo Ribeiro-da-Silva, Journal of Comparative Neurology, *under revision*

**Manuscript 2:**
Limited changes in spinal lamina I dorsal horn neurons following the cytotoxic ablation of non-peptidergic C fibers. Abeer W. Saeed and Alfredo Ribeiro-da-Silva, Journal of Comparative Neurology, *submitted*

**Manuscript 3:**
Non-peptidergic primary afferents are presynaptic to lamina I projection neurons in rat spinal cord. Abeer W. Saeed and Alfredo Ribeiro-da-Silva, Molecular Pain, *submitted*

**Responsibilities of authors and co-authors**
The following statements describe the responsibility of all authors of the above co-authored manuscripts:

**Dr. Alfredo Ribeiro-da-Silva:** Supervisor of the doctoral thesis and principal investigator of all the projects described in the thesis. For all of the manuscripts, he was the main intellectual influence, contributed significantly to the original ideas and edited the writing of the manuscripts.

**Abeer Saeed:** Designed and performed all the experiments described in the thesis, collected all of the data and performed all of the quantitative analyses presented in the manuscripts, and wrote the first version and assembled all of the figures of all the manuscripts included in this thesis.

# Chapter 1

General Introduction

## 1.1 Epidemiology of Pain

Imagine being awakened, night after night, by a burning sensation running down your arm. You try to get out of bed, but any movement only makes the pain worse. You reach out for the medication bottle on your bedside table and take one pill, then another. You lie down, hoping for the pain to subside. After a couple of hours, you muster the courage to move again. The pain has not quite dissipated, still lingering as a tingling sensation, but at least it is not unbearable. You put on a light, baggy t-shirt, because anything else makes you feel like you are being stuck with sharp pins.

How many days have you endured this agony? You have isolated yourself from family and friends to avoid their concerned eyes and from burdening them with your complaints about the constant pain. You feel trapped in your own house, in your own body. When will this suffering end?

Approximately 1 million people in Canada alone suffer every day from some or all of the symptoms of burning, tingling, shooting, crawling pain which are all characteristics of chronic neuropathic pain. Physiologically, pain is a critical alarm system that warns of injury or protects damaged tissue until fully healed. However, there are times when the pain persists for months or years after full recovery from injury. Such pain, known as chronic pain, is a disease in its own right.

Neuropathic pain is defined by the International Association for the Study of Pain (IASP) as "pain caused by a lesion or disease of the somatosensory nervous system". It is a major neurological problem, affecting around 1.5-3% of people worldwide (Moulin et al., 2007; Taylor, 2006) and is cited as the 14$^{th}$ most common pain complaint seen in general practice (Hasselstrom et al., 2002). Nevertheless, until recently, neuropathic pain has been largely neglected by doctors.

Neuropathic pain is characterized by spontaneous pain, allodynia (pain induced by normally innocuous stimuli), hyperalgesia (an exaggerated pain response to a painful stimulus) and affective disorders, such as depression and anxiety.

Neuropathic pain results from nerve damage in the peripheral or central nervous systems and can be a consequence of nerve trauma, herpes zoster infection, chemotherapy or spinal cord injury among many others. Unfortunately, because we lack an understanding of the underlying etiology, effective and efficient analgesics have yet to be developed.

Before we can devise a successful therapeutic approach, not only must we investigate the changes that lead to and maintain chronic neuropathic pain, but we also have to understand the role played by the various peripheral and central components of the pain system in physiological conditions.

The spinal cord acts as an important relay center for pain-related (nociceptive) transmission, where information from the periphery is modified and modulated before being conveyed to supraspinal centers in the brainstem and brain for processing. Therefore, understanding the changes that occur at the level of the spinal cord, an important site in the initiation or maintenance of chronic neuropathic pain, could help in finding therapeutic targets.

This thesis gives an overview of the literature currently available, three original research manuscripts and a general discussion aimed at integrating the work and addressing the significance of the findings.

## 1.2 Pain Transmission Circuitry

René Descartes, a French philosopher and scientist of the 17$^{th}$ century, was the first to use the term "pain pathway" in his book, Treatise of Man (*Traité de l'homme).* Descartes described how a noxious stimulus initiates the transmission of a "pain" signal from the skin, through the spinal cord, to be perceived in the brain (Figure 1).


**Figure 1** – L'homme de René Descartes (Descartes, 1664).

With the progress of

scientific experimentation, the pain pathway has proven to be more complex than put forward by Descartes, nevertheless, the skin, spinal cord and brain remain key components of the nervous system responsible for the pain sensation.

In the following sections, each part of the pain pathway and its role in the transmission and modulation of the nociceptive signal will be discussed in more detail.

### 1.2.1 The Skin

The skin, the largest organ in the body, protects the body from the outside environment and external pathogens and provides sensory information about the surrounding environment. The skin is innervated by a variety of sensory neurons which are sensitive to touch, temperature and pain-related noxious stimuli.

The skin is composed of an outer layer of stratified squamous epithelium known as the epidermis and an inner, thicker layer, known as the dermis.

#### *1.2.1-1 The Epidermis*

Approximately 90% of the epidermis is composed of keratinocytes, whose primary function is to act as a barrier against environmental damage, such as intrusion of pathogens and water loss (Kanitakis, 2002). Other components of the epidermis include melanocytes, Langerhans cells and Merkel cells.

Melanocytes produce melanin, a pigment responsible for the color of skin and the protection of the nuclei of keratinocytes from harmful ultraviolet radiation (Kobayashi et al., 1998). Langerhans cells are mediators of immunologic responses within the skin, playing a role in the capture, uptake and processing of antigens (Girolomoni et al., 2002). Merkel cells associate with sensory afferents to form Merkel receptors, responsive to light touch (Maricich et al., 2009).

#### *1.2.1-2 The Dermis*

The dermis is composed of two layers, the papillary and the reticular (Kanitakis, 2002). The papillary layer sends projections into the epidermis which bring blood capillaries and tactile receptors into closer contact with the epidermal

cells. The reticular dermis is composed of collagen and elastin, thereby providing elasticity and retractile properties to the skin; moreover, blood vessels and nerves endings are dispersed throughout this layer.

*1.2.1-3 Peripheral Sensory Receptors*

Santiago Ramón y Cajal (1909) was the first to describe the sensory receptors of the somatosensory system which, based on their morphology, can be grouped into the two major populations of encapsulated receptors and free nerve endings. Encapsulated receptors consist of nerve endings surrounded by one or more layers of non-neural cells; they include Meissner's corpuscles, which respond to low frequency vibrations, light pressure and discriminative touch, Pacinian corpuscle, which are sensitive to deep pressure and high frequency vibrations, and Ruffini endings, which are sensitive to stretch or distortion of the skin connective tissue (Iggo and Andres, 1982). Free nerve endings are "non-specialized", unencapsulated terminal endings, with no complex sensory structures, that detect changes in temperature (thermoreceptors), mechanical stimuli (mechanoreceptors), or respond selectively to damaging or potentially damaging stimuli (nociceptors) (Lele and Weddell, 1956).

In this thesis, we will focus on the types and functions of nociceptors.

1.2.2 Nociceptors

In 1906, Sherrington (1906) discovered that intense stimulation of some nerves results in autonomic responses and pain. He gave the name *nociceptors* to the receptors activated by these intense stimuli.

Some nociceptors can selectively respond to either noxious thermal, chemical or mechanical stimuli. However, most nociceptors are classified as polymodal, whereby they can be activated by all three types of noxious stimuli (Bessou and Perl, 1969; Torebjork, 1974; Willis, 2007). Moreover, some are termed "silent" nociceptors, because they only respond to noxious stimuli following injury to the surrounding tissue (Handwerker et al., 1991; Lynn and Carpenter, 1982).

*1.2.2-1 Peripheral Signal Transduction*

Nociceptors express transducer proteins which selectively respond to intense chemical, mechanical and thermal stimuli. Once activated, this stimulus energy is transduced into the exchange of sodium and potassium ions. If the resulting current is sufficient (i.e. reaches the threshold value), an action potential is generated and a neural impulse is transmitted through the nociceptive primary afferents to the spinal cord (Crill and Schwindt, 1984).

Nociceptors are generally electrically silent (Woolf and Ma, 2007) and transmit all-or-none action potentials only when stimulated. However, the activation of nociceptors does not necessarily lead to the perception of pain. Pain perception depends also on the frequency of action potentials in primary afferents, the temporal summation of pre- and postsynaptic signals in the spinal cord, as well as processing of the signal by higher brain structures (Coggeshall and Willis, 2004).

Unlike other receptors, repeated or strong stimulation causes nociceptors to become sensitized (Bessou and Perl, 1969). Sensitized nociceptors are characterized by a lowered response threshold and an amplified response to previously effective stimuli (Bessou and Perl, 1969; LaMotte et al., 1982; Meyer and Campbell, 1981).

*1.2.2-2 Dorsal Root Ganglia*

Nociceptive sensory neurons are pseudounipolar, as a single process exits the cell body and splits into two branches. The first is a peripheral branch which terminates in skin and deeper structures such as viscerae, muscle and joints as free nerve endings. And the second is a central branch, which synapses with neurons in the spinal dorsal horn. The cell bodies of the nociceptive neurons are located in the dorsal root ganglia or the trigeminal ganglia (Kandel et al., 1991). The trigeminal ganglia possess neurons that specifically innervate the face, while the dorsal root ganglia (DRG) associate with the rest of the body.

Nociceptive sensory neurons can be divided into distinct populations based on their peptide content (Hokfelt et al., 1976; New and Mudge, 1986; Price, 1985) and the diameter and degree of myelination of their primary afferents. This will be discussed in a later section.

### 1.2.3 Nociceptive Primary Sensory Neurons

Primary sensory neurons correspond to four types of primary afferents: Aα, Aβ, Aδ and C fibers (Willis, 1985; Willis and Coggeshall, 1991). Aα and Aβ are fast-conducting, myelinated fibers involved in proprioception and discriminative touch, respectively.

Aδ and C fibers comprise the nociceptive primary afferents; Aδ fibers are thinly myelinated afferents, 1-5 μm in diameter with a conduction speed between 5-35 m/s, whereas C fibers are unmyelinated afferents, measuring 0.2-1.5 μm in diameter with a conduction speed of 0.5-2 m/s. This difference in myelination and therefore conduction speed, gives way to two phases of pain sensation; a "fast" sharp and piercing pain, transmitted by Aδ fibers, followed by a "slow" dull and aching pain, transmitted by C fibers (Julius and Basbaum, 2001). Unmyelinated, nociceptive C fibers are further differentiated into two populations, the peptidergic and the non-peptidergic fibers.

#### *1.2.3-1 Peptidergic C Fibers*

Peptidergic C fibers are nerve growth factor (NGF)-dependent (Averill et al., 1995; McMahon et al., 1994; Snider and McMahon, 1998), and express peptides, such as substance P (SP) and calcitonin gene related-peptide (CGRP). Peripherally, peptidergic fibers innervate the epidermis, blood vessels, glands and hair follicles, as well as muscle and joint (Fundin et al., 1997; Grelik et al., 2005; Ruocco et al., 2001) and centrally, these fibers terminate in lamina I and outer lamina II of the spinal dorsal horn (Cuello et al., 1993; Ribeiro-da-Silva, 2004) where they establish simple axo-dendritic or axo-somatic contacts with dorsal horn projection neurons (McLeod et al., 1998; Ribeiro-da-Silva, 2004; Ribeiro-da-Silva et al., 1989).

All SP-containing primary afferents have been shown to be nociceptors (Lawson et al., 1997). Peptidergic fibers express heat-evoked currents larger than those of non-peptidergic fibers (Stucky and Lewin, 1999) and their predominant excitatory neurotransmitter is glutamate (Battaglia and Rustioni, 1988; Merighi et al., 1991).

*1.2.3-2 Non-peptidergic C Fibers*

During the early postnatal period, non-peptidergic C fibers differentiate from a common pool they initially share with the peptidergic population. The non-peptidergic fibers switch off the expression of the NGF receptor, TrkA and begin expressing Ret which is the glial cell-derived growth factor (GDNF) receptor (Bennett et al., 1998; Molliver et al., 1997; Woolf and Ma, 2007), shifting dependency from NGF to GDNF for survival. About 50% of the C fiber population is composed of non-peptidergic afferents (Averill et al., 1995; Molliver et al., 1995), which do not express peptides, although demonstrating fluoride-resistant acid phosphatase (FRAP) activity (Coimbra et al., 1970; Hunt and Rossi, 1985; Knyihar et al., 1974; Nagy and Hunt, 1982). This FRAP has been recently shown to represent the transmembrane isoform of prostatic acid phosphatase (Zylka et al., 2008). The non-peptidergic afferents also express the purinergic P2X3 receptor (Guo et al., 1999; Snider and McMahon, 1998) and the G-protein coupled receptor, Mrgprd (Dong et al., 2001; Zylka et al., 2003; Zylka et al., 2005), and bind the *Griffonia simplicfolia* isolectin B4 (IB4) (Alvarez and Fyffe, 2000; Silverman and Kruger, 1988).

Peripherally, the non-peptidergic afferents innervate the epidermis, upper dermis and hair follicles (Ambalavanar et al., 2003; Lu et al., 2001; Zylka et al., 2005) and terminate mainly in inner lamina II of the dorsal horn in complex arrangements named synaptic glomeruli (Ribeiro-da-Silva and Cuello, 1990).

Compared with peptidergic afferents, the non-peptidergic afferents have longer action potentials and more tetrodotoxin (TTX)-resistant sodium channels (Stucky and Lewin, 1999). However, their predominant excitatory neurotransmitter is also glutamate (Battaglia and Rustioni, 1988; Merighi et al.,

1991).

IB4-positive primary afferents are implicated in the phenomenon of hyperalgesic priming such that the occurrence of an acute inflammation episode induces a long-lasting sensitivity to inflammatory mediators, prompting the development of chronic pain in a later episode of inflammation (Aley et al., 2000; Joseph and Levine, 2010; Levine et al., 1993; Reichling and Levine, 2009). Moreover, non-peptidergic afferents seem to play a role in developing and maintaining oxaliplatin-induced hyperalgesia (Joseph et al., 2008). Oxaliplatin is a cancer chemotherapy agent with a dose-limiting side effect of the development of acute onset, painful peripheral neuropathy.

In spite of what is said above, the relative role of peptidergic and non-peptidergic afferents in normal nociception and in chronic pain is still not well understood.

#### *1.2.3-3 Discrepancies in C Fiber Classifications in Rat vs. Mouse*

Rodents, especially mice and rats, are frequently utilized in pain research. However some features of these animals have to be taken into consideration when projecting results across species. For example, the peptidergic and non-peptidergic C fiber populations in the mouse form separate entities. This separation is not as clear cut in the rat (Molliver et al., 1997; Woodbury et al., 2004; Zwick et al., 2002). In mice, there is a localization of the capsaicin-sensitive, transient receptor potential vanilloid receptor-1 (TRPV1) only in the peptidergic fibers (Dirajlal et al., 2003; Woodbury et al., 2004; Zwick et al., 2002), whereas in rats, TRPV1 is present in both C fiber populations (Guo et al., 1999). Moreover, while the C fiber population expressing CGRP and SP in the mouse is distinct from that exhibiting IB4-binding (Molliver et al., 1997; Woodbury et al., 2004; Zwick et al., 2002), several studies indicated in the rat the colocalization of CGRP and SP in neuronal populations that bind IB4 (Fang et al., 2006; Petruska et al., 2002; Petruska et al., 2000; Price et al., 2005).

### 1.2.4 Substance P

SP is an excitatory neuropeptide discovered in 1931 in extracts of brain and intestine (V. Euler and Gaddum, 1931). The "P" term is designated for the "powder" containing the active agent obtained from tissue extracts (Gaddum and Schild, 1934). It was 30 years later, in 1971, that the 11 amino acid sequence of SP; Arg- Pro- Lys- Pro- Gln- Gln- Phe- Phe- Gly- Leu- Met- NH2, was established (Chang et al., 1971).

SP is a member of the tachykinin family, which also includes neurokinin A (NKA) and neurokinin B (NKB). SP and NKA are derived from the same pre-pro-tachykinin gene, PPTA. Through a process of alternative splicing, the PPTA gene yields three messenger ribonucleic acid (mRNA) transcripts encoding distinct precursor proteins (α-,β- and γ-pre-pro-tachykinins). The process of alternative splicing is modulated and controlled in a tissue-specific manner (Nawa et al., 1984). The α-pre-pro-tachykinin mRNA only encodes SP and occurs mainly in the central nervous system, while the β- and γ-pre-pro-tachykinin mRNA encode both SP and NKA and occur in both the central nervous system and the peripheral tissues. These precursor proteins then undergo further post-translational proteolytic processing to become mature forms of SP and NKA (Figure 2).

Mature SP molecules are packaged into dense core synaptic vesicles (Cuello et al., 1977) together with CGRP (Merighi et al., 1991) which are then transported to the presynaptic terminal from which they are then released outside the synapses (Bean et al., 1994). Indeed, it has been suggested that SP acts on its receptor through volume transmission, rather than through synapses (Zoli and Agnati, 1996). The co-release of CGRP with SP potentiates the action of the latter by inhibiting its breakdown (Le Greves et al., 1985). Moreover, De Biasi and Rustioni (1988) demonstrated the co-existence of SP and glutamate in the same primary afferent terminals, which supported previous studies describing the modulatory effect of SP on the excitatory action of glutamate (Urban and Randic, 1984). In the spinal cord, the highest density of SP immunoreactivity is found in lamina I and outer lamina II of the dorsal horn (Ribeiro-da-Silva and Hokfelt, 2000).

Once released from the pre-synaptic membrane, SP either binds to its preferred receptor, the neurokinin-1 receptor, activating several second messenger systems or its biological activity is terminated by the action of a neutral metalloendopeptidase (Benuck and Marks, 1975) or an angiotensin I converting enzyme (Skidgel et al., 1984).

*1.2.4-1 Neurokinin-1 Receptor*

The neurokinin-1 receptor (NK-1r) is a member of the class I (rhodopsin-like) cell surface G protein coupled receptor (GPCR) superfamily, which also includes the neurokinin-2 (NK-2r) and neurokinin-3 (NK-3r) receptors, which are the preferential receptors for NKA and NKB, respectively (Shigemoto et al., 1990; Yokota et al., 1989). The NK-1r consists of a seven hydrophobic α-helical transmembrane domains with a glycosylated extracellular N-terminus and an intracellular C-terminus (Figure 3). The human NK-1r has a 92% homology with mouse and rat NK-1r (Fong et al., 1992).

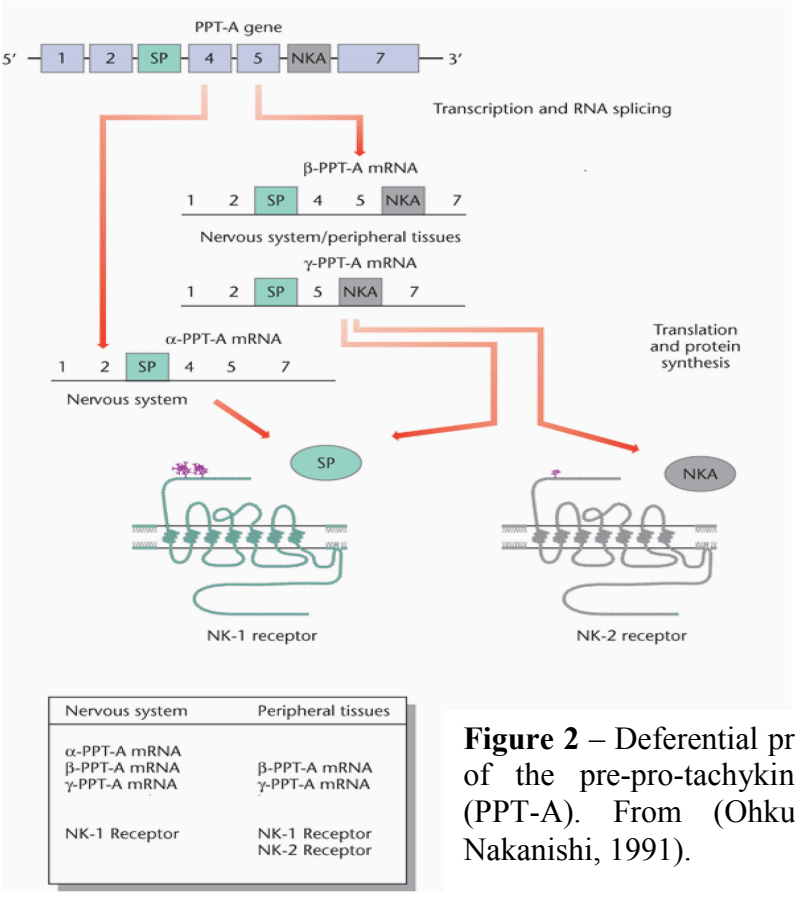


**Figure 2** – Deferential processing of the pre-pro-tachykinin gene (PPT-A). From (Ohkubo and Nakanishi, 1991).

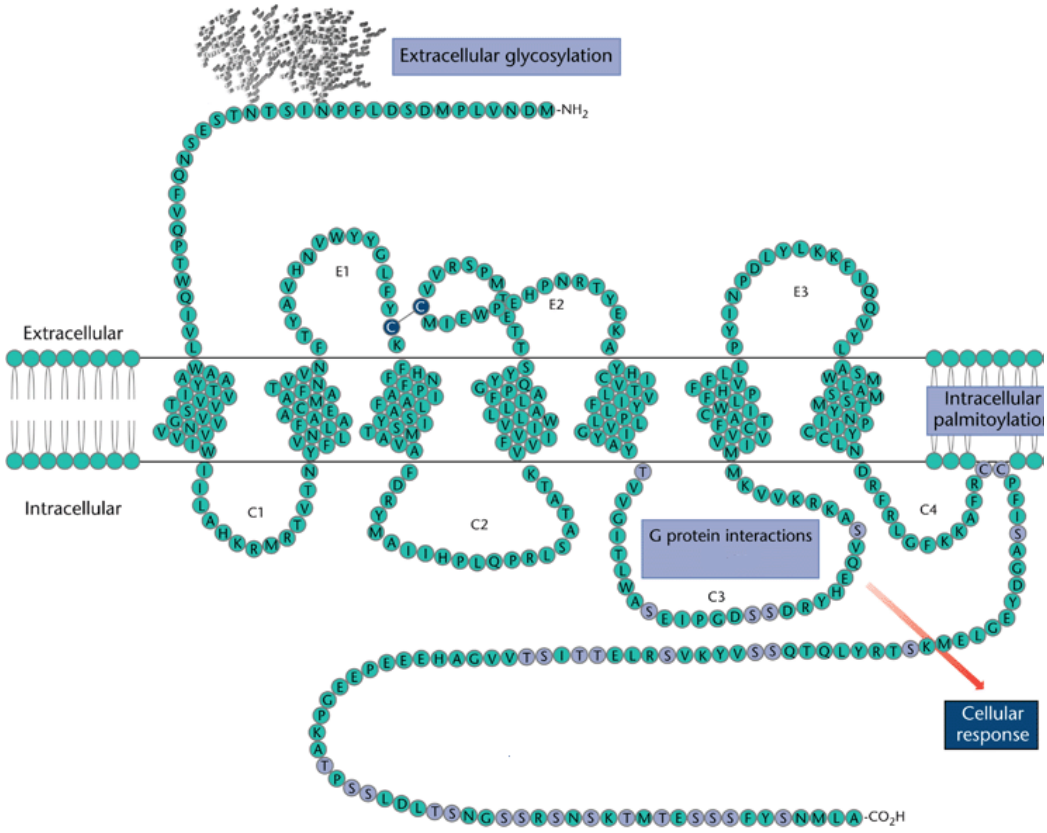


**Figure 3** - Schematic model of the rat NK-1r. From (Bremer and Leeman, 2001).

*1.2.4-2 NK-1r: Signal Transduction*

The binding of SP to the NK-1r, coupled to Gαq and/or Gα11, activates the phospholipase C β (PLCβ) pathway, causing the hydrolysis of phosphatidylinositol 4,5-bisphosphate ($PIP_2$) and the generation of two intracellular second messengers inositol 1, 4, 5-triphosphate (IP3) and diacylglycerol (DAG) (Mitsuhashi et al., 1992; Nakajima et al., 1992). IP3 stimulates the mobilization of calcium from endoplasmic reticulum stores causing an increase in intracellular calcium concentrations, which in turn causes cellular responses through the activation of calcium-calmodulin (Ca2+/CAM) kinase. DAG activates the protein kinase C (PKC) pathway which leads to protein phosphorylation and activation of various gene transcription factors. Furthermore, activation of PKC stimulates the catalytic activity of phospholipase $A_2$ ($PLA_2$) and

the subsequent release of arachidonic acid (AA) (Garcia et al., 1994), which is a precursor to inflammation-inducing agents (Figure 4).

Alternatively, activation of NK-1r coupled to Gαs protein, leads to the stimulation of membrane bound adenyl cyclase (AC), which results in the formation and accumulation of cyclic AMP (cAMP) (Mitsuhashi et al., 1992; Nakajima et al., 1992). cAMP in turn activates protein kinase A (PKA) which results in the phosphorylation of cAMP-response-element-binding protein(CREB), and the subsequent regulation of gene expression (Figure 5).

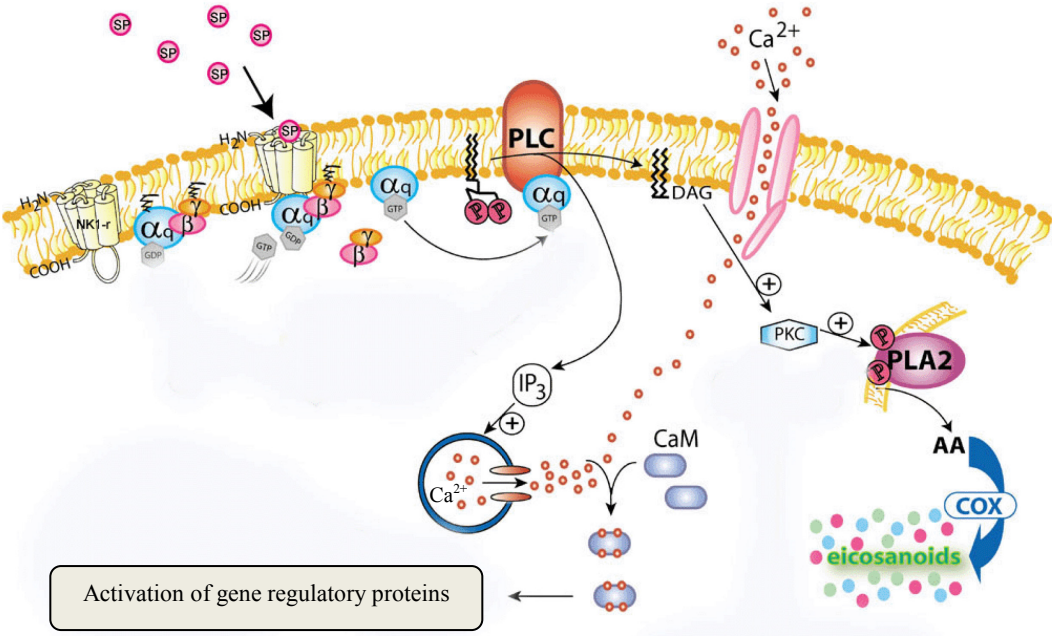


**Figure 4** – NK-1r-Gαq-PLCβ pathway. From (Billington and Penn, 2003).

*1.2.4-3 NK-1r Regulation*

To stop the signaling of the SP/NK-1r complex, the C terminus of the NK-1r is phosphorylated by G protein-coupled receptor kinase (GRK) (Premont and Gainetdinov, 2007) which subsequently leads to the recruitment of β-arrestins and the internalization of the phosphorylated NK-1r complex into clathrin-coated pits (Ferguson et al., 1996). In the endosome, the NK-1r dissociates from the SP

ligand and the β-arrestins, and is dephosphorylated, and the resensitized receptor is recycled back to the cell surface. Alternatively, the receptor may be trafficked to lysosomes for degradation and downregulation (Marchese et al., 2008) (Figure 6).

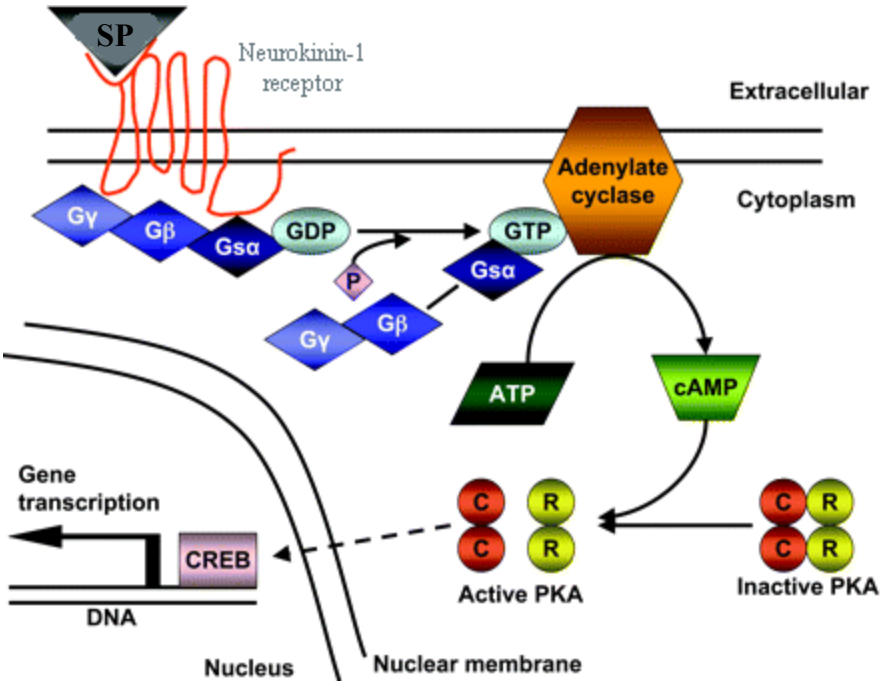


**Figure 5** – NK-1r-Gαs-cAMP pathway. From (Soon et al., 2008).

*1.2.4-4 SP, NK-1r and Clinical Disease States*

SP and its receptor are widely distributed in the nervous system and in peripheral tissues (Bremer and Leeman, 2001) which implicates them in the pathophysiology of various conditions, including pain, asthma, cystitis, inflammatory bowel disease, migraine, anxiety and emesis (Quartara and Maggi, 1998). Some of these conditions are discussed below.

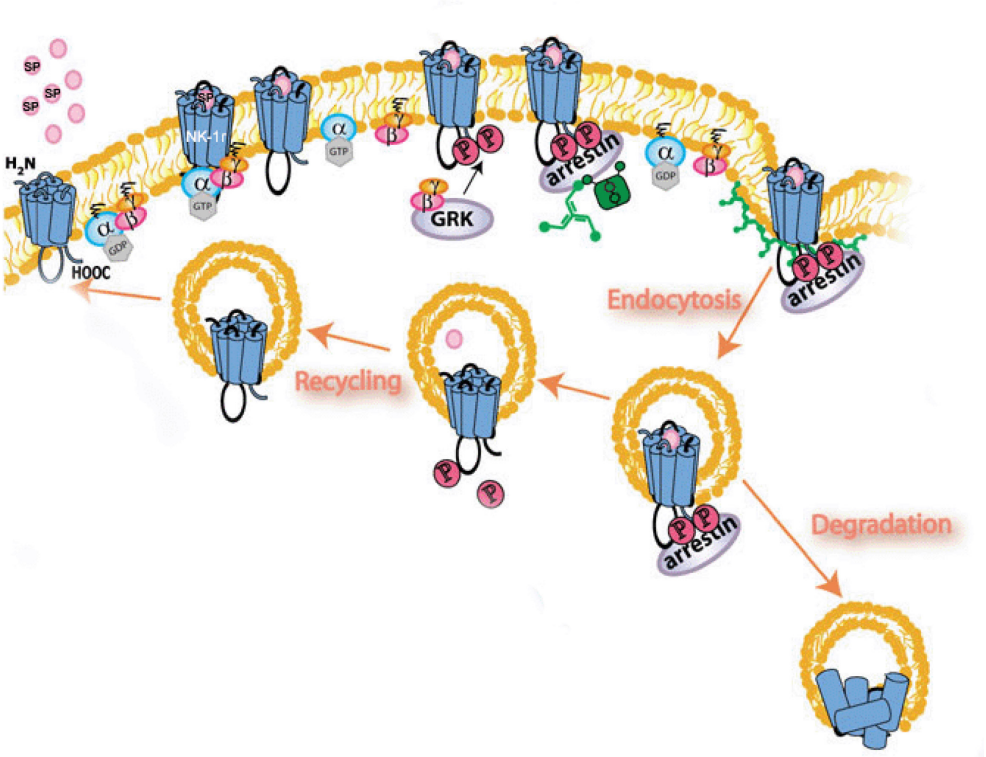


**Figure 6 –** NK-1r regulation through recycling or degradation. From (Billington and Penn, 2003).

*1.2.4-4a SP and Pain*

In the DRG, 20-30% of the neuronal populations contain SP (Otsuka and Yoshioka, 1993). SP is involved in the propagation of painful stimuli primarily in the thinly myelinated Aδ fibers and the unmyelinated peptidergic C fibers (Lawson et al., 1997; McCarthy and Lawson, 1989). SP exerts its action both peripherally and centrally. Peripherally, SP participates in the regulation of inflammatory, immune and wound-healing responses (Bolton and Clapp, 1986; Carolan and Casale, 1993; Devillier et al., 1986; Hartung et al., 1986; Hokfelt et al., 1975; Lundberg et al., 1983; Marasco et al., 1981; McGovern et al., 1995; Nilsson et al., 1985; Roch-Arveiller et al., 1986; Sakamoto et al., 1993; Stanisz et al., 1986; Tanaka et al., 1988; Vishwanath and Mukherjee, 1996). Although most of these effects are mediated by the NK-1r (Ribeiro-da-Silva et al., 2000), the effects on mast cells are not (Bueb et al., 1990). Centrally, SP binds to its receptor, present on spinal dorsal horn projection neurons, where it acts as a co-

transmitter with glutamate, facilitating the transmission of nociceptive stimuli towards supraspinal regions (Marshall et al., 1996).

The role of SP and NK-1r in nociceptive transmission has been investigated by several groups. Application of SP onto the dorsal horn by iontophoresis resulted in the excitation of nociceptive neurons (Radhakrishnan and Henry, 1991; Salter and Henry, 1991), while the intrathecal administration of SP elicited behavioral pain responses (biting, scratching, licking) (Seybold et al., 1982). Moreover, a strong SP input from stimulated peptidergic primary afferents resulted in the internalization of NK-1r (Allen et al., 1999) and the intrathecal injection of the cytotoxic saporin conjugated to SP selectively destroyed NK-1r-expressing spinal lamina I neurons, which attenuated behavioral responses to noxious mechanical and thermal stimuli (Khasabov et al., 2002; Mantyh et al., 1997; Nichols et al., 1999; Suzuki et al., 2002b; Vierck et al., 2003; Wiley et al., 2007; Wiley and Lappi, 2003).

*1.2.4-4b SP and Inflammation*

SP is present at the peripheral endings of peptidergic primary sensory afferents. Its release induces inflammation, known as neurogenic inflammation, which plays a role in the pathogenesis of some diseases such as rheumatoid arthritis (Gronblad et al., 1988) and fibromyalgia (Salemi et al., 2003).

Release of SP, and its binding to the NK-1r, activates resident immune cells such as macrophages, leucocytes and mast cells (Ho et al., 1996; Maggi, 1997), enhances the release of pro-inflammatory mediators such as cytokines, histamine, an inflammatory transcription factor, nuclear factor-κB (NFκB), interleukin-1β (IL-1β), and tumor necrosis factor α (TNF-α) (Bost, 2004; Ho et al., 1997; Holzer and Holzer-Petsche, 1997), and acts on the endothelial cells of blood vessels to increase vascular permeability, vasodilatation, and plasma extravasations of neutrophils and monocytes (Pernow, 1983; Smith et al., 1993). Taken together, these three effects result in the amplification of the inflammatory response, subsequent edema formation and further release of SP. It was

demonstrated that the administration of NK-1r antagonists blocks the neurogenic inflammation in animal models of rheumatoid arthritis (Decaris et al., 1999).

*1.2.4-4c SP and Emesis*

The vomiting reflex center, which is found in the medulla oblongata, expresses the NK-1r and is densely innervated by SP immunoreactive nerve fibers (Helke et al., 1984; Yamazoe et al., 1984). These SP-positive afferents are closely associated with gastric efferent neurons that project to the stomach (Ladic and Buchan, 1996). It was demonstrated that the central administration of SP induces emesis (Gardner et al., 1994). Moreover, several studies indicate the inhibitory effect of NK-1r antagonists on a broad spectrum of emetic stimuli (Bountra et al., 1993; Gardner et al., 1995; Rudd et al., 1999; Tattersall et al., 1993; Watson et al., 1995). Clinically, chemotherapy-induced nausea and vomiting is among the worst side effects in chemotherapy (Coates et al., 1983). The 2003 FDA- approved, NK-1r antagonist, aprepitant (EMEND®, Merck & Co, Inc), is the only anti-emetic available on the market that has significantly improved the quality of life of cancer patients (Hargreaves et al., 2011).

1.2.5 Cytoarchitecture of the Spinal Cord

The grey matter in the spinal cord is mainly composed of neuronal cell bodies and glia. Rexed divided the grey matter in the spinal cord of the cat into ten laminae (Rexed, 1952), and his lamination was subsequently adapted to other species. Lamina I to VI, designated as the dorsal horn of the spinal cord, receive sensory input from the periphery (Figure 7). The superficial layers of the dorsal horn, composed of lamina I, known as the marginal layer, and lamina II, known as the substantia gelatinosa, receive the majority of the primary termination of the nociceptive Aδ and C fibers originating from the skin (Light and Perl, 1979; Ribeiro-da-Silva and De Koninck, 2008; Sugiura et al., 1986).

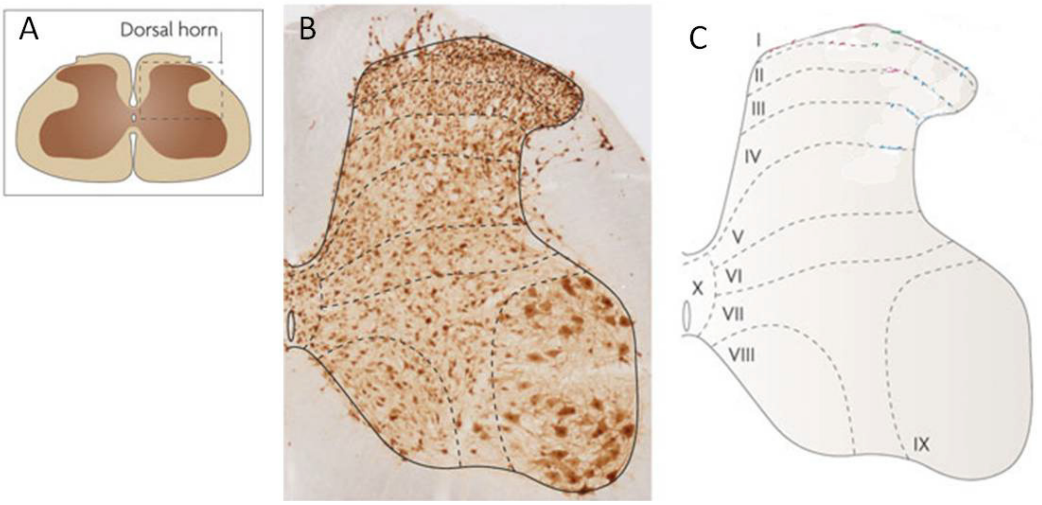


**Figure 7 –** Rexed laminar division of the spinal cord. **A:** Illustration of a transverse section of the spinal cord. **B:** Immunostaining with NeuN antibody which specifically labels neurons. **C:** Schematic illustration of the laminar organization of the spinal dorsal horn. From (Todd, 2010).

Among the lamina I spinal dorsal horn neurons are two populations, namely, neurons which give rise to axons that ascend the spinal cord named projection neurons and interneurons with very spiny, ventrally oriented dendrites (Cordero-Erausquin et al., 2009). Lamina I interneurons comprise more than 90% of the laminar neuronal population and were described to be presynaptic to lamina I spinoparabrachial projection neurons (Cordero-Erausquin et al., 2009), suggesting a role in the modulatory control of the signal conveyed supraspinally from the spinal cord.

Of interest to this thesis are the projection neurons, which will be further discussed below.

*1.2.5-1 Projection Neurons*

Neurons projecting supraspinally to the brainstem and brain are found in both lamina I and deeper laminae. The majority of these neurons travel in contralateral ascending pathways.

*1.2.5-1a Lamina I NK-1r-immunoreactive Neurons*

Approximately 80% of lamina I projection neurons express the NK-1r (Marshall et al., 1996; Todd et al., 2000). This population represents less than 5% of the total spinal dorsal horn neurons (Mullen et al., 1992; Suhonen et al., 1996). These projection neurons are excitatory and do not contain the inhibitory neurotransmitters, gamma-aminobutyric acid (GABA) or glycine (Littlewood et al., 1995). Their role in transmitting noxious pain was demonstrated by several studies. Acute noxious stimuli causes receptor internalization (Mantyh et al., 1995) or c-fos expression (Doyle and Hunt, 1999) in NK-1r-expressing lamina I neurons. Moreover, all dorsal horn neurons activated by SP were also activated by noxious stimulation (Henry, 1976) and selective ablation of NK-1r-immunoreactive neurons in lamina I via injection of the cytotoxin SP-saporin, resulted in alleviating pain sensitivity in neuropathic and inflammatory pain models (Mantyh et al., 1997; Nichols et al., 1999).

*1.2.5-1b Deep Laminae NK-1r-immunoreactive Neurons*

NK-1r-expressing neurons present in deeper laminae (LIII – LV) have large neurons with dorsally directed dendrites which terminate within the superficial laminae (Bleazard et al., 1994; Brown et al., 1995; Littlewood et al., 1995). These neurons receive synaptic input from both unmyelinated SP-positive peptidergic and myelinated primary afferents (Naim et al., 1997; Naim et al., 1998). Like lamina I projection neurons, these neurons also project rostrally to the parabrachial nucleus and other brainstem nuclei (Todd et al., 2000).

*1.2.5-1c Classification of Lamina I Projection Neurons*

Lamina I projection neurons are divided into three populations based on their morphological and correlated physiological properties, a classification that is consistent across species.

Morphologically, this classification relies on the cell body shape and the dendritic arborization pattern when viewed in the horizontal plane. Fusiform neurons have elongated, spindle-shaped soma with a primary dendrite at each end. Multipolar neurons consist of an irregularly-shaped cell body with four or more

dendrites. Pyramidal neurons have a triangular soma with one primary dendrite arising from each of the three corners of the cell body (Almarestani et al., 2007; Craig, 2003; Yu et al., 2005; Yu et al., 1999; Zhang and Craig, 1997; Zhang et al., 1996) (Figure 8). This classification has been shown to be consistent across species (rats, cats and monkeys) (Yu et al., 2005; Yu et al., 1999; Zhang and Craig, 1997; Zhang et al., 1996).

The physiological characteristics of these neuronal populations were identified based on their response to cutaneous input. Nociceptive specific (NS) neurons respond only to noxious heat and pinch. Polymodal nociceptive (HPC) neurons respond to noxious heat, pinch and cold, as well as to innocuous cold. Innocuous thermoreceptive (COLD) neurons respond to innocuous cooling only (Craig and Bushnell, 1994; Craig and Kniffki, 1985; Dostrovsky and Craig, 1996; Han et al., 1998).

A study by Han et al. (1998) demonstrated that the morphological and physiological properties of lamina I projection neuronal populations are correlated. All fusiform cells were NS, pyramidal cells were all COLD and multipolar cells consisted of both HPC and NS neurons. Moreover, these observations suggest a role for fusiform and multipolar neurons in nociceptive signaling, which is supported by studies in rats and monkeys, that demonstrate the expression of NK-1r by these two populations, while pyramidal neurons seldom express the NK-1r, in agreement with a non-nociceptive role for these neurons (Almarestani et al., 2007; Yu et al., 2005; Yu et al., 1999).

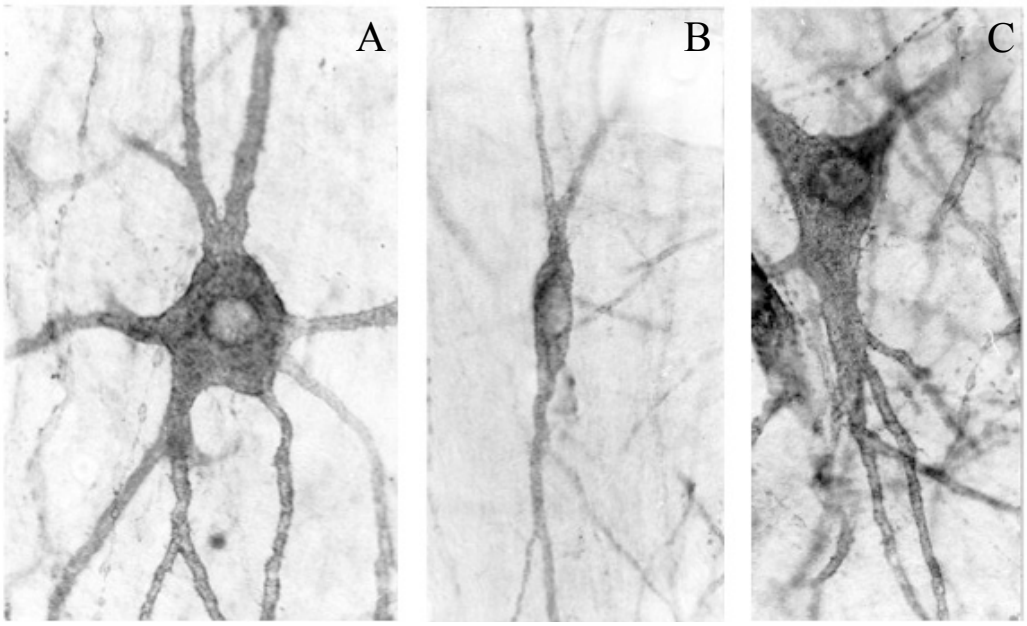


**Figure 8 –** The three populations of lamina I projection neurons in horizontal sections of primate spinal cord. **A:** Multipolar neurons **B:** Fusiform neurons **C:** Pyramidal neurons. From (Yu et al., 1999).

*1.2.5-2 Ascending Pain Pathways*

As mentioned earlier, projection neurons are mainly concentrated in lamina I of the spinal dorsal horn. The axons of these projection neurons cross the midline and then travel in the contralateral white matter to the thalamus and various brainstem nuclei including the lateral parabrachial nucleus (PB), caudal ventrolateral medulla (CVLM), periaqueductal grey (PAG) and nucleus tractus solitarius (NTS) (Al-Khater and Todd, 2009). According to Todd's group, at lumbar levels in the rat, the majority of lamina I projection neurons project to the PB and CVLM (Al-Khater and Todd, 2009; Spike et al., 2003), with less than 5% projecting to the thalamus (Al-Khater et al., 2008) (Figure 9). However, data from our laboratory in both rat and primate indicate a considerably higher percentage of lamina I neurons projecting to the thalamus from lumbar levels (Yu et al., 2005; Yu et al., 1999).

These target nuclei have diverse functions in processing the different components of pain. The CVLM modulates the cardiovascular responses to nociceptive inputs and contributes to the supraspinal descending pathways (Lima

et al., 2002). The PAG is a central site for analgesic action and is involved in organizing coping mechanisms to deal with pain and contributes to the descending modulation of the dorsal horn (Heinricher et al., 2009). The NTS is a site of convergence for both nociceptive and cardiorespiratory afferents and modulates respiratory activity, blood pressure and other autonomic responses that result from a noxious stimulation (Boscan et al., 2002). The thalamic neurons projecting to the primary and secondary somatosensory cortices are involved in the sensory-discriminative pain aspects, while those projecting to the insular cortex and amygdala contribute to the limbic processing of pain (Gauriau and Bernard, 2004).

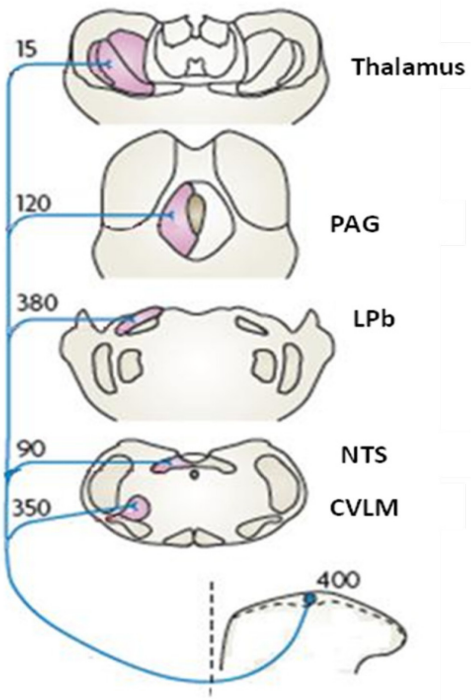


**Figure 9 –** Supraspinal projections of lamina I neurons. From (Todd, 2010).

The lateral parabrachial is the target nucleus in work presented in this thesis. Therefore, the spinoparabrachial pathway will be discussed in more detail.

*1.2.5-2a Spinoparabrachial Pathway*

Spinoparabrachial (spino-PB) neurons represent the majority of the NK-1r-immunoreactive lamina I projection neurons with up to 80-90% of all lamina I projection neurons either projecting exclusively to the PB or sending collaterals to the PB through the spino-thalamic pathway (Al-Khater and Todd, 2009; Ding et al., 1995; Hylden et al., 1989; Spike et al., 2003).

Lamina I spino-PB neurons encode nociceptive and thermoceptive information (Bester et al., 2000; Keller et al., 2007; Nakamura and Morrison, 2008b). Subsequently, neurons from the PB area project in turn to the amygdala

and the hypothalamus in the forebrain, and the PAG and CVLM in the brainstem (Bester et al., 2000; Gauriau and Bernard, 2002; Jasmin et al., 1997).

Therefore, the spino-PB pathway is implicated in the emotional and homeostatic control mechanisms in response to pain (Craig, 2003; Gauriau and Bernard, 2002).

### 1.2.6 The Cerebral Cortex

Nociception is encoded for by activation of the neural circuitry by noxious stimuli. However, the perception of pain emerges in the cortex as a result of multidimensional integration of sensory discriminative, affective motivational and cognitive evaluative components (Melzack and Casey, 1968). The cortex is an active regulator of nociceptive transmission, such that, depending on the behavioral context, the nociceptive signals could receive enhanced attention or be subordinated to other needs of higher priority (Bolles and Fanselow, 1982; Koyama et al., 2005; Quevedo and Coghill, 2007). Functional imaging techniques helped researchers recognize that pain perception in the cortex occurs via an interacting cortical matrix, rather than there being a specific pain center (Apkarian et al., 2005). The intensity and affective quality of the perceived pain is the net result of the interaction between ascending inputs and descending modulation.

The descending pathways from the brain to the superficial lamina I dorsal horn, which were mentioned earlier, are discussed more fully below.

### 1.2.7 Descending Pain Pathways

Spinal nociceptive processing is subject to inhibitory and facilitatory influences descending from the brain (McMahon and Wall, 1988). The balance between descending inhibition and facilitation is dynamic but it could be disrupted under certain behavioural, emotional or pathological states.

#### *1.2.7-1 Descending Facilitation*

Descending serotonergic facilitatory pathways originate in the rostral ventromedial medulla (RVM) (Urban and Gebhart, 1999; Urban et al., 1996;

Zhuo and Gebhart, 1997). This pathway exerts its facilitatory influences via the release of serotonin onto the central terminals of small diameter primary afferents activating ionotropic 5-hydroxytryptamine receptors (5-HT3) (Zeitz et al., 2002). Activation of the descending facilitatory pathways results in enhanced pain processing and intensified responses to noxious stimulation, which, in physiological states, allows for the enhanced detection, localization and reaction to potentially harmful stimuli (Ali et al., 1996; Green et al., 2000; Oyama et al., 1996).

*1.2.7-2 Descending Inhibition*

The descending inhibitory pathway plays a part in the negative feedback loop to dorsal horn neurons. A painful stimulus is transmitted to supraspinal regions, activating the descending inhibitory pathway, which in turn prevents excessive pain awareness by attenuating the successive painful signals (Fields and Basbaum, 1978).

There are two major descending inhibitory pathways, the PAG-RVM-dorsal horn circuitry and the noradrenergic inhibitory pathway. Both of these descending inhibitory pathways are subject to emotional and cognitive regulation, which in turn modulate the responses to nociceptive signalling of spinal neurons.

*1.2.7-2a The PAG-RVM-dorsal horn Pathway*

The RVM acts as the final relay station for descending anti-nociceptive action from a number of supraspinal sites, since it receives direct projections from the spinal dorsal horn and projects directly to the spinal cord (Newman, 1985). The RVM connects directly to the PAG (Behbehani and Fields, 1979; Gebhart et al., 1983) which in turn is heavily interconnected with the prefrontal cortex, hypothalamus, and amygdala (Gebhart et al., 1983). Stimulation of this pathway contributes to the spinal anti-nociceptive effect by release of pain inhibitory monoamines, amino acids and neuropeptides, such as GABA and serotonin from descending axon terminals in direct contact with spinal projection neurons (Giesler et al., 1981a; Jensen and Yaksh, 1984; Westlund et al., 1990).

*1.2.7-2b The Noradrenergic Pathway*

Descending noradrenergic pathways originating from brainstem nuclei, such as the locus coeruleus exert inhibitory influences onto spinal cord neurons through the release of norepinephrine which acts predominantly at spinal α2-adrenoceptors (Millan, 2002; Pertovaara, 2006), therefore, inhibiting the release of neurotransmitters from the primary afferent terminals and reducing the excitability of spinal dorsal horn projection neurons (Budai et al., 1998; Stanfa and Dickenson, 1994; Sullivan et al., 1992; Suzuki et al., 2002a).

## 1.3 Theories on Development of Chronic Pain

Chronic neuropathic pain is associated with multiple alterations in the peripheral and central nervous system that lead to maladaptive plasticity of the nociceptive system. These alterations are linked to peripheral and central sensitization mechanisms, characterized by a lowering in stimulation thresholds, an increase in the suprathreshold responses and spontaneous activity. Several of these mechanisms will be briefly discussed below.

### 1.3.1 Peripheral Sensitization

Peripheral sensitization refers to the upregulation in primary afferent activity. It can occur due to several mechanisms, some of which will be discussed below.

*1.3.1-1 Wallerian Degeneration*

Wallerian degeneration is a pathological process that occurs following nerve injury and was first described in frogs by Augustus Waller (1851). It is identified by the progressive degeneration and phagocytosis of myelin and axons distal to the site of injury (Stoll et al., 2002). Although Wallerian degeneration is a necessary prerequisite for the regeneration of injured peripheral nerves, only its role in the development of neuropathic pain will be discussed in this thesis.

Following nerve injury, resident Schwann cells, endothelial cells and mast cells release pro-inflammatory cytokines, including TNF-α. TNF-α stimulates the upregulation of other pro-inflammatory cytokines, activates the phagocytic activity of Schwann cells and recruits macrophages to the site of injury, all of which contribute to the process of nerve fiber degeneration (Lisak et al., 1997). Furthermore, the local release of TNF-α from Schwann cells causes spontaneous electrophysiological activity in the surviving nociceptive fibers, which contributes to the phenomenon of ectopic firing (Sorkin et al., 1997)

*1.3.1-2 Ectopic Activity in Primary Afferents*

Ectopic activity of primary afferents can lead to ongoing spontaneous pain or paroxysmal shooting pain that originates at sites other than the peripheral terminals. Ectopic impulses may originate from the dorsal root ganglion of the injured nerve, the site of nerve injury (either from the spontaneous activity of the injured nerves or from the heightened sensitivity of the intact nerves to stimuli) and/ or from the demyelinated axons of the injured nerve (Devor and Bernstein, 1982; Tal and Eliav, 1996; Wall and Devor, 1983). Several studies have demonstrated a correlation between spontaneous firing and nerve injury in both humans (Gracely et al., 1992; Nystrom and Hagbarth, 1981) and in animal models (Seltzer et al., 1991).

*1.3.1-3 Sensory-sympathetic Coupling*

The partial denervation of the skin following nerve injury may induce noradrenergic hypersensitivity of the intact primary afferents, whereby contributing to stimulus-independent spontaneous activity. The sympathetically-maintained pain following nerve damage is a result of the sprouting of sympathetic post-ganglionic fibers so as to form “baskets” around DRG cells or to make non-synaptic contacts with the intact primary afferents in the periphery that acquired functional adrenergic receptors (Janig et al., 1996; McLachlan et al., 1993; Ruocco et al., 2000; Yen et al., 2006).

*1.3.1-4 Abnormal Expression of Voltage-gated Sodium Channels*

Voltage-gated sodium channels (VGNC) are important for the generation of action potentials. Following nerve injury, these channels undergo a change in their expression as well as their distribution (Devor et al., 1993; England et al., 1996). Dorsal root ganglion neurons are known to express two types of VGNC, fast-inactivating TTX-sensitive (Nav1.3 and Nav1.7) and slow-inactivating TTX-resistant (Nav1.8 and Nav1.9) channels (Dib-Hajj et al., 1999b; Waxman et al., 1999). Nerve injury decreases the expression of Nav1.8 and Nav1.9 in the DRG soma, yet it increases its expression in the periphery, suggesting that nerve injury may trigger the translocation of Nav1.8 to the injury site, (Dib-Hajj et al., 1999a; Novakovic et al., 1998; Okuse et al., 1997). Moreover, nerve injury induces the re-expression of Nav1.3 in DRG cells, a VGNC usually expressed during embryonic development and down-regulated in the adult (Black et al., 1999; Dib-Hajj et al., 1999a; Waxman et al., 1994). The translocation and accumulation of Nav1.8 VGNC to the injury site may account for the inappropriate action potential initiation and subsequent hyperexcitability of afferents in the region of demyelination (Burchiel, 1980; Devor et al., 1993). The re-activation of Nav1.3 in the DRG may induce abnormal repetitive firing in the injured neurons, thus acting as ectopic impulse generators (Matzner and Devor, 1994).

1.3.2 Central Sensitization

Central sensitization is a result of increased release of excitatory neurotransmitters, such as glutamate and SP, and enhanced synaptic efficacy. Once central sensitization is established, the central nervous system amplifies the degree, duration and spatial extent of the pain, to a level that does not reflect the specific qualities of peripheral noxious stimuli. There are several mechanisms implicated in central sensitization at the level of the spinal dorsal horn and some of these will be discussed here.

*1.3.2-1 Pathological Sensitization of Spinal Cord Neurons*

Nerve injury-triggered peripheral ectopic firing of primary afferents causes a prolonged or massive input into the spinal cord, which enhances the response of dorsal horn neurons to all subsequent input (Woolf and Wall, 1986) in a phenomenon referred to as "wind-up". This process reduces the nociceptive threshold of dorsal horn neurons and leads to their subsequent sensitization. Moreover, peripheral nerve injury, pre-synaptically, causes the down-regulation of inhibitory μ-opioid receptors (Kohno et al., 2005) and the upregulation of voltage-gated calcium channel activity (Li et al., 2004) in the spinal dorsal horn, consequently leading to an enhanced release of glutamate onto post-synaptic dorsal horn neurons. Post-synaptically, a peripheral nerve injury results in a change in the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-methyl-D-aspartate (NMDA) glutamate receptors, such that there is an increased recruitment of AMPA receptors to the cell surface (Harris et al., 1996) and activation of NMDA receptors. This increase in glutamate receptor activity results in increased calcium influx (Dougherty et al., 1993; Duggan, 1995; Yoshimura and Yonehara, 2006), which causes the release of neuropeptides and glutamate and the subsequent nitric oxide synthesis, prostaglandin production, protein phosphorylation and central glial activation. All these factors lead to the increased spontaneous activity of dorsal horn neurons, expansion of their receptive fields and the decreased threshold to subsequent afferent input.

*1.3.2-2 Disinhibition*

Disinhibition is a decrease in synaptic inhibition, resulting in increased excitability or activation of normally silent pathways. Disinhibition may occur as a result of alterations in supraspinal descending pathways, such as a reduced descending noradrenergic inhibition (Rahman et al., 2008) or an enhanced descending serotonergic facilitation (Bee and Dickenson, 2008; Vera-Portocarrero et al., 2006). Alternatively, disinhibition may result from changes to local GABA-ergic inhibition, due to loss of GABA-ergic interneurons (Moore et al., 2002; Scholz et al., 2005) or downregulation of potassium chloride cotransporter isoform 2 (KCC2) resulting in increased intracellular chloride ion concentration,

whereby the activation of $GABA_A$ receptors may induce depolarization instead of hyperpolarization, therefore provoking spontaneous activity, enhancing the excitability of the cell and effectively reducing the strength of GABA-ergic inhibition (Coull et al., 2005; Coull et al., 2003; Keller et al., 2007).

*1.3.2-3 Glia Activation*

Peripheral nerve injury triggers the early glial response via the activation of spinal microglia. This activation is prompted by adenosine triphosphate (ATP) binding to the purinergic receptors, P2X4 and P2X7. Activated microglia release cytokines (IL-6, IL-10, IL-1β and TNF-α), nitric oxide, brain derived neurotrophic factor (BDNF) and excitatory amino acids, all of which contribute to the sensitization of dorsal horn neurons (Inoue, 2006). The microglial cytokines directly modulate the activity of the central terminals of primary afferents and the dorsal horn neurons, while BDNF produces a depolarizing shift in the anion reversal potential, thereby inducing an inversion of inhibitory GABA-ergic currents mentioned above (Coull et al., 2005).

The early microglial activation is followed by a later, longer-sustained increase in the activation and proliferation of astrocytes, which in turn releases several neurotransmitters and enhance synaptic activity (Coyle, 1998; Ren and Dubner, 2008; Zhuang et al., 2005).

1.3.3 Reorganization in Supraspinal Regions

Nerve injury may result in an increase in neuronal excitability and reorganization of neuronal connections in the thalamus and cortex (Lenz et al., 2000). Functional magnetic resonance imaging studies in humans show that light touch, in patients with neuropathic pain, results in the activation of a complex cortical network of nociceptive, motor and cognitive areas not seen in normal subjects (Maihofner et al., 2006; Schweinhardt et al., 2006). This suggests a change in the processing of the cutaneous stimuli at supraspinal centers.

1.4 The Sciatic Nerve

The animal models used throughout this thesis involve the manipulation of the sciatic nerve of Sprague Dawley rats. The sciatic nerve is the largest and longest nerve in all vertebrates. Several studies have demonstrated the existence of variations in the anatomy of the sciatic nerve depending on the rodent species and strain (Asato et al., 2000; Rigaud et al., 2008; Swett et al., 1991). The sciatic nerve of Sprague Dawley rats enters through the sciatic foramen and projects mostly to the L4 and L5 lumbar segments of the spinal cord, with also a small contribution to L6, mainly through the L4 and L5 spinal nerves, and to a smaller extent the L6 spinal nerve (Asato et al., 2000; Decosterd and Woolf, 2000; Rigaud et al., 2008; Swett et al., 1991). Peripherally, proximal to the spinal cord, the sciatic nerve forms a large bundle of nerve fibers encased in a connective tissue sheath called the epineurium. While distal to the spinal cord, the sciatic nerve subdivides into three fasciculi, each enclosed in connective tissue known as perineurium. The three main branches of the sciatic nerve are tibial, sural and common peroneal and these innervate distinct regions of the dorsal and plantar surfaces of the hind paw (Swett and Woolf, 1985; Takahashi et al., 1994). The tibial nerve innervates the middle zone

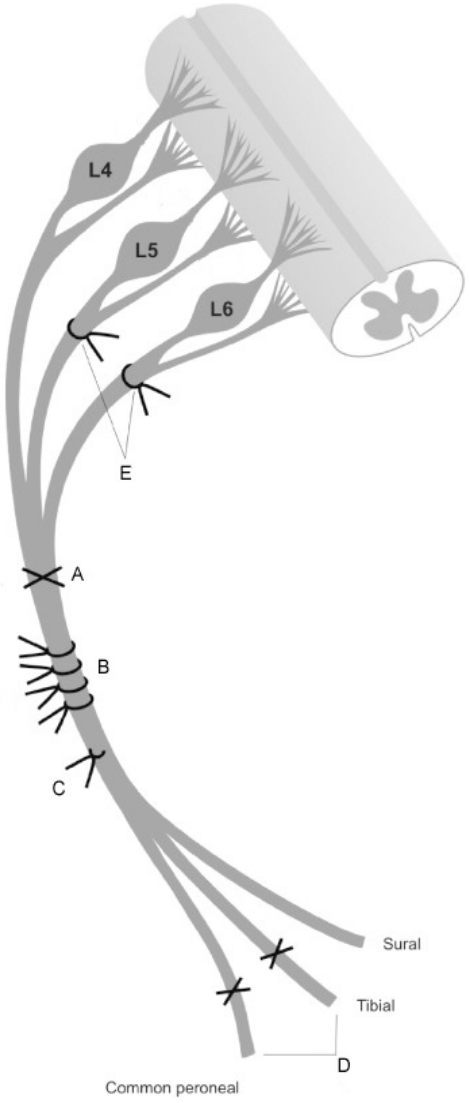


**Figure 10 –** Schematic illustration of models of peripheral nerve injury. **A:** Sciatic nerve transection. **B:** Chronic constriction injury. **C:** Partial sciatic nerve ligation. **D:** Spared nerve injury model. **E:** Spinal nerve ligation. From (Klusakova and Dubovy, 2009).

of the plantar surface, the sural nerve innervates the lateral zone of the hind paw and the common peroneal innervates the middle zone of the dorsal aspect of the hind paw (Figure 11). The primary afferents of the sciatic nerve terminate in the medial three-fourths of the superficial dorsal horn in a highly detailed, topographic pattern which correlates to the topography of the innervated skin surface, such that afferents from the common peroneal terminate in a zone between the medially situated tibial zone and the more laterally located sural zone (Swett and Woolf, 1985).

## 1.5 Experimental Animal Models

Since carrying out experimental studies in humans is unethical and the human experience of pain is far too complex to be fully reproduced in one model, a number of animal models have been developed to mimic human neuropathic pain etiologies and to study the aspects of various neuropathic pain conditions. Some of these models will be described below:

The first neuropathic pain model, designed in 1979 (Wall et al., 1979), comprised the complete transection of the sciatic nerve which resulted in approximately 60% reduction of primary afferent neurons and a high degree of autotomy (Figure 10A).

The model of chronic constriction injury (CCI) (Bennett and Xie, 1988) is an animal model of peripheral mononeuropathy in which the sciatic nerve is loosely constricted with four chromic gut sutures (Figure 10B). It results in a significant, but not complete, loss of primary afferents, many of which are large myelinated axons (Coggeshall et al., 1993). However, this model presents a considerable inflammatory response, owing to the use of chromic suture material, which may contribute to the neuropathic symptoms (Bennett and Xie, 1988).

The partial sciatic nerve ligation (PSNL) model (Seltzer et al., 1990) involves the tight ligation of half of the sciatic nerve with a silk suture (Figure 10C). However, this procedure does not involve a total denervation of the sciatic innervation territory and it is difficult to control the number and types of afferents injured between experiments.

The spared nerve injury (SNI) model (Decosterd and Woolf, 2000) involves the tight ligation of the tibial and common peroneal nerves, which comprise two of the three distal branches of the sciatic nerve, while the sural nerve is left intact (Figure 10D). The damaged sensory neurons innervate a restricted area in the periphery. Nevertheless, there is a considerable interaction between the somas of the injured and non-injured primary afferents in the DRGs.

The spinal nerve ligation (SNL) model (Kim and Chung, 1992) involves the tight ligation of one or two (L5 only or L5 and L6) of the spinal segmental nerves that contribute to the sciatic nerve (Figure 10E). This procedure results in the complete loss of afferents from the ligated spinal nerves. However, there is a risk of exposure of the intact L4 spinal nerve to the inflammatory components released from the ligated L5 spinal nerve, which lies in close proximity to the L4 spinal segmental nerve.

For the interest of this thesis, two animal models were used, namely the chronic constriction injury model using polyethylene cuff (cuff) and the IB4-saporin model of cytotoxic ablation of a primary afferent population, both of which will be further examined below.

### 1.5.1 Cuff Model

The first cuff model was developed by Mosconi and Kruger (1996) and involves the placement of short, fixed-diameter cuffs of polyethylene tubing around the main branch of the sciatic nerve. The model was behaviorally characterized in rats (Pitcher and Henry, 2004) and in mice (Benbouzid et al., 2008), both of which showed behavior that persisted for at least two months. With this model of peripheral neuropathy there is less variability in performing the procedure among experimenters than with the original CCI model, which allows for minimal variability in the degree of nerve constriction among animals. Therefore, it leads to a consistent nerve injury and in theory permits easier comparisons with other published studies (Cahill and Coderre, 2002; Cahill et al., 2003; Coull et al., 2005; Coull et al., 2003; Fisher et al., 1998).

1.5.2 IB4-saporin Model

Saporin is a 30-kDa ribosomal inactivating protein first described in 1983 by Stirpe et al. (1983). Saporin is a stable molecule with a very specific enzymatic activity. Once inside the cell, the saporin inactivates the ribosome by removing a single adenine base from the large ribosomal subunit, which inhibits protein synthesis and consequently results in cell death (Wiley and Lappi, 1994). This all-or-none approach is favorable since only target neuronal populations are affected, producing reliable lesions, while leaving other neighboring populations intact and thereby creating an effective tool for investigating a specific component in complex pathways.

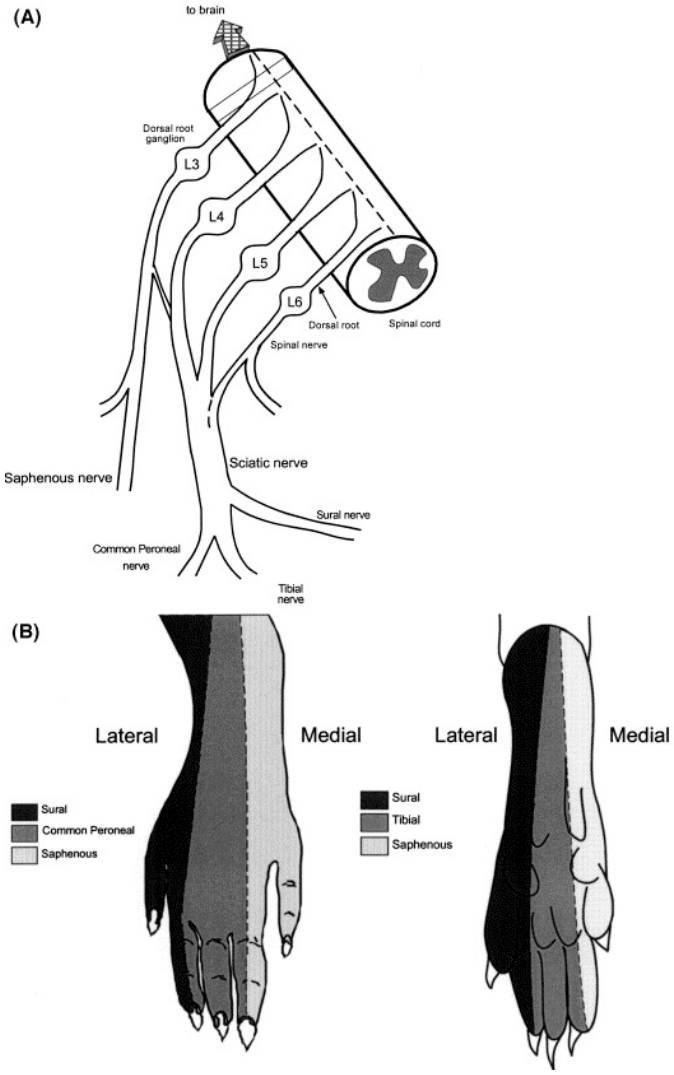


**Figure 11 –** Diagram of region of entry of the sciatic nerve into the spinal cord **(A)** and the zones of peripheral innervation by the branches of the sciatic nerve **(B)**. From (Decosterd and Woolf, 2000).

When saporin is conjugated to IB4, the toxin becomes specifically targeted to IB4-positive, non-peptidergic primary afferents through the binding of IB4 to cell surface α-D- galactose moities. It has been documented by several groups that injection of IB4-saporin into the sciatic nerve will lead to the elimination of the non-peptidergic primary afferent population, with no significant long term changes in behavioral responses

(Tarpley et al., 2004; Vulchanova et al., 2001). The lesion site is restricted to the medial three-fourths of the substantia gelatinosa, which corresponds to the central terminal fields of the tibial, common peroneal and sural branches of the sciatic nerve (Swett and Woolf, 1985).

Since there are no hyperalgesia or allodynia observed in the IB4-saporin model, it is not considered a model of neuropathic pain. However, it is a useful tool to study the role played by the non-peptidergic population of primary afferents under diverse conditions.

## 1.6 General Objectives and Thesis Rationale

Although extensive research is taking place in the chronic pain field, we are still far from identifying all the circuits contributing to the development and maintenance of chronic pain in the spinal cord due to the insufficient knowledge of the intrinsic dorsal horn circuitry. Therefore, to understand how chronic pain is initiated and maintained, studies of the pathophysiological mechanisms and physiological alterations that take place in pain models are important. However, at least as important are studies of the normal dorsal horn synaptic circuitry as well as of the normal molecular and physiological mechanisms involved in the processing of pain-related information. The general objectives of the work described in this thesis are two: a) to study changes in spinal lamina I cell populations and innervation in an animal model of neuropathic pain; b) to contribute to our knowledge of the synaptic connections and function of one of the main populations of primary afferents related to the transmission of pain-related information.

This thesis covers three experimental chapters, namely:

1. A combination of behavioral testing and confocal imaging was utilized to examine the phenotypic changes in the spinal lamina I projection neurons in an animal model of neuropathic pain. Furthermore, the spinal dorsal horn system, in chronic pain state, was challenged by an intradermal injection of capsaicin to test the functionality of the phenotypic changes.

2. The possible role of the non-peptidergic nociceptive afferents in inducing phenotypic changes in the spinal dorsal horn was studied using behavioral and confocal imaging techniques in the IB4-saporin model of selective cytotoxic ablation of non-peptidergic C fibers.
3. Confocal imaging and electron microscopy techniques were used to investigate the direct innervation of lamina I projection neurons by non-peptidergic unmyelinated primary afferents in naïve animals.

# Chapter 2

*De novo* expression of neurokinin-1 receptors by spinoparabrachial lamina I pyramidal neurons following a neuropathic lesion

**Abeer W. Saeed and Alfredo Ribeiro-da-Silva**

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## 2.1 Abstract

Lamina I of the spinal dorsal horn is a major site of integration and transmission to higher centers of nociceptive information from the periphery. One important primary afferent population that transmits such information to the spinal cord expresses substance P (SP). These fibers terminate in contact with lamina I projection neurons which express the SP receptor, also known as the neurokinin-1 receptor (NK-1r). Three types of lamina I projection neurons have been described: multipolar, fusiform and pyramidal. The first two types are thought to be nociceptive and express the NK-1r, whereas the pyramidal type has been described as non-nociceptive and seldom expresses the NK-1r.

In this immunocytochemical and behavioral study, we induced a neuropathic lesion in the rat by means of a polyethylene cuff placed around the sciatic nerve. We document that this lesion led to a *de novo* expression of NK-1r on pyramidal neurons as well as a significant increase in SP-immunoreactive innervation onto these neurons. These phenotypic changes were evident at the time of onset of neuropathic pain-related behavior. Additionally, we show that following a noxious stimulus (intradermal capsaicin injection), these NK-1r on pyramidal neurons were internalized, providing evidence that these neurons become responsive to peripheral noxious stimulation. We suggest that the changes following nerve lesion in the phenotype and innervation pattern of pyramidal neurons are of significance for neuropathic pain and/or limb temperature regulation.

## 2.2 Introduction

Chronic neuropathic pain occurs as a consequence of damage to either the central (e.g. spinal cord injury or multiple sclerosis) or the peripheral (e.g. post herpetic neuralgia or painful diabetic neuropathy) nervous systems. However, in spite of considerable progress, the mechanisms which trigger and maintain neuropathic pain are still poorly understood. Following lesions to peripheral nerves, some changes thought to be related to neuropathic pain occur at the level of the peripheral nervous system (Grelik et al., 2005; Peleshok and Ribeiro-da-

Silva, 2011; Taylor and Ribeiro-da-Silva, 2011; Yen et al., 2006), but some develop in the dorsal horn of the spinal cord where the modulation of incoming pain-related signals occurs (Abbadie et al., 1996; Bailey and Ribeiro-da-Silva, 2006; Castro-Lopes et al., 1993; Coull et al., 2003; Keller et al., 2007).

Spinal lamina I projection neurons play a major role in the forwarding of pain-related information to higher centers. Therefore, changes in the properties of these neurons may occur in neuropathic pain states and may be important for the triggering and/or maintenance of the pain-related condition. These lamina I neurons project mainly to the lateral parabrachial nucleus (LPb) (Bernard et al., 1995; Cechetto et al., 1985; Craig, 1995; Feil and Herbert, 1995; Wiberg and Blomqvist, 1984) and to the thalamus (Carstens and Trevino, 1978; Craig and Burton, 1981; Giesler et al., 1979; Giesler et al., 1981b; Willis et al., 1979). Lamina I projection neurons have been classified into three populations, based on their morphological properties; fusiform, multipolar and pyramidal (Yu et al., 2005; Yu et al., 1999; Zhang and Craig, 1997; Zhang et al., 1996). An intracellular physiological study allowed the correlation of morphological and physiological characteristics. Fusiform neurons were nociceptive-specific (NS) and responded to noxious heat and pinch; multipolar neurons were either NS or responded to noxious heat, pinch and noxious and innocuous cold (HPC); in contrast, pyramidal neurons were non-nociceptive, and responded to innocuous cooling (COOL) (Han et al., 1998). In agreement with their responsiveness to noxious stimuli, both fusiform and pyramidal neurons were shown to be immunoreactive for the main substance P (SP) receptor, the neurokinin-1 receptor (NK-1r). However, in agreement with their lack of response to noxious stimuli, pyramidal neurons seldom express NK-1r (Almarestani et al., 2007; Yu et al., 2005; Yu et al., 1999).

Importantly, we found that NK-1r were expressed *de novo* by pyramidal neurons, in an animal model of inflammatory arthritis (Almarestani et al., 2009). However, such expression started only at 2 weeks after the injection of complete Freund's adjuvant (CFA) into the plantar surface of the rat hind paw (Almarestani et al., 2009), coincident with the onset of extensive joint and bone damage, as

assessed by imaging approaches (Almarestani et al., 2011). Interestingly, we detected that, in normal animals, pyramidal neurons are hardly innervated by peptidergic nociceptive primary afferents, as revealed by SP immunoreactivity, but became abundantly innervated as from 2 weeks post-CFA injection (Almarestani et al., 2009). This is an important observation, because in past studies in the cat, combining intracellular physiology and injection of a marker with immunocytochemistry, it was shown that dorsal horn neurons which were abundantly innervated by SP were nociceptive, whereas non-nociceptive neurons were scarcely innervated by SP (De Koninck et al., 1992; Ma et al., 1996). It was also shown that, in the rat, neurons expressing the NK-1r are abundantly innervated by SP immunoreactive (-IR) primary afferents and display c-fos immunoreactivity following noxious stimulation (Todd et al., 2002). Based on the above, there seems to be a correlation between the presence of nociceptive responses and the abundance of SP innervation and expression of NK-1r by these neurons. Therefore, the lack of SP innervation of lamina I pyramidal neurons and the lack of expression of NK-1r by most of them in naïve animals, and the drastic change in NK-1r expression and SP innervation in these neurons in the presence of arthritis would suggest the switch from a non-nociceptive to a nociceptive phenotype of these cells (Almarestani et al., 2009).

In this study, we investigated whether the induction of neuropathic pain would trigger the *de novo* expression of NK-1r by lamina I pyramidal cells and alter their innervation by SP-IR nociceptive afferents.

## 2.3 Materials & Methods

The guidelines of the Canadian Council on Animal Care for the care and use of experimental animals were thoroughly followed in all the experiments described. Furthermore, the studies were conducted following approval by the McGill University Faculty of Medicine Animal Care Committee and followed the guidelines of the International Association for the Study of Pain.

A total of 74 male Sprague Dawley rats (Charles River, Quebec, Canada) weighing 215-225g were used for the experiments. The number of animals used

and their suffering was kept to the minimum necessary for the conduction of the study. The number of animals per group varied between four and eight animals. All animals were exposed to 12hr light/dark cycle and given food and water *ad libitum*. The cages housed four animals each and were fitted with soft bedding and a plastic tube for an enriched environment.

### 2.3.1 Animal Preparation

*Surgeries*

Animals were anesthetized with 5% isoflurane in oxygen using a gas chamber. The left femoral muscle of the thigh was exposed using blunt dissection. The sciatic nerve was exposed and freed from surrounding connective tissue using a glass pipette. In the experimental group, a 2mm PE60 polyethylene tubing (Intramedic, Fisher Scientific, Whitby, ON, Canada) was loosely placed on the sciatic nerve using 90-degree bent tip tweezers, ensuring minimal manipulation of the nerve. In the sham group, the sciatic nerve was exposed in the same manner as above but was not manipulated in any way. The incision was then sutured in two layers (muscles and skin, respectively). Animals were returned to their cages to recover. There was no difference in weight gain between experimental and sham groups at all time points studied.

*Injection of Tracers*

For all experiments requiring retrograde tracing, animals were anesthetized using 5% isoflurane in oxygen and placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA) and stabilized with non-perforating ear bars. With Bregma as the reference point, the coordinates for the parabrachial nucleus (rostral/caudal: -9.12; medial/lateral: -2.1; dorsal/ventral: -6.3) were calculated from the Paxinos & Watson Rat Brain Atlas (Paxinos and Watson, 2005). A small hole was drilled through the skull at the target point, exposing the dura mater. A glass micropipette (Wiretrol II, Drummond Scientific Company, Broomall, PA) was lowered to the stereotaxic position of the parabrachial nucleus.

Two µl of 1.0% solution of cholera toxin B (CTb) (List, Campbell, CA) were slowly injected into the parabrachial nucleus over a period of 20 minutes. A 10 minute waiting period was imposed before the micropipette was retracted from its position to minimize leakage of the tracer. CTb was injected seven days prior to sacrificing the animals.

*Capsaicin Injection*

As a noxious stimulus, a 20µl intra-dermal injection of a 0.1% capsaicin solution (Sigma/Aldrich, St-Louis, MO) in 20% ethanol, 7% TWEEN 80 and 73% physiological saline, was given between the interdigital pads of the left hind paw. The sham group received an intra-dermal vehicle injection of 20% ethanol, 7% TWEEN 80 and 73% physiological saline. Animals were sacrificed at 12 min post-injection.

2.3.2 Behavior Testing

Animals were always habituated to the testing apparatus for 30 minutes prior to testing. Baseline behavioral thresholds for both tests were measured for two consecutive days prior to surgical treatments.

*Testing for Mechanical allodynia*

The testing apparatus consisted of clear plastic enclosures elevated on a mesh grid, which allowed complete access to the ventral side of the animal. Animals were tested using the up-down method described previously by Chaplan et al. (Chaplan et al., 1994). In brief, von Frey filaments of increasing stiffness were applied with 5 seconds delay between each presentation, starting with the 0.008g filament. The filament was applied to the mid-plantar area of the hind paw, avoiding the pads of the foot, until it buckled, and maintained for 10 seconds or until an obvious behavior (paw withdrawal, flicking or licking) occurred. A positive response prompted the next weaker filament to be presented, while a negative response prompted the presentation of the next stronger filament. This process was repeated 6 times and the number of positive reactions was noted and

an average threshold was calculated. After the right paw for all the animals was tested, the left paw was tested in the same manner.

*Testing for Thermal hyperalgesia*

The Hargreaves test (Hargreaves et al., 1988) was employed to measure heat nociceptive thresholds. Clear plastic enclosures were set on top of a glass floor. The light source was directed onto the skin area of the paw in contact with the glass. When the paw was lifted, the light source automatically turned off. Testing included three trials per paw with each trial being completed for all the animals before the start of the next trial, ensuring a 30 minute wait before the start of the next trial to minimize desensitization effects. The time from turning on of the light source until withdrawal was noted and an average of the three trials per paw was calculated.

2.3.3 Animal Perfusion

At the end of each time point (7, 10, 15, 21, 28 days), the animals were deeply anesthetized with 0.3ml/100g of body weight of Equithesin (6.5mg chloral hydrate and 3mg sodium pentobarbital i.p.). They were then perfused through the left cardiac ventricle with perfusion buffer - for composition see (Côté et al., 1993) - for one minute followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4, for 30 minutes. The brain and spinal cord segments L4-L5 were extracted and post-fixed for 4 hours and 2 hours, respectively, in 4% paraformaldehyde in PB. The specimens were then cryoprotected in 30% sucrose in PB overnight at 4°C.

2.3.4 Immunohistochemistry

To examine the injection site at the level of the parabrachial nucleus, serial 100µm-thick coronal sections of the relevant brain region were obtained. Furthermore, the dorsal aspect of the L4-L5 spinal cord segment was cut into serial 50µm thick horizontal sections, to examine lamina I. All specimens were cut using a freezing-sledge microtome (Leica, Richmond Hill, Ontario) and

collected as free-floating in phosphate-buffered saline (PBS) with 0.2% Triton-X 100 (PBS+T).

Sections were incubated in 10% normal donkey serum (Jackson, West Grove, PA) in PBS+T for one hour at room temperature to block unspecific staining. Then, spinal cord sections were incubated with the primary antibodies: goat anti-CTb (List Biological Laboratories, Campbell, CA; product #703, lot #7032A6) at 1:5000 dilution and rabbit anti-NK1-r (Sigma/Aldrich, St-Louis, MO; product # S8305; lot 084K4845) at 1:10000 dilution in PBS+T containing 5% normal donkey serum for 48 hours at 4°C. Next, sections were washed several times with PBS+T and incubated for 2 hours at room temperature with a biotinylated donkey anti-goat IgG (1:250, Jackson, West Grove, PA), and, after further washing in PBS+T incubated with streptavidin conjugated to AlexaFluor 568 (Molecular Probes) and donkey anti-rabbit AlexaFluor 488 in 5% normal donkey serum and PBS+T for 2 hours at room temperature. Finally, sections were washed with PBS, mounted on gelatin-subbed slides and coverslipped with an anti-fading mounting medium (Aqua Polymount; Polysciences, Warrington, PA). Slides were stored in the dark at -4°C.

Brainstem sections of the injection site were incubated with anti-CTb antibody followed by biotinylated donkey anti-goat IgG and streptavidin conjugated to AlexaFluor 568. They were mounted and coverslipped as described above.

Spinal cord sections for the quantification of SP-IR input to lamina I neurons originated from a different cohort of animals (n=6). Sections were cut and processed as described above, but the primary antibody mixture included a rat anti-SP monoclonal antibody [spent tissue culture supernatant diluted 1:10, kindly supplied by A. Claudio Cuello; (Cuello et al., 1979); Medimabs, Montreal, Qc, Canada]. Furthermore, the secondary antibody mixture also included a donkey anti-rat IgG conjugated to Cy5 (Jackson, West Grove, PA).

2.3.5 Antibody Specificity

As controls for immunocytochemistry, some sections were processed by omitting the primary antibodies or by pre-absorption with the peptide used to generate the antibody. In all cases, there was a complete loss of immunoreactivity.

The goat anti-CTb antibody was generated against CTb and its specificity was demonstrated by the lack of any staining in animals not injected with CTb.
The rabbit anti-NK-1r antibody was generated against a synthetic peptide corresponding to amino acids 393-407 of the C-terminus region of the rat NK-1r, and purified by ion-exchange chromatography. In Western blots from rat brain, it recognizes a single band at 46 kDa, whose staining is specifically inhibited by incubation with the blocking peptide (data supplied by the manufacturer). Furthermore, it was shown that it does not produce any staining in NK-1r knockout mice, although it recognizes the receptor in wild type mice (Ptak et al., 2002).

The anti-substance P antibody has been extensively used in the literature by us (e.g. (Almarestani et al., 2009) and others. Although we obtained it directly from Professor Cuello, it is available commercially (e.g. MediMabs, BD Biosciences Pharmingen, Abcam). It is a monoclonal antibody (coded NC1/34) generated in the rat by immunization against the entire sequence of substance P conjugated to bovine serum albumin with carbodiimide, and fusing the spleen cells from these animals with a myeloma cell line (Cuello et al., 1979). One clone (NC1/34.HL) was chosen and characterized by radioimmunoassay; it did not recognize enkephalins, somatostatin or β-endorphin, and did recognize the C-terminal sequence of the substance P molecule (Cuello et al., 1979). The characterization of this antibody was described in detail in previous publications from our laboratory (Almarestani et al., 2009; McLeod et al., 2000). In short, although this antibody should not be able to differentiate substance P from the other two mammalian tackykinins, neurokinin A and neurokinin B, it does not recognize neurokinin B in the concentrations used for immunocytochemistry.

2.3.6 Morphological Identification and Quantification of Lamina I Neurons

Our criteria of identification and quantification of lamina I neurons have been described extensively in previous publications from our laboratory [see e.g. (Almarestani et al., 2009)]. In brief, in the current study, six 50 µm-thick serial horizontal sections were cut from the dorsal part of the L4-L5 spinal segments. Four rats were used per time point. Sections were examined with a PlanFluotar 40X oil immersion objective on a Zeiss Axioplan 2e imaging fluorescence microscope. We only counted neurons ipsilateral to the lesion side which had visible nuclei, and with the cell body entirely located within the plane of the section, as assessed with the fine focus of the microscope. Lamina I neurons were classified according to their cell body shape and dendritic arborization into multipolar, fusiform, pyramidal and unclassified types (Almarestani et al., 2009).

2.3.7 Quantification of Substance P-IR Boutons on Pyramidal Neurons

Sections were examined using a Zeiss LSM 510 confocal scanning laser microscope. We used a multi-track scanning method and appropriate filters for the separate detections of AlexaFluor 488, AlexaFluor 568 and Cy5. Images used for quantification represented serial optical sections obtained along the z-axis (z-stacks), using a 63X plan-apochromatic oil-immersion objective. Furthermore, for an unbiased representation of the images taken, all the parameters of laser power, pinhole size and image detection were kept constant for all samples. All neurons classified as pyramidal neurons based on the criteria described above were scanned, independently of being immunoreactive for the NK-1r or labeled with the retrograde tracer only. Because the CTb labeled reliably and consistently only the cells bodies and primary dendrites, the analysis of the SP-IR innervation was restricted to these compartments of the cells. The images obtained were converted to TIFF files. To calculate the density of SP-IR boutons per unit length of neuronal membrane, the length of membrane in the soma and proximal dendrites was measured with the help of an MCID Elite Image Analysis System (Imaging Research, St.Catharines, ON, Canada) and the number of appositions from SP-IR

boutons onto it was counted. This was done on alternate optical sections from the z-stack, to avoid counting twice the same boutons.

### 2.3.8 Statistics

Graphpad Prism 5 (Graphpad Software, San Diego, CA) was used to perform all statistical tests. Two-way ANOVA followed by Bonferroni correction was applied to compare differences in pain-related behavior and lamina I cell populations between sham and experimental groups across the experimental time points studied. Unpaired t-test was used to compare the substance P-IR boutons in direct apposition to lamina I neurons following vehicle or capsaicin intradermal injection. All values are expressed as means ± SEM. Significance level for all the tests was set at $p<0.05$.

## 2.4 Results

### 2.4.1 Development of mechanical allodynia and thermal hyperalgesia in neuropathic animals

We confirmed the development of neuropathic pain in our polyethylene cuff model of chronic constriction injury ("cuff") by means of behavioral testing. Animals in the cuff group developed mechanical allodynia, as revealed by testing with the von Frey filaments (Figure 1A). This allodynia developed at 10 days after cuff placement, and was still present at 28 days. These results were significantly different from the sham group.

Regarding changes in thermal sensitivity, we detected a decrease in withdrawal latency to heat when compared to the sham group, beginning at 10 days and that was still present at the 28 days time point (Figure 1B). There was no difference from baseline in the contralateral side data, either in sham-operated or cuff animals (data not shown).

2.4.2 CTb injection into the parabrachial nucleus

The morphological observation of the CTb injection site in the parabrachial nucleus revealed a similar distribution to that observed by us in previous studies (Almarestani et al., 2007, 2009). The injection site covered most of the parabrachial complex, including the lateral parabrachial nucleus (LPb). Retrogradely labeled lamina I neurons were mostly found on the contralateral side, although a few were also present on the side ipsilateral to the CTb injection. CTb labeling of spinoparabrachial lamina I neurons included the cell body and primary dendrites (Figure 2).

2.4.3 Classification of lamina I cells

As in previous publications from our laboratory, the different lamina I spinoparabrachial neuronal populations were identified based on their dendritic arborization and cell body shape when viewed in the horizontal plane. Multipolar neurons possess four or more primary dendrites arising from the cell body, independently of the shape of the soma (Figure 2A and 2B). Fusiform neurons have a spindle-shaped soma with one dendrite arising from each end of the cell body (Figure 2C and 2D). In the horizontal plane, pyramidal neurons have a triangular-shaped soma with a primary dendrite originating from each corner (Figure 2E and 2F). A relatively small number of neurons displayed features that were transitional between two of the cell types described above and did not meet the criteria for the main types because of their atypical appearance. Furthermore some neurons had a portion of the cell body and/or proximal dendritic tree truncated due to sectioning. All these cells were not classifiable and were considered as "unclassified" for the purposes of our quantitative analyses.

2.4.4 Changes in lamina I neurons following cuff application

As previously described (Almarestani et al., 2007, 2009), lamina I spinoparabrachial neurons, in control animals, identified by CTb retrograde labeling, often expressed NK-1r. Indeed, most lamina I spinoparabrachial neurons of the fusiform and multipolar types expressed the receptor (Figures 2A-D and

3B, C). However, pyramidal neurons seldom expressed NK-1r in sham-operated animals (Figures 2E and 3A). Following cuff application, pyramidal neurons showed a *de novo* expression of NK-1r in a high proportion of cells (Figures 2F and 3A), whereas there were no changes in NK-1r expression by fusiform and multipolar neurons (Figures 2B, D and 3B-C). This *de novo* expression of NK-1r was detected from day 10 post-cuff (Figure 3A).

2.4.5 Quantification of substance P innervation

We confirmed that fusiform and multipolar neurons receive abundant innervation from SP-IR fibers whereas pyramidal neurons were scarcely innervated, as we had previously observed (Almarestani et al., 2009). In cuff animals, we observed a significant increase in the number of appositions from SP-IR fibers at the 21 day time point when compared with pyramidal neurons of sham animals (Figures 4 and 5). This quantification was carried out in the cell bodies and proximal dendrites of neurons, independently of NK-1r expression. No significant change in SP-IR innervation was observed in the multipolar and fusiform neuronal populations (data not shown).

2.4.6 NK-1 receptor internalization in pyramidal cells following capsaicin injection

Injection of capsaicin intradermally into the hind paw of animals with cuff injury at the 21 day time point led to the internalization of the NK-1r from the cell surface to the cytosol of pyramidal neurons (Figure 6B). However, solvent injection did not induce NK-1r internalization (Fig 6A). NK-1r on multipolar and fusiform neurons were also internalized following capsaicin but not solvent injections (data not shown).

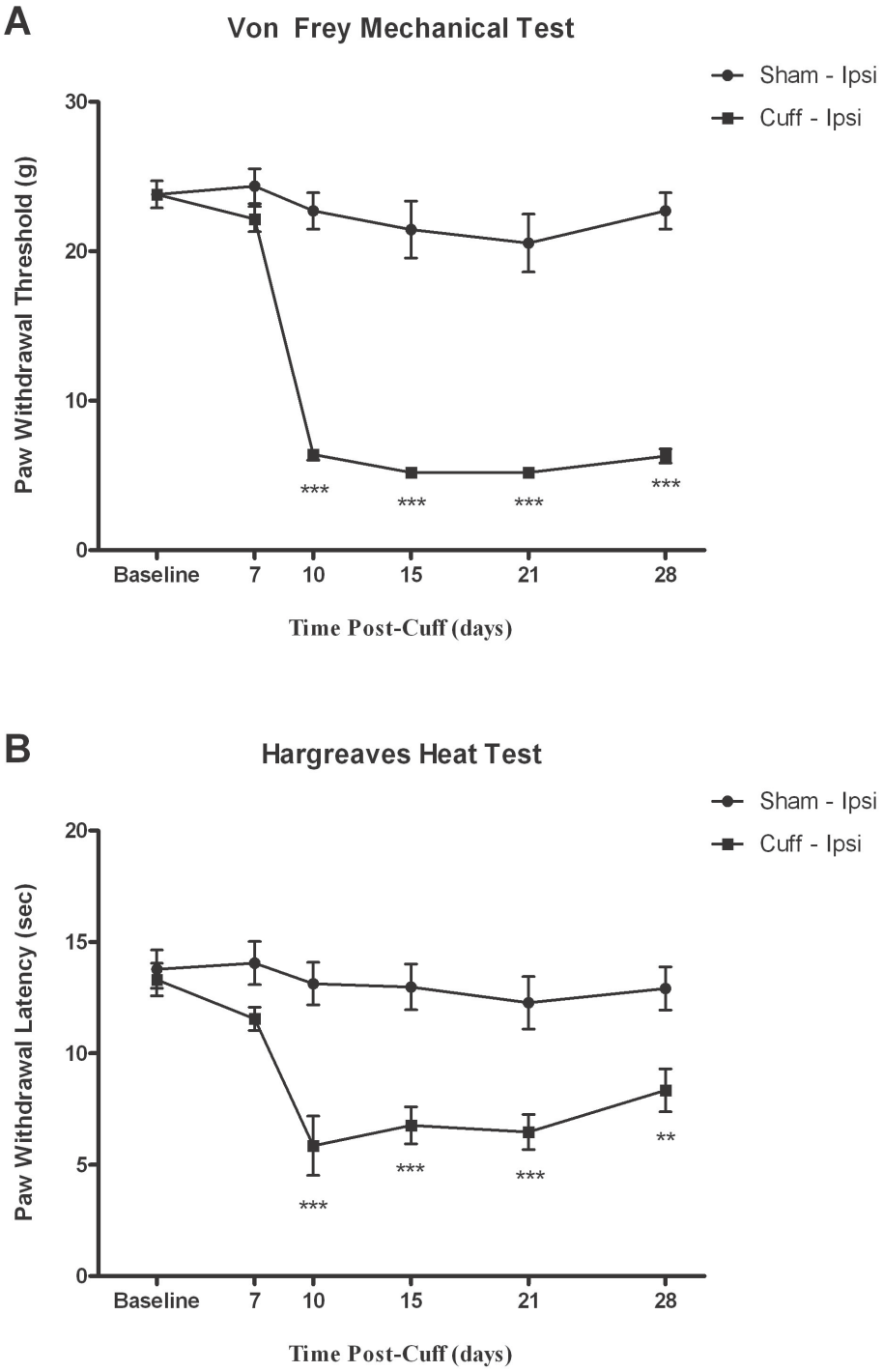
A
Von Frey Mechanical Test
Sham - Ipsi
Cuff - Ipsi
Paw Withdrawal Threshold (g)
30
20
10
0
***
***
***
***
Baseline
7
10
15
21
28
Time Post-Cuff (days)
B
Hargreaves Heat Test
Sham - Ipsi
Cuff - Ipsi
Paw Withdrawal Latency (sec)
20
15
10
5
0
***
***
***
**
Baseline
7
10
15
21
28
Time Post-Cuff (days)

Figure 1

**Figure 1: Behavioral changes following the cuff lesion. A:** Assessment of tactile allodynia using von Frey filaments in cuff and sham-operated animals. Neuropathic animals showed a decrease in withdrawal threshold by 10 days post-cuff. No changes from baseline were detected in the Sham group. N=10, 2-way ANOVA with Bonferroni correction. (*** $P<0.001$) **B:** Assessment of thermal hyperalgesia using the Hargreaves test in cuff and sham groups. Neuropathic animals displayed a significant decrease in withdrawal latencies starting at 10 days post-surgery. No significant reduction in withdrawal latencies was detected for the sham group. N=10, 2-way ANOVA with Bonferroni correction. (** $P<0.01$, *** $P<0.001$)

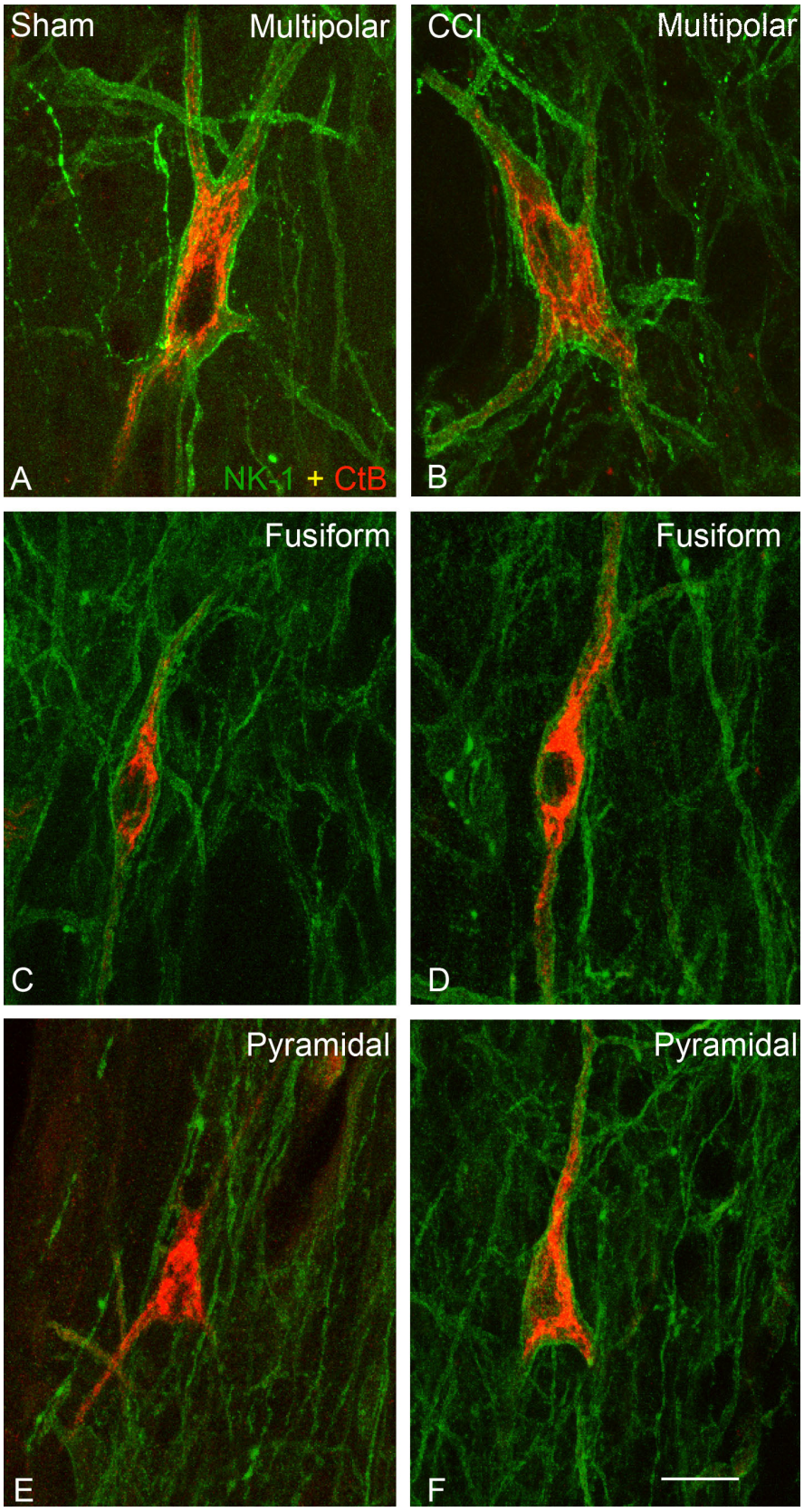
Sham
Multipolar
A
NK-1 + CtB
CCI
Multipolar
B
Fusiform
C
Fusiform
D
Pyramidal
E
Pyramidal
F

Figure 2

**Figure 2:** Confocal images showing the morphology and NK-1r expression of lamina I spinoparabrachial neuronal populations, in sham-operated and cuff groups, at the 21 days time point. Unlike multipolar and fusiform neurons, pyramidal neurons normally did not express NK-1r (A, C, E). However, neuropathic animals developed a *de novo* expression of NK-1r on pyramidal neurons (F), while multipolar and fusiform neurons maintained their normal levels of expression of these receptors (B, D). Scale bar = 20 μm

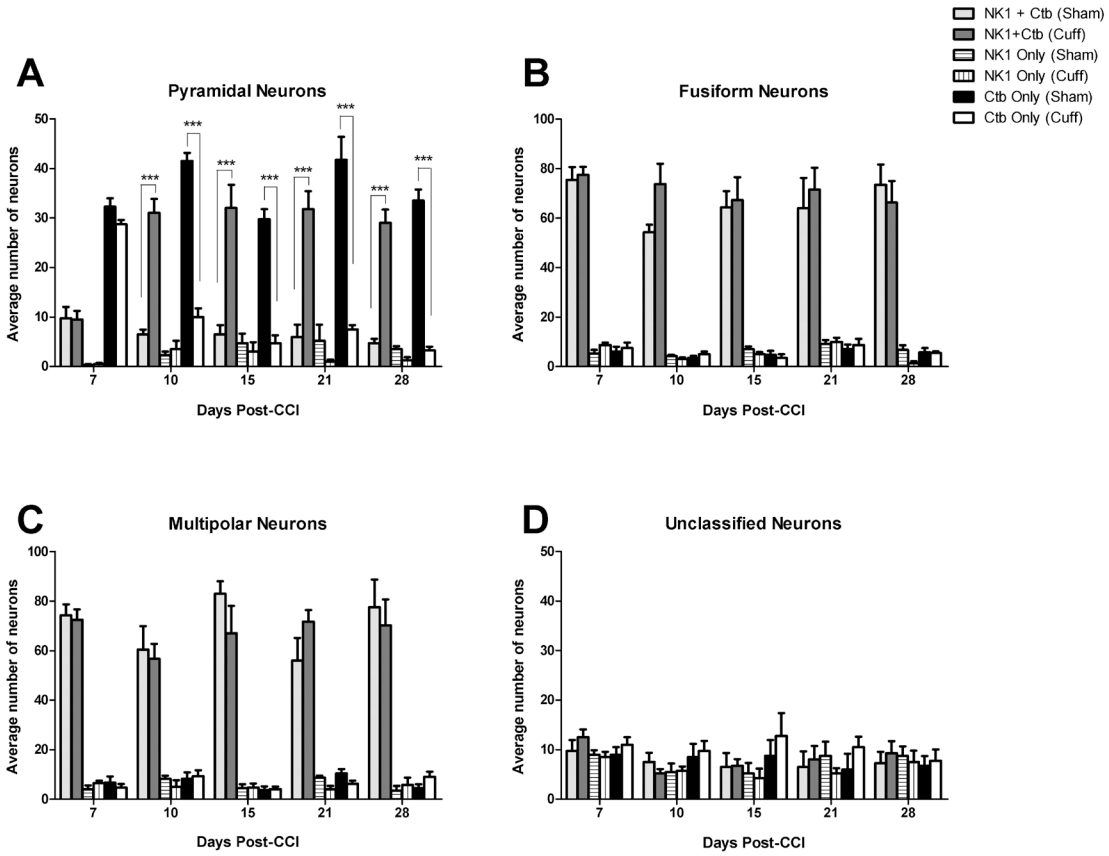
A
Pyramidal Neurons
B
Fusiform Neurons
C
Multipolar Neurons
D
Unclassified Neurons
Average number of neurons
Days Post-CCI
NK1 + Ctb (Sham)
NK1+Ctb (Cuff)
NK1 Only (Sham)
NK1 Only (Cuff)
Ctb Only (Sham)
Ctb Only (Cuff)
***

Figure 3

**Figure 3:** Comparison of the distribution of neurons of each morphological type which were immunoreactive for the NK-1r (NK1 Only), retrogradely labeled from the parabrachial nucleus (CTb Only) or double labeled (NK1 + CTb), ipsilaterally to the lesion or sham operation, at 7, 10, 15, 21 and 28 days post-surgery. A) Pyramidal neurons; B) Fusiform neurons; C) Multipolar neurons; D) Unclassified neurons. In A, note that no change was detected at any time point in the number of neurons that expressed only the NK-1r; at 7 days the number of pyramidal neurons which expressed the NK-1r and were also CTb-positive was very low, but at and after the 10 day time point, there was a marked increase in the number of neurons which were immunoreactive for both the NK-1r and CTb, accompanied by a parallel decrease in the number of neurons which are not immunoreactive for NK-1r and were positive for CTb. In B, C and D, note that there were no changes at any time point in the number of fusiform, multipolar and unclassified neurons which were immunoreactive or not for the two markers we used. Only cells with visible nuclei were counted. Values represent average number of neurons (±SEM) counted per animal. N =4. Comparisons were made by means of a 2-way ANOVA with Bonferroni post-hoc tests (*** $P<0.001$).

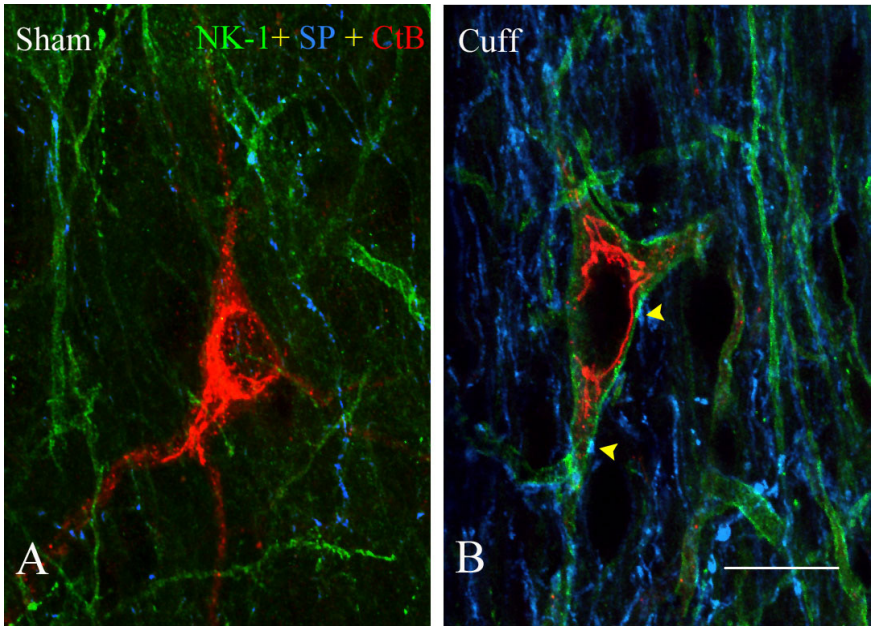


**Figure 4:** Confocal triple-labeling images showing increased substance P innervation of pyramidal neurons and *de novo* NK-1r expression following a cuff lesion, at the 21 days time point. We observed a substantial increase in the number of substance P boutons apposed to lamina I spinoparabrachial pyramidal neurons that had *de novo* NK-1r expression (B) compared to neurons in the sham-operated group with no NK-1r expression (A). Substance P – SP (in blue); NK-1r - NK-1 (in green), spinoparabrachial neurons - CTb (in red). Scale bar = 20 μm

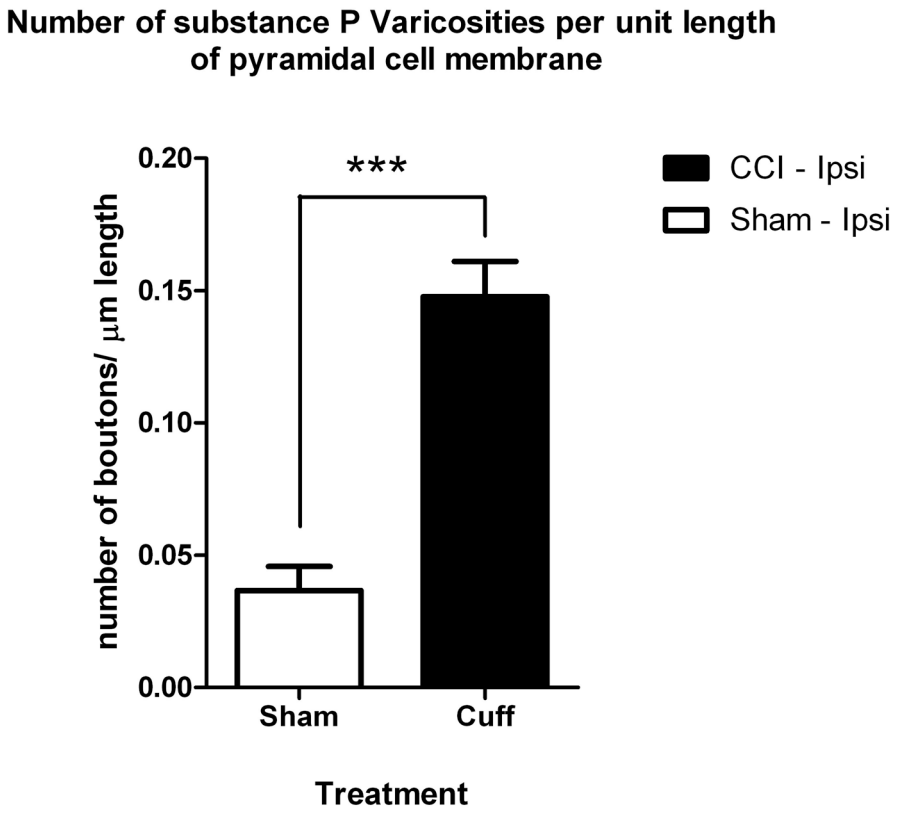


**Figure 5:** Quantification of substance P-IR boutons in direct apposition to lamina I spinoparabrachial pyramidal neurons. At the 21 day time point, there was a significant increase in the number of substance P boutons in close apposition with the pyramidal neurons, when compared to the sham-operated group, independently of the presence or absence of NK-1r staining. Because of limitations in the CTb filling of neurons, boutons counted were only those apposed to the soma membrane and membranes of the primary dendrites. N= 6. Data compared by unpaired t-test. *** $P<0.001$

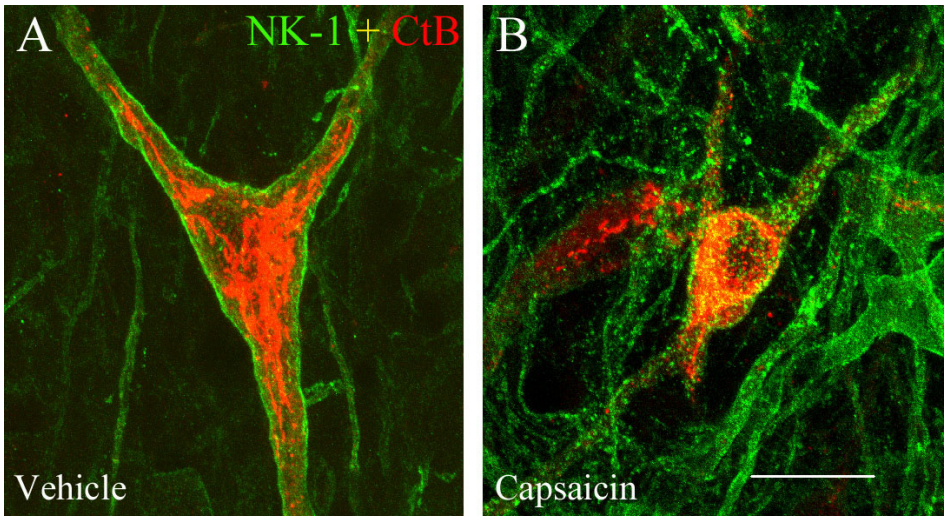


**Figure 6:** Demonstration of NK-1r internalization following noxious stimulation. Intradermal injection of capsaicin in the ipsilateral hind paw at the 21 day time point caused internalization of surface NK-1r (B). No internalization was detected with vehicle injections at the same time point in cuff animals (A). N=4. Scale bar = 20 μm

## 2.5 Discussion

In this study, we show that, in the cuff model of neuropathic pain, lamina I spinoparabrachial pyramidal neurons develop a *de novo* expression of functional NK-1r. Furthermore, we also found a substantial increase in the number of appositions from axons immunoreactive for SP onto pyramidal neurons, raising the possibility of a functional switch of these neurons.

### 2.5.1 Properties of pyramidal neurons

There is evidence from the literature that there are three main functional types of lamina I neurons in the cat and primate. These have been identified on the basis of their response to cutaneous inputs: a) nociceptive specific neurons (NS) are responsive only to noxious heat and pinch; b) polymodal nociceptive neurons (HPC) are responsive to noxious pinch and heat as well as to innocuous and noxious cold; and c) innocuous thermoreceptive neurons (COLD) are responsive only to innocuous cooling (Craig and Bushnell, 1994; Craig and Kniffki, 1985; Dostrovsky and Craig, 1996; Han et al., 1998). Interestingly, in the cat, a correlation was found between the physiological and morphological properties of lamina I projection neurons, in a study combining intracellular recording from lamina I cells with intracellular labeling (Han et al., 1998). Indeed, in the above study, fusiform cells were all NS, pyramidal cells were all COLD, and multipolar cells were divided among HPC and NS neurons. In agreement with the finding that pyramidal cells are non-nociceptive, a study from our laboratory in the primate revealed that spinothalamic pyramidal neurons seldom expressed NK-1r, whereas fusiform and multipolar cells were normally NK-1r immuno-positive (Yu et al., 1999). Subsequent studies from our laboratory demonstrated that, in the rat, spinothalamic and spinoparabrachial pyramidal neurons were seldom immunoreactive for the NK-1r, in contrast with fusiform and multipolar neurons which were mostly immunoreactive for the receptor (Almarestani et al., 2007; Yu et al., 2005). There is ample evidence that nociceptive cells express NK-1r (Mantyh et al., 1995; Mantyh et al., 1997). Therefore, the observation that in naïve animals pyramidal neurons seldom express NK-1r concurs with a non-

nociceptive role for these cells. However, our data on the absence of expression of NK-1r by pyramidal neurons in the rat spinal cord contrasts with the results from another laboratory. Indeed, the group of Andrew J. Todd did not find any differences among the three types of lamina I projection neuron in the expression of NK-1r (Todd et al., 2002). This group also found that pyramidal neurons should be nociceptive, as they display not only NK-1r immunoreactivity but also respond to noxious stimulation with c-fos expression (Todd et al., 2002). The reasons for this discrepancy were discussed at length in one of our previous publications (Almarestani et al., 2009). In short, the differences were certainly not caused by the antibody used, as we obtained the same results with three different antibodies, nor animal strain. The only possible explanation for the different results obtained by our laboratory compared to Todd's lab is the application of different cell classification criteria. A key element used by our lab to distinguish pyramidal from multipolar neurons is the number of primary dendritic trunks when viewed in the horizontal plane. Indeed, pyramidal neurons have four primary dendrites, three of which are visible on a confocal optical section cut to show the cell nucleus. This is due to the fact that the $4^{th}$ dendrite is oriented towards the white matter. We consider as multipolar all cells that unequivocally display 4 or more main dendritic trunks in the horizontal plane. We stress this point, because some main dendrites branch very close to the cell body (Almarestani et al., 2009). An important point in support of the accuracy of our neuronal classification is that we observed in an animal model of arthritis that a considerable number of pyramidal neurons started to express detectable levels of NK-1r at day 15 after a subcutaneous injection of complete Freund's adjuvant (CFA) in the hind paw skin (Almarestani et al., 2009). Such expression started only at a time point in which there were already obvious signs of joint and bone damage, as detected by imaging methods (Almarestani et al., 2011).

Our current study expands these observations to a model of neuropathic pain. In the current study we observed that at 10 days post-cuff, but not at 7 days, there was a significantly higher number of pyramidal neurons expressing NK-1r than in sham operated animals. This increased receptor expression persisted for at

least up to 28 days post-cuff, the last time point examined, and this receptor expression was strictly ipsilateral (contralateral data not shown).

2.5.2 Significance of increased peptidergic fiber innervation on pyramidal neurons

In the current study, we confirmed quantitatively previous qualitative data from our laboratory revealing that non-NK-1r-IR pyramidal neurons in control animals were almost devoid of SP innervation (Almarestani et al., 2009). This information is in agreement with the concept that these cells are non-nociceptive. Indeed, electrophysiological studies have provided evidence that non-nociceptive dorsal horn cells do not respond to SP (Henry, 1976) and studies combining intracellular recording and injection with HRP of neurons with ultrastructural immunocytochemistry in the cat have shown that non-nociceptive neurons are scarcely innervated by SP (De Koninck et al., 1992; Ma et al., 1996). In contrast, the same studies have shown that spinal dorsal horn neurons with strong nociceptive responses were abundantly innervated by SP-IR fibers (De Koninck et al., 1992; Ma et al., 1996). In agreement with that, in the current study we confirmed our previous observation that fusiform and multipolar neurons, which have been considered as nociceptive, were abundantly innervated by SP (Almarestani et al., 2009). Interestingly, we also confirmed that the few pyramidal neurons which were NK-1r-IR in control rats were abundantly innervated by SP-IR fibers. If we exclude the discrepancy on what should be considered a pyramidal neuron, our data concurs with the results from Andrew J. Todd's lab in that lamina I neurons which are immunoreactive for the NK-1r are abundantly innervated by SP and respond to noxious stimulation (Todd et al., 2002).

Importantly, as shown in figure 5, we detected that following a cuff lesion there was a very significant increase of SP-IR appositions on pyramidal neurons. Because we did not discriminate between pyramidal neurons which were NK-1r positive and those that were not when performing our counts, we cannot confirm whether this high innervation by SP of pyramidal neurons was restricted to the population that became NK-1r-IR. However, our visual assessment would suggest that NK-1r expression and abundant innervation by SP are associated. Our data

support our previous qualitative only observation in the CFA arthritis model of a *de novo* innervation of pyramidal neurons by SP-IR fibers (Almarestani et al., 2009).

2.5.3 Significance of NK-1 receptor internalization

NK-1r internalization has been used as a measure of the receptor's activation (Abbadie et al., 1997; Adelson et al., 2009; Allen et al., 1997; Mantyh et al., 1995; Marvizon et al., 2003). In the current study, we tested the functionality of the NK-1r expressed on pyramidal neurons, following development of neuropathic pain, by injecting capsaicin intradermally in the hind paw. We used capsaicin because it has been described previously as a potent noxious stimulus (Afrah et al., 2001; Go and Yaksh, 1987; Lever and Malcangio, 2002; Marvizon et al., 2003). In neuropathic animals, we observed the internalization of NK-1r in all three lamina I neuronal populations following capsaicin injection, however, vehicle injection did not induce NK-1r internalization. This NK-1r internalization provides strong evidence that in neuropathic animals the majority of pyramidal neurons respond to noxious stimulation.

2.5.4 Role in nociception

As mentioned above, there is evidence indicating that SP-positive innervation is abundant on neurons with nociceptive responses when compared to neurons lacking such responses (De Koninck et al., 1992; Ma et al., 1996), and that neurons which express the NK-1r are selectively innervated by SP (McLeod et al., 1998; Todd et al., 2002) and are nociceptive (Todd et al., 2002). The integration of the above information with the data of this study would suggest that following neuropathic pain development, pyramidal neurons with *de novo* NK-1r expression may have undergone a functional change.

There is evidence from the literature that, under normal physiological conditions, pyramidal neurons represent COOL cells, activated by innocuous cooling of the skin but unresponsive to other stimuli (Craig and Bushnell, 1994;

Craig and Hunsley, 1991; Craig and Kniffki, 1985; Craig et al., 2001; Craig and Serrano, 1994; Dostrovsky and Craig, 1996; Han et al., 1998). Our results would suggest that in situations accompanied by a chronic pain state, such as in arthritis (Almarestani et al., 2009) and in neuropathic pain (current study), a considerable number of pyramidal cells would be converted into HPC cells, and therefore responsive to noxious pinch and heat as well as to innocuous and noxious cold. This hypothesis is at present based only on speculation, as it would have to be confirmed electrophysiologically. It would also require that pyramidal cells have direct and/or indirect connections with thalamic areas involved in nociception, what is far from clear, as it has been proposed that these cells project to a thalamic area involved in thermoregulation (Han et al., 1998).

### 2.5.5 Role in thermoregulation

Based on the above, it is possible that pyramidal cells in chronic pain states, although activated by noxious stimuli, might still be involved only in thermoregulation, through either an indirect thalamic connection (see above) or direct or indirect connections from the parabrachial nucleus to the preoptic area (POA), where the thermoregulatory center resides (Nakamura and Morrison, 2008b). We can speculate that, following neuropathy, the increased activity of the pyramidal neurons would be perceived as cooling causing a shift from homeostatic temperatures, which would activate the thermoregulatory center leading to thermogenesis (Nakamura and Morrison, 2008b) and cutaneous vasoconstriction (Osborne and Kurosawa, 1994). This possibility would need to be investigated, by measuring peripheral and core temperatures and documenting any increase in food consumption. At present, it is more speculative than based on facts.

## 2.6 Conclusions

In this study, we described a *de novo* expression of NK-1r on pyramidal neurons and an associated increase in SP-IR innervation of these cells in a model of chronic neuropathic pain. We also demonstrated the internalization of these

receptors following a noxious stimulus, indicating that these cells become responsive to noxious stimuli. These data, together with the results from our work on an arthritis model, suggest that in chronic pain states, either lamina I pyramidal cells become nociceptive or they trigger abnormal cooling-related autonomic responses following noxious stimulation. Our results may aid in better understanding the development of cold allodynia or changes in homeostatic basal levels following nerve injury.

2.7 Acknowledgements

The authors would like to thank Manon St-Louis for laboratory expertise and Jacynthe Laliberté for confocal microscopy assistance. We are also particularly grateful to Dr. Claudio Cuello for helpful discussions and the supply of the anti-substance P monoclonal antibody and to Dr. Terence Coderre for the use of his animal behavior facility.

## Connecting text: Chapter 2 to Chapter 3

In the previous chapter, we illustrate how a chronic nerve injury resulted in the *de novo* expression of NK-1r on lamina I pyramidal neurons, a switch concomitant with the development of pain-related behavior. This phenotypic switch had been previously documented in our lab in a model of CFA-induced inflammation (Almarestani et al., 2009). Therefore, we thought that it would be interesting if this *de novo* expression of NK-1r could be used as a marker for the development of chronic neuropathic pain, which could be later used to test efficacy of newly developed treatments for chronic pain, an issue we indirectly address in Chapter 3.

In the next chapter, we demonstrate how an experimental model, of cytotoxic ablation of the non-peptidergic afferents, which does not lead to a pain state, can be used to investigate the necessity of a chronic pain state for the *de novo* NK-1r expression on lamina I pyramidal neurons. One possibility was that the loss of non-peptidergic afferents would trigger the expression of NK-1r by pyramidal cells as a compensatory mechanism.

# Chapter 3

Limited changes in spinal lamina I dorsal horn neurons following the cytotoxic ablation of non-peptidergic C-fibers

**Abeer W. Saeed and Alfredo Ribeiro-da-Silva**

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3.1 Abstract

Non-peptidergic nociceptive neurons are a sub-population of small diameter primary sensory neurons that comprise approximately 50% of the C fiber population. Together with the peptidergic sub-population, they transmit nociceptive information from the periphery to the superficial dorsal horn of the spinal cord. Despite the numerous studies investigating the role of the non-peptidergic primary afferents, their role in normal nociception and in pain remains poorly understood. Our lab has previously demonstrated that, in rat models of neuropathic and inflammatory pain, there is a *de novo* expression of substance P receptors (NK-1r) by lamina I pyramidal projection neurons, a neuronal population that normally does not express these receptors. In this study, we used a ribosomal toxin, saporin, conjugated to the lectin IB4 (IB4-SAP) to selectively ablate the non-peptidergic nociceptive C fibers, to investigate if the loss of these fibers was enough to induce a change in NK-1r expression by lamina I projection neurons. IB4-saporin treatment led to the specific and permanent ablation of the IB4-positive afferents. However, no changes in responses to acute thermal and mechanical stimuli were detected. An overall increase in immunoreactivity for NK-1r was observed in lamina I projection neurons, although the number of pyramidal neurons immunoreactive for the receptor remained low. These results suggest that a chronic inflammatory or neuropathic component is essential to trigger the expression of NK-1r by pyramidal neurons.

3.2 Introduction

Most of the pain-related (nociceptive) information is relayed from the periphery to the spinal dorsal horn via small diameter primary afferents, which represent either unmyelinated (C) or thinly myelinated (Aδ) axons, with only a minority being of larger diameter [for reviews see (Alvarez and Fyffe, 2000; Ribeiro-da-Silva and De Koninck, 2008)]. Usually, the smaller diameter nociceptive afferents are classified into two mostly independent subpopulations, the peptidergic and non-peptidergic (Hunt and Mantyh, 2001; Hunt and Rossi, 1985). The peptidergic fibers, express substance P (SP) and calcitonin gene-

related peptide (CGRP) and depend on nerve growth factor (NGF) for survival post-natally, while the non-peptidergic fibers, are devoid of neuropeptides, bind the Griffonia simplicifolia isolectin-B4 (IB4), express the purinergic receptor P2X3 and depend on glial-derived neurotrophic factor for post-natal survival (Molliver et al., 1997; Silverman and Kruger, 1990; Stucky and Lewin, 1999). There is some evidence that these two subpopulations differ in their physiological properties. The action potentials of non-peptidergic nociceptive neurons had higher activation thresholds and were of longer duration than those of peptidergic neurons, characteristics attributable to the presence of a greater density of tetrodotoxin-resistant $Na^+$ channels on the IB4-positive neurons (Stucky and Lewin, 1999; Wu and Pan, 2004b). It has been proposed that, in the mouse, peptidergic fibers would be responsible for the transmission of thermal nociception, whereas the non-peptidergic would convey noxious mechanical stimuli (Cavanaugh et al., 2009). These populations also differ in their central termination in the spinal cord. Indeed, the peptidergic afferents terminate mostly in lamina I and outer lamina II, while the non-peptidergic project to inner lamina II (Bradbury et al., 1998; Ribeiro-da-Silva, 2004; Ribeiro-da-Silva and De Koninck, 2008). In the mouse, it has been suggested, based on experimental evidence, that the non-peptidergic afferents have connections to a completely different ascending pathway from the peptidergic, as they would connect to lamina V cells that would project to areas of the brain like the amygdala, as opposed to the peptidergic that would connect to lamina I expressing substance P receptors (NK-1r) (Braz et al., 2005; Scherrer et al., 2009). However, these observations need confirmation and it is questionable whether they are applicable to other species, such as the rat and higher species.

Lamina I projection neurons comprise three populations; fusiform, multipolar and pyramidal, which have distinctive morphology (Yu et al., 2005; Yu et al., 1999; Zhang and Craig, 1997; Zhang et al., 1996), and function (Han et al., 1998). Fusiform neurons have an elongated soma with two dendrites at each end; physiologically, in the cat, they have been shown to be nociceptive specific (NS), and respond to noxious heat and pinch. Multipolar neurons have four or

more dendrites arising from an irregularly-shaped soma and have been shown to be either NS or HPC (polymodal neurons responding to a variety of stimuli, including noxious heat, pinch and noxious and innocuous cold) (Han et al., 1998). Pyramidal neurons have a triangular-shaped soma with one dendrite at each of the three tips; these neurons do not respond to noxious stimuli, however, they respond to innocuous cooling (COOL cells) (Han et al., 1998). Fusiform and multipolar neurons express NK-1r, in agreement with the observation in the cat that they are nociceptive, while pyramidal neurons seldom express such receptors in rat and primate, in agreement with the concept that they are non-nociceptive under physiological conditions (Almarestani et al., 2007; Yu et al., 2005; Yu et al., 1999). However, it was demonstrated in rat that in chronic CFA-induced arthritis (Almarestani et al., 2009) and in an animal model of neuropathic pain (Saeed and Ribeiro-da-Silva, 2012) there is a *de novo* expression of NK-1r by a considerable number of pyramidal neurons. This novel expression was accompanied by a novel innervation of these pyramidal neurons by SP-immunoreactive fibers, further providing evidence of a phenotypic change in these neurons in both arthritis and neuropathic pain models, indicating that these neurons would participate in nociceptive responses in chronic pain conditions (Almarestani et al., 2009; Saeed and Ribeiro-da-Silva, 2012).

Although there is not yet a specific tool to selectively ablate the peptidergic fibers, the injection of IB4 conjugated to the ribosomal toxin, saporin, into the sciatic nerve selectively and permanently ablates the non-peptidergic C fibers, sparing the other fiber populations (Vulchanova et al., 2001). The above study revealed a transient elevation of the thermal and mechanical nociceptive thresholds, with a return to normal by 3 weeks post-IB4-saporin injection, in contrast with an expected dramatic elevation of nociceptive thresholds, as a consequence of the loss of approximately half of the nociceptive C afferents. Importantly, our preliminary data using IB4-saporin injections revealed an overall increase in NK-1r expression in lamina I. In the current study, our main objective was to investigate whether the selective cytotoxic ablation of IB4-binding afferents led to a *de novo* expression of NK-1r by pyramidal neurons or whether

the reported increase in NK-1r was a result of increased immunoreactivity in the neurons that normally express it. Our working hypothesis was that a *de novo* expression of NK-1 receptors by lamina I pyramidal neurons was triggered following the ablation of the non-peptidergic C-fiber population as an adaptive mechanism to maintain homeostasis of the nociceptive circuit. Our data did not confirm the hypothesis.

## 3.3 Materials & Methods

The experimental procedures followed the guidelines for the Care and Use of Experimental Animals of the Canadian Council on Animal Care and the International Association for the Study of Pain (IASP). All protocols were approved by the McGill University Faculty of Medicine Animal Care Committee.

Forty-two male Sprague Dawley rats (Charles River, Quebec, Canada), weighing between 220-230g, were used. Animals were divided into 7 groups of 6 animals each. The number of animals used and their suffering was kept to the minimum necessary for the conduction of the study. Animals were exposed to 12hr light/dark cycles with food and water available *ad libitum* and were housed four animals to a cage fitted with soft bedding and a plastic tube for an enriched environment.

### 3.3.1 Animal Preparation

*Surgeries*

Animals were anesthetized with 5% isoflurane in oxygen. Unilateral lesions were carried out on the left sciatic nerve. Using blunt dissection, the left femoral muscle was exposed. Care was taken to minimize the stretching of the sciatic nerve when it was separated from the surrounding connective tissue. Experimental animals received a total of six µL of an 800 µg/mL solution of IB4-saporin (Advanced Targeting Systems, San Diego CA, USA) in 0.2M Phosphate Buffered Saline (PBS) and Fast Green Dye (Sigma, Missouri, USA) injected at three injection sites into the sciatic nerve proximal to its branching point using

calibrated glass micropipettes (Wiretrol II, Drummond Scientific Company, Broomall, PA). The Fast Green dye was used to monitor the accuracy of the injection. The control group was injected with vehicle solution of six µL 0.2M PBS in Fast Green dye using the same method. The incision was sutured in two layers using 4-0 Vicryl sutures (Ethicon Inc, New Jersey USA). Animals were returned to their cages to recover. No difference in weight gain between experimental and sham groups was observed at any time point throughout the study.

*Injection of tracer*

To retrogradely trace projection neurons, animals were first anesthetized using 5% isoflurane in oxygen, placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA) and the head stabilized with non-perforating ear bars. The coordinates for the parabrachial nucleus (rostral/caudal: -9.12; medial/lateral: -2.1; dorsal/ventral: -6.3) were calculated, with Bregma as the reference point, from the Paxinos & Watson Rat Brain Atlas (Paxinos and Watson, 2005). A small hole was drilled through the skull at the target point, exposing the dura mater. A glass micropipette (Wiretrol II, Drummond Scientific Company, Broomall, PA) was lowered to the stereotaxic position of the parabrachial nucleus and 2 µl of 1.0% solution of cholera toxin subunit B (CTb) (List, Campbell, CA) was slowly injected over a period of 20 minutes. To minimize leakage of the tracer, a 10 minute waiting period was imposed before the micropipette was retracted from its position. CTb was injected seven days prior to sacrificing the animals.

### 3.3.2 Behavior Testing

Animals were always habituated to the testing apparatus for 30 minutes prior to testing. Baseline behavioral thresholds were measured for two consecutive days prior to surgical treatments.

*Testing for mechanical allodynia*

Animals were placed in clear plastic enclosures elevated on a mesh grid, which allowed complete access to the ventral side of the animal. Animals were

tested using the up-down method previously described by Chaplan et al. (Chaplan et al., 1994). Filaments of increasing stiffness were applied to the mid-plantar area of the hind paw, avoiding the foot pads, until an obvious withdrawal or flicking/licking behavior occurred. In case of a positive response, the next weaker filament was presented, however, in the case of a negative response; the next stronger filament was applied. This process was repeated until 6 responses were recorded and a mean threshold was calculated. The testing was performed on the right paw of all the rats followed by the testing of the left paw in the same manner.

*Testing for mechanical hyperalgesia*

Mechanical hyperalgesia was assessed using the pin prick method described by Coderre et al. (Coderre et al., 2004). The point of a blunted 23 gauge needle was applied to the skin of the heel (touching, but not penetrating). Behavioral responses to the pin prick were rated according to the following scale: 0=no response; 1=rapid paw flicking, stamping, or shaking (less than 1 s); 2=repeated paw stamping, shaking, or paw lift less than 3 s; 3=above behaviors or hind paw licking for more than 3 s; 4=above behaviors for more than 3 s and hind paw licking for more than 3 s. An additional point was added if any vocalizations occurred. The mean of the reaction for each paw was calculated.

*Testing for thermal hyperalgesia*

The Hargreaves test (Hargreaves et al., 1988) was used to measure thermal nociceptive thresholds. Clear plastic enclosures were set on top of a glass floor. The light source was directed onto skin area of the paw in contact with the glass. The time from turning on the light source until withdrawal was noted. Testing included three trials per paw with each trial being completed for all the animals before the start of the next trial. This ensured there was a 30 minute wait before the start of the next trial to minimize desensitization effects. The average of the three trials per paw was calculated.

### 3.3.3 Animal Perfusion

At each time point, the animals were deeply anesthetized with Equithesin (6.5mg chloral hydrate and 3mg sodium pentobarbital in a volume of 0.3 ml, i.p., per 100g body weight). They were then perfused through the left cardiac ventricle with perfusion buffer (for composition see (Côté et al., 1993)) for one minute, followed by 30 minutes of 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. The brain (at 21 day timepoint) and spinal cord segments L4-L5 were extracted and postfixed for 4 hours and 2 hours, respectively, in 4% paraformaldehyde in PB. The specimens were then cryoprotected in 30% sucrose in PB overnight at 4°C.

### 3.3.4 Immunohistochemistry

Serial 100µm-thick coronal sections of the brain were obtained, to examine the site of injection at the level of the parabrachial nucleus. L4-L5 spinal cord segments were trimmed and cut serially at 50µm thickness. Both horizontal and coronal sections were cut using a freezing-sledge microtome (Leica, Richmond Hill, Ontario). Sections were collected as free-floating in phosphate-buffered saline (PBS) with 0.2% Triton-X 100 (PBS+T).

To examine lamina I projection neurons, horizontal sections were incubated in 10% normal donkey serum (Jackson, West Grove, PA) in PBS+T for one hour at room temperature to block unspecific staining. Subsequently, spinal cord sections were incubated, for 48 hours at 4°C, with the primary antibodies: goat anti-CTb at 1:5000 dilution (List), rabbit anti-NK1-r, raised against residues 376-407 of the C-terminal sequence of the rat receptor, at 1:10000 dilution (Sigma) and IB4 conjugated to AlexaFluor 647 (Molecular Probes) in PBS+T containing 5% normal donkey serum. Then, the sections were washed several times with PBS+T and incubated for 2 hours at room temperature with secondary antibodies preabsorbed with 10mg/ml acetone rat brain powder: donkey anti-goat Rhodamine Red X (1:200, Jackson, West Grove, PA) and donkey anti-rabbit

AlexaFluor 488 (Molecular Probes). Finally, sections were washed with PBS, mounted on gelatin-subbed slides and coverslipped with an anti-fading mounting medium (Aqua Polymount; Polysciences, Warrington, PA). Slides were stored at -4°C. Control sections were processed by omitting the primary antibody which resulted in complete loss of immunoreactivity. The above protocol was also followed for the comparison of NK-1 receptor immunoreactivity in sham- and IB4-saporin-treated groups in transverse sections and the labeling of non-peptidergic afferents in horizontal sections, however in these two cases IB4 was conjugated to AlexaFluor 568 (Molecular Probes) instead of AlexaFluor 647.

To examine the primary afferent populations in the spinal dorsal horn, sections were processed as described above except that they were incubated for 48 hours with IB4 conjugated to AlexaFluor 568, at 1:200 dilution (Molecular Probes) and rabbit anti-CGRP, at 1:2000 dilution (Sigma) followed by incubation with goat anti-rabbit AlexaFluor 488 (Molecular Probes).

Brainstem sections of the injection site were incubated with anti-CTb antibody (List) followed by biotinylated donkey anti-goat IgG (Jackson, West Grove, PA) and streptavidin conjugated to AlexaFluor 568 (Molecular Probes). All sections were mounted and coverslipped as described above.

### 3.3.5 Antibody Specificity

As controls for immunocytochemistry, some sections were processed by omitting the primary antibodies or by pre-absorption with the peptide used to generate the antibody, in both cases resulting in a complete loss of immunoreactivity.

The goat anti-CTb antibody (List Biological; 703, lot 7032A5) was raised against purified CTb and its specificity was demonstrated by the lack of any staining in animals not injected with CTb. The rabbit anti-NK-1r antibody (Sigma; S8305, lot 084K4845) was generated against a synthetic peptide corresponding to amino acids 393-407 of the C-terminus region of the rat NK-1r conjugated to KLH as the immunogen and purified by ion-exchange chromatography. In Western blots from rat brain, the antibody was found to label

only a specific band at 46 kDa, whose staining is specifically inhibited by incubation with the blocking peptide (data supplied by the manufacturer). Furthermore, it was shown that it does not produce any staining in NK-1r knockout mice, although it recognizes the receptor in wild type mice (Ptak et al., 2002). The rabbit anti-CGRP antibody (Sigma; C8198, lot 070M4835) was generated against synthetic rat CGRP conjugated to KLH as the immunogen. Using dot-blot immunoassay, the antibody was found to recognize rat CGRP conjugated to bovine serum albumin (BSA); it only shows cross-reactivity with CGRP (human) and β-CGRP (human) (data supplied by the manufacturer). Specific staining was abolished by pre-incubating the antiserum with rat CGRP. This antibody was previously used by us as a marker for peptidergic fibers in rat skin, dorsal root ganglia and spinal cord (Bailey and Ribeiro-da-Silva, 2006; Peleshok and Ribeiro-da-Silva, 2011; Taylor et al., 2009; Yen et al., 2006).

### 3.3.6 Morphological Characterization and Quantification of Lamina I Neurons

Our criteria of identification and quantification of lamina I neurons have been described extensively in previous publications from our laboratory [see e.g. (Almarestani et al., 2009)]. In brief, in the current study, six serial, 50 µm-thick horizontal sections were cut from the dorsal part of the L4-L5 spinal segments. Six rats were used per time point. Sections were examined under a PlanFluotar 40X oil immersion objective on a Zeiss Axioplan 2e imaging fluorescence microscope. Only neurons with visible nuclei, ipsilateral to the injection side, and with the cell body entirely located within the plane of the section, as assessed with the fine focus of the microscope, were included in our quantifications. Lamina I neurons were classified according to the shape of their cell body and their dendritic arborization in the horizontal plane. Fusiform neurons have two primary dendrites with one arising from each end of an elongated, spindle-shaped soma. Multipolar neurons have an irregularly-shaped cell body with four or more primary dendrites arising from the cell body. Pyramidal neurons have a triangularly-shaped soma with three primary dendrites arising from each of the cell body's corners, in some cases, a fourth primary dendrite, oriented toward the

white matter, was visible by confocal reconstruction or by adjusting the fine focus of a conventional fluorescence microscope. Neurons were classified as "unclassified" if they exhibited features transitional between any of these types, as they did not meet the required criteria.

To obtain images for the illustrations and to confirm the data obtained with conventional fluorescence microscopy, some sections were examined using a Zeiss LSM 510 confocal scanning laser microscope, using a multi-track scanning method and appropriate filters for the separate detections of AlexaFluor 488, AlexaFluor 568 or Rhodamine Red X and AlexaFluor 647. Low magnification images represent single optical sections, whereas images of individual neurons represent serial optical sections obtained along the z-axis (z-stacks), using a 63X plan-apochromatic oil-immersion objective.

### 3.3.7 Statistics

One-way analyses of variance (ANOVA) followed by Dunnett's post hoc test was used to compare the control group to each of the time points studied for each cell type population, and were applied to compare the differences within each neuronal population at the 21 day time point post-IB4-saporin injection. Two-way ANOVA followed by Bonferroni correction were applied to compare differences in pain-related behavior at each time point between the sham and experimental groups. Values were expressed as means ± SEM. The significance level was set at $P < 0.05$. All data were analyzed using GraphPad Prism 5 for Windows (GraphPad Software, San Diego, CA).

### 3.3.8 Figure Preparation

All immunofluorescence images were obtained with the confocal microscope. They were saved in the Zeiss LSM format, exported as TIFF files and prepared for publication using Adobe Photoshop 7.0 (San Jose, CA). The original images were optimized for brightness and contrast, and pseudo colors were assigned to the markers (green to NK-1 and CGRP, and red to IB4 and CtB), to

ensure uniformity throughout the study, but no other image manipulation was done.

## 3.4 Results

### 3.4.1 Behavioral assessment following injection of IB4-saporin

Animals injected with IB4-saporin into the sciatic nerve did not display any changes in evoked thresholds to the application of von Frey hairs (Figure 1A), to an acute noxious stimulus (pin prick) (Figure 1B) or to noxious thermal stimuli (Figure 1C) up to 21 days following IB4-saporin injection when compared to vehicle-injected sham animals.

There was no difference from baseline in the contralateral side, either in IB4-saporin-injected or vehicle-injected groups.

### 3.4.2 Changes in spinal dorsal horn following IB4-saporin injection

21 days after the injection of IB4-saporin into the sciatic nerve, we detected a virtually complete loss of non-peptidergic afferents on the side ipsilateral to the injection at the level of the L4-L5 spinal segments compared to shams which had no loss (Figure 2). The area of loss of staining corresponded to the inner two thirds of the dorsal horn. The labeling persisting in the lateral third corresponded to non-sciatic afferents. There was no significant loss of peptidergic fibers, as detected by CGRP immunoreactivity when comparing the IB4-saporin group to sham (Figure 2, A and D). There were no contralateral changes in either IB4 or CGRP labeling (not shown). The dramatic loss of IB4 staining was even better noticeable in horizontal sections of inner lamina II; indeed, as the lateral part of the layer was present in either another confocal plane or an adjacent section, the ipsilateral loss of IB4 labeling was complete in IB4-saporin treated rats (Figure 3).

Interestingly, we observed a dramatic ipsilateral increase in NK-1r immunoreactivity in lamina I in IB4-saporin-treated rats when comparing them to sham animals (Figure 4).

3.4.3 Observations in Lamina I spinoparabrachial neurons

As in previous publications from our laboratory, spinoparabrachial lamina I neuronal populations were identified based on the dendritic arborization and cell body shape as viewed in the horizontal plane. Multipolar neurons possess four or more primary dendrites arising from an irregularly-shaped cell body (Figure 5A and 5D). Fusiform neurons have one dendrite arising from each end of an elongated soma (Figure 5B and 5E). Pyramidal neurons have a triangularly-shaped soma with a primary dendrite originating from each of the three corners (Figure 5C and 5F). Some cells displayed features transitional between two of the cell types described above or had a portion of the cell body and / or proximal dendritic tree truncated due to sectioning. These latter neurons were not classifiable and were considered as "unclassified".

In sham animals, we confirmed our previous observations that spinoparabrachial fusiform and multipolar neurons displayed immunoreactivity for NK-1r in a high proportion of cells, whereas pyramidal neurons were almost never immunoreactive for the receptor (Figures 5 and 6). We observed an increase in the intensity of NK-1r staining in multipolar and fusiform neurons, when comparing the ipsilateral side of the spinal dorsal horn of IB4-saporin-injected animals (Figure 5, D and E) to the ipsilateral side of vehicle-injected sham animals (Figure 5, A and B), although there was no change in the proportion of neurons expressing immunoreactivity for the receptor (Figure 6). However, the number of pyramidal neurons expressing detectable NK-1r immunoreactivity remained very low (Figure 6).

We also performed a time-course study of the number of neurons of each cell type immunoreactive for the NK-1r in animals that were not injected with the retrograde tracer. We did not observe any change from 2 to 8 weeks post-IB4-saporin injection in the number of neurons of each type immunoreactive for the NK-1r (Figure 7), meaning that number of pyramidal neurons which were immunoreactive for the receptor remained very low at all time points studied.

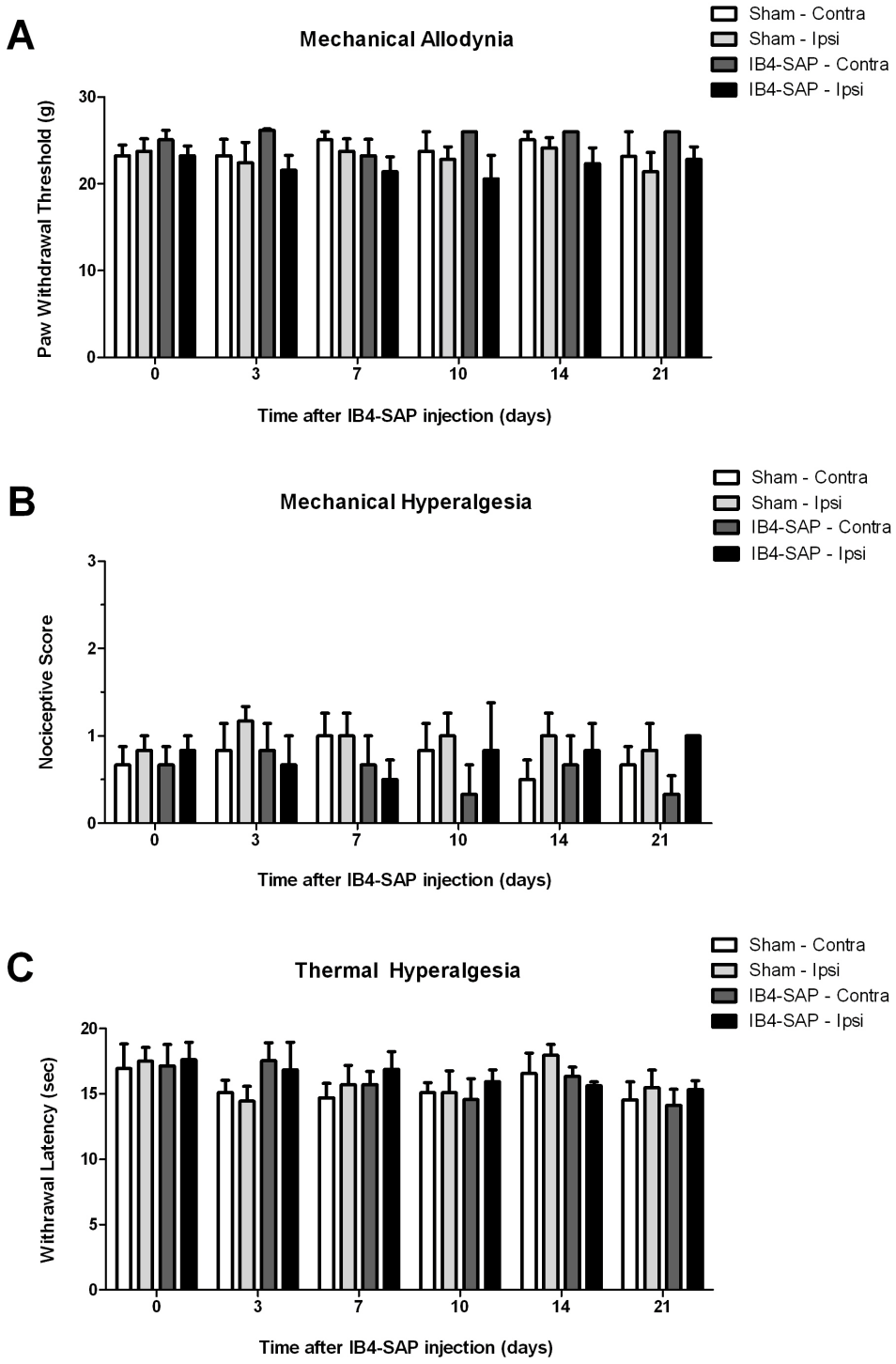
A
Mechanical Allodynia
Sham - Contra
Sham - Ipsi
IB4-SAP - Contra
IB4-SAP - Ipsi
Paw Withdrawal Threshold (g)
30
20
10
0
0
3
7
10
14
21
Time after IB4-SAP injection (days)
B
Mechanical Hyperalgesia
Sham - Contra
Sham - Ipsi
IB4-SAP - Contra
IB4-SAP - Ipsi
Nociceptive Score
3
2
1
0
0
3
7
10
14
21
Time after IB4-SAP injection (days)
C
Thermal Hyperalgesia
Sham - Contra
Sham - Ipsi
IB4-SAP - Contra
IB4-SAP - Ipsi
Withrawal Latency (sec)
20
15
10
5
0
0
3
7
10
14
21
Time after IB4-SAP injection (days)

Figure 1

**Figure 1:** Behavioral characterization at several time points following IB4-saporin injection. A: Assessment of mechanical allodynia using von Frey filaments in IB4-saporin-treated and sham animals; mechanical thresholds were not significantly different from the sham group. B: Assessment of mechanical hyperalgesia using pin prick in IB4-saporin-injected and sham groups; there was no significant difference in mechanical nociceptive rating between IB4-saporin-injected and sham groups. C: Assessment of thermal hyperalgesia using the Hargreaves test in IB4-saporin-injected and sham groups; IB4-saporin-injected animals did not display a significant difference in withdrawal latencies at any of the time points studied following IB4-saporin injection when compared with the sham group. N=6, 2-way ANOVA with Bonferroni post-hoc.

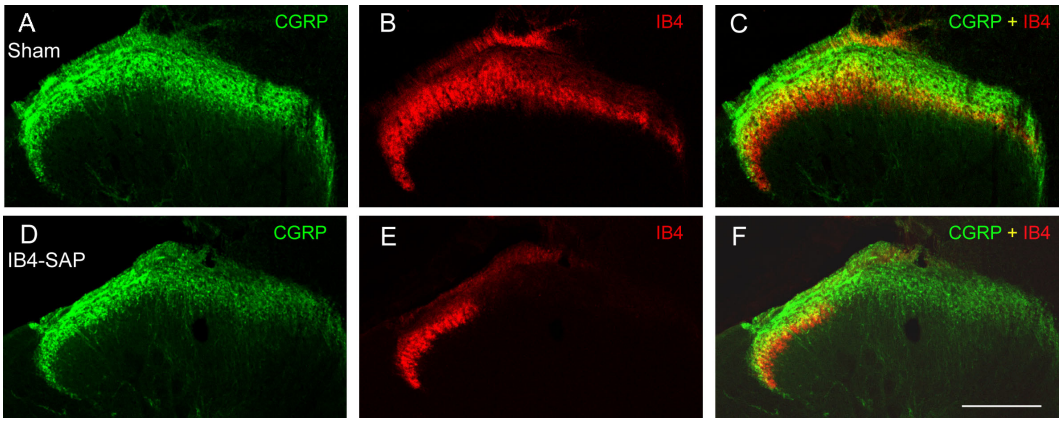


**Figure 2:** Confocal low magnification images from transverse sections comparing the ipsilateral side of the dorsal horn of vehicle-injected animals (A-C) with the ipsilateral side of IB4-saporin- injected (D-F) rats, at 21 days post-injection. In the lower panels, note the virtually complete depletion of IB4 staining (in red), except in the lateral third of the dorsal horn, that does not receive sciatic afferents. It is important to note that although in this particular section there seems to be a slight reduction of CGRP immunoreactivity (in green) in the area of the lesion, we did not observe any consist change in CGRP-IR fibers in the area of the lesion in IB4-saporin treated animals compared to sham. Scale bar = 100µm

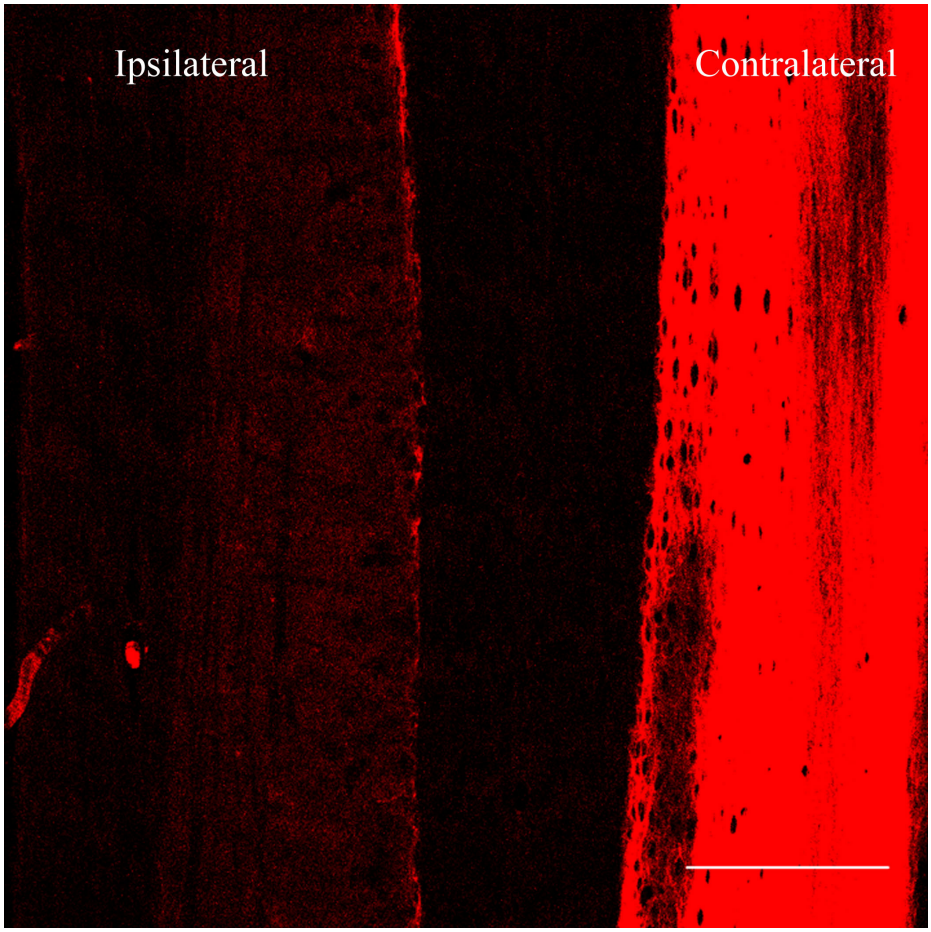


**Figure 3:** Confocal image at low magnification of a horizontal section from inner lamina II comparing the ipsilateral and contralateral sides of the spinal cord of an IB4-saporin injected animal at 21 days post-injection. Note that the ipsilateral side is completely devoid of IB4 labeling (red) compared to the contralateral side. The lateral part of lamina II, where IB4 staining persisted, was not present in this confocal plane. Scale bar = 500µm.

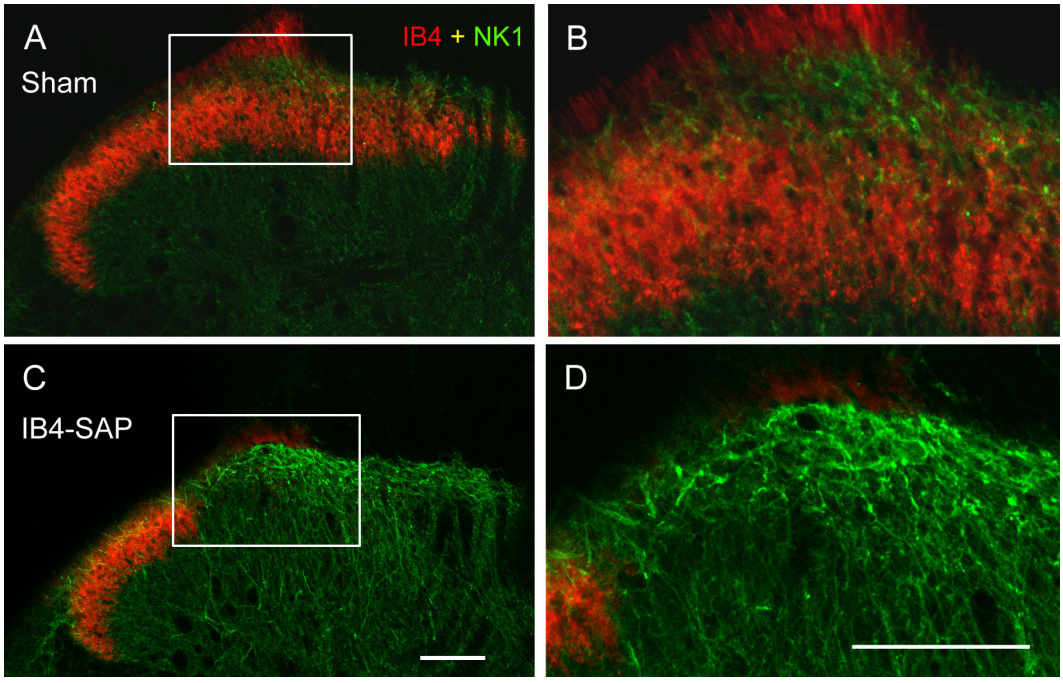


**Figure 4:** Confocal images comparing NK-1r immunoreactivity in the ipsilateral side of vehicle-injected (A, B) and IB4-saporin- injected (C, D) animals, in transverse sections, at 21 days. NK-1 receptors – NK-1 (in green), non-peptidergic afferents – IB4 (in red). The framed region in A and C is enlarged in B and D. Scale bar = 100μm

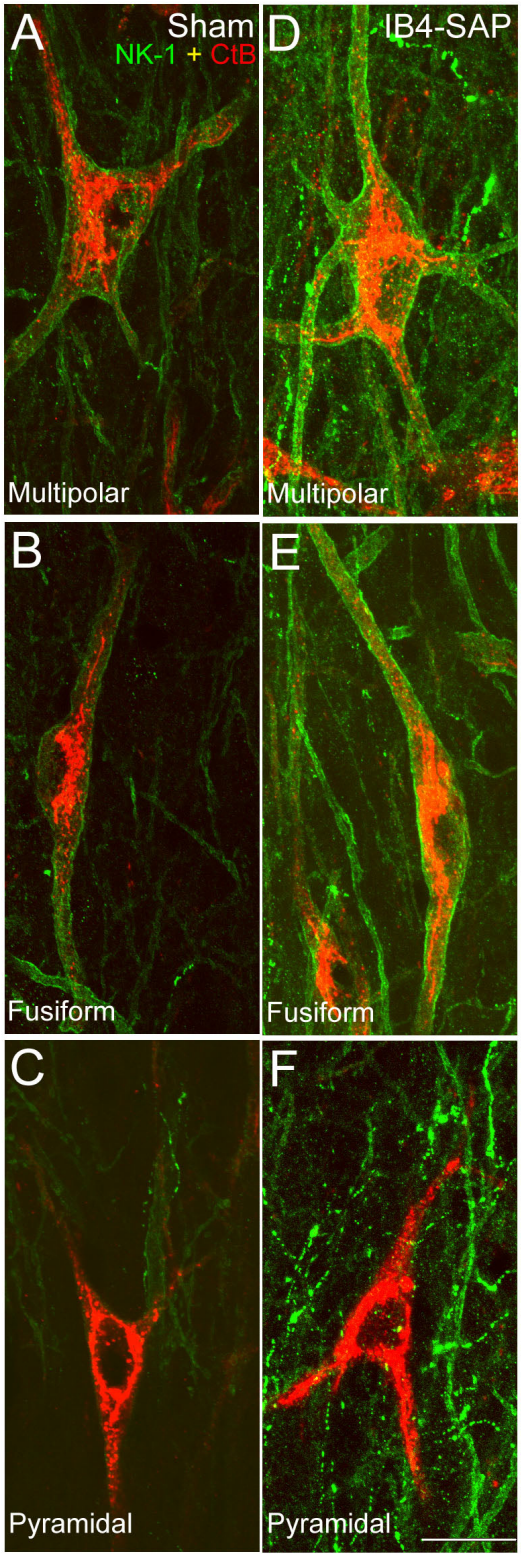
A
Sham
NK-1 + CtB
Multipolar
D
IB4-SAP
Multipolar
B
Fusiform
E
Fusiform
C
Pyramidal
F
Pyramidal

Figure 5

**Figure 5:** Confocal images at high magnification showing the morphology and NK-1r expression by the lamina I spinoparabrachial neuronal population in IB4-saporin-injected animals compared to the vehicle-injected sham group, at the 21 days time point. Both multipolar and fusiform neurons showed an increase in NK-1r immunoreactivity in each cell (D, E), compared to the sham group (A, B). However, the great majority of pyramidal neurons did not display NK-1r immunoreactivity following IB4-saporin injection at the 21 days time point (C, F). NK-1 receptor - NK-1 (in green), retrograde tracer CTb (in red). Scale bar = 20μm.

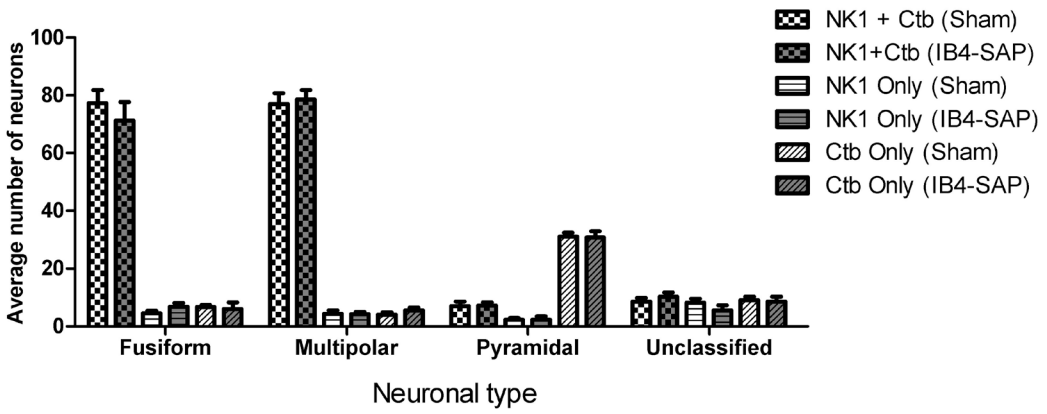


**Figure 6:** Quantitative comparison of the lamina I neuronal populations immunoreactive for the NK-1r (NK-1 only), retrogradely labeled from the parabrachial nucleus (CTb only) or double labeled (NK-1 + CTb), on the ipsilateral side of IB4-saporin-injected or sham group at 21 days. There were no changes in the number of fusiform, multipolar, or pyramidal neurons immunoreactive for one or both of the markers when comparing IB4-saporin-injected to sham groups. Only cells with visible nuclei were counted. Values represent average number of neurons (±SEM) counted per animal. N =6, one-way ANOVA.

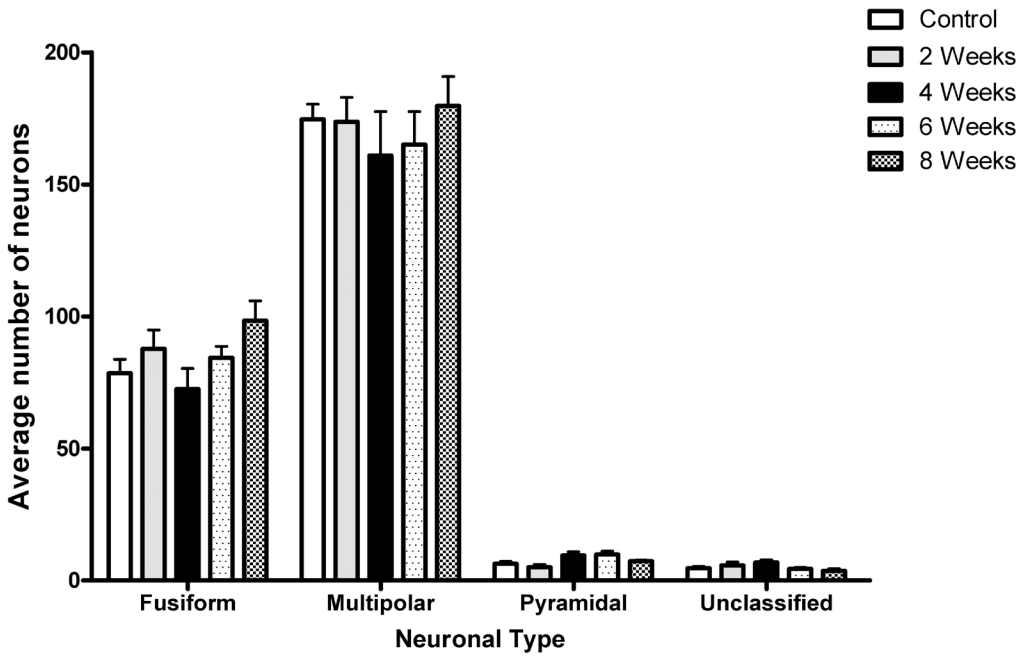


**Figure 7:** Quantitative comparison of lamina I neuronal populations expressing the NK-1r, on the ipsilateral side of IB4-saporin-injected and vehicle-injected (sham) groups at 2, 4, 6, and 8 weeks post-injection. There were no detectable changes in the number of fusiform, multipolar, or pyramidal neurons immunoreactive for NK-1r when comparing IB4-saporin-injected rats to the sham group. Only cells with visible nuclei were counted. Values represent average number of neurons (±SEM) counted per animal. N =6, one-way ANOVA with Dunnett's post hoc.

## 3.5 Discussion

In this study, we observed that the selective ablation of non-peptidergic nociceptive primary afferents by means of IB4-saporin injection into the sciatic nerve did not cause any change in pain-related behavior compared to vehicle-injected sham animals. This raises the possibility that normal behavioral function was maintained either due to redundancy in the IB4 afferent population or due to a compensatory mechanism in the spinal cord. We detected an overall increase in the expression of NK-1r in populations of lamina I projection neurons that already expressed the NK-1r, which would support the idea of spinal compensatory mechanisms. Nevertheless, we did not observe any *de novo* expression of NK-1r by pyramidal neurons or any change in the total number of lamina I neurons that expressed the NK-1r.

### 3.5.1 Effect of IB4-saporin injection on non-peptidergic primary sensory neurons

When injected in a peripheral nerve, IB4-saporin is known to cause a selective loss of primary afferents binding the plant lectin IB4 (Tarpley et al., 2004; Vulchanova et al., 2001). This is the result of selective binding of the lectin IB4 from the conjugate to the α-D-galactoside of versican, an extracellular matrix protein expressed exclusively on the non-peptidergic afferents, and of the retrograde transport to the DRG neurons of the conjugate, where the saporin component induces cell death through mitochondrial toxicity. Several studies agree that the maximal loss of IB4 binding occurs by 21 days post IB4-saporin injection and that this loss persists indefinitely as it results from cell death (Tarpley et al., 2004; Vulchanova et al., 2001).

### 3.5.2 Behavioral changes following IB4-saporin injection

Following IB4-saporin injection in the sciatic nerve, we did not detect any changes in the pain thresholds for innocuous mechanical or noxious mechanical and thermal stimuli. Our results differ from those of two other groups (Tarpley et al., 2004; Vulchanova et al., 2001) who previously injected IB4-saporin into the sciatic nerve of rats in that we did not observe a transient elevation in mechanical

and thermal thresholds at early time points (up to day 14 post-IB4-saporin injection). However, our data concurs with that of the two above studies in that by 21 days post-injection thermal and mechanical thresholds were not different from control levels. A previous study from our laboratory in which the IB4-saporin was injected bilaterally into the mental nerves did not reveal any difference from controls when mechanical thresholds were tested in the lower lip skin (Taylor and Ribeiro-da-Silva, 2012). Our data also concurs with a previous, unpublished, study from our laboratory with intra-sciatic nerve injection of IB4-SAP (Bailey et al., 2005). These results are in agreement with previous work that describes the lack of behavioral changes following ablation of IB4-positive epidermal innervations (Lindfors et al., 2006). However, the fact that both thermal and mechanical responses were temporarily affected (Tarpley et al., 2004; Vulchanova et al., 2001) or not affected at all (our studies) following the ablation of the non-peptidergic population of nociceptive afferents contradicts the observation in the mouse that non-peptidergic and peptidergic fibers mediate specific modalities, mechanical and thermal nociception, respectively (Cavanaugh et al., 2009; Scherrer et al., 2009). The discrepancy may be due to species differences in the properties of peptidergic and non-peptidergic afferents. These concern in particular the expression of the vanilloid receptor TRPV1, which is known to have an important role in thermal nociceptive mechanisms (Julius and Basbaum, 2001). Indeed, in mouse TRPV1 receptors are expressed exclusively by peptidergic primary afferents (Zwick et al., 2002). In contrast, in rat both peptidergic and non-peptidergic afferents express TRPV1 (Michael and Priestley, 1999).

### 3.5.3 Changes in dorsal horn lamina I projection neurons following IB4-saporin injection

Based on the normal responses to noxious stimuli in the absence of one of the two main populations of nociceptive primary afferents, the logical reasoning would be that the other main population would be able to compensate for such loss. This would imply the occurrence of adaptive compensatory changes in the other population. Surprisingly, previous studies (Tarpley et al., 2004; Vulchanova

et al., 2001) reported a slight decrease in substance P immunoreactivity following IB4-saporin treatment, which might suggest an increased release of substance P vesicles. However, in the present study, we did not detect any consistent decrease in CGRP immunoreactivity, nor did we observe a change in the peptidergeric population in the recent study in the trigeminal system (Taylor and Ribeiro-da-Silva, 2012). However, we did observe an overall increase in NK-1r immunoreactivity following IB4-saporin injection (see Figure 4). As our laboratory had previously detected a novel expression of NK-1r by pyramidal neurons in chronic pain models (Almarestani et al., 2009) (Saeed and Ribeiro-da-Silva, submitted), we investigated if this NK-1r immunoreactivity increase was due to an upregulation in neurons already expressing it or if it was the result of a *de novo* synthesis by a new neuronal population. Our quantitative study of NK1r expression by the three lamina I projection neuronal populations did not reveal any novel expression of NK-1r by pyramidal neurons or even an increased number of cells of the other two populations displaying NK-1r immunoreactivity (Figure 6). This indicated that the increased NK-1r immunoreactivity we detected was caused by an increase in the number of receptors by cell populations already expressing it. Furthermore, we did not detect any change in the number of lamina I neurons of each type which were NK1-r-positive over 8 weeks (Figure 7).

## 3.6 Conclusions

In this study, we have demonstrated that the selective lesioning of the non-peptidergic afferents of the sciatic nerve do not cause any significant pain-related behavior change for up to 21 days after lesion. In the spinal dorsal horn, changes were limited to the loss of the non-peptidergic afferents and a strong increase in overall immunoreactivity for the NK-1r. However, contrary to what we might expect, the overall number of neurons expressing the NK-1r did not increase and there was no *de novo* expression of the receptor by pyramidal neurons. These results suggest that a persistent pain state may be required to trigger the NK-1r expression by pyramidal neurons.

3.7 Acknowledgements

The authors would like to thank Manon St-Louis for laboratory expertise and Jacynthe Laliberté for confocal microscopy assistance. We are also particularly grateful to Dr. Gary Bennett for the use of his animal behavior facility.

## Connecting text: Chapter 3 to Chapter 4

In the previous chapter, we found that the cytotoxic ablation of an entire population of nociceptive primary afferents, the non-peptidergic C fibers, did not trigger the expression of NK-1r by pyramidal neurons, although it induced an over-expression of these receptors by fusiform and multipolar cells. During the course of this study we used IB4 labeling as a way to identify the efficacy of our neurotoxin lesions. Surprisingly, we verified that, in control animals, there was some direct innervation of lamina I neurons expressing the NK-1r by non-peptidergic, IB4-binding, afferents. This observation in the rat contradicted studies by Basbaum's group which indicated, in a mouse model, that the non-peptidergic afferents do not form directly or indirect contacts with lamina I neurons expressing the NK-1r (Braz et al., 2005).

Therefore, in the next chapter, we performed a systematic study of the termination of non-peptidergic afferents in lamina I, in naïve rats, and analyzed quantitatively their innervation of the different populations of lamina I projection neurons. We confirmed that non-peptidergic afferents are less abundant in lamina I than the peptidergic, although they still contributed significantly to the direct innervation of lamina I projection neurons. We also confirmed at the ultrastructural level that these non-peptidergic afferents, identified by P2X3 immunoreactivity, established *bona fide* synapses on lamina I neurons expressing the NK-1r.

# Chapter 4

Non-peptidergic primary afferents are presynaptic to lamina I projection neurons in rat spinal cord

**Abeer W. Saeed and Alfredo Ribeiro-da-Silva**

Submitted to Molecular Pain, 2012

4.1 Abstract

**Background:** Pain-related (nociceptive) information is carried from the periphery to the dorsal horn of the spinal cord mostly by two populations of small diameter primary afferents, the peptidergic and the non-peptidergic. The peptidergic population expresses neuropeptides, such as substance P and calcitonin gene-related peptide (CGRP), while the non-peptidergic fibers are devoid of neuropeptides, express the purinergic receptor P2X3, and bind the isolectin B4 (IB4). Although it has been known for some time that in rat the peptidergic afferents terminate mostly in lamina I and outer lamina II and non-peptidergic afferents in inner lamina II, the extent of the termination of the latter population in lamina I was never investigated as it was considered as very minor. Because our preliminary evidence suggested otherwise, we decided to re-examine the termination of non-peptidergic afferents in lamina I, in particular with regards to their innervation of projection neurons expressing substance P receptors (NK-1r). We used retrograde labeling of neurons from the parabrachial nucleus combined with lectin IB4 binding and immunocytochemistry. Samples were examined by confocal and electron microscopy.

**Results:** By confocal microscopy, we studied the termination of non-peptidergic afferents in lamina I using IB4 binding and P2X3 immunoreactivity as markers, in relation to CGRP immunoreactivy, a marker of peptidergic afferents. The number of IB4 or P2X3-labeled fibers in lamina I was much higher than expected, although they were less abundant than CGRP-labeled afferents. There were very few fibers double-labeled for CGRP and either P2X3 or IB4. We found a considerable number of IB4-positive fiber varicosities in close apposition to NK-1r-positive lamina I projection neurons, which were distinct from peptidergic varicosities. Furthermore, we confirmed at the ultrastructural level that there were bona fide synapses between P2X3-immunoreactive non-peptidergic boutons and neurokinin-1 receptor-positive lamina I dendrites.

**Conclusions:** These results indicate the presence of direct inervation by non-peptidergic nociceptive afferents of lamina I projection neurons expressing

NK-1r. Further investigations are needed to better understand the role of these connections in physiological conditions and chronic pain states.

## 4.2 Introduction

Unmyelinated C afferents comprise approximately 70% of all primary afferents fibers (Nagy and Hunt, 1983). The majority of such fibers transmit nociceptive information from the periphery to the spinal dorsal horn and have been divided into two main populations, the peptidergic and the non-peptidergic (Hunt and Rossi, 1985). The peptidergic population synthesizes neuropeptides, such as substance P (SP) and calcitonin gene related peptide (CGRP), expresses the high affinity nerve growth factor receptor, trkA, and terminates mainly in lamina I and outer lamina II of the spinal dorsal horn (Averill et al., 1995; Cuello et al., 1993; Skofitsch and Jacobowitz, 1985). The non-peptidergic unmyelinated afferents, although devoid of neuropeptides, express the purinergic P2X3 receptor, bind the plant isolectin B4 (IB4), express receptors for glial cell-derived neurotrophic factor (GDNF) and terminate mainly in inner lamina II of the spinal dorsal horn (Bennett et al., 1998; Silverman and Kruger, 1988; Vulchanova et al., 1998). These two populations can also be differentiated based on the ultrastructural properties of their central terminals in the spinal dorsal horn; the peptidergic terminate mostly as simple axo-dendritic boutons, containing several large granular vesicles (LGV), and occasionally as central terminals of type Ib glomeruli (CIb), which are rich in LGV (Ribeiro-da-Silva, 2004; Ribeiro-da-Silva et al., 1989). In contrast, the non-peptidergic afferents terminate most often as the central boutons of type Ia glomeruli, which are poor in LGV, have a very dense matrix and are often postsynaptic to GABA-positive neurons at axo-axonic or dendroaxonic synapses (Ribeiro-da-Silva, 2004).

Regarding peptidergic afferents, their dorsal horn termination has been investigated in particular in relation to neurons which express the SP receptor, the neurokinin-1 receptor (NK-1r) (Liu et al., 1994; McLeod et al., 1998; Todd et al., 2002). It has been shown at the confocal and electron microscopic levels that SP-immunoreactive (IR) terminals innervate preferentially neurons which display

NK-1r immunoreactivity (McLeod et al., 1998; Todd et al., 2002). In lamina I, an abundant innervation by SP-IR primary afferents of projection neurons, which are immunoreactive for the NK-1r and are activated by noxious stimuli, has been detected (Todd et al., 2002). Unfortunately, our knowledge concerning the central terminations of the non-peptidergic C fiber population is limited. In rat, as mentioned above, they have been shown to terminate mostly in inner lamina II as central terminals of type Ia glomeruli. However, besides the fact that they are postsynaptic to GABAergic interneurons, their synaptic connections are not well known. Studies in rat utilizing whole-cell recordings suggest that these non-peptidergic afferents form indirect connections with lamina I projection neurons through interneurons in lamina II (Lu and Perl, 2003, 2005), while other studies, using transgenic mice, proposed that the non-peptidergic afferents synapse onto inner lamina II interneurons which in turn would connect with deep lamina V projection neurons (Braz et al., 2005). However, it was never investigated whether lamina I projection neurons receive direct synapses from non-peptidergic afferents, likely because it was assumed that the termination of such afferents in lamina I was negligible. In this study, we examined quantitatively the innervation of NK-1r-positive lamina I projection neurons by non-peptidergic unmyelinated primary afferents, using confocal microscopy, and also provided ultrastructural evidence of direct synapses of these afferents on NK-1r-IR lamina I neurons.

## 4.3 Results

### 4.3.1 Termination of non-peptidergic afferents in lamina I

We examined the termination of non-peptidergic afferents using either IB4 binding or P2X3 immunoreactivity. We used sections cut in the three planes: transverse, parasagittal and horizontal (Figures 1-3), and focused on the region equidistant from the lateral and medial limits of the dorsal horn. In transverse sections, when comparing the distribution of IB4-binding (IB4+) and P2X3-IR fibers at low magnification, it was apparent that the band of intense IB4+ labeling extended more ventrally than the P2X3 band (Figure 1). Overall, some IB4+ and

P2X3-IR terminals co-localized CGRP immunoreactivity (in yellow in Figures 1 and 3), although the majority did not. A surprising observation was that the number of boutons in lamina I that were IB4+ or P2X3-IR and did not co-localize CGRP immunoreactivity was much higher than what could be expected based on the literature. Some IB4+ and P2X3-IR boutons which did not co-localize CGRP immunoreactivity could be observed in transverse sections (Figure 1 C-D; arrowheads). However, these boutons were particularly apparent in parasagittal (Figure 2) and horizontal sections (Figure 3). In horizontal sections, we could be absolutely certain that the boutons from non-peptidergic afferents were located in lamina I since we used serial sections, and the confocal images were obtained with a very small pinhole corresponding to an optical slice of ~0.5 µm adjacent to the white matter. These sections revealed a considerable innervation of lamina I by boutons immunoreactive for P2X3, although they were clearly less abundant than the CGRP-IR (Figure 3A). The comparison with optical slices from inner lamina II allowed us to assess how much more abundant P2X3-IR boutons were in that layer (Figure 1D, 2 and 3B), confirming that inner lamina II is the main termination site for non-peptidergic afferents.

4.3.2 Innervation of lamina I projection neurons by non-peptidergic afferents

To study the innervation of lamina I projection neurons by non-peptidergic afferents, we labeled neurons retrogradely by means of a stereotaxic CTb injection in the lateral parabrachial nucleus. The injection site covered most of the parabrachial complex, including the lateral parabrachial nucleus, an observation that is comparable to distributions previously reported by us (Almarestani et al., 2007, 2009). The majority of the retrogradely labeled lamina I neurons were found on the contralateral side, although a few were also present on the side ipsilateral to the CTb injection. CTb labeling of spinoparabrachial lamina I neurons included the cell body and primary dendrites (Figures 4 and 5).

We also performed a labeling of these cells with antibodies against the SP, receptor, the NK-1r. Indeed, it has been previously described (Almarestani et al., 2007, 2009) that lamina I, fusiform and multipolar spinoparabrachial neurons,

identified by retrograde tracing of CTb, often express NK-1r immunoreactivity, while lamina I pyramidal neurons seldom express NK-1r immunoreactivity. These neuronal populations can be identified based on their dendritic arborization and cell body shape when viewed in the horizontal plane. Multipolar neurons have four or more dendrites arising from an irregularly-shaped soma (Figure 4A). Fusiform neurons possess two dendrites, each arising from one end of a spindle-shaped soma (Figure 4B and 5). Lastly, pyramidal neurons have a triangular soma with one primary dendrite arising from each of the three tips of the soma (Figure 4C). In this study, we observed IB4+ non-peptidergic varicosities in apparent direct apposition to all three populations of lamina I projection neurons, multipolar (Figure 4A), fusiform (Figure 4B) and pyramidal (Figure 4C). These boutons were distinct from CGRP-positive peptidergic boutons, and from boutons of the small proportion of primary afferents which are simultaneously CGRP-positive and bind IB4, as described previously (Alvarez and Fyffe, 2000; Priestley et al., 2002) (Figure 5).

### 4.3.3 Quantification of non-peptidergic varicosities apposed to NK-1r-positive lamina I projection neurons

Although peptidergic innervation is associated mainly with multipolar and fusiform lamina I neurons (Yu et al., 2005), the quantitative data in the present study, which was carried out in the cell bodies and proximal dendrites of neurons, demonstrates the presence of a non-peptidergic innervation on all three populations of lamina I projection neurons (Figure 6). Since the main objective of this study was to give an account of the non-peptidergic innervation, no quantification was performed for the peptidergic innervation on lamina I neurons, which has been previously investigated (Charlton and Helke, 1985; Ma et al., 1996; McLeod et al., 1998).

### 4.3.4 Ultrastructural demonstration of synapses between non-peptidergic primary afferents and lamina I neurons

Because the resolution of confocal microscopy is insufficient to demonstrate the presence of bona fide appositions of two structures and synapses between them, we performed an ultrastructural study using P2X3 immunoreactivity. We had to omit glutaraldehyde from the fixative because it completely blocked the immunostaining, even in very low concentration, which affected the quality of the ultrastrucure. In lamina I, all P2X3-IR boutons established simple axo-dendritic or axo-somatic synapses (Figure 7A). In inner lamina II, most P2X3-IR boutons were involved in complex synaptic arrangements, as predicted from the literature (Ribeiro-da-Silva and De Koninck, 2008), forming the central element of type Ia glomeruli (Figure 7B).

We used a double labeling for P2X3 and NK-1r to demonstrate direct appositions and synapses between P2X3 immunoreactive boutons and NK-1r dendritic profiles (Figure 8).

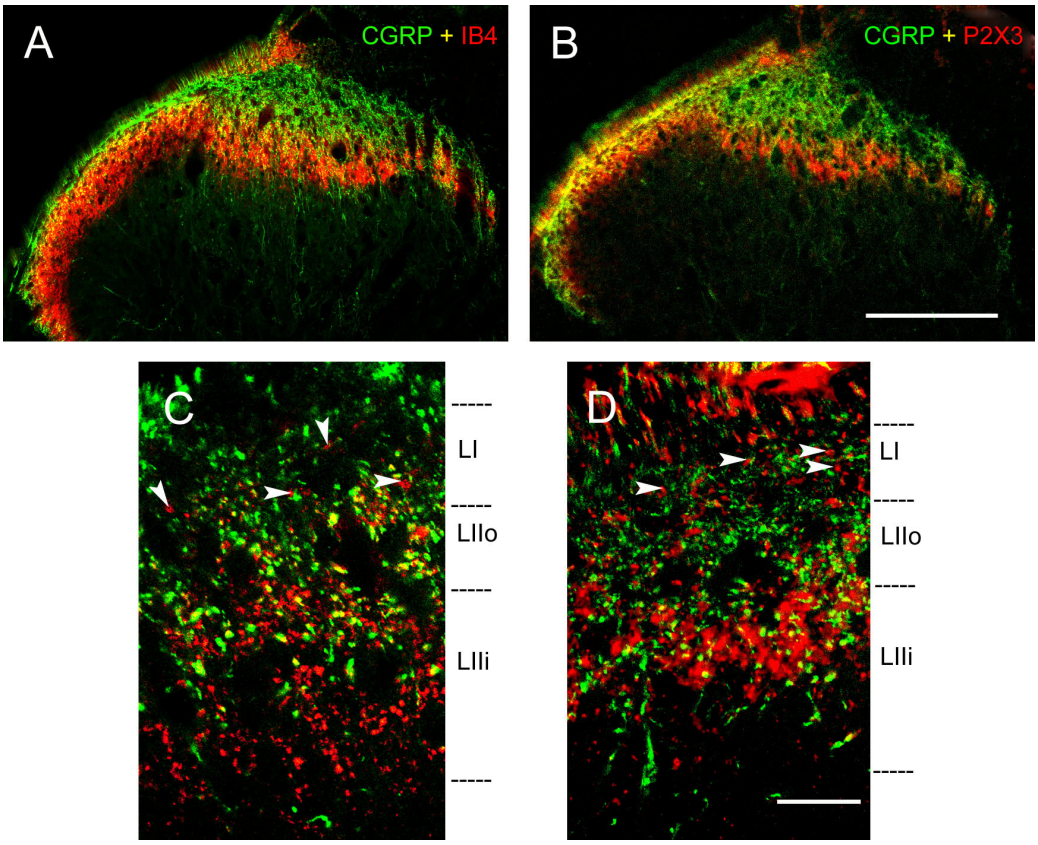


**Figure 1:** CGRP, IB4 and P2X3 staining in transverse spinal cord sections. **A** and **B** show low magnification confocal images of CGRP-IR and IB4-positive (A) or P2X3-IR (B) fibers. **C** and **D** represent high magnification confocal images from the middle third of the latero-medial extent of the superficial dorsal horn. In **C**, note that there is limited co-localization of IB4 and CGRP (in yellow). Arrowheads show axonal varicosities (boutons) from non-peptidergic fibers in lamina I, which do not co-localize CGRP immunoreactivity. Calcitonin gene related peptide – CGRP (in green); isolectin B4 – IB4 (in red); purinergic receptor P2X3 – P2X3 (in red). Scale bar (**A**, **B**) = 200 μm; scale bar (**C**, **D**) = 20 μm.

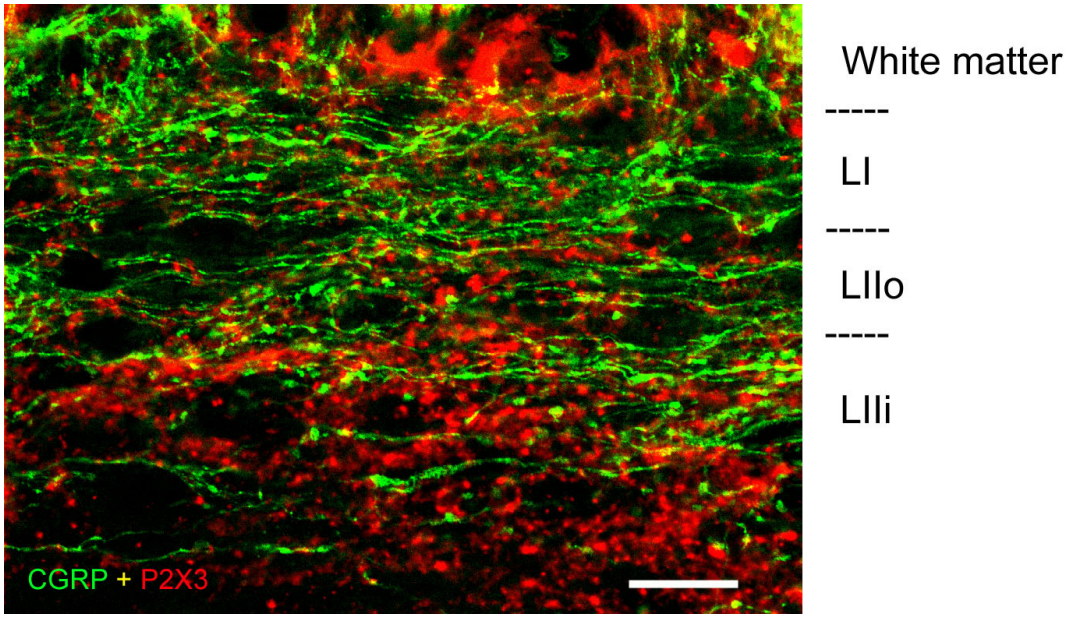


**Figure 2:** Confocal images at high magnification obtained from parasagittal spinal cord sections showing CGRP-IR (green) and P2X3-IR (red) varicosities in the superficial dorsal horn. P2X3-IR varicosities were present in considerable number in lamina I (LI) but were more highly concentrated in inner lamina II (LIIi). Scale bar = 20 µm.

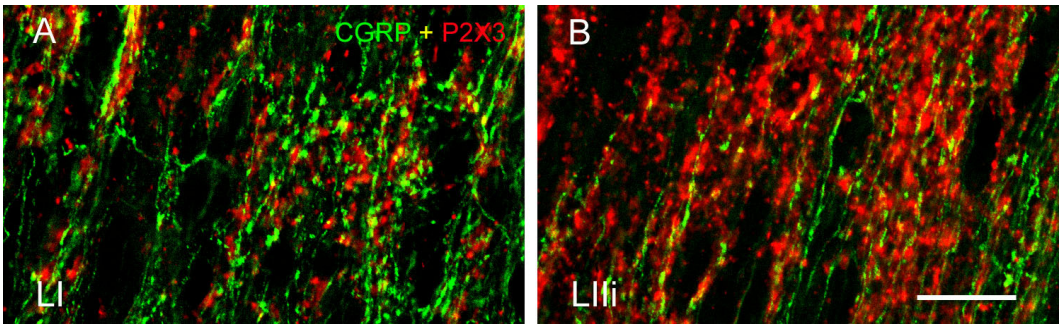


**Figure 3:** Confocal images at high power obtained from horizontal spinal cord sections. In a confocal optical section from lamina I adjacent to the white matter (A), note the relatively abundant P2X3-IR fibers with varicosities (boutons). CGRP-IR fibers and boutons were considerably more abundant in this lamina. In a confocal optical section from inner lamina II (B), note the very high density of P2X3-IR fibers and varicosities, higher than that of CGRP-IR fibers in lamina I. Note that most varicosities display either P2X3 or CGRP immunoreactivity, although some co-localization is observed (yellow). Scale bar (A, B) = 20 μm.

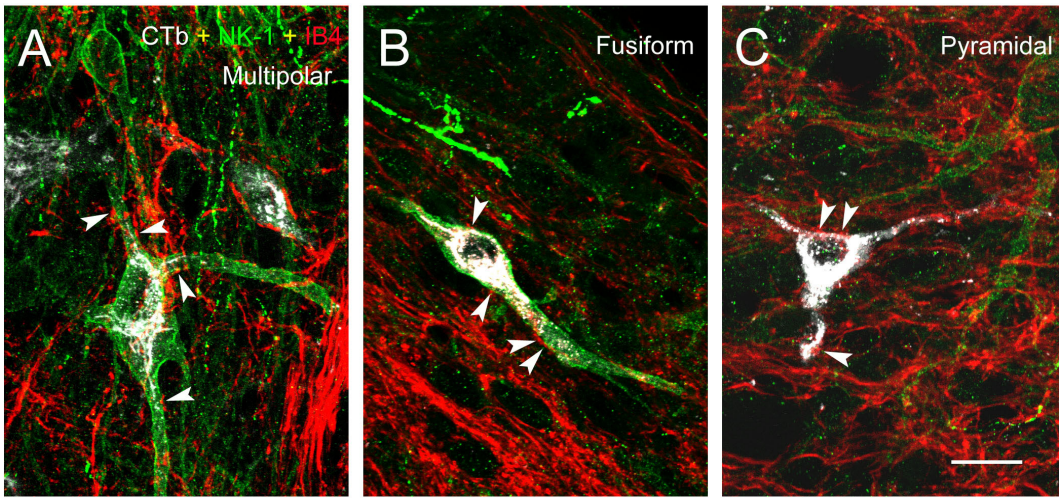


**Figure 4:** Confocal triple-labeling image showing the innervation of lamina I spinoparabrachial (A) multipolar, (B) fusiform and (C) pyramidal neurons by IB4+ varicosities (indicated by arrowheads). IB4 (red); NK-1r (green); spinoparabrachial neurons labeled with CTb (white). Scale bar (A-C) = 20 µm.

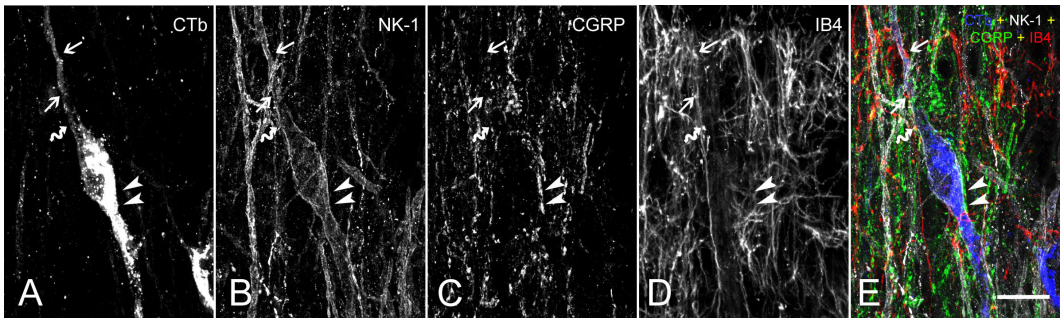


**Figure 5:** Example of a quadruple labeling observed by confocal microscopy at the confocal level using a multi-track approach. In this image the following signals were simultaneously detected: CGRP (green); IB4 binding (red); CTb transported retrogradely from the parabrachial nucleus (blue); NK-1r (white). A fusiform neuron, double labeled with CTb and NK-1r, is innervated by CGRP-IR boutons (arrowhead) and IB4+ (arrow) boutons, which represent distinct populations. However, a small population of varicosities co-labeled for CGRP and IB4 (curved arrow) was detected. Scale bar (A-E) = 20 μm.

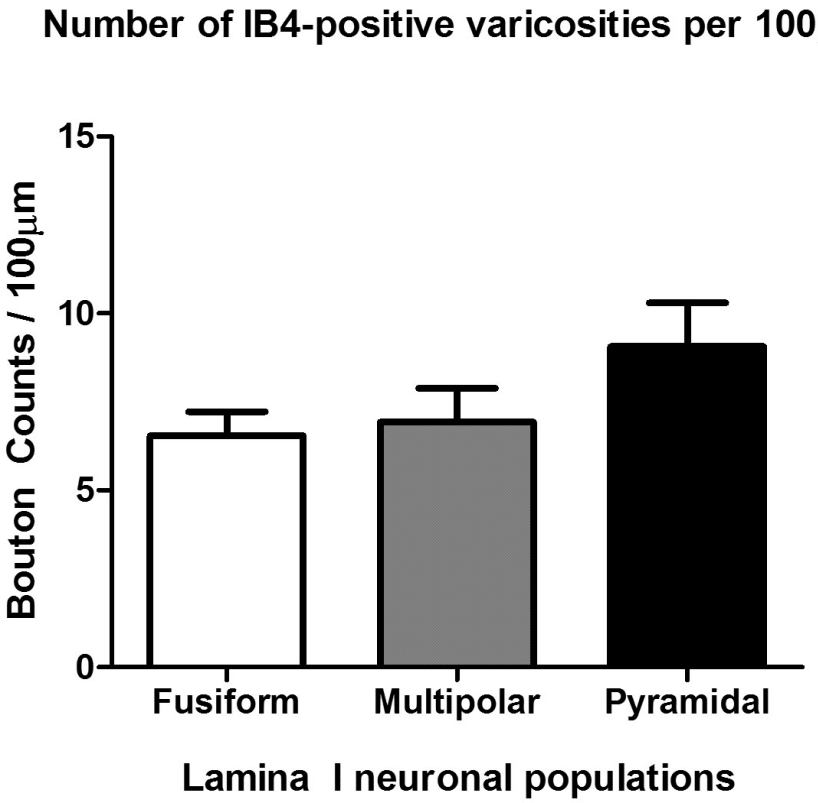


**Figure 6:** Quantification of IB4-positive boutons in direct apposition to lamina I spinoparabrachial neurons. Because of limitations in the CTb filling of neurons, we limited the quantification to boutons apposed to the soma membrane and membranes of primary dendrites. N= 6.

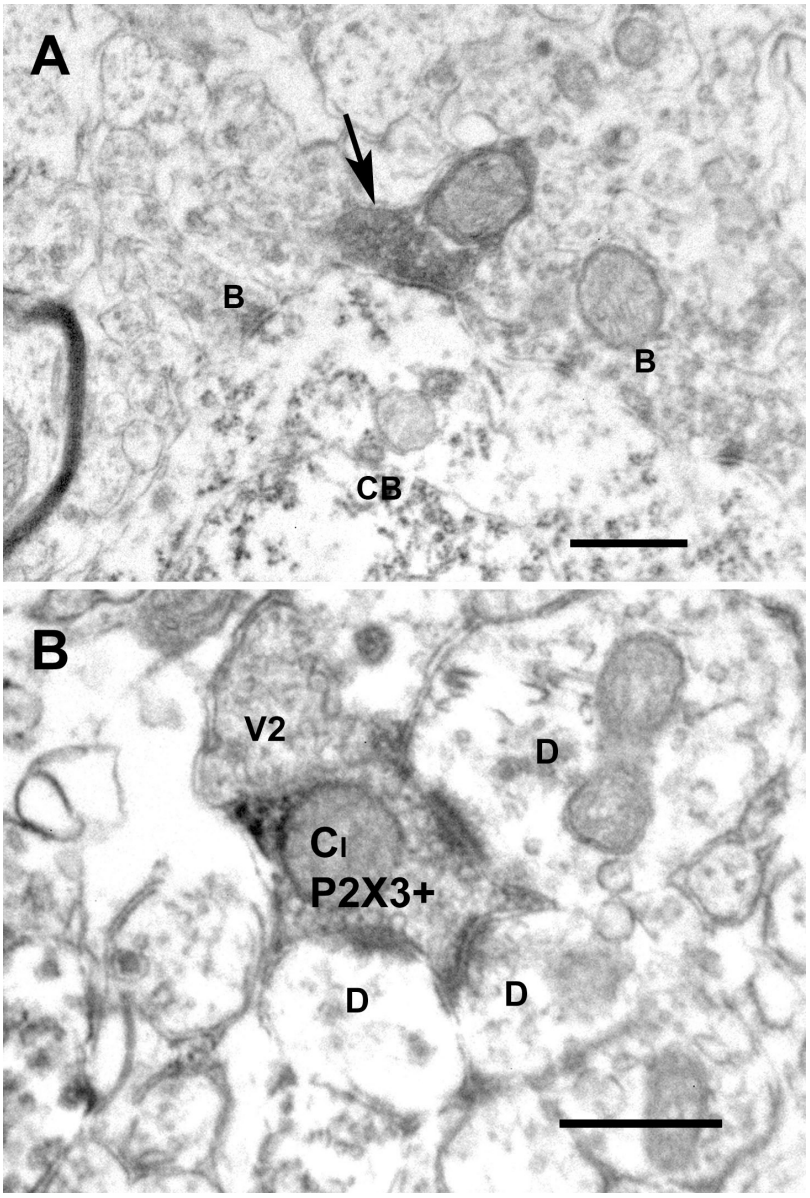


**Figure 7:** Electron micrograph showing: in **A**, a P2X3-IR bouton (arrow) forming an axo-somatic contact onto a lamina I cell body (CB); in **B**, a P2X3-IR central bouton of a type Ia glomerulus (CI P2X3+) presynaptic to 3 lamina I dendrites (D) and apposed to an axonal bouton (V2). Scale bar = 0.5 µm.

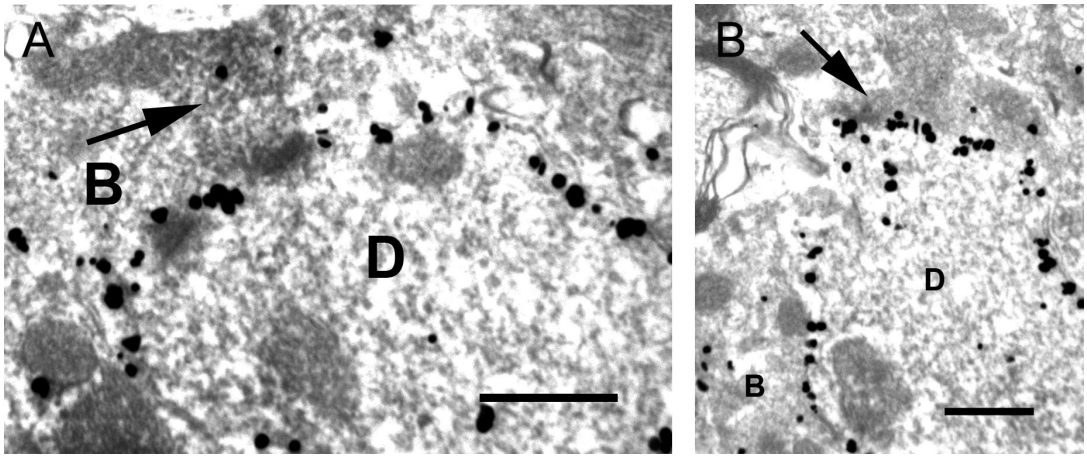


**Figure 8:** Electron micrographs showing axo-dendritic contacts between P2X3-positive boutons (arrows) and NK-1r-positive dendrites (D) (**A** and **B**). Note the silver-intensified gold particles along the plasma membrane of the dendrites, representing NK-1r-IR sites. B: unlabeled axonal bouton. Scale bar = 0.5 μm.

## 4.4 Discussion

In this study, we demonstrate the presence in lamina I of a significant number of boutons originating from non-peptidergic afferents immunoreactive for P2X3 or binding the lectin IB4. We also provide evidence at the confocal microscopy level that IB4+ boutons are in apposition to lamina I projection neurons which express the NK-1r. Lastly, we provide ultrastructural evidence of synapses between P2X3-IR boutons and lamina I dendritic profiles immunoreactive for the NK-1r.

### 4.4.1 Technical considerations

Because most of this study was carried out using confocal microscopy, we could not fully ensure that boutons from non-peptidergic afferents were presynaptic to neurons in lamina I. For this reason, we carried out an ultrastructural study using antibodies against P2X3 and provided direct evidence of synapses between P2X3-IR axonal boutons and dendrites and cell bodies in lamina I. We also performed at the ultrastructural level a double labeling for P2X3 and the NK-1r that demonstrated that some of the structures innervated by these boutons expressed the NK-1r, in agreement with what was assumed from the confocal data. We could observe unequivocal evidence of synaptic contacts, in spite of the fact that we had to use a fixative without glutaraldehyde and pre-treatment of the sections with a detergent for a short period to obtain P2X3 staining at the ultrastructural level. It should be pointed out that Naim et al. (Naim et al., 1997) examined at the ultrastructural level ultrathin sections recut from thicker sections previously examined by confocal microscopy. Naim et al.'s study showed that immunoreactive varicosities seen in close apposition to the membrane of NK-1r-IR under the confocal microscope actually formed synapses when viewed under the electron microscope. Therefore, we are confident that the IB4+ varicosities in apposition to NK-1r-IR lamina I projection neurons should establish synapses in a high proportion of cases.

It is known that in rat there is a small proportion of peptidergic sensory fibers in lamina I and II that colocalize CGRP and somatostatin, do not respond

to NGF and bind IB4 (Alvarez and Fyffe, 2000; Priestley et al., 2002). This obliged us to investigate the co-localization of IB4 binding and P2X3 immunoreactivity, our markers of non-peptidergic nociceptive C fibers, with CGRP immunoreactivity. Indeed, as predicted, we found a limited level of co-localization of either marker of non-peptidergic afferents with CGRP immunoreactivity. However, most varicosities that we observed in lamina I which were IB4+ or P2X3-IR did not co-localize CGRP immunoreactivity, what reassured us regarding the validity of our findings regarding the overall innervation of lamina I by non-peptidergic afferents. But it was still possible that a subpopulation of these afferents innervating NK-1r-IR lamina I projection neurons would represent exactly this minor subpopulation that is simultaneously CGRP-IR and IB4+. Therefore, we carried out a quadruple labeling in which we detected CTb (the retrograde label), NK-1r, CGRP and IB4 binding. Unfortunately, because of antibody incompatibilities, we could not perform P2X3 staining and had to use the lectin IB4 conjugated to a fluorochrome. Although our confocal microscope can identify 4 separate signals reliably using the multi-track approach, it was technically impossible to perform a quantitative analysis of the innervation of lamina I cells using more than 3 signals (IB4, CTb and NK-1r) because of photobleaching during the performance of the Z-stacks. However, we examined enough cells using the quadruple labeling to ensure that the proportion of axonal boutons colocalizing IB4 binding and CGRP immunoreactivity in apposition to lamina I neurons was low. An example of the quadruple labeling is given on Figure 4.

4.4.2 Innervation of lamina I projection neurons by non-peptidergic C fibers

The main objective of this study was to investigate the issue of the innervation of lamina I projection neurons by non-peptidergic C fibers because to our knowledge a systematic study combining labeling of these sensory fibers with labeling of these lamina I neurons had never been done. This is an important issue for the reasons given below. Previous work by Lu and Perl (Lu and Perl, 2005), using simultaneous whole-cell recordings from pairs of neurons, provided

some evidence that input from primary afferent C fibers terminating in inner lamina II may reach lamina I projection neurons via interposed interneurons. Since the great majority of C fibers innervating inner lamina II are non-peptidergic, it has been later suggested that the above pathway may be important for pain-related information conveyed by non-peptidergic C fibers to reach lamina I projection neurons which then project to supraspinal levels. If this is true, lamina I nociceptive projection neurons would receive direct input from peptidergic afferents and polysynaptic input from the non-peptidergic afferents (Ribeiro-da-Silva and De Koninck, 2008).

Alternatively, it has been proposed that the non-peptidergic afferents are part of a distinct and parallel pathway from that of their peptidergic counterpart. This view obtained some support from a study by Braz et al. (Braz et al., 2005) in a transgenic mouse, which demonstrated that the termination of non-peptidergic afferents on lamina II would be on excitatory interneurons, which in turn would synapse on deep lamina V projection neurons with ascending connections to the amygdala, hypothalamus and bed nucleus of stria terminalis. As they found minimal connection with lamina I neurons expressing the NK-1r, the above group proposed the involvement of the non-peptidergic afferents in the affective/emotional component of pain. Alternatively, other studies also in the mouse, proposed that distinct subsets of primary sensory afferents selectively mediate responses to different stimulus modalities. These studies provided some evidence suggesting that, in the mouse, non-peptidergic afferents play a particular role in transmitting mechanical pain, as opposed to the peptidergic which would be involved in conveying thermal pain (Cavanaugh et al., 2009; Scherrer et al., 2009). This idea of divergent pain pathways can be criticized since all the studies supporting it were performed in mice, which have been shown to demonstrate an explicit dichotomy between the C fiber populations that does not apply to rats and higher order species such as primates. In particular, the vanilloid TRPV1 receptors are localized only in the peptidergic fibers in mice (Woodbury et al., 2004; Zwick et al., 2002) whereas they are present in both peptidergic and non-

peptidergic afferents in the rat and higher species (Guo et al., 1999; Tominaga et al., 1998).

Our data provides evidence that non-peptidergic primary afferents establish direct connections with lamina I projection neurons, in addition to the possible indirect connections via an interposed interneuron suggested in previous studies (Lu and Perl, 2005). Although the non-peptidergic primary afferent termination in lamina I may seem as a minor contribution to the lamina I synaptic circuitry when compared to their termination in inner lamina II, they may prove to have a significant role in the transmission of nociceptive signals. Indeed, the terminals of these fibers in inner lamina II represent the central bouton of type Ia glomeruli, and are involved in complex modulatory circuits involving GABAergic presynaptic inhibition (Ribeiro-da-Silva and De Koninck, 2008). A direct termination of non-peptidergic afferents on lamina I cells would bypass such modulation. Since non-peptidergic afferents have a more extensive distribution in the epidermis than peptidergic afferents (Taylor et al., 2009), show larger and more sustained responses to capsaicin than peptidergic afferents (Liu et al., 2004) and signal mainly via glutamate without the presence of neuropeptide co-transmitters, the activation of lamina I projection neurons by these non-peptidergic afferents would result in a different signal transmitted than when activated by the peptidergic afferents.

Although the focus of this study was mainly on NK-1r-positive projection neurons in lamina I, a previous study has demonstrated that the non-peptidergic afferents contact the NK-1r-positive superficial dorsal dendrites of lamina III-IV neurons (Sakamoto et al., 1999). These results together with our findings support the idea that non-peptidergic afferents transmit information supraspinally via spinal dorsal horn NK-1r-positive projection neurons.

## 4.5 Conclusion

These results expand our previous knowledge concerning the termination in dorsal horn of non-pepdergic primary afferents by providing evidence that they terminate directly on lamina I neurons expressing the NK-1r.

## 4.6 Materials & Methods

The guidelines of the Canadian Council on Animal Care for the care and use of experimental animals and of the International Association for the Study of Pain were followed in all the experiments. Furthermore, all studies were previously approved by the McGill University Faculty of Medicine Animal Care Committee.

A total of 26 male Sprague Dawley rats (Charles River, Quebec, Canada) weighing 225-250g were used for the experiments. The number of animals used and their suffering were kept to the minimum necessary for the conduction of the study. Animals were exposed to 12hr light/dark cycle and given food and water *ad libitum*. The cages were fitted with soft bedding and a plastic tube for an enriched environment and the animals were housed in cages of four.

### 4.6.1 Animal Preparation

*Injection of Tracers*

For the immunohistochemistry experiments requiring retrograde tracing, six animals were anesthetized using 5% isoflurane with oxygen and placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA) and stabilized with non-perforating ear bars. The coordinates for the parabrachial nucleus (rostral/caudal: -9.12; medial/lateral: -2.1; dorsal/ventral: -6.3) were calculated from the Paxinos & Watson Rat Brain Atlas (Paxinos and Watson, 2005) with Bregma as the reference point. A small hole was drilled through the skull at the target point, exposing the dura mater and a glass micropipette (Wiretrol II, Drummond Scientific Company, Broomall, PA) was lowered to the stereotaxic position of the parabrachial nucleus. Two µl of 1.0% solution of cholera toxin B (CTb) (List, Campbell, CA) were slowly injected into the parabrachial nucleus over a period of 20 minutes, followed by a waiting period of 10 minute before the micropipette was retracted from its position to minimize leakage of the tracer. CTb was injected seven days prior to sacrificing the animals.

4.6.2 Animal Perfusion

For immunohistochemistry, eighteen animals were deeply anesthetized with 0.3ml/100g of body weight of Equithesin (6.5mg chloral hydrate and 3mg sodium pentobarbital i.p.). They were then perfused through the left cardiac ventricle with perfusion buffer (for composition see (Côté et al., 1993)) for one minute, followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB), pH 7.4, for 30 minutes. The spinal cord segments L4-L5, as well as the brains from animals injected with the retrograde tracer, were extracted and post-fixed for 1 or 2 hours, respectively, in the same fixative.

For examination under the electron microscope, eight animals were anesthetized as described above, and then perfused with perfusion buffer for one minute, followed by a 30 minutes perfusion with a mixture of 4% PFA and 15% picric acid in 0.1M PB. The spinal cord segments L4-L5 were taken out and post-fixed for one hour in the same fixative.

All specimens were immersed overnight in 30% sucrose in PB at 4°C for cryoprotection.

4.6.3 Tissue Processing

*Confocal microscopy*

The injection site at the level of the parabrachial nucleus was examined by cutting serial, 100µm-thick, coronal sections of the relevant brain region. The dorsal aspect of the L4-L5 spinal cord segment was cut into serial, 50µm-thick horizontal sections (n=10), 50µm-thick parasagittal sections (n=4) or 50µm-thick transverse sections (n=4). All sections were cut using a freezing sledge microtome (Leica, Richmond Hill, Ontario, Canada) and collected as free-floating in phosphate-buffered saline (PBS) with 0.2% Triton-X 100 (PBS+T). To block unspecific staining, all spinal cord sections were incubated, for one hour, in 10% normal donkey serum (NDS) (Jackson, West Grove, PA) in PBS+T at room temperature. Subsequently, the sections were placed in primary antibodies (or

conjugated lectin IB4 - see below) for 48 hours at 4$^{o}$C. We used a mixture of 2 or 4 primary antibodies (each raised in a different species), or IB4, in PBS+T containing 5% NDS. Next, the sections were washed in PBS+T and then incubated in species-specific secondary antibodies that were raised in donkey and conjugated to either AlexaFluor 488, AlexaFluor 405, Rhodamine RedX or biotin. The sections were incubated in 3 different cocktails: #1) rabbit anti-CGRP at a 1:200 dilution (Sigma, St Louis, MO) and lectin IB4 conjugated to AlexaFluor 568 at a 1:200 dilution (Molecular Probes); #2) rabbit anti-CGRP and guinea pig anti-P2X3 at a 1:25,000 dilution (Neuromics, Edina, MN); #3) goat anti-CTb at a 1:5000 dilution (List Biological), rabbit anti-NK-1r at a 1:10000 dilution (Sigma, St Louis, MO), guinea pig anti-CGRP at a 1:8000 dilution (Peninsula, San Carlos, CA) and lectin IB4 conjugated to AlexaFluor 647 at a 1:200 dilution (Molecular Probes). All the sections were washed with PBS+T and then (for #1) incubated for 2 hours at room temperature with donkey anti-rabbit AlexaFluor 488; (for #2) incubated for 90 minutes in a biotin conjugated donkey anti-guinea pig IgG (Jackson Immunoresearch, West Grove, PA, 1:200). Further signal amplification for P2X3 signaling was achieved by treating the sections with 1 hour incubation in an avidin-biotin (A+B) complex (Vectastain Elite ABC kit, Vector Laboratories) followed by tyramide (Perkin-Elmer, Norwalk, CT, 1:75) for 7 minutes. Sections were then incubated in streptavidin conjugated to AlexaFluor 568 (Molecular Probes, Eugene, OR, 1:200) and donkey anti-rabbit AlexaFluor 488; or (for #3) incubated for 2 hours at room temperature with secondary antibodies: donkey anti-goat Rhodamine Red X, donkey anti-rabbit AlexaFluor 488, and donkey anti-guinea pig AlexaFluor 405. Finally, sections were washed with PBS, mounted on gelatin-subbed slides and coverslipped with an anti-fading mounting medium (Aqua Polymount; Polysciences, Warrington, PA). Slides were stored at -4°C until further processing.

To evaluate the injection site in the parabrachial nucleus, brainstem sections were incubated with anti-CTb antibody followed by a biotinylated donkey anti-goat IgG and streptavidin conjugated to AlexaFluor 568. They were then mounted and coverslipped as described above.

*Electron Microscopy*

Spinal cord specimens from the L4-L5 region were freeze-thawed in liquid nitrogen for 30 seconds, then cut into 50μm-thick transverse sections on a Vibratome (TPI, St. Louis, MO, USA) and collected as free-floating in PBS. To increase further antibody penetration, sections were incubated for 15 minutes in PBS+T, but all further incubations were carried out in PBS without Triton. Following a short PBS wash, the sections were incubated with 50% ethanol for 30 minutes. They were then incubated for 30 minutes in 0.5% BSA (bovine serum albumin) and subsequently incubated for one hour in 10% NDS. Sections were incubated for 48 hours, at 4°C, either with a primary antibody mixture of guinea pig anti-P2X3 at dilution 1:200,000 and rabbit anti-NK-1r at dilution 1:4000, or the guinea-pig anti-P2X3 antibody (at the same dilution) alone, in 5% NDS and 0.1% BSA.

Sections were then washed several times with PBS and then incubated for 90 minutes in a biotinylated donkey anti-guinea pig IgG antibody at dilution of 1:1000 in PBS and processed for tyramide amplification as described above. Afterwards, the sections were incubated, for one hour, in A+B enzyme complex. Labeling for the P2X3 antibodies was revealed following the incubation of the section with intensified DAB (3', 3'diaminobenzidine with 1% cobalt chloride and 1% nickel ammonium sulfate) to which 1% hydrogen peroxide was added (Ribeiro-da-Silva et al., 1993). The reaction was stopped by two washes in PBS. Sections processed for P2X3 staining were then osmicated (see below). Sections previously incubated in two primary antibodies (anti-P2X3 and anti-NK-1r) were placed for 10 minutes in washing buffer (0.8% BSA and 0.5% fish gelatin in PBS; pH 7.4) and then incubated overnight at 4°C with anti-rabbit IgG conjugated to gold particles (1.4nm diameter, Nanoprobes, Yaphank, NY) in washing buffer, at dilution of 1:200. The following day, all sections are washed for 3 minutes in washing buffer, rinsed for 3 minutes in PBS, and then immersed for 10 minutes in 1% glutaraldehyde in PBS. The sections were washed with de-ionized water for 2 minutes and then incubated for 8 minutes with an HQ silver enhancement

reagent following manufacturer's instructions (Nanoprobes, Yaphank, NY). The sections were then thoroughly rinsed with de-ionized water and then washed with 0.1M PB.

The sections were then post-fixed in 1% osmium tetroxide in PB for 1 hour at room temperature and, subsequently, dehydrated in ascending concentrations of ethanol and propylene oxide, followed by flat-embedding in Epon (Ribeiro-da-Silva et al., 1993). The Epon-embedded sections were observed at low power with a light microscope and selected areas were trimmed and re-embedded in Epon blocks. The Epon blocks were then trimmed, cut into ultrathin sections (60 nm) on a Reichert-Jung ultramicrotome (Nussloch, Germany) using a diamond knife and placed onto formvar-coated one-slot grids. Finally, sections were counterstained with uranyl acetate and lead citrate and examined using a Philips/FEI CM120 electron microscope equipped with a digital camera.

### 4.6.4 Image acquisition and quantification

Immunofluorescence sections were examined using a Zeiss LSM 510 confocal scanning laser microscope, equipped with Argon, HeNe1 and HeNe2 lasers, plus a Titanium-Sapphire multiphoton laser (MIRA 900F pumped by a Verdi-V5, Coherent Canada, Mississauga, ON, Canada). W e used a multi-track scanning method and appropriate filters, for the separate detections of AlexaFluor 488, streptavidin conjugated to AlexaFluor 568 or Rhodamine Red X, IB4-conjugated to AlexaFluor 647 and/or AlexaFluor 405, respectively. Images used for quantification represent serial optical sections obtained along the z-axis (z-stacks), using a 63X plan-apochromatic oil-immersion objective. Furthermore, for an unbiased representation of the images taken, all the parameters of laser power, pinhole size and image detection were kept constant for all samples. The images obtained were converted to TIFF files. Our criteria of identification and quantification of lamina I neurons have been described extensively in previous publications from our laboratory (Almarestani et al., 2009) (see also Results section). To calculate the density of IB4-positive boutons per unit length of neuronal membrane, the length of membrane in the soma and proximal dendrites

was measured with the help of an MCID Elite Image Analysis System (Imaging Research, St.Catharines, ON, Canada) and the number of appositions from IB4-positive boutons onto it was counted. This was done on alternate optical sections from the z-stack, to avoid counting the same boutons twice.

## 4.7 Acknowledgements

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# Chapter 5

General Discussion

## 5.1 Overview

In this thesis, we presented experimental work that expands on our current understanding of spinal dorsal horn pain-related circuitry and identifies changes that occur in the spinal cord following a nerve lesion. These changes may play a role in the development and maintenance of chronic neuropathic pain.

In the second chapter of this thesis, we described the phenotypic changes in the projection neurons of the spinal dorsal horn that occur following a peripheral nerve lesion. We described a *de novo* expression of NK-1r on the pyramidal lamina I projection neurons following a neuropathic lesion, which was associated with a marked increase of SP innervation on those cells. Furthermore, we demonstrated that after lesion pyramidal neurons became responsive to noxious stimulation, as they internalized the NK-1r following an intradermal injection of capsaicin into the hind paw. In the third chapter, we concluded that the loss of the non-peptidergic afferents alone was not sufficient to cause a phenotypic alteration in the neuronal populations of the spinal dorsal horn, since there were neither behavioral changes in response to acute stimuli nor changes in NK-1r expression following the cytotoxic ablation of the non-peptidergic population of nociceptive fibers. This observation would suggest that the pain-related dorsal horn circuitry comprises compensatory mechanisms that safeguard against the disruption of the circuitry. Finally, in the fourth chapter, we illustrated the presence of axonal boutons from non-peptidergic fibers in lamina I of the spinal dorsal horn, innervating all three populations of the lamina I projection neurons. Although this layer previously has been thought to be devoid of non-peptidergic innervation, our results show otherwise and suggest an added level of complexity in the processing of pain-related signals in the spinal dorsal horn.

In this chapter, we will discuss how these new findings corroborate with the currently available literature on pain mechanisms. Furthermore, we will outline several future experiments that may be interesting to this line of research.

### 5.1.1 Involvement of Lamina I pyramidal cells in thermoregulation

In chapter 2, we mentioned evidence from the literature that pyramidal cells normally are non-nociceptive and respond only to innocuous cooling (Han et al., 1998). However, we also presented evidence that, as of 10 days following a neuropathic lesion, pyramidal cells start to express NK-1r and develop an abundant innervation by peptidergic afferents. These observations are similar to those observed in our lab in an arthritis model, in which similar alterations were observed in pyramidal neurons starting only at a time point in which obvious joint damage was observed (Almarestani et al., 2009). The internalization of NK-1r in pyramidal cells following a noxious stimulus would suggest that these cells become nociceptive. However, such a conclusion is not legitimate based only on the data we have, as a response to noxious stimulation does not imply that the message forwarded to the brain by these cells is a nociceptive one. Indeed, we advanced an alternative explanation suggesting a role for these changes in thermoregulation-based homeostatic mechanisms. In this discussion we decided to expand further on this latter concept.

There is evidence that lamina I pyramidal neurons may be implicated in the thermoregulatory system, inducing defensive responses such as shivering, non-shivering thermogenesis and cutaneous vasoconstriction, in response to a drop in skin temperature before it affects core body temperature. The observation in Chapter 4 that in normal animals, lamina I pyramidal neurons are directly innervated by non-peptidergic afferents reinforces the concept that these afferents are involved in thermoregulation as well (see below).

*5.1.1-1 Non-peptidergic afferents and thermoregulation*

The transient receptor potential (TRP) melastatin-8 (TRPM8) channel is one of the temperature sensitive channels expressed on primary sensory neurons (Latorre et al., 2011; Lumpkin and Caterina, 2007). TRPM8 is implicated in innocuous cooling, as it is activated by moderate cooling of the ambient temperature to temperatures between 22-27°C and application of exogenous "cooling-mimetic" compounds such as menthol (McKemy et al., 2002; Peier et al., 2002). Recently TRPM8 was implicated in controlling cold avoidance

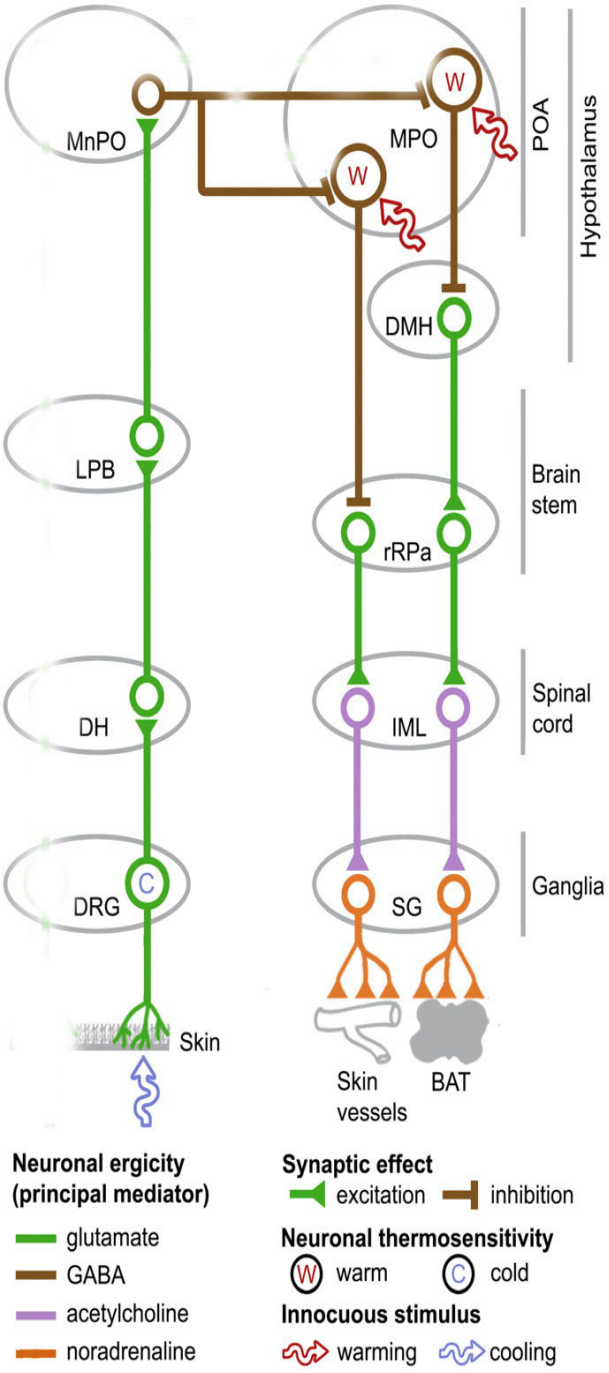


**Figure 1 –** Schematic of the neural pathways activated in response to innocuous thermal stimulation. DH: dorsal horn; LPB: lateral parabrachial nucleus; IML: intermediolateral column; SG: sympathetic ganglia. From (Romanovsky et al., 2009).

behavior, tail-skin vasoconstriction and thermogenesis (Almeida et al., 2012). While some studies demonstrated the expression of TRPM8 exclusively by non-peptidergic afferents (Dhaka et al., 2008; Peier et al., 2002; Story et al., 2003), others showed the presence of TRPM8 channels in both C fiber populations (Babes et al., 2004; Okazawa et al., 2004; Takashima et al., 2007; Xing et al., 2006).

### *5.1.1-2 Pyramidal lamina I projection neurons and thermoregulation*

As mentioned in Chapter 2, lamina I pyramidal neurons have been identified as COLD neurons and are implicated in the transmission of innocuous cooling signals to supraspinal brain structures including the lateral Pb (Craig and Bushnell, 1994; Craig and Kniffki, 1985; Dostrovsky and Craig, 1996; Han et al., 1998). Indeed, it is known that, in

addition to its previously described role in nociception, the lateral Pb plays a role in thermoregulation (Kobayashi and Osaka, 2003; Nakamura and Morrison, 2008b). Pyramidal lamina I neurons, activated by thermosensory information from primary afferents, send glutamatergic projections to the lateral Pb. At this level, there is activation of third-order excitatory neurons which in turn activate GABA-ergic neurons in the median preoptic nucleus (MnPO) of the preoptic hypothalamic area (POA). The MnPO neurons then inhibit the warm-sensitive inhibitory neurons in the medial preoptic area (MPO) of the POA (Nakamura and Morrison, 2008a, b). The warm-sensitive inhibitory neurons in the MPO tonically suppress the sympathoexcitatory premotor neurons in the dorsomedial hypothalamus (DMH) and the rostral raphe pallidus nucleus (rRPa) (Morrison et al., 2008; Nakamura and Morrison, 2007; Rathner et al., 2008), therefore suppressing brown adipose tissue (BAT) thermogenesis and skin vasoconstriction (Osaka, 2004). Inhibiting the MPO tonic suppression results in increased BAT thermogenesis and skin vasoconstriction thus increasing core body temperature (Figure 1).

*5.1.1-3 Is there impaired thermoregulation following nerve injury?*

Chronic nerve injury was characterized, among other features, by a loss of primary afferents, including non-peptidergic afferents (Peleshok and Ribeiro-da-Silva, 2011) and by a phenotypic switch in pyramidal lamina I neurons, resulting in a *de novo* expression of NK-1r as described in the Chapter 2 of this thesis. Both, the non-peptidergic afferents and pyramidal lamina I neurons have been implicated in thermoregulation. Therefore, the changes that occur after peripheral nerve injury, in particular those we described in Chapter 2 may result in thermoregulatory alterations.

The abovementioned loss of primary sensory afferents may play a part in the thermoregulatory disturbances, such that a retraction of the primary sensory afferents may result in a decrease in the number of TRPM8 channels available to detect environmental cooling. Therefore, this loss of feed forward mechanism and dissociation from external changes in environmental temperatures would led to a

suppression of body core temperature defenses and inhibition of BAT thermogenesis. These changes, if unopposed, may result in the decrease in core body temperature, leading to hypothermia (Nakamura and Morrison, 2007, 2008b, 2010).

On the other hand, the *de novo* expression of NK-1r by pyramidal lamina I neurons and the much increased innervation by nociceptive afferents of these neurons following nerve injury, described in Chapter 2, might have the opposite effect. Therefore, these changes may represent an important compensatory mechanism to prevent hypothermia. Indeed, an enhanced thermoregulatory signal projected to the lateral Pb may result in an increased suppression of the MPO warm-sensitive neurons, therefore activating the sympathoexcitatory neurons in the DMH and rRPA and increasing non-shivering BAT thermogenesis and cutaneous vasoconstriction, therefore, leading to the increase of core body temperature (Morrison et al., 2012; Morrison et al., 2008; Nakamura and Morrison, 2008a). However, further investigations would be required to confirm this idea.

Although the changes in thermoregulation following peripheral nerve injury are still to be fully investigated, earlier work demonstrated a lowered threshold for the onset of shivering following nerve injury (Stoner, 1971). This would indicate that thermoregulatory defenses are activated at warmer temperatures, which would be in agreement with our concept that there is altered thermoregulation following peripheral nerve injury.

### 5.1.2 NK-1r antagonists and negative clinical trials as analgesics

Throughout this thesis, the NK-1r was used as a marker for nociceptive neurons to identify and examine changes in the pain-related circuitry, at the level of the spinal dorsal horn. Although these receptors have been found to be critical for development of pain-related sensitization (Hunt and Mantyh, 2001; Khasabov et al., 2002), clinical trials of NK-1r antagonists failed to demonstrate any analgesic efficacy.

It was argued that the clinical studies were bound to fail as the evidence for the role of SP in nociception was circumstantial (Hill, 2000). Therefore, if SP did not play a dominant role in nociception, we could not expect that the specific pharmacological blockade of its receptor would produce any substantial analgesia. It was also suggested that the nociceptive effects of SP release demonstrated in preclinical studies may have been a result of the release of CGRP or other neuropeptides that co-exist with SP. Finally, it was suggested that the behavior tests used to measure pain levels are rather a measure of animal distress, which in addition to pain incorporates other factors such as stress. Therefore, the observed alleviation of pain-related behavior following the administration of NK-1r antagonists in animals models may alternatively be due to a reduced response to stressful stimuli, which although significant in animal models, was not sufficient to result in clinical pain relief (Hill, 2000).

However, on the other hand, it was argued that the failed clinical studies do not justify ruling out the development of NK-1r antagonists for the treatment of chronic neuropathic pain, since some of the non-peptide NK-1r antagonist compounds employed in the published clinical trials were demonstrated to perform poorly and for short durations in the animal models tested, and to have poor penetration of the blood-brain barrier (Urban and Fox, 2000). Moreover, the number of published clinical trials available to the scientific community is limited, many of which were only presented in abstract form or as unpublished observations. Furthermore, several studies tested NK-1r antagonists in dental pain models, which were argued to be acute, rather than chronic pain models, which would explain the lack of efficacy in these trials. Finally, the most potent and effective NK-1r antagonist (SDZ-NKT-343; (2-nitrophenyl-carbamoyl-(S)-prolyl-(S)-3-(2-napthyl)alanyl-*N*-benzyl-*N*-methylamide) in animal models has yet to be tested clinically for its anti-nociceptive effects. Furthermore, a wider range of pain states are yet to be examined, which may prove helpful in determining the efficacy of NK-1r antagonists.

Another point to consider is that SP is not the main transmitter of the peptidergic afferents, but rather glutamate, with which it is co-localized (De Biasi

and Rustioni, 1988). This means that with an NK-1r antagonist we are blocking the effect of a co-transmitter or modulator, not of the main transmitter. This fact may suggest that monotherapy with NK-1r antagonists may not result in any clinical efficacy. To this effect, development of a poly-receptor targeting strategy, where several compounds acting on different receptor populations are tested for their synergistic effect, may prove to be successful in attenuating chronic neuropathic pain.

### 5.1.3 Other neurokinin receptors

As mentioned in the introduction, SP is a member of the tachykinin family, including NKA and NKB, which preferentially bind to the NK-2r and NK-3r, respectively (Maggi, 1995). Both NKA and NKB play a role in the nociceptive signaling at the level of the spinal dorsal horn, which will be further discussed. The relevance of this for the thesis is that interfering with the signaling of these two other tachykinins may prove in the future to be of clinical relevance.

#### *5.1.3-1 NKA and NK-2r*

NK-2r are mainly distributed in lamina I, IX and X of the spinal cord and expressed on astroglial leaflets, which infiltrate between and surround neuronal processes (Zerari et al., 1998). NKA released from the central terminals of primary sensory C fibers in response to peripheral noxious stimuli, is capable of diffusing over large distances therefore activating astroglial NK-2r (Duggan et al., 1988; Duggan et al., 1990; Hope et al., 1990). Activation of astroglial NK-2r results in the release of cytokines, prostaglandins and excitatory amino acids therefore activating surrounding neurons and modulating the transmission of nociceptive sensory information. The role of NK2-r and NKA in nociceptive signaling is suggested by the prolonged decrease in nociceptive threshold and increased spinal cord excitability following the intrathecal injection of NKA or selective NK-2r agonists (Couture et al., 1993; Cridland and Henry, 1986; Fleetwood-Walker et al., 1990; Laneuville et al., 1988).

*5.1.3-2 Effect of NKA and SP on NK-1r*

NKA and SP are colocalized and co-released from the central terminals of C fibers in the spinal dorsal horn (Dalsgaard et al., 1985; Hua et al., 1986; Hua et al., 1985; Ogawa et al., 1985) as a response to noxious stimuli (Duggan et al., 1988; Duggan et al., 1990; Hope et al., 1990). Two studies suggested that NKA and SP have comparable NK-1r affinity at similar concentrations in the dorsal horn (Hastrup and Schwartz, 1996; Trafton et al., 2001). Although most investigations reveal that SP has a higher affinity for the NK-1r (Ribeiro-da-Silva et al., 2000). Furthermore, the physiological effects of the two neurokinins differ since NKA is not as susceptible to endopeptidase degradation as SP (Nyberg et al., 1984; Theodorsson-Norheim et al., 1987), therefore while SP exerts short term effects on NK-1r available at a discrete and focused area at the site of release, NKA exerts a more prolonged effect that is distributed over a greater rostrocaudal distance (Trafton et al., 2001).

Moreover, the two neurokinins bind distinct sites of the NK-1r, such that SP increases the levels of both cAMP and IP3, while NKA only affects IP3 levels (Sagan et al., 1996), therefore resulting in different signaling and gene transcription effects.

Therefore, it is important to keep in mind that NKA does not act only through its cognate receptor the NK-2r, which is virtually absent from the dorsal horn, but also through the NK-1r. Indeed, the NKA-mediated effect on the NK-1r activation and signaling should be considered when developing SP-NK-1r system-targeted therapeutic drugs, since sequestering SP, but not NKA, using selective anti-SP agents might result in more prolonged, widespread hyperalgesia mediated by the action of NKA on the then more accessible NK-1r.

*5.1.3-3 NKB and NK-3r*

NK-3r are found in lamina I-III of the superficial dorsal horn of the spinal cord (Mantyh et al., 1989; Yashpal et al., 1990), where they are expressed by two neuronal populations; GABA- and nitric oxide synthase-containing inhibitory interneurons and μ-opioid receptor-expressing excitatory interneurons (Ding et

al., 2002; Seybold et al., 1997). NKB, unlike NKA and SP, is not produced by primary sensory afferents (Moussaoui et al., 1992; Ogawa et al., 1985). NKB is found in intrinsic spinal neurons (Ogawa et al., 1985; Warden and Young, 1988), present throughout lamina I-III of the spinal dorsal horn (McLeod et al., 2000; Polgar et al., 2006; Too and Maggio, 1991). These NKB-expressing cells were never GABA-IR and contained the vesicular glutamate transporter (VGLUT2) (McLeod et al., 2000; Polgar et al., 2006), suggesting their involvement in glutamatergic synaptic transmission (Varoqui et al., 2002). NKB mediates its anti-nociceptive effects in the superficial dorsal horn via the activation of the NK-3r, which in turn causes the release of endogenous opioids, therefore limiting nociceptive transmission (Laneuville et al., 1988).

### 5.1.4 Glia and SP

The role of SP and NK-1r in neuropathic pain has been discussed from the perspective of their relationship with dorsal horn neuronal populations. However, the spinal glia are also affected by the action of SP and NK-1r. Since the neuroimmune activation of glia, including astrocytes and microglia, in the spinal cord is an important element in the transition of pain from acute to chronic (DeLeo et al., 2004; Marchand et al., 2005; Saab et al., 2008; Watkins and Maier, 2002), the relationship of glia and SP will be addressed for the sake of completeness and because exploiting it further in the future may be an interesting avenue in relation to neuropathic pain.

#### *5.1.4-1 Astrocytes*

Spinal astrocytes were demonstrated to express functional NK-1r (Marriott et al., 1991; Palma et al., 1997). SP released from nerve terminals activates astroglial NK-1r. Stimulation of the NK-1r triggers the release of $Ca^{2+}$ from inositol 1,4,5-triphosphate (IP3)-sensitive intracellular concentrations calcium stores through activated phospholipase C (PLC) pathway and induces the influx of extracellular $Ca^{2+}$ via canonical transient receptor potential-3 (TRPC3) channels. Together, these changes lead to the increase of intracellular $Ca^{2+}$ concentrations

and the consequential release of glutamate from astrocytes (Miyano et al., 2010), resulting in synaptic transmission. However, the levels of expression of NK-1r by astroglia are likely not high, as we did not observe any NK-1r immunostaining in cells with the morphology of astrocytes in our studies.

*5.1.4-2 Microglia*

Microglia express NK-1r, and the release of SP from nociceptive primary afferents has been shown to activate the NK-1r on these microglial cells (Rasley et al., 2002). The activated microglia would then increase their expression of membrane bound tumor necrosis factor alpha (mTNF-α). Unlike activation of toll-like receptor 4 (TLR4), NK-1r stimulation does not activate the TNF-α converting enzyme (TACE), therefore mTNF-α is not converted to the soluble form of TNF-α (sTNF-α). mTNF-α activates TNF-α receptor (TNF-αR) on neighboring, unstimulated microglial cells via direct cell-cell contact. Stimulation of TNF-αR results in an increase in OX-42 levels and induces the release of chemokine ligand 2 (CCL2) and other substances, which in turn activates adjacent spinal neurons and potentiate excitatory synaptic transmission in the spinal cord (Zhou et al., 2010) (Figure 2). We have never specifically looked at NK-1r in microglia, what should be done by a double labeling of NK-1r and a microglia marker. If there would be an upregulation of NK-1r in microglia in neuropathic pain, it could have implications in triggering further loss of GABAergic inhibition, besides the mechanism described above. It still needs to be shown whether the activation of NK-1r in microglia leads to the release of BDNF in neuropathic pain conditions, as has been shown for P2X4 receptor activation (Coull et al., 2005)

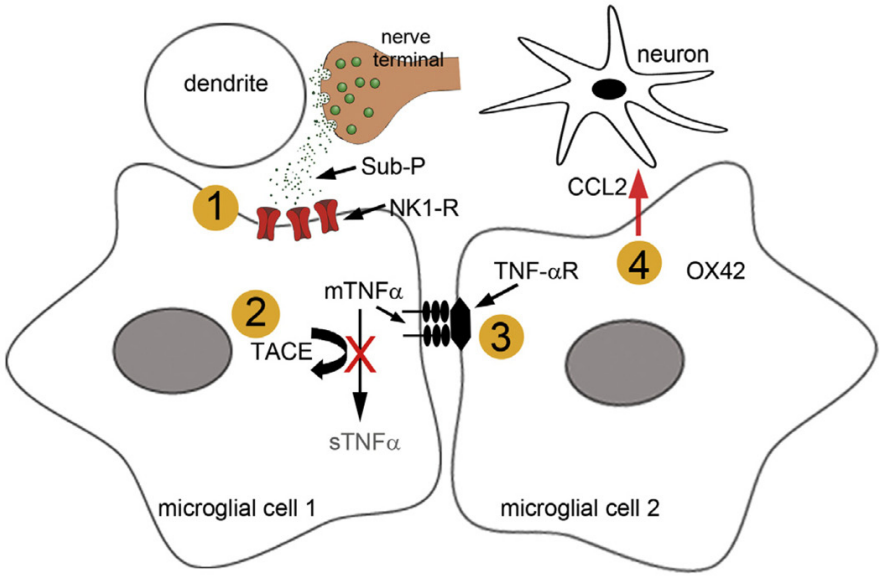


**Figure 2 –** Activation of spinal neuronal cells via microglial cell-cell signaling. From (Jasmin and Ohara, 2010).

5.1.5 Changes following loss of non-peptidergic afferents

A recent study from our lab has shown that non-peptidergic afferents in the spinal cord recover very slowly from an initial loss following a cuff lesion like the one we have used in Chapter 2 (Lorenzo et al., to be submitted). This loss may be associated with structural changes in the non-peptidergic afferent circuitry. These changes may include the persisting loss of E-cadherins, which are members of synaptically enriched transmembrane glycoproteins mediating adhesion (Geiger and Ayalon, 1992). E-cadherins are specifically associated with non-peptidergic afferents and play a critical role in the targeting of axonal terminations (Brock et al., 2004), therefore their loss could slow down the regrowth of the non-peptidergic fibers into their proper lamina area.

On the other hand, the majority of non-peptidergic afferents form connections with spinal interneurons, which have also been demonstrated to be lost following peripheral nerve injury (Meisner et al., 2010). Whether the loss of non-peptidergic afferents triggers trans-synaptic GABAergic interneuron loss in the spinal cord or not is still a matter of debate (Moore et al., 2002; Polgar et al., 2004; Scholz et al., 2005). However, a study has shown that an inhibitor of apoptosis prevented inhibitory interneuron cell loss and loss of inhibition in

lamina II following a neuropathic lesion (Scholz et al., 2005). If that is confirmed, the preventive administration of an anti-apoptotic agent could block the development of neuropathic pain. However, more important than the occurrence or not of inhibitory cell loss is what happens to the terminals of the GABAergic interneurons. Indeed, recovery of inhibition could come from the re-establishment of inhibitory synaptic circuitry through sprouting. Along this line, a study from our lab has shown that, following a cuff lesion, there was a loss of IB4-binding terminals in the area of distribution of the sciatic nerve, which was accompanied by a loss of terminals from GABAergic interneurons, as revealed by GAD immunostaining; interestingly, after an initial loss, the number of GAD+ terminals recovered substantially by 1.5 months post-lesion, accompanying a partial recovery of IB4 terminals and an improvement in pain-related behavior (Lorenzo et al., to be submitted). This finding would indicate that re-establishing inhibitory circuits is important to resolve neuropathic pain.

*5.1.5-1 IB4-saporin versus peripheral nerve injury*

Although a peripheral nerve injury and intra-sciatic injection of IB4-saporin both result in loss of the non-peptidergic afferents, peripheral nerve injury is associated with damage to all the primary sensory afferents in the injured nerve, release of inflammatory substances and activation of glia in the spinal cord. However, injection of IB4-saporin into the sciatic nerve results in the progressive loss of non-peptidergic afferents in a system with a minimal inflammatory component, which may allow time for the undamaged peptidergic afferents to compensate for the loss of the other population. In this regard, it might be interesting to check how much activation of glia the IB4-saporin model triggers, as this has never been investigated. However, we should predict that it will trigger less activation of glia than the cuff model of neuropathic pain.

5.1.6 Direct activation of lamina I projection neurons by non-peptidergic afferents

The two primary sensory C fiber populations differ in both their neurochemical composition and electrophysiological properties, as discussed

earlier (see Chapter 1). Therefore, the direct innervation of non-peptidergic afferents onto lamina I projection neurons described in chapter 4 of this thesis indicates that non-peptidergic afferents can activate monosynaptically lamina I projection neurons, besides a very likely role in the activation of these cells polysynaptically, as had been described earlier (see Chapter 4 for a discussion). This may be important because it represents a different form of monosynaptic activation when compared to that by peptidergic afferents. Not only is the direct non-peptidergic innervation lacking the neuromodulation by such neuropeptides as SP (Snider and McMahon, 1998), but the non-peptidergic DRG neurons exhibit higher activation thresholds and longer duration of action potentials than peptidergic DRG neurons, a difference attributed to the higher density of N-type $Ca^{2+}$ channel currents (Wu et al., 2004; Wu and Pan, 2004a), TTX-R $Na^{+}$ channel currents (Stucky and Lewin, 1999) and A-type voltage-gated $K^{+}$ channel currents (Vydyanathan et al., 2005). Therefore, although a higher activation threshold may require a stronger stimulus to be activated (suggesting non-peptidergic afferents may play a more significant role in chronic rather than acute pain), the longer action potential duration can allow for more calcium influx, resulting in an enhanced release of glutamate and other excitatory transmitters from the central terminals in the spinal dorsal horn (Scroggs and Fox, 1992) leading to a stronger activation of the projection neurons. However, the above is based mostly on speculation, and would need to be tested directly. Furthermore, the difference in the signal transmitted to the lamina I projection neurons by the non-peptidergic primary sensory afferents which are directly presynaptic to them and via interposed excitatory interneurons remains to be investigated by electrophysiological studies. Indeed, understanding the physiological role, and subsequent alterations following nerve injury, of the non-peptidergic primary sensory afferents which establish direct synapses onto lamina I projection neurons seems of particular relevance. Furthermore, comparing these to what we know of the function and changes of the peptidergic sensory afferents may aid in mapping a more detailed representation of the pain-related circuits in the dorsal horn of the spinal cord in normal conditions and in chronic pain.

## 5.2 Future Experiments

The results presented in this thesis provide a background for further investigations in the field. In the following text, we will discuss some experiments that may be interesting to pursue in the future.

### *5.2.1 Effects of opioid analgesics on NK-1r expression*

Chronic pain alters the psychological/ affective state (Costigan and Woolf, 2000; Melzack and Wall, 1965), in addition to the aberrations in sensory processing (Jensen et al., 2005), with anxiety and depression being the most prominent affective states linked to chronic pain (Frank et al., 1988; Rhudy and Meagher, 2000). Several studies have demonstrated the effectiveness of morphine as an anti-nociceptive and anxiolytic drug in animal models of neuropathic pain (Roeska et al., 2008; Wallace et al., 2008) and since the spino-parabrachial lamina I projection neurons constitute part of the sensory/discriminative and emotional/affective pathways of pain, it would be interesting to investigate the effect of morphine on the *de novo* expression of NK-1r on pyramidal neurons described in chapter 2. Since the *de novo* expression of NK-1r on pyramidal neurons may play a role in the development and maintenance of chronic pain, it would be interesting to investigate if the behavioral changes induced by morphine are associated with a reversal of NK-1r expression by pyramidal cells. Indeed, we think that the *de novo* expression of NK-1r by pyramidal neurons can be used as a marker for the maladaptive changes in the central nervous system and therefore, useful in the preclinical screening for future therapies targeting chronic pain. Similar experiments assessing the role of clinically used anti-depressants in neuropathic pain on preventing or reversing the NK-1r expression by pyramidal cells may also help understand the role that the affective/emotional pathway plays in development and maintenance of chronic pain following nerve injury.

### *5.2.2 Loss of non-peptidergic afferents in a neuropathic pain model*

A recent study from our lab reported that the selective loss of non-peptidergic afferents in the mental nerves (trigeminal system) resulted in further decrease in mechanical thresholds when coupled with a chronic constriction injury (Taylor and Ribeiro-da-Silva, 2012). However, we cannot extrapolate directly this data for the sciatic nerve model. Indeed, the sciatic nerve is a mixed nerve, composed of both sensory and motor fibers, while the mental nerve is a purely sensory nerve. Moreover, while a cuff lesion of the sciatic nerve results in the non-peptidergic afferents return to sham levels only by 16 weeks (Peleshock et al., to be submitted), the non-peptidergic afferents return within 4 weeks following a comparable injury in the mental nerve (Taylor and Ribeiro-da-Silva, 2011), which indicates an inherent difference between the two regions. This difference in regeneration of the non-peptidergic afferents may be due to the difference in the levels of GDNF available after nerve lesion, such that an injury to the sciatic nerve results in relatively low levels of GDNF (Leclere et al., 2007) compared with an injury to the mental nerve, where GDNF levels increased significantly by 4 weeks (Taylor and Ribeiro-da-Silva, 2011). Therefore, it will be interesting to study the thermal and mechanical behavioral changes and the changes in NK-1r expression on lamina I projection neurons and their primary afferent innervation in a model of chronic neuropathic pain following the injection of IB4-saporin into the sciatic nerve. In this study, a period of 14 days should elapse between the IB4-saporin injection and the induction of nerve injury, to ensure the complete loss of the non-peptidergic afferents prior to nerve damage, as it was done in the previous study of our lab (Taylor and Ribeiro-da-Silva, 2012).

*5.2.3 Retrograde tracing with adenoviruses*

To better map the complex neuronal networks in the spinal dorsal horn, a viral tracing technique has been developed and validated in a previous publication from our laboratory (Cordero-Erausquin et al., 2009). In this study, two replication-deficient adenoviruses (Ad) were used to retrogradely label neurons from the parabrachial nucleus. One virus encoded for the red fluorescent protein

(RFP), which retrogradely labeled in a Golgi-like manner the projection neurons. Indeed, the dendritic spines, distal dendrites and axons of these neurons were clearly labeled, a characteristic that exceeds the limitations of retrograde tracing with CTb, which only labels the cell body and proximal dendrites. The second virus encoded for both green fluorescent protein (GFP) and the non-toxic fragment of the tetanus toxin, TTC. The TTC allows for the transport of the adenovirus trans-synaptically, however, due to its impaired replication, the adenovirus did not label beyond the second order neurons. The Ad-GFP-TTC virus allowed for the complete labeling of the dendrites and axons of interneurons presynaptic to lamina I projection neurons. The use of this technique in association with the various markers for primary afferents (P2X3 and CGRP for non-peptidergic and peptidergic afferents, respectively) together with those used to identify interneuronal populations (GAD65 and VGluT2 for inhibitory and excitatory interneurons, respectively) will help unravel the synaptic relationship between the incoming primary afferents and neuronal populations of the spinal dorsal horn.

## 5.3 Summary

In this thesis, we provide novel experimental data and attempt to show how our results provide a further understanding of normal pain-related dorsal horn circuitry and of changes that occur following peripheral nerve injury.

We described how the *de novo* expression of functional NK-1r on pyramidal lamina I projections neurons following chronic nerve injury is part of complex pathophysiological changes. Such changes include alterations in glia and other transmitter systems and neuronal populations, which result in changes in nociception as well as thermoregulation.

We have also illustrated how the selective loss of non-peptidergic afferents as a consequence of an intra-sciatic IB4-saporin injection differs from the loss of non-peptidergic afferents following peripheral nerve injury. These differences may explain the absence of changes in pain-related behavior and phenotype of lamina I cells in the IB4-saporin model. Therefore, challenging the

IB4-saporin model with a peripheral nerve injury may provide data contributing to a better understanding of the role of the non-peptidergic afferents in pain-related circuitry.

Finally, we demonstrate the presence of *bona fide* synapses between non-peptidergic afferents and lamina I projection neurons, providing evidence that these lamina I neurons receive monosynaptic input from the peripheral structures innervated by these afferents. This may be of significance, as in contrast with the non-peptidergic afferents terminating as central elements in synaptic glomeruli, the primary afferent signal, from this novel monosynaptic pathway, is not modified presynaptically by the influence of GABAergic interneurons. However, further quantitative and experimental analyses are required to understand the significance of these connections under normal and pathophysiological states.

We hope that the results described in this thesis will help in giving a better picture of the complexity of the physiological pain-related circuitry in the dorsal horn of the spinal cord and provide some insight into the alterations that occur following chronic peripheral nerve injury. We also hope that what is described here may provide some clues for the development of new therapeutic targets to alleviate chronic neuropathic pain.

## 5.4 Contributions to Original Knowledge

In this thesis we describe some novel observations that in our view make original contributions to knowledge. These are summarized below.

1. De novo expression of neurokinin-1 receptors by spinoparabrachial lamina I pyramidal neurons following a neuropathic lesion. Abeer W. Saeed and Alfredo Ribeiro-da-Silva, Journal of Comparative Neurology, *under revision*

   This work describes the changes that occur at the level of lamina I projection neurons following nerve lesion. We show for the first time a *de novo* expression of NK-1r by pyramidal neurons in a neuropathic pain model.

There is evidence in the literature that pyramidal neurons under normal physiological conditions are non-nociceptive, hence do not express the NK-1r. We were also the first to show that the NK-1r expressed *de novo* on pyramidal neurons are functional, by demonstrating their internalization following an intradermal injection of capsaicin, a noxious stimulus. We also demonstrate quantitatively for the first time that pyramidal neurons, normally scarcely innervated by SP, receive abundant SP innervation after the lesion. Taken together, these results suggest that the phenotypic switch of pyramidal neurons and their increased SP innervation may play a role in chronic neuropathic pain.

2. Limited changes in spinal lamina I dorsal horn neurons following the cytotoxic ablation of non-peptidergic C fibers. Abeer W. Saeed and Alfredo Ribeiro-da-Silva, Journal of Comparative Neurology, *submitted*

This work provided evidence that the cytotoxic ablation of the non-peptidergic population of nociceptive primary afferents did not cause any phenotypic switch in pyramidal neurons, although there was a significantly increased expression of NK-1r by the other two populations of lamina I neurons that normally express the receptor. This indicates that under physiological conditions, in the absence of a nerve lesion, there are compensatory mechanisms in the spinal dorsal horn capable of balancing out the loss of the non-peptidergic fibers, without the need of a phenotypic switch in lamina I pyramidal neurons. From this work, we may propose that the development of chronic pain following a nerve lesion is dependent on factors other than the disruption of primary afferent populations.

3. Non-peptidergic primary afferents are presynaptic to lamina I projection neurons in rat spinal cord. Abeer W. Saeed and Alfredo Ribeiro-da-Silva, Molecular Pain, *submitted*

In this work, we provide the first description of IB4-positive and P2X3-IR non-peptidergic primary afferent boutons in lamina I of the rat spinal dorsal horn. We demonstrated, using the confocal microscopy, that these terminals are distinct from their CGRP-IR peptidergic counterpart and that they innervate directly fusiform, multipolar and pyramidal lamina I projection neurons. We also described, using the electron microscope, the presence of *bona fide* synapses between the non-peptidergic terminals, as detected by P2X3 immunoreactivity, and NK-1r-positive neuronal profiles in lamina I. Our observations suggest the presence of an unmodulated pathway for signaling monosynaptically via the non-peptidergic afferents contacting lamina I projection neurons which express the NK-1r.

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