A behavioural and morphological analysis of compromised primary afferent C-fibre input to the superficial dorsal horn of the rat spinal cord

By
Andrea Lee Bailey

A thesis submitted to the Faculty of Graduate Studies and Research of McGill University in partial fulfillment of the requirements for the Degree of Doctor of Philosophy

May, 2009

Department of Pharmacology and Therapeutics
McGill University
Montreal, Quebec

This thesis is dedicated to my Mother and Father
Words cannot begin to construe my deepest appreciation for their unwavering support, motivation, guidance, love and pride. They are not only my best friends, but also my role models for strength, integrity and perseverance.

This thesis is also dedicated to Kirk Lee Foon
My life and my love,
My best friend and soul mate.

I owe part of this thesis to my Brother
who has shown me the true value of being a good person and for that I shall be eternally grateful.

Abstract

Over 27 million Canadians suffer from chronic pain, interfering with the capacity to perform daily activities. At present, therapeutic strategies offer moderate pain relief, however are accompanied by side effects such as sedation, tolerance and the risk of dependence. A deeper understanding of the fundamental, latent pathological processes of the genesis and maintenance of specific chronic pain conditions is crucial in designing not only the appropriate pharmacopoeia but also the unique clinical strategies each patient deserves.

Neuropathic pain, a chronic pain syndrome, is caused by direct or indirect damage of the central nervous system (central neuropathic pain) or to the primary afferent fibres (PAF) in the case of most peripheral neuropathic pain (IASP association on taxonomy). Comprised of a heterogeneous population of axons derived from cells residing in the dorsal root ganglion (DRG), the small diameter C-fibres (C-PAF) are of great interest as they are responsible for the transduction of pain-related signals arising from the periphery. The adult C-fibre neuronal population is comprised of a heterogeneous group of cells distinguished by their neuropeptide expression profile and their nutritive source (growth factors). The peptidergic population, as the name implies, consists of neurons which express high levels of neuropeptides such as substance P (sP) and calcitonin gene-related peptide (CGRP). Nutritive support for the peptidergic class of neurons is provided by nerve growth factor (NGF). The non-peptidergic class of C-fibre neurons expressing cell surface glycoproteins which bind to the plant lectin, Isolectin B_4 (IB4) is characterized by a paucity of neuropeptides. Growth factor support is supplied by glial-derived neurotrophic factor (GDNF). Non-peptidergic afferents are known to play a role in nociceptive transmission; however our understanding of the nature of this information and how it is processed by these afferents in the spinal cord is limited.

The main objective of this dissertation focused on examining the morphological, neurochemical and behavioural consequences of compromised C-PAF input to the superficial dorsal horn. Our approach was two-fold: examining the effects of a constriction injury of the common sciatic nerve and the more

specific approach of selectively ablating the IB4-binding sub-population of C-PAFs (intra-sciatic injection of IB4-Saporin).

The peripheral nerve lesion study was aimed at examining the time-related effects of a constriction neuropathy on the peptidergic and non-peptidergic C-PAFs using neurochemical markers for each neuronal population. Observations of IB4-binding PAF terminals in lamina II revealed the transient loss of IB4-binding resultant from the degeneration of C1a synaptic glomeruli (Bailey, AL and Ribeiro-da-Silva, A., 2006). In addition, we evaluated paw withdrawal thresholds in these animals to peripherally applied noxious heat and innocuous mechanical and cold stimuli. Specifically, paw withdrawal latencies to noxious heat stimuli were significantly reduced in neuropathic animals throughout the 3 week time course. Sensitivity to innocuous cold stimuli (acetone spray) was also significantly altered in neuropathic animals. Furthermore, application of acetone to the uninjured paw elicited a withdrawal response in the neuropathic paw denoted as a crossed withdrawal reflex. Paw withdrawal thresholds to innocuous and noxious mechanical stimulation were significantly reduced in neuropathic animals. Probative analysis of CGRP-immunoreactivity in the initial 3 weeks following cuff application revealed an absence in any detectable change in the density of labeled varicosities within the superficial spinal dorsal horn (L4-L5) (Bailey, A.L., Ribeiro-da-Silva, A., 2006). Ultrastructural examination of glomerular and non-glomerular peptidergic boutons at the L4-L5 lumbar level showed no apparent signs of central terminal atrophy. In contrast, there was a significant decline in IB4-labelled non-peptidergic terminals perceptible by the 5th post-operative day which peaked by day 10. By the 15th day following nerve lesion, IB4-binding was restored. Ultrastructural analysis confirmed the presence of degenerated varicosities representing the core element of type Ia synaptic glomeruli, with signs of regeneration apparent at day 15 post-operative.

The intrasciatic injection of IB4-Saporin resulted in the significant loss of IB4-binding neurons in the DRG as well as their central terminals within the superficial dorsal horn. Examination of neurochemical markers for the peptidergic C-fibre population and their postsynaptic contacts within lamina I of the dorsal horn revealed that sP and CGRP immunoreactivity were differentially affected by

the loss of IB4-binding afferent input; sP-immunoreactivity declined within the first 2 weeks following lesion formation however CGRP-immunoreactivity remained unchanged for several weeks, manifesting a decline in levels only by the 7^{th} week post-operative. Furthermore, IB4-Saporin treatment resulted in a marked increase in NK1-receptor immunoreactivity perceptible at the 2 week time point following lesioning persisting until the end of the observation period at 7 weeks. The differential decline in neuropeptide levels and the increase in NK1-receptor expression are indicative of compensatory mechanisms engaged pursuant to the permanent loss of IB4-binding terminals in the dorsal horn. IB4-Saporin treatment did not precipitate a deficit in paw withdrawal responses to innocuous and nocuous mechanical and thermal stimuli. Furthermore, the loss of IB4-binding C-fibres had no effect on inflammatory hyperalgesia and allodynia. Similarly, IB4-Saporin treatment did not affect nociceptive behaviours in all phases of the formalin test. However, in a model of noxious heat-provoked conditioned place avoidance, the loss of IB4-binding afferent input to the SG resulted in a deficit in avoidance learning lending support to the hypothesis that postsynaptic neurons in the SG communicate with projection neurons in the deep dorsal horn in contact with affective-motivational regions of the brain.

Résumé

La douleur chronique affecte plus de 27 millions de Canadiens et représente un obstacle dans la poursuite de leurs activités quotidiennes. Les stratégies thérapeutiques actuelles offrent un soulagement modéré de la douleur et s'accompagnent d'effets secondaires tels la sédation et les risques de tolérance et de dépendance. Une plus grande compréhension des processus pathologiques fondamentaux et latents sous-tendant la formation et le maintien des douleurs chroniques spécifiques est essentielle pour l'élaboration d'agents pharmacologiques plus appropriés et pour la conception de traitements cliniques plus ciblés aux besoins des patients.

La douleur neuropathique, un syndrome de douleur chronique, est causée par des dommages directs ou indirects des fibres afférentes primaires. Composées d'une population hétérogène d'axones dont les corps cellulaires sont situés dans les ganglions rachidiens de la racine postérieure (DRG [dorsal root ganglion]), les fibres nerveuses de type C à petits diamètres sont d'un intérêt particulier étant responsables de la transmission des influx douloureux provenant de la périphérie. La population de fibres nerveuses C chez l'adulte est constituée d'un groupe hétérogène de cellules se distinguant par leur profil d'expression de neuropeptides et par leur source neurotrophique (facteurs de croissance). La population neuronale peptidergique, comme son nom implique, se compose de neurones exprimant des niveaux élevés de neuropeptides comme la substance P (sP) et le peptide lié au gène de la calcitonine (CGRP [calcitonin gene-related peptide]); son soutien nutritif est assuré par le facteur de croissance des cellules nerveuses (NGF [nerve growth factor]). La population neuronale non peptidergique exprimant des glycoprotéines de surface ayant la capacité de se lier à la lectine de plante Isolectine B_4 (IB4) se caractérise par une présence plus faible de neuropeptides; son soutien nutritif est assuré par le facteur neurotrophique dérivé des cellules gliales (GDNF [glial-derived neurotrophic factor]). Bien que le rôle joué par les fibres afférentes non peptidergiques dans la transmission nociceptive soit connu, notre compréhension de la nature de l'information et des mécanismes impliqués

dans le traitement de celle-ci par ces fibres afférentes dans la moelle épinière restent limitée.

Dans cette thèse, notre but premier était d'étudier les conséquences morphologiques, neurochimiques et comportementales des dommages effectués au niveau des fibres afférentes primaires de type C (C-PAFs [C-primary afferent fibres]) de la corne postérieure superficielle. Cet effort fut mené de deux façons: soit par des lésions produites par constriction du nerf sciatique, soit par suppression sélective, induite par injection intra-sciatique d'IB4-Saporin, de la sous-population de C-PAFs se liant à IB4.

La réalisation de lésions nerveuses périphériques visait à comprendre l'effet relatif du temps sur les C-PAFs peptidergiques et non peptidergiques consécutif à des neuropathies provoquées par constriction, et ce, au moyen des marqueurs neurochimiques associés à chacune de ces populations neuronales**.** L'observation des terminaisons des PAFs se liant à IB4 dans la lame II révélait la perte passagère du marquage d'IB4 résultant de la dégénérescence des glomérules synaptiques C1a (Bailey, AL and Ribeiro-da-Silva, A., 2006). De plus, des tests mesurant les temps de retrait de patte en réponse à des stimulations mécaniques, thermiques chaudes et froides appliquées à la périphérie furent effectués chez ces animaux. Spécifiquement, les seuils de retrait étaient plus faibles lors des tests de nociception thermique effectués au cours des trois semaines d'étude. La réactivité aux stimulations froides (produites par vaporisation d'acétone) était également significativement altérée chez les animaux neuropathiques. En outre, l'application d'acétone sur la patte non lésée produisait une réponse de retrait de la patte lésée, dénotant un réflexe de retrait croisé. Les seuils de retraits de patte vis-à-vis les stimulations mécaniques étaient significativement réduits chez les animaux neuropathiques. L'analyse de l'immunoréactivité de CGRP durant les trois premières semaines suivant l'enserrement du nerf dans un manchon ne montrait aucun changement de densité du marquage de varicosités dans la corne postérieure superficielle (L4-L5) (Bailey, A.L., Ribeiro-da-Silva, A., 2006). Sur le plan ultrastructural, l'examen des boutons peptidergiques glomérulaires et non glomérulaires au niveau lombaire L4-L5 ne révélait aucun signe apparent d'atrophie des terminaisons. En revanche, dans les terminaisons non

peptidergiques se liant à IB4 le marquage d'IB4 subissait une baisse passagère cinq jours après la réalisation de la lésion, atteignant un creux dix jours après. Le marquage d'IB4 se rétablissait à partir du jour 15. L'analyse ultrastructurale confirmait la présence de varicosités dégénérées représentant l'élément central des glomérules synaptiques de type Ia dont les signes de régénération devenaient évidents à partir du quinzième jour post-opératoire.

L'injection intra-sciatique d'IB4-Saporin provoquait une perte significative des neurones se liant à IB4 tant dans les DRG que dans les terminaisons centrales de la corne postérieure superficielle. Comme le révélait l'examen des marqueurs neurochimiques associés aux populations peptidergiques des fibres C et à leurs contacts post-synaptiques dans la lame I de la corne postérieure, l'immunoréactivité de sP suivant la rupture des transmissions afférentes se liant à IB4 était différente de l'immunoréactivité de CGRP; celle de sP diminuait au cours des deux premières semaines suivant la réalisation de la lésion alors que celle de CGRP restait stable durant plusieurs semaines pour ne décliner qu'à partir de la semaine 7. Par ailleurs, le traitement à l'IB4-Saporin causait une nette augmentation de l'immunoréactivité des récepteurs NK1 observable à partir de la deuxième semaine suivant la lésion et persistant jusqu'à la fin de la période d'observation de sept semaines. La baisse des niveaux de neuropeptides combinée à la hausse des niveaux de récepteurs NK1 signalaient l'existence de mécanismes compensatoires découlant de la disparition permanente du marquage d'IB4 dans les terminaisons de la corne postérieure. Le traitement à l'IB4-Saporin n'entraînait aucune baisse des seuils nociceptifs vis-à-vis des stimulations thermiques et mécaniques. De plus, la disparition des fibres C se liant à IB4 n'affectait ni l'hyperalgésie inflammatoire, ni l'allodynie. La sensibilité nociceptive non plus n'était pas modifiée par l'injection d'IB4-Saporin, et ce, à quelque phase que ce soit du test au formol. Cependant, les effets de baisse de performance dans l'apprentissage d'évitement, consécutifs à la rupture des transmissions afférentes se liant à IB4 vers la substantia gelatinosa (SG) et enregistrés lors d'expériences d'évitement de place conditionné induit par la chaleur, tendent à soutenir l'hypothèse postulant le relai d'informations à partir des neurones post-synaptiques dans la SG qui projettent dans les couches

profondes des cornes postérieures de la moelle d'où grimpe l'influx douloureux vers les régions du cerveau impliquées dans les états motivationnels et affectifs.

Table of Contents

Index of Figures and Tables

Chapter I: General Introduction

Chapter II

Chapter III

Chapter IV

List of Abbreviations

AMP: adenosine monophosphate
AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AP: Acid phosphatase
ATP: adenosine triphosphate
Aβ: A-Beta primary afferent neurons
Aδ: A-Delta primary afferent neurons
BDNF: brain-derived neurotrophic factor
BNST: bed nucleus of the stria terminalis
CCI: chronic constriction injury of the sciatic nerve
CGRP: Calcitonin gene related peptide
C_{Ia}: central bouton of type Ia synaptic glomerulus
CI_b: central bouton of type Ib synaptic glomerulus
CII: central bouton of type II synaptic glomerulus
CIPA: Congenital insensitivity to pain with anhydrosis
CNS: central nervous system.
COLD: Lamina I neurons responsive to innocuous cooling.
C-Ret: GDNF receptor transduction domain
CRPS: Complex regional pain syndrome (I and II).
CVLM: caudal ventrolateral medulla
DAMGO:[D-Ala^2-*N*-Me-Phe^4, Gly^5-ol]
DCV: Dense core vesicles
DRG: dorsal root ganglion.
DRt: dorsal reticular nucleus
EM: Electron microscope (microscopy)
EM-1/EM-2: endomorphin 1 and 2 receptors
ENK: Enkephalin
FRAP: Fluoride resistant acid phosphatase
GABA: Gamma-aminobutyric acid
GAD: Glutamate acid decarboxylase
GDNF: Glial derived neurotrophic factor
GFR: GDNF receptor ligand binding domain
GTP: Guanine Triphosphate
HPC: Neurons responsive to noxious heat and pinch and noxious and innocuous cooling.
HTM: High threshold mechanoreceptors
IB4: Isolectin B_4 *Griffonia Simplicifolia*
IB4-SAP: IB4-Saporin
IR: Immunoreactive
LGV: Large granular vesicle
LI: Lamina I of the superficial dorsal horn, referred to as the marginal zone
LIIi: Lamina II inner of the superficial dorsal horn
LIII: Lamina III of the superficial dorsal horn, referred to as the nucleus proprius
LIIo: Lamina II outer of the superficial dorsal horn
LIV: Lamina IV of the superficial dorsal horn
LTM: Low threshold mechanoreceptors
LV: Lamina V of the superficial dorsal horn
MRF: medullary reticular formation,
NGF: Nerve growth factor
NGS: Normal Goat Serum
NHS: Normal Horse Serum
NK1: Neurokinin 1 receptor
NMDA: N-methyl D-aspartate
NS: nociceptive specific neurons.
NT3: Neurotrophin 3

OCT: Optimal cutting temperature embedding medium for frozen tissue specimens
$P2X_3$: Purinergic P2X receptor 3
PAF: primary afferent fiber.
PAG: periaqueductal grey matter
PB: 0.2 M phosphate buffer
PBN: parabrachial nucleus
PBS: 0.2M Phosphate buffered saline
PBST: Phosphate buffered saline with 0.2 % triton X-100
PE: Polyethylene tubing
PFA: paraformaldehyde
PNS: peripheral nervous system.
PWL: Paw withdrawal latencies
PWT: Paw withdrawal threshold
QA: quisqualate
RA: rapidly adapting
RIP: Ribosome inactivating protein
ROS: reactive oxygen species
RNA: Ribonucleic acid
SAP: Sapori
rRNA: ribosomal ribonucleic acid
S: Svedberg unit
SA: slowly adapting
SDH: superficial dorsal horn.
SG: Substantial Gelatinosa.
SOM: Somatostatin.
sP: Substance P.
ssP-Sap: [$Sar^9(O2)^{11}$]-substance P-saporin
SV: Synaptic vesicle.
TrkA: Tyrosine receptor kinase A receptor.
TRPV1: Transient receptor potential vanilloid receptor 1
TUNEL: terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labelling
TSA: Tyramid system amplification
V1: presynaptic dendrite in a synaptic glomerulus
V2: peripheral axon in a synaptic glomerulus
vFH: von Frey Hair

Acknowledgements

First and foremost, I would like to thank my supervisor, Dr. Alfredo Ribeiro-da-Silva for his unwavering support over the years. You have taught me so much more than just science, you showed me what I was capable of achieving and thus you brought out the best in me.

I would like to thank Dr. Gary Bennett for being my mentor over the years. You taught me how to think like a scientist.

I would like to extend my sincerest appreciation to Dr. Claudio Cuello, my advisor and mentor. I want to thank you for keeping me on track and for providing me with guidance and kind words of support.

I will forever be in a debt of gratitude to Manon St-Louis, technician extraordinaire. I could not have achieved my goals if it wasn't for your continuous support and technical expertise. Moreover, you have been a great friend in times of laughter and tears. Your support and guidance have been indispensable.

I would like to extend a huge thank you to Vanessa Partridge for her friendship and support as well as her technical expertise. You shine like a star in the night sky. Never change.

My sincere gratitude to Jacynthe Laliberté for her technical mastery of the confocal microscope and for the wonderful talks we've shared.

To the past and present members of the Ribiero-da-Silva lab, I thank you for your unfaltering patience and faith in me over the years. Special thanks goes out to Lina Almarestani (my little palm tree) and Abeer Wael Saeed; you are like a burst of sunshine on a rainy day.
Many thanks to Laurie Sellings for the laughter and the awesome times we have shared during this period of growth, and Simon Allard for your unconditional friendship and help.

I am in a great debt of gratitude to the administrative staff for their help and support over the years. A special thank you is extended to Tina Tremblay, Héléne Duplessis and Pam Moore.

I owe a debt of gratitude to Officer Alain Dionne and the rest of the team at Station 5 of the Montreal Police for going beyond the call of duty.

Au Jean-Sebastien Austin, je vous remercie pour votre dedication et travailler dur que vous avez dévoué à la traduction de la résumé de mon thèse.

I would like to thank the Louise Edwards Foundation for their financial support as well as the Canadian Institute of Health Research (CIHR) and the Pain M2C CIHR training program.

Last, but not least, I would like to thank my rats for the sacrifices they have made in the name of science.

GUIDELINES OF THESIS PREPARATION

The following is reproduced in full from with the "Guidelines Concerning Thesis Preparation":

> Candidates have the option of including, as part of the thesis, the text of one or more papers submitted or to be submitted for publication, or the clearly-duplicated text of one or more published papers. These texts must be bound as an integral part of the thesis.
>
> If this option is chosen, **connecting texts that provide logical bridges between the different papers are mandatory**. The thesis must be written in such a way that it is more than a mere collection of manuscripts; in other words, results of a series of papers must be integrated.
>
> The thesis must still conform to all other requirements of the "guidelines for Thesis Preparation". **The thesis must include**: A Table of Contents, an abstract in English and French, an introduction which clearly states the rationale and objectives of the study, a review of the literature, a final conclusion and summary, and a thorough bibliography or reference list.
>
> Additional material must be provided where appropriate (e.g. in appendices) and in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.
>
> In the case of manuscripts co-authored by the candidate and others, **the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent.** Supervisors must attest to the accuracy of such statements at the doctoral oral defence. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of all the authors of the co-authored papers.

Statement of Authorship

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

Manuscript 1: Transient loss of terminals from Non-peptidergic nociceptive fibres in the substantia gelatinosa of spinal cord following chronic constriction injury of the sciatic nerve.
A.L. Bailey and A. Ribeiro-da-Silva, Neuroscience 138: 675-690, 2006.

Manuscript 2: Selective lesioning of IB4-binding primary afferent fibres: a morphological and behavioural analysis of the effects of intra-sciatic nerve IB4-Saporin treatment.
A.L.Bailey and A. Ribeiro-da-Silva, Pain (to be submitted).

Manuscript 3: Deficient Associative Learning Exhibited in Rats Treated with Isolectin B_4-Saporin.
A.L. Bailey, A. W. Saeed and A. Ribeiro-da-Silva, to be submitted.

Responsibilities of authors and co-authors:

The following statements describe the responsibilities of all the authors of the above co-authored manuscripts:

Dr A. Ribeiro-da-Silva: Principal investigator of all the projects and the main intellectual influence for the manuscripts reported in this thesis. In addition, processed confocal images for manuscript 1 and conducted the semi-quantitative analysis of spinal cord sections prepared for electron microscopy, including the processing of the images for manuscript 1.

A.L.Bailey: Investigator and intellectual influence on all of the projects reported in this thesis. Planned all animal experiments, performed all the immunocytochemical stainings, acquired all of the data, obtained all of the confocal micrographs and performed all of the quantification presented in this

thesis. A.L Bailey wrote the first version of all of the manuscripts included in this thesis which were subsequently edited by the Dr. A. Ribeiro-da-Silva and co-authors to their final versions presented herein.

A.W. Saeed: Assisted in the planning and execution of several behavioural tests (conditioned place avoidance and walking tract) for manuscript 3.

Chapter I
General Introduction

Statement of problem and purpose of investigation.

"Perfer et obdura; dolor hic tibi proderit olim"

"Be patient and tough; some day this pain will be useful to you."

Ovid (43 B.C-17 A.D)

Many polytheistic ancient civilizations worshiped Gods and Goddesses of love, fertility, fire and water. Among these beatific figureheads were the more formidable Deities of war and death and in some civilizations, those of suffering and distress, such as *Poena* (from the Latin: punishment, pain) the Roman Goddess of pain and punishment. From ancient civilizations through the Ages of Reason, Enlightenment and Romanticism, the ever-evolving science of medicine relinquished the concept of pain as a form of divine punishment for the more empirical approach. Throughout the middle ages, Christian doctrine revered pain as a divine gift from God (Natali, J-P, 1992). Believed to admonish impending injury, or inflicted as a form of punishment for moral turpitude, pain was believed to be a defining human characteristic bestowed upon man by The Creator (Duby G, 1992). This concept is not unique to Christianity; the Hindu faith, for example, accepts pain as part of an individuals Karma, the consequence of past inappropriate behaviour and is thus tolerated without complaint (Whitman S.D, 2007).

Culture and religion greatly influenced societies prevailing sentiments regarding pain and individual suffering**.** Many of the world's cultures utilize painful rituals as a form of personal growth (Anderson, R.T., and Anderson, S.T., 1992). By the acceptance of pain as a natural consequence of divine punishment or as a method of declaring one's transcendence from childhood to adulthood, spirituality bestows great value to the pain experience. Throughout the ages, philosophers questioned the ontological origins of pain and the meaning of suffering; was it so that we can truly experience pleasure? Is it good? Does it have value? The search for value in pain transcends the ages.

The Enlightenment era marked a transition in the societal acceptance of pain. The secular regard of pain which dominated the 17th and 18th century yielded to a more sympathetic desire to reduce suffering (Porter R, 1992). Dissatisfied with the Christian tenet that pain was part of the Original Sin and was to be accepted without question or complaint, philosophy became centered on the diminution of pain. Consequently, medical science followed suit and the investigation of the physiological processes which caused pain began.

Pain has often been considered as two separate entities. Aristotle (384-322 B.C) believed pain to be an emotion (see Perl, E.R., 2007) whereas Epicurus (342-270 B.C) defined pain as a specific sensation. The French philosopher, mathematician, scientist and writer Réne Descartes (1596-1650) has been entitled the "Father of Modern Science and Mathematics". His notorious statement "*cogito ergo sum*" (I think, therefore I am) resides at the core of his Dualism Theory of the human mind and body, whereby they exist as separate entities; the mind an ephemeral substance and the body, a tangible object (Benini, A and DeLeo, J.A., 1999). Descartes postulated that involuntary reactions to external stimuli was initiated and effectuated by the stimulation of the peripheral terminals of *nerve fibrils*. Peripheral stimulation displaces the central terminations of the *nerve fibrils* resulting in the re-arrangement of the *interfibrillar space*. This disruption in the structured *interfibrillar space* liberates the flow of *animal spirits* in the appropriate nerves ultimately culminating in the physical reaction to the stimulus (Descartes, R., 1664). The displacement of these nerve fibrils, such that they separate from their structure of origin, the brain will then perceive the experience as painful (Descartes, R., 1664).

Descartes later broadened his theory which states that the intensity of the pain experienced is proportional to the extent of the injury (Specificity theory) (Benini, A. and DeLeo, J.A., 1999). In this scenario, the body has influence over the mind. Descartes selected the pineal gland as the seat of the soul, the site where the mind and body interact. His supposition was derived from the fallacious assumption that the solitary pineal gland was unique to humans, as it was hypothesized that

humans possessed the capacity to think, however lower mammals did not (matter he referred to as *res extensa* (Benini, A and DeLeo, J.A., 1999). Descartes' comprehensible explanation of involuntary reaction, acknowledged as The Reflex Theory is eloquently depicted in three illustrations, the mind-body dichotomy (1662), *L'homine* (1662) and *L'homme de Réne Descartes* by Charles Angot (1664) (Descartes, R. 1664).

Figure 1

L'homme de Réne Descartes by Charles Angot (1664)

L'Homme de Rene Descartes (Treatise of Man)

Translated by T.H. Steele

Harvard University Press, Cambridge Massachusetts, 1972

The Cartesian Reflex theory and Specificity theory of pain transmission and perception were a truly insightful hypothesis during a time when the fundamental principles of medical science in the 17th century applied the ancient Galen diagnostics of the Four Humors: blood, phlegm, bile and black bile. The

symptomatic treatment of pain by means of crude pharmacological preparations of plant extracts from *Salix Alba* (Willow Bark)*, Ephedra Equisetina E. Sinica* (Ephidrine) *and Panx Ginseng* (Ginseng) were common in the ancient world and formulations and preparations are still in use today (Calixto, J.B. et al., 2000).

The dualistic approach to the study of mind and body can still be applied to physiological study today. Pain, a simple word is by no means simply defined. The International Association for the Study of Pain (IASP) has defined pain as the following: "An unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" (International Association for the Study of Pain (IASP)(Merskey, H and Bogduk, N., 1994, IASP task force on taxonomy). The use of the words *unpleasant* and *emotion* characterize pain as a perception and not merely the physiological processes of neurons and neurotransmitters. Removing the perceptive component of pain we are left with nociception, the neuroanatomical and neurophysiological aspects of pain bringing us back to the mind/body dichotomy.

Statistics Canada reported that 27,131,964 Canadians suffered from pain or discomfort ranging from mild to severe in 2005. Of this population, 2.7 % reported severe pain or discomfort, equal to over 734,782 individuals aged 12 or over. In the same year, the province of Quebec reported over 6,473,413 people afflicted with pain or discomfort with 1.7% of this population reporting pain or discomfort which interferes with most of their daily activities (of a total population of 7, 782,561) (Statistics Canada, Canadian Community Health Survey [CCHS] 2.1 and 3.1, 2005)

Notwithstanding the laborious discovery of new drug targets simultaneous with the continued sophistication of in-market pharmaceuticals to second and third generation improvements, patients frequently feel limited betterment, once again bypassing an opportunity to vanquish the cycle. The number of physicians, psychiatrists, anesthesiologists, nurses and therapists schooled in the clinical management of chronic pain and the co-related illnesses is small relative to the enlarging group of chronic pain patients. The increasing elderly population will push the numbers of those afflicted with pain higher with the increased prevalence

of age-related painful illnesses such as osteoarthritis. In 2001, one in eight Canadians were aged 65 or over. This proportion is projected to increase to one in five by the year 2026 (Health Canada, Cat.H39-608, 2002). The physiological decline in the elderly requires unique care, thus exacerbating the need for appropriate, effective therapeutic strategies to conquer the debilitating disease called Chronic Pain.

Neuropathic pain is a chronic pain syndrome, which can be incited by a direct or indirect casualty of the primary afferent fiber (PAF). PAFs are responsible for the transduction of peripheral noxious stimuli to the central nervous system (spinal cord). Such injury may arise from physical trauma to the nerve (e.g. accident, physiological disorder), metabolic disorders (e.g. diabetes), viral infections (e.g. Herpes Zoster) and medications for treatments of diseases such as acquired immunodeficiency syndrome (AIDS) and cancer (Fricker, B., et al., 2008).

A deeper understanding of the fundamental, latent pathological processes of the genesis and maintenance of specific chronic pain conditions such as Painful Diabetic Neuropathy, Post-Herpetic Neuralgia, cancer pain and arthritis is crucial in designing not only the appropriate pharmacopoeia but the unique clinical strategies each patient deserves. In the search for such knowledge, basic researchers have developed animal models which closely mimic the symptomology and pathology of various acute and chronic pain conditions (see Fricker, B. et al., 2008 for review). The models in current practice have demonstrated the effects of subtlety; the location of the injury, the material used to induce the injury, animal strain and diet; variations of a theme which can produce dramatic and distinctive pathological consequences.

Primary afferent sensory fibers are a heterogeneous population of axons derived from cells residing in the dorsal root ganglion, which is located just outside the spinal cord. Of this diverse collection of cells, the small diameter fibers are of great interest as they are responsible for the transduction of pain-related signals arising from the periphery. Neurons with small diameter axons can

be segregated into subclasses based primarily on their expression of neuropeptides; the peptidergic and the non-peptidergic. Although non-peptidergic afferents are known to play a role in imparting nociceptive information (Gerke, M.B. and Plenderleith, M.B., 2001), our understanding of the nature of this information and how it is processed by these afferents in the spinal cord is limited. Using nerve injury models, examination of the aberrant effects on the expression of specific neurotransmitters or ion channels as well as the viability of these afferents brings us one step closer to solving the mystery of the non-peptidergic sensory neuron.

1.2 Anatomical basis of nociception in the superficial dorsal horn.

1.2.1 Sensory input to the superficial dorsal horn.

Innocuous and noxious sensory stimuli are imparted from peripheral tissues to the central nervous system (CNS). The pseudounipolar primary afferent neuron (PAN), derived from the trunk neural crest, is localized to the dorsal root ganglia (DRG), a nodule situated proximal to the spinal cord within the region of the dorsal roots and in close proximity to the ventral root junction. The DRG is comprised of a heterogeneous population of PANs each with distinct properties and characteristics. Three discrete sensory neuron classes are classified according to cell soma diameter, conduction velocity and the presence of a myelin sheath: C-fibres, A-delta (Aδ) fibres and A-Beta (Aβ) fibres (Millan, M., 1999). Surrounding the cell bodies of PANs within the DRG are small satellite cells which rest upon the basal lamina of the endoneurium.

Previous studies using light and electron microscopy have investigated the ingress in the dorsal horn and arborization patterns of primary afferents of several types. These studies unraveled a differential termination pattern of large, intermediate and small or fine caliber afferents in circumscribed layers of the superficial region of the dorsal horn in multiple species **(**Light, A.R., Perl, E.R. 1979**;** Brown, A.G. 1982**;** Maxwell, D.J., Réthelyi, M, 1987; for recent review see Ribeiro-da-Silva, A and De Koninck, Y., 2008). Ultrastructural studies have

revealed that most primary afferent terminals establish simple axo-dendritic or axo-somatic contacts in the dorsal horn. However, studies combining electron microscopy with lesions of primary afferents or with intracellular labeling have revealed that a considerable number of primary afferents terminate as the central elements of complex synaptic arrangements named synaptic glomeruli (for review see Ribeiro-da-Silva, A., 2004). To qualify as a synaptic glomerulus, the central bouton (C) of primary afferent origin (containing round agranular vesicles) has to appear in an isolated electron micrograph as surrounded by at least four dendritic profiles (one or more can be replaced by a peripheral axonal bouton), and two synapses have to be visible between the C and the peripheral profiles (Ribeiro-da-Silva, A and Coimbra, A., 1982; Ribeiro-da-Silva, A., 2004). The surrounding dendritic or axonic structures may consist of (1) dendrites devoid of synaptic vesicles (D), (2) synaptic vesicle containing dendrites also named presynaptic dendrites (V_1) and (3) peripheral axonal boutons (V_2) (Ribeiro-da-Silva, A., 2004).

Two classes of synaptic glomeruli were identified in lamina I-III of the rat spinal cord (Ribeiro-da-Silva, A., Coimbra, A. 1982). Examination of the glomeruli clearly indicated differences in the contour of the central terminal, the number of mitochondria and the relative density of the synaptic vesicles and their morphology, thus necessitating the categorizing the glomeruli into types I and II (Ribeiro-da-Silva, A., Coimbra, A. 1982). It was later determined that the central terminals of most type I synaptic glomeruli had a unique acid phosphatase activity (Ribeiro-da-Silva, A. et al., 1986) and could bind the plant lectin IB4 (Alvarez, F.J. et al., 2004). However, a minority of them contained dense-core vesicles and were immunoreactive for neuropeptides (see below). This occurrence of these two types of central terminal prompted a change in the nomenclature: Type Ia and Ib (see Ribeiro-da-Silva 2004). Type I glomeruli likely represent mostly the termination of C-fibres (Ribeiro-da-Silva, 2004; Coimbra, A. et al., 1984). The prevalence of C-fibre terminals forming type Ib synaptic glomeruli in lamina I of the superficial dorsal horn in rat is low (approximately 20% of all type I glomerular endings in lamina II) (Ribeiro-da-Silva, A., 2004). In rat, the primary afferents corresponding to thicker, non-nociceptive afferents often terminate as

synaptic glomerular of type II; these differ from type I in that they have larger central terminals, which have sparser synaptic vesicles which are more uniform in size, a lighter matrix and a higher density of mitochondria (Ribeiro-da-Silva, A and Coimbra, A., 1982; Ribeiro-da-Silva, A., 2004; Ribeiro-da-Silva, A and De Koninck, Y., 2008). A more detailed description of synaptic glomeruli is given later in this introduction (see section 1.3.1 (Figure 5) and 1.4.1 (Figure 6) for peptidergic and non-peptidergic synaptic glomeruli respectively).

1.2.1.1 Primary Afferent A-Fibres.

Primary afferent sensory neurons residing in the dorsal root ganglion are classified according to a variety of physical and functional characteristics. A-fibres are sub-divided into three categories: Aδ-fibres, Aα fibres and Aβ fibres. The classification criteria are based upon conduction velocities (Tandrup, T., 2004).

Functionally A-fibres are organized in accord with their conduction velocities and are therefore relegated to one of several groups; muscle afferents: I-III; cutaneous afferents: Aα, Aβ and Aδ (Djouhri, L and Lawson, S.N., 2004). Cutaneous A-fibres can be differentiated according to their response to noxious or innocuous stimuli. Non-nociceptive A-fibres respond to low threshold mechanical stimuli (LTM) such as the light brushing of the skin and blunt mechanical pressure (Djouhri, L and Lawson, S.N., 2004). Aδ-LTMs are found in hairy skin and are commonly referred to as D-hair or down-hair units. Highly sensitive to very slow displacement of the hair, these afferents also respond to stretching and occasionally to cooling of the skin (Djouhri, L and Lawson, S.N., 2004). Aα and Aβ LTMs are characterized by their responses to sustained mechanical pressure as rapidly adapting and slowly adapting LTM's (RA and SA respectively) (Djouhri, L and Lawson, S.N., 2004). The receptive fields of RA LTMs are concentrated on the tips of the toes and the footpads and have been identified in the glabrous skin of the medial edge of the saphenous dermatome of the foot (Leem, J.W., Willis, W.D., Chung, J.M., 1993). RA LTMs respond to low frequency vibration in the

range of 5-40 Hz, particularly at the tips of the toes (Leem, J.W., Willis, W.D., Chung, J.M., 1993). SA LTM units are classified as type I and II based upon their different receptive field (RF) locales. SAI units are localized to the toe tips and footpads akin to RA units. SA II units in contrast are found in the skin overlying the toe joints and ankle joints and are responsive to movement of the ankle and of the fourth and fifth toes. In glabrous skin, these afferents respond to the bending of the proximal joint of the digits (Leem, J.W., Willis, W.D., Chung, J.M., 1993)

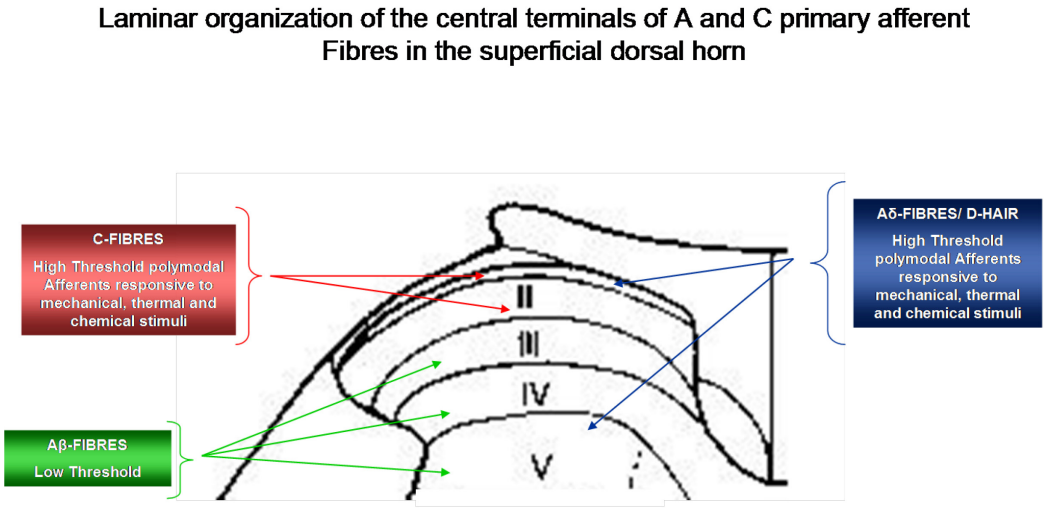


Figure 2

Diagrammatic representation of the separate classes of primary afferent input to the superficial laminae of the dorsal horn.

(Adapted from Sah, D.W.Y., et al., 2003).

Nociceptive A-fibres, differentiated by their stimulus modality, respond to high threshold stimulation. Mechano-heat responsive A fibres (MH) respond to noxious mechanical pressure and heat (>53ºC). High threshold mechano-sensitive (HTM) fibres have been shown to respond weakly to thermal stimuli when sensitized. Aδ-HTMs represent approximately 73% of Aδ nociceptors. They respond exclusively to noxious mechanical stimuli (Leem, J.W., Willis, W.D., Chung, J.M., 1993)

Stimulation of Aβ afferents is commonly thought to transduce innocuous stimuli; however in recent studies examining the compound action potentials induced in

peripheral nerves in response to noxious stimuli, conduction velocities which are too quick to be attributed to slower conducting Aδ or C fibres indicate that some Aβ afferents are responsive to high threshold stimuli (Djouhri L, Lawson, S.N., 2004). Indeed, approximately 20% of large diameter neurons in the DRG have been identified as nociceptive based upon their expression of the nociceptive sodium channel $Na_v1.9$ in addition to their conduction velocities (Djouhri, L., et al., 2002, Fang, X., Djouhri, L., Black, J.A., Dib-Hajj, S.D., Waxman, S.G., Lawson, S.N., 2002).

1.2.1.2 Primary afferent C-fibres

Classified as a cutaneous nociceptor, C-fibres are considered as type IV cutaneous afferents. The diameter of the cell soma of C-fibre neurons measures approximately 12μm. They are characterized as slowly conducting primary afferents, a consequence of their lack of myelin-coated peripheral and central axonal processes (0.5-2.0 m/s) (Almeida, T.F., Roizenblatt, S and Tufik S.., 2004). On account of their slow response to stimuli, C-fibres are accountable for the second phase of the pain response.

The adult C-fibre neuronal population is comprised of a sundry group of characteristic cells distinguished by their neuropeptide expression profile and their nutritive source (growth factors) (Molliver, D.C. , Radeke, M.J., Feinstein, S.C., Snider, W.D.., 1995; Priestley, J.V., Michaels, G.J., Averill, S., Liu, M., Willmott, N.., 2002 Luo, W., Wickramasinghe, S.R., Savitt, J.M., Griffin, J.W., Dawson, T.M,, Ginty, D.D., 2007). The peptidergic population, as the name implies, consists of neurons which express high levels of neuropeptides such as substance P (sP) and calcitonin gene-related peptide (CGRP), which have been identified in large-dense core vesicles (DCV) in terminals located in the spinal cord (see Ribeiro-da-Silva, A., 2004). Nutritive support for the peptidergic class of neurons is provided by nerve growth factor (NGF) (Bennett, DL. Michael, GJ. Ramachandran, N. Munson, JB. Averill, S. Yan, Q. McMahon, SB. Priestley, J.V., 1998; Priestley, J.V., Michaels, G.J., Averill, S., Liu, M., Willmott, N., 2002). Neurons within this class express the NGF high affinity receptor tyrosine kinase (RTK) receptor TRKA as well as the low affinity receptor p75 (Wright and

Snider, 1995; Kashiba et al., 2003). The non-peptidergic class of C-fibre neurons is characterized by a paucity of neuropeptides in addition to the absence of LDCV. (see Ribeiro-da-Silva, A., 2004). Growth factor support is supplied by glial-derived neurotrophic factor (GDNF) and, consequently, these neurons express the signal transduction domain of the GDNF receptor C-Ret and the ligand binding glycosylphosphatidyl inositol (GPI)-linked growth factor receptor domain alpha 1 and 2 (GFRα1,GFRα2 and GFRα3) (Bennett, DL. Michael, GJ. Ramachandran, N. Munson, JB. Averill, S. Yan, Q. McMahon, SB. Priestley, JV; Honda, T., Takahashi, M., Sugiura, Y. et al., 1999; Orozco et al., 2001; Stucky, C.L., Rossi, J., Airaksinen, M.S., Lewin, G.R., 2002).

The precise formation of distinct neuronal populations and their apt synaptic connections in the superficial dorsal horn, concomitant with the appropriate innervation of peripheral target tissues, is essential for CNS integrity and function.

In rat DRG, neonatal studies have shown that small dark cells in the DRG (B cells) develop after large light neurons (A cells) in the rat (E13-E15) (Kitao, Y., Robertson, B., Kudo, M., Grant, G., 2002). Neuronal soma size and peripheral target tissues have been hypothesized to influence the relative birthdates of large and small sensory neurons (Kitao, Y., et al., 2002). However, a recent study demonstrated that growth factors are essential in the formation of the appropriate neurochemical and receptor phenotype characterized by cells of certain size profiles (Luo, W., Wickramasinghe, S.R., Savitt, J.M., Griffin, J.W., Dawson, T.M,, Ginty, D.D., 2007). Indeed, the formation and development of DRG neurons is mediated by the presence of specific neurotrophic factors within the CNS which include neurotrophin 3 (NT-3), brain-derived neurotrophic factor (BDNF) and NGF (Marmigere & Ernfors 2007, Luo, W., Wickramasinghe, S.R., Savitt, J.M., Griffin, J.W., Dawson, T.M,, Ginty, D.D., 2007). Large DRG neurons can be identified by the neurofilament marker NF200 by embryonic day 12.5 (E12.5) (Fitzgerald, M and Macdermott, A., 2005). A NGF-dependent population of cells expressing TRKA is present in the majority of embryonic DRG neurons (E12) (Luo, W., Wickramasinghe, S.R., Savitt, J.M., Griffin, J.W., Dawson, T.M,, Ginty, D.D., 2007). Messenger RNA (mRNA) for the signal transduction domain of the GDNF receptor C-Ret can be detected in

approximately 12% of DRG neurons, specifically confined to large diameter cells at E11.5 (Silos-Santiago, I., Molliver, D.C., Ozaki,S., Smeyne, R.J., Fagan, A.M., Barbacid, M., Snider, W.D., 1995). C-Ret protein appears in the same population by E13 (Baudet, C., Mikaels, A., Westphal, H., Johansen, J., Johansen, T.E., Ernfors, P., 2000). The pre-natal expression of C-Ret is confined to large diameter neurons (~187μm) until E15.5 (Molliver, D.C., Wright, D.E., Leitner, M.L., Parsadanian, A.S., Doster, K., Wen, D., Yan, Q., Snider, W.D., 1997). At E16 a proportion of small and medium diameter neurons co-express TRKA and C-Ret receptors (Silos-Santiago, I., Molliver, D.C., Ozaki,S., Smeyne, R.J., Fagan, A.M., Barbacid, M., Snider, W.D., 1995; Luo, W., Wickramasinghe, S.R., Savitt, J.M., Griffin, J.W., Dawson, T.M,, Ginty, D.D., 2007). Shortly thereafter, C-Ret expression is refined, occurring in small somata. The co-expression of both trophic factor receptors persists until birth, when, over the course of the first two to three weeks of life [post-natal days (P) 1-21] TRKA expression in the C-Ret expressing neurons undergoes extinction (Silos-Santiago, I., Molliver, D.C., Ozaki,S., Smeyne, R.J., Fagan, A.M., Barbacid, M., Snider, W.D., 1995). By adulthood, the distinction between peptidergic and non-peptidergic primary sensory neurons is apparent; 90% of C-Ret-positive neurons are non-peptidergic (Silos-Santiago, I., Molliver, D.C., Ozaki,S., Smeyne, R.J., Fagan, A.M., Barbacid, M., Snider, W.D., 1995).

A recent study by Luo and colleagues proposed that the post-natal extinction of TRKA in the C-Ret subpopulation of small sensory neurons is a C-Ret-dependent process (Luo, W., Wickramasinghe, S.R., Savitt, J.M., Griffin, J.W., Dawson, T.M,, Ginty, D.D., 2007). Employing a Cre-recombinase knock-out of the C-Ret gene ($Ret^{f/f}$•Wnt1-Cre mouse), investigators demonstrated an essential role for C-Ret for the proper recession of TRKA in non-peptidergic neurons as mutant post-natal (at P14) mice maintained TRKA expression in non-peptidergic neurons identified by the binding of Isolectin B_4 (IB4; see section on non-peptidergic neurons). Of particular intrigue, the authors further demonstrated that the embryonic expression of C-Ret in large and, subsequently, small neurons in the DRG is an NGF-dependent phenomenon. TRKA-positive neurons in NGF-null mice undergo apoptosis; however NGF-null mice lacking the Bax gene do not

exhibit the same phenotype due to the absence of the pro-apoptotic Bax gene. Utilizing $NGF^{-/-}$•$Bax^{-/-}$ double knock-out mice, C-Ret expression was dramatically reduced in the early C-Ret expressing population of large, TRKA-negative neurons at P0. Moreover, the expression of the ligand binding GFRα1 and GFRα2 receptors were undetected in the double knock-out. Furthermore, GDNF receptor expression in the embryonic rat does not signal for cell survival, but rather for cell phenotype (shift of C-Ret expression to large neurons) (Baudet, C., et al., 2000; Luo, W., Wickramasinghe, S.R., Savitt, J.M., Griffin, J.W., Dawson, T.M,, Ginty, D.D., 2007). Shortly after birth, non-peptidergic cell viability is GDNF-dependent (Baudet, C., et al., 2000).

In the absence of adequate growth factor support, the processing of nociceptive stimuli in baseline and pathological states is disrupted resulting in the abnormal perception of pain (Indo Y., 2002). Mutations in the TrkA gene contribute to the formation of congenital insensitivity to pain with anhidrosis (CIPA) (Indo, Y., et al., 2002). Transgenic mouse models with similar mutations to those witnessed in humans results in mice displaying characteristic symptoms of congenital insensitivity to pain without anhidrosis (Indo et al., 1996; Koltzenburg M., 1999). NGF-null mice die shortly after birth, exhibiting substantial loss of both sensory and sympathetic neurons (Smeyne, R.J., Klein, R., Schnapp, A., Long, L.K., Bryant, S., Lewin, A., Lira, S.A., Barbacid, M., 1994).

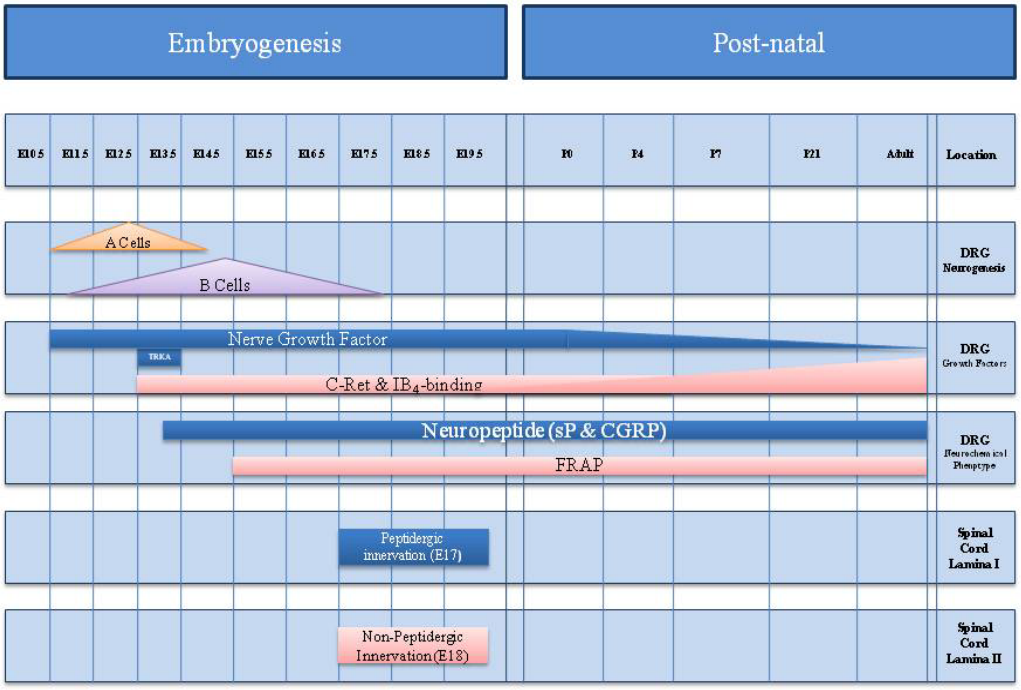


Figure 3

Timeline representing the development and differentiation of primary afferent neurons from embryogenesis to adulthood.

(Adapted from Fitzgerald, M and MacDermott, A, 2005).

The embryonic refinement of C-Ret expression and the post-natal extinction of TRKA in a sub-lineage of PANs which do not express neuropeptides ultimately results in the formation of two populations of C-fibres: the peptidergic and the non-peptidergic. This embryonic and post-natal divergence prompts the question: what is the significance of two subclasses of cutaneous C-fibres? The presence of both peptidergic and non-peptidergic neurons in the DRG has provoked the curiosity of many researchers.

Extensive immunocytochemical and electrophysiological studies revealed further distinctive characteristics, establishing an obvious dichotomy (see peptidergic and non-peptidergic sub-headings below). Many have hypothesized that PAF C-fibres comprises a parallel system of nociceptive input from cutaneous peripheral targets (Hunt, S.P., Rossi, J,. 1985); Koltzenburg M., 1999; Hunt, S.P., Mantyh, P.W., 2001; Zylka, 2005; Braz et al., 2005)

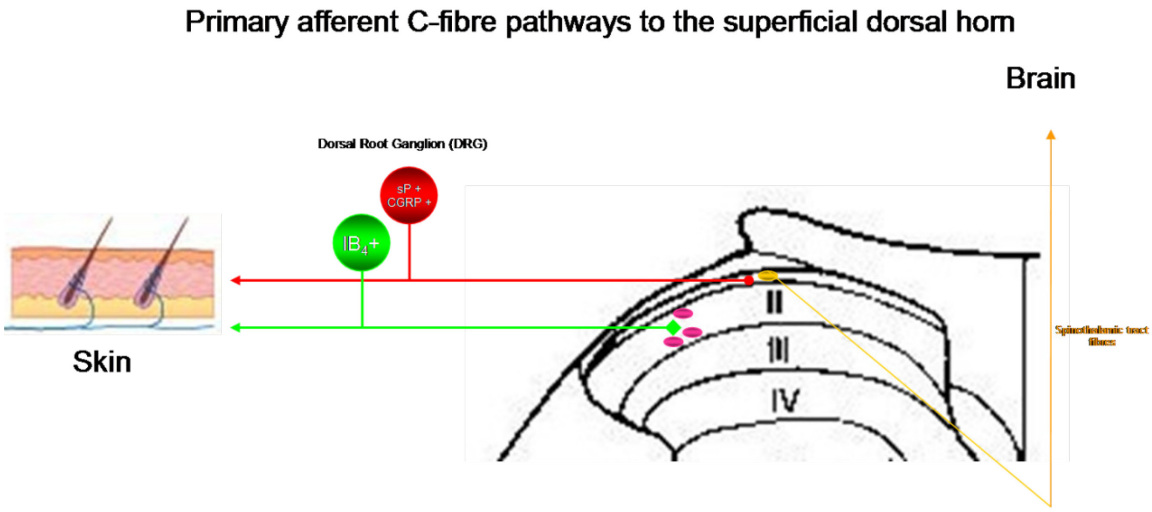


Figure 4

Primary afferent input to the superficial dorsal horn.

Diagrammatic representation of peptidergic and non-peptidergic input to the superficial dorsal horn.

(Adapted from Zylka, M.J. et al., 2005).

Antecedent reasoning notwithstanding, the neuroanatomical and neurochemical differences between peptidergic and non-peptidergic PAFs led to the hypothesis that they represented a reciprocating, supporting role in the transmission of nociceptive information (Hunt, S.P., Rossi, J,. 1985). Congruent with this hypothesis, the anticipated outcome following the selective lesioning of one or the other C-fibre subclass would be one of profound analgesia (Hunt, S.P., Rossi, J,. 1985). The elimination of a significant proportion C-fibres can be achieved using the neonatal application of the pungent capsaicinoid Capsaicin (genus capsicum).

More recent studies have employed the use of intrathecally applied capsaicin to selectively lesion the central terminals of TRPV1-expressing peptidergic terminals (Cavanaugh, D.J., Lee, H., Lo, L., Shields, S.D., Zylka, M.J., Basbaum, A.I., Anderson, D.J., 2009) Technological advancements within the past twenty years have refined such approaches with the development of specific ligands or antibodies conjugated to neurotoxic compounds such as ricin or saporin which permit the researcher to extirpate a particular neuronal population at any point within the course of the experiment (Wiley, R.G, Kline, IV R.H, 2000).

Albeit the large quantity of data obtained in recent years examining the distinguishing features of non-peptidergic PANs, a true understanding of their task in the transmission of nociceptive information is at present incomplete.

1.3 Peptidergic primary afferent sensory fibres.

Nobel Prize laureate Dr. Ulf S. von Euler (1905-1983) accompanied by Sir John Henry Gaddum (1900-1965) discovered one of the most well known neuropeptides, substance P, in 1931. Substance P (sP) is an 11 amino acid peptide localized (but not restricted) to small sensory neurons in the DRG in rat (Ju, G., Hökfelt, T., Brodin, E., Fahrenkrug, J., Fischer, J.A., Frey, P., Elde, R.P., Brown, J.C., 1987; Smith, G.D., Seckl, J.R., Harmar, A.J., 1993; Yoon, Y.S., Hwang, I.K., Lee, I.S., Suh, J.G., Shin, J.W., Kang, T.C., Oh, Y.S., Won, M.H., 2003). Substance P belongs to a family of similar peptides named neurokinins, all which share a common sequence, Phe-X-Gly-Leu-Met-NH_2 (Nakanishi, S., 1991). The nociceptive role of sP was later suggested by Dr. Fred Lembeck and confirmed by Dr. J. L Henry (Henry J. L., 1976; Bromovsky, P.R., 2005)

The co-expression of other neuropeptides such as calcitonin gene-related peptide (CGRP) with sP in the central terminals of small caliber afferents in the superficial layers of the dorsal horn established the presence of peptide-rich primary afferent fibres as a well defined population of primary sensory fibres (Ju, G., Hökfelt, T., Brodin, E., Fahrenkrug, J., Fischer, J.A., Frey, P., Elde, R.P., Brown, J.C.,1987; Cuello, A.C., Ribeiro-da-Silva, A. Ma, W. De Koninck, Y and Henry, J.L., 1993; Smith, G.D., Seckl, J.R., Harmar, A.J., 1993; Ribeiro-da-Silva,

A., 1995a; Ribeiro-da-Silva, A. and Hökfelt, 2000; Yoon, Y.S., Hwang, I.K., Lee, I.S., Suh, J.G., Shin, J.W., Kang, T.C., Oh, Y.S., Won, M.H., 2003).

1.3.1 Morphology

Peripheral terminals

Located in the DRG, the cell somata of peptidergic neurons are typically small diameter sensory neurons which are heterogeneously distributed amongst medium and large sensory neurons (McCarthy, P.W., Lawson, S.N. 1989; McCarthy, P.W., Lawson, S.N. 1990). As pseudounipolar cells, these neurons extend small caliber (~1µm thick), unmyelinated peripheral and central axons (Cruz, F., Lima, D., Coimbra, A. 1993). Peptidergic PAFs innervate a variety of peripheral tissues. Sensory fibres expressing sP, CGRP have been observed in the bone marrow of the rat knee joint (Iwasaki A, Inoue K, Hukuda S. 1995). sP-immuno-labelled fibres are apparent in the lining cell layer of the synovium, with some branches extending into the joint space. Peri-vascular networks localized in the sub-lining cell layer of the synovium displayed sP, CGRP and NPY-positive fibres (Bjurholm, A., Kreicbergs, A., Ahmed, M., Schultzberg, M. 1990; Iwasaki A, Inoue K, Hukuda S. 1995). Neuropeptide-positive sensory fibres (sP and CGRP-positive) have been identified in the periosteum of both membranous bone (mandible and calvaria) and in long bones (tibia) in the rat (Hill, E.L., Elde, R. 1991). In the superficial layers of the periosteum, sP-immunoreactive fibres presented as a fine varicose network (Hill, E.L., Elde, R. 1991). Neuroanatomical survey studies of the sensory innervation of the intervertebral disc in rat suggest a predominant peptidergic contribution to the sensory detection of nociceptive stimuli (Aoki, Y., Ohtori, S., Takahashi, K., Ino, H., Douya, H., Ozawa, T., Saito, T., Moriya, H., 2005). Specifically, 54.5 ± 5.0% of fluorogold-retrogradely traced lumbar disc afferents were CGRP-immunoreactivity whereas non-peptidergic afferents, as identified by IB4-binding (see below; non-peptidergic afferents) were virtually absent in the disc (Aoki, Y., Ohtori, S., Takahashi, K., Ino, H., Douya, H., Ozawa, T., Saito, T., Moriya, H., 2005).

Peptide-rich primary afferents have also been observed in visceral tissues. Analysis of the neurochemical composition of uterine cervical afferents of the hypogastric nerve revealed high CGRP content (Tong, C., Conklin, D., Clyne, B.B., Stanislaus, J.D., Eisenach, J.C.M., 2005). Fluorogold retrograde labeling of cervical afferents confirmed the provenance (sensory) of these CGRP-immunoreactivity varicosities, originating from the DRG (Papka, R.E., 1990).

Considerable research has been conducted in regards to the sensory innervation of the integument. Neuropathic pain conditions such as complex regional pain syndrome (CRPS) type I and II present symptoms of altered skin texture, temperature and hair growth. These symptoms are the result of altered sensory and sympathetic innervation of both glabrous (plantar surface) and hairy skin (lower mandibular region) (Coderre, T.J., Xanthos, D.N., Francis, L., Bennett, G.J. (2004). Peptidergic afferents are highly represented in both hairy and glabrous skin (Carlton, S.M., Zhou, S., Coggeshall, R.E., 1996; Ruocco et al., 2001; Yen, L.D. et al., 2006). Small caliber C-fibre peripheral projections terminate in free nerve endings (FNEs) in both cutaneous tissue types (Kruger, 1989). The fine terminal endings of cutaneous afferents are characterized by distinct morphological features. FNEs of C-fibres typically lack Schwann cell sheaths. In 1973, Cauna described the Schwann cell-free FNEs as “pencillate nerve endings” which flank the basal lamina and form intimate contacts with the epithelium (Cauna, N., 1973; Kruger, L., et al., 1981). The high content of vesicles and organelles provide a basis of identification (Cauna, N., 1973). The FNEs of PAF in hairy skin possess similar characteristics as those found in dermatoglyphic glabrous palmar and plantar skin of the rat (Munger, B.L, Ide, C., 1988)

CGRP-immunoreactive axons inhabit the dermal papillae proximal to vascular loops and Meissner corpuscles (Kruger, L., Silverman, J.D., Mantyh, P.W., Sternini, C., Brecha, N.C., 1989). Moreover, peptidergic axons enter the epidermis in glabrous skin and project collaterals into the stratum spinosum (Kruger, L., Silverman, J.D., Mantyh, P.W., Sternini, C., Brecha, N.C., 1989). Patterns of peptidergic sensory innervation as identified by CGRP-immunoreactivity are similar in hairy skin (Kruger, L. et al., 1989).

Central terminals

The peptidergic sensory afferents project to the superficial lamina of the dorsal horn of the spinal cord, specifically lamina I and outer lamina II (LI and LIIo respectively). Projections to the deep dorsal horn, specifically lamina V (LV) have also been observed (Ribeiro-da-Silva, A. and Cuello, A.C., 1995b). The morphological features of small diameter peptidergic terminals have been described in detail (Ribeiro-da-Silva, A. et al, 1989; Ribeiro-da-Silva, A. 2004). The boutons frequently exhibited a scalloped border and contained round agranular synaptic vesicles (SV) as well as a smaller number of large granular vesicles or dense-core vesicles (DCV). The synaptic associations of these terminals were inelaborate, establishing simple axo-dendritic synapses with large or small dendritic profiles, or axo-somatic contacts with neuronal cell bodies (Ribeiro-da-Silva, A et al., 1989; McLeod, A., et al., 1998; Ribeiro-da-Silva, A., et al., 2004). In a small but significant number of cases (see section 1.2.1 above), the terminals of peptidergic afferents were observed to represent the core element of synaptic glomeruli (as shown in figure 5 below) (Ribeiro-da-Silva, A., et al., 1989; Ribeiro-da-Silva A., 2004). These peptidergic synaptic glomeruli, termed type Ib, are decidedly simpler than non-peptidergic synaptic glomeruli (type Ia) in that they are never post-synaptic to their surrounding profiles and are therefore not involved in presynaptic inhibition (Ribeiro-da-Silva, A., 2004).

Several morphological studies have suggested that regulation of primary afferent signaling is mediated by presynaptic inhibitory connections from local spinal GABAergic or glycinergic neurons (Todd, A.J., 1996; Todd, A.J., Watt, C., Spike, R.C., Sieghart, W., 1996).

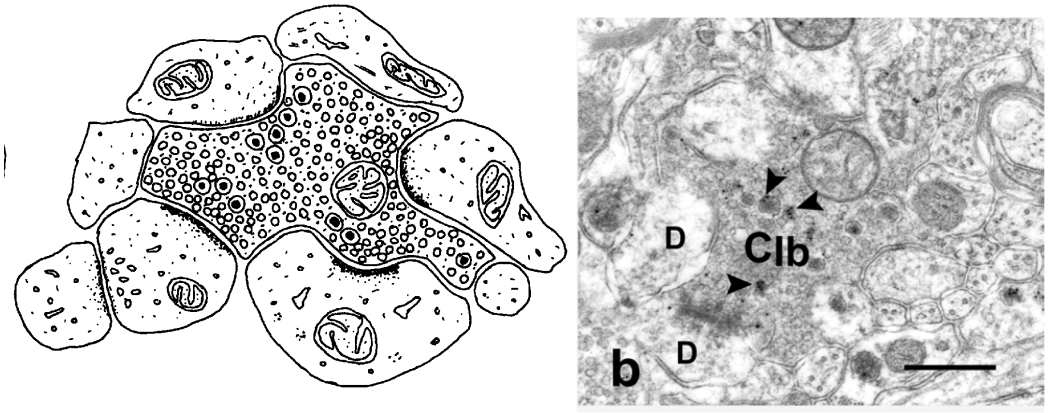


Figure 5

Diagrammatic representation of a type Ib synaptic glomerulus. Note the simple synaptic architecture as there are no pre-synaptic connections with the central bouton.CIb: central bouton, D: dendrite, Arrows indicate the presence of dense core vesicles.
Courtesy of Dr. A. Ribeiro-da-Silva

1.3.2 Neurochemistry

Early studies examined the presence of neuropeptides in small diameter sensory neurons and their respective terminals in the superficial dorsal horn of the rat. McCarthy and Lawson, in a double-labeling immunohistochemical study, detected sP immunoreactivity in 70% small dark cells. Furthermore, sP immunoreactivity was identified in 50% of C-fibre neurons characterized by their conduction velocity (McCarthy, P.W., Lawson, S.N., 1989). Similarly, CGRP immunoreactivity was detected in 46.5% of all neurons. In the small dark cell population, 68% showed CGRP-immunoreactivity. The primary afferent origin of these neuropeptide-expressing terminals has been determined by dorsal rhizotomy and by the neonatal systemic application of capsaicin (Jessell, T., Tsunoo, A., Kanazawa, I., Otsuka, M., 1979; Lawson S. N and Nickels S.M., 1980; Nagy, J.I.,

Vincent, S.R., Staines, W.A., Fibiger, H.C., Reisine, T.D., Yamamura, H.I., 1980; Jancsó, G., Hökfelt, T., Lundberg, J.M., Kiraly, E., Halász, N., Nilsson, G., Terenius, L., Rehfeld, J., Steinbusch, H., Verhofstad, A., Elde, R., Said, S., Brown, M., 1981; Nagy, J.I., Hunt, S.P., Iversen, L.L., Emson, P.C., 1981; Ribeiro-da-Silva, A., Coimbra, A. , 1982 and Coimbra, A., Ribeiro-da-Silva, A., Pignatelli, D., 1984). Dorsal rhizotomy leads to the degeneration of primary afferent terminals as well as a 50-80 % depletion of sP in the superficial dorsal horn (Jessell, T., Tsunoo, A., Kanazawa, I., Otsuka, M., 1979; Pohl, M., Benoliel, J.J., Bourgoin, S., et al., 1990). Similarly, dorsal rhizotomy produced an 85 % reduction of CGRP immunoreactivity in the superficial dorsal horn indicative of the purely sensory contribution of CGRP-positive terminals in lamina I and outer lamina II (Pohl et al., 1990). Systemic application of capsaicin in newborn rats resulted in a marked decrease in sP and somatostatin (SOM) in dorsal roots and a similar marked loss of sP and SOM in the dorsal horn. This partial decline in sP and SOM following both rhizotomy and capsaicin-induced injury to PAFs is indicative of both sP and SOM expressing neurons residing in the superficial dorsal horn (Pohl, M., et al., 1990; Ribeiro-da-Silva, A., 1995a). Neonatal capsaicin treatment resulted in a 60% decline in CGRP-immunoreactivity, an indication that not all CGRP afferents express the capsaicin receptor (Pohl et al., 1990). Later studies examining the co-existence of neuropeptides in primary afferent terminals questioned whether multiple neuropeptides co-exist in synaptic vesicles or whether different boutons of a primary afferent axon expressed specific neuropeptides (Plenderleith, M.B., et al., 1990). Ultrastructural analysis of peptidergic afferents using post-embedding immunogold determined the presence of galanin, sP and CGRP-immunoreactivity in the same dense core vesicles (Zhang, X., Nicholas, A.P., Hökfelt, T., 1993).

Supplemental to neuropeptides expressed in the peptidergic C-fibre terminal, studies have demonstrated the presence of classical neurotransmitters such as glutamate (De Biasi S, and Rustioni A., 1998). In post-embedded, immunogold-labelled spinal cord preparations, glutamate immunoreactivity presented in dark, scalloped-edged terminals containing agranular vesicles and occasional large granular vesicles as well in light, neurofilament-rich terminals (De Biasi S, and

Rustioni A., 1988; Todd, A.J. and Ribeiro-da-Silva, A., 2005). Colloid gold particles 20nm (glutamate) and 10nm (sP) in diameter demonstrated the co-existence of sP and glutamate in the small dark terminals; dorsal rhizotomy confirmed the origin of these terminals as arising from dorsal roots (De Biasi S, and Rustioni A., 1998). Upon release from synaptic vesicles, glutamate functions as a fast, excitatory neurotransmitter. Glutamate receptors localized to the plasma membrane of postsynaptic neurons fall into two categories: ionotropic and metabotropic receptors. Activation of the ionotropic N-methyl D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kianate receptors results in the opening of the ion channel to permit the flow of sodium (Na^+) and potassium (K^+) and in the case of the NMDA receptor, calcium (Ca^{2+}) cations.

The co-expression of sP and glutamate within primary afferent terminals is suggestive of their co-release upon afferent stimulation. The co-delivery of sP may augment the postsynaptic response initiated by activation of ionotropic glutamate receptors (Merighi, A., Polak, J.M., Theodosis, D.T., 1991).

Rusin and compeers (1992, 1993) demonstrated such augmentation, using whole cell-voltage clamp recordings from spinal cord slices. Substance P and Neurokinin A (NKA) potentiated NMDA and AMPA/quisqualate (QA)-induced currents in dorsal horn neurons (Rusin, K.I., Ryu, P.D., Randic, M., 1992; Rusin, K.I, Jiang, M.C., Cerne, R., Randic, M., 1993). Application of the non-selective tachykinin antagonist Spantide II blocked the potentiation of NMDA postsynaptic currents induced by the administration of 2nM sP. In an effort to determine whether the enhancement of NMDA currents by activation of tachykinin receptors was due to an interaction with the regulatory effects of glycine on the NMDA receptor, the authors examined the responses to applications of NMDA alone or in the presence of sP in dorsal horn neurons bathed in different concentrations of glycine. The application of glycine (0.1μM) prevented such potentiation by sP, ascertaining the influence of the tachykinin on NMDA receptors. Moreover, sP appears to influence the release of glutamate from primary afferent terminals; primary afferent stimulation produced the sustained, enhanced release of both excitatory amino acids significantly (Rusin, K.I, Jiang, M.C., Cerne, R.,

M., 1993; Zhang, Q., Zhao, Y., Guo, Y., Cao, D.Y., Tian, Y.L., Yao, F.R., Wang, H.S., 2006).

Peptidergic sensory neurons and their distal terminals are known to express receptors functionally implicated in the transmission of nociceptive signals (Ruocco, I., Cuello, A.C., Ribeiro-da-Silva, A., 2000; Tsukagoshi, M., Goris, R.C., Funakoshi, K., 2006). One of them, a member of the transient receptor potential (TRP) family of ion channels is of particular interest. The proficient, degenerative effect of the systemic administration of capsaicin on primary afferent terminals proved providential. The ostensible selectivity of capsaicin precipitated the classification of primary sensory neurons as capsaicin-sensitive or capsaicin-insensitive. Such selectivity infers the presence of a capsaicin receptor, expressed in a specific population of primary sensory neurons. Indeed TRPV1, initially named VR1, identified and cloned by M. Caterina and associates (Caterina, M.J., Schumacher, M.A., Tominaga, M., Rosen, T.A., Levine, J.D., Julius, D,. 1997), is activated by capsaicin. The receptor is a tetrameric, non-selective cation channel permeable to sodium (Na^+) and calcium (Ca^{2+}). Aside from capsaicin, the ion channel pore can be opened at ambient temperatures greater than 43ºC (Caterina, M.J., Leffler, A., Malmberg, A.B., Martin, W.J., Trafton, J., Petersen-Zeitz, K.R., Koltzenburg, M., Basbaum, A.I., Julius, D., 2000) and by high local concentration of protons (H^+) (tissue pH <6.8) **(**Pedersen, S.F, Owsianik, G., Nilius, B., 2005**).** In basal conditions, channel opening can be elicited by strong membrane depolarization, consistent with voltage-gated ion channels. Increases in temperature induce a leftward shift in the voltage-dependent activation threshold of the channel (Pedersen, S.F, Owsianik, G., Nilius, B., 2005). Protons, activation of Protein Kinase C (PKC) and a decrease in the cellular concentration of phosphatidylinositol (4,5) bisphosphate (PI(4,5)P2) all decrease the temperature threshold for TRPV1 activation and potentiate channel activation in response to capsaicin (Pedersen, S.F, Owsianik, G., Nilius, B., 2005).

In the rat DRG, more than 50% of the neurons expressed the TRPV1 receptor (Guo, A., Vulchanova, L., Wang, J., Li, X., Elde, R., 1999). Receptor expression was confined to small and medium diameter neurons. In the spinal cord TRPV1

immunoreactivity occurs in Lissauer's tract, lamina I and LIIi. Remarkably, receptor expression was completely absent in outer lamina II (Guo, A., Vulchanova, L., Wang, J., Li, X., Elde, R., 1999). The TRPV1 receptor is expressed by both peptidergic and non-peptidergic sensory neurons in the rat. In the mouse, TRPV1 is localized almost exclusively on peptidergic afferents (Leffler, A. Mönter, B and Koltzenburg, M., 2006).

1.3.3 Synaptic interaction in the superficial dorsal horn.

In the hopes of understanding nociceptive transmission and processing in the spinal cord, identification of the neurons postsynaptic to the peptidergic C-fibre terminal is essential. Discerning the receptor for sP was conducted in preparations of soluble sP receptor isolated from the chicken, rat, and bovine brains (Nakanishi, S., 1991). In soluble form, the receptor maintained high affinity to sP. The inhibition of the receptor by guanine triphosphate (GTP) appropriated the receptor to the G-protein coupled receptor (GPCR) class. The receptor with the highest affinity for sP was named Neurokinin 1 or NK1 for short (Nakanishi, S., 1991). Evaluation of the localization of the NK1 receptor determined its presence on local lamina I and outer lamina II neurons (Bleazard, L., Hill, R.G., Morris, R., 1994; Vigna, S.R., Bowden, J.J., McDonald, D,M., Fisher, J., Okamoto, A., McVey, D.C., Payan, D.G., Bunnett, N.W., 1994; Brown, J.L., Liu, H., Maggio, J.E., Vigna, S.R., Mantyh, P.W., Basbaum, A.I., 1995; Littlewood, N.K., Todd, A.J., Spike, R.C., Watt, C., Shehab, S.A., 1995; Marshall, G.E., Shehab, S.A., Spike, R.C., Todd, A.J., 1996 and Todd, A.J. et al., 1998).

In cat dorsal horn, neurons activated by sP were found to be excited by noxious stimuli (Henry, J.L, 1976). The interaction of sP with the NK1 receptor promotes the internalization of the complex by a clathrin-mediated mechanism into early endosomes. Specifically, phosphorylation of intracellular residues promotes the binding of β-arrestin, uncoupling NK1 from G-protein receptor complex. Subsequently, the β-arrestin-coupled receptor complex is endocytosed into clathrin-coated pits. Substance P disassociates from the NK1 receptor in the endosomal compartment and undergoes degradation. The NK1 receptor is then

recycled back to the plasma membrane (Garland, A.M, Grady, E.F., Lovett, M., Vigna, S.R., Frucht, M.M., Krause, J.E, and Bunnett, N.W., 1996; Mantyh P., 2002).

Studies from the 1970's identified the importance of lamina I or the marginal zone of the superficial dorsal horn in nociception (Christensen, B.N., Perl, E.R., 1970; Light, A.R., Trevino, D.L., Perl, E.R., 1979). Neuroanatomical characterization of lamina I cells identified morphologically distinct neuronal populations. Based on cell soma shape and their pattern of dendritic arborization, three predominant lamina I neurons can be identified in the horizontal plane; Fusiform, Pyramidal and Multipolar (Lima, D., Coimbra, A., 1983, 1986; Lima, D., Mendes-Ribeiro, J.A., Coimbra, A., 1991; Zhang, E.T., Han, Z.S., Craig, A.D., 1996; Zhang, E.T., Craig, A.D, 1997; Yu, X.H., Zhang, E.T., Craig, A.D., Shigemoto, R., Ribeiro-da-Silva, A., De Koninck, Y., 1999; Todd, A.J., McGill, M.M., Shehab, S.A., 2000; Spike, R.C., Puskár, Z., Andrew, D., Todd, A.J., 2003; Castro, A.R., Pinto, M., Lima, D., Tavares, I., 2004; Todd, A.J., McGill, M.M., Shehab, S.A., 2000; Spike et al, 2003; Castro, A.R., Pinto, M., Lima, D., Tavares, I., 2004; Todd, A.J., Spike, R.C., Young, S., Puskár, Z., 2005). Fusiform neurons possess an elongated (ovoid) cell body from which a primary dendrite projects from each pole. Pyramidal neurons have, as their name implies, a pyramid-shaped cell soma, extending a primary dendrite from each corner. Multipolar neurons issue four or more dendrites with no regard to cell soma morphology.

The electrophysiological properties of marginal zone neurons revealed idiosyncratic properties which could be correlated to cellular morphology (Craig, A.D., Kniffki, K.D., 1985; Burstein, R., Dado, R.J., Cliffer, K.D., Giesler, G.J. Jr., 1991; Craig, A.D, Bushnell, M.C., 1994; Dostrovsky, J.O., Craig, A.D., 1996; Han, Z-S., Zhang, E-T., Craig, A.D., 1998). Characterized in cat and in monkey, lamina I neurons were classified as: polymodal neurons receptive to noxious heat and pinch in addition to innocuous and noxious cold (termed HPC neurons), nociceptive specific neurons (NS) and innocuous thermoreceptive neurons responsive to innocuous cooling (COLD). When combined with morphology, it became apparent that fusiform cells were NS. Pyramidal neurons responded primarily to innocuous cooling (COLD) and multipolar neurons could be

classified as HPC or NS (Han, Z-S., Zhang, E-T., Craig, A.D., 1998; Doyle, C.A and Hunt, SP., 1999). Morphological studies coupled with immunocytochemistry affirm the expression of the sP receptor on fusiform and multipolar cells, consistent with their electrophysiological profiles. The lack of NK1 receptors on pyramidal cells is in keeping with their innocuous phenotype (Yu, X.H., Zhang, E.T., Craig, A.D., Shigemoto, R., Ribeiro-da-Silva, A., De Koninck, Y., 1999; Yu, X.H., Ribeiro-da-Silva, A., De Koninck, Y., 2005).

Retrograde labeling from brain regions in cat and monkey by Trevino, D.L and Carstens, E. (1979) in the interest of identifying the spinothalamic tract established lamina I as a predominant source of projection neurons to the brain (Todd, A.J., 2002). Pursuant studies demonstrated projections from the marginal zone to the caudal ventrolateral medulla (CVLM), lateral parabrachial area (LPb) and periaqueductal grey matter (PAG), areas that are known to process nociceptive information (Lima, D., Mendes-Ribeiro, J.A., Coimbra, A., 1991; Todd, A.J., 2002; Zhang, E.T., Han, Z.S., Craig, A.D., 1998; Zhang, E.T., Craig, A.D, 1997; Almarestani, L.,Waters, S.M., Krause, J.E., Bennett, G.J., Ribeiro-da-Silva, A., 2007).

1.3.4 Functional implications in acute and chronic pain

Peptidergic C-PAFs have been highly implicated in the processing of nociceptive information. The abundance of pro-inflammatory neuropeptides (sP and CGRP) stored in synaptic vesicles within their central and peripheral terminals are suggestive of an important role in the transmission of inflammatory pain and neurogenic inflammation. Immunocytochemical and radioimmunoassay (RIA) methods have been used to evaluate neuropeptide levels in animal models of chronic neuropathic pain induced by peripheral nerve injury. Specifically, in a comparative analysis of the effects of chronic constriction injury and sciatic nerve transection, RIA levels of sP and CGRP in the ipsilateral spinal cord were significantly decreased at 10 and 31 days following nerve transection (Kajander, K.C. and Xu, J., 1995). Following CCI, levels of sP were significantly decreased in the ipsilateral dorsal horn 60 days following the induction of nerve injury

(Kajander, K.C. and Xu, J., 1995). The role these neuropeptides play in the pathological (behavioural) manifestations such as hyperalgesia and allodynia have been examined using antagonists for the NK1 and CGRP receptors. Intrathecal administration of both receptor antagonists significantly reduced paw withdrawal thresholds to mechanical stimuli in an animal model of spinal nerve transection for a period of 20 minutes following administration (Lee, S.E., and Kim, J-H., 2007).

The effects of nerve injury on pepetidergic fibre innervation of glabrous skin of the plantar hind paw and the hairy skin of the lower mandibular region has been examined. Examination of CGRP and sP-immunoreactive sensory fibres in the glabrous skin of the plantar surface of the hind paw demonstrated a rapid decline in the density of innervation peaking two weeks following chronic constriction injury of the sciatic nerve (Ma, W., and Bisby, M.A., 2000; Yen, L., Bennett., G.J., Ribeiro-da-Silva, A., 2006). By the 4^{th} week following nerve injury, the density of CGRP-immunoreactive fibres present in the upper dermis increased with fibre density peaking above sham levels by the 8^{th} week post-operative (Yen, L., Bennett, G.J., Ribeiro-da-Silva, A., 2006). Peptidergic sensory innervation of the hairy skin of the lower mandibular region in the rat demonstrated a similar transitory pattern of denervation followed by hyperinnervation (Grelik, C., Bennett, G.J., Ribeiro-da-Silva, A., 2005).

1.4 Non-peptidergic Primary afferent C-fibres.

The physiological relevance of the existence of the non-peptidergic class of C-fibres has been excogitated by some of the greatest minds in the field (Hunt, S.P., Rossi, J,. 1985); Hunt, S.P., Mantyh, P.W., 2001; Zylka M.J., 2005). Formulated predominantly on the neuroanatomical and neurochemical attributes, the regnant hypothesis affirms that non-peptidergic PAFs complement their peptidergic counterpart, operating in parallel to communicate pain signals to the spinal cord (Zylka M.J., 2005). Contemporary data acquired from in depth studies examining the electrophysiological properties of receptors and their responses to chemical

stimuli (capsaicin) suggest that these afferents play a distinct role in nociceptive transmission.

1.4.1 Morphology

Peripheral terminals

The projection targets for non-peptidergic primary afferent terminals have been studied extensively (Plenderleith, M.B., Snow, P.J., 1993; Ivanavicius, S.P., Blake, D.R., Chessell, I.P., Mapp, P.I. et al., 2004; Aoki, Y., Ohtori, S., Takahashi, K., Ino, H., Douya, H., Ozawa, T., Saito, T., Moriya, H., 2005). Early identification of non-peptidergic primary afferent sensory neurons in the periphery and the CNS was difficult, requiring the enzymatic reaction of acid phosphatase (see below: central terminals). Subsequent studies discovered the preferential binding of the afferents to certain plant lectins, facilitating their identification (see below). Retrograde labeling techniques applied to peripheral nerves demonstrated that lectin-labelled, non-peptidergic afferent project predominantly to cutaneous tissues with minimal representation in visceral or muscle afferents (Plenderleith, M.B., Snow, P.J., 1993). Analysis of the sensory innervation of joints such as the rat knee joint, the intervertebral disc and the rat wrist joint yield similar results; the non-peptidergic contribution of sensory input is negligible, 0.6, 1 and 3% of retrogradely labeled neurons projected afferents to these regions respectively (Ivanavicius, S.P., Blake, D.R., Chessell, I.P., Mapp, P.I. et al., 2004; Aoki, Y., Ohtori, S., Takahashi, K., Ino, H., Douya, H., Ozawa, T., Saito, T., Moriya, H., 2005; Kuniyoshi, K., Ohtori, S., Ochiai, N., et al., 2007).

Afferent projections from non-peptidergic sensory neurons to visceral organs appear to be tissue specific. Retrograde labeling and lectin-binding of non-peptidergic afferents from the urinary tract have also demonstrated regional variations in non-peptidergic innervations. Forty-nine percent of primary afferents innervating the distal urethra were lectin-positive whereas only18 to 22 % of those innervating the bladder or proximal urethra were lectin-labelled (Yoshimura, N., Seki, S., Erickson, K.A., Erickson, V.L., Hancellor, M.B., de Groat, W.C., 2003).

Notwithstanding the negligible representation of non-peptidergic PAF in joints and their marginal presence in the bladder, these afferents are highly expressed in cutaneous tissues (O'Brien, C, Woolf, C.J., Fitzgerald, M., Lindsay, R.M., Molander, C., 1989; Bennett, D.L., Dmietrieva, N., Priestley, J.V., Clary, D., McMahon, S.B., 1996; Perry, M.J., Lawson, S.N., 1998; Lu, J., Zhou, X.F., Rush, R.A., 2001; Ambalavanar, R., Moritani, M., Haines, A., Hilton, T., Dessem, D., 2003; Aoki, Y., Ohtori, S., Takahashi, K., Ino, H., Douya, H., Ozawa, T., Saito, T., Moriya, H., 2005; Zylka, M.J., Rice, F.L., Anderson, D.J., 2005). The recently identified sensory neuron-specific GPCR subclass called Mas-related G-protein coupled receptors d (Mrgprd) has been shown to be expressed in non-peptidergic afferents projecting to hairy and glabrous skin (Zylka, M.J., Dong, X., Southwell, A.L., Anderson, D.J., 2003; Zylka, M.J., Rice, F.L., Anderson, D.J., 2005).

Identification of non-peptidergic terminals in the dermis and epidermis aided by Isolectin B_4 (IB4)-binding (see non-peptidergic section for lectin binding and IB4-specificity below) has proved to be an arduous task. Epidermal cells bound IB4 readily, precluding the detection of IB4-binding epidermal fibres (Petruska, J.C., Streit, W.J., Johnson, R.D., 1997)

Akin to peptidergic afferents, non-peptidergic afferents terminate in the epidermis as FNEs (Petruska, J.C., Streit, W.J., Johnson, R.D., 1997). The pan-neuronal marker protein gene product 9.5 (PGP9.5) (a member of the ubiquitin hydrolase family of proteins) combined with IB4 facilitated the eduction of IB4-labelled axons from IB4-binding to epidermal cells. Deduced from this method, IB4-labelled axons in the dermis penetrate the epidermis as single axons derived from sub-epidermal axon bundles (Petruska, J.C., Streit, W.J., Johnson, R.D., 1997). IB4-positive axons (alone or in bundles) travel along the dermal-epidermal border parallel to the skin surface. IB4-positive axons were shown to innervate vascular structures in the deep dermis. The origin of these axons were speculated to be autonomic or sensory, however, in the absence of any further neurochemical marker for either subpopulation, the ganglia of origin remains speculation (Petruska, J.C., Streit, W.J., Johnson, R.D., 1997; Taylor, A.M., Peleshok, J.C., Ribeiro-da-Silva, A. 2009).

Central terminals

The central terminals of non-peptidergic PAFs are localized primarily to the middle third (dorsal-ventral) of lamina II (LII) of the superficial dorsal horn (also known as the substantia gelatinosa of Rolandi due to its diaphanous and gelatinous physiognomy in fresh tissue) (for a more detailed description of the substantia gelatinosa, see section 1.5.2; see Cervero, F., Iggo, A., 1980 for review).

The dorsal border of lamina II can be differentiated from lamina I by the presence of a considerable number of thinly myelinated fibres in the latter. Lamina II has been further subdivided into an outer (dorsal) region of high cellular content (LIIo or LII_A) and an inner (ventral) territory (LIIi or LII_B) of slight cellular composition (see Ribeiro-da-Silva, A., 2004 for review).

The structure and synaptic organization of the *en passant* and terminal varicosities of non-peptidergic afferents residing in the SG have been described in great detail as they form the central or core element of synaptic glomeruli (Coimbra, A., Magalhães, M.M., Sodré-Borges, B.P., 1970; Coimbra, A., Sodré-Borges, B.P., Magalhães, M.M., 1974; Knyihár E, Gerebtzoff, M.A., 1973; Cruz, F. Lima, D. Zieglgansberger,W. Coimbra, A., 1991), which were later classified as being of type Ia (see Ribeiro-da-Silva A. 2004). A striking feature of the SG is the dominant presence of synaptic glomeruli in the ventral 2/3 of the lamina (Ribeiro-da-Silva, A. 2004). The frequency of synaptic glomeruli within the SG is far greater than in lamina I in the rodent (Ribeiro-da-Silva, A. 2004). Approximately 80% of primary afferent axon terminals within LII form the core of type I_a glomeruli. Moreover, the outer part of LII contains few type I_a glomeruli whereas they are abundant in the middle third of lamina II (Ribeiro-da-Silva, A., Coimbra, A., 1982; Ribeiro-da-Silva, A., 2004).

In type Ia synaptic glomeruli the scalloped-shaped central bouton (CI_a), approximately 1.5-2.5 μm in diameter, contains an abundant supply of round agranular synaptic vesicles of varying diameter (35-70nm) (Coimbra, A, Sodré-Borges, B.P., Magalhães, M.M., 1974; Ribeiro-da-Silva, A., Coimbra, A.., 1982). Mitochondria are sparse, limited to a maximum of 2 or 3 within each central terminal profile. Apposed dendritic and axonal profiles consist of regular

dendrites devoid of synaptic vesicles (D) as well as vesicle-rich or pre-synaptic dendrites (Ribeiro-da-Silva, A., 2004). Asymmetrical synapses between the central bouton and contiguous dendrites (D) are common in synaptic glomeruli (Ribeiro-da-Silva, A., 2004). Pre-synaptic dendrites (denoted as V1 terminals) often establish postsynaptic contacts with the central bouton at asymmetrical synapses. However, V1 profiles also establish presynaptic relationships with the central terminal and with adjacent dendrites at symmetrical synapses. In contrast, in type Ia glomeruli, the less frequent V_2 terminals containing abundant round and flattened vesicles of variable sizes have been interpreted as axonal boutons from local circuit neurons. These V_2 profiles are pre-synaptic to the central terminal at symmetrical synapses and are also pre-synaptic to other dendrites; however they are never post-synaptic to the central bouton. A diagrammatic representation of type Ia synaptic glomeruli is shown in figure 6.

Of noteworthy mention is the near complete absence of large granular vesicles contained within these CI_a terminals (figure 6). The paucity of DCV suggests that they do not express the neuropeptides common to peptidergic CI_b terminals. The neurochemical characterization of the CI_a terminal confirms the absence of neuropeptides, making these terminals indeed, non-peptidergic (see below: Neurochemistry). Furthermore, the peptidergic $C1_b$ boutons are never postsynaptic to V_1 or V_2 profiles, unlike the type $_{CIa}$ terminals which are prevalent in lamina II. In addition to the high content of type I_a synaptic glomeruli, a number of *en passant* oval varicosities from myelinated afferents enter lamina IIi ventrally and form flame-shaped arbors. Thin, unmyelinated undulating fibres rich in mitochondria can also be observed in deep lamina Iii. Varicosities derived from these fibres possess a sinusoid contour encompassing round, consistent sized synaptic vesicles and infrequent large granular vesicles (see Cruz, F. Lima, D. Zieglgansberger, W., Coimbra, A., 1991 for review).

Differing in structural properties and location, type CII synaptic glomeruli possess a round central bouton larger in size than their CI counterpart. Abundant in mitochondria and loosely packed synaptic vesicles, these CII glomeruli are localized to the ventral-most ⅓ rd of lamina II as well as in lamina III and are thought to be the central terminations of non-nociceptive primary sensory fibres

(Ribeiro-da-Silva, A., Coimbra, A., 1982; for review see Ribeiro-da-Silva, A., 2004).

The primary afferent origin of all synaptic glomeruli has been determined by dorsal root transection experiments (Coimbra, A., Sodré-Borges, B.P., Magalhães, M.M., 1974, Coimbra et al., 1984). Subsequent to dorsal root transaction, the central bouton of the synaptic glomeruli demonstrated signs of degeneration. These include the delayed deterioration of synaptic connections with adjacent profiles and the clustering of synaptic vesicles to the centre of the terminal (Coimbra, A., Ribeiro-da-Silva, A., Pignatelli, D., 1984).

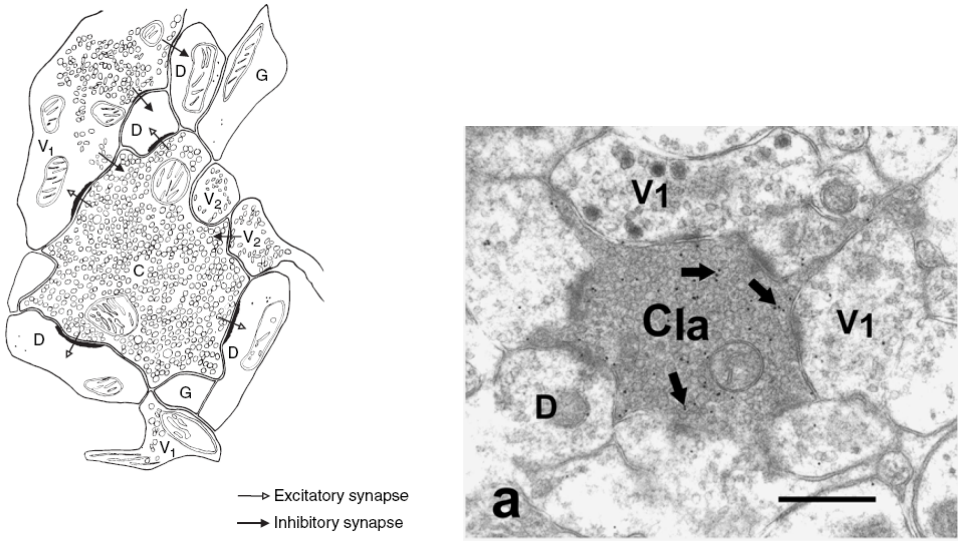


Figure 6 *Central Terminal of a CIa glomerulus*

Picture on the left represents a diagram of a type Ia synaptic glomerulus. Note the complex synaptic architecture. C, central bouton; V1, presynaptic dendrites; D, regular dendrites; V2, peripheral axonal bouton; G, glia. Picture on the right represents an electron micrograph of the same glomerular type processed for the demonstration of glutamate immunoreactivity (arrows indicate gold particles representing glutamate sites in the central bouton – CI_a).

Courtesy of Dr. A. Ribeiro-da-Silva

1.4.2 Neurochemistry.

Acid phosphatase (AP) is a hydrolase enzyme typically detected in the lysosomal compartment of the cell. The acidification of the endosome following fusion with the lysosome permits the AP enzyme to catalyze of an orthophosphoric monester into alcohol and hydrogen phosphate. In sensory ganglia, extra-lysosomal AP has been detected in a subset of small neurons

H. J Colmant, in the late 1950's, discovered the presence of AP in small dark DRG neurons (Colmant, H.J., 1959). Ultrastructural analysis determined the topography of AP reaction product in synaptic glomeruli the SG; localized within the middle one third of lamina II (Coimbra, A., Sodré-Borges, B.P., Magalhães, M.M., 1974; Ribeiro-da-Silva, A., Castro-Lopes, J.M., Coimbra, A., 1986). Incubations of spinal cord sections in sodium fluoride solutions prior to initiation of the AP reaction demonstrated the resistance of the enzyme to these ions, prompting the name fluoride resistant acid phosphatase (FRAP) (Knyihár, E. and. Gerebtzoff. M. A., 1973). At the ultrastructural scale, FRAP appears as an electron dense precipitate in most synaptic glomeruli; 89% of C_{Ia} terminals residing in the intermediate zone of lamina IIi, were FRAP-positive (Nagy, J.I., Hunt, S.P., 1982; Ribeiro-da-Silva, A., Castro-Lopes, J.M., Coimbra, A., 1986; Molander et al., 1987; Silverman, J.D., Kruger, L. 1988b). FRAP enzymatic precipitate was localized around synaptic vesicles and along the perimeter of the axon membrane (Ribeiro-da-Silva, A., Castro-Lopes, J.M., Coimbra, A., 1986). Dorsal root transection results in the time-dependent decrease in AP reaction product, which appeared in degenerated CI_a terminals, clustered amongst decaying synaptic vesicles (Coimbra, A., Sodré-Borges, B.P., Magalhães, M.M., 1974). Recently, it was identified that FRAP seems to be indeed the transmembrane isoform of prostatic acid phosphatase (Zylka, M. J., Sowa, N. A., Taylor-Blake, B, Twomey, M. A., Herrala, A., Voikar, V and Vihko P., 2008).

The arduous histochemical identification of CI_a, non-peptidergic terminals by way of FRAP enzymatic reaction product yielded to the less cumbersome method

of lectin-binding. Complex oligosaccharide structures composed of specific side chain epitopes were discovered to be differentially expressed by identified subpopulations of sensory neurons (Dodd, J., Jessell, T.M., 1985). Specifically, two types of lactoseries carbohydrate backbone structures, differentiated by their binding to monoclonal antibodies. N-acetyl-lactosamine type 2 was expressed by 45% of small and medium diameter sensory neurons. In the spinal dorsal horn, monoclonal antibodies directed to type 2 oligosaccharide structures (antibody A5) labelled a dense plexus of PAFs in the intermediary region between LII_A and LII_{Bd} (Dodd, J., Jessell, T.M., 1985). Furthermore, monoclonal antibodies raised against distinct side chain epitopes demonstrated the neuron-specific expression of particular carbohydrate antigens. The monoclonal antibody LA4 identified neurons expressing the carbohydrate side chain *Galactose*α1-*Galactose*β1-4*N-acetylglucosamine* (Galα1-3Galβ1-4GlcNAc-R). Double-labelling with FRAP determined the co-expression of LA4 with the enzyme reaction product. Greater than 75% of DRG neurons labelled with LA4 were FRAP-positive.

In lieu of monoclonal antibodies, plant-derived lectins, known to bind with a high degree of specificity to cell surface glycoprotein structures can be conjugated to either horseradish peroxidase (HRP) or a fluorochrome. Lectins isolated from the seeds of an African shrub of the binomial name *Bandeiraea Simplicifolia (BS)* are composed of four glycoprotein structures of varying combinations. Type B BS lectins display potent specificity for α-D-galactopyranosyl residues. Five isolectins from the B family have been classified. Isolectin $I\text{-}B_4$ (IB4) has been determined to bind preferentially to α-D-galactose residues, specifically to monosaccharide chains. Non-specific binding to longer side chain neoglycoconjugate di- and tri-saccharides can be isolated and removed by treating tissue samples with 1% aqueous periodic acid (Streit, W.J., Schulte, B.A., Balentine, D.J., Spicer, S.S., 1985). Histochemical analysis of galactose-glycoconjugates in sensory neurons of the rat using a variety of lectins including IB4 observed IB4-binding in the small dark B cell population in the DRG (Streit, W.J., Schulte, B.A., Balentine, D.J., Spicer, S.S., 1985). Approximately 50% of DRG neurons bind IB4 (Bergman, E., Carlsson, K., Liljeborg, A., Manders, E., Hökfelt, T., Ulfhake, B., 1999). Appraisal of lectin-bound profiles in the rat spinal

cord demonstrated the presence of IB4-binding afferent terminals in the SG. Furthermore, IB4 was determined to bind to glial profiles in the grey and white matter of the spinal cord (Streit, W.J., Schulte, B.A., Balentine, D.J., Spicer, S.S., 1985; Nakagawa, F., Schulte, B.A., Spicer, S.S., 1986). Additionally, IB4-binding was observed in the Nodes of Ranvier of myelinated afferents (Streit, W.J., Schulte, B.A., Balentine, D.J., Spicer, S.S., 1985; Nakagawa, F., Schulte, B.A., Spicer, S.S., 1986).

J.D Silverman and L. Kruger (1990) conducted thorough analysis of α-D-galactose- and GlcNAc-expressing peripheral autonomic, gustatory, visceral and enteric neurons, with particular attention paid to their co-expression with FRAP and CGRP. Comparative analysis of IB4-binding, FRAP enzyme reaction product and CGRP-immunoreactivity was conducted using serial sections of the selected tissues. In the DRG, IB4-binding was localized to a subpopulation of small neurons (Silverman, J.D, Kruger, L., 1990). Lectin-binding was confined to the Golgi cisternae, the plasmalemma and the cytoplasm. Earlier results by Nakagawa et al. (1986) reported a similitude of IB4-binding in the rat trigeminal ganglion (TG). Substantial co-localization of IB4 with FRAP-precipitate was observed. Comparisons to CGRP-immunoreactivity demonstrated the differential labeling of separate neuronal subpopulations; with only 10% of the neurons examined co-labelled with both CGRP-immunoreactivity and IB4-binding (Silverman, J.D, Kruger, L., 1990). Observations in the spinal cord revealed the presence of rather diffuse IB4-binding in the SG. FRAP precipitate presented as a refined band in the inner aspect of the SG. CGRP-immunoreactivity, in contrast was primarily located lamina I and outer lamina II. Fine structural analysis of localization of IB4-binding in the SG confirmed the presence of IB4-specific lactoseries carbohydrate complexes in C_{Ia} axon terminals (Kitchener et al., 1993; Gerke, M.B., Plenderleith, M.B., 2004). Furthermore, unlabelled D, V_1 and V_2 contiguous profiles indicated that IB4 does not cross synapses (Gerke and Plenderleith, 2004).

IB4-binding is not restricted to primary sensory neurons. Examination of the lectin-binding galactose and N-acetylglucosamine carbohydrates in autonomic

ganglia revealed the small presence of IB4-binding neurons in the superior sympathetic ganglion. In the parasympathetic sphenopalatine ganglion, numerous perisomatic axons bound IB4; similar to observations made in the otic and ciliary ganglia (Silverman, J.D, Kruger, L., 1990). Additionally, the authors report abundant IB4-labelled neurons in the gustatory geniculate ganglion which also labelled positive for FRAP precipitate. These observations are unique as later studies confine the neuronal expression of IB4-specific glycoconjugates to sensory neurons projecting predominantly to the integument (Plenderleith, M.B., Snow, P.J., 1993)

Neurochemical expression surveys of C-fibre neurons indicating the co-expression of neuropeptides with IB4-binding created the dialectic of "peptidergic" and "non-peptidergic" phenotyping. The debate surrounds the issue as to the degree of IB4-binding in primary afferent terminals which express sP or CGRP. A recent publication by Price and Flores (2006) reported a significant degree of co-localization of sP and CGRP with IB4-binding in both the DRG and the TG. Approximately 45% of CGRP-immunoreactivity neurons also labelled IB4-positive. A smaller population of sP-immunoreactivity neurons co-localized with IB4-binding (29.8 ± 2.86%) in the adult rat DRG. Approximately 30 and 20 % of IB4-binding neurons also displayed CGRP-immunoreactivity and SP-immunoreactivity respectively (Price, T.J and Flores, C.M., 2007). In the TG, neuropeptide overlap with IB4-binding was apparent in fewer neurons than in the DRG. In the TG, 27.5 ± 1.44% of CGRP-immunoreactivity neurons and 30.1± 4.05% of sP neurons were also IB4-positive (Price, T.J and Flores, C.M., 2007). The percentage of IB4-positive neurons displaying CGRP-immunoreactivity was similar to that observed in the DRG with 24.37 ± 4.66 % of the neurons co-expressing both neuronal markers. However, discordant with the DRG, fewer TG IB4-binding neurons (12.9 ± 3.11 %) exhibited sP-immunoreactivity (Price, T.J and Flores, C.M., 2007).

A comprehensive evaluation of the neurochemical phenotype of sensory neuron populations in young adult and aged (30 months) rats conducted by Bergman and compeers (1999) reported the significant co-localization of sP and CGRP in IB4-

labelled neurons. In young rats, 53% and 20% of IB4-binding neurons were CGRP- and sP-positive respectively (Bergman, E., Carlsson, K., Liljeborg, A., Manders, E., Hökfelt, T., Ulfhake, B., 1999). Of the total population of CGRP-immunoreactivity neurons in the DRG (43 ± 1.34%), 59% expressed the IB4-binding phenotype. Similarly, 55% percentage of sP neurons (17 ±1.80 % of DRG neurons labelled), were also IB4-positive. The extensive overlap described in these studies is in stark contrast to data reported from the laboratories of J.V Priestley (1991) and J.D Silverman and L. Kruger (1988a, 1990). Overlap of CGRP-immunoreactivity with the monoclonal antibody LA4 in the TG was observed in a mere 8% of the total neurons examined (Alvarez, F.J., Morris, H.R., Priestley, J.V., 1991). Silverman and Kruger (1990) documented that only 10% of CGRP-immunoreactivity neurons in the DRG co-labelled with IB4 (Silverman, J.D, Kruger, L., 1990). Moreover, sensory innervation of the rat meninges is supplied by CGRP-immunoreactivity afferents conjointly with IB4-binding fibres; decidedly asunder populations supported by their disparate target structures (blood vessels and meninges respectively) (Soygüder, Z., 1999). Furthermore, Remak bundles (Schwann cell-ensheathed unmyelinated C-fibre bundles assembled within a single basal lamina) are comprised of separate CGRP-and IB4-positive axons (Murinson, B.B., Hoffman, P.N., Banihashemi, M.R., Meyer, R.A., Griffin, J.W., 2005).

In an electrophysiological survey of 24 small diameter sensory neurons, Petruska and associates identified a population of IB4-binding neurons that did not express sP or CGRP (of the 20 neurons of this type (Type 2 neurons) that were neurochemically characterized, all 20 were distinctly non-peptidergic) (Petruska, J.C., Napaporn, J., Johnson, R.D., Gu, J.G., Cooper, B.Y., 2000).

The divergent reports regarding the specificity of the lectin IB4 for primary afferent neurons dearth of neuropeptides is disconcerting. Isolectin B_4 displays marked specificity to Galα1-3 and Galα1-4 Galβ1-4GlcNAc (Wu, A.M., Song, S.C., Wu, J.H., Kabat, E.A., 1995). Primary afferent terminals in lamina I and dorsal lamina II bind lectins bearing affinity (yet not exclusively) for Galβ1-4GlcNAc (Dodd and Jessell, 1985). Overlap of IB4-binding with neuropeptides could be the result of the presence of Galα1-3Galα1-4GlcNAc-R glycoconjugates

in low concentrations on peptide-expressing neurons, accounting for weak IB4-binding to peptide-containing neurons. The greater the density of cell surface α-D-Galactosides, the more intense the labelling with IB4 (Kirkeby, S and Moe, D., 2001, 2002). Other factors that influence the binding of lectins to their preferred carbohydrate are temperature (high temperatures enhance binding) and the presence of divalent ions (higher concentration of the necessary divalent ions promote binding) (Kirkeby, S and Moe, D., 2001, 2002).

The immunocytochemical analyses presented in this thesis concur with studies reporting limited overlap of IB4 with neuropeptides. In the DRG, there is no apparent overlap observed in our immunocytochemical studies. Similarly, in the spinal cord, IB4 labelled a distinct population of axon terminals from sP. In regards to CGRP, a narrow band of overlap could be discerned at the LIIo/LIIi border; the territory of closely apposed axon terminal from both neuronal populations.

As previously mentioned, approximately half of the C-fibre neuronal population expresses the NGF receptor TrkA and the low affinity receptor p75. The residual half responds to GDNF, expressing both the C-Ret receptor and GFRα1 (Molliver, D.C, Snider, W.D., 1997). Cytochemical analysis combined with *in situ* hybridization has elucidated the selective co-expression of TrkA mRNA in peptidergic sensory neurons (Averill, S., McMahon, S.B., Clary, D.O., Reichardt, L.F., Priestley, J.V., 1995). It has been reported that 95% of TrkA-immunoreactivity neurons expressed CGRP whereas only 14% bound IB4 (Averill, S., McMahon, S.B., Clary, D.O., Reichardt, L.F., Priestley, J.V., 1995). Moreover, IB4-binding neurons display specificity for C-Ret expressing neurons (Bennett, D.L., Michael, G.J., Ramachandran, N., Munson, J.B., Averill, S., Yan, Q., McMahon, S.B., Priestley, J., 1998). Bennett and associates report that 95% of IB4-binding neurons in the DRG are C-Ret-positive. Furthermore, 79% of C-Ret neurons are capable of binding IB4 (Bennett, DL. Michael, GJ. Ramachandran, N. Munson, JB. Averill, S. Yan, Q. McMahon, SB. Priestley, J.V., 1998). In nerve lesion studies, GDNF was capable of preventing the loss of IB4-binding in the SG (Bennett, DL. Michael, GJ. Ramachandran, N. Munson, JB. Averill, S. Yan, Q.

McMahon, SB. Priestley, J.V., 1998). These studies indicate that IB4-binding neurons are exclusively responsive to GDNF, whereas, peptidergic sensory neurons are supported by NGF. Indeed, Molliver and colleagues demonstrate the early post-natal shift in nutritive support from NGF (TRKA-expressing neurons) to GDNF (C-Ret-expressing neurons) was co-incidental with IB4-binding neurons in the DRG (Bennett, DL. Michael, GJ. Ramachandran, N. Munson, JB. Averill, S. Yan, Q. McMahon, SB. Priestley, J.V., 1998).

Adenosine 5'-triphosphate (ATP) can be considered the molecular currency of energy transfer in the cell. In the nervous system, ATP is released from synapses by an activity-dependent mechanism. Purinergic receptors with high affinity for ATP have been identified on sensory neurons and in the spinal cord (Jahr CE, Jessell TM, 1983; Fyffe, R.E., Per,l E.R., 1984; Li, J., Perl, E.R., 1995). The ionotropic P2X family of purinergic receptor contains 7 members. In the interest of nociception, the $P2X_3$ receptor is particularly alluring. This non-selective, calcium permeable cation channel is allocated to small diameter, nociceptive DRG neurons (Chen, Y., Li, G.W., Wang, .C, Gu, Y., Huang, L.Y., 2005; Burnstock G, Wood JN., 1996). Co-expression studies have demonstrated the near exclusive expression of $P2X_3$ receptors on IB4-binding sensory neurons (Vulchanova, L., Riedl, M.S., Shuster, S.J., Stone, L.S., Hargreaves, K.M., Buell, G., Surprenant, A., North, R.A., Elde, R., 1998; Petruska, J.C., Cooper, B.Y., Gu, J.G., Rau, K.K., Johnson, R.D., 2000). In the rat DRG, 94% of $P2X_3$-immunoreactivity neurons have been shown to bind IB4. Diminutive co-expression with sP and SOM (3% and 7% respectively), as well as CGRP, further demonstrates their restricted expression (Vulchanova, L., Riedl, M.S., Shuster, S.J., Stone, L.S., Hargreaves, K.M,, Buell, G., Surprenant, A., North, R.A., Elde, R., 1998; Petruska, J.C., et al., 2000). In the superficial dorsal horn, $P2X_3$-immunoreactivity is localized to lamina IIi, reminiscent of the regional binding of the IB4 lectin. Neonatal capsaicin treatment and dorsal rhizotomy resulted in the significant decrease of $P2X_3$-immunoreactivity in the SG (Vulchanova, L., Riedl, M.S., Shuster, S.J., Stone, L.S., Hargreaves, K.M,, Buell, G., Surprenant, A., North, R.A., Elde, R., 1998). The $P2X_3$ receptor displays rapid activation and

deactivation in the presence of ATP (Petruska, J.C., Napaporn, J., Johnson, R.D., Gu, J.G., Cooper, B.Y., 2002).

Evidence indicates that endogenous ligand for the purinergic receptor $P2X_3$, ATP operates as a co-transmitter. The exocytotic release of ATP from synaptic vesicles with other neurotransmitters such as glutamate and γ-aminobutyric acid (GABA) suggests that ATP functions as a co-transmitter and neuromodulator (Burnstock, G., 2007). The combined release of excitatory ATP and the inhibitory GABA is demonstrative of the delicate balance of excitation and inhibition in the central nervous system (see below).

Once released, ATP can be rapidly metabolized in the synaptic cleft by extracellular, membrane-bound ectonucleotidases (Burnstock, G., 2007). Adenosine released from non-peptidergic afferent terminals exerts its effect by binding to pre- and postsynaptic adenosine (A) receptors. Of particular interest is the adenosine 1 (A1) receptor. Activation of this GPCR results in postsynaptic inhibition mediated by hyperpolarization of the postsynaptic cell by activation of potassium (K^+) channels (Sawynok, J and Liu, X.J., 2003). Activation of the A1 receptor is thought to contribute to spinal analgesia, demonstrated by the use of receptor agonists and antagonists. A1 receptor immunoreactivity is distributed throughout the spinal cord; however, dense labelling is remarkably apparent in LIIi, in close apposition to IB4-binding primary afferent terminals (Sawynok, J and Liu, X.J., 2003; Schulte, G., Robertson, B., Fredholm, B.B., DeLander, G.E., Shortland, P., Molander, C.., 2003). The metabotropic glutamate receptors mGluR1 and mGluR2/3 were found in a subset of A1-positive neurons (Schulte et al., 2003).

Akin to peptidergic neurons, IB4-binding, non-peptidergic neurons express the TRPV1 receptor. In the superficial dorsal horn of the rat, intense TRPV1immunoreactivity is strikingly apparent in LIIi. Co-expression studies determined the co-localization of TRPV1 with IB4 in LIIi. Moreover, a significant number of TRPV1 receptors were co-expressed with $P2X_3$ in inner lamina II (Guo, A., Vulchanova, L., Wang, J., Li, X., Elde, R., 1999). Of the 50% of small diameter neurons in the DRG that were TRPV1immunoreactive, 75 and 78% co-

label with IB4 and $P2X_3$ respectively (Guo, A., Vulchanova, L., Wang, J., Li, X., Elde, R., 1999).

When stimulated with capsaicin, IB4-binding cultured DRG neurons demonstrated robust inward currents (Liu, M., Willmott, N.J., Michael, G.J., Priestley, J.V., 2004). The currents produced are greater in magnitude and amplitude in comparison to those elicited in IB4-negative neurons. Moreover, the kinetic profiles of the currents elicited by the application of capsaicin were remarkably different than those produced in non IB4-binding neurons (Liu, M., et al., 2004). The majority of IB4-positive neurons displayed sustained inward currents sensitive to capzasepine. The capsaicin induced increase in intracellular Ca^{2+} was greater in IB4-positive neurons than in IB4-negative neurons (Liu, M., et al., 2004). Moreover, the authors found that the magnitude of TRPV1immunoreactivity was greater in IB4-binding cells.

1.4.3 Synaptic interaction in the superficial dorsal horn.

The unique architecture of the non-peptidergic type Ia synaptic glomerulus, particularly the postsynaptic relationship with presynaptic axons and dendrites (see figure 6), has prompted researchers to consider the functional implications on nociceptive transmission in the superficial dorsal horn (Ribeiro-da-Silva, A., 2004; Ribeiro-da-Silva, A. and De Koninck, Y, 2007).

Understanding the nature of this relationship may enlighten our current comprehension of the avocation of non-peptidergic sensory neurons.

The neurochemical identity of the dendrites and axons forming presynaptic connections with IB4-binding CIa glomerular terminals have been ascertained as being inhibitory; containing GABA alone or co-localized with enkephalin (ENK) or acetylcholine (ACh) (Todd, A.J., Lochhead, V., 1990; Ribeiro-da-Silva, A., 2004)

Whole cell voltage-clamp recordings from LIIo and LIIi neurons stimulated by application of capsaicin or by direct electrical stimulation of attached dorsal roots produced interesting results. Capsaicin application elicited glutamatergic miniature excitatory postsynaptic currents (mEPSC) in both LIIo and LIIi neurons, however the amplitude and frequency of recorded mEPSCs from LIIo

neurons was significantly increased in the presence of capsaicin compared to neurons in LIIi (Pan, Y.Z., Pan HL., 2004). Furthermore, the peak amplitude of EPSCs produced by electrical stimulation of dorsal roots was higher in LIIo neurons than those recorded from LIIi neurons. These results are indicative of the presynaptic inhibition of CI_a terminals residing in inner lamina II.

1.4.4 Functional implications in the processing of acute and chronic pain.

Studies of the electrophysiological properties of IB4-binding DRG neurons confirmed their nociceptive nature. Small diameter sensory neurons (≤ 26µm) exhibited a characteristic inflection in the descending phase of the action potential; a property of nociceptive neurons (Koerber, H.R., Druzinsky, R.E., Mendell, L.M., 1988; Traub, R.J., Mendell, L.M., 1988; Gold, M.S., Shuster, M.J., Levine, J.D., 1996). Stucky and Lewin (1999), in a study analyzing the electrophysiological features of IB4-positive and negative DRG neurons from mouse, observed this inflection in 89% of IB4-binding neurons (Stucky, C.L. and Lewin, G.R., 1999). In vivo cellular recordings from IB4-positive and negative neurons in rats further differentiated the two populations; IB4-positive neurons exhibited slow-rising, longer duration action potentials (Fang, X., Djouhri, L., McMullan, S., Berry, C., Waxman, S.G., Okuse, K., Lawson, S.N., 2006). Moreover, IB4-positive neurons displayed slower conduction velocities and possessed membrane potentials that were -10mv (median) more negative than IB4-negative neurons. The sodium channels $Na_v1.8$ and $Na_v1.9$ were also differentially expressed with $Na_v1.9$ channels demonstrating greater intensity on IB4-binding neurons (Fang, X., et al., 2006).

The expression of the ion channels $P2X_3$ and TRPV1 are evidence of the involvement of the IB4-binding, non-peptidergic primary afferent in nociception. The effects of peripheral nerve injury on these afferents have been examined at length (Chen, Y., Li, G.W., Wang, C., Gu, Y., Huang, L.Y., 2005; Meisner JG, Reid AR, Sawynok J., 2008). At present, it is clear they are important in the transmission of pain-related signaling by the effects of antagonist for both ion channels; however, the exact nature of this role is still in the stages of conjecture

(McGaraughty, S., Wismer, C.T., Zhu, C.Z., Mikusa, J., Honore, P., Chu, K.L., Lee, C.H., Faltynek, C.R., Jarvis, M.F., 2003; McGaraughty, S., Jarvis, M.F., 2005; Roberts and Connor, 2006).

The effects of dorsal root transection on non-peptidergic primary afferent fibres by the examination of FRAP precipitate has been evaluated at length. FRAP reaction product undergoes nascent dissipation within 24 hours. FRAP precipitate was localized to the plasma membrane of the central bouton in the early stages of deterioration. Later, FRAP-activity dissipated throughout the terminal. At the ultrastructural level, incipient degeneration of CI_a terminals was apparent within 20 hours of dorsal root transection or DRG removal (Knyihár E, László I, Tornyos S., et al., 1974). At the onset of atrophy, osmophilic degeneration bodies, clustering of synaptic vesicles and deteriorated mitochondria were evident. In the nascent stages of atrophy, synaptic connections to the surrounding dendrites and axon profiles were imperforate. As the terminal underwent deterioration, it became electron dense. Ultimately, the terminal succumbs to glial invasion and engulfment (Knyihár, E., et al., 1974). The dissipation of FRAP-activity was attributed to the cessation in the production of the enzyme in the cell bodies (Knyihár, E and Csillik, B., 1976).

Following peripheral nerve injury, FRAP-activity in the DRG declined within the initial week following sciatic nerve section (Tenser, R.B., 1985). In the second week post-injury, FRAP-activity in the DRG returned to near normal levels. The reappearance of enzymatic activity in the DRG pre-dated the return of the enzyme in the SG (Tenser, R.B., 1985). By the 40^{th} post-operative day, the onset of restitutive FRAP-activity in the SG could be observed (Csillik, B., and Knyihár-Csillik, E., 1981). The origin of the resurgent enzyme is of great significance; it may represent invading primary sensory axons sprouted from lamina I or III now capable of synthesizing the enzyme or, it may be indicative of the regeneration of decayed CI_a terminals. The latter hypothesis was supported by the emergence of growth cones derived from primary afferent axons re-initiating synaptic associations with intrinsic SG neurons (Knyihár-Csillik E, Csillik B., 1981). The infliction of a second nerve injury (transection) subsequent to the completion of synaptogenesis in the SG following the initial nerve trauma (crush)

demonstrated the primary afferent origin of the recrudescent FRAP-positive terminals (Csillik, B and Knyihár-Csillik, E., 1981). Crushing of the sciatic nerve produced the same degenerative processes observed resultant to sciatic nerve transection (Csillik, B and Knyihár-Csillik, E., 1981). The temporal pattern of atrophy was comparative to transection. The spatial regeneration of FRAP-activity was initiated medially (at the midline) and proceeds laterally (Csillik, B and Knyihár-Csillik, E., 1981).

Consonantly, nerve transection or crush elicited the prompt disappearance and gradual restoration of IB4-binding in the SG. Molander and colleagues (1996) reported this phenomenon resultant to nerve transection and nerve crush. The temporal kinetics of the decline and resurgence of IB4-binding in transection differed from nerve crush (Molander, C., et al., 1996). One week following transection of the sciatic nerve, IB4-binding in the SG was in the initial stages of waning. By the second week, the decline in IB4-binding in the SG peaks; remaining in a diminutive state for as long as 20 weeks (Molander, C., et al., 1996). Akin to FRAP-activity, a remigration of IB4-binding was observed; however, the incipience of IB4-binding in the SG was a protracted process; 8 months after nerve transection the intensity of IB4-binding had increased considerably, yet remains below control levels (Molander, C., et al., 1996). The onset of the decline in IB4-binding following nerve crush was comparable to nerve transection; within two weeks, IB4-binding was significantly diminished. Per contra to nerve transection, however, the intensity of lectin labelling in the SG has approached control levels within 10 weeks of the injury (Molander, C., et al., 1996).

In a modified model of chronic constriction injury (CCI) whereby a fixed-diameter polyethylene (PE) cuff of standard length is placed over the sciatic nerve, our laboratory demonstrated a decline in IB4-binding in LIIi in the L4 lumbar spinal cord as early as 5 days after cuff application (Bailey, AL and Ribeiro-da-Silva, A., 2006). Restored IB4-binding in the SG reached control levels three weeks from the date of surgery; pre-dating the observed recovery reported by Molander and associates (Molander, C., et al., 1996; Bailey, AL and Ribeiro-da-Silva, A., 2006; see chapter 2 of this thesis).

Fine structural examination of CI_a varicosities at various time intervals subsequent to cuff application revealed the same degenerative atrophy reported by Knyihár et al (1974); clustered synaptic vesicles, electron dense bodies and the dissolution of synaptic contacts with adjacent dendrites and axons were apparent (Bailey, AL and Ribeiro-da-Silva, A., 2006). Moreover, CI_a terminals examined 15 and 21 days post-surgery displayed evidence of regeneration; growth cone-like structures could be observed not unlike those reported by Knyihár and colleagues (1974).

As the onset of the decline in lectin-binding and FRAP-activity was comparable in all studies, the mechanism of this phenomenon appears to be independent of the nerve injury performed. However, the reported variations in restored binding and enzyme activity may be indicative of model-dependent mechanisms in regeneration. Transection of the sciatic nerve separates the cell bodies in the DRG from their peripheral terminals whereas crush and constriction of the nerve compromises axon transport. *In vitro* studies have demonstrated the importance of growth factors on neuronal regeneration (Tucker, B.A., Rahimtula, M., Mearow, K.M.., 2006).

The number of IB4-binding neurons in the DRG following the induction of various types of nerve injury (rhizotomy, nerve ligation, nerve transection and combinations of rhizotomy with nerve transection or ligation) was examined (Guseva, D and Chelyshev Y., 2006). When the DRG were examined 30 and 60 days after rhizotomy, ligation and transection of the peripheral nerve, there was a marked decrease in the total number of IB4-positive neurons in the L5 DRG. The combined injuries of rhizotomy and ligation or transection resulted in a further decrease in the number of neurons binding IB4 (Guseva, D and Chelyshev Y., 2006). These results were supported by Hammond and colleagues (2004). Spinal nerve ligation effectively eliminated IB4-binding in the L5 DRG one week after surgery (Hammond, D.L., Ackerman, L., Holdsworth, R., Elzey, B., 2004). As observed in the SG, restoration of IB4-binding in the L5 DRG was apparent 20 weeks later (Hammond, D.L., Ackerman, L., Holdsworth, R., Elzey, B., 2004). Axotomy resulted in a 50% decrease of $P2X_3$ expression in the L4 and L5 DRG (Bradbury, E.J, Burnstock, G., McMahon, S.B et al., 1998). Intrathecal

application of exogenous GDNF reversed the effects of axotomy on $P2X_3$ expression (Bradbury, E.J., et al., 1998). In a spinal nerve ligation model (L5/L6 spinal nerve ligation), the number of neurons responding to the ATP analog αβ-methyl-ATP was significantly reduced (Kage, K., Niforatos, W., Zhu, C.Z., Lynch, K.J., Honore, P., Jarvis, M.F., 2002). Similar to the effects of rhizotomy, $P2X_3$ expression diminished following ligation of the spinal nerves (Kage, K., Niforatos, W., Zhu, C.Z., Lynch, K.J., Honore, P., Jarvis, M.F., 2002). These studies illustrate the effects of peripheral nerve injury on non-peptidergic afferents. However, they do not provide information regarding the exact role they play in the pathogenesis of chronic or acute pain. Cytotoxic agents, when conjugated to specific neuronal ligands, may be the key to solving the mystery.

Ribosome inactivating proteins (RIP) are cytotoxic compounds which exert their effect on the large ribosomal subunit. By removing a crucial adenine residue, these proteins render the ribosome unable to continue protein synthesis culminating in cell death by apoptosis (Wiley, R.G, Kline, IV R.H, 2000; Wiley RG, Lappi DA, 2003). An example of such a RIP is saporin, an endoglycosidase purified from soapwort *Saponaria officinalis*.

Numerous Saporin conjugates have been synthesized, including IB4-Saporin (IB4-SAP) (See section 1.7.2 for details). The advent of axonally transported targeted cytotoxic compounds is providential; indeed, the selective lesioning of a targeted population of neurons bestows the opportunity to examine the consequence of such lesion on the organism, thus assisting in the determination of their function.

Vulchanova and colleagues (2001) demonstrated the effects of IB4-SAP in a published report in *Neuroscience*. The study, a behavioural and morphological assessment of the effects of the absence of IB4-positive neurons, revealed a delayed transitory increase in acute pain thresholds to mechanical and heat stimuli. This transitory effect is interesting, if not confusing; suggesting that in this brief period (4-5 days), other sensory fibres are not responsible for the transmission of acute mechanical or heat stimuli. The return of the pain sensitivity levels to near baseline further suggest that acute pain sensitivity is mediated by some compensatory mechanism. What did this study reveal about non-peptidergic

afferents? It would seem that they may be responsible for the transduction of acute heat and mechanical impulses from the periphery. Could it possible that non-peptidergic sensory neurons are the sole afferent pathway designated to impart acute noxious information; and in their absence, compensatory alterations (central or peripheral) are capable of returning sensitivity thresholds to homeostatic conditions? These questions warrant answers if we are to understand not only the function of non-peptidergic sensory neurons, but the nature and ability of compensatory modifications.

1.5 Homeostatic balance of excitation and inhibition in the superficial dorsal horn.

Like a metronome keeping pace for the pianist, inhibitory and excitatory interneurons in the superficial dorsal horn maintain a certain level of synaptic activity in resting conditions. In the event of a shift towards excitation or inhibition, synaptic activity may be enhanced or suppressed resulting in a state of central hyper-excitability or hypo-excitability respectively; akin to a pianist off count by an over or under paced metronome if you will. The neurochemical players consist of the excitatory amino acid glutamate, the neuropeptide sP, and the purine ATP (Jahr CE, Jessell TM, 1983; Fyffe, R.E., Perl, E.R. , 1984; Hökfelt, T., 1991). Inhibition is mediated by GABA, glycine, and opioid peptides. The location of their respective receptors on primary afferent terminals and intrinsic dorsal horn neurons regulate signal transduction and create an environment in balance.

1.5.1 Lamina I

Although most lamina I neurons are local circuit neurons, many project to higher levels, sending up axons that terminate in several regions of the brain (Marshall et al., 1996; Todd, A.J., McGill, M.M., Shehab, S.A., 2000; Spike, R.C., Puskár, Z., Andrew, D., Todd, A.J., 2003). Regions of the brain that receive input from lamina I projection neurons include the caudal ventrolateral medulla (CVLM), the thalamus, the dorsal reticular nucleus (DRt), the medullary reticular formation

(MRF), the parabrachial nucleus (PBN) and the periaqueductal grey (PAG). It appears as though projections from lamina I neurons to brain regions are non-specific; neurons of particular morphological classes (fusiform, mutipolar, pyramidal) do not exhibit a preference for a specific brain region (Spike, R.C., Puskár, Z., Andrew, D., Todd, A.J., 2003). Yet, previous work from Lima and Coimbra (1989) and Lima and colleagues (1991) are not in agreement with the reported non-specific projection patterns observed by Spike and associates (2003). Per contra to the non-specific projection patterns regarded by Spike, R.C et al (2003), the Lima reports (1989, 1991) assert that fusiform neurons project predominantly to the CVLM or the PBN; 80% of the lamina I neurons retrogradely labelled from the CVLM were fusiform, a stark contrast to the 35% observed by Spike and colleagues; a finding supported by observations from cat lamina I cells labelled from the CVLM by Andrew and associates (Lima, D., Mendes-Ribeiro, J.A., Coimbra, A., 1991; Andrew, D., Krout, K.E., Craig, A.D., 2003; Spike, R.C., Puskár, Z., Andrew, D., Todd, A.J., 2003). Furthermore, pyramidal neurons demonstrated a predilection for projections to the PAG; an observation not shared by Spike et al, who report few neurons projecting to this brain region (Spike, R.C., Puskár, Z., Andrew, D., Todd, A.J., 2003).

Neurons located in deeper regions of the dorsal horn (lamina III-IV) have been shown to express the NK1-receptor (Todd, A.J., McGill, M.M., Shehab, S.A., 2000). These NK1-receptor expressing neurons also contribute significantly to ascending projections to the brainstem as retrograde tracing demonstrated a marked presence of NK1-immunoreactive axons derived from neurons residing in lamina III and IV of the rat dorsal horn in the contralateral lateral reticular nucleus in addition to the contralateral LPBN (Todd, A.J., McGill, M.M., Shehab, S.A., 2000).

Supraspinal projections from the superficial and deep dorsal horn transfer spinally-modulated nociceptive information to brainstem and cortical regions responsible for the cognitive processing of pain (Newman HM, Stevens RT, Apkarian AV., 1996; Price, D.D, 2000, 2002; Willis, WD. Jr., 2007). Ascending pathways are segregated according to their neuroanatomical origin and their functional role. The lateral pain pathway, derived from ascending projections

originating in the superficial and deep dorsal horn, is tasked with communicating nociceptive signals to brainstem, thalamic and cortical nuclei associated with the sensory discriminative aspects of pain (intensity, location) (Bernard, J.F and Besson, J.M., 1990). In contrast, the medial pain pathway, originating primarily from more ventral regions of the dorsal horn (LVII, VIII), terminates in cortical regions associated with fear, anxiety and memory (Treede, R.D., Kenshalo, D.R., Gracely, R.H., Jones, A.K., 1999; Sewards, T.V and Sewards, M.A., 2002).

The lateral pain pathway is comprised of two major ascending tracts derived from the spinal dorsal horn, the lateral and medial spinothalamic tract (lSTT and mSTT respectively) (Willis, W.D., Westlund, K.N., 1997). The lateral spinothalamic tract (lSTT), comprised of long ascending axons originating from neurons located in laminae I, IV-VII, VIII, X and the lateral spinal nucleus of the spinal cord which establish monosynaptic contacts within the thalamus (Trevino, D.L and Carstens, E., 1973, 1975; Carstens, E and Trevino, D.L., 1978; Willis et al., 1979; Giesler, G.J., et al., 1979; Lima, D., Coimbra, A.A., 1980; Burstein, R., Dado, R.J., Cliffer, K.D., Giesler, G.J. Jr., 1991; Lima D., 2007). The majority of these projections are contralateral with a significant ipsilateral contribution from the lateral spinal nucleus and deep dorsal horn laminae (Lima, D and Coimbra, A., 1988). Lesion studies of lateral thalamic nuclei have ascribed the lSTT the role of transmitting nociceptive information to somatosensory nuclei responsible for the sensory discriminative aspect of pain (Bowsher, D., 1957; Melzack, R, Casey, K.L, 1968). In contrast, several aspects of the mSTT illustrate a potential role in the transmission of pain-related information to affective regions of the cortex (Bowsher. D., 1957; Melzack, R, Casey, K.L, 1968) (For review, see Lima D., 2007). The sensory discriminative processing of pain is mediated by several nuclei of the thalamus (ventral posterior lateral (VPL), ventral posterior medial (VPM) and ventral posterior inferior (VPI) in addition to somatosensory regions of the cortex (SI and SII) (Treede, R.D., Kenshalo, D.R., Gracely, R.H., Jones, A.K., 1999; Lima, D., 2007). Input derived from caudal (lumbar) regions of the spinal cord project to the lateral-most aspect of the above mentioned thalamic nuclei whereas the medial region of these nuclei receive axonal input from rostral (cervical) segments. This somatotopic map of incoming information permits

spatial discrimination of encountered stimuli (Lima D, 2007) Furthermore, the small peripheral receptive fields of lSTT neurons (ex: single digits) allows for accurate stimulus localization exemplifying the importance of this pathway for sensory discrimination (Giesler, G.J Jr, Spiel, H.R., Willis, W.D., 1981b). Nociceptive (LI, IV and V) and wide dynamic range (LIV and V) neurons which contribute axons to the lSTT encode for stimulus intensity (Kenshalo Jr., et al., 1979; Ferrington, D.G., Sorkin, L.S., Willis, W.D. Jr., 1986; Surmeier et al., 1986 a, b).

In addition to the lSTT, indirect ascending projections relay through brainstem nuclei (Cechetto, D.F., Standaert, D.G., Saper, C.B. 1985). Retrograde labelling from the lateral parabrachial nucleus has shown the LI, NK1-expressing neurons send projections to this region of the brainstem (Bernard, J.F and Besson, J.M., 1990; Gauriau, C and Bernard, J.F, 2002; Almarestani, L., et al., 2007). Termed the spino-parabrachial pathway, connections from the LPBN to limbic centers such as the amygdala evince a role in the affective aspect of pain (Bernard, J.F and Besson, J.M., 1990).

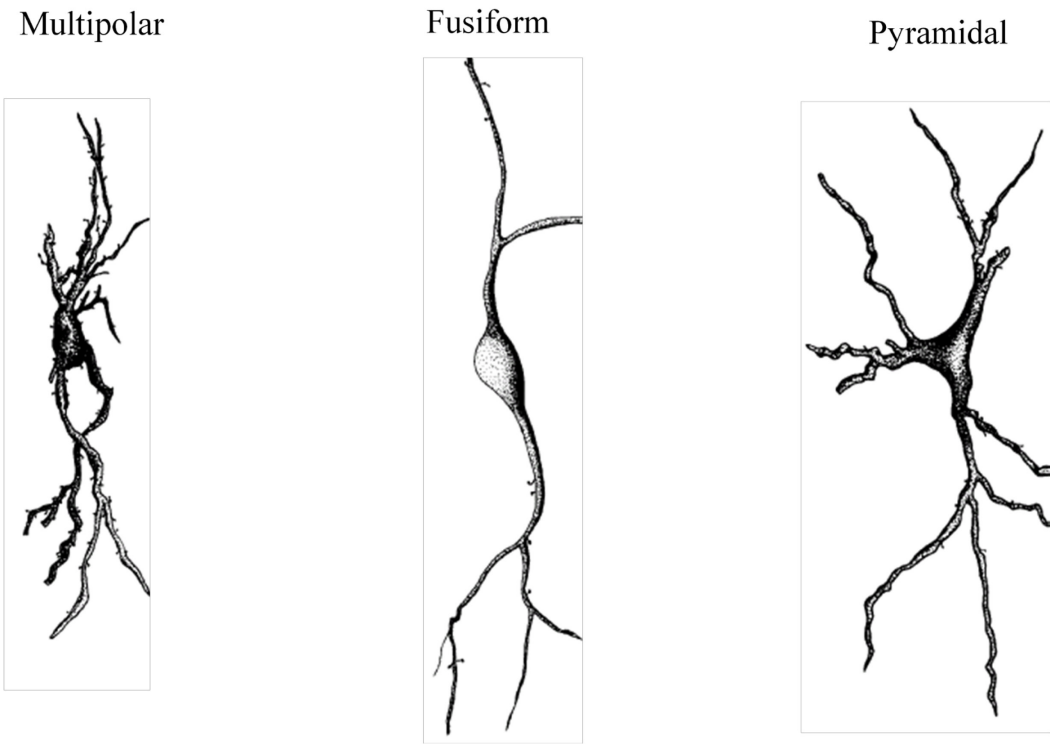


Figure 7

Morphogical classification of Lamina I neurons.

Representation of the three common morphological classes of Lamina I neurons

A similar dialectic surrounds the proportion of lamina I neurons of specific morphological classes expressing the NK1 receptor. In monkey, Yu et al (1999) reported that 6% of pyramidal neurons were NK1-immunoreactive. Furthermore, 75% of the pyramidal neurons that project to the thalamus did not express the NK1 receptor (Yu, X.H., Zhang, E.T., Craig, A.D., Shigemoto, R., Ribeiro-da-Silva, A., De Koninck, Y., 1999). However, the proportions observed in rat by another group are inconsistent with Yu's findings; Todd, A.J. and associates reported that 80% of lamina I pyramidal neurons retrogradely labelled from the CVLM were NK1-immunoreactive; the same percentage reported by Spike and colleagues (Todd, A.J., Puskár, Z., Spike, R.C., Hughes, C., Watt, C., Forrest, L.,

2002; Spike, R.C., Puskár, Z., Andrew, D., Todd, A.J., 2003). However, work conducted in our lab confirmed that lamina I pyramidal neurons normally do not express the NK1 receptor (Yu, X.H., Ribeiro-da-Silva, A., De Koninck, Y., 2005; Almarestani, L. Waters, SM. Krause, JE. Bennett, GJ. Ribeiro-da-Silva, A., 2007; Almarestani, L., et al., 2009). This disparity in the cell expression of NK1 and the nature of the cell which expresses the receptor is ostensibly due to the criteria by which lamina I neurons are segregated morphologically. Indeed, many of the pyramidal neurons classified by Todd and collaborators would be re-classified as multipolar cells using the criteria defined by Craig, A.D and colleagues (Zhang, E.T., Han, Z.S., Craig, A.D., 1996; Zhang, E.T., Craig, A.D., 1997; Almarestani, L., et al., 2009). The classification of this particular morphological type of lamina I neurons is highly important, as several studies have demonstrated using intracellular physiology and neurochemical labeling that the pyramidal neuron is non-nociceptive (Han, Z-S., Zhang, E-T., Craig, A.D., 1998).

All combined, data collected so far indicates that lamina I of the superficial spinal cord is the predominant site of origin of pain-related supraspinal projections. It has been determined that morphologically distinct classes of lamina I projection neurons respond to specific noxious and innocuous stimuli (see above).

1.5.1.1 Excitation

Released sP (from peptidergic PAF terminals) bind to NK1 receptors on the postsynaptic lamina I neuron. Coupling of the receptor and ligand prompts the immediate internalization of the NK1 GPCR by clathrin mediated endocytosis into early endosomes (Quartara, L., Maggi, C.A., 1998). The NK1 receptor is then recycled back to the plasma membrane while sP is degraded in perinuclear vesicles. Such receptor internalization following ligand or agonist binding is postulated to reduce signal transduction by desensitizing the postsynaptic neuron. However, it has been suggested that phosphorylation and uncoupling of the receptor from the associated G-proteins is the underlying cause of desensitization of NK1 expressing neurons (Quartara, L., Maggi, C.A., 1998). Inhibitors of

endocytosis and recycling mechanisms prevent resensitization of the postsynaptic neurons.

Substance P released into the synaptic cleft is thought to interact with the NK1 receptor located on the postsynaptic neuron within the immediate vicinity. However, early inspection of the location of the receptor in relation to the synapse revealed the extrasynaptic placement of the receptor supporting the hypothesis that the mechanism of excitation mediated by sP was that of volume transmission (Liu, H., Brown, J.L., Jasmin, L., Maggio, J.E., Vigna, S.R., Mantyh, P.W., Basbaum, A.I., 1994). Re-examination of the issue by McLeod and colleagues (1998), in our lab, demonstrated a close relationship between the site of sP release and the postsynaptic location of the NK1 receptor indicating that volume transmission across considerable distances is unlikely, although the study did not rule our the possibility of extra-synaptic action of sP on the NK1 receptor as it has been demonstrated that most NK1 receptors are located extra-synaptically (McLeod, A., et al., 1998).

The interaction of sP with the NK1 receptor initiates an intracellular cascade of signal transduction. The receptor, coupled to G11/Gq G protein promotes phospholipase C (PLC) and phosphatidyl inositol (Pi) turnover resulting in the mobilization of calcium from intracellular stores and from the extracellular space. The binding of sP to the NK1 receptor results in the depolarization of the postsynaptic neuron. Analysis of the postsynaptic responses in NK1-positive lamina I neurons was analyzed by Cheunsuang and colleagues (2002) following primary afferent stimulation. Primary afferent stimulation produced long duration, large amplitude excitatory postsynaptic potentials in NK1-positive neurons (Cheunsuang, O., Maxwell, D., Morris, R., 2002).

In addition to the excitatory effect of sP, primary afferent terminal in lamina I express and release glutamate. Glutamate, an excitatory amino acid is stored in synaptic vesicles localized at asymmetric synapses (Fremeau, R.T. Jr, Troyer, M.D., Pahner, I., Nygaard, G.O., Tran, C.H., Reimer, R.J., Bellocchio, E.E., Fortin, D., Storm-Mathisen, J., Edwards, R.H., 2001). The high concentration of glutamate within the synaptic vesicles permits the direct identification of glutamate-immunoreactive sites from the transmitter pool located within primary

afferent terminals (De Biasi S, and Rustioni A., 1988). The co-localization of glutamate and sP within primary afferent terminals has been demonstrated using double-labelling EM post-embedding immunocytochemistry (De Biasi S, and Rustioni A., 1988). The accumulation of glutamate in synaptic vesicles is an energy-driven process requiring synaptic vesicle transporters (Alvarez, F.J, Villalba, R.M., Zerda, R., Schneider, S.P., 2004**).** At present, three vesicular glutamate transporters have been identified: $VGluT_1$, $VGluT_2$ and $VGluT_3$ (Bellocchio et al., 2000; Takamori et al., 2000; Bai et al., 2001; Fremeau, R.T. Jr., et al., 2001; Gras C, Herzog E, Bellenchi GC, Bernard V, Ravassard P, Pohl M, Gasnier B, Giros B, El Mestikawy S., 2002; Li, J.L., Xiong, K.H., Dong, Y.L., Fujiyama, F., Kaneko, T., Mizuno, N., 2003; Todd, A.J., Hughes, D.I., Polgár, E., Nagy, G.G., Mackie, M., Ottersen, O.P., Maxwell, D.J., 2003). Essential to the synaptic accumulation of glutamate, vesicular glutamate transporters present in primary afferent boutons asseverates glutamate as the primary fast excitatory neurotransmitter utilized by these terminals. Complementary promulgations from Li and colleagues (2003) and Alvarez, F.J. et al (2004) indicate the presence of $VGluT_1$ in primary afferent terminals in LIII and LIV, with strong immunoreactivity in the inner one third of LII (Li, J.L., et al., 2003; Alvarez, F.J., et al., 2004). $VGluT_2$-immunoreactivity can be observed in the superficial dorsal horn, specifically in lamina I and LIIo/i with weak labelling in LIII and LIV (Li, J.L., et al., 2003; Alvarez, F.J. et al., 2004). Using dorsal rhizotomy, both groups ascertained the primary afferent origin of the VGluT-immunoreactivity. Co-localization with known primary afferent markers such as sP, CGRP, SSEA4 and IB4 demonstrated the differential expression of the transporters in primary afferent terminals. Both $VGluT_1$ and $VGluT_2$ were found to co-localize with sP-immunoreactivity in lamina I and lamina IIo (Li, J.L., et al., 2003; Alvarez, F.J. et al., 2004). However, IB4-binding was found to co-localize with $VGluT_2$ (Li, J.L., et al., 2003; Alvarez, F.J. et al., 2004). The reason for the differential expression of the transporters within primary afferent terminals is speculative; Fremeau (2001) surmised that $VGluT_1$ was present in terminals with a low synaptic vesicle-release probability (Fremeau, R.T. Jr., et al., 2001). However, $VGluT_1$immunoreactivity in LIII/IV was determined to be attributed to terminals

of low-threshold mechanoreceptors; afferents likely to release neurotransmitters frequently due to their sensitivity to low-threshold stimuli (Alvarez, F.J. et al., 2004).

Upon the synaptic release of glutamate, interaction with either metabotropic or ionotropic glutamate receptors promotes the rapid depolarization of the postsynaptic target neuron (Zieglgänsberger and Puil E.A., 1973; Schneider, S.P., Perl, E.R. 1985; Jeftinija et al., 1991). Ionotropic glutamate receptor family consists of the NMDA receptor, the high affinity AMPA/low affinity kainate receptor and the high affinity kainate receptor (Tölle, T.R., Berthele, A., Zieglgänsberger, W., Seeburg, P.H., Wisden, W., 1993). Comprised of multiple subunits, receptor composition is speculated to play a role in the postsynaptic effects initiated upon receptor-ligand interaction. Within the superficial dorsal horn, *in situ* hybridization studies have demonstrated the regional distribution of receptor subunits for all ionotropic receptor classes (Furuyama, T., Kiyama, H., Sato, K., Park, H.T., Maeno, H., Takagi, H., Tohyama, M., 1993) et al., 1993; Tölle, T.R., et al., 1993). Messenger RNA for the NR1 subunit of the NMDA receptor is profusely distributed throughout the superficial dorsal horn (Tölle, T.R., et al., 1993). Moreover, the NR1 subunit of the NMDA receptor is shown to be expressed in NK1-positive lamina I neurons at multiple levels of the rat spinal cord (Benoliel et al., 2000). Functional NMDA receptors have been identified on primary afferent terminals in the superficial dorsal horn (Liu, H., 1994; Lu, C.R., Hwang, S.J., Phend, K.D., Rustioni, A., Valtschanoff, J.G., 2003). Similarly studies have demonstrated the presence of presynaptic AMPA receptors (Lee, C.J., Bardoni, R., Tong, C.K., Engelman, H.S., Joseph, D.J., Magherini, P.C., MacDermott, A.B., 2002).

The AMPA/low affinity kainate subunit GluRA is preferentially expressed in LI and LIIo, with GluRB mRNA levels evenly distributed throughout LI, LIIo/i and LIII, and mRNA for the high affinity kainate receptor GluR5 was present in LI neurons whereas mRNA expression for GluR7 was consistent throughout the superficial dorsal horn (Tölle, T. R., et al., 1993). Calcium permeable AMPA receptors have been identified in LI and LII neurons in close approximation to capsaicin-sensitive primary afferent terminals (Engelman et al., 1999). Within

lamina I, a small margin of lamina I NK1-positive neurons express the Ca^{2+} permeable AMPA receptor. The AMPA receptor antagonist 6-nitro-7-sulphamoylbenzol[*f*]quinoxaline-2,3-dione disodium (NBQX) has been shown to block (in a dose-dependent manner) the postsynaptic excitation induced by electrical stimulation suggesting the presence of AMPA receptors on LI neurons (Seagrove, L.C., Suzuki, R., Dickenson, A.H., 2004).

1.5.1.2 Inhibition

In resting conditions, inhibitory mechanisms control the propagation of excitatory signals, maintaining a steady state of synaptic activity. GABAergic interneurons in lamina I comprise approximately 25% of the total neuron population (Todd, A.J., McKenzie, J. 1989; Polgár, E., Hughes, D.I., Riddell, J.S., Maxwell, D.J., Puskár, Z., Todd, A.J., 2003). Studies conducted in transgenic mice expressing enhanced green fluorescence protein (EGFP) under regulation by the GABA synthetic enzyme glutamic acid decarboxylase (GAD) isoform 67, revealed that 73% of lamina I neurons expressing EGFP-GAD67 were also GABA-immunoreactive (Dougherty KJ, Sawchuk MA, Hochman S., 2005). Similarly, 68% of GABA-immunoreactivity lamina I neurons were positive for EGFP-GAD67. The morphological properties of lamina I interneurons is not as well known as that of projection neurons, so the the issue of morphological properties of GABAergic cells is still controversial.

When released, low concentrations of GABA have been shown to bind preferentially to presynaptic $GABA_B$ receptors in the superficial dorsal horn (Chéry, N., De Koninck, Y., 2000). The presence of presynaptic $GABA_B$ receptors is suggestive of their involvement in the regulation of neurotransmitter release from presynaptic terminals (Riley, R.C., Trafton, J.A., Chi, S.I., Basbaum, A.I., 2001). This has been confirmed in regards to the regulation of neurokinin release from presynaptic terminals. Activation of presynaptic $GABA_B$ receptors by baclofen inhibited NK1 receptor internalization following electrical stimulation of dorsal roots presumeably by the inhibition of sP release from primary afferent terminals (Malcangio, M. Bowery, N.G. 1993; Riley, R.C., Trafton, J.A., Chi, S.I., Basbaum, A.I., 2001; Lao, L and Marvizón, JC., 2005).

Pre-and postsynaptic $GABA_A$ receptors modulate excitatory transmission in lamina I. As GABA and glycine are co-released from synaptic vesicles, the inhibitory postsynaptic currents (IPSC) are induced by both neurotransmitters suggesting the presence of glycine and $GABA_A$ receptors on lamina I neurons (Chéry, N., De Koninck, Y., 1999). Miniature IPSCs recorded in lamina I neurons have demonstrated the extra-synaptic localization of $GABA_A$ receptors (Chéry, N., De Koninck, Y., 1999).

1.5.1.3 Regulation of synaptic activity.

Excitatory and inhibitory receptors, present on pre-and postsynaptic neurons, serve as mechanisms to regulate signal transduction. These receptors may exert their effects by modulating the release of neurotransmitters from presynaptic terminals or by inhibiting postsynaptic neurons. It should be noted that the presence of re-uptake transporters such as the excitatory amino acid (EAA) transporters and also play a modulatory role.

The release of sP from peptidergic afferent terminals is not ungoverned. Using NK1 receptor internalization as a method to monitor the release of sP, it would appear that activation of presynaptic NMDA receptors promote the release of sP (Liu, H., Mantyh, P.W., Basbaum, A.I., 1997; Marvizón, J.C., Martínez, V., Grady, E.F., Bunnett, N.W., Mayer, E.A., 1997). Capsaicin-induced release of sP from primary afferent terminals was shown to be inhibited by the administration D-APV, an NMDA receptor antagonist (Afrah, A.W., Stiller, C.O., Olgart, L., Brodin, E., Gustafsson, H., 2001). The release of glutamate from primary afferent terminals is also thought to be regulated by presynaptic NMDA receptors (Bardoni, R., Torsney, C., Tong, C.K., Prandini, M., MacDermott, A.B., 2004). Electrically stimulated AMPA-mediated EPSCs recorded in LII neurons are inhibited by the application of NMDA; a phenomenon termed PAD or primary afferent depolarization mediated inhibition of presynaptic terminals (Rudomin, P., Schmidt, R.F, 1999; Willis, W.D. Jr. 1999; Lee, C.J., et al., 2002). Monitoring NK1-receptor internalization, application of the $GABA_A$ receptor agonist isoguvacine increased NK1-receptor internalization induced by capsaicin (Lao, L and Marvizón, J.C., 2005). Picrotoxin, reduced Ca^{2+} and Cl^-, blockade of $Ca2^+$

channels and NMDA receptors inhibited the $GABA_A$ facilitation of NK1-receptor internalization, as did the application of baclofen, a $GABA_B$ receptor antagonist. The conclusions drawn from these experiments were that $GABA_B$ receptors located on the presynaptic primary afferent terminal inhibit sP release, and that upon binding to $GABA_A$ receptors located on the postsynaptic neurons inhibit this presynaptic inhibitory regulation of sP release resulting in the internalization of the NK1 receptor by released neurokinins (Lao, L and Marvizón, JC., 2005).

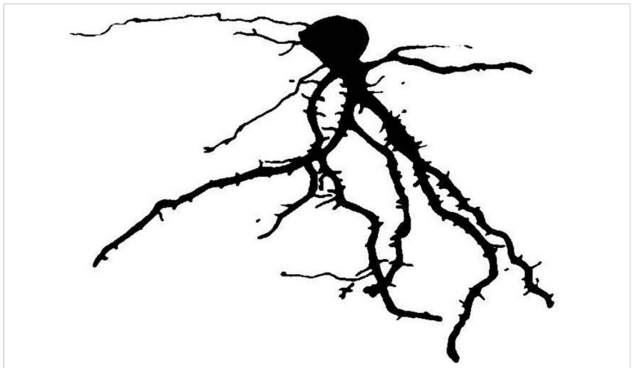
1.5.2 Lamina II

Lamina II is comprised of local circuit neurons forming synaptic connections hypothesized to modulate signal transduction by amplifying and integrating primary afferent input (Lu,Y and Perl, E.R., 2003, 2005). At the end of the first decade of the 1900s, Ramon y Cajal (1909) identified three neuronal classes in lamina II in the young cat: limitroph cells, central cells and transverse cells. Gobel, S. (1975, 1978) characterized numerous morphologically distinct cells in the cat trigeminal subnucleus caudalis: islet cells, stalked cells, arboreal cells and border cells. Gobel's stalked cells bore striking resemblances to Cajal's limitrophe cells whereas Gobel's islet cells were similar in morphology to Cajal's central cells. Notwithstanding a large number of publications which aimed to characterize and classify lamina II neurons, the cellular morphology of lamina II neurons is complicated, with many studies introducing new cells classified according to an ever expanding and overlapping set of criteria (a thorough historical review of the cells of the SG by Cervero and Iggo (1980) is certainly worth the read). Beal and Cooper (1978) remarked in their report on the cells of the SG in monkey that due to the extensive variation in morphology, it was impossible to form any system of classification (Beal, J.A., Cooper, M.H., 1978). Later, A.J Todd, A.J. and S. G Lewis (1986) identified islet cells and stalked cells in the SG of the rat spinal dorsal horn, which were further characterized at the ultrastructural scale by Todd, A.J. (1988).

Islet cells, reminiscent of Cajal's central cells, were first characterized by Gobel (1975) in the trigeminal nucleus caudalis (meduallary dorsal horn) of the cat and

described as possessing a rounded or fusiform cell body approximately 20μm in diameter located in both inner and outer lamina II or at the border between the two sub-regions (Gobel, S., 1975). Islet cells, as described by Gobel, display dendritic projections spanning the width of the SG (150 μm) yet remaining confined within the lamina. As with the impressive dendritic arbor, the axon, which may issue from the soma or from a primary dendrite, projects rostral-caudally to distances up to 1000μm (Gobel S., 1975). In the rat SG, Golgi-stained islet cells possessed an extensive dendritic tree projecting in the rostral-caudal plane as seen in the cat. The somata of islet cells were found throughout the dorsal-ventral width of the SG. Islet cell somata lay approximately 35-102 μm deep to the white matter, with smaller sized cells (128-149μm^2) located within 35-78μm from the white matter whereas larger cells (208-362μm^2) were found deeper (80-102μm) (Spike, R.C., Todd, A.J., 1992). The dendritic arbors of islet cells were restricted to narrow bands oriented rostral-caudally (Todd, A.J., Lewis, S.G., 1986). Of particular interest was the presence of presynaptic dendrites issued from the fusiform-shaped soma of islet cells. Containing round or oval agranular synaptic vesicles, the presynaptic dendrites formed symmetrical synapses onto dendritic spines and shafts. The vesicular pre-synaptic dendrites participated in both simple and complex synaptic arrangements, with complex synaptic compositions consisting of triads and glomeruli (Todd, A.J., 1988). Synaptic triads were formed by two dendrites in which one dendrite was presynaptic to the other and an axon, which could be the central bouton of a synaptic glomerulus. In no case were Golgi-labelled dendrites identified as forming presynaptic contacts with an axon bouton (Todd, A.J., 1988). Dendrites participating in synaptic glomeruli were postsynaptic to the central axon varicosity of type I or II glomeruli at both symmetrical and asymmetrical axo-dendritic synapses. Axons of islet cells possessed boutons containing round, loosely packed agranular vesicles (Todd, A.J., 1988). Three types of islet cells were characterized based upon the location of the soma in reference to the dendritic tree: cells with the soma lying ventral to the dendritic arbor, somata lying dorsal to the dendritic arbor and cells in which the soma and the dendritic tree lay within the same plane (Todd, A.J., Lewis, S.G., 1986).

Islet Cell



Stalked Cell

Figure 8
Camera Lucida reconstructions of Lamina II Islet and Stalked cells
(Adapted from Todd, A.J. and Lewis, S.G., 1986)

The limitrophe cells described by Cajal, named as such as they were predominantly localized to the border of the marginal zone and the SG whereas central cells are distributed throughout the SG (Cajal, R., 1909; Cervero, F., Iggo, A., 1980). Later studies by Gobel (1975, 1978) and Todd, A.J. (1986, 1988) identified cells with similar morphology to Cajal's limitrophe cells, the stalked cells (Gobel S., 1975, 1978; Beal and Cooper, 1978; Todd, A.J., Lewis, S.G., 1986; Todd, A.J., 1988). Stalked cells possess spindle-shaped or round cell bodies from which primary dendrites extend in the rostral-caudal orientation (Cervero, F., Iggo, A., 1980; Todd, A.J., Lewis, S.G., 1986). The distinguishing feature which permits the differentiation of stalked cells from islet cells is the length of the dendritic tree in the rostral-caudal direction, with stalked cells possessing shorter dendritic trees (Todd, A.J., Lewis, S.G., 1986). In many cases, the somata

of stalked cells are located dorsal to their dendritic arbors giving them a cone-shaped appearance in the sagittal plane (Todd, A.J., Lewis, S.G.,, 1986). Stalked cell dendrites do not establish presynaptic contacts. Postsynaptic dendrites form both symmetrical and asymmetrical synapses. As in the case of islet cells, stalked cell dendrites are observed participating in complex arrangements such as synaptic glomeruli where they are found postsynaptic to the primary afferent central axon at asymmetrical contacts (Todd, A.J. 1988).

Lamina II neurons identified and characterized by Grudt T.J., and Perl, E.R (2002) in the hamster spinal dorsal horn exhibited features similar to the cells described by Gobel (1975, 1978) and Todd, A.J. (1988). Islet cells defined by Grudt, T.J., Perl, E.R. were similar in morphology to the large islet cells of Todd and Lewis (1986), whereas their central cells, which possessed rostral-caudally oriented dendrites much shorter than islet cells were akin to the small islet cells characterized by Todd and Lewis (1986). Moreover, vertical cells, predominant in outer lamina II, were characterized by their vertically oriented dendrites, which in many instances were located ventral to the cell body with some neurons possessing dorsally oriented dendrites (Grudt, T.J., Perl, E.R., 2002). These cells resemble Cajal's limitrophe cells or Gobel's stalked cells. The radial cells of Grudt and Perl (2002) reside at the margin between outer and inner lamina II and have dendrites projecting in a circular fashion from the cell soma; the dendritic arbor of these cells is typically longer in the rostral-caudal direction than in the dorsal-ventral and medial-lateral planes, and axon branches descend ventrally into the superficial nucleus proprius (Grudt, T.J., Perl, E.R., 2002). The morphology of these cells was similar to the stellate cells described by Shoenen J (1982) in the human spinal cord.

In the early 1960's, identification and characterization of LII neurons demonstrated the preponderance of small neurons displaying dendritic arbors restricted to the confines of the SG. At this time, the hypothesis was that the SG is a *closed system*, a widely accepted conjecture originally proposed by Szentágothai in 1964 (Cervero, F., Iggo, A., 1980). The presence of axons confined to the SG

and the description of axons derived from lamina II neurons which entered Lissauer's tract (or lateral fasciculus proprius) where they would migrate rostrally or caudally for a short distance to reflect back to the SG suggested to Szentágothai that the SG received primary afferent input, but demonstrated a lack of forward communication (Szentágothai, J., 1964; Cervero, F., Iggo, A., 1980). The presence of dendrites from neurons originating from the deep dorsal horn within the SG was the only possible source of forward connection suggested by Szentágothai at the time. It is important to note that Szentágothai included lamina III as part of the SG.

Later observations by Ritz and Greenspan (1985) and others (Sedivec, M.J., Capowski, J.J., Mendell, L.M., 1986; Woolf, C.J., King, A.E. 1987) provided evidence that many deep dorsal horn neurons receiving primary afferent nociceptive information did not direct dendrites dorsally into lamina II (Todd, A.J., 1989). However, some deep dorsal horn neurons (LIII-LV) indeed issued superficially directed dendrites which terminated in the marginal zone and the SG; labelled in the monkey, they were identified as spinothalamic tract cells (Todd, A.J., 1989). Todd, in the interest of determining whether neurons of the nucleus proprius (LIII) and lamina IV issue dorsally directed dendrites into lamina II, conducted a morphological study which in addition, aimed to determine whether the dorsally directed dendrites received monosynaptic information from nociceptive primary afferent terminals (Todd, A.J., 1989). Five Golgi-labelled lamina III or superficial lamina IV neurons were examined at the light and electron microscopic levels; three cells originated from de-afferented (root transected) animals and the remaining two cells were derived from intact animals. Of the three cells examined from root transected animals, two of the cells exhibited prolific dendritic trees which spanned the entire thickness of LII. The remaining cell projected highly branched dendrites into the ventral-most aspect of LII. Degenerated primary afferent boutons were observed to form both symmetrical and asymmetrical synapses with the shaft and on spines of the LIII/LIV derived dendrites (Todd, A.J., 1989). In intact animals, primary afferent boutons identified as constituents of synaptic glomeruli formed axo-dendritic

synapses (asymmetrical and symmetrical) with the dorsally directed dendritic arbors issued from LIII/IV neurons (Todd, A.J., 1989).

Stalked cells, as characterized by Gobel (1980), have been observed to issue dorsally directed dendrites which penetrated the ventral aspect of lamina I. Furthermore, axons of stalked cells arising from the cell body or from the primary dendrite displayed prolific branching within the marginal zone (Bennett, G.J., Abdelmoumene, M., Hayashi, H., Dubner, R., 1980).

The substantial rostral-caudal orientation of the dendritic arbors of many SG neurons is highly indicative of the propagation of information to proximal segments of the spinal cord. The implications of this segmental dissemination are illustrated in a physiological study of primary afferent-evoked EPSP and IPSP in lamina II neurons (Kato G, Furue H, Katafuchi T, Yasaka T, Iwamoto Y, Yoshimura M., 2004). Examination of primary afferent-evoked EPSPs in the L2 segment of the lumbar spinal cord following the stimulation of the L5 dorsal root resulted in the suppression of the rostral excitation (Kato G, Furue H, Katafuchi T, Yasaka T, Iwamoto Y, Yoshimura M., 2004).

In the late 1970's, a number of studies suggested that axons derived from the SG projected supraspinally as cells of the SG could be retrogradely labelled from the contralateral thalamus in the monkey (Willis, W.D., Leonard, R.B., Kenshalo, D.R Jr.1978; Willis, W.D., Kenshalo, D.R. Jr, Leonard, R.B. 1979). Injections of cholera toxin B subunit (CTb) into the lateral reticular nucleus (adjacent to the ventral aspect of the trigeminal nucleus) retrogradely labelled lamina II cells in the cervical and lumbar enlargements (Lima, D., Coimbra, A., 1991). Lamina II cells retrogradely labelled in 10 coronal sections comprised 28% and 21% of all cells labelled in the cervical and lumbar enlargements respectively. Cells displaying ascending projections fit the morphological criteria of radial and stalked cells. This study illustrates the presence of a spino-fugal pathway derived from lamina II neurons (Lima, D., Coimbra, A., 1991).

Based upon the identification of extra-laminar and supraspinal projections from lamina II in addition to the presence of deep dorsal horn dendrites within the SG, the *'closed system'* hypothesis required re-evaluation. The expanded theory advocated the SG as a region where primary afferent signals were modulated,

integrated and relayed to marginal zone neurons which would project the transmogrified information to supraspinal targets (Kumazawa, T and Perl, ER., 1978; Wall P.D, 1978, 1980; Price, D.D., Hayashi, H., Dubner, R., Ruda, M.A., 1979; Cervero, F., Iggo, A., 1980). At the core of the hypothesis was the tenet that SG was comprised of inhibitory interneurons. The existence of excitatory neurons within the SG had not been entirely dismissed however data ascertaining their subsistence was lacking. Electrophysiological recordings from LII neurons, an arduous task due to the small size and location of the neurons, provided considerable information regarding the functional properties of SG interneurons.

Extracellular recordings of single unit activity elicited in lamina II neurons by innocuous and noxious stimulation of the appropriate peripheral receptive field demonstrated that islet cells confined to inner lamina II responded to low threshold mechanical stimulation. Stalked and islet cells resident to outer lamina II responded exclusively to noxious stimuli (nociceptive specific) or were characterized as wide dynamic range neurons (Bennett, G.J., Abdelmoumene, M., Hayashi, H., Dubner, R., 1980). These results are reminiscent of those reported by Light and colleagues (1979), in which it was reported that cells possessing dendritic arbors confined to outer LII were largely responsive to thermal or nociceptive stimuli; however, neurons with their soma and dendritic tree located within inner lamina II and neurons with dendritic trees which entered the superficial layers of lamina III responded preferentially to low threshold mechanical stimuli (Light, A.R., Trevino, D.L., Perl, E.R., 1979). These studies were among the first to characterize the electrophysiological or functional properties of specific classes of morphologically identified SG neurons.

As technology advanced, so has our understanding of the physiological properties of morphologically defined classes of superficial dorsal horn neurons. Neurons resident to the SG can be classified according to their firing patterns: tonic firing neurons (TN), adaptive firing neurons (AN) and delayed firing neurons (DN) (Melnick, I.V., Santos, S.F., Safronov, B.V., 2004, Melnick, I.V., Santos, S.F., Szokol, K., Szûcs, P., Safronov, B.V., 2004; Santos et al., 2004). Tonic firing neurons display tonic spike activity during a protracted depolarization (500ms) elicited by current injection. The mean firing threshold for

these neurons is -50mv. Adaptive firing neurons feature burst-like spike activity at the immediate onset of current-evoked depolarization, demonstrating an average firing threshold of -50mv.

The onset of spike activity in delayed firing neurons emerge following a considerable period of quietude, typically at the end of the current pulse. These neurons exhibited a transient K^+ current which significantly influenced spike activity. Their average firing threshold of -40mv was markedly higher than in both tonic and adaptive neurons and required significantly greater magnitude of current to initiate spike activity (Santos et al., 2004). In some neurons, the pattern of delayed firing activity varied with strong stimulation. These variations consisted of neurons exhibiting consistent discharges during the entire pulse duration (DN_1), discontinuous burst-like activity (DN_2) and a single spike discharge (DN_3) (Santos et al., 2007).

Sharp electrode and tight seal recording techniques have demonstrated that morphologically classified SG neurons can be associated with the aforementioned patterns of spike activity (Grudt, T.J., Perl, E.R., 2002; Melnick, I.V., Santos, S.F., Safronov, B.V., 2004, Melnick, I.V., Santos, S.F., Szokol, K., Szûcs, P., Safronov, B.V., 2004; Santos et al., 2007). Grudt, T.J., Perl, E.R. (2002) merged electrophysiology and morphology in a comprehensive study which aimed to associate morphologically classified SG neurons in the hamster with distinct electrophysiological properties such as action potential firing pattern, frequency of spontaneous miniature discharges and current-voltage relationships. Action potential firing patterns elicited by a 1ms pulse elucidated several categories of neurons. Briefly, islet cells demonstrated a tonic firing pattern reminiscent of patterns exhibited by TN as identified by Santos et al., 2004. Vertical neurons (stalked cells) displayed tonic firing patterns or delayed firing. Of the delayed firing vertical cells (stalked), a portion manifested DN_1 or DN_2 patterns. Central neurons (small islet cells) elicited either adaptive firing (transient central cells which presented an outward current at the end of hyperpolarizing steps termed I_A and in non-I_A cells) or tonic patterns. Medial-lateral cells exhibited tonic firing patterns (Grudt, T.J., Perl, E.R., 2002).

From the original descriptions of Rolando to the contemporary detailed morphological and physiological properties of the neuropile, our understanding of the cellular composition of the SG has greatly evolved. Szentágothai surmised from the available anatomical evidence of the time, that the SG, an immure region receiving primary afferent input, lacked the morphological features necessary for forward communication. Lamina II cells have been shown to project outside the SG and in some cases, project to supraspinal targets. The central boutons of primary afferent fibres, which form the central element of synaptic glomeruli, establish synaptic contacts (either directly or through interneurons) with deep dorsal horn neuronal dendrites as well as forming synapses with lamina II islet and stalked cells. Combined with the unique firing patterns elicited in SG neurons, we can appreciate the complexity of its circuitry.

1.5.2.1 Excitation

Primary afferent stimulation (Aδ and C-fibres) produces fast excitatory postsynaptic potentials in approximately 70% of SG neurons (Yoshimura, M., Jessell, T., 1990; Kato et al., 2004). CNQX, the AMPA receptor antagonist effectively blocked afferent-evoked EPSPs in 70% of SG neurons. In the presence of low concentrations of the voltage-gated sodium channel inhibitor tetrodotoxin (TTX), only Aδ-evoked EPSPs were blocked and not C-fibre induced depolarization of SG neurons. Higher concentrations of TTX resulted in the inhibition of C-fibre induced EPSPs (Yoshimura, M., Jessell, T.,, 1990). In a later study, Aδ-evoked EPSCs in SG neurons were abolished by the administration of CNQX (Yoshimura, M., Nishi, S. 1993). Application of the selective NMDA antagonist 2-amino-5-phosphonovaleric acid (APV) was ineffective at suppressing spontaneous mEPSCs, confirming the results obtained in the 1990 study (Yoshimura, M., Jessell, T.,, 1990; Yoshimura, M., Nishi, S. 1993).

The exclusive expression of the purinergic, non-selective cation channel $P2X_3$ on non-peptidergic primary afferent terminals in the SG is suggestive of the importance of ATP as a neurotransmitter or neuromodulator in this region. In tight-seal, whole cell patch-clamp recordings from lamina II neurons, bath application of ATP resulted in activation of fast inward currents as well as the

potentiation of glutamate-evoked currents. Suramin, an antagonist selective for P_2 purinergic receptors, markedly reduced ATP-induced fast inward currents; both CNQX and theophylline, a non-selective P_1 antagonist, were ineffective at this task (Li, J., Perl, E.R., 1995). Furthermore, glutamate-evoked EPSCs were appreciably enhanced subsequent to priming by ATP. Similarly, the antecedent application of ATP substantially increased glutamate-evoked fast currents relative to the sole administration of glutamate (Li, J., Perl, E.R., 1995).

It has been estimated that approximately 68% of lamina II neurons are excitatory (Santos et al., 2007). This approximation is based on *in situ* hybridization studies which documented the presence of vesicular glutamate transporters (VGlut1-3) in the marginal zone and lamina II; 38%, 33% and 6% of superficial dorsal horn neurons expressed VGluT1, 2 and 3 mRNA respectively (Landry M, Bouali-Benazzouz R, El Mestikawy S, Ravassard P, Nagy F., 2004). Of the neurons expressing either VGluT1 or VGluT2, 14% expressed both transporters, bringing the estimated number of excitatory interneurons to 68% (Santos et al., 2007). Moreover, evaluation of the regional distribution of tritiated (^{3}H) AMPA, glutamate and the NMDA blocker MK-801 in the superficial dorsal horn revealed the concentration of these glutamate receptors in the substantia gelatinosa (Henley, J.M., Jenkins, R., Hunt, S.P., 1993).

Santos and colleagues, in an impressive physiological study using tight seal whole cell patch-clamp recording techniques, recorded from 1500 SG neurons and determined that of 102 monosynaptic connections between lamina II neurons and those located in lamina I or lamina III, 87 were glutamatergic and 15 were inhibitory (Santos et al., 2007). Recordings from pairs of neurons established the presence of monosynaptic connections between lamina II neurons. Furthermore, monosynaptic connections between resident SG neurons and neurons located in either the marginal zone or in lamina III were evident. The conclusions drawn from the data obtained in this study were that excitatory interneurons of the SG for predominantly intra-laminar connections whereas inhibitory interneurons establish connections frequently outside of the SG (lamina I or lamina III). Furthermore, neurons displaying adaptive or tonic discharge

patterns were excitatory and neurons which exhibit delayed spiking may be excitatory or inhibitory (Santos et al., 2007). Antecedent to the study by Santos and associates, a study by Lu,Y and Perl, E.R., (2005) presented morphological and physiological data documenting monosynaptic, excitatory synaptic interactions between excitatory SG neurons with lamina I projection and non-projection neurons (Lu,Y.,Perl, E.R. , 2005). Specifically, in 7 pairs of neurons, 6 pairs consisted of an outer lamina II vertical cell forming monosynaptic contacts with a cell residing in lamina I. Dorsal root stimulation of the vertical cell exhibited conduction velocities were indicative of Aδ-fiber input. In three of the vertical cell-lamina I pairs, the exogenous administration of sP evoked an excitatory inward current in the postsynaptic lamina I neuron. The sP-sensitive lamina I neurons were regarded as projection neurons as they displayed gap-firing patterns in response to depolarizing steps; a feature of spinobrachial and spino-periaqueductal grey projecting marginal zone neurons (Lu, Y., Perl, E.R. , 2005).

1.5.2.2 Inhibition

As previously mentioned, one of the dominant characteristics of type Ia (non-peptidergic) synaptic glomeruli is the presence of peripheral profiles which form presynaptic contacts with the central axon terminal (Ribeiro-da-Silva, A., Coimbra, A., 1982; Ribeiro-da-Silva, A., Pignatelli, D., Coimbra, A., 1985). Presynaptic dendrites were proposed to function as a mechanism of stimulus suppression by inhibiting the primary afferent terminal at the centre of the participating glomerulus (Ribeiro-da-Silva, A., Pignatelli, D., Coimbra, A., 1985). The morphological features of islet cells establishes them as a contender for the source of presynaptic dendrites; in the cat, islet cell dendrites form presynaptic contacts with the central primary afferent bouton of synaptic glomeruli (Barber RP, Vaughn JE, Roberts E., 1982; Gobel S, Falls WM, Bennett GJ, Abdelmoumene M, Hayashi H and Humphrey E., 1980). It was determined that 31% of lamina II neurons were GABAergic, and 43% of these GABA-containing cells also contained glycine (Todd AJ, Sullivan AC., 1990). Light and electron microscopic analysis of islet cells in the SG by Todd, A.J. and Spike, R.C. (1992) demonstrated the existence of GABA-immunoreactivity islet cells, some of which

were also glycine-immunoreactivity. Derived from the observations within this study, the authors suggested the existence of two populations of islet cells; those with large perikarya and relatively extensive dendritic arbors in the rostral-caudal plane which contain GABA and in some cases glycine and small islet cells which are GABA and glycine negative (Todd AJ, Sullivan AC., 1990; Todd, A.J., Russell, G., Spike, R.C. 1992). In the SG, primary afferent stimulation evoked EPSCs in islet cells which in turn elicited IPSCs in central transient cells (Lu,Y., Perl, E.R., 2003). This study sketches a rudimentary picture of the synaptic architecture of the substantia gelatinosa by revealing one of many neuronal circuits composite of the SG neuropile.

A-δ primary afferent stimulation has been shown to induce long and/ or short duration IPSPs in SG neurons (Yoshimura, M., Nishi, S. 1995). In a "di-synaptic" arrangement, the Aδ-fiber-evoked release of glutamate activated postsynaptic AMPA receptors located on an inhibitory interneuron in the SG. Activation of the inhibitory cell resulted in the generation of long and/ or short IPSPs in a contiguous interneuron within the SG (Yoshimura, M., Nishi, S. 1995). The afferent-evoked short IPSPs were reversibly blocked by the application of the glycine receptor antagonist strychnine. The long duration IPSPs were blocked in a reversible fashion by the $GABA_A$ receptor antagonist bicuculline. Thus, AMPA-mediated excitation of lamina II GABAergic interneurons by Aδ-PAFs results in the generation of $GABA_A$ receptor-mediated IPSPs in second order LII neurons.

Studies have demonstrated the predominant expression of $GABA_B$ receptors in the SG of the superficial dorsal horn (Bowery, N.G., Hudson, A.L., Price, G.W., 1987; Yang, K., Feng, Y., Li, Y., 2001). Bowery et al (1987) reported high concentrations of $GABA_B$ receptors in the SG with uniform distribution of $GABA_A$ receptors throughout the grey matter of the spinal cord (Bowery, N.G., Hudson, A.L., Price, G.W., 1987). Neonatal application of capsaicin results in a 40-50% reduction in $GABA_B$ receptors indicating the presynaptic expression of the receptor on primary afferent terminals (Price, G.W., Wilkin, G.P., Turnbull, M.J., Bowery, N.G., 1984; Yang, K., Feng, Y., Li, Y., 2001). Presynaptic $GABA_B$ receptors have been shown to reduce primary afferent-evoked release of neurotransmitters and neuropeptides (Kangrga et al., 1991; Malcangio et al.,

1993; Marvizón et al., 1999; Ataka et al., 2000; Yang, K., Feng, Y., Li, Y., 2001). Intrathecal administration of baclofen, a $GABA_B$ receptor agonist has been shown to exhibit pain enhancing effects at low doses and antinociceptive effects at high doses (Iyadomi, M., Iyadomi, I., Kumamoto, E., Tomokuni, K., Yoshimura, M., 2000). Application of baclofen reduced the frequency but not the amplitude of miniature EPSCs and IPSCs in SG neurons thought to be mediated by the binding of the agonist to excitatory and inhibitory neurons expressing presynaptic $GABA_B$ receptors. The effect is thought to be mediated by the reduction of neurotransmitter release from presynaptic terminals (Iyadomi, M., Iyadomi, I., Kumamoto, E., Tomokuni, K., Yoshimura, M., 2000).

Other neurotransmitter systems have been shown to modulate GABA-mediated inhibition in the superficial dorsal horn. The cholinergic system has been implicated in the facilitation or suppression of GABAergic inhibition in the SG (Li, D.P, Chen, S.R., Pan, Y.Z., Levey, A.I., Pan, H.L., 2002; Kawasaki, Y., Kumamoto, E., Furue, H., Yoshimura, M., 2003). In a whole cell patch-clamp study, the amplitude of dorsal root stimulation-evoked EPSCs in lamina II neurons was reduced in a dose-dependent manner by the application of acetylcholine (ACh) (10-100μM) (Li, D.P., et al., 2002). Non-NMDA (presumably AMPA)-mediated mEPSC frequencies were reduced significantly by Ach, an effect which could be antagonized by the non-selective muscarinic receptor antagonist atropine indicating the presence of presynaptic muscarinic receptors (Li, D.P., et al., 2002). Interestingly, ACh facilitated the frequency of $GABA_A$-receptor mediated mIPSCs, an effect which could also be blocked by atropine. The effects of ACh on excitatory and inhibitory postsynaptic currents in lamina II neurons was determined to be mediated by presynaptic M2 muscarinic receptors; dorsal rhizotomy effectively reduced the density of M2 receptors in the superficial dorsal horn (LI-III) by nearly 55% and similarly by the application of the capsaicin receptor agonist resiniferatoxin (RTX) (47.2 ±9.2 % reduction in M2-receptor density) (Li, D.P., et al., 2002).

Spinal adenosine can be derived by the intracellular and extracellular metabolism of purine nucleotides (ATP and AMP). Bidirectional nucleoside transporters export adenosine into the extracellular milieu (Sawynok, J., Liu, X.J.,

2003). Adenosine is not stored in exocytotic vesicles, release of adenosine occurs in response to cell stress and depolarization. Substance P and glutamate have been shown to increase the cellular release of adenosine (Sawynok, J., Liu, X.J., 2003). Of the three classes of G-protein-coupled adenosine receptors (A1, A2a and A2b and A3), the A1 receptor is highly expressed in the SG of the rat spinal dorsal horn (Sawynok, J., Liu, X.J., 2003; Schulte, G., Robertson, B., Fredholm, B.B., DeLander, G.E., Shortland, P., Molander, C., 2003). Binding of adenosine to the Gi/Go coupled receptor results in the decrease in adenylyl cyclase, as well as inhibiting N-type Ca^{2+} channels (Sawynok, J., Liu, X.J., 2003). In the SG, application of adenosine reversibly reduced the amplitude of electrically-evoked GABA and glycine-mediated IPSCs; an effect mimicked by the A1 receptor agonist N^6-cyclopentyladenosine (CPA) (Yang, K., Fujita, T., Kumamoto, E., 2004). Furthermore, adenosine reduced the frequency, but not the amplitude of GABA and glycine-mediated spontaneous IPSCs suggesting these effects are mediated by presynaptic A1 receptors. Indeed, in paired-pulse experiments, the ratio of the peak amplitude of the second IPSC to the first IPSC evoked by electrical stimulation was increased by adenosine; in the case of a postsynaptic mechanism, both the first and second IPSC would be equally diminished resulting in no change in the ratio of their amplitudes. These data suggest that presynaptic A1 receptors, located on GABAergic and glycinergic SG neurons, suppress inhibition mediated by these neurotransmitters (Yang, K., Fujita, T., Kumamoto, E., 2004).

Opioid peptides are known to elicit spinal analgesia by exerting their effect on spinal cord neurons and primary afferent fibres. Pre- and postsynaptic opioid receptors μ, κ, δ are highly expressed in lamina I and II of the cervical dorsal horn (Besse, D, Lombard, M.C., Zajac, J.M., Roques, B.P., Besson, J.M., 1990). Descending enkephalinergic pathways arising from the PAG and the nucleus raphe magnus facilitate inhibition in the superficial dorsal horn (Li, H., Wu, L., Li, Y.Q., 2003). Opioid peptides have also been demonstrated to exert an effect on the response of dorsal horn neurons to GABA. Immunohistochemical studies suggest that more than 70% of methionine-enkephalin (Met-Enk) immunoreactivity neurons in the superficial dorsal horn also co-localize GABA

(Li, H., Wu, L., Li, Y.Q., 2003; Ribeiro-da-Silva, A., 2004). Met-Enk is capable of depressing $GABA_A$ receptor mediated inward chloride (Cl^-) inhibitory postsynaptic currents in approximately 65% of the dorsal horn neurons examined (Li, H., Wu, L., Li, Y.Q., 2003). Agonists for the μ, κ, δ-opioid receptors ([D-Ala^2-*N*-Me-Phe^4, Gly^5-ol] enkephalin (DAMGO), Dynorphin A and DPDPE respectively) mimicked the effect of Met-Enk on $GABA_A$-mediated IPSCs (Li, H., Wu, L., Li, Y.Q., 2003).

The selective μ-opioid receptor agonist endomorphin-2 (EM-2) is expressed in primary afferent terminals within the dorsal horn; EM-2-immunoreactive axon terminals establish asymmetrical synapses with peripheral profiles immunoreactive for the μ-opioid receptor (Martin-Schild, S., Gerall, A.A., Kastin, A.J., Zadina, J.E., 1998; Wu SY, Ohtubo Y, Brailoiu GC, Dun NJ., 2003). Application of the opioid peptides endomorphin-1 (EM1) and EM2 on spinal cord slices elicited outward currents or hyperpolarization in SG neurons; a phenomenon mediated by inward rectifying K^+ channels (Wu SY, Ohtubo Y, Brailoiu GC, Dun NJ., 2003). Furthermore, EM1 or EM2 application suppressed short-latency EPSCs and long-latency IPSCs in SG neurons. Met-Enk and the μ-opioid receptor agonist DAMGO depressed short-latency EPSCs and long-latency IPSCs. The frequency of mEPSCs and mIPSCs were reduced by the presence of EM1, but not the amplitude suggesting the effects of EM1 is mediated by presynaptic EM1 receptors (Wu SY, Ohtubo Y, Brailoiu GC, Dun NJ., 2003).

At present, it is difficult to formulate a realistic map of the circuitry of the local and “long-distant” connections of SG neurons due to methodological limitations. The promising approach of electrophysiological recording from synaptically linked neuronal pairs is an ambitious and operose task; few morphologically identifiable pairs in spinal cord slices which form monosynaptic connections are considered as reliable synaptic units (Lu, Y., Perl, E.R., 2003, 2005). However, we can learn a great deal from these studies. These studies certainly characterize the strong inhibitory nature of the SG. The commission of the SG is still speculative; ‘modulation’, ‘integration’ and ‘relay’ are all terms which have been used to describe the presumable function of this region of the superficial dorsal

horn. The high incidence of inhibitory neurotransmitters and receptors coupled with presynaptic control mechanisms certainly support the modulation and integration hypothesis. In light of the strong evidence suggesting that the SG functions as a regulatory system of noxious synaptic transmission, alterations in the cellular composition and mechanisms within this region may contribute significantly to the pathogenesis of neuropathic pain. Indeed many studies have suggested that loss of inhibitory tone in the superficial dorsal horn may account for increased excitability documented in the spinal cord. Several studies have reported a significant loss of GABAergic interneurons in the superficial dorsal horn and specifically in the SG (Sugimoto et al., 1989, 1990; Castro-Lopes, J.M., Tavares, I., Coimbra, A., 1993; Ibuki, T., Hama, A.T., Wang, X.T., Pappas, G.D., Sagen, J., 1997; Eaton, M.J., Plunkett, J.A., Karmally, S., Martinez, M.A., Montanez, K., 1998; Whiteside, G.T., Munglani, R, 2001; Moore, K.A et al., 2002).

The hypothesized mechanism underlying the occurrence of cell death in the superficial dorsal horn following the induction of several different types of peripheral nerve injury (see below) is apoptotic cell death induced by excitotoxicity (Sugimoto et al., 1984, 1985, 1986, 1987a, b, 1989 and 1990; Castro-Lopes, J.M, J.M., et al., 1993; Coggeshall et al., 2001; Petrenko and Shimoji, 2001; Moore, K.A, et al., 2002). Apoptotic cell death induced by an intolerable increase in cell excitation is a well documented phenomenon (Mattson M.P, 2003). In regards to chronic pain, neuronal loss in the superficial dorsal horn, particularly LI-II, has been documented following the induction of a peripheral neuropathy (Sugimoto et al., 1984, Sugimoto, T., Takemura, M., Okubo, J., Sakai, A., 1985; Sugimoto, T., Takemura, M., Sakai, A., Ishimaru, M., 1986; Sugimoto, T., Takemura, M., Sakai, A., Ishimaru, M., 1987a, Sugimoto, T., Takemura, M., Sakai, A., Ishimaru, M., 1987b; Sugimoto, T., Bennett, G.J., Kajander, K.C., 1989 and Sugimoto, T., Bennett, G.J., Kajander, K.C., 1990; Castro-Lopes, J.M et al., 1993; Coggeshall et al., 2001; Petrenko and Shimoji, 2001; Moore, K.A et al., 2002). In early studies conducted by Sugimoto and colleagues (Sugimoto, T., et al., 1984, 1985, 1986, 1987a, b), peripheral nerve injury (transection) of the inferior alveolar branch of the trigeminal nerve resulted

in the increased incidence of neurons undergoing degeneration indicated by the increased affinity for Toluidine Blue ('dark neurons') in the medullary dorsal horn. Dark neurons highly stained with Toluidine Blue exhibit signs of degeneration: shallow invaginations in the nuclear membrane and shriveled cell membranes. Administration of the glycine receptor antagonist strychnine enhanced the incidence of dark neurons in the medullary dorsal horn significantly suggesting that a reduction in glycinergic inhibition is one of the mechanisms underlying the induction of dark, degenerative neurons in the dorsal horn (Sugimoto, T., et al., 1987a,b). These dark neurons were presumed to be GABAergic inhibitory neurons of the SG. Indeed, studies have reported significant alterations in GABA tissue levels, the number of GABA-immunoreactivity neurons and the expression of GABA and glycine receptors in the SDH following peripheral nerve injury (Castro-Lopes, J.M, 1993; Ibuki, T., Hama, A.T., Wang, X.T., Pappas, G.D., Sagen, J., 1997; Eaton MJ, Plunkett JA, Karmally S, Martinez MA, Montanez K., 1998; Moore, K.A et al., 2002).

Following chronic constriction injury (CCI) or spared nerve injury (SNI), the proportion of neurons which responded with IPSCs induced by primary afferent stimulation was reduced 17% and 28% respectively (Moore, K.A et al., 2002). Furthermore, the amplitude and kinetics of the evoked IPSCs decreased significantly in CCI and SNI animals. In CCI-operated rats which lacked primary afferent-evoked IPSCs, the frequency of sIPSC were significantly decreased (Moore, K.A et al., 2002). In this study, the combined administration of bicuculline and strychnine ($GABA_A$ and glycine receptor antagonists respectively) was required to completely block primary afferent-evoked IPSCs in naïve animals; the kinetics of the $GABA_A$-mediated component of the IPSCs differed remarkably from the glycinergic component. Reminiscent of the results reported by Yoshimura and Nishi (1995), the GABAergic element of the IPSC is characterized by a slow rise time to peak amplitude and a long decay time. In contrast, the glycinergic constituent exhibits significantly quicker rise and decay times compared to the GABA-mediated component of the IPSC (Yoshimura, M., Nishi, S. 1995; Moore, K.A et al., 2002). This suggests that the $GABA_A$-mediated

portion of the primary afferent induced IPSCs is the dominant factor in the elicited inhibitory potential. Sciatic nerve injury induced a shift in the kinetics of the primary afferent-induced IPSCs towards those resembling glycine suggesting that nerve injury alters GABAergic transmission (Moore, K.A et al., 2002). Examination of $GABA_A$-mediated sIPSCs revealed a significant reduction in frequency indicative of a presynaptic alteration in either GABA release or diminished activity-dependent receptor ($GABA_A$) induced sIPSCs. The absence of any detectable change in $GABA_A$-receptor-generated sIPSC amplitude is evidence in support of the former possibility. Evaluation of GAD isozyme 65 (GAD_{65}) in the superficial dorsal horn of nerve lesioned animals revealed a 20-40% reduction in GAD_{65} protein levels observable 6 days following nerve injury and persisting up to 4 weeks (CCI) or beyond (SNI) (Moore, K.A et al., 2002). Moreover, the authors of this study demonstrated the increased incidence of apoptotic neurons in LI and LII determined by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labelling (TUNEL). Taken together, these data provides credence to the cell-death-induced disinhibition hypothesis.

Increased spontaneous discharges from injured primary afferent fibres are thought to contribute to increases in excitatory signaling in the dorsal horn witnessed in neuropathy models (Wall, P.D., Waxman, S., Basbaum, A.I.., 1974; Wall, P.D and Gutnick, M., 1974; Govrin-Lippomann and Devor, 1978; Sugimoto, T., et al., 1989, 1990; Moore, K.A et al., 2002). Neuromas formed at the proximal stump of transected peripheral nerves have been documented to elicit ectopic discharges (Kajander and Bennett, 1992; Nakamura, S and Atsuta, Y., 2004). It has been shown that the onset of the symptoms associated with peripheral nerve injury (CCI) occurs simultaneously with increased spontaneous and ectopic firing from injured Aβ and Aδ primary afferent fibres (Kajander and Bennett., 1992). As several studies have demonstrated, primary afferent signaling is mediated by AMPA receptors (Yoshimura, M., Jessell, T., 1990; Yoshimura, M., Nishi, S. 1993). Increase AMPA signaling results in the removal of the voltage-sensitive Mg^{2+} block of NMDA receptors prompting a substantial augmentation of intracellular Ca^{2+} (Mattson, M.P., 2003). The opening of voltage-dependant Ca^{2+} channels, combined with the metabotropic glutamate receptor-

induced release of Ca^{2+} from the endoplasmic reticulum, further increases cytosolic calcium levels. Activation of presynaptic NMDA receptors, as well as the decrease in the presynaptic uptake of synaptic glutamate results in the increased release of neurotransmitters (glutamate) and neuropeptides (sP, CGRP) from the presynaptic terminal, enhancing excitatory tone in the superficial dorsal horn. Excessive intracellular Ca^{2+} can lead to excitotoxicity (Mattson, M.P., 2003). Enhanced intracellular calcium can induce the activation of calcium-dependant proteolytic enzymes such as calpains which degrade membrane receptors and cytoskeletal proteins. Furthermore, enhanced cytosolic calcium concentrations lead to the activation of Ca^{2+}/calmodulin protein kinases and Ca^{2+} dependent endonucleases (Mattson MP, 2003). The activation of these Ca^{2+}-dependent processes can lead to oxidative stress; increased reactive oxygen species (ROS) ultimately culminate in the loss of ion gradients across the mitochondrial and cellular membranes and, in healthy conditions (pro-apoptotic and anti-apoptotic processes in homeostasis), the cell undergoes programmed cell death mediated in part by Ca^{2+} induced activation of a number of pro-apoptotic genes such as Bax and P53 (for an excellent review on the mechanisms of excitotoxicity see Mattson MP, 2003).

1.6 Sensory Discriminatory and Affective Emotional processing of pain

The affective-emotional component of pain underlies the unpleasantness associated with pain: fear, anxiety, (Price, D.D, 2000, 2002). The medial pain pathway is responsible for the transmission of nociceptive information to cortical structures associated with the limbic system (Sewards, T.V and Sewards, M.A., 2002). Derived from neurons located primarily in the deep dorsal horn (LV) (Giesler G.J., et al., 1978; Burstein, R., Potrebic, S., 1993), the medial pain pathway conveys nociceptive information to several thalamic nuclei such as the posterior ventromedial nucleus (VMpo), the centrolateral nucleus (CL), the ventrocaudal medial dorsal nucleus (MDvc) and the parafascicular nucleus (PF) (Treede, R.D., Kenshalo, D.R., Gracely, R.H., Jones, A.K., 1999). Cortical targets such as the insula and the anterior cingulate cortex (ACC) receive direct input

from the VMpo and the MDvc, PF and CL respectively (Treede, R.D., Kenshalo, D.R., Gracely, R.H., Jones, A.K., 1999).

The insula and ACC have been shown to be highly involved in the motivational aspect of pain, specifically in the formation associative learning in the context of pain. Lesions of the ACC produced significant deficits in conditioned place avoidance to formalin injections implicating this region in establishing an association between environmental cues with a paired noxious stimulus (Gao, Y.J., Ren, W.H., Zhang, Y.Q., Zhao, Z.Q., 2004). Similarly, lesions of the amygdala, a component of the limbic system responsible for fear conditioning, produced a significant reduction in the magnitude of avoidance behaviour in response to formalin (Gao, Y.J., Ren, W.H., Zhang, Y.Q., Zhao, Z.Q., 2004). Direct input from the spinal cord to the amygdala has been observed in several retrograde and anterograde tracing studies (Burstein et al., 1991; Cliffer et al., 1991; Menetréy and Pommery, 1991; Burstein and Potrebic; 1993). Specifically, fluorogold injections into the CeA resulted in the retrograde labelling of spinal cord neurons within the grey matter, the majority of which were located in the deep dorsal horn reticulated area (lamina V), the lateral spinal nucleus, near the central canal, the intermediate zone and the ventral horn (Burnstein and Potrebic, 1993). Examination of neurons residing in the superficial dorsal horn revealed an absence of labelled neurons. Unilateral injections of the CeA resulted in a large proportion of labelled neurons in the contralateral spinal cord (Burstein and Potrebic, 1993).

Sensory information originating in cutaneous tissues is transmitted to the superficial laminae of the spinal dorsal horn by both peptidergic and non-peptidergic sensory fibres (Hunt, S.P and Rossi, J, 1985; Zylka, M.J, 2005). Information carried by peptidergic afferents is transferred to marginal zone neurons where the information is sent to supraspinal sensory and affective brain regions via the spinothalamic tract and the spino-parabrachio-amygdaloid pathways respectively (Burnstein et al., 1991, Burnstein and Potrebic, 1993; Jasmin et al., 1997). In contrast, information transduced to the substantia gelatinosa via non-peptidergic primary afferents is significantly modulated by local inhibitory neurons which establish presynaptic contacts with the central

bouton of non-peptidergic synaptic glomeruli (for review see Ribeiro-da-Silva, A., 2004). The lack of projection neurons within this region of the superficial dorsal horn has long suggested that the SG functions as a region of signal modulation (Szentágothai 1964; Rethelyi and Szentágothai, 1969; Cervero and Iggo, 1980). Due to the large presence of synaptic glomeruli, it has been further suggested that this region also functions as a centre for signal amplification (Kumazawa and Perl., 1978; Wall P.D, 1978, 1980; Price et al., 1979; Cervero and Iggo, 1980). Neuroanatomical studies have documented synaptic interactions between SG neurons as those located in lamina I and the deep dorsal horn, thus implying that modulated signals from non-peptiderigic PAF input may reach supraspinal targets through indirect ascending pathways (Todd and Lewis, 1986; Todd, A.J., 1989; Lu and Perl., 2005). As several morphological studies have demonstrated, lamina II interneurons possess expansive dendritic arbors in the rostral-caudal plane, but restricted dendritic projections in the dorsal-ventral orientation (Bennett et al., 1980; Todd and Lewis, 1986; Todd A.J., 1988). Neurons resident to lamina III have been documented issuing profuse dendritic branching in lamina II which were observed in several instances establishing pre and postsynaptic contacts with afferent boutons of synaptic glomeruli. Moreover, these lamina III neurons possessed ventrally directed processes which enter the deep dorsal horn (Todd A.J., 1989). Furthermore, wide dynamic range neurons in lamina V respond to C-fibre intensity stimulation of the sciatic nerve indicating either a direct or indirect contact with primary afferent terminals (Woolf, C.J., King, A.E, 1987). These WDR neurons project profuse dorsal-ventral dendritic arbors which in some instances penetrate the ventral borders of lamina II and I (Woolf, C.J., King, A.E. 1987). Furthermore, many WDR neurons issue processes which cross the spinal cord via the dorsal and ventral commissures (Woolf, C.J., King, A.E, 1987).

1.7 An integrative summary and perspective.

We have learned from a number of neurodegenerative disorders that a shift in the balance of inhibition and excitation within a system can result in neurotoxic events. In many cases the shift is toward enhanced excitation by either increased excitatory tone or decreased inhibitory control. In respect to chronic neuropathic pain, we have clear evidence that suggests that both events may underlie the pathological and the symptomological manifestations of the disorder; enhanced primary afferent excitatory input to the superficial dorsal horn (LI-LII) and reduced inhibitory control caused in part by the death of postsynaptic, local GABAergic interneurons. Factoring in the occurrence of aberrant expression of inhibitory and excitatory neurotransmitters and their respective receptors on pre- and postsynaptic terminals, we can begin to piece together pathophysiological mechanisms and strategically formulate potential therapeutics. It is important that we understand the normal processing of nociceptive information in the superficial dorsal horn so that we can strive to predict which pathways are likely to be altered in the event of a nerve injury. It is therefore important that we identify these pathways and the relay stations encountered along the way. As the above sections have described, there are two important junctions in the network from the periphery to the CNS: the connection between the primary afferent terminal and the postsynaptic projections of dorsal horn neurons and the subsequent connection from these cells/terminals to other intrinsic or extrinsic (LI, LIII, supraspinal projections) profiles. Of the three classes of primary afferent fibres, the unmyelinated C-fibre population is an important highway, transporting nociceptive information from cutaneous and deep tissues to lamina I and II of the superficial dorsal horn (see sections 1.2.1.2, 1.3 and 1.4). We have learned a great deal about the nature of C-fibres; the non-peptidergic/peptidergic dichotomy in particular. The existence of these two remarkably distinct avenues for afferent information has raised many questions and has provided the impetus for a vast array of studies into their properties namely their morphology, their neurochemical phenotypes, their electrophysiological idiosyncrasies and their synaptic neighbours. Our knowledge of these properties has advanced

considerably in the last 40 years; these two afferent pathways differentially communicate and process nociceptive information from the periphery to the spinal dorsal horn and yet they function in concert to provide accurate information regarding the nociceptive stimulus encountered. The non-peptidergic primary afferent subclass exhibit interesting morphological and physiological features which set them apart from their peptidergic companion: presynaptic inhibitory control from intrinsic GABAergic SG neurons and a unique neurochemical phenotype lacking neuropeptides and expressing unique presynaptic receptors/ion channels. Their synaptic territory in the dorsal horn is also intriguing. The substantia gelatinosa has captivated many scientists over the years. The apparent lack of forward communication observed by Szentágothai in the mid-1960s, led to the 'closed system' hypothesis which was later put to rest in light of confuting anatomical data illustrating synaptic connections between SG neurons and neurons residing in the marginal zone (LI) and the nucleus proprius (LIII), in addition to supraspinal projections. Thousands of cells in the SG have been pierced by recording electrodes in search of EPSCs and IPSCs stimulated by electrical and mechanical manipulation of C-fibres and their peripheral receptive fields in normal and pathological conditions. Despite the vast compendium of data collected thus far, a clear picture of the information transmitted by non-peptidergic C-fibres and the modulation of this information in the SG are lacking. In the face of such a lack of information arises ingenuity; new animal models and technological approaches facilitate the quest to fill in the blanks.

1.8 Animal models

Of the variant causes of neuropathic pain, compression of a peripheral nerve such as the median nerve in carpal tunnel syndrome, are the most frequently reported painful neuropathies (England J. D., 1999; Fricker, B., Muller, A., René, F., 2008). Nerve compression may arise from the entrapment of a nerve at bony prominences or in narrow canals such as in the cases of ulnar or median nerve compression neuropathies respectively (England J. D., 1999). The variability in the originating cause and ensuing pathology demonstrates the need for multiple animal models. Employing different experimental methods of peripheral nerve

entrapment, these animals provide the opportunity to evaluate the pathological processes and behavioural manifestations as well as the occasion to test new therapeutics (Fricker, B., Muller, A., René, F., 2008).

Animal models of peripheral neuropathies are commonly performed on the sciatic nerve, its constituent spinal nerves or its peripheral branches (Bennett and Xie, 1988; Shir, Y and Seltzer, Z., et al., 1990; Kim, S.H and Chung, J.M., 1992; Mosconi, T and Kruger, L., 1996; Decosterd, I and Woolf, C.J, 2000). Procedurally, each model consists of the snug ligation of the nerve of interest. Methodologically, the models are similar, however several crucial differences exist; injury location, ligature material, animal strain and lesion severity greatly influence the pathogenesis of the resultant neuropathy (Maves, T.J., Pechman, P.S., Gebhart, G.F., Meller, S.T., 1993). Several models have been developed to examine the regenerative capacity of peripheral nerves following severe nerve trauma. Nerve transection models are commonly used to study the effects of growth factors and nerve grafts on regeneration (IJkema-Paassen, J., Jansen, K., Gramsbergen, A., Meek, M.F., 2004).

Model	Location	Methodology	Material	Reference
Chronic Constriction Injury (CCI)	Common sciatic nerve proximal to trifurcation	Application of 4 loose-fitting ligatures 1 mm apart	4-0 Chromic Gut sutures	Bennett, G.J. and Xie,Y.K., 1988.
Spinal Nerve ligation (SNL)	L5 and/ or L6 spinal nerves	The ligation of either the L5 and L6 spinal nerves.	3-0 silk thread	Kim, S.H and Chung, J.M., 1992.
Partial Nerve Ligation (PNL)	Common sciatic nerve distal to the posterior semitendinuous nerve	The dorsal ½ to ⅓ of the nerve is ligated	8-0 silicon-treated silk thread	Shir, Y and Seltzer, Z., 1990.
Spared Nerve Injury (SNI)	Tibial and Common peroneal nerves	Both nerves are tightly ligated and a 2-4mm section distal to the ligation is sectioned	5-0 Silk thread	Decosterd, I and Woolf, C.J, 2000.
Polyethylene Cuff Model of Chronic Constriction	Common sciatic nerve proximal to trifurcation.	One or several fixed diameter polyethylene cuffs are applied to the sciatic nerve	Polyethylene tubing Inner diameter: 0.0762 cm	Mosconi, T and Kruger, L., 1996.

Table I:

Animal models of neuropathic pain.

Two distinct animal models were employed for the purpose of the studies described in this thesis. The Mosconi and Kruger model of chronic constriction injury was used to examine the effects of such an injury on various properties of both C-fibre populations as well as on the expression of certain neurochemical targets within the superficial dorsal horn. The second animal model consists of a neurotoxic lesion targeted to selectively ablate the non-peptidergic population of primary afferent fibres to permit the evaluation of the consequence of their absence on their peptidergic counterpart. Moreover, this animal model provided the opportunity to examine changes in the expression of markers for lamina I neurons and inhibitory interneurons in the SG.

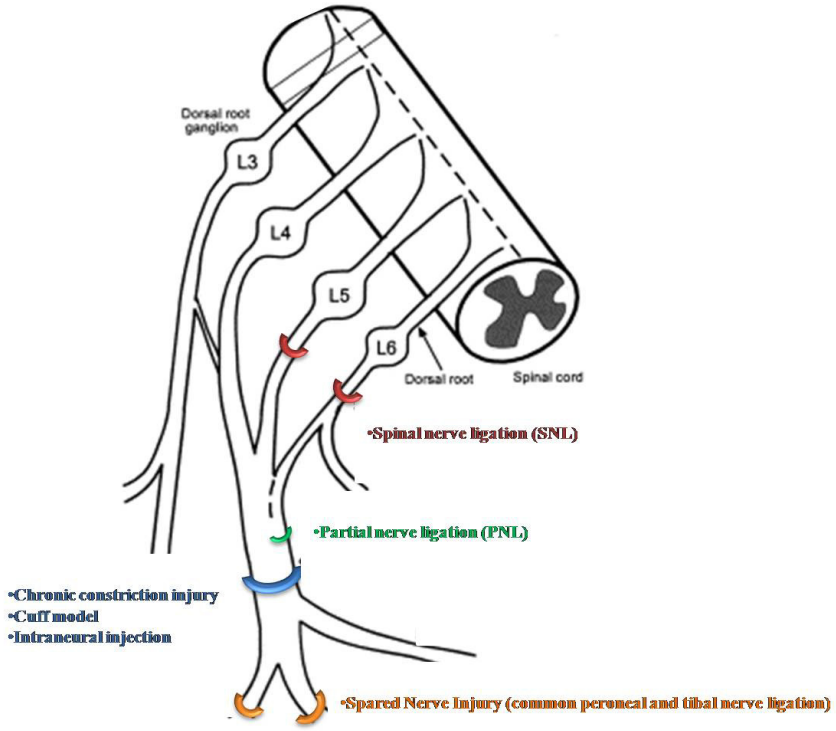


Figure 9

Diagrammatic representation of the common animal models of peripheral nerve injury.

(Adapted from Decosterd and Woolf, 2000)

1.8.1 The Mosconi and Kruger model of chronic constriction injury.

It is essential that an animal model accurately reproduces the symptoms commonly reported by patients in the clinic. Patient suffering from peripheral neuropathies often present with the following symptoms: allodynia (pain in response to an innocuous stimuli), dysesthesia (spontaneous pain), hyperalgesia (enhanced pain response to a noxious stimuli, anesthesia (loss of normal sensation in the affected region) and referred pain. Neuropathy paradigms typically consist of one or several, snug-fitting (but not tight), ligatures applied to a peripheral

nerve or its constituent branches. The most common model of peripheral constriction neuropathy is the chronic constriction injury (CCI) developed by Bennett and Xie in the late 1980's (1988). This model effectively reproduces the pathology observed in the clinic (Bennett, G.J., Xie, Y.K., 1988). The paradigm consists of tying 4 chromic gut suture ligatures around the common sciatic nerve. The resultant compression is caused by the extensive swelling of the constricted sciatic nerve, effectively self-strangulating against the ligatures.

Similar to the CCI paradigm, the model designed by T. Mosconi and L. Kruger consists of the application of 4 fixed-diameter poly-ethylene cuff around the sciatic nerve (Mosconi T, Kruger L., 1996). Due to the fixed diameter of the cuff, the intra-animal variability seen with the individual tying of ligatures is no longer an issue. The spectrum of fiber injury is far more constant compared to CCI (Mosconi T, Kruger L, 1996). In lieu of the 4 smaller polyethylene cuffs placed around the nerve, our model consists of the application of a single, slightly longer cuff (see Pitcher, G.M., Ritchie, J., Henry, J.L. 1999). The symptoms demonstrated by cuff-treated animals are akin to those exhibited by CCI animals as well as patients (Bailey, AL and Ribeiro-da-Silva, A., 2006).

1.8.2 Neurotoxic lesioning

Termed "molecular neurosurgery", the use of neurotoxic agents to selectively lesion a specific population of neurons has proven efficient. Exploiting the natural process of receptor-mediated endocytosis, neurotoxic agents conjugated to a specific ligand may enter the cell and upon its transport to the cell body, induce apoptosis (Wiley, R.G and Lappi, D.A., 2003). Ribosome inactivating proteins such as Saporin effectuate cell death by inducing apoptosis. Upon binding to the large ribosomal subunit, Saporin, an endoglycosidase, removes a crucial adenine (#4324) rendering the ribosome unable to associate with elongation factor 2 resulting in the arrest of protein synthesis (Wiley, R.G and Lappi, D.A., 2003). Remarkably few ribosomes are required to be inactivated (10%) in order to elicit cell death. Binding to the ribosomal subunit and the resultant inactivation of protein synthesis is a rapid process, occurring within minutes to hours upon reaching the cell soma.

Intraneural (intra-sciatic) administration of IB4 conjugated to saporin was used to selectively lesion the IB4-binding, non-peptidergic C-fibre neurons as well as their peripheral and central terminals. Several pilot studies are required in order to determine the specifics of the protocol. These include determining the minimal effective dose of the conjugate, the time required to produce the desired lesion, the extent of the lesion and the appropriate controls. These pilot studies have been conducted prior to initiating the appropriate experiments needed to disprove the null hypothesis.

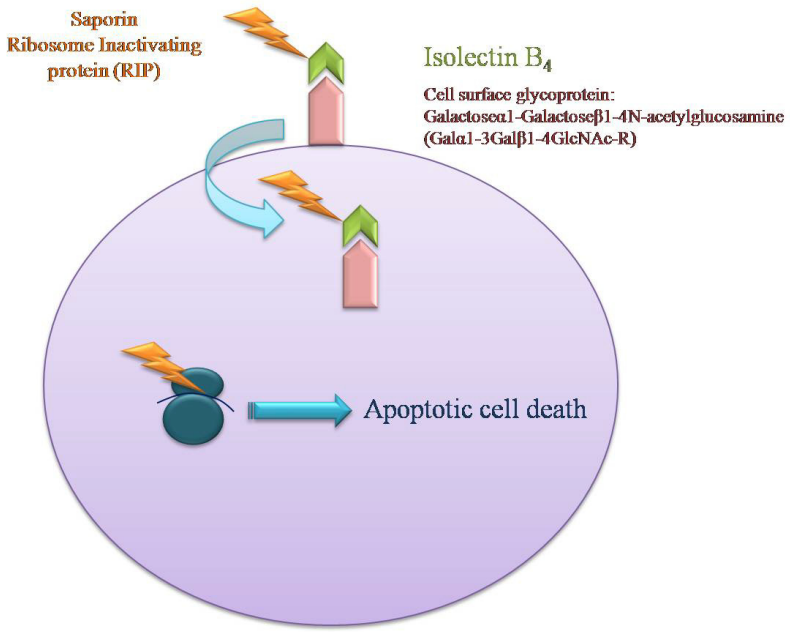


Figure 10

Mechanism of action of IB4-Saporin.

Diagramatic representation of the internalization of IB4-conjugated to saporin and the cytotoxic effect on protein synthesis.

(Adapted from Adavnced Targeting Systems Bio (ATS Bio)

Retrieved from www.atsbio.com)

1.9 Objectives and Rationale

From peripheral receptive fields to higher brain centers, the passage of noxious stimuli along primary afferent fibres to the first relay station in the superficial spinal dorsal horn holds important information about how peripheral signals are enhanced, inhibited or modulated prior to their supraspinal voyage. At present, a clear picture of these processes is but a sketch in progress.

Before we can truly comprehend the pathological consequences of peripheral neuropathies, a firm grasp on the routine processing of nociceptive information is essential. However, examination of the consequence of injury to or the selective loss of a constituent within a system can provide a great deal of information regarding the functionality of said constituent.

Non-peptidergic primary afferents constitute nearly half of the C-fibre population of sensory fibres and yet information pertaining to their contribution to nociception is incomplete. Therefore, I proffer that further comprehension of the nature of information passed from primary afferents to spinal dorsal horn neurons can only be fully understood when we determine the influence of non-peptidergic primary afferent C-fibres on the normal and abnormal processing of nociceptive information.

In defining the objectives of this thesis, we took into account: 1) that the effect of peripheral nerve injury induced by the application of a fixed-diameter cuff on non-peptidergic afferents has yet to be characterized, 2) that the nature of the effect of such nerve injury on the central terminals of non-peptidergic afferents has yet to be determined, 3) that the effect of the selective ablation of non-peptidergic afferents by IB4-Saporin treatment on nociceptive behaviour and neurochemical markers for peptidergic afferents is not fully characterized and 4) that the effect of IB4-Saporin treatment on the neurochemical markers for lamina I neurons and SG inhibitory neurons remains undetermined. Based on the above premises, we used light and electron microscopy to determine the effects of peripheral nerve injury on both peptidergic and non-peptidergic afferents and their immediate postsynaptic targets. Furthermore, we used behavioural analyses combined with light microscopy to examine the consequence of the selective loss

of non-peptidergic afferents on nociceptive behaviour as well as on neurochemical markers for peptidergic primary afferent fibres and postsynaptic neurons in lamina I and II. The following working hypotheses were formulated:

Hypothesis I:

Peripheral nerve injury will differentially affect non-peptidergic (IB4-binding) and peptidergic primary afferents.

Hypothesis II

The selective ablation of IB4-binding, primary afferent fibres will produce alterations in nociceptive responses to acute peripherally applied noxious stimuli.

Hypothesis III

The absence of non-peptidergic, IB4-binding input to the superficial dorsal horn will disrupt spinal pathways projecting to affective regions of the brain involved in the formation of associative learning in the context of a nociceptive stimulus.

Chapter II

Transient loss of terminals from non-peptidergic nociceptive fibres in the substantia gelatinosa of spinal cord following chronic constriction injury of the sciatic nerve. A.L. Bailey and A. Ribeiro-da-Silva, Neuroscience 138: 675-690, 2006.

Transient loss of terminals from non-peptidergic nociceptive fibres in the substantia gelatinosa of spinal cord following chronic constriction injury of the sciatic nerve.

Andrea L. Bailey[1,2] and Alfredo Ribeiro-da-Silva[1,2,3]

1. *Department of Pharmacology & Therapeutics, McGill University, 3655 Promenade Sir William Osler, Montreal Quebec, Canada H3G 1Y6.*
2. *Alan Edwards Center for Research on Pain, McGill University, 740 Doctor Penfield Avenue, Montreal, Quebec, Canada H3A 2B2.*
3. *Department of Anatomy & Cell Biology, McGill University, 3640 University Street, Montreal Quebec, Canada H3A 2B2.*

Corresponding author:

Dr. A. Ribeiro-da-Silva, Department of Pharmacology & Therapeutics, McGill University, 3655 Promenade Sir William Osler, Montreal, Quebec, H3G 1Y6, Canada. Tel: 514.398.3619, Fax: 514.221.3207, E-mail:alfredo.ribeirodasilva@mcgill.ca

Keywords:

IB4-binding; synaptic glomeruli; neuropathic pain; CGRP; confocal microscopy; electron microscopy

Acknowledgements:

This research was support by CIHR grant #38093 to A. Ribeiro-da-Silva. Andrea L. Bailey acknowledges a studentship from the Louise Edwards Foundation. We are grateful to Gary J. Bennett and Véronique Kéna-Cohen for their help with the animal behaviour, to Sid Parkinson for editorial assistance, and to Manon St. Louis, Vanessa Partridge and Johanne Ouellette for technical expertise.

Abstract

It is well known that following peripheral nerve injury, there are numerous changes in neurotransmitter and neuropeptide expression in the superficial dorsal horn, the dorsal root ganglion and the periphery. Of particular interest are the relative contributions of two sub-types of unmyelinated C-fibres in the initiation and maintenance of chronic pain, the peptidergic, and the non-peptidergic. Evidence gathered in recent years has led researchers to believe that the non-peptidergic nociceptive primary afferents are functionally distinct from their peptidergic counterpart. For our study, we used a well established animal model of constriction neuropathy (the Kruger model) and studied Wistar rats at 5, 7, 10, 15 and 21 days after nerve lesion caused by the application of a fixed diameter polyethylene cuff to the left sciatic nerve. Animals were assessed for the onset and evolution of mechanical allodynia using calibrated von Frey filaments and were additionally tested for thermal (heat and cold) hypersensitivity. Immunocytochemical detection of calcitonin gene-related peptide (CGRP) and Isolectin B4 (IB4) binding was used to visualize the dorsal horn distribution of the boutons from the peptidergic and non-peptidergic fibres respectively. Using confocal microscopy and image analysis, we detected a significant decrease in the density of IB4-labelled boutons, ipsilateral to the lesion, at 7 and 10 days following nerve injury. The density of IB4-labelled varicosities retuned to control levels by 15 days. There were no significant changes in the density of CGRP-labelled varicosities at all time points examined. Applying EM, we initially detected degenerative changes in the central elements of type I glomeruli and then a considerable reduction in their number followed by recovery at 15 days post-lesion. As the central boutons of type Ia represent varicosities from the fibres which bind IB4, the EM changes confirmed that there was a bona fide transient loss of varicosities, not simply a loss of IB4 binding. These data indicate that, in this animal model, morphological changes in the nociceptive C-fibre input of the rat dorsal horn are restricted to the non-peptidergic sub-population and are transient in nature. Furthermore, such changes do not correlate with the time-course of the allodynia.

Introduction

Following peripheral nerve injury, there are changes in neurotransmitter and neuropeptide expression in the superficial dorsal horn, the dorsal root ganglion (DRG) and the periphery. Of particular interest are the relative contributions of two sub-types of unmyelinated C-fibres in the initiation and maintenance of chronic pain. In fact, it has been proposed that pain related information is conveyed by two unmyelinated primary afferent fibre populations (Hunt, S.P and Rossi, J., 1985). Of these two populations, one contains neuropeptides and terminates predominantly in lamina I and outer lamina II, whereas the other possesses fluoride resistant acid phosphatase activity (FRAP), binds the isolectin B_4 from *Griffonia simplicifolia* (IB4) and terminates predominantly in inner lamina II (Hunt, S.P and Mantyh, P.W., 2001; Alvarez, F.J and Fyffe, R.E., 2000; Ribeiro-da-Silva, A., Tagari, P., Cuello, A.C., 1989). In addition, the two populations differ in the ultrastructural properties of their central terminals. The non-peptidergic population terminates mostly as central varicosities of type Ia synaptic glomeruli (C_{Ia}) whereas the peptidergic terminate, in the great majority, as simple axo-dendritic terminals with only a small number functioning as the central bouton of glomeruli of type Ib (with large dense-core vesicles in the central terminal) (Ribeiro-da-Silva, A., Tagari, P., Cuello, A.C., 1989; Ribeiro-da-Silva, A., 2004). The idea of two distinct sub-populations of C-fibres would suggest the occurrence of parallel processing in pain pathways, yet the functional role of the non-peptidergic afferents in the transmission of pain related information remains poorly understood (Alvarez, F.J and Fyffe, R.E., 2000; Hunt, S.P and Mantyh, P.W., 2001). However, there is some evidence to indicate that these fibres may be functionally distinct from the peptidergic population (Stucky, C.L and Lewin, G.R., 1999). During development, all small diameter DRG neurons are responsive to nerve growth factor (NGF) and likewise express the high affinity NGF receptor TrkA and the low affinity neurotrophin receptor p75 (Silos-Santiago, I., et al., 1995). However, during the early postnatal period, approximately half of the small diameter DRG neurons down regulate these

receptors while concomitantly up regulating their expression of c-ret, the signal transduction domain of the GDNF receptor (Bennett, D.L., Averill, S., Clary, D.O., Priestley, J.V., McMahon, S.B., 1996; Molliver, D.C and Snider, W.D., 1997). It is these GDNF-responsive, non-peptidergic fibres that subsequently bind the lectin IB4 (Plenderleith, M.B., Wright, L.L., Snow, P.J., 1992; Molliver, D.C and Snider, W.D., 1997).

Studies using animal models of peripheral nerve injury have attempted to analyze the role of the non-peptidergic C-fibres in conditions of chronic pain. Molander et al. (1996) showed that, following sciatic nerve transection, there is a dramatic decrease in IB4-labelling in the superficial dorsal horn, reaching maximal depletion 2 weeks post-lesion. However, over extended periods of time (8 months), IB4-labelling was restored, albeit to levels below controls. Similar results were obtained following nerve crush, with restoration of IB4-labelling occurring much sooner. These studies have provided some insight into the possible functional role of the non-peptidergic fibres, more specifically, their potential involvement in mechanical and thermal nociception. However, only a few studies have attempted to examine directly the responses of both IB4-positive and IB4-negative neurons to certain stimuli. Liu et al (2004) have shown that cultured rat IB4-positive DRG neurons showed robust inward currents following capsaicin application. These responses were greater in magnitude than those seen in IB4-negative neurons and exhibited distinctive current kinetics. Taken together, this evidence confirms that the non-peptidergic afferents may belong to a functionally distinct class of unmyelinated C-fibres.

The majority of these studies conducted qualitative assessments of the changes in peptide expression or IB4-labelling, limiting observations to major alterations in immunoreactivity. However, we know that small changes in peptide or receptor expression can result in functional alterations. For example, an incomplete antisense knockdown of the $P2X_3$ receptor, one of many receptors expressed by the IB4-binding fibres, resulted in a significant attenuation of mechanical hyperalgesia induced following nerve ligation (Barclay et al., 2002).

Our study was designed to fill in some of the gaps in our knowledge by providing a quantitative analysis of the changes induced in peptide expression and

IB4-labelling in two sub-populations of primary afferent nociceptive C-fibres following nerve injury. We supplemented our study with EM observations, which allowed us to confirm that the changes we observed at the light microscopic level corresponded to a bona fide transient degeneration of terminals, not just to a transient loss IB4-labelling.

Materials and Methods

The guidelines contained in the Care and Use of Experimental Animals of the Canadian Council, volumes I and II were rigorously followed. Furthermore, all protocols were reviewed and approved by the McGill University Animal Care Committee before experimentation began.

Surgical Procedures

Sixty-three male Wistar rats weighing 170-200g were used in all experiments. Prior to surgery, animals were anaesthetized with 0.5 ml/kg Ketamine (Vetalar) and 0.25 ml/kg Xylazine (Bayer). On the left side, below the pelvis, each animal was shaved to reveal an obvious groove. An incision 2 cm in length was made at the site. The Bicep femoralis and Gluteus superficialis muscles, which overlie the sciatic nerve, were separated with forceps. A polyethylene cuff (PE), 2 mm in length with an inner diameter of 0.75 mm, was placed on the exposed nerve. The wound was sutured, and an antibiotic ointment applied. Sham operated animals underwent the same procedure; however no cuff was placed on the nerve. These animals served as controls. Four to six animals per surgical treatment (sham and neuropathic) were used for each time period. Fifteen additional animals were used to analyze thermal hyperalgesia (heat and cold) (7 sham-operated and 8 neuropathic animals). A separate group of animals was processed for electron microscopy.

Behavioural Testing

Mechanical allodynia

In order to study the nocifensive behaviour induced by mechanical stimuli, Von Frey filaments 4, 8 and 15 grams in force were applied. Prior to surgical treatment, animals were habituated to the testing apparatus for 30 minutes. Baseline thresholds were taken on the two consecutive days before surgery. For testing, rats were placed on a metal mesh platform under a plastic enclosure which allowed access to all four paws. On each test day, animals were briefly habituated to the test environment for 15 minutes. Testing consisted of the application of the von-Frey filament to the plantar surface of the left and right hind paws between the interdigital pads, in the awake and unrestrained animal. Testing was initiated with the 4 gram von-Frey filament, testing the left paws of all rats first, followed by the right paws. Once each series was complete, the next von-Frey filament was applied (8 and then 15 grams). Each filament was applied, slightly buckling, 10 times and the number of obvious withdrawals was recorded. Acute withdrawal, biting, licking or shaking of the left hind limb and vocalization were considered to be positive responses. This test is designed to measure the extent of the tactile allodynia seen in neuropathic animals.

Heat Hyperalgesia

Evaluation of thermal nociceptive thresholds was carried out using the Hargreaves test (Hargreaves et al, 1988). Animals were placed in a transparent plastic enclosure on top of an acclimatized glass surface. Animals were habituated to the testing apparatus for 30 minutes a day on the two consecutive days prior to baseline threshold acquisition. Thresholds were measured using a radiant light source connected to an automatic timer: when the hind paw was lifted, the light is immediately shut off and the latency is measured. Measurements were taken from the plantar surface of both the left and right hind paws. The cut off time was set at 20.48 seconds to prevent tissue injury. Baseline thresholds were taken 3 days prior to surgery. Means represent the average of three trials taken at 5 minute intervals.

Cold allodynia

To test for signs of altered sensitivity to cold stimuli, animals were tested using an acetone spray. Briefly, animals were placed in the same test apparatus used for mechanical stimulation. Baseline thresholds were acquired prior to surgical treatment. Testing consisted of the application of 50µl of acetone to the plantar surface of the hind paw via a 1mL syringe equipped with a blunted 20 gauge needle. Response to acetone application was recorded on an ordinal scale as follows: 0 indicated no response, a quick stamp or flick was recorded as 1, repeated flicking was recorded as 2 and licking or biting of the paw was recorded as a 3. Animals were given 20 seconds in which to respond. A response of 1 or 2 resulted in an additional 20 seconds. The final response was recorded. Both left and right hind paws were tested and a total of three trials were conducted. The median was graphed. Statistical analysis was conducted using the Friedman's test followed by Dunn's post hoc tests.

Tissue Preparation

At the end of the desired time period (5, 7, 10, 15 and 21days post surgery) the animals were deeply anaesthetized with Equithesin (6.5mg chloral hydrate and 3 mg sodium pentobarbital in a volume of 0.3ml, i.p., per 100 g body weight). Perfusion of the rats was carried out through the left cardiac ventricle, with 4% paraformaldehyde (PFA) in 0.1M phosphate buffer (PB), pH 7.4. After 30 minutes, the spinal cords were removed and immersed in 4% PFA and 0.1M PB for 2 hours after which they were transferred into 30% sucrose and PB at 4°C. The following day, 50 µm thick transverse sections were cut on a freezing sledge microtome (Leica). These sections were from the 4^{th} and 5^{th} lumbar segments (L4 and L5) of the spinal cord, the region corresponding to the sciatic nerve central projections. The sections were collected as free-floating in phosphate buffered saline with 0.2% Triton X-100 (PBS+T).

Double Labelling

Sections, once collected, were incubated for 1 hour in 10% normal horse serum (NHS) (Vector Laboratories). Then, following washes in PBS+T, we added a rabbit anti-CGRP antibody, in a 1:2000 dilution (Sigma), and Isolectin B_4 conjugated to Alexa Fluor 488 (Molecular Probes), in a 1:200 dilution. 5% NHS and PBS+T were then added. The plate with the sections was wrapped in foil and placed on a shaker overnight at 4°C. On the second day, a fluorochrome preparation was made consisting of an anti-rabbit IgG antibody conjugated to Rhodamine Red X in a 1:50 dilution (Jackson Immunoresearch Laboratories Inc.) in 5% NHS and 10mg/ml of rat acetone brain powder (Sigma) in PBS+T for half an hour at 37°C. This preparation was vortexed every ten minutes, followed by centrifugation at 4000 rpm for 20 minutes at 16°C. Sections were washed extensively in PBS +T and the fluorochrome preparation was added for an incubation period of 2 hours. At the end of the incubation, the sections were washed in PBS and mounted on gelatine-subbed slides. They were left to dry overnight at 4°C. The following day they were coverslipped with Vectashield, the edges of the coverslip were sealed with nail polish and the slides were stored at -20°C until examined under the microscope.

Microscope observations and quantification

Sections from control and neuropathic animals were examined using a Zeiss LSM 510 confocal scanning laser microscope with argon and helium neon lasers. Appropriate filters were selected for the separate detection of Alexa 488 and Rhodamine Red-X, using a multitrack scanning method to completely separate the detection of the two signals. This approach allows for the, complete separation of settings regarding lasers, filter combinations and detection thresholds for the Alexa 488 and Rhodamine RedX channels. The settings are alternately and automatically changed by the digital signal processor at the end of scanning each line.

To obtain all images to be used for quantification, a 63X plan-apochromatic oil immersion objective was utilized. The images obtained represent

optical sections approximately 0.5 μm thick in each channel, as determined by the pinhole sizes we used for each channel. To ensure that we obtained a representative sampling, all the parameters of laser power, pinhole size and image detection were kept unchanged when obtaining all images used for quantification. Furthermore, in each field, we always scanned the focal plane that corresponded to the apparently highest density of immunolabelled structures, as detected in a quick scanning of the section along the Z axis. The penetration of the immunoreagents throughout the entire thickness of the 50 μm sections was excellent. The images obtained were converted to TIFF files and quantified using an MCID Elite image analysis system (Imaging Research Inc., St. Catharines, ON, Canada). For quantification, each confocal image was analyzed with the MCID Elite software, using a modified version of functions originally designed for radioautographic grain counting, so that individual axonal varicosities were detected. The area counted per section was a rectangle 100 X 60 μm, and was placed equidistantly from the dorsal and medial edges of the dorsal horn (Fig. 1). In each animal, a total of 5 tissue sections were measured bilaterally (both in the ipsilateral and contralateral sides relative to the lesion). Four animals per survival time and four sham operated animals were used for this analysis. In order to compensate for the lack of counting of overlapped and clustered varicosities, a correction was performed by the MCID software. In brief, for areas of overlap, the computer divided the areas of clustered staining by the average size of axonal varicosities, giving a better estimate of the number of varicosities present in the field. The same segmentation limits were used for all samples. The means and SEM for each treatment group (control and neuropathic) per side (ipsilateral and contralateral) and signal (IB4 and CGRP) were calculated. Test of significance was carried out on GraphPad Prism version 4. Unpaired T-tests were performed when analyzing control animals with neuropathic animals. Paired t-tests were used for side to side comparisons. Significance was set at $p<0.05$.

Electron microscopy

In order to determine whether there was a transient loss in IB4-labelling or a decrease in the number of IB4-labelled terminals in the spinal dorsal horn following cuff application, the spinal cord from some animals was processed for electron microscopic observation. Briefly, animals underwent surgeries for cuff application as described above or were sham operated. At the end of the desired time point (5, 7, 10 and 15 days), animals were perfused transcardially with 1% glutaraldehyde and 1% paraformaldehyde in 0.1M phosphate buffer. Spinal cords were removed and post-fixed for 2 hours in the same fixative and then transferred into a 30% sucrose solution in 0.1M phosphate buffer. Transverse sections of the L4-L5 spinal cord were cut 50 μm thick on a Vibratome (TPI). Alternate Vibratome sections were either directly osmicated as described below or washed and then treated with 1% sodium borohydride, followed by overnight incubation in biotinylated-IB4 (1:200) (Molecular Probes). The following day, sections were incubated in an Avidin-Biotin complex (1:400) (from a Vector Elite ABC kit) for 1 hour. Labelling was revealed using a double intensified DAB reaction using cobalt chloride and nickel ammonium sulphate (Ribeiro-da-Silva, A., Priestley, J.V., Cuello, A.C., 1993). All sections were then treated for 1 hour in 1% osmium tetroxide in 0.1 M phosphate buffer. Following dehydration in ascending alcohols and propylene oxide, the sections were flat-embedded in Epon. In order to select fields corresponding to the CNS termination area of sciatic nerve afferents, the coverslips containing the ipsilateral side of the spinal cord of rats 7 and 10 days after lesion were observed at low power with a microscope and photographed. The micrographs were used as a guide to identify, in all Vibratome sections, the area to be trimmed and cut for EM observation. At this stage, all sections were re-embedded in Epon and, after polymerization, the blocks trimmed, and ultrathin sections were cut on a Reichert ultramicrotome using a diamond knife. The trimmed area in all blocks had a mediolateral extension slightly less than the region of termination of sciatic afferents in the corresponding segment. The ultrathin sections were collected onto one-slot, formvar-coated grids, counterstained with uranyl acetate and lead citrate and examined with a Philips 410 EM equipped with a digital camera.

In order to evaluate the average number of synaptic glomeruli per ultrathin section, two animals per group were used. From each animal, four Epon blocks were used, two corresponding to the ipsi- and two to the contralateral side. One ultrathin section was cut per block. These sections were scanned under the electron microscope and the number of synaptic glomeruli of each morphological type were counted in lamina II after being identified using well established criteria (Ribeiro-da-Silva, A., Tagari, P., Cuello, A.C., 1989; Ribeiro-da-Silva, A., 2004). In brief, to be considered as a synaptic glomerulus, a synaptic arrangement had to fulfil all of the following criteria when observed on isolated ultrathin sections: a) possess a central axonal bouton full of granular synaptic vesicles; b) this central bouton has to be surrounded by at least 4 neuronal profiles, mostly regular dendrites, but some representing presynaptic dendrites (V_1) or axonal boutons (V_2); c) two or more synapses have to be observed between the central bouton and the peripheral profiles. The identification of individual glomerular types was based primarily on the properties of the central boutons. Type Ia glomeruli (non-peptidergic) were easy to identify because they possess small, electron-dense and scalloped central boutons (C_{Ia}), packed with small agranular vesicles of non-uniform diameter, and were often postsynaptic to V_1 and/or V_2 profiles. In contrast, type Ib synaptic glomeruli (peptidergic) differed because of the presence of numerous larger dense-core vesicles in the central bouton, where they co-existed with the agranular vesicles. In addition they are not postsynaptic to V_1 or V_2 profiles, being surrounded almost entirely by dendrites. Type II glomeruli (non-nociceptive) had central boutons which were rounder, larger, richer in mitochondria and more electron-lucent than those in types Ia and Ib glomeruli; as with type Ia, they were frequently postsynaptic to V_2 and V_1 profiles (Ribeiro-da-Silva, A., Tagari, P., Cuello, A.C., 1989; Ribeiro-da-Silva, A., 2004). The observations made in ultrathin sections cut from the superficial part of Vibratome slices from material processed for IB4 binding confirmed that only type Ia glomeruli were labelled and that glomeruli of other types were not. Therefore, as the morphological preservation was clearly superior in sections not processed for IB4 binding, it was only in this material that the counts of synaptic

glomeruli were made. The observer who performed the EM observations was blinded to the experimental groups.

Results

Analysis of behavioural changes in sham-operated and neuropathic animals

Qualitative assessment of animal behaviour following cuff application revealed that animals in the neuropathic group exhibited signs of nociceptive behaviour including limping and guarding of the left (ipsilateral) hind paw. Guarding consisted of maintained withdrawal of the paw in an everted position with the toes together and slightly ventroflexed. Neuropathic animals exhibited signs of increased mechanical sensitivity at the 4 gram threshold by the 7^{th} day following cuff application. This reached statistical significance by day 10 when compared to baseline ($p< 0.05$, paired t-test) (Fig. 2). The percent response to von-Frey filament application at this threshold increased steadily over the course of the 21 days following cuff application, with the percent response the highest at 21 days post-operatively reaching 56% ($p<0.05$ vs Baseline).

Examination of mechanical allodynia at 8 and 15 gram thresholds showed a similar pattern of behaviour, with the initial increase in the percent response appearing at post-operative day 5

(18.7 % and 31.3% respectively). By day 10, post-operative thresholds reached statistical significance and remained significant until the end of the time point. Post-operative withdrawal frequency to the 15 gram von-Frey filament reached 97.75% by day 21, indicating that cuff-operated animals were extremely mechano-sensitive. In contrast, sham-operated animals showed no signs of neuropathy. Withdrawal thresholds remained similar to that of baseline thresholds for all three grams of force applied throughout the course of the study. In addition, we examined the contralateral hind paw (right) to observe whether cuff treated animals exhibited signs of bilateral hypersensitivity. The right paws of neuropathic animals showed no signs of allodynia or hyperalgesia nor the sham operated animals.

In addition to changes in mechanical thresholds, animals showed a significant decrease in withdrawal latencies to noxious thermal stimuli (Fig. 3).

By the fifth post-operative day, neuropathic animals showed a significant decrease in withdrawal latencies compared to baseline ($p<0.001$). Withdrawal latencies remained significantly reduced compared to baseline ($p<0.001$) until the end of the survival period (21 days). Sham operated animals showed no significant change in thresholds over time ($p>0.05$). Right (contralateral) paw withdrawal latencies were also assessed in both sham and neuropathic animals. Right paw withdrawal thresholds were not significantly different compared to baseline ($P>0.05$) for both treatment groups. The exception was a left paw thresholds in sham animals on day 10 compared to baseline (decreased threshold) ($p<0.05$).

Recent evidence indicates that nerve injured animals also display aberrant sensitivity to cold stimuli. We used a 50µl acetone spray administered to the hind paw to determine if there were indications of altered sensitivity to cold. Neuropathic animals exhibited signs of increased sensitivity to cold stimuli by post-operative day 7 (Fig. 4; ($p<0.01$, Friedman's test followed by Dunn's post-hoc compared to baseline). Responses to cold stimuli remained significantly increased compared to baseline throughout the course of the study (Fig. 4). Sham-operated animals showed no alterations in cold thresholds for the duration of the study compared to baseline ($p<0.05$). Right paw thresholds for both sham and neuropathic animals did not show any signs of aberrant sensitivity to acetone application however we did observe an interesting phenomenon. Stimulation of the right, uninjured hind paw with acetone resulted in the brisk withdrawal of the operated, injured hind paw in neuropathic animals. This phenomenon was observed in sham-operated animal, however to a much lesser extent than in neuropathic animals.

IB4 labelling in neuropathic and sham-operated animals.

In sham-operated animals, IB4-binding was confined to the substantia gelatinosa (lamina II) with particularly intense labelling in the inner lamina II (IIi) (Fig.5A). IB4 labelling in the superficial dorsal horn in sham animals remained unchanged in all time periods examined. At higher magnification, IB4 labelling was shown to represent fibres with dilated portions, representing axonal varicosities, throughout inner lamina II (Fig.5D). By the fifth day post-surgery,

neuropathic animals exhibited a slight decrease in the density of IB4-labelled varicosities compared to control animals ($p<0.05$), which was evident in the middle third of lamina II ipsilateral to nerve lesion (Fig.6A). This drop in the number of IB4-labelled varicosities was marked at day 7 following nerve lesion and remained low until day 10 ($p<0.05$) (Figs. 6B and 6C). By days 7 and 10, the density of IB4-labelled varicosities was shown to be significantly decreased ipsilateral to nerve injury compared to control animals ($p<0.05$) (Figs. 5B, 5G, 6B and 6C). The loss in IB4 labelling was restricted to the middle third of the superficial dorsal horn (Fig. 5B), the region innervated by the sciatic nerve (Swett, J.E., and Woolf, C.J., 1985).

Interestingly, by day 15, neuropathic animals showed resurgent IB4 labelling in the middle third of lamina II, with a density of varicosities similar to that of control animals ($p>0.05$) (Fig. 5C, 5J and 5D). Levels of IB4-binding continued to remain at near normal levels 21 days after surgery ($p>0.05$) (not shown). Quantification of IB4-binding in the contralateral dorsal horn revealed no change in IB4-binding at all time periods ($P>0.05$; side to side and between treatment groups) (Figs. 6A-D).

CGRP immunoreactivity in neuropathic and sham-operated animals

In sham-operated animals, CGRP-IR fibres were mostly located in lamina I and outer lamina II, with some fibres extending ventrally to terminate as far as lamina V (Fig. 5A). At high magnification, CGRP immunoreactivity could be seen in punctate structures throughout lamina I and outer lamina II, with some labelling of fibres travelling into deeper laminae (Fig.5E). We did not detect any changes in the density of CGRP-IR varicosities when comparing sham-operated and neuropathic animals at any of the survival periods studied ($p>0.05$ for all time periods; Figs. 6E-H).

Electron microscopy examination of non-peptidergic terminals

We confirmed that IB4 binding occurred on the plasma membrane of rather electron-dense unmyelinated axons and of varicosities which represented the central terminals of type Ia synaptic glomeruli (Fig. 7A). The latter structures

could be easily identified even in preparations which were not stained for the demonstration of IB4 binding (Figs. 7B and 7A) based on typical electron density, shape, low number of mitochondria and properties of synaptic vesicles (Ribeiro-da-Silva, A., 2004). However, in the interest of accuracy, both sections prepared for pure morphology and for IB4 labelling at the EM level were examined, although counts of synaptic glomeruli were performed in the former only, as the preservation was superior. IB4 labelling was seldom found in axonal boutons with dense core vesicles, and was never found in the central boutons of type Ib glomeruli, which possessed a considerable number of dense core vesicles varicosities in the central terminal (Fig. 7C), or in the bigger, richer in mitochondria and less electron dense terminals of type II glomeruli (Fig. 7D). The observation of ultrathin sections from the side ipsilateral to the lesion in neuropathic animals across all time periods revealed evidence of type Ia central terminal degeneration and depletion from days 5 to 10 post-lesion (Figs. 8B, 9A, 9C and 9D), with apparent regrowth and reconstitution of glomeruli by day 15 (Figs 9D and 10) . However, the classical signs of dense-type degeneration of the entire terminal were seldom observed. More specifically, at post-surgery days 5 and 7, there was a decrease in the number of structures detected as type Ia glomeruli (Fig. 11), based on counts performed using pre-established criteria of identifying type Ia glomerulus as a complex structure with an electron-dense and scalloped central bouton (C_{Ia}) surrounded by at least 4 neuronal profiles, either dendrites or vesicle-containing axon terminals, with at least two synaptic contacts visible (Ribeiro-da-Silva, A., Tagari, P., Cuello, A.C., 1989; Ribeiro-da-Silva, A., 2004). Signs of central terminal degeneration could be detected in some of the C_{Ia} terminals, such as mitochondrial vacuolization, clustering and swelling of synaptic vesicles and loss of synaptic connections with adjacent profiles (Fig. 9A). Some terminals appeared considerably shrunken and had depletion of synaptic vesicles and loss of synapses with adjacent terminals (Fig. 9B). At the 7 and 10 day time points, some electron-dense axons, similar to those connecting the central boutons of type Ia glomeruli in control animals, were seen connecting small, electron-dense, non-glomerular varicosities (Figs. 8B and 9C). These occurred in the region where normally central varicosities of type Ia should be

rather abundant (Fig. 8B). At 10 days post-lesion, the number of structures that could be identified as type Ia glomeruli was further decreased to approximately 1/3 of control levels (Figs. 8A and 11); very few glomeruli with signs of degeneration similar to those at the 5 and 7 days time points were observed. At 15 days, no signs of degeneration were detected in type Ia glomeruli and a higher number of glomeruli of this type could be observed again (Figs. 10 and 11); some of the type Ia glomeruli had a normal structure, but occasionally growth cone-like structures could be observed at the end of electron-dense axons (Fig. 9D). These structures were tentatively identified as growth cones because they represented dilated parts at the end of axons, with filopodia extending from the cone and extending in between adjacent profiles, with almost no synaptic vesicles but with the presence of cisterns and vesicles of the smooth endoplasmic reticulum (not visible in Fig. 7D), and neurotubules which were not oriented along their major axis (Csillik, B and Knyihár-Csillik, E., 1981; Knyihár-Csillik, E., Rakic, P., Csillik, B., 1985; Burry, R.W., Lah, J.J., Hayes, D.M., 1992).

Discussion

In this study, we confirmed that, following a chronic constriction injury of the sciatic nerve, there is a transient loss of IB4 binding in the dorsal horn of the spinal cord ipsilateral to the lesion. Furthermore, by expanding our study to the ultrastructural level, we were able to demonstrate that there was a bona fide loss of varicosities from non-peptidergic unmyelinated fibres, not simply a loss of binding sites, followed by an apparent restoration of the synaptic structure.

Neuropathic animals exhibit altered thresholds to noxious and innocuous stimuli.

Following the application of a cuff to the left sciatic nerve, animals exhibited signs of neuropathic behaviour, evident by the 5th post-operative day. Paw withdrawal thresholds to innocuous mechanical stimuli revealed that animals were allodynic. Neuropathic animals maintained low thresholds to noxious and innocuous stimuli throughout the time course of this study (3 weeks), which is in agreement with previously reported decreases in withdrawal thresholds in a model

of sciatic nerve ligation (CCI) lasting up to 8 weeks (Attal et al., 1994). Similar results were reported by Lindenlaub and Sommer (2000) showing that, following CCI, 50% withdrawal thresholds were low for a period of 38 days. In contrast, in a nerve crush model, low withdrawal thresholds were moderate and short in duration, with recovery by day 7 (Attal et al., 1994).

Neuropathic animals also showed a significant drop in withdrawal latencies to noxious heat by post-operative day 5 compared to baseline ($p<0.001$) and remained significantly decreased throughout the entire survival period of 21 days ($P<0.001$). Interestingly, in other studies using animal models of nerve crush and sciatic nerve injury (SNI) there were no changes in withdrawal latencies to noxious heat (Decosterd, I., Woolf, CJ., 2000). Lack of changes in withdrawal latencies to noxious heat were also detected in a study in the mouse examining changes in thermal and mechanical thresholds in three neuropathy models: the Seltzer model, the spared tibial nerve injury model and the spared peroneal nerve injury model (Shields, S.D., Eckert, W.A. III., Basbaum, A.I., 2003). It is likely that the heterogeneity in the injury produced by each neuropathy model resulted in differences in the onset, duration and severity of nociceptive behaviour. This point is illustrated by the recent demonstration of substantial differences in spontaneous pain behaviours in 5 different models of neuropathy, specifically in the chronic constriction model where animals showed the most dramatic evidence of spontaneous paw lifting (Dowdall, T., Robinson, I., Meert, T.F., 2005). This study also shows differences in the severity, duration and rate of onset of paw lifting in hot and cold plate tests across the models. In addition to differences in the model employed, variations in behaviour thresholds may result from any number of factors including animal strain, housing conditions, animal gender and experimenter (Chesler, E.J., Wilson, S.G., Lariviere, W.R., Rodriguez-Zas, S.L., Mogil, J.S., 2002). Moreover, there are many different protocols available to test for evidence of mechanical allodynia, thermal hyperalgesia and spontaneous pain and therefore the technique used to evaluate these pain behaviours may result in discrepancies.

In addition to changes in mechanical and heat thresholds following nerve injury, we observed increased sensitivity to acute cold stimuli. Numerous

neuropathy models have shown that peripheral nerve injury results in increased sensitivity to innocuous cold (Attal et al., 1990; Won, R., Jung, S.J., Park, Y.G., Chung, S.S., Lee, B.H., 2004). In an animal model of peripheral mononeuropathy (CCI), Attal et al. (1990) observed significant decreases in withdrawal latency to innocuous cold (10ºC water bath) in addition to decreases in vocalization thresholds. Cold sensation is mediated by two known receptors, TRPM8 and TRPA1 (Tominaga, M and Caterina, M.J., 2005; Reid, G., 2005). Both belong to the TRP family of ion channels which includes the well known TRPV1 (VR1) receptor, the capsaicin receptor. TRPM8, a non-selective cation channel with high permeability to calcium is activated by cold stimuli in the range of 25-28 ºC and is localized in small diameter C-fibres. Studies of the co-expression of TRPM8 with TRPV1 are inconclusive with some studies indicating no overlap in expression, while Okazawa et al. (2004) showed moderate (29%) co-expression in cultured rat DRG neurons. Whether there is a bona fide co-expression remains to be clarified. The threshold of activation for TRPA1 is much lower (17 ºC) and can be activated by isothiocyanate compounds such as mustard oil. Similar to TRPM8 receptors, the TRPA1 receptor is localized on small diameter neurons, however, unlike TRPM8; co-expression with TRPV1 has been ascertained along with sP and CGRP. Alterations in cold sensitivity following peripheral nerve injury are thought to reflect changes in both TRPM8 and TRPA1 receptors. More specifically, the percentage of cold-responsive neurons was increased following peripheral nerve injury (Djouhri, L., Wrigley, D., Thut, P.D., Gold, M.S., 2004). This was attributed to either an increase in receptor expression on uninjured neurons, or decreases in a slowly inactivating K^+ current which is thought to mask cold responses in some cold-receptive neurons (Djouhri, L., Wrigley, D., Thut, P.D., Gold, M.S., 2004).

In our study, along with the ipsilateral increase in acute cold sensations, there was a contralateral effect as well. More specifically, application of acetone to the right, uninjured paw resulted in the brisk withdrawal of the injured paw. This phenomenon has been observed in other models of unilateral nerve injury and is known as the crossed withdrawal reflex. Won et al. (2004) examined the effects of stimulation of the uninjured (contralateral) hind paw with von-Frey filaments and

acetone on withdrawal frequencies of the injured (ipsilateral) hind paw following ligation of the tibial and sural nerves. Results observed were similar to those reported in our study; stimulation of the uninjured hind paw with acetone induced increased withdrawals in the injured paw. This crossed withdrawal reflex is thought to be attributed to increased activity in crossed afferents in the spinal cord that, under normal conditions are quiet or silent (Sotgiu, M.L., Brambilla, M., Valente, M., Biella, G.E., 2004).

Transient loss of IB4 labelling following nerve lesion

As pointed out in the introduction, the physiological role of the non-peptidergic, IB4-binding fibres is far from being understood. Despite the fact that their role in normal nociception remains elusive, numerous animal models of peripheral nerve injury have been used to examine the morphological changes in the non-peptidergic afferents in conditions of chronic pain, as detected by the binding of the lectin IB4 (Plenderleith, M.B and Snow, P.J., 1990; Molander, C., Wang, H.F., Rivero-Melián, C., Grant, G., 1996; Sugimoto et al., 2000; Akkina, S.K., Patterson, C.L., Wright, D.E., 2001; Li L, and Zhou XF, 2001). Following sciatic nerve transection, IB4-labelling in the ipsilateral dorsal horn was reduced as early as one week post lesion, remaining low for a duration of 8 months post-lesion (Molander, C., Wang, H.F., Rivero-Melián, C., Grant, G., 1996). Interestingly, in the same study, levels of IB4-labelling in the ipsilateral dorsal horn were similarly depleted following sciatic nerve crush, although the degree of depletion in IB4-binding reported was less than that seen following complete nerve transection. We show that, following the application of a fixed diameter polyethylene cuff to the left sciatic nerve, the depletion and subsequent restoration of IB4-labelling in the ipsilateral dorsal horn occurs at a very different rate than the one reported by Molander et al. (1996) using sciatic nerve transection or crush. Indeed, studies employing different animal models of peripheral nerve injury (ligation, crush) have shown different rates in IB4- binding restoration despite similar rates in the depletion of IB4-binding. It is difficult at the moment to speculate on the cause of this discrepancy.

Non-peptidergic central terminals undergo degeneration and subsequent regeneration following cuff application.

In an effort to determine if there was a bona fide loss of non-peptidergic terminals from the substantial gelatinosa, we performed an electron microscopy study in a small subset of animals examining both IB4-labelling and synaptic glomeruli morphology. The examination of non-peptidergic terminals at the ultrastructural level revealed IB4 labelling almost exclusively on the plasma membrane of electron-dense small unmyelinated axons and in central boutons of type Ia glomeruli in inner lamina II. The examination of the IB4-binding central boutons of type Ia synaptic glomeruli in neuropathic animals revealed that in the early stages of the lesion (days 5 and 7 after surgery), terminals showed marked signs of degeneration. Moreover, particularly at post operative day 10, there was a marked decrease in the number of type Ia glomeruli. What is of particular interest is that at day 7, and more often at day 10 post-surgery, we observed numerous dense axons with slightly dilated, vesicle-containing portions, which established only sparse synaptic connections (Figs. 9C and 10). These structures occurred in the area where type Ia glomeruli are normally found and likely represent the atrophic terminal arborizations of the IB4-binding afferents. Upon verification, they were found to display weak IB4-labelling (not illustrated). Interestingly, there were little or no changes in the central boutons of peptidergic afferents. Despite the small sample size, the data was very consistent, with extremely little variation between the animals. We are thus confident that a larger n would have produced the same results.

It has been shown that the transient loss of FRAP enzymatic activity as a result of transganglionic degenerative atrophy induced by peripheral nerve injury (crush) is restored through a process by which axonal growth cones of primary afferent sensory fibres establish synaptic connections with dendritic growth cones of lamina II cells (Csillik, B and Knyihár-Csillik, E., 1981). It was thought that regeneration of central terminals from injured axons was an unlikely event, and the reoccurrence of FRAP enzymatic activity following peripheral nerve injury could be accounted for by collateral sprouts from uninjured primary afferents. This was shown not to be the case, as a secondary peripheral nerve injury was

able to completely abolish the FRAP activity restored following the primary lesion (Csillik, B and Knyihár-Csillik, E., 1981). Our results agree with this study, in that we saw an atrophy of terminals followed by a re-growth. However, this transient change followed a completely different time course, probably due to the different lesion model used. Our EM data confirms unequivocally that the transient loss of IB4-labelled boutons is not caused by a loss of binding capability by terminals that are still present but rather by an actually terminal loss followed by re-growth. The temporal pattern of changes in pain sensitivity levels observed in this study did not parallel the morphological changes exhibited in the spinal dorsal horn, more specifically the degeneration and restoration of non-peptidergic synaptic terminals. Thus, it is unlikely that the hyperalgesia and allodynia induced by cuff application can be attributed exclusively to the transient degeneration of the C_{Ia} terminals. There are a myriad of constituents which are at play in the initiation and maintenance of chronic pain symptoms, making it improbable that pain is the product of a single pathophysiological process. These include sensitization of primary afferent fibres, alterations in inhibitory control within the spinal dorsal horn and central sensitization.

We can speculate that such terminal degeneration and subsequent restoration (plasticity) contribute in part to the development of pain behaviours. However, within the scope of this study, we can not make a direct association between our observed morphological and behavioural outcomes.

CGRP immunoreactivity is unaffected by cuff application.

Despite the significant changes in IB4-labelling, levels of CGRP immunoreactivity remained unchanged in all time periods examined. Similar results have been observed in animal models for diabetic neuropathy. Streptazotocin-induced diabetes in mice resulted in no changes in CGRP immunostaining in the spinal dorsal horn (Akkina, S.K., Patterson, C.L., Wright, D.E., 2001). Similar results were obtained in animal models of CCI. Cameron et al. (1997) reported that up to 70 days following sciatic nerve ligation, there were no changes in CGRP labelling in the superficial dorsal horn. Similarly, Villar et al. (1989) showed that, following sciatic nerve crush, there were no changes in

CGRP immunoreactivity. Nevertheless, studies have shown long-term changes in other neuropeptides expressed by peptidergic afferents in nerve injury including sP. We limited our study to the examination of CGRP, a neuropeptide expressed exclusively in primary afferents complimented by our examination of the central terminals of peptidergic afferents at the ultrastructural level. Although we did not detect any changes in CGRP-IR in this study does not imply that the peptidergic terminals were not affected by the nerve injury, but that either the degree of changes was below our level of detection, or outside the temporal boundaries of our study.

Alterations in non-peptidergic afferents following cuff application may be growth factor dependent

It is well known that expression of both NGF and GDNF are altered following peripheral nerve injury (Wells, M.R., Vaidya, U., Schwartz, J.P., 1994; Lee et al., 1998; Holstege et al., 1998; Shen, H., Chung, J.M., Chung, K., 1999; Oh, E.J., Yoon, Y.W., Lee, S.E., Hong, S.K., 2000; Takahashi, N., Nagano, M., Suzuki, H., Umino, M., 2003). In normal conditions, NGF and GDNF are target-derived, with only low levels of expression in the DRG (Thoenen and Barde, 1980; Anand, 2004). However, following peripheral nerve injury, levels of NGF and GDNF are known to be altered in the DRG (Lee et al., 1998; Oh, E.J., Yoon, Y.W., Lee, S.E., Hong, S.K., 2000). It has been hypothesized that, following peripheral nerve injury, retrograde transport of growth factors from the periphery is disrupted (Lee et al., 1998), leading to the accumulation of growth factors in the distal portion of the ligated nerve (Oh, E.J., Yoon, Y.W., Lee, S.E., Hong, S.K., 2000; Ohta, K., Inokuchi, T., Gen, E., Chang, J., 2001). Over time, protein levels of GDNF and NGF have been detected in the proximal portion of the nerve indicating their increased expression proximal to the lesion. Proposed sources of de novo synthesis are Schwann cells in nerve and satellite cells in the DRG (Rind, H.B., Von Bartheld, C.S., 2002). However, NGF and GDNF syntheses following peripheral nerve injury are not changed in the same way. More specifically, constriction of the sciatic nerve resulted in the up-regulation of GDNF mRNA in the DRGs and in Schwann cells by post-operative day 8 (Ohta, K., Inokuchi, T.,

Gen, E., Chang, J., 2001). Although increases were reported in one study in the DRG for both NGF mRNA and protein levels (Herzberg et al., 1997), most studies report only little change in NGF expression (Takahashi, N., Nagano, M., Suzuki, H., Umino, M., 2003), or rapid and short lived (2 days-long) decreases in NGF protein levels (Lee et al., 1998). These data indicate that growth factor expression is variably affected following partial nerve injury, with alterations in NGF expression being less marked than those of GDNF .

On the other hand, our data strongly suggests that, in this animal model, the non-peptidergic, GDNF-dependant afferents are more sensitive to the effects of the lesion than the NGF-dependant peptidergic, resulting in a transient marked decrease in IB4-labelled varicosities and little change in CGRP-IR boutons. The recovery of the non-peptidergic varicosities in the spinal dorsal horn that we observed might be caused in part by the increased levels of GDNF observed in DRG and peripheral nerve proximal to the lesion, which would be greater than those observed for NGF. However, this has never been directly documented in the model we used. Therefore, we need to examine changes in the level of expression and the location of GDNF and NGF in the sciatic nerves and DRG of cuff-treated and sham-operated animals. These experiments are currently underway.

Conclusions

In this study, using a well established neuropathic pain animal model, we detected for the first time a transient but selective loss of boutons from non-peptidergic nociceptive afferents which represent the central terminals of type I glomeruli in the spinal cord of these animals. The significance of these changes for the evolution of the pain is at present unclear, although they may play a role in the initial stages of the development of the hyperalgesia and allodynia.

It is also evident that there are model-dependant changes in both peptidergic and non-peptidergic primary afferent C-fibres. Evaluating the anatomical, neurochemical and behavioural changes in different animal models will provide critical information regarding the sequence of events responsible for the initiation

of chronic pain symptoms, which may have profound implications for the way in which patients with chronic neuropathic pain are treated.

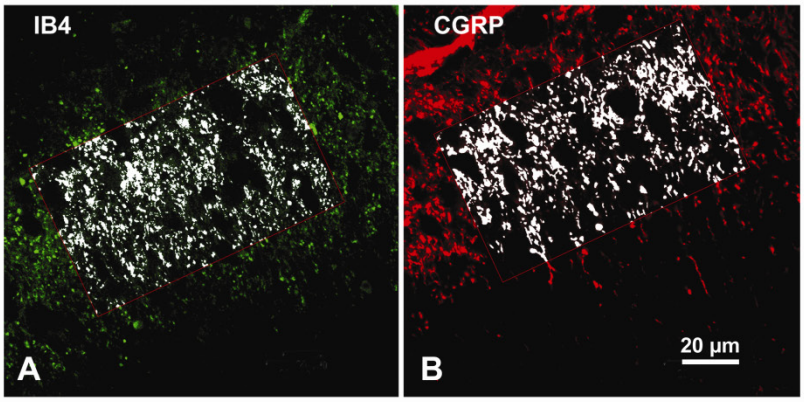


Figure 1

Illustration of the detection of axonal varicosities displaying IB4 binding (A) and CGRP immunoreactivity (B) using the approach described in the Methods section. Images represent 0.5 µm-thick confocal optical sections, obtained with a 63X plan-apochromatic oil immersion objective. In the image analysis system, a rectangle of 100 X 60 µm (with a red outline in figure) was placed equidistantly from the dorsal and medial edges of the dorsal horn and the features automatically detected inside it (in white). The software feature we used was originally devised for radioautographic grain counting and allows us to define a minimal size for a varicosity to be counted (to eliminate cut axons) and compensate for overlapped varicosities. See Methods for details.

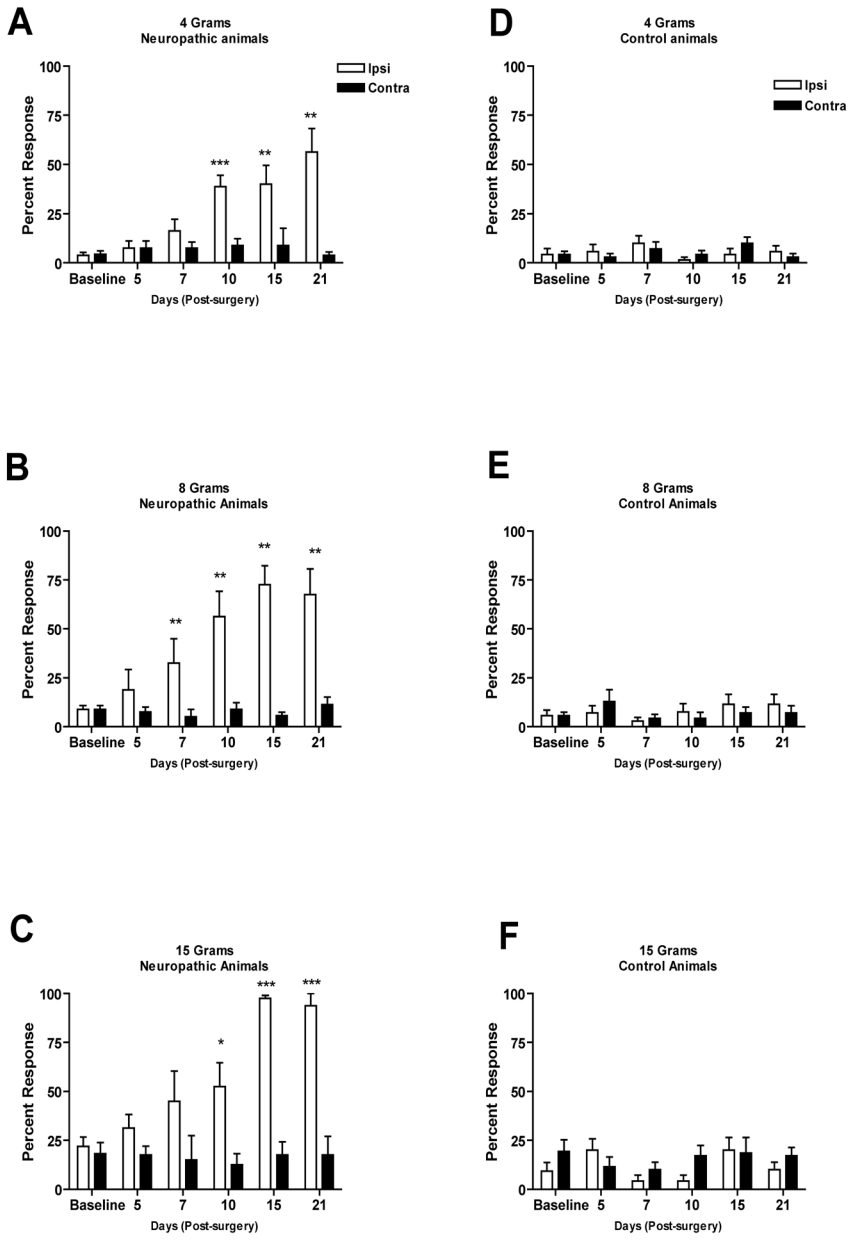

A
4 Grams
Neuropathic animals
Ipsi
Contra
Percent Response
100
75
50
25
0
Baseline 5 7 10 15 21
Days (Post-surgery)
***
**
**
D
4 Grams
Control animals
B
8 Grams
Neuropathic Animals
**
**
**
**
E
8 Grams
Control Animals
C
15 Grams
Neuropathic Animals
*
***
***
F
15 Grams
Control Animals

Figure 2

Figure 2

A-F. Behavioural assessment of mechanical allodynia in lesioned animals (A-C) and sham-operated animals (D-F) at 4, 8 and 15 gram thresholds. **A-C**: Neuropathic animals showed a substantial increase in percent response by post-operative day 7 at all three thresholds. Percent response to vFH at all three thresholds continued to increase throughout the time course of the study. Right paw thresholds in lesioned animals showed no evidence of hypersensitivity to mechanical stimuli at all thresholds tested for the entire duration of the study. **D-F**: Sham operated animals showed no evidence of mechanical allodynia at all thresholds compared to baseline. In addition, right paw thresholds were indistinguishable from left paw thresholds in sham operated animals (*$p<0.05$. **$p<0.01$, ***$p<0.001$).

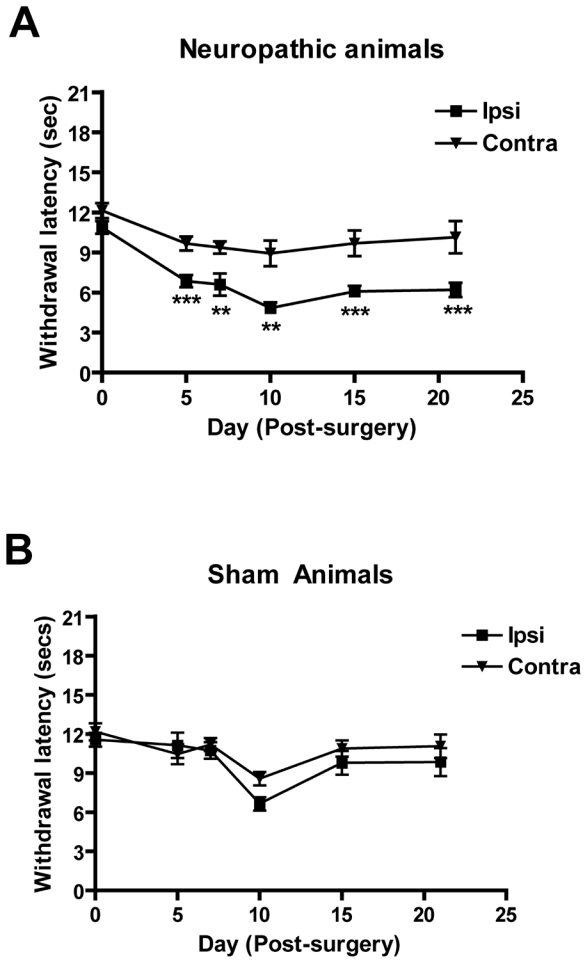

A
Neuropathic animals
Withdrawal latency (sec)
Day (Post-surgery)
Ipsi
Contra
***
**
**
***
***
B
Sham Animals
Withdrawal latency (secs)
Day (Post-surgery)
Ipsi
Contra

Figure 3

Figure 3

Assessment of thermal hyperalgesia using the Hargreaves test in lesioned (**A**) and sham operated animals (**B**). By the fifth post-operative day, neuropathic animals showed a significant decrease in withdrawal latencies compared to baseline ($p<0.001$). Withdrawal latencies remained significantly reduced compared to baseline ($p<0.01$) until the end of the survival period (21 days). No significant reduction in latencies was observed in sham-operated animals. Right paw thresholds in both sham and lesioned animals remained unremarkable for the entire duration of the study (*$p<0.05$. **$p<0.01$, ***$p<0.001$).

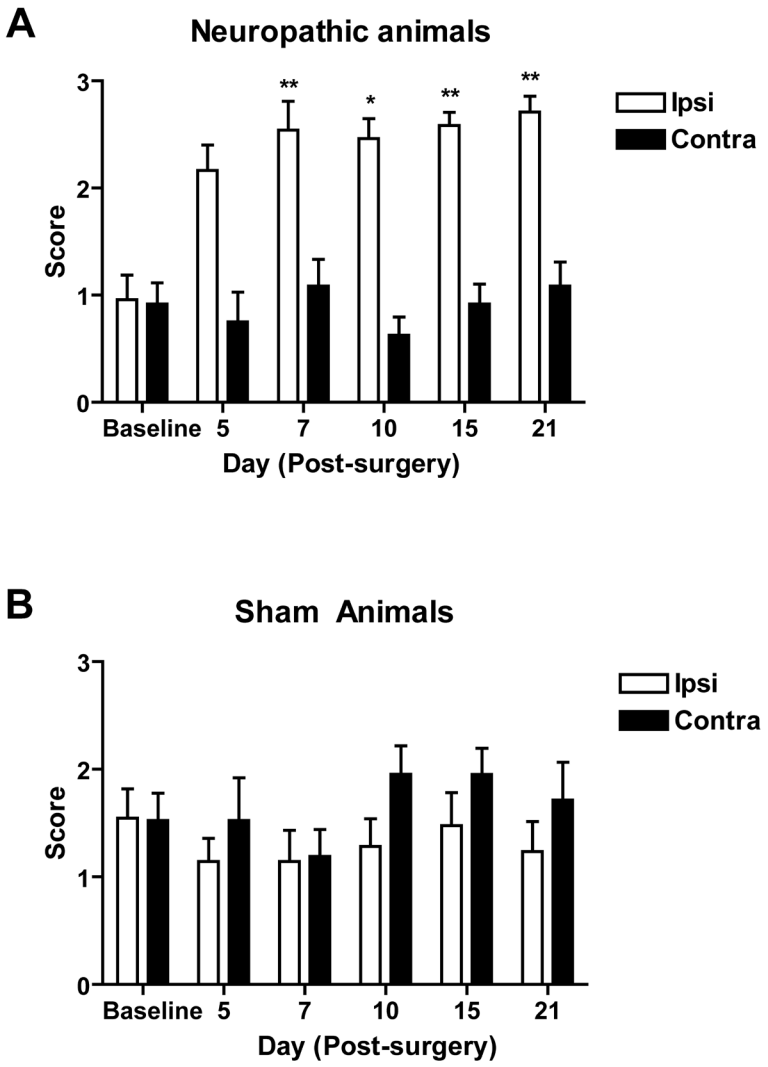


Figure 4

Assessment of cold sensitivity in lesioned (**A**) and sham operated animals (**B**). **A:** Lesioned animals showed a substantial increase in cold sensitivity evident at post-operative day 5 and reaching significant by day 7 compared to baseline ($p<0.01$). Cold sensitivity scores remained significantly high compared to baseline at all time points in the study (compared to baseline, Freidmann's test, Dunn's Post hoc test). In contrast, right paw sensitivity scores were not significantly affected. **B:** Sham operated animals showed no alterations in cold sensitivity at any point during the study. Right paw scores remained unchanged compared to baseline throughout the course of the study ($p>0.05$).

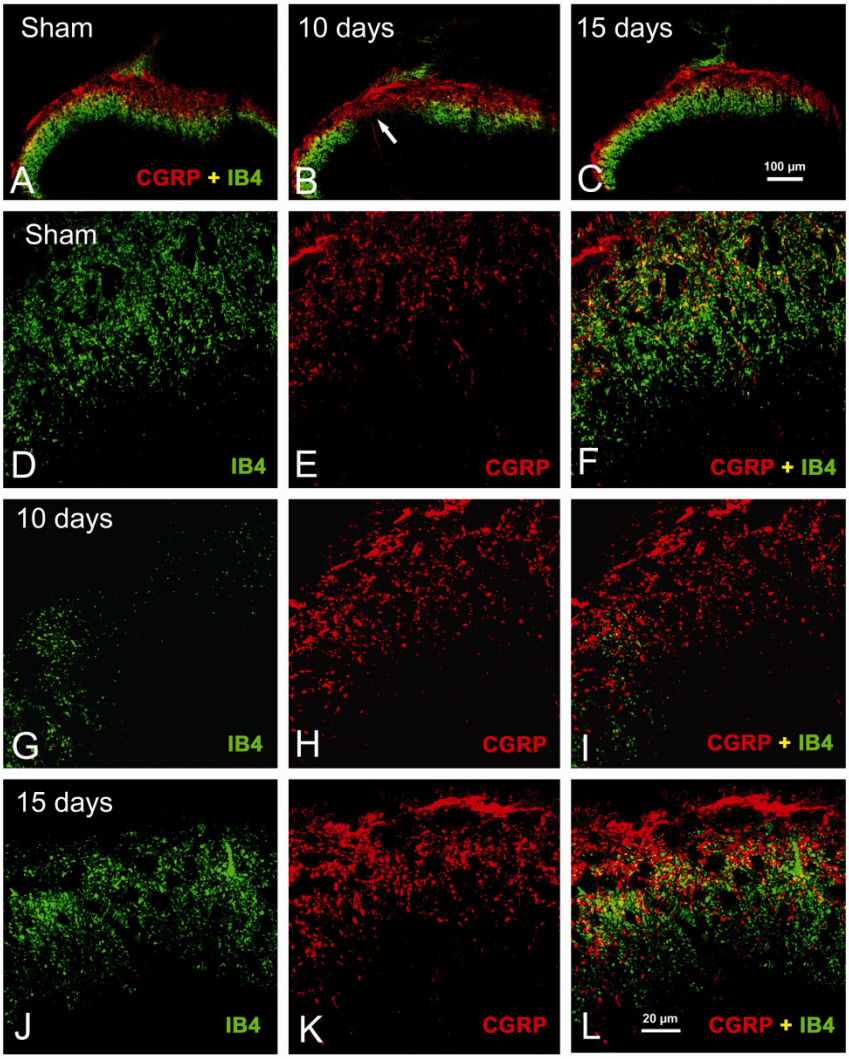

Sham
10 days
15 days
A
CGRP + IB4
B
C
100 µm
Sham
D
IB4
E
CGRP
F
CGRP + IB4
10 days
G
IB4
H
CGRP
I
CGRP + IB4
15 days
J
IB4
K
CGRP
L
20 µm
CGRP + IB4

Figure 5

Figure 5

Confocal microscopy images from the dorsal horn of sham-operated and lesioned animals *ipsilateral* to the surgery. IB4-labelling is shown in green and CGRP immunoreactivity in red. All images were obtained with a multitrack approach, allowing the simultaneous detection of both labels. ***A*** to ***C*** represent low magnification images from sham-operated (***A***), and neuropathic animals 10 days (***B***) and 15 days (***C***) after surgery, respectively. Note that in ***B*** there is a region of depletion of IB4 staining (arrow), and that in ***C*** this area of depletion is not seen. ***D*** to ***L*** represent high magnification (oil immersion) confocal images from sham-operated (***D***-***F***), and neuropathic animals, 10 (***G***-***I***) and 15 days (***J***-***L***) after surgery, respectively. In each row, the image on the left represents the IB4 labelling channel only, the one in the middle the CGRP channel only and the one on the right both channels simultaneously. At this higher magnification, the depletion of IB4-labelled varicosities is evident at 10 days post-surgery (***G***), as well as the restoration of IB4-labelled varicosities at day 15 (***J***).

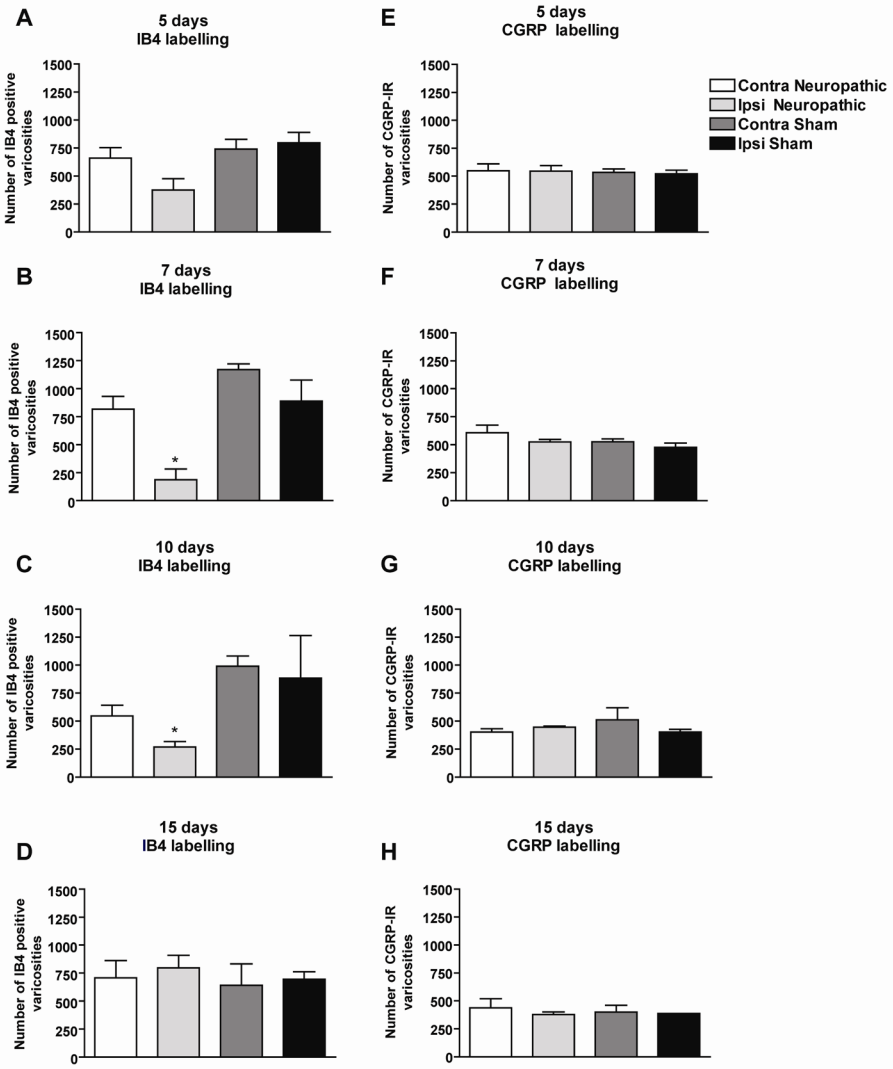

A
5 days
IB4 labelling
B
7 days
IB4 labelling
C
10 days
IB4 labelling
D
15 days
IB4 labelling
E
5 days
CGRP labelling
F
7 days
CGRP labelling
G
10 days
CGRP labelling
H
15 days
CGRP labelling
Number of IB4 positive varicosities
Number of CGRP-IR varicosities
1500
1250
1000
750
500
250
0
*
Contra Neuropathic
Ipsi Neuropathic
Contra Sham
Ipsi Sham

Figure 6

Figure 6

Quantitative analysis of the changes in the number, per 6000 μm^2, of IB4-positive and CGRP-IR varicosities within the region corresponding to the sciatic territory (area pointed with an arrow in Figure 5B) in the ipsilateral (ipsi) and contralateral (contra) dorsal horn. ***A***-***C***: Isolectin-B_4-labelling ipsilateral to nerve injury was decreased 5, 7 and 10 days following cuff application compared to control animals, and the differences were significantly different from the ipsilateral side of sham-operated animals (*$p<0.05$). Fifteen days after nerve injury (***D***), IB4-labelling did not differ significantly from control animals ($p>0.05$, side to side comparison; $P>0.05$, ipsilateral control vs. ipsilateral neuropathic). ***E***-***F***: Quantitative analysis of CGRP-IR in sham and neuropathic (NP) animals revealed no significant changes 5, 7, 10 or 15 days post-lesion ($p>0.05$ for side to side and control vs. neuropathic comparisons).

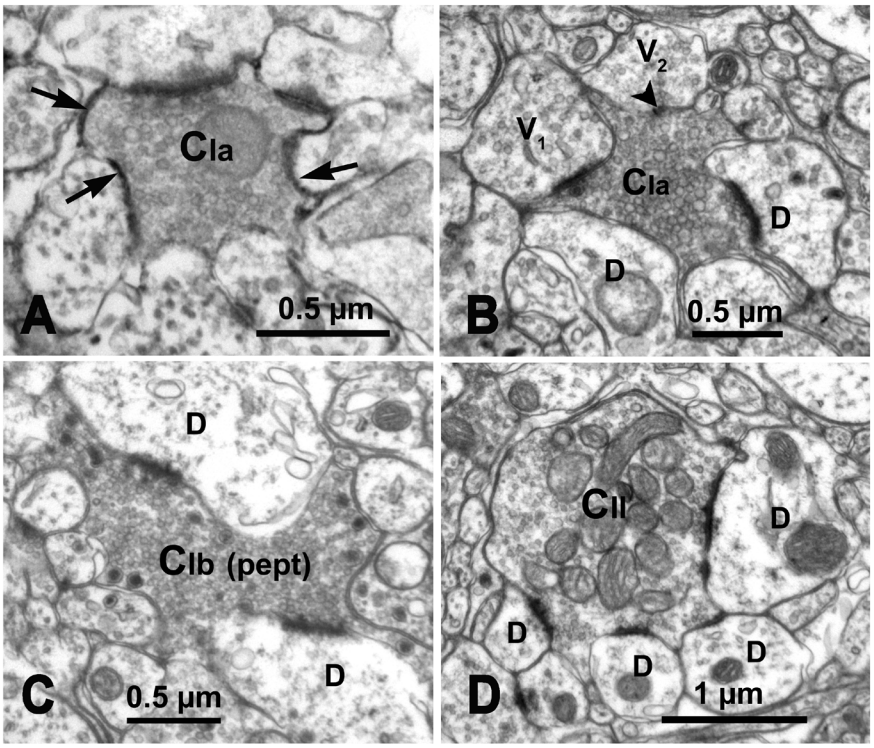


Figure 7

Figure 7

Normal morphology of synaptic glomeruli, as observed in sham-operated animals. ***A*** shows IB4 labelling of the plasma membrane (arrows) around the central terminal (C_{Ia}) of a type Ia synaptic glomerulus. To facilitate the detection of the dense precipitate, the section was not counterstained with uranyl and lead, however, the main characteristics of the central ending of type Ia glomeruli are easily identified, such as the high density of agranular synaptic vesicles with a wide variation of diameters, the almost absolute absence of large dense-core vesicles and the low number of mitochondria, as well as the irregular contour. These characteristics are all detected in ***B***, which shows a glomerulus of the same morphological type in a section which was not processed for IB4 labelling and was counterstained with uranyl and lead. Note also the presence of vesicle containing profiles, identified tentatively as a presynaptic dendrite (V_1) and a peripheral axon (V_2) presynaptic to the C_{Ia} bouton at an axo-axonic contact (arrowhead). IB4 labelling was never detected on type Ib (***C***) and type II (**D**) glomeruli, here shown in micrographs from sections not processed for IB4 binding. C_{Ib} (pept), central bouton of a type Ib glomerulus (rich in dense-core vesicles), which corresponds to the termination of peptidergic afferents (Ribeiro-da-Silva, A., Tagari, P., Cuello, A.C., 1989); C_{II}, central bouton of a type II glomerulus, easy to identify because of the larger size, light matrix and high density of mitochondria (Ribeiro-da-Silva and Coimbra, 1982). D, glomerular dendrites.

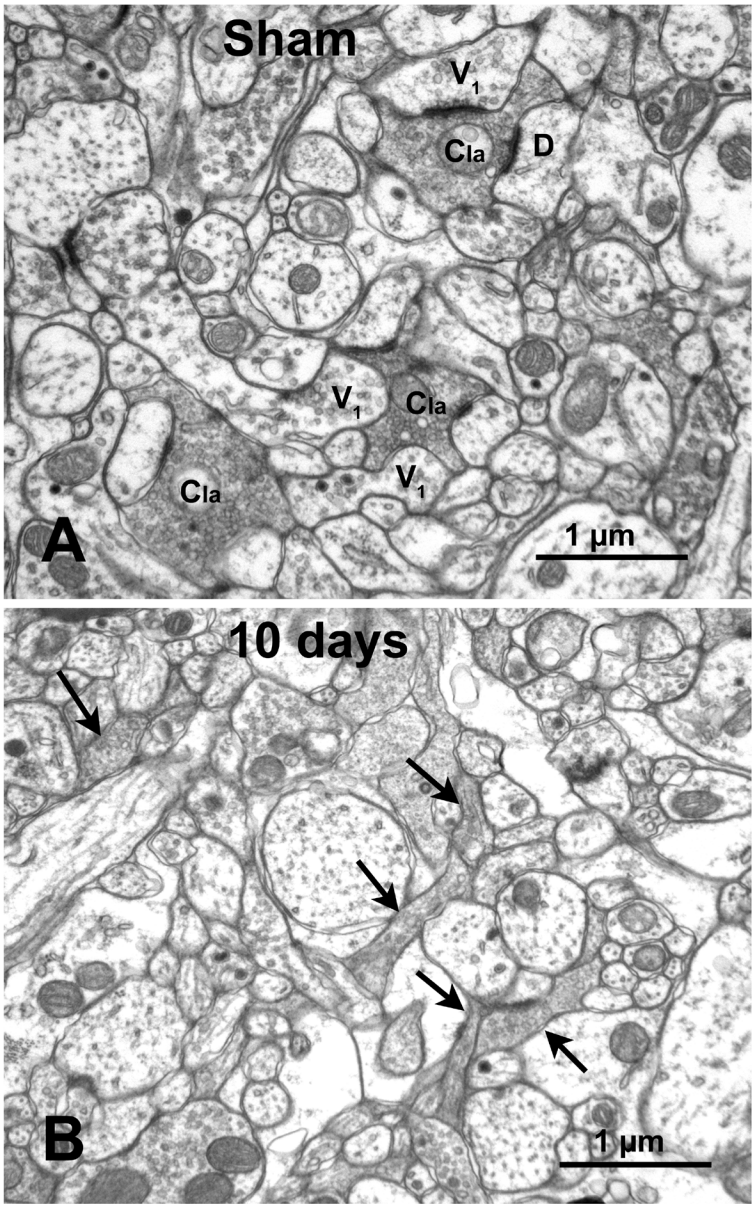


Figure 8

Figure 8

Electron micrographs of areas where normally type Ia glomeruli occur in high density in a sham-operated (***A***) and a neuropathic animal (***B***) 10 days after surgery. In the sham-operated animal, note the presence in the field of three type Ia glomeruli, identified by the characteristic C_{Ia} central boutons. Note also that regular (D) and presynaptic dendrites (V_1) surround the central bouton. In the neuropathic animal, note that type Ia glomeruli are not detected in this field; however, it appears that the C_{Ia} boutons have been replaced by dense axons (arrows) with some slightly dilated, vesicle-containing parts, which seldom established synaptic contacts.

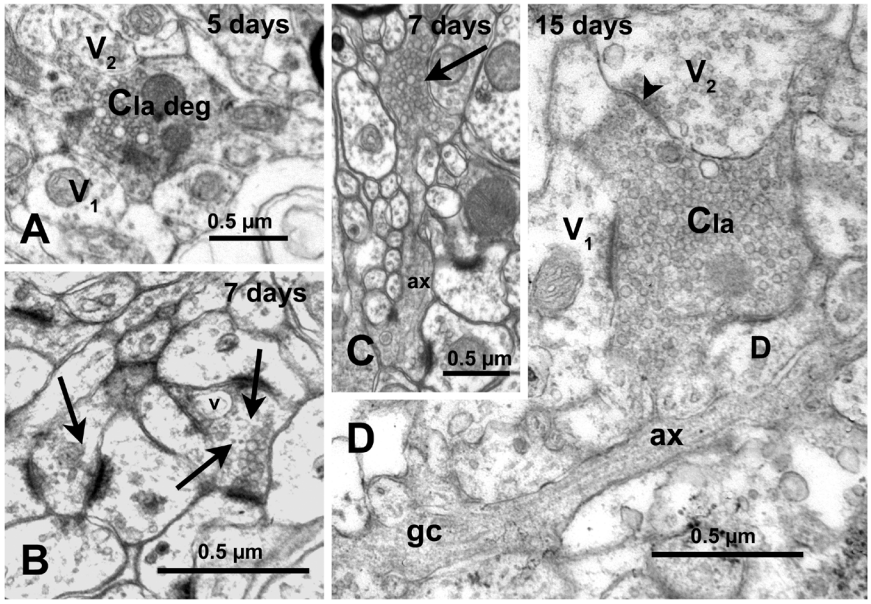

5 days
V2
CIa deg
V1
A
0.5 µm
7 days
ax
C
0.5 µm
15 days
V2
V1
CIa
D
ax
D
gc
0.5 µm
7 days
v
B
0.5 µm

Figure 9

Figure 9

Ultrastructure of degenerative changes in type Ia glomeruli at 5 and 7 days following the placement of the cuff. ***A***. Five days after the lesion, note clustering of the synaptic vesicles in a restricted area of the central bouton of a type Ia glomerulus (C_{Ia}), dilated vesicles and areas of densification of the matrix of the terminal; C_{Ia} boutons with these changes were almost never observed in sham-operated or control animals and were abundant in lesioned animals at 5 days post-surgery. ***B.*** In some C_{Ia} boutons, the entire terminal would become rather lucent and atrophic in size (arrows), with a partial or complete loss of synaptic vesicles and some vacuoles (v), as in this micrograph from a neuropathic animal 7 days post-lesion. Note that the terminal indicated with a single arrow has almost no synaptic vesicles, although the synaptic contacts are surprisingly still observed. ***C***. In this micrograph, observe at higher power a dense axon (ax) like the ones that normally connect C_{Ia} boutons, here virtually devoid of the dilated parts, which were small (arrow) and seldom established contacts with surrounding structures; this observation was confirmed in serial section analysis and is similar to what is illustrated at lower power in Fig. 5B. ***D***. At 15 days post-lesion, note that the glomerular structure seems to be rebuilt, with a normal appearing C_{Ia} bouton, which is post-synaptic to a peripheral axon (V_2) at a symmetric synapse (arrowhead) and pre-synaptic to a presynaptic dendrite (V_1) and a regular dendrite (D). However, note in the lower part of the field a dense axon with a dilated part in the lower left part of the field, which was interpreted as a growth cone (gc).

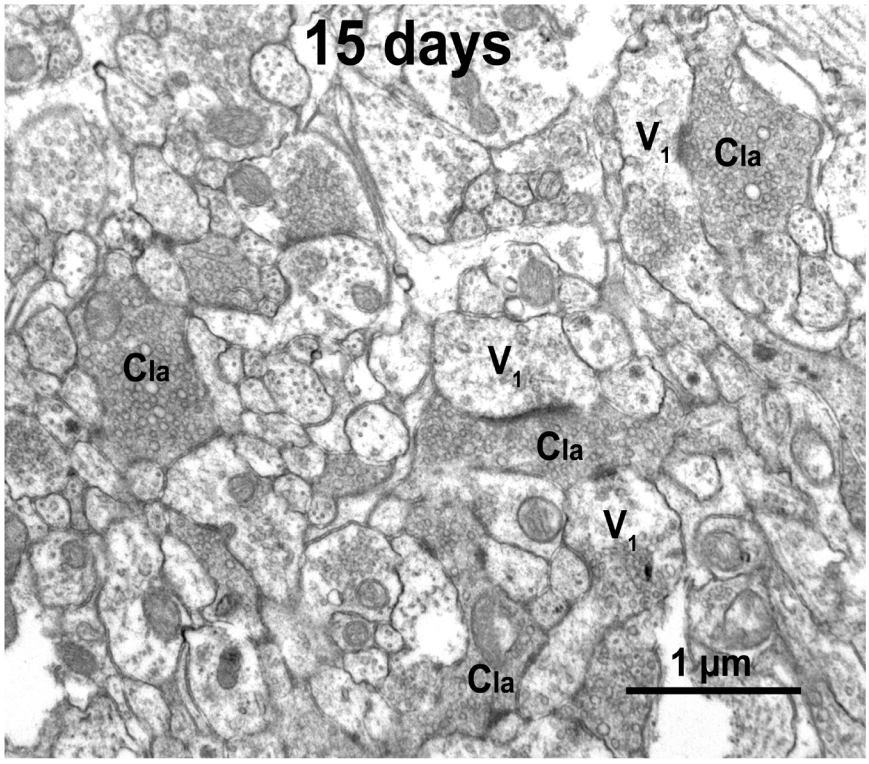


Figure 10

Ultrastructure of the region of termination of non-peptidergic fibres at 15 days post-lesion. Note that type Ia glomeruli now occur again with similar density to control animals, but that some of their central boutons (C_{Ia}) are elongated (see the two C_{Ia} boutons in the central lower part of micrograph). This observation is frequent in parasagittal but is not normally seen this frequently in transverse sections of control animals. V_1, presynaptic dendrites.

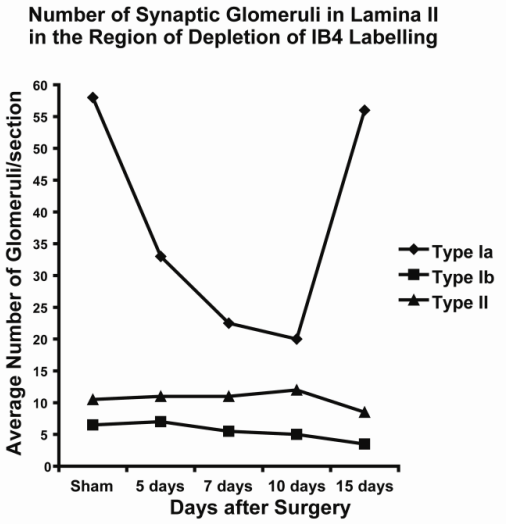


Figure 11

Average number of synaptic glomeruli of each morphological type per section in lamina II of sham-operated and neuropathic animals 5, 7, 10 and 15 days after surgery. The EM blocks were trimmed so that only the region of the medio-lateral extent of lamina II, corresponding to the area of central projection of sciatic afferents, was kept and observed under the EM. Note that at 7 and 10 days post-surgery the number of type Ia glomeruli was 1/3 of the values for sham-operated and neuropathic animals at 15 days post-surgery.

Chapter II-III

The primary objective of the neuropathy study focused on evaluating neurochemical and morphological changes in primary afferent C-fibre subpopulations in a time-dependent manner. Moreover, we examined the development and maintenance of allodynia and hyperalgesia to innocuous and noxious thermal and mechanical stimuli.

The observed population-specific transitory degenerative atrophy of IB_4-binding, non-peptidergic primary afferent C-fibre varicosities in the superficial dorsal horn following the induction of chronic neuropathic pain lends substantial support to the current tenet that the non-peptidergic C-fibre subsidiary population participate in the transmission of peripherally derived nociceptive information in a manner distinct from their peptidergic counterpart (Hunt, S.P., Rossi, J., 1985; Zylka, M.J., 2005). A number of studies employing a wide range of experimental approaches have demonstrated anatomical, neurochemical and physiological properties unique to the non-peptidergic (IB_4-positive) sensory neuron population; distinct current kinetics in response to capsaicin, IB_4-binding neuron-specific intracellular signalling pathways, unique sodium channel subtype- specific response patterns, action potential firing properties influenced by A-type voltage-gated potassium channels and tophic factor support requirements for neurite outgrowth (Liu, M., Willmott, N.J., Michael, G.J., Priestley, J.V., 2004; Hucho, T.B., Dina, O.A., Levine, J.D., 2005; Fang, X., Djouhr,i L., McMullan, S., Berry, C., Waxman, S.G., Okuse, K., Lawson, S.N., 2005; Vydyanathan, A., Wu, Z.Z., Chen, S.R,, Pan, H.L., 2005; Tucker BA, Rahimtula M, Mearow KM., 2006 respectively).

Yet, despite our ongoing evaluation of the idiosyncrasies of non-peptidergic C-fibres, our understanding of their specific role in the transmission of nociceptive information is still in its infancy. In the quest to better comprehend the functional role these afferents partake in nociception, we examined the behavioural, morphological and neurochemical consequence of the selective ablation of IB_4-binding afferents using a targeted neurotoxin in the setting of acute pain.

Chapter III

Selective lesioning of IB4-labeled primary afferent fibres: a morphological and behavioural analysis of the effects of intra-sciatic nerve IB4-Saporin treatment
Andrea L. Bailey and A. Ribeiro-da-Silva, Pain (to be submitted).

Selective lesioning of IB4-labeled primary afferent fibres: a morphological and behavioural analysis of the effects of intra-sciatic nerve IB4-Saporin treatment

Andrea L. Bailey[1, 2] and A. Ribeiro-da-Silva[1, 2, 3].

1. *Department of Pharmacology & Therapeutics, McGill University, 3655 Promenade Sir William Osler, Montreal Quebec, Canada H3G 1Y6.*
2. *Alan Edwards Center for Research on Pain, McGill University, 740 Doctor Penfield Avenue, Montreal, Quebec, Canada H3A 2B2.*
3. *Department of Anatomy & Cell Biology, McGill University, 3640 University Street, Montreal Quebec, Canada H3A 2B2.*

Corresponding author:

Dr. A. Ribeiro-da-Silva, Department of Pharmacology & Therapeutics, McGill University, 3655 Promenade Sir William Osler, Montreal, Quebec, H3G 1Y6, Canada. Tel: 514.398.3619, Fax: 514.221.3207, E-mail:alfredo.ribeirodasilva@mcgill.ca

Acknowledgements.

This research was supported by CIHR grant # 79411 to A. Ribeiro-da-Silva. Andrea L. Bailey acknowledges a studentship from the Louise Edwards Foundation. We are grateful to Veronique Kena-Cohen and Alison Leja for their assistance with the behavioural studies, Dr. Gary Bennett for his assistance and advice and Manon St-Louis for her technical expertise.

***Key Words**: Isolectin B (IB4), Saporin, Calcitonin gene-related peptide (CGRP), Substance P (sP), Neurokinin-1 receptor (NK1 receptor), allodynia, hyperalgesia, neurotoxic lesions.*

Abstract

Small diameter nociceptive primary afferent fibres are subdivided into two neurochemically distinct subpopulations, the peptidergic and the non-peptidergic. However, the precise nature of the involvement of non-peptidergic C-fibres in the transmission and processing of pain-related information remains elusive. In particular, it is not known whether the peptidergic and non-peptidergic afferents function as one unit, or whether they represent independent avenues of nociceptive input to the superficial dorsal horn. Advances in neurotoxic lesion methodologies provide the possibility of selectively ablating neurochemically defined neuronal populations. In this study, we used saporin conjugated to the lectin IB4 (IB4-Saporin) to selectively ablate the non-peptidergic subpopulation of primary afferents and determine the consequence of their loss on peptidergic primary afferents and behavioural responses to acute nociceptive stimuli. Using IB4 binding to detect non-peptidergic afferents, we observed a marked loss of IB4-labeled cell bodies in the L5-L6 dorsal root ganglia (DRG) two weeks following the intra-sciatic injection of IB4-Saporin, with no apparent loss of peptidergic neurons as detected by calcitonin gene-related peptide (CGRP)-immunoreactivity. Similarly, using IB4-labeling and $P2X_3$-immunoreactivity as markers of non-peptidergic afferent terminals within the superficial dorsal horn, we detected a remarkable loss of non-peptidergic terminals in lamina II with no appreciable change in CGRP-immunoreactive peptidergic terminals in laminae I and II. In contrast, examination of substance P (sP)-immunoreactive profiles within the ipsilateral dorsal horn revealed a substantial decline in sP immunoreactivity concomitant with a pronounced increase in immunoreactivity for the sP receptor (the NK1 receptor) in lamina I. Qualitative analysis of immunoreactivity for the GABA synthetic enzyme glutamic acid decarboxylase (GAD) revealed an appreciable reduction in GAD-positive profiles within the superficial dorsal horn, suggesting a compensatory decline in inhibitory signaling following the loss of non-peptidergic input to lamina II. Evaluation of these changes seven weeks following the intra-sciatic injection of IB4-Saporin revealed a diminution in CGRP-immunoreactive terminals in lamina I not observed at the two week time point. Evaluation of paw withdrawal thresholds to acute thermal

(heat and cold) and mechanical (von Frey hair) stimuli revealed no significant changes compared to baseline and control treated animals. Likewise, following the induction of acute inflammation by the intraplantar injection of complete Freund's adjuvant (CFA), examination of inflammatory heat hyperalgesia and mechanical allodynia were unaffected by IB4-Saporin treatment. Similarly, IB4-Saporin treatment was ineffective at altering formalin-induced pain in both the first and second phase of the formalin test. In contrast, the duration of hind paw lifting induced by the intraplantar administration of the purinergic agonist αβ-Methyl-ATP was markedly reduced. Furthermore, capsaicin-induced heat hyperalgesia was completely absent in IB4-Saporin treated animal 30 and 60 minutes following capsaicin administration. These results suggest that compensatory changes in neuropeptide signalling within the peptidergic subpopulation take place following the loss of non-peptidergic primary afferents, as well as alterations in inhibitory signalling, as demonstrated by the decline in GAD-immunoreactivity. Furthermore, these compensatory changes persisted for several weeks following the initial injury. Behavioural analyses suggest that the loss of non-peptidergic input to the superficial dorsal horn does not influence nocifensive behaviours in response to acute noxious stimuli nor acute inflammatory hyperalgesia. However, the absence of capsaicin-mediated heat hyperalgesia in IB4-Saporin treated animals lends support to previously published data suggesting that notwithstanding the expression of the capsaicin transient receptor potential vanilloid 1 (TRPV1) by both peptidergic and non-peptidergic neurons in the rat, non-peptidergic neurons demonstrate a more robust response to capsaicin than their peptidergic counterpart.

Introduction

Axonally transported cytotoxins offer the unique opportunity to examine the morphological and functional consequences of the targeted ablation of a particular neuronal population. These cytotoxins share some of the advantages provided by genetic knock-out methods, in the absence of the unavoidable developmentally-induced compensatory changes. Termed "molecular neurosurgery", the use of cell-specific ligands conjugated to toxins such as the ribosome inactivating protein (RIP) Saporin results in the targeted cell death of the neuronal population of interest. These conjugates can help clarify the specific functional role of sensory afferents, the relationship with other neuronal populations and the immediate compensatory processes resulting from the population loss.

A population of sensory neurons for which their contribution to the transmission of nociceptive signals remains not fully understood is the non-peptidergic subpopulation of small diameter sensory neurons. Although during development all small diameter primary sensory neurons are nerve growth factor responsive, the non-peptidergic population of sensory neurons gets defined following an early post-natal phenotypic shift in growth factor receptor expression. Indeed, after birth, approximately half of the small diameter primary sensory neurons stop expressing nerve growth factor receptors TrkA and p75 and start expressing the glial cell line-derived neurotrophic factor (GDNF) co-receptor C-ret (Bennett, DL. Michael, GJ. Ramachandran, N., Munson, J.B., Averill, S., Yan, Q., McMahon, SB., Priestley, J.V., 1998). The non peptidergic afferents possess a unique cell surface glycoprotein which binds with high specificity to the plant lectin Isolectin B4 derived from the plant *Bandeiraea Simplicifolia* (IB4) (Silverman J.D and Kruger, L., 1990; Kitchener et al., 1993; Gerke, M.B and Plenderleith, M.B., 2004), besides displaying several unique neurochemical, anatomical and physiological properties, which differente them from their peptidergic counterpart.

The most prominent morphological distinction between these two primary afferent subtypes is the presence of dense core vesicles rich in pro-inflammatory neuropeptides such as substance P (sP) and calcitonin gene-related peptide

(CGRP) in the peptidergic population and their absence in the non-peptidergic. Due to this absence of neuropeptides, it has been suggested that the non-peptidergic class of sensory C-fibres plays a minor role in inflammatory pain, with greater attention focused on their potential involvement in neuropathic pain.

In rat, the IB4-labelled afferents terminate mostly in the dorsalmost part of inner lamina II (LIIi) of the dorsal horn (Alvarez, F. J and. Fyffe, R. E., 2000; Hunt S. P and Mantyh P. W., 2001; Ribeiro-da-Silva, A., 2004). The architecture of the central terminals of the IB4-labeled, non-peptidergic afferents is well known; indeed, the majority of their terminals form the core element of type Ia synaptic glomeruli (C_{Ia}) (Hunt S. P and Mantyh P. W., 2001; Ribeiro-da-Silva, A., 2004). Peptidergic axon terminals project to lamina I and outer lamina II (LIIo) and, in contrast to the non-peptidergic, are mostly non glomerular, forming simple axo-dendritic or axo-somatic contacts; indeed, only a small number of them are located in the center of synaptic glomeruli (Ribeiro-da-Silva, A., 2004). Most noteworthy, the central boutons of non-peptidergic synaptic glomeruli receive presynaptic contacts from dendrites and axons, a feature uncharacteristic of the peptidergic population (Ribeiro-da-Silva, A., 2004). These presynaptic connections are hypothesized to play a crucial role in spinally mediated signal regulation as studies have established their neurochemical phenotype as inhibitory in nature (Todd, A. J and Lochhead, V., 1990; Ribeiro-da-Silva, A., 2004).

Besides the differences in the anatomical structure of the central terminations in the and synaptic organization within the dorsal horn, there is also evidence from recent studies demonstrating that the IB4-binding non-peptidergic population differs from the peptidergic in neurotransmitter release patterns and unique physiological properties (Fang, X., Djouhri, L., McMullan, S., Berry, C., Waxman, S.G., Okuse, K., Lawson, S.N., 2006; Matsuka, Y., Edmonds, B., Mitrirattanakul, S., Schweizer, F.E., Spigelman, I., 2007). This evidence strongly suggests that the non-peptidergic sub-population of sensory neurons may play a specific role in the transmission of noxious stimuli. The nociceptive nature of IB4-binding, non-peptidergic, primary afferents has been confirmed by numerous studies (Petruska, J.C., et al., 2001; Petruska, J.C., 2002). Evaluation of their electrophysiological responses to peripherally applied noxious stimuli categorized

them as polymodal nociceptors, as they responded to thermal, mechanical and chemical stimuli in the noxious range (Gerke, M.B., and Plenderlieth, M.B., 2001). It should be stressed that in rat as well as in other species, the non-peptidergic afferents are known to co-express the purinergic ion channel $P2X_3$ and the capsaicin-sensitive transient receptor potential vanilloid 1 receptor (TRPV1), both highly implicated in nociceptive signaling (Vulchanova, L., Riedl, M.S., Shuster, S.J., Stone, L.S., Hargreaves, K.M., Buell, G., Surprenant, A., North, R.A., Elde, R.., 1998; Guo, A., Vulchanova, L., Wang, J., Li, X., Elde, R., 1999). In addition to responding to noxious thermal stimuli above 42°C, voltage-gated TRPV1 receptors are sensitive to a variety of chemical substances, most notably, the pungent compound capsaicin and to high local concentrations of protons (Julius, D and. Basbaum. A. I., 2001). Electrophysiological recordings from neurochemically defined cultured rat dorsal root ganglion (DRG) neurons suggest that, although expressed by both peptidergic and non-peptidergic sensory neurons in the rat, population specific differences in the sensitivity and response properties (current patterns) of the TRPV1 receptor to capsaicin and protons have been documented (Liu, M. Willmott, N.J., Michaels, G.J. and Priestley, J.V., 2004). Specifically, bath application of capsaicin produced an inward current greater in magnitude and evoked a larger increase in intracellular calcium in IB4-positive neurons compared to IB4-negative neurons. Moreover, in acidic conditions, IB4-negative neurons responded preferentially, demonstrating greater amplitudes in response to protons (Liu, M., Willmott, N.J., Michaels, G.J. and Priestley, J.V., 2004). These effects were attributed to the differential expression levels of the TRPV1 receptor by peptidergic and non-peptidergic neurons (Julius, D and. Basbaum. A. I., 2001) However, these results may be better explained by the presence of TRPV1 receptor isoforms exhibiting variable sensitivities to capsaicin and protons (Julius, D and. Basbaum. A. I., 2001).

In the current study, the elimination of the IB4-labelling, non-peptidergic population of sensory neurons, achieved by the intra-sciatic administration of IB4 conjugated to Saporin (IB4-SAP), provided the opportunity to examine the effects of such loss on the peptidergic population. Using behaviour and immunocytochemical assays, we determined that the loss of IB4-labelling sensory

neurons and their central projections prompted compensatory changes in SP, CGRP and the neurokinin 1 receptor (NK1)-immunoreactivity in the superficial dorsal horn. Behavioural analyses demonstrated that the loss of non-peptidergic primary afferent fibers did not influence acute pain sensation (thermal and mechanical). Notwithstanding a marked deficit in αβ-Methyl-ATP-induced paw lifting and a complete loss of capsaicin-evoked heat hyperalgesia, IB4-SAP treatment was ineffective at altering pain levels in the formalin test.

The results of this study demonstrate that in the absence of non-peptidergic sensory fibres, neuropeptide expression by the peptidergic class of nociceptive afferents is altered in a time-dependent manner suggesting the presence of compensatory mechanisms.

Materials and Methods

The guidelines contained in the Care and Use of Experimental Animals of the Canadian Council, volumes I and II were rigorously followed. All protocols were reviewed and approved by the McGill University Animal Care Committee prior to experimentation. Animals were housed 4 per cage (2 experimental and 2 control animals). Food and water were provided *ad libitum*. Animals were maintained on a 12h/12h light/dark cycle. All surgical and behavioural procedures were carried out during the light cycle. Animals were given time to acclimatize to housing conditions prior to experimentation. Care was taken to ensure the number of animals used in this study were restricted to those of necessity.

Surgical Procedures

Intra-neural injections of IB4-Saporin and Vehicle control.

Male Sprague-Dawley rats (225-250g) (Charles River Laboratories, Saint-Constant, Quebec, Canada) were used in all experiments. Animals were randomly assigned to treatment groups (GraphPad QuickCalcs, GraphPat Software Inc.). Animals were anesthetized by rapid induction with isoflurane gas (99.9% isoflurane USP, Pharmaceutical Partners of Canada Inc., Ontario, Canada) followed by maintenance of anaesthesia using a mixture of 3.5-5% isoflurane and oxygen. All unilateral lesions were conducted on the left sciatic nerve. The lateral

aspect of the thigh was shaved and a 2cm-long incision was made at the level of the sciatic notch. The underlying muscles, the gluteus superficialis and the bicep femoralis, were separated, exposing the sciatic nerve proper. The nerve was carefully isolated from the surrounding connective tissue and stabilized using a blunt probe, with special care taken to minimize stretching of the nerve. A 27½ gauge needle, attached to a 25µL Hamilton syringe (Hamilton Company, Nevada, USA) with polyethylene (PE) 20 tubing (Benton Dickson, New Jersey, USA) 20cm in length, was inserted into the sciatic nerve proximal to its trifurcation and fasciculation. The Hamilton syringe was placed in a syringe pump operated by a foot pedal. Rate of injection was set at 13.3 mL/hour. Retraction of the syringe plunger to a specified volume draws the same volume of solution into the needle and tubing at the distal end. Six µL of an 800 µg/mL solution of IB4-Saporin (IB4-SAP) (Advanced Targeting Systems, San Diego CA, USA) in 0.2M Phosphate Buffered Saline (PBS) and Fast Green Dye (Sigma, Missouri, USA) was injected. The wound was sutured in layers using 4-0 Vicryl sutures (Ethicon Inc, New Jersey USA). Animals were returned to their home cages and were allowed to recover for 2 weeks prior to behaviour experimentation. Vehicle injections (6µL of 0.2M PBS and Fast green dye), unconjugated SAP (6µL of 800 µg/mL SAP in 0.2M PBS in Fast green dye), simulated injections (insertion of needle devoid of solution; Sham) and naïve animals served as controls.

Behaviour Testing

Prior to surgical treatment, baseline behavioural thresholds were taken. Post-operative testing was conducted periodically with consistent inter-test intervals (once every 3 days per stimulus). Animals were always habituated (defined as the cessation of stress-related behaviours such as frantic exploration, defecation and grooming) prior to testing.

Mechanical Allodynia.

Animals were habituated to the behavioural apparatus for 20 minutes per day on 2 consecutive days prior to baseline threshold acquisition and on each day prior to testing. Animals were placed on an elevated mesh grid in clear plastic enclosures

(Allentown Caging Equipment, New Jersey, USA), where all 4 paws were accessible to the experimenter. Sensitivity to innocuous punctate mechanical stimulation was conducted using calibrated von Frey filaments (Stoelting Co. Illinois, USA). Briefly, von Frey filaments of varying thresholds, 4 g, 8 g and 15 g, were applied to the plantar surface of the hind paw, specifically, the receptive field of the sciatic nerve. Care was taken not to stimulate close to the interdigital pads. Each filament was presented 10 times and the number of obvious paw withdrawals were recorded and converted into a percentage of response. The left paws of all rats were tested at one threshold followed by the right paws, after which, the next filament threshold was applied. Stimulation was initiated with the 4 g filament followed by the 8 g and then the 15 g filament.

Heat Hyperalgesia

Following habituation, baseline thresholds were taken on 3 consecutive days and the average calculated. For testing, rats were placed in plastic enclosures on the acclimatized glass surface (30°C) of a plantar heat hyperalgesia test apparatus (University of California, San Diego, CA, USA). Paw withdrawal thresholds were measured using a mobile radiant light source connected to an automatic timer. In this device, sensitivity to the applied thermal stimulus is measured as the latency to withdrawal of the paw from the stimulus source. Once the hind paw is lifted away from the light source, the light and timer is immediately turned off, and the time until withdrawal is reported. Paw withdrawal thresholds for both the left and right hind paws were obtained. For each hind paw, three measurements taken at 5 minute intervals were recorded and averaged.

Cold Allodynia

Sensitivity to cold stimuli was assessed using acetone application to the plantar surface of the rat hind paw. Briefly, rats were placed in the same test apparatus used for the analysis of mechanical allodynia. Twenty µL of acetone (Fisher Scientific, Ottawa, Canada) were applied to the plantar hind paw, and the ensuing behavioural responses were scored on an ordinal scale. Specifically, the absence of a response was scored as 0; a rapid flick or stamp of the hind paw was scored

as 1; repeated flicking of the paw was scored as 2, and licking of the hind paw was recorded as 3. Animals were given 20 seconds to respond to the acetone. When a response of 1 or 2 was evident, the rats were given an additional 20 seconds. The final behaviour displayed by the animals was recorded. If the rat responded by licking the hind paw following acetone application, then the behaviour was scored as 3, and no additional time was given.

Nociceptive responses to αβ-Methyl-ATP

Immediately following intraplantar injection 100 µL of a 2.47nM solution of αβ-Methyl-ATP, rats were placed in clear plastic enclosures (Allentown Caging Equipment, New Jersey, USA). The duration of hind paw elevation was measured over the course of 10 minutes, in two minute time intervals. Hind paw elevation was defined as the complete absence of contact with the floor.

Complete Freund's Adjuvant treatment

In an effort to determine whether the selective loss of IB4-labelling, non-peptidergic PAFs had an effect on nociceptive thresholds in the setting of acute inflammation, on the 14^{th} day following intra-sciatic administration of IB4-SAP or control treatment, animals received an subcutaneous intraplantar injection of 100 µL of a 1mg/mL solution of complete Freund's adjuvant (CFA; Sigma-Aldrich Canada LTD, Oakville, Ontario, Canada). Withdrawal thresholds to noxious heat, mechanical and innocuous mechanical stimuli were evaluated for 72 hours at 24 hour intervals using the techniques mentioned above.

Intraplantar Capsaicin

In order to examine the effect of the loss of TRPV1 receptors secondary to the loss of IB4-labeled primary afferent fibers, both IB4-SAP and control rats received an intraplantar injection of 10µL of a 0.1% solution of Capsaicin dissolved in Ethanol, Tween 80 and physiological saline and were evaluated for behavioural responses to both noxious and innocuous mechanical stimuli, in addition to noxious heat stimuli, at 30 and 60 minutes prior and subsequent to capsaicin administration.

Intraplantar Formalin

Assessment of the consequence of IB4-SAP-induced lesions on the typical biphasic patterned response to a 50 μL intraplantar injection of 5% formalin was conducted on the 14th day post-IB4-SAP administration. Formalin-induced nocifensive behaviours were evaluated using the weighted score assessment system (Dubuisson, D and Dennis, S.G., 1977; Coderre, T.J., Fundytus, M.E., McKenna, J.E., Dalal, S., Melzack. R., 1993). Briefly, immediately following the sub-cutaneous administration of formalin to the hind paw, spontaneous pain behaviours were scored using the following ordinal scale: 0= no obvious signs of favouring of the injected paw, 1= weight bearing on the injected paw was minor or absent, 2= elevation of the injected hind paw, without contacting any surface and 3= the injected hind paw was licked or bitten (Dubuisson, D and Dennis, S.G., 1977; Coderre, T.J., Fundytus, M.E., McKenna, J.E., Dalal, S., Melzack. R., 1993). The duration of time spent expressing each of the behavioural categories was measured over a 60 minute period divided into time intervals of 5 minutes (300 seconds). Weighted scores were acquired using the following equation:

$$\frac{T_1 + 2T_2 + 3T_3}{\text{Time bin (300 seconds)}}$$

Where T represents the amount of time spent in each behavioural category (indicated by the subscript number).

RotaRod

In order to determine the effect of IB4-SAP on motor function, rats were tested on an accelerated rota rod. Animals were habituated to the rota rod apparatus (Ugo Basile, Comerio VA, Italy) on three separate occasions until drop latencies were between 250-300 seconds. Post-operative testing was conducted periodically to evaluate the motor function in each animal. Drop latencies were recorded and averaged.

Immunocytochemistry

At the end of each time point, animals were deeply anaesthetized with Equithesin (6.5mg chloral hydrate and 3 mg sodium pentobarbital in a volume of 0.3ml, i.p., per 100 g body weight). Perfusion of the rats was carried out through the left cardiac ventricle, with 4% paraformaldehyde (PFA) in 0.1M phosphate buffer (PB), pH 7.4. Spinal cords and the left and right L4, L5 and L6 dorsal root ganglia (DRG) were removed and stored in 30 % sucrose (spinal cords) at 4ºC; DRG were frozen on dry ice in optimal cutting temperature (OCT) embedding medium for frozen tissue specimens (Sakura Finetek USA Inc. Torrance CA, USA) and stored at -20ºC until needed. Fifty (50) μm thick transverse sections of the L5 and L6 region of the spinal cord were cut on a freezing sledge microtome (Leica, New Jersey, USA). The sections were collected as free-floating in phosphate buffered saline with 0.1% Triton X-100 (PBS+T). DRGs were cut on a cryostat (Leica, Germany) at a thickness of 7 μm thick onto gelatin subbed slides. Spinal cord sections were incubated in 10 % normal horse or goat serum (NHS, NGS) (Vector Laboratories, Burlingame, CA, USA) for 1 hour. Sections were then incubated overnight in the following primary antibodies or reagents: guinea pig anti-$P2X_3$ antibody (1:7500) (Neuromics, Edina, MN, USA), lectin IB4 conjugated to Alexa 488 (1:200) (Molecular Probes, Burlington, ON, Canada), rat anti-SP monoclonal antibody (1:10) (gift from A.C. Cuello; Cuello, A., et al., 1978; MediMabs, Montreal, Canada), mouse anti-glutamic acid decarboxylase 65 (GAD_{65}) monoclonal antibody (1:1000; Chemicon International, Temecula, CA, USA), rabbit anti-NK1 receptor antibody (gift from J. Krause; Ardelt, A. A., Karpitskiy V. V,, Krause, J. E and. Roth K. A., 1996) and rabbit anti-CGRP antibody (1:2000) (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada). The following day sections were washed in PBS+T and processed differently depending on the initial incubation. Sections to be processed for $P2X_3$ immunoreactivity were incubated in a donkey anti-guinea pig biotinylated IgG (1:200) (Jackson Immunoresearch Laboratories Inc, West Grove, PA, USA) for one hour and then washed in PBS+T. Sections were then incubated in a 1:250 dilution of avidin and biotin (Vector Laboratories, Burlingame, CA, USA) for 1 hour after which they were

thoroughly washed in PBS+T. Shortly after, the Tyramide system amplification (TSA) was used. Briefly, sections were incubated for 7 minutes in biotinylated Tyramide (1:75) (PerkinElmer, Boston, MA, USA) and then washed repeatedly in PBS+T. The tissue was then incubated for 1 hour in Streptavidin-conjugated Alexa 488 (1:200) (Molecular Probes, Burlington, ON, Canada), washed repeatedly in PBS and then mounted on gelatine coated slides. For CGRP and IB4 double-labeling, sections were washed in PBS+T and then incubated in donkey anti-rabbit IgG conjugated to Rhodamine Red-X (1:50) (Jackson Immunoresearch Laboratories Inc, West Grove, PA, USA), after which they were washed in PBS and mounted on gelatin coated slides. Tissue processed for SP and NK1-receptor double-labelling was incubated for 1 hour in a mixture of donkey anti-rabbit antibody conjugated to Rhodamine Red-X (1:50) (Jackson Immunoresearch Laboratories Inc, West Grove, PA, USA) and goat anti-rat IgG conjugated to Alexa 488 (Molecular Probes, Burlington, ON, Canada**).** The sections were then washed and mounted as indicated above. Sections processed for GAD immunoreactivity were incubated in a donkey anti-mouse IgG Rhodamine Red-X (1:50) (Jackson Immunoresearch Laboratories Inc, West Grove, PA,USA). DRG sections were processed in a similar manner as above, however all incubations were conducted on slides. The concentration of antibodies and the duration of the incubations were identical to that of free-floating sections. Controls of immunostaining were carried out by pre-adsorbing the primary antibodies with the control blocking peptides prior to incubations; no immunostaining was observed.

Confocal Microscopy

Sections from control and IB4-SAP treated animals were examined using a Zeiss LSM 510 confocal scanning laser microscope with argon and helium neon lasers (Carl Zeiss Canada, Toronto, ON, Canada). Appropriate filters were selected for the separate detection of Alexa-488 and Rhodamine Red-X, using a multitrack scanning method to completely separate the detection of the two signals. Images were first examined at low magnification followed by observations at higher magnification (using a 63X plan-apochromatic oil immersion objective).

Qualitative assessment of immunoreactivity was conducted using five or six sections per side (contralateral/ipsilateral) and per rat; these were initially assessed at low magnification, followed by acquisition of images at high magnification (63X magnification under oil immersion). Pixel saturation was carefully avoided.

Captured images were saved as LSM files and the contrast and brightness adjusted prior to converting the files to TIFF format. The figures based on confocal images were prepared using Adobe Photoshop 7.1.

Results

Morphological observations

Dorsal Root Ganglion

Intra-sciatic injection of IB4-SAP resulted in anterograde transport of the toxin to the cell body located in the DRG. Examination of the L5 DRG 14-days following IB4-SAP injection revealed a substantial decrease in the number of IB4-labeled neuronal cell bodies compared to SAP-only injected animals (Figure 1)**.** Peptidergic neuronal cell bodies, as identified by CGRP immunoreactivity were unaffected by IB4-SAP treatment (Figure 1).

Spinal Cord: 14 day time point

Saporin, a type I ribosome inactivating protein (RIP) inhibits protein synthesis by the catalytic depurination of the 28 Svedberg unit (S) large ribosomal ribonucleic acid (28SrRNA), by specifically removing a crucial adenine residue in the α-sarcin-ricin loop required for protein elongation. Pilot studies were conducted to determine injection protocol parameters; specifically, to find the required concentration of IB4-SAP to produce a target-specific lesion and was maximally effective, in the absence of extraneous damage such as non-specific cell death in non-targeted neuronal populations (Wiley, R.G., Lappi, D.A., 2001). These initial studies revealed that a 6 µL injection of an 800µg/mL IB4-SAP (in 0.2M PBS, pH 7.4) was sufficient at producing a substantial depletion of IB4-labeled primary afferent terminals in the inner aspect of the substantial gelatinosa (SG) at the L5-L6 spinal level. Lesions were restricted to the central terminal fields of the tibial

and sural branches of the sciatic nerve, which are located in the medial two thirds of the SG (Figure 2). IB4-positive primary afferent terminals localized to the residual (outer) third of the SG, corresponding to the terminal field of the posterior cutaneous nerve of the thigh (see Swett, J. E., and Woolf, C.J., 1993), were unaffected by IB4-SAP treatment (Figure 2).

Characteristically, in the ipsilateral dorsal horn there was a complete loss of IB4-binding in the region corresponding to the spinal cord distribution of the afferents from the tibial and sural nerves. However, other lesion patterns were observed in several animals. These patterns presented as a succinct island of markedly diminished IB4-labelling localized to the medial or central aspect of the tibial nerve region of the SG. Residual IB4-labelling within the tibial nerve region (in small islands) may represent the terminal field of the superficial peroneal branch of the sciatic nerve observed at higher lumbar spinal cord levels (lower L4-upper L5). Identification of non-peptidergic, primary afferent terminals by the expression of the purinergic receptor $P2X_3$ revealed a substantial loss of $P2X_3$ immunoreactive (IR) profiles within the SG analogous to the lesions characterized with IB4-labelling.

Time-course studies indicated that 14-days following IB4-SAP injection, lesions were optimal (based on maximum mediolateral extension and degree of loss of IB4-labelled terminals). Our results are in accordance with the suggested required post-injection period indicated for lectin-based, axonally transported toxins (Wiley, R.G and Oeltmann, T.N., 1986). Therefore, behavioural experiments were conducted on or after day 14 post-injection of IB4-SAP.

Injections in control animals with SAP, PBS or insertion of the needle produced a narrow, circumscribed band devoid of IB4-positive terminals situated on the border between the sural nerve territory and the territory of the posterior cutaneous nerve of the thigh. The mediolateral lengths of the lesions in control animals ("sham lesions") were, by comparison, significantly shorter than those of IB4-SAP-induced lesions. Lesions exhibited by the control groups were caused by insertion of the needle, as demonstrated by the presence of identical lesions in animals following insertion of needle in the nerve in the absence of any injection. This is in agreement with the known fact that insertion of bevelled needles

intrafascicularly invariably damages axons and Schwann cells within the peripheral nerve (Maruyama, M., 1997). To minimize this effect, we carried out all injections with the utmost care.

Identification of peptidergic terminals in the marginal layer of the superficial dorsal horn was conducted using antibodies directed against CGRP. CGRP immunoreactivity remained unchanged compared to controls in lamina I and lamina II at 14 days following IB4-SAP treatment (Figure 3A-B). In contrast, there was an apparent decrease in SP immunoreactivity in area same area (Figure 3C-D). This apparent decrease in SP immunoreactivity was uniform throughout the entire mediolateral extent of lamina I-II and was not restricted to the area of the lesion. In contrast, NK1 receptor immunoreactivity was considerable increased in lamina I on the ipsilateral side subsequent to IB4-SAP administration (Figure 4A-B). Examination of glutamic acid decarboxylase (GAD)-immunoreactivity revealed an appreciable reduction in GAD immunoreactivity in the ipsilateral dorsal horn of IB4-SAP treated animals (Figure 4C-D).

Spinal cord: seven week time point

Seven weeks following IB4-SAP injection, IB4-labelling in the superficial dorsal horn remained unchanged from observations taken at the 14 day time point. Indeed, IB4-labeling was markedly absent from the sciatic nerve territory of the dorsal horn. Immunoreactivity for SP (Figure 3G-H) and the NK1 receptor (not shown) were similar to that observed at the 14 day time point, with SP-immunoreactivity remarkably diminished in the ipsilateral dorsal horn, and NK1 receptor immunoreactivity considerably increased. Similarly, GAD immunoreactivity remained low. In contrast, CGRP immunoreactivity was now decreased in the ipsilateral dorsal horn, but remained unchanged in the contralateral dorsal horn (Figure 3E-F).

Behaviour

Rota Rod performance

Evaluations of pre- and post-surgical motor performance, by means of an accelerated RotaRod, were conducted to exclude the potential influence of

abnormal motor function on elicited withdrawal responses. Baseline drop latencies for all groups were similar (Figure 5). Post-injection latencies, acquired on days 6, 10 and 13, decreased slightly in all groups from their respective baselines, yet did not reach significance levels ($p>0.05$) (Figure 5). Furthermore, the decline in drop latencies was equivalent in all groups.

Nociceptive responses to αβ-Methyl-ATP

Intraplantar administration of αβ-methyl-ATP, a $P2X_3$ receptor agonist, in PBS-injected control animals produced a marked hyperalgesic response indicated by the immediate elevation of the injected paw. Moreover, control animals displayed heightened hyperalgesia, demonstrated by prolonged elevation of the hind paw (Figure 6), culminating 4-6 minutes after injection (0-2 minutes: 10.42%; 4-6 minutes: 17.5%). The duration of sustained hind paw guarding waned 8-10 minutes following injection (4.07%), representative of αβ-methyl-ATP treatment. By comparison, IB4-SAP treated animals displayed signs of attenuated hyperalgesia (Figure 6). Specifically, IB4-SAP-treated animals expended considerably less time in a guarded position, the proportion of which was consistent for the duration of the observation period (2-4 minutes: 7.865%; 6-8 and 8-10 minutes: 6.15%).

Heat hyperalgesia

During the development phase of the lesion, evaluations of acute withdrawal responses to noxious thermal stimuli were performed to elucidate the role of IB4-positive fibers in acute thermal pain. Paw withdrawal latencies (PWL) taken prior to surgical treatment (baselines) were invariable among control groups (SAP: 12.24 ± 0.8562, $n = 7$; PBS: 13.87 ± 0.8930, $n = 8$; Sham: 13.09 ± 0.9529, $n = 8$ and Naïve: 12.48 ± 0.7422, $n = 8$; IB4-SAP: 12.74 ± 0.7022, N = 8). Post-operative testing, performed periodically (every 3rd day), revealed no perceptible deviation in thermal sensitivity from baseline in control groups throughout the post-operative test period (Figure 7). Remarkably, IB4-SAP treatment was inefficacious in shifting paw withdrawal latencies from baseline (Baseline: 12.74 ± 0.7022; P.O day 4: 13.03 ± 1.004; P.O day 10: 14.52 ± 1.198; $p>0.5$). Post-

treatment thresholds observed in IB4-SAP treated animals were comparable to those of control animals (Figure 7).

Mechanical Allodynia

Sensitivity to innocuous and noxious (punctuate) mechanical stimulation was conducted during the process of lesion development. Calibrated von Frey filaments were presented at various thresholds ranging from innocuous (4 g), intermediate (8 g) and noxious (15 g). Baseline thresholds at the 4 gram threshold were invariable among all groups ($p>0.05$) (Figure 8). Post-operative thresholds of the control groups remained consistent throughout the testing period, with no evidence of deviation from baseline ($p>0.05$, all groups). Similarly, IB4-SAP treated animals did not exhibit any changes in paw withdrawal frequency at any time during the course of testing, remaining at baseline. These results were consistent at both the 8 and 15 g thresholds; both control and IB4-SAP groups showed consistent withdrawal frequencies throughout the 14-day period, unchanged from their respective baselines (Figure 8).

Cold Allodynia

The plantar application of acetone, prior to surgery, elicited modest sensitivity scores in both control and IB4-SAP animals (Figure 9). Post-operative scores for control animals were unremarkable, apart from a transitory increase in sensitivity on day 5 in the SAP control group. Sensitivity scores for IB4-SAP treated animals exhibited an unsubstantial, yet perceptible increase, evident by day 5, and again at day 14.

Capsaicin

Paw withdrawal thresholds for both mechanical and heat stimuli were assessed prior to capsaicin treatment. Heat paw withdrawal latencies were similar in all treatment groups ($p>0.05$) (Figure 10)**.** Following capsaicin treatment, paw withdrawal latencies to noxious heat stimuli in PBS control animals were significantly lower at both 30 and 60 minutes post-injection than in animals treated with IB4-SAP ($p<0.01$).

Complete Freund's Adjuvant

Following CFA treatment, both control (PBS) and IB4-SAP treated animals displayed the classical features of adjuvant induced pain: redness, swelling and nocifensive behaviours such as paw lifting. Paw withdrawal thresholds to noxious heat were evaluated at 24, 48 and 72 hours after CFA administration (Figure 11). Control animals (SAP-injected) exhibited a significant decrease in paw withdrawal latencies at the 24 hour time point which continued to decline until the end of the 72 hour observation period. At 24 hours following CFA treatment, withdrawal latencies in both IB4-SAP and SAP treated animals were significantly lower than paw withdrawal latencies taken prior to CFA treatment (day 10) ($p<0.5$, $p<0.01$ respectively).

Formalin

The intraplantar administration of 5% formalin resulted in the immediate swelling of the injected hind paw and the onset of nocifensive behaviours (lifting, guarding and licking/biting). The extent of swelling, determined by the measurement of hind paw thickness, were similar in both IB4-SAP and control animals (Figure 12). Following the administration of formalin, control animals (PBS) exhibited the classical biphasic response pattern typical after intraplantar formalin injection (Figure 12). Similarly, IB4-SAP treated animals demonstrated the same biphasic response with no significant difference in weighted score compared to the PBS-formalin group (Figure 12).

Discussion

Our results show that the selective lesioning of the non-peptidergic population of nociceptive primary afferents did not cause any significant deficit in nociceptive responses nor hyperalgesia. Furthermore, we detected changes in neurotransmitter/neuromodulator markers in the dorsal horn that may be indicative of compensatory responses.

Immunoreactivity

The results of this study demonstrate that the intra-sciatic injection of IB4-SAP resulted in the selective ablation of IB4-labeled, non-peptidergic primary sensory neurons. Furthermore, IB4-SAP administration produced marked, time-dependent alterations in immunoreactivity for sP and CGRP, for the neuropeptide receptor NK1 and for the GABA synthetic enzyme GAD in the superficial dorsal horn.

We observed that at 14 days post-lesion there were no changes in CGRP immunoreactivity but a substantial decrease in sP immunoreactivity, whereas both neuropeptide immunoreactivities were decreased at 7 weeks. There were no changes in the contralateral side of the dorsal horn at any time point or in control animals. The early (at two weeks) decline in SP immunoreactivity may be the result of several possible compensatory mechanisms. For example, increased release of vesicular stores of the peptide from the central terminals of the afferents may account for low neuropeptide immunoreactivity levels. However, as several studies have documented the co-localization or co-storage of sP and CGRP within the same synaptic vesicle, a parallel decline in both neuropeptide immunoreactivities would be expected (Tuchscher, M.M. and Seybold, V.S, 1989; Plenderleith M.B., Haller, C.J., Snow, P.J., 1990; Ribeiro-da-Silva, A. 1995). Our data at the 7 week time point showed a decline in CGRP immunoreactivity, lending further support to a mechanism of time-dependent peptide-specific changes in synthesis and storage. This observation should be confirmed by the examination of neuropeptide levels within the DRG and superficial dorsal horn by western blot analyses. The simultaneous increase in NK1-receptor immunoreactivity may be indicative of ligand-mediated regulation of receptor expression, as low concentrations of synaptically released neurotransmitters

typically induce the post-synaptic availability in receptors in an attempt to maintain baseline signalling levels

The observed decline in neuropeptide immunoreactivity following IB4-SAP treatment is counter-intuitive, as the loss of a substantial proportion of small diameter sensory neurons would presumably produce an increase in neurotransmitter synthesis in the effort to accommodate the increased signal traffic. It is possible that such increases did occur in our model; however, they were not detected due to the large temporal intervals between time points. Accordingly, a more controlled time course should be conducted to determine more precisely the temporal patterns of the changes in sP and CGRP immunoreactivities. However, it is also possible that the detected decreases in peptide immunoreactive may indicate a high turnover of peptides. Due to their limited storage in the terminals, an augmentation in the rate of release could result in decreased peptide immunoreactivity. However, sP and CGRP are only released following noxious stimulation, this interpretation is unlikely because these animals do not display hyperalgesia and allodynia.

The decreased GAD immunoreactivity that we observed ipsilateral to the lesion at both time points suggests an effect of the loss of non-peptidergic input on inhibitory neurotransmission within the SG. The loss of inhibitory tone within the superficial dorsal horn has attracted considerable interest in recent years. Significant alterations in spinal cord inhibitory mechanisms in the setting of chronic pain have been observed (Moore, K.A., Kohno, T., Karchewski, L.A., Scholz, J., Baba, H., Woolf, C.J., 2002). Specifically, following peripheral nerve injury, a significant reduction in the population of GABAergic neurons resident to the SG has been reported, suggesting that as a result of the loss of afferent input inhibitory cells undergo trans-synaptic cell death (Moore, K.A., et al., 2002). Due to the high incidence of GABAergic pre-synaptic inhibitory contacts established with the terminals of non-peptidergic primary afferents (Todd A. J. and. Lochhead. V., 1990; Ribeiro-da-Silva, A., 2004), deafferentation-induced loss of GABAergic interneurons may be a likely cause of the decreased GAD immunoreactivity we observed following IB4-SAP treatment.

Behaviour: Response to acute mechanical stimuli

The analysis of acute nociceptive behaviours in animals lacking IB4-labeled afferents revealed the absence of any affect on paw withdrawal thresholds to innocuous and noxious stimuli, but for a marginally (but non-significantly) elevated responses to acute cold stimuli in IB4-SAP treated animals initiated 5 days after IB4-SAP administration and peaking at post-injection day 11.

The results of our study are in disagreement with results previously reported by Vulchanova and collaborators (2001). In contrast to the absence of a significant effect on nociceptive withdrawal behaviours we observed in our study, the authors reported a transitory increase in withdrawal thresholds in IB4-SAP treated animals compared to SAP-injected controls (Vulchanova, L., Olson, T.H., Stone, L.S., Reidl, M.S., Elde, R., Honda, C.N., 2001; Tarpley, J.W., Kohlet, M.G., Martin, M.J., 2004). The reasons for the discrepancies are at best speculative. However, the behavioural results reported in the current study have been reproduced several times in our laboratory by more than one experimenter; therefore, we are confident that we have accurately assessed the effect of IB4-SAP on acute nociceptive withdrawal responses.

Effects of IB4-Saporin treatment on paw withdrawal latencies in response to acute noxious heat, CFA-induced inflammation and capsaicin-induced heat hyperalgesia

Paw withdrawal latencies to acute noxious heat (as tested by the Hargreaves method) remained unaltered compared to baseline and control group latencies over the course of the 14 days following IB4-SAP treatment. However, examination of paw withdrawal latencies to noxious heat in IB4-SAP treated animals (day 14) 30 and 60 minutes following an intraplantar injection of capsaicin revealed a complete block of capsaicin-induced heat hyperalgesia. These data suggest that TRPV1 receptors expressed by non-peptidergic, IB4-labeled neurons are required for capsaicin-induced heat hyperalgesia, lending support to the *in vitro* data reported by Liu, M and colleagues (2004) which demonstrated a neuronal population-specific sensitivity of the TRPV1 receptor to capsaicin. Moreover, the above authors demonstrated that TRPV1 receptors

expressed by IB4-negative neurons displayed enhanced sensitivity to protons (Liu, M et al., 2004). Accordingly, we observed that IB4-SAP treatment was ineffective at altering CFA-induced heat hyperalgesia over a 72 hour period. Taken together, the results of our behaviour experiments are compatible with the above reported *in vitro* data suggesting that IB4-positive and IB4-negative neurons express TRPV1 receptors possessing different sensitivities to capsaicin and protons.

Response to chemical irritants

The typical biphasic response observed following intraplantar formalin was unaltered by IB4-SAP treatment. This result suggests that IB4-labeled afferents are not involved in the formation of formalin-induced pain. It is possible that other nociceptive afferents (peptidergic C-fibres and Aδ-fibres) can compensate for the loss of non-peptidergic afferents.

The biphasic nature of the formalin test has been attributed to phase-specific activation and sensitization of primary afferents and dorsal horn neurons (McCall, W.D., Tanner, K.D., Levine, J.D., 1996; Martindale, J, Bland-Ward, P.A, Chessell, I.P., 2001). Analysis of C-fibre activity following formalin injection demonstrated a biphasic activation of small diameter primary afferents (McCall W.D., et al., 1996). Formalin is known to activate Aδ and C-fibres, however, an in depth analysis of the neuronal phenotype of activated C-fibre neurons in response to formalin administration has yet to be conducted. Administration of the selective TRPV1 receptor antagonist iodo-resiniferotoxin (I-RTX) resulted in a significant, dose-dependent reduction (91% and 76%) in the number of flinches in the first and second phase of the formalin test respectively (Kanai, Y, Hara, T and Imai, A, 2005). Moreover, intrathecal administration of I-RTX markedly reduced spinal release of CGRP, suggesting the effects of I-RTX is mediated through TRPV1-expressing peptidergic neurons (Kanai, Y, Hara, T and Imai, A., 2005). Thus, despite the absence of non-peptidergic C-fibres following IB4-SAP treatment, activation of peptidergic TRPV1 receptors is still possible.

αβ-Methyl-ATP

Intraplantar application of αβ-Methyl-ATP, a non-selective P2X receptor agonist, produced profound pain-related behaviour in control animals as indicated by the sustained elevation of the hind paw exhibited immediately post-injection. In contrast, IB4-SAP treatment produced a marked attenuation in the duration of paw lifting over the 10 minute observation period. These results suggest that IB4-SAP produced a significant loss in non-peptidergic innervation of the plantar skin within the sciatic dermatome.

General conclusions

The results from this study suggest that, in the absence of IB4-labeled primary afferent input to the superficial dorsal horn, nociceptive responses to acute innocuous and noxious stimuli remained for the most part normal. However, we observed that responses to capsaicin-induced heat hyperalgesia were blocked in IB4-SAP treated animals, an observation that supports *in vitro* data suggesting that peptidergic and non-peptidergic C-fibre afferents express varieties of TRPV1 receptors with differential sensitivities to capsaicin and protons (Liu, M et al., 2004). Furthermore, the lack of altered paw withdrawal latencies to adjuvant-induced heat hyperalgesia lends further support to this hypothesis, as tissue pH levels are lowered following the induction of inflammation.

In light of these findings, our current perspective on the functional role of non-peptidergic nociceptors requires a re-evaluation. It is hard to understand that the lesioning of a population of primary afferents which is known to be nociceptive and participates in complex synaptic arrangements named glomeruli would lead to so few functional changes, despite the loss of GABAergic presynaptic inhibition (see above). It should be kept in mind that the loss of non-peptidergic afferents was not immediate but was complete only at the end of two weeks. Therefore, this progressive loss would differ from the acute pharmacological blockade of P2X3 receptors on these afferents, which has been shown to reduce inflammatory and neuropathic pain (Jarvis, M.F., Burgard, E.C., McGaraughty, S., Honore, P., Lynch, K., Brennan, T.J., et al., 2002). A consequence of our data would be that peptidergic afferents would be able to compensate for most of the consequences

of a progressive loss of the non-peptidergic afferents. However, it is still possible that a major loss in function may have remained undetected by our studies. Specifically, an anterograde tracing study using transgenic mice expressing wheat-germ agglutinin (WGA) in IB4-positive, $Na_V1.8$-positive subpopulation of primary afferent fibres revealed a possible supraspinal pathway to limbic cortical regions engaged by IB4-labeled, non-peptidergic afferents (Braz, J.M., et al., 2005). Indeed, by the trans-synaptic transfer of WGA from IB4-labeled afferents to neurons located in LII of the dorsal horn, such study revealed a putative circuit connecting LII neurons with neurons residing in the deep dorsal horn (LV) which projected supraspinally to several cortical regions such as the amygdala, the bed nucleus of the stria terminalis (BNST) and the hypothalamus (Braz, J.M., et al., 2005). This pathway, shown to be independent of the lamina I spino-thalamic and spino-parabrachial pathways, suggests that afferent input from non-peptidergic fibres would communicate with higher brain centers associated with the affective-motivational component of pain. It would therefore be of particular interest to examine the effects of the loss of non-peptidergic input to the dorsal horn on affective behaviours in response to nociceptive stimulation.

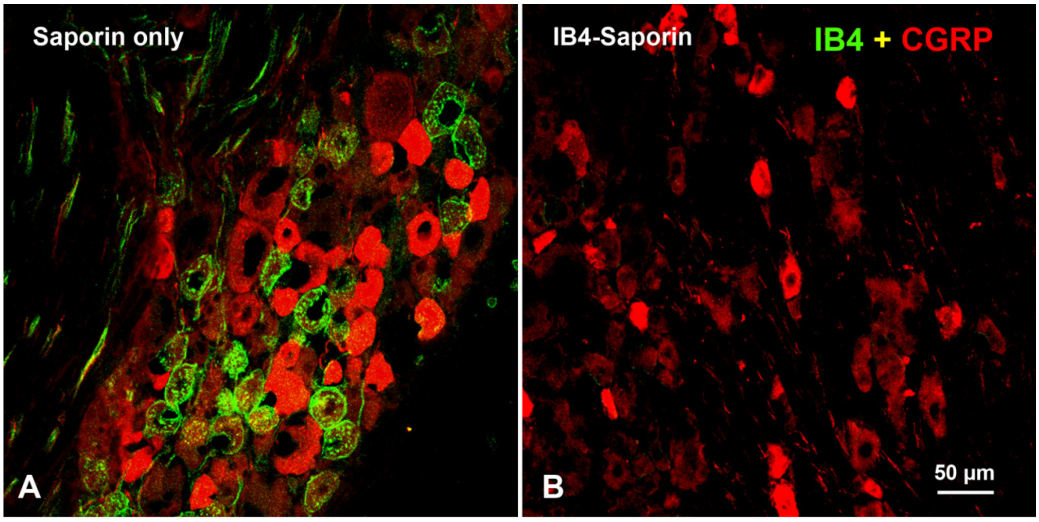


Figure 1: Confocal image of the L5 dorsal root ganglion from Saporin control (A) and IB4-Saporin treated (B) animals 14 days following intra-sciatic nerve injections. IB4-Saporin treatment (B) resulted in the selective loss of IB4-labeled (green) neurons with no remarkable change in CGRP (red) immunoreactivity. Control injections consisting of Saporin or vehicle (PBS) did not alter IB4 or CGRP stainings (A).

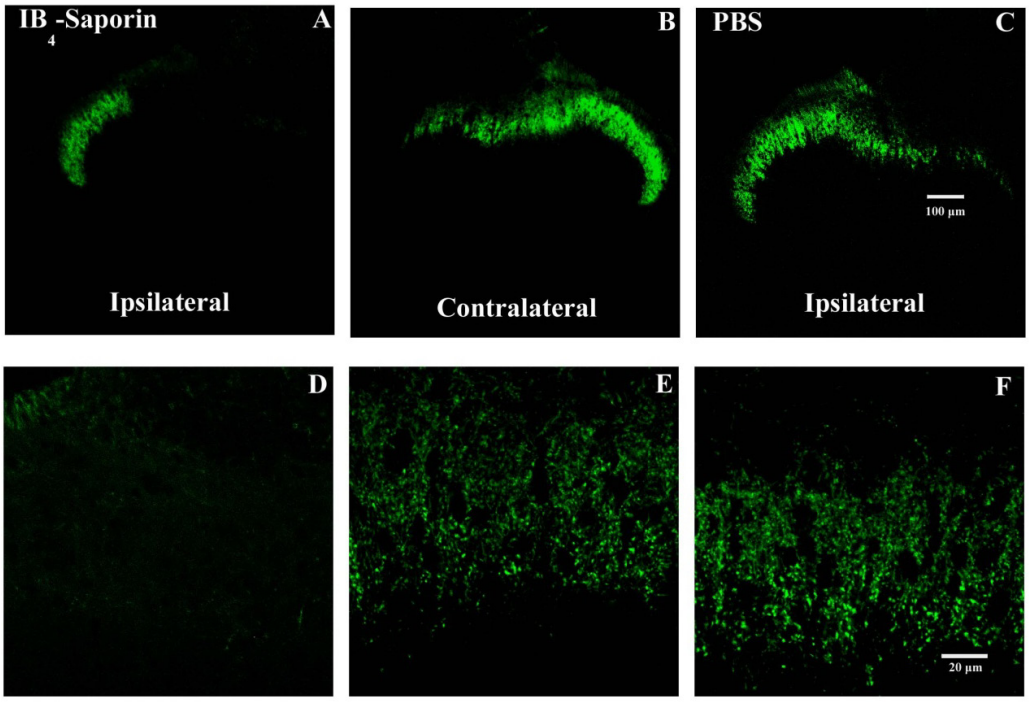


Figure 2: IB4-labelling in the superficial dorsal horn 14 days following IB4-Saporin or vehicle treatment. **A-C**:Low magnification confocal images demonstrating the regional loss of IB4-labelling within the sciatic nerve territory in the superficial dorsal horn compared to the contralateral side (B) and vehicle-injected animals (C). **D-E**: High magnification confocal images of IB4-labeled varicosities in the substantia gelatinosa of IB4-Saporin treated and vehicle treated animals.

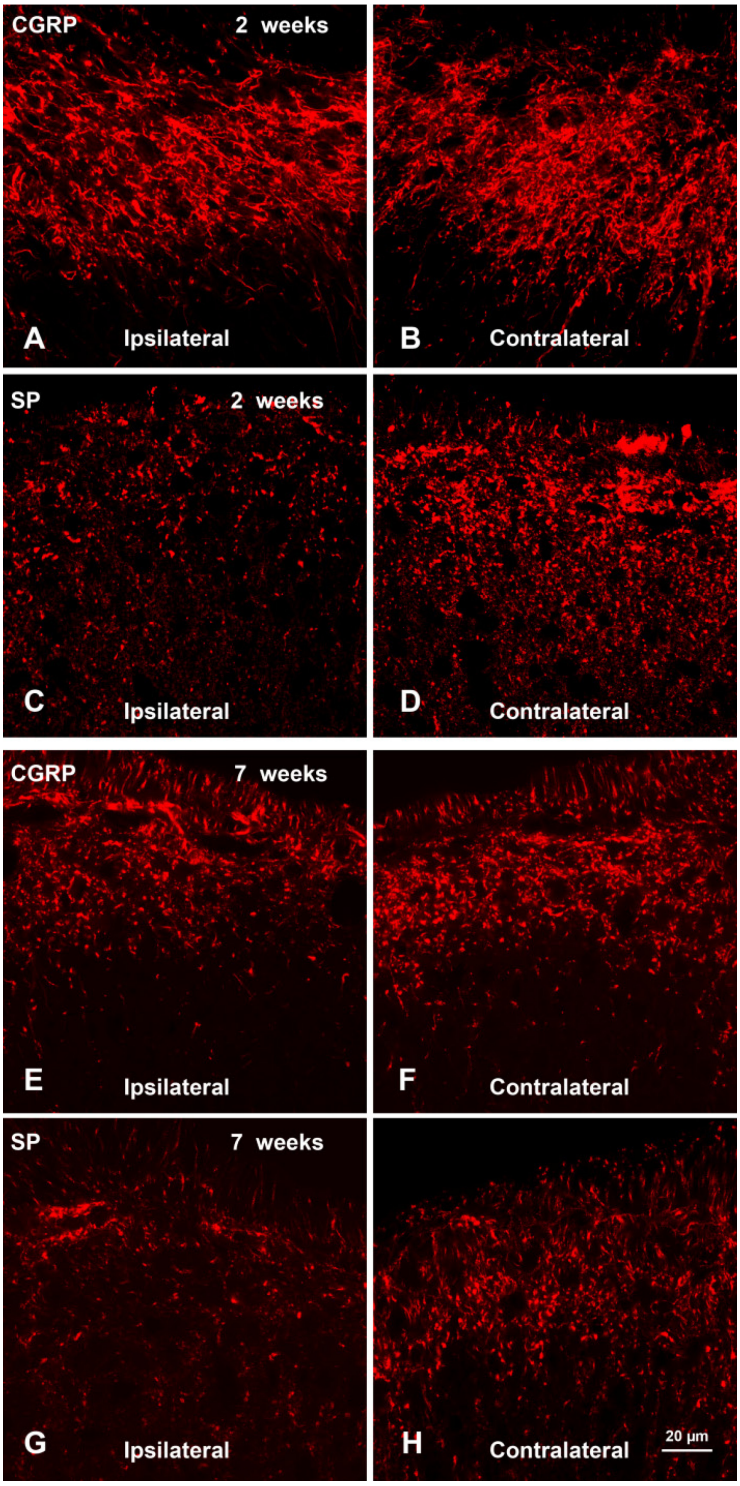

CGRP
2 weeks
A
Ipsilateral
B
Contralateral
SP
2 weeks
C
Ipsilateral
D
Contralateral
CGRP
7 weeks
E
Ipsilateral
F
Contralateral
SP
7 weeks
G
Ipsilateral
H
Contralateral
20 µm

Figure 3

Figure 3: CGRP and SP-immunoreactivity in the ipsilateral and contralateral dorsal horn 14 days (A-D) and 7 weeks (E-H) following IB4-Saporin treatment. At 14 days following IB4-SAP injection, CGRP immunoreactivity remained unchanged from contralateral and control (not shown) levels (**A-B**), whereas SP-immunoreactivity was substantially decreased in the ipsilateral dorsal horn (**C-D**). Interestingly, CGRP immunoreactivity was reduced at the 7 week time point in the ipsilateral dorsal horn (**E-F**), and substance P immunoreactivity remained similar to observations made at the 2 week time point (**G-H**).

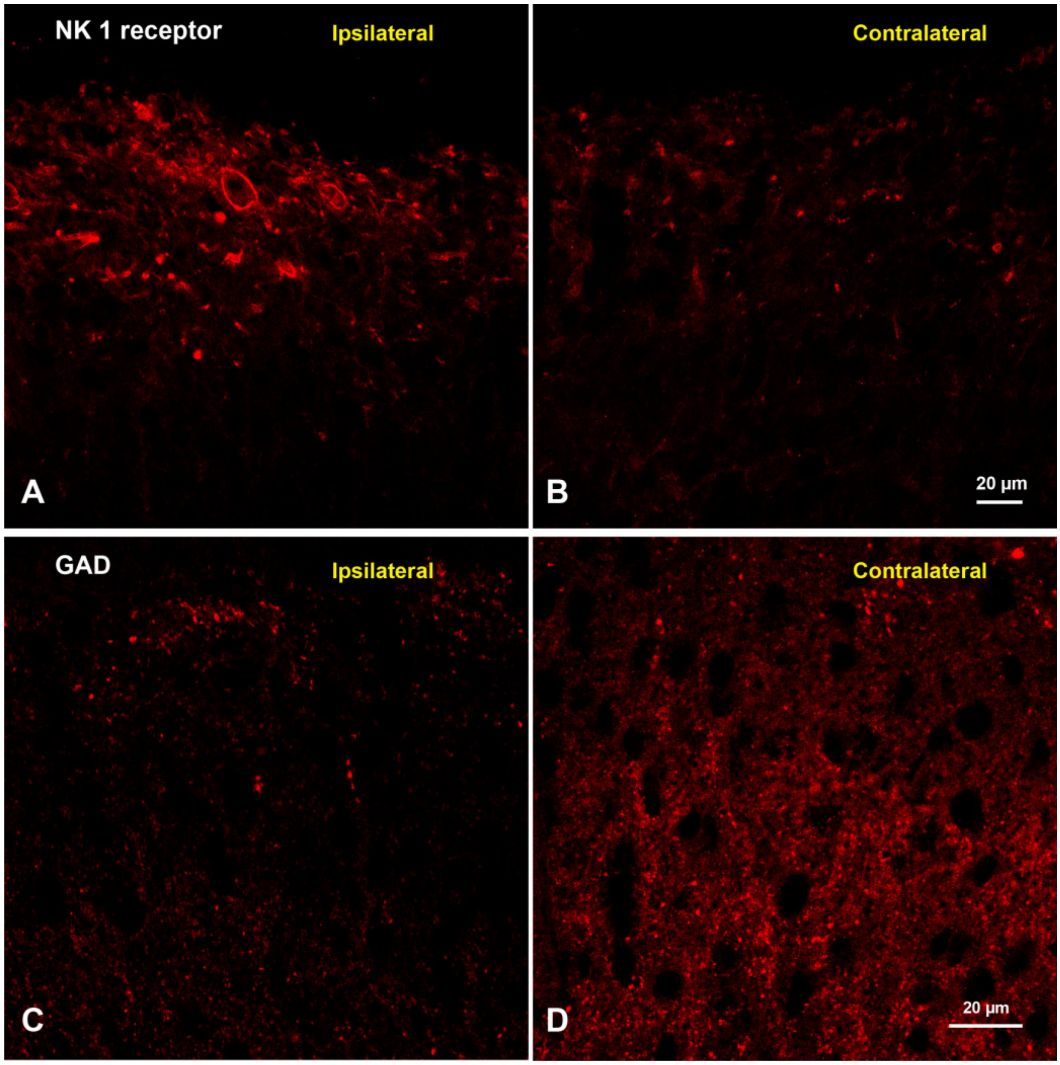


Figure 4: NK1 receptor and GAD immunoreactivity in the ipsilateral and contralateral superficial dorsal horn 14 days following IB4-Saporin injection. **A-B**: High magnification confocal images demonstrating the marked increase in NK1 receptor immunoreactivity in lamina I in the ipsilateral side compared to the contralateral. **C-D**: High magnification confocal images showing a diminution of GAD-immunoreactive profiles in the ipsilateral (compared to the contralateral) dorsal horn in IB4-Saporin treated animals 14 days following the induction of the lesion.

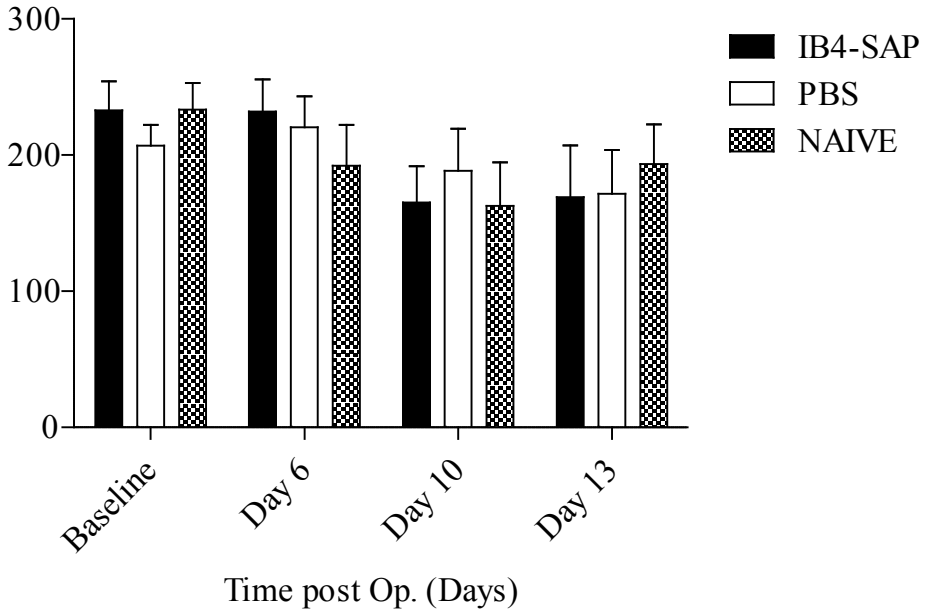


Figure 5: Rota Rod performance in IB4-Saporin and control animals (vehicle and naïve control groups). Motor performance was evaluated on an accelerated Rota Rod at several time points following the intra-sciatic injection of IB4-Saporin. Drop latencies measured in seconds revealed normal motor performance in all treatment groups (between and within group comparisons). Bars represent mean ± SEM. N=7. $p<0.05$. Two-way repeated Measures ANOVA

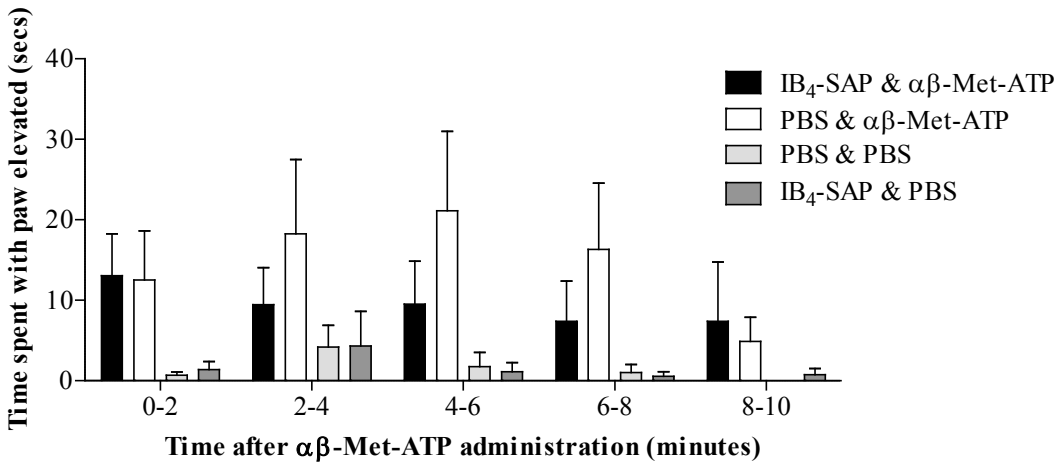


Figure 6: αβ-Methyl-ATP-induced nociceptive behaviours in IB4-Saporin (IB4-SAP) and control treated rats 14 days following IB4-SAP injection. The duration of hind-paw lifting induced by αβ-Methyl-ATP was assessed over a 10 minute interval in 2 minute time intervals IB4-SAP treatment (black bars) reduced the duration of hind-paw lifting evoked by the intraplantar administration of αβ-Methyl-ATP compared to PBS (IB4-SAP) controls (dark gray bars). Bars represent the mean duration of paw-lifting per time bin ± SEM. N=8 rats per group. p value set a $p<0.05$.

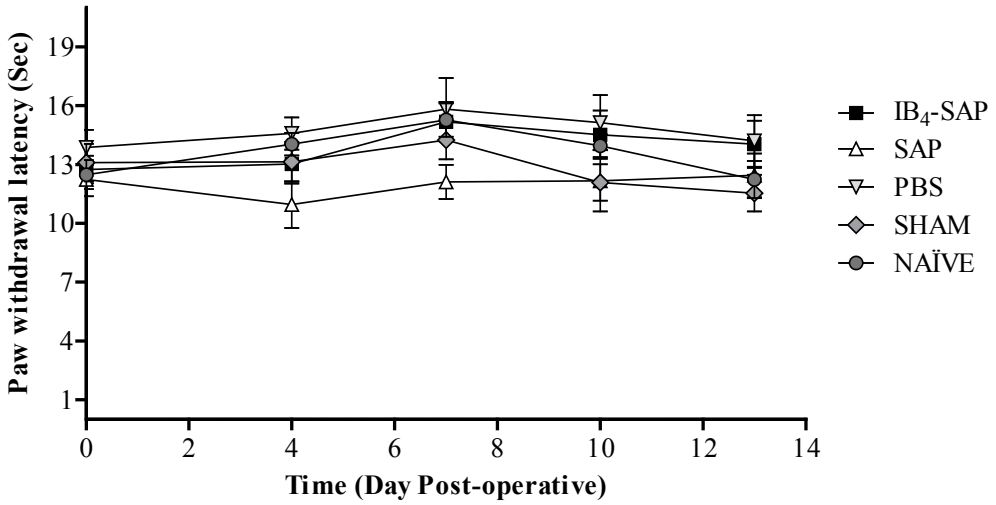


Figure 7: Evaluation of paw withdrawal latencies to acute noxious radiant heat in IB4-Saporin (IB4-SAP) treated and control animals. Paw withdrawal latencies in IB4-SAP treated animals (black squares) remained near baseline throughout the 14 day observation period following IB4-SAP injection. Compared to control groups (SAP open triangle; PBS vehicle, shaded inverse triangle; sham, shaded diamond and naïve, darkened circle), IB4-SAP treatment had no effect on withdrawal latencies over the course of the 14 day post-operative period. Values represent mean ±SEM. N=8 rats per group. $p<0.05$ Two-way repeated measures ANOVA with Post-Hoc tests (Bonferroni corrected).

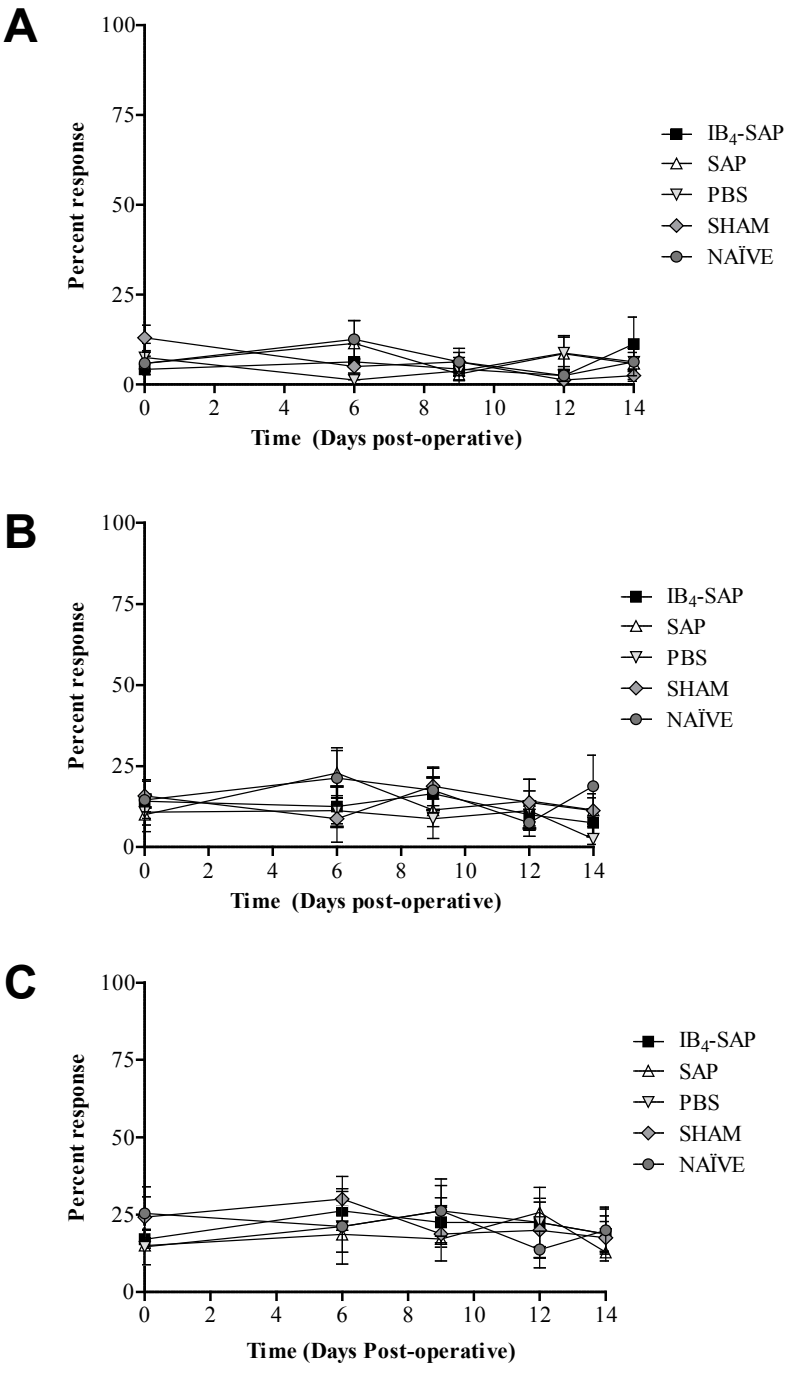

A
100
75
50
25
0
Percent response
0
2
4
6
8
10
12
14
Time (Days post-operative)
IB4-SAP
SAP
PBS
SHAM
NAÏVE
B
100
75
50
25
0
Percent response
0
2
4
6
8
10
12
14
Time (Days post-operative)
IB4-SAP
SAP
PBS
SHAM
NAÏVE
C
100
75
50
25
0
Percent response
0
2
4
6
8
10
12
14
Time (Days Post-operative)
IB4-SAP
SAP
PBS
SHAM
NAÏVE

Figure 8

Figure 8: Hind-paw sensitivity to innocuous and noxious mechanical stimuli in IB4-Saporin (IB4-SAP) and control animals evaluated periodically following IB4-SAP or control treatment (sham, vehicle). A-C: The frequency of withdrawal responses to von Frey filament application to the plantar surface of the left hind paw measured at A) 4, B) 8 and C) 15 grams of force. At all three thresholds, IB4-SAP treatment had no effect on withdrawal latencies over the course of the 14 day post-operative period compared to control groups (SAP open triangle; PBS vehicle, shaded inverse triangle; sham, shaded diamond and naïve, darkened circle). Symbols represent mean ± SEM. N=8 rats per group. $p<0.05$ Two-way repeated measures ANOVA with Post-Hoc tests (Bonferroni corrected).

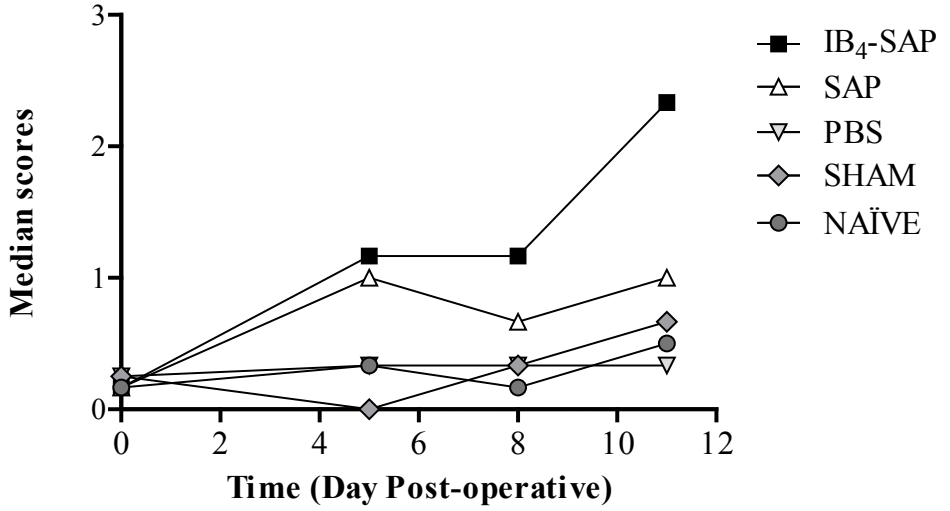


Figure 9: Evaluation of acute cold sensitivity in IB4-Saporin (IB4-SAP) and control rats in response to acetone spray. Baseline sensitivities to acetone spray were similar in all groups. Sensitivity to acute cold stimuli increased non-significantly in IB4-SAP (black squares) treated animals over the course of the 14 day post-operative period compared to control groups (SAP open triangle; PBS vehicle, shaded inverse triangle; sham, shaded diamond and naïve, darkened circle). Symbols represent the median response. N=8 rats per group. ($p>0.05$ Friedman's test).

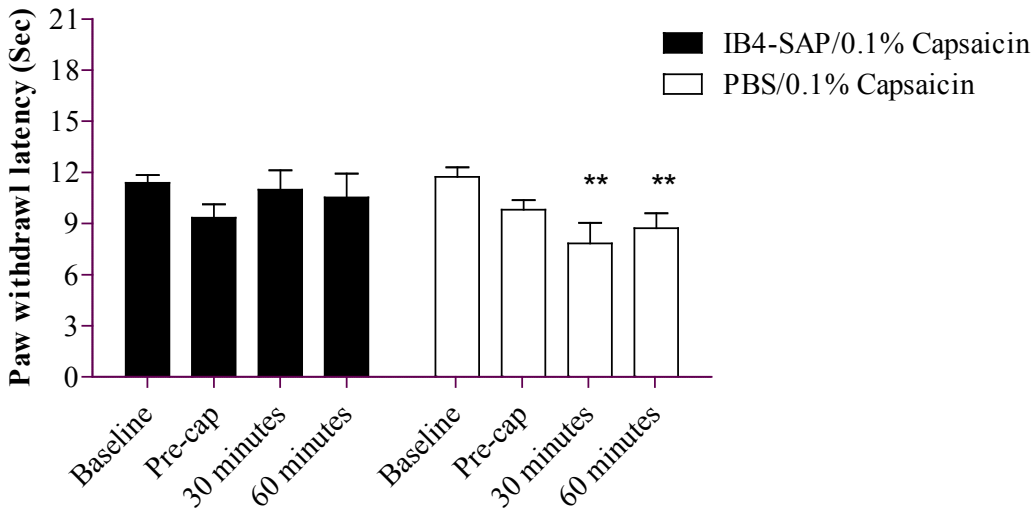


Figure 10: Evaluation of Capsaicin-evoked heat hyperalgesia in IB4-Saporin (IB4-SAP) and vehicle (PBS) control groups 14 days following IB4-SAP treatment. Baseline latencies represent paw withdrawal latencies taken prior to IB4-SAP injection. IB4-SAP treatment (black bars) produced a complete inhibition in capsaicin induced heat hyperalgesia 30 and 60 minutes following intraplantar capsaicin compared to vehicle control (white bars). Pre-cap, before capsaicin treatment. Bars represent mean ± SEM. N=10 rats per group. **$p<0.01$, Two-way repeated measures ANOVA with Post-hoc comparisons.

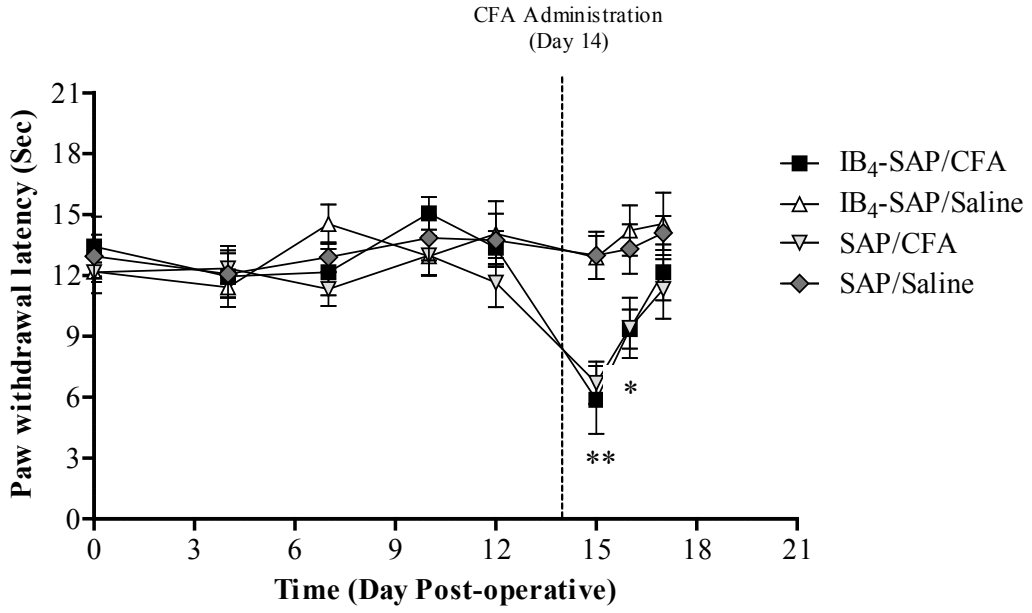


Figure 11: Evaluation of paw withdrawal latencies in IB4-Saporin (IB4-SAP) and control animals following intraplantar complete Freund's adjuvant (CFA). Over the course of the 14 days following IB4-SAP treatment (black squares), paw withdrawal latencies to noxious radiant heat did not differ significantly from vehicle controls. On the 14th day following IB4-SAP or vehicle injection (dotted line), animals received an intraplantar injection of CFA or saline. Paw withdrawal latencies were taken at 24, 48 and 72 hours post-CFA or saline treatment. CFA treatment produced a profound drop in withdrawal latencies 24 hours after CFA administration, increasing towards baseline over the course of the following 48 hours (shaded inverse triangle). Compared to vehicle controls (shaded inverse triangle), IB4-SAP treatment (black squares) did not elicit a deficit in CFA-induced heat hyperalgesia. N=7 rats per group. Values are represent as mean ± SEM, p value set at $p<0.05$. Two-way repeated measures ANOVA with Post-hoc comparisons. Bonferroni corrected.

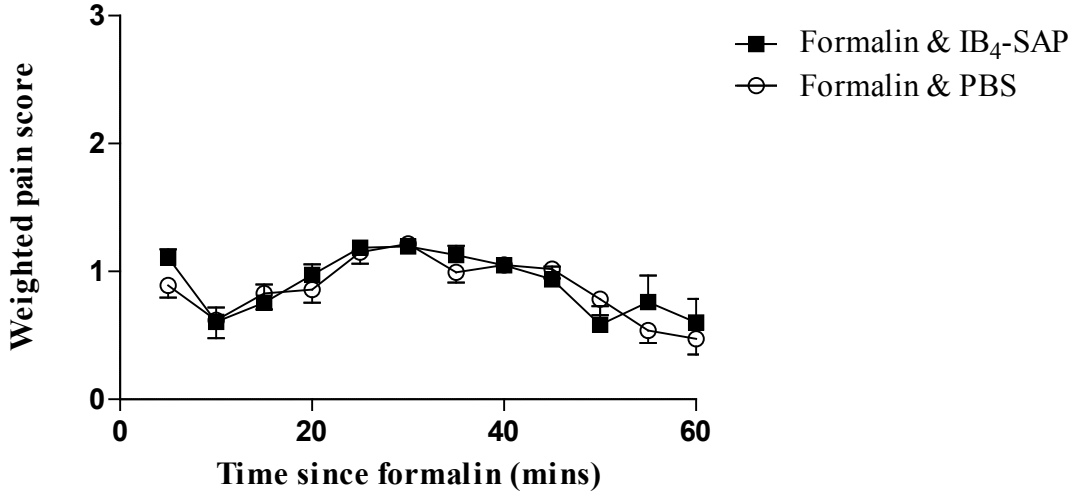


Figure 12: Examination of the biphasic response induced by intraplantar formalin treatment in IB4-Saporin (IB4-SAP) and vehicle control rats on the 14th day following IB4-SAP treatment. Intraplantar formalin (open circles) resulted in a typical biphasic response demonstrated by the presence of two distinct phases of nociceptive behaviours separated by an interval of quiescence. IB4-SAP treatment (black squares) did not produce a change in formalin-induced nociceptive behaviours in any phase of the formalin test. N=5-6 rats per group. Values represent mean ± SEM, p value set at $p<0.05$

Chapter III-Chapter IV

The intraneural injection of IB_4-Saporin, resulting in the selective ablation of the IB_4-binding, non-peptidergic population of small diameter sensory neurons revealed, that in response to acutely applied innocuous and noxious stimuli, the absence of this neuronal subpopulation did not affect withdrawal response thresholds. Notwithstanding compensatory alterations in sP, CGRP, NK1 and GAD-immunoreactivity, this data suggests that the functional role of non-peptidergic afferents is not tightly linked to sensory discrimination.

The central terminals of non-peptidergic varicosities form unique synaptic arrangements with local substantia gelatinosa neurons **(**see Ribeiro-da-Silva, A., 2004 for review**)**. At present, the circuitry of this region of the superficial dorsal horn remains latent. Comprised of excitatory and inhibitory local circuit interneurons which send axonal and dendritic projections to dorsal and ventral lamina, it appears as though LII plays an important role in transferring modulated afferent input to the marginal zone as well as to the deep dorsal horn.

In an effort to deduce the synaptic relationships forged between non-peptidergic primary afferents and their post-synaptic targets within the substantia gelatinosa in addition to their downstream circuitry, Braz, J.M and colleagues (2005), using a cre-lox-P, transgenic mouse model to express wheatgerm agglutin (WGA), a lectin capable of synaptic transfer, in a sub-population of IB_4-binding sensory neurons expressing the $Na_V1.8$ channel, revealed a putative synaptic connection between LII neurons which received IB_4-binding input with neurons localized to LV of the deep dorsal horn. Moreover, the authors demonstrated that these deep dorsal horn neurons projected supraspinally to cortical regions such as the amygdala, the hypothalamus and the bed nucleus of the stria terminalis; all of which play an active role in the affective-motivational aspect of the pain experience (Gao, Y.J., Ren, W.H., Zhang, Y.Q., Zhao, Z.Q., 2004; Deyama, S., Nakagawa, T., Kaneko, S., Uehara, T., Minami, M.,2008).

Using IB_4-Saporin to selectively lesion IB_4-binding C-PAFS, we examined the effects of the loss of non-peptidergic input to the substantia gelatinosa in a conditioned place avoidance paradigm designed to evaluate avoidance learning to a noxious stimulus. The results demonstrate that in the absence of IB_4-binding C-

PAF input to the substantia gelatinosa, rats displayed a significant deficit in avoidance learning, thus lending substantial support to the proposed periphrastic pathway of Braz, J.M et al (2005).

The results of this study imply that information processed within the substantia gelatinosa is capable of reaching supraspinal targets, independent of the lamina I-spino-parabrachial pathway, advocating a potential role imparting afferent input to affective centers of the cortex.

Chapter IV

Deficient Associative Learning Exhibited in Rats Treated with Isolectin B4-Saporin, A. L. Bailey, A. W. Saeed and A. Ribeiro-da-Silva, to be submitted.

Deficient Associative Learning Exhibited in Rats Treated with Isolectin B4-Saporin

Andrea L. Bailey[1,2], Abeer W. Saeed[1,2] and Alfredo Ribeiro-da-Silva[1,2,3]

1) Department of Pharmacology and Therapeutics, McGill University, McIntyre Medical Building, 3655 Promenade Sir William Osler, H3G 1Y6 Montreal, Quebec, Canada.
2) Alan Edwards Centre for Research on Pain, McGill University, Strathcona Anatomy & Dentistry Building, 3640 University Street, H3A 2B2 Montreal, Quebec, Canada
3) Department of Anatomy and Cell Biology, McGill, University, Strathcona Anatomy & Dentistry Building, 3640 University Street, H3A 2B2 Montreal, Quebec, Canada.

Acknowledgements

This research was supported by CIHR grant MOP-79411 to A. Ribeiro-da-Silva. Andrea L. Bailey acknowledges a studentship from the Louise Edwards Foundation. We are grateful to Dr. Gary Bennett for granting us access to his animal behaviour testing facilities, as well as for all his help on this project.

Key Words

Non-peptidergic, Primary afferent, C-Fibres, Amygdala, Conditioned place avoidance, IB4-Saporin, Peptidergic, Nociception, Affective-emotional, Sensory discrimination.

Abstract

Nociceptive stimuli from cutaneous tissues are transmitted to the superficial laminae of the spinal dorsal horn by both peptidergic and non-peptidergic primary afferent C- fibres (C-PAFs). Sensory information carried by peptidergic afferents is transduced to marginal zone neurons where it is sent to supraspinal sensory and affective brain regions via the spinothalamic tract and the spino-parabrachio-amygdaloid pathways respectively. In contrast non-peptidergic C-PAFs transmit nociceptive information to lamina II where their central terminals form the core element of type Ia synaptic glomeruli. Within this region of the superficial dorsal horn, non-peptidergic boutons establish synaptic contacts with local excitatory and inhibitory neurons. The lack of projection neurons within lamina II has long suggested that the region is involved in the local modulation of incoming nociceptive information. However, neuroanatomical studies have documented synaptic interactions between lamina II neurons with those located in lamina I and the deep dorsal horn, thus implying that modulated signals from non-peptidergic PAF input may reach supraspinal targets through indirect ascending pathways to limbic regions involved in the affective-emotional processing of pain. Specifically, a recent study using a transgenic mouse model provided evidence of downstream synaptic circuitry involving non-peptidergic C-fibres. Wheat germ agglutinin (WGA), a lectin capable of trans-synaptic transport was expressed in non-peptidergic afferents under the control of the $Na_{v1.8}$ channel promoter. Lamina II neurons which received input from non-peptidergic WGA-positive fibres synaptically transferred the WGA to neurons located in lamina V. Further evaluation revealed significant labelling in several regions of the brain which process the affective-emotional component of pain including the amygdala, a region of the brain responsible for associative learning behaviours in a painful context.

In an effort to determine whether this proposed pathway engaged by non-peptidergic C-PAFs contributes substantially to avoidance learning, we evaluated such behaviours in rats which received bilateral intra-sciatic nerve injections of the selective neurotoxic conjugate IB4-Saporin which lesioned specifically the non-peptidergic C-PAF population. Avoidance learning was evaluated using a

light/dark shuttle box paradigm in which the dark compartment was paired with a noxious stimulus (heated floor, 50°C). Following habituation, IB4-Saporin and control (vehicle injections of phosphate buffered saline, PBS) animals were exposed to the shuttle box for 7 consecutive days in which they were monitored for the duration of time they spent in the lighted compartment. Results demonstrated that control animals efficiently learned to avoid the darkened environment whereas IB4-Saporin treated animals were incapable of forming the association between the darkened compartment and the noxious stimulus. Latencies to the onset of nocifensive behaviours in response to the heated floor were not significantly different between treatment groups indicating that altered sensitivity to the noxious stimulus was not a factor in the failure to learn. Due to the presence of a contracture of the hind paws in IB4-Saporin treated animals, walking track analysis and foot-print parameters were examined to ensure that IB4-Saporin animals were capable of establishing complete contact with the noxious surface. The results demonstrated no significant difference in these measures between IB4-Saporin and control animals eliminating the possibility that the deficit in avoidance learning was caused by the inability of the animals to maintain complete contact with the heated surface.

These results suggest that non-peptidergic C-PAFs engage a supraspinal pathway projecting to associative learning brain regions and that, in the absence of non-peptidergic input to lamina II, this behaviour is significantly diminished.

Introduction

Small diameter sensory afferents originate as a single population of nerve growth factor (NGF)-dependent neurons. In the post-natal period, they begin to diverge into two separate groups of primary afferents marked by a shift in growth factor support from NGF to glial derived neurotrophic factor (GDNF) in approximately half of the neuronal population (Molliver et al., 1995; Priestley et al., 2002; Luo et al., 2007). The newly formed GDNF-dependent subgroups of afferents additionally lose their peptidergic phenotype, giving rise to a non-peptidergic group of nociceptive afferents (Silos-Santiago et al., 1997). Given the marked differences in neurochemistry and neuroanatomy, it has been proposed that the two each subpopulations of small diameter nociceptive fibres have separate functions in regards to nociceptive input to the superficial dorsal horn (Hunt, S.P., Rossi, J., 1985).

Several studies have attempted to elucidate the importance of either peptidergic or the non-peptidergic population of nociceptive afferents in the transmission of peripherally derived noxious stimuli in the setting of acute and chronic pain (Molander et al., 1996; Bailey, A., et al., 2006.). Due to the abundant expression of pro-inflammatory neuropeptides such as substance P and CGRP, the peptidergic population has been implicated in the transmission of pain related sensory information in the setting of acute and chronic inflammatory conditions (Quartara L, Maggi CA., 1998) The presence of peptidergic peripheral terminals in structures such as the joints, that are often involved in inflammatory pathology, lends support to the hypothesis that they are important in the genesis and maintenance of inflammation. In contrast, the non-peptidergic afferents lack neuropeptides and are mostly absent from joint tissues, what would suggest that they are minimally involved in the pathogenesis of inflammatory conditions (O'Brien et al., 1989; Bennett et al., 1996; Perry and Lawson., 1998; Lu et al., 2001; Ambalavanar et al., 2003; Aoki et al., 2005; Zylka et al., 2005). The non-peptidergic afferents are often identified histologically by lectin-binding using the selective isolectin B4 (IB4) and express nociception-related ion channels such as TRPV1 and $P2X_3$, which has been taken as evidence of their implication in the transmission of pain-related information (Vulchanova, L., et al., 1998; Petruska,

J.C., Cooper, B.Y., Gu, J.G., Rau, K.K., Johnson, R.D., 2000). The complex structural arrangement of their central terminals in the substantia gelatinosa, where they represent the central element of synaptic glomeruli and receive inhibitory pre-synaptic contacts, illustrate a possible role of the non-peptidergic afferents in the modulation of primary sensory input, however, their precise role in pain transmission remains not fully understood (Lu,Y. ,Perl, E.R., 2003, 2005; Ribeiro-da-Silva A., 2004).

Examination of the morphological and neurochemical consequences of nerve injury on both peptidergic and non-peptidergic sensory fibres have demonstrated a differential response to nerve lesions (Hökfelt, T., Broberger, C., Xu, Z.Q., Sergeyev, V., Ubink, R., Diez, M 2000; Bailey, A., et al., 2006.). Time dependent changes in neuropeptide levels (Kajander, K.C., Xu, J., 1995; Ma,W., Bisby, M.A., 1998) and ion channel expression (TRPV1 and $P2X_3$ respectively) (Caterina, M.J., et al., 2000; McGaraughty, S., et al., 2003; McGaraughty, S., Jarvis, M.F., 2005) are indicative of their importance in pain transmission. However, these studies did not elucidate the independent contribution of either fibre subtype in the pathogenesis of chronic pain, nor did they provide valuable information pertaining to the relative relationship of the peptidergic and non-peptidergic C-fibres within the setting of acute and chronic pain.

Several studies applying selective neurotoxin conjugates have demonstrated the effects of the directed ablation of either non-peptidergic afferents or spinal lamina I projection neurons on normal and abnormal nociceptive processing (Mantyh, P.W., 1997; Vulchanova, L., Olson, T.H., Stone, L.S., Riedl, M.S., Elde, R., Honda, C.N., 2001; Vierck, C.J. Jr, Kline, R.H, Wiley, R.G., 2003; Wiley R.G et al., 2007; Wiley R.G., 2008). Evaluation of sensory withdrawal responses to innocuous and noxious stimuli in the absence of either substance P receptor (NK1 receptor)-expressing lamina I neurons or non-peptidergic afferents have contributed somewhat to the clarification of the relative role of peptidergic and non-peptidergic afferents in nociception.

The indirect loss of peptidergic afferent processing induced by the intrathecal application of substance P (sP) conjugated to the neurotoxin saporin revealed distinct sensory abnormalities which consisted of attenuated mechanical

and thermal sensitivity in the setting of inflammation and nerve injury (Wiley R.G et al., 2007; Wiley R.G., 2008). Furthermore, sP-saporin induced a deficit in an animal model of mustard oil-induced hyperalgesia operant escape, indicating an effect on lamina I neurons projecting to brain regions involved in fear and anxiety (Wiley R.G et al., 2007; Wiley R.G., 2008). The results of these studies illustrate the importance of the ascending nociceptive specific pathways arising from the marginal zone, the region of the superficial dorsal horn which receives afferent input directly from peripheral targets via the peptidergic subpopulation of primary afferents fibres.

The injection into the sciatic nerve of saporin conjugated to IB4 resulted in the near complete loss of IB4-binding non-peptidergic primary afferent fibres. Unlike the effects of sP-saporin, which induced a lesion in spinal cord projection neurons, IB4-Saporin (IB4-SAP) eliminated afferent input to lamina II. In contrast to the sensory deficits observed following intrathecal administration of sP-SAP, IB4-SAP treatment did not produced any alterations in baseline withdrawal latencies to acute noxious thermal stimuli nor any deficit in withdrawal thresholds to innocuous or noxious mechanical stimuli (Bailey, A.L and Ribeiro-da-Silva, A, manuscript in preparation). In an acute model of complete Freund's adjuvant induced inflammation, withdrawal responses to noxious mechanical and thermal stimuli were identical to inflamed animals which did not receive treatment with IB4-SAP (Bailey, A.L and Ribeiro-da-Silva, A, manuscript in preparation). However, deficits in sensitivity were observed following intraplantar capsaicin injection in these animals. Indeed, IB4-SAP treated rats demonstrated a complete lack of capsaicin induced thermal hypersensitivity. Furthermore, IB4-SAP treated rats revealed an attenuated response to intraplantar αβ-methyl-ATP. These results confirmed the loss of TRPV1, $P2X_3$-expressing non-peptidergic cutaneous terminals (Bailey, A.L and Ribeiro-da-Silva, A, manuscript in preparation).

Lamina II mostly contains local circuit neurons, which are thought to modulate incoming peripheral signals (Lu, Y., Perl, E.R., 2003, 2005). Neuroanatomical studies have demonstrated the presence of projection neurons resident in this region of the superficial dorsal horn, however their numbers are very low (Al-Khater, K.M., Kerr, R., Todd, A.J., 2008). However, several studies

have documented the occurrence of intra-laminar connections to lamina I and III via dendritic projections which penetrate the dorsal and ventral border of lamina II (Light, A.R., Kavookjian, A.M., 1988). Thus modulated nociceptive signals within lamina II may be capable of ascending indirectly to supraspinal targets via marginal zone or deep dorsal horn projections neurons.

Using a Cre-LoxP transgenic mouse model, investigators were able to trace a downstream synaptic circuitry initiated by primary afferent input by non-peptidergic afferents. In these mice, wheat germ agglutinin (WGA), a lectin capable of transsynaptic transport was expressed in non-peptidergic afferents under the control of the $Na_{v1.8}$ channel promoter; it subsequently labelled neurons in lamina II which connected to lamina V cells. Further evaluation revealed significant labelling in several motor and affect-related regions of the brain including the amygdala, a region of the brain responsible for nociceptive conditioned behaviour (Braz, J.M., Nassar, M.A., Wood, J.N., Basbaum, A.I., 2005).

Based on the above, we decided to apply bilateral intra-sciatic injections of IB4-SAP to study the effects of the selective ablation of non-peptidergic input to lamina II on conditioned avoidance behaviour. In a typical shuttle box paradigm, rats were conditioned to avoid entering a dark compartment paired with a continuous noxious thermal stimulus (a hot plate set at 52 °C).

Materials and Methods

Intra-sciatic injections of IB4-Saporin and Vehicle control.

A total of 12 male Sprague-Dawley rats (225-250g) (Charles River Laboratories, Saint Constant, Qc, Canada) were used in all experiments (6 rats per treatment group). Animals were randomly assigned to treatment groups (GraphPad QuickCalcs, GraphPad.com). Animals were anesthetized by rapid induction with Isoflurane gas (99.9% isoflurane USP, Pharmaceutical Partners of Canada Inc., Ont, Canada) followed by maintenance of anesthesia using a mixture of 3.5-5% isoflurane and oxygen. All injections were performed bilaterally (in both left and

right sciatic nerves). On each side, the lateral aspect of the thigh was shaved and a 2cm incision was made at the level of the sciatic notch. The underlying muscles; the gluteus superficialis and the bicep femoralis were separated, exposing the sciatic nerve proper. The nerve was carefully isolated from the surrounding connective tissue and stabilized using a blunt probe, with special care taken to minimize stretching of the nerve. A 27½ gauge needle, attached to a 25µL Hamilton syringe (Hamilton Company,Nevada, USA) with PE 20 polyethylene tubing (Benton Dickson, New Jersey, USA) 20 cm in length, was inserted into the sciatic nerve proximal to the trifurcation and fasciculation. The Hamilton syringe was placed in a syringe pump operated by a foot pedal. Rate of injection was set at 13.3 mL/hour. Retraction of the syringe plunger to a specified volume draws the same volume of solution into the needle and tubing at the distal end. Six µL of an 800 µg/mL solution of IB4-SAP (Advanced Targeting Systems, San Diego CA, USA) in 0.2M phosphate buffered saline (PBS) and Fast Green Dye (Sigma, Missouri, USA) were injected. Vehicle injections consisted of 6µL of 0.2M PBS and Fast green dye. The wound was sutured in layers using 4-0 Vicryl sutures (Ethicon Inc, New Jersey USA). Animals were returned to their home cages and were allowed to recover for 2 weeks prior to behaviour experimentation.

Conditioned Place Avoidance.

Apparatus

Animals were conditioned to associate a darkened environment, the conditioned stimulus (CS) with an aversive nociceptive thermal, unconditioned stimulus (US). The avoidance apparatus consisted of a modified shuttle box measuring 60.9 x 20.32 x 20.32 cm made of 0.635 cm thick clear acrylic (Cyro Acrylic FF, Cyro Industries; New Jersey, USA). A 20.32 x 20.32 cm septum divided the box into two equal sized chambers. Movement amid the compartments was facilitated by an 8.26 x 8.26 cm opening located and the bottom center of the septum. A 20.32 x 15.24 cm top with six, 2.5 cm diameter ventilation holes was fastened by hinges to each chamber. The chamber functioning as the conditioned stimulus (CS), including the chambers tops and the septum, were painted solid black. The contralateral compartment remained

transparent. The floor surfaces of either compartment were identical. The floor of the darkened compartment (Unconditioned Stimulus; US) was heated by a thermally regulated hot plate (LabLine Slide Warmer). The temperature of the floor surfaces of both compartments was taken after each conditioning trial per rat and was measured using digital thermometer (Thermocoupler Thermometer Type T, Cole Parmer; Quebec, Canada) equipped with a surface probe (Physitemp Instruments; New Jersey, USA). The thermally neutral compartment was brightly lit by an 60 watt halogen bulb.

Conditioning trials

Prior to conditioning, animals were habituated to the conditioning apparatus, in the absence of the thermal stimuli. Specifically, animals were placed in the neutral, brightly lit compartment in the center facing outward. Each rat was given 2 minutes to freely explore both compartments. The amount of time spent in either chamber was recorded.

Conditioning was initiated on the following day and was conducted daily. The floor temperature (US) of the conditioning chamber (CS) was set at 50 ±1ºC and was measured after each conditioning trial. Rats were placed in the apparatus in the same manner as for the habituation. Each animal was given two minutes to move from the neutral environment to the dark compartment. Once the rat moved into the CS, a barrier was placed over the septal opening preventing the animal from escaping. The latency to enter the CS was recorded. Once inside the CS, animals were exposed to the US for 25 seconds. The latency to obvious hind paw pain behaviour (rapid flicking, stamping and licking) was measured, and the type of behaviour was recorded. Animals were immediately removed from the CS at the end of the 25 second exposure period. Results are shown as the mean and standard error of the mean (SEM).

Walking track analysis

Gross observation of bilaterally injected IB4-SAP into the sciatic nerve revealed the presence of abnormal hind paw positioning whilst in a resting position. In order to determine whether the altered hind paw placement interfered

with the animals' ability to detect the noxious stimulus within the dark compartment, each rat underwent gait analysis in order to verify the degree of contact established between the plantar surface of the hindpaw and the floor during conditioning trials.

Gait analysis was performed using footprint tracking. Briefly, rats were conditioned to walk continuously along a lit corridor (86.9 cm in length) to a darkened dead end. The floor of the corridor was lined with white 9.07kg weight paper. Following the final day of conditioning, the hind paws of each rat were dipped in non-toxic water-based poster paint and placed at the beginning of the track. Three walking track trials were obtained per rat.

Analysis of the foot prints were conducted by examining the following parameters: Toe spread (distance between 1^{st} and 5^{th} digit), intermediate toe spread (distance between 2^{nd} and 4^{th} digit) and paw length (measured from top of 3rd digit to the base of the heel print). Several prints were analysed per rat and the mean and SEM were reported.

Withdrawal latency analysis

During the conditioning trials, the paw withdrawal latency to the noxious thermal stimulus was measured in order to examine whether IB4-SAP treatment resulted in a deficit in sensory discrimination. Withdrawal latencies were obtained by measuring the onset of withdrawal from the moment the animal entered the dark compartment. Hindpaw withdrawal was defined by lifting, rapid stamping and alternate licking of the hindpaws.

Results

Hindpaw withdrawal latencies

Comparative evaluation of hindpaw withdrawal latencies to the noxious thermal stimulus of the hot plate floor of the darkened compartment revealed no significant differences between PBS control animals and those which received bilateral IB4IB4-SAP injections. Interestingly, paw withdrawal latencies increased non-significantly in both treatment groups over the 7 day conditioning period (Figure 1).

Walking track analysis

Animals which received bilateral injections of the vehicle (PBS) did not display any abnormalities in hind paw positioning at rest or during movement (rearing or walking). In contrast, IB4-SAP treated rats displayed an abnormal eversion (outward turning) of both hind paws when at rest (resting on haunches), however, it did not impair their ability to walk or rear. When engaged in a walking gait, IB4-SAP rats did not display the contraction. Footprint analysis of several parameters demonstrated a lack of any significant difference in toe spread ($p>0.05$), intermediate toe spread ($p>0.05$) and print length ($p>0.05$) either hind paw (Figures 2a, b and 3). Comparative analysis of print parameters within lesion and control groups demonstrated the lack of any significant differences in toe spread, intermediate toe spread or print length between left and right paws ($p>0.05$).

Conditioned Avoidance

Prior to conditioning (post-surgery day 14), the characteristic tendency of rodents to seek the safety of an dark environment was confirmed. All animals displayed a preference for the darkened compartment ($P<0.0001$ for both control and lesion animals) (Figure 4). Control animals (PBS-injected), demonstrated competent learning, exemplified by a persistent increase in entrance latencies over the course of the conditioning phase (7 days) (Figure 5). The learning curve in control animals was evident by their increasing hesitancy to enter into the darkened environment demonstrated over the course of the conditioning phase. On each subsequent conditioning trial, control animals spent less time investigating the entrance to the dark compartment, resting near to the entrance of the room, but avoiding it completely (Figure 5).

In contrast, IB4-SAP-treated animals did not display investigative behaviours throughout the course of the conditioning phase; rather, these animals persistently exited the lit compartment within moments of being placed (Figure 5). The profound deficit in associative learning exhibited by IB4-SAP-treated rats, notwithstanding repeated conditioning trials, demonstrated that these animals

displayed a significant difficulty in forming an association between the darkened compartment and the noxious stimulus (significance of the effect of treatment, $p<0.0001$). The results of a two way repeated measure ANOVA demonstrated that there was a significant interaction between treatment (IB4-SAP) and time (conditioning days) ($p<0.01$).

Discussion

Our data shows that the selective ablation of non-peptidergic primary afferent input to the superficial dorsal horn resulted in the significant impairment of passive avoidance learning in a model of pain-induced condition place avoidance.

Conditioned place avoidance and non-peptidergic primary afferent input to the superficial dorsal horn.

Evaluation of conditioned place avoidance behaviour following the bilateral intra-sciatic injection of IB4-SAP resulted in a significant deficit in avoidance learning to a noxious thermal stimulus (52°C) paired with a darkened environment. The results of this study suggest that IB4-binding, non-peptidergic primary afferent fibres connect with an ascending pathway to affective and motivational regions of the cortex.

The premise for the present study was derived from a transgenic mouse anterograde tracing study where wheat germ agglutinin (WGA), a lectin capable of crossing the synapse, was expressed in a subpopulation of IB4-binding primary afferents which possess the voltage gated sodium channel Nav1.8 employing a cre-recombinase system (Braz, J.M., Nassar, M.A., Wood, J.N., Basbaum, A.I., 2005). Examination of synaptically transferred WGA to dorsal horn neurons revealed the presence of WGA+ neurons in laminas II-V, with scant labelling in lamina I (Braz, J.M., Nassar, M.A., Wood, J.N., Basbaum, A.I., 2005). Further examination of several brain regions demonstrated the presence of WGA-positive neurons in the hypothalamus and the amygdala (Braz, J.M., Nassar, M.A., Wood, J.N., Basbaum, A.I., 2005). In light of the negligible labelling of lamina I neurons

(the few labelled were NK1-negative), in addition to the lack of WGA+ neurons in the parabrachial nucleus, the authors proposed a parallel pathway derived from IB4+/Nav1.8+ non-peptidergic afferents to limbic areas. This pathway would be independent of the spino-parabrachial-amygdaloid pathway engaged by peptidergic primary afferent fibres in lamina I of the superficial dorsal horn (Braz, J.M., Nassar, M.A., Wood, J.N., Basbaum, A.I., 2005). The pathway proposed suggested that IB4+/Nav1.8+ non-peptidergic afferents contacted lamina II neurons which contact deep dorsal horn neurons in lamina V, a site of known supraspinal projections (Burstein R, Potrebic S., 1993). Thus, it is likely that non-peptidergic afferent input to lamina II is transferred to deep dorsal horn neurons by either direct contact with dorsally directed dendrites of lamina V neurons or by indirect contact with dorsally projecting dendrites of lamina III and IV neurons which establish synaptic contact with lamina V cells.

Nociceptive pathways from the substantia gelatinosa to higher brain centers.

There is morphological evidence that sensory information transmitted to lamina II via non-peptidergic primary afferents is significantly modulated by local inhibitory neurons which establish presynaptic contacts with the central boutons of non-peptidergic synaptic glomeruli (for review see Ribeiro-da-Silva, A., 2004). The relative lack of projection neurons within this region of the superficial dorsal horn has long suggested that lamina II functions as a region of signal modulation (Szentagothai, J., 1964; Réthelyi, M., Szentágothai, J., 1969; Cervero, F., Iggo, A., 1980). Due to the presence of numerous synaptic glomeruli, it has been further suggested that this region also functions as a centre for signal amplification (Kumazawa, T and Perl, E.R., 1978; Wall P.D, 1978, 1980; Price, D.D., Hayashi, H., Dubner, R., Ruda, M.A., 1979; Cervero, F., Iggo, A., 1980). Neuroanatomical studies have documented synaptic interactions between lamina II neurons as those located both in lamina I and in the deep dorsal horn, thus implying that modulated signals from non-peptidergic PAF input may reach supraspinal targets through indirect ascending pathways (Todd and Lewis, 1986; Todd, A.J., 1989; Lu, Y., Perl, E.R.., 2005). It is known that sensory information originating in cutaneous tissues is transmitted to the superficial laminae of the

spinal dorsal horn by both peptidergic and non-peptidergic sensory fibres (Hunt, S.P., Rossi, J., 1985; Zylka, M.J, 2005). Information carried by peptidergic afferents is transferred to marginal zone neurons where the information is sent to supraspinal sensory and affective brain regions via the spinothalamic tract and the spino-parabrachio-amygdaloid pathways, respectively (Burstein, R., Dado, R.J., Cliffer, K.D., Giesler, G.J. Jr., 1991, Burstein R, Potrebic S., 1993; Jasmin, L., Burkey, A.R., Card, J.P., Basbaum, A.I.., 1997).

As several morphological studies have demonstrated, lamina II interneurons possess extensive dendritic arbors in the rostral-caudal plane, but restricted dendritic projections within the dorsal-ventral orientation (Bennett, G.J., Abdelmoumene, M., Hayashi, H., Dubner, R., 1980; Todd, A.J., Lewis, S.G., 1986; Todd A.J., 1988). Neurons residing in lamina ;III have been documented to display profuse dendritic branching in lamina II, where they have shown in several instances establishing pre and postsynaptic contacts with the central boutons of synaptic glomeruli, which are of known primary afferent origin (Todd, A.J., Lochhead, V., 1990; Todd AJ, Sullivan AC., 1990; Todd, A.J., Russell, G., Spike, R.C., 1992). Moreover, these lamina III neurons possessed ventrally directed processes which enter the deep dorsal horn (Todd A.J., 1989). Furthermore, wide dynamic range (WDR) neurons in lamina V respond to C-fibre intensity stimulation of the sciatic nerve indicating either a direct or indirect contact with primary afferent terminals (Woolf, C.J., King, A.E., 1987). These WDR neurons are known to project profuse dorsal-ventral dendritic arbors which in some instances penetrate the ventral borders of lamina II and I (Woolf, C.J., King, A.E., 1987). Furthermore, many WDR neurons issue processes which cross the spinal cord via the dorsal and ventral commissures (Woolf, C.J., King, A.E., 1987). Therefore, the pathway proposed by Braz et al. (2005) is compatible with the existing literature on dorsal horn connections.

Indeed, our observations concur with the findings of Braz et al. (2005) that indicate that the non-peptidergic IB4-binding primary afferents have completely different ascending connections, compared to the peptidergic. The results of that study, if confirmed, would provide extensive support to the concept that the non-peptidergic afferents would be related more to the affective components of pain,

whereas the peptidergic would be related more to the sensory-discriminative aspects. Indeed, the results of our study demonstrate that the selective loss of non-peptidergic, cutaneously derived input to lamina II had no effect on withdrawal latencies to the noxious thermal stimulus, indicating a lack of effect on sensory discrimination. In contrast, the profound effects on the passive avoidance test in the current study would indicate a preferential role of the non-peptidergic afferents on us caring about the pain and perception of it as unpleasant. This would be in agreement with a major ascending projection of non-nociceptive afferents to the amygdala rather than to sensory-discriminative areas of the CNS. It is important to note that the subjects used in our study were rats whereas the neuroanatomical data from which we based our investigation was obtained in mice. Notwithstanding the consistency of our behavioural data with the neuroanatomical pathways outline by Braz and collegues, the neuroanatomical and neurochemical differences between these two species of rodent, specifically in regards to the nociceptive system, are significant (for example see: Rigaud, M., Gemes, G., Barabas, M.E., Chernoff, D.I., Abram, S.E., Stucky, C.L., Hogan, Q.H., 2008). Given the robustious deficit in avoidance learning observed in IB4-SAP treated rats, it is likely that the pathway charted by Braz et al occurs in the rat.

.

Conclusion

The results of this study support the existence of a separate, indirect supraspinal pathway from non-peptidergic afferents to limbic brain regions such as the amygdala, as originally proposed by Braz et al (2005). If confirmed by other studies, the existence of such a pathway would provide considerable insight into the functional role of non-peptidergic, IB4-binding sensory neurons in the transmission of nociceptive information. Indeed, the deficit in pain-related associative learning we detected following the selective loss of IB4-binding primary afferent C-fibres is highly suggestive of their role in transducing cutaneous noxious stimuli to affective brain centres.

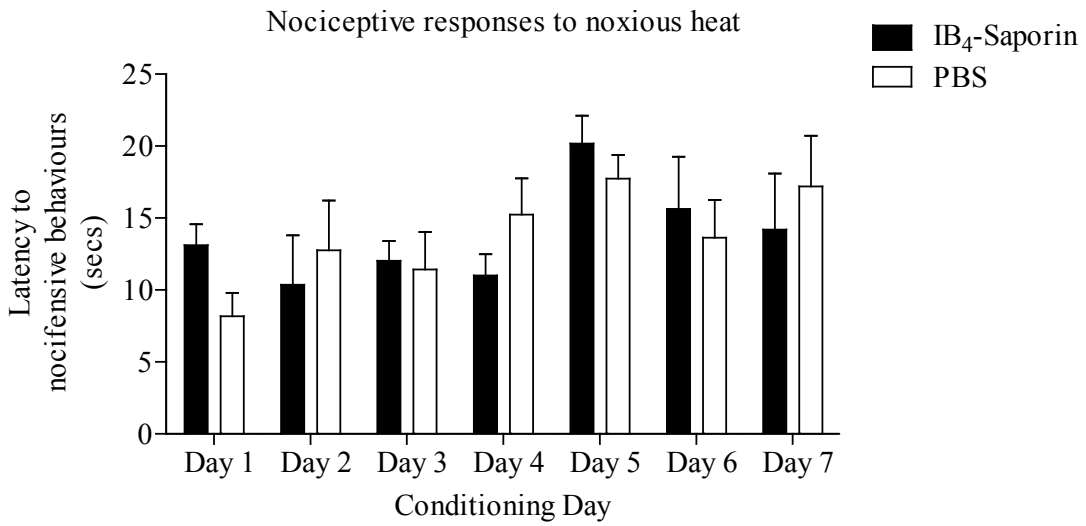


Figure 1: Latencies to nocifensive behaviours in response to noxious heat evaluated during conditioning trials initiated at the 14th day following bilateral IB4-Saporin injection or vehicle (PBS) into the sciatic nerves. Nocifensive behaviours consisted of hind paw stamping, escape behaviour and licking. Bars represent mean ± SEM (n=6 rats per group).

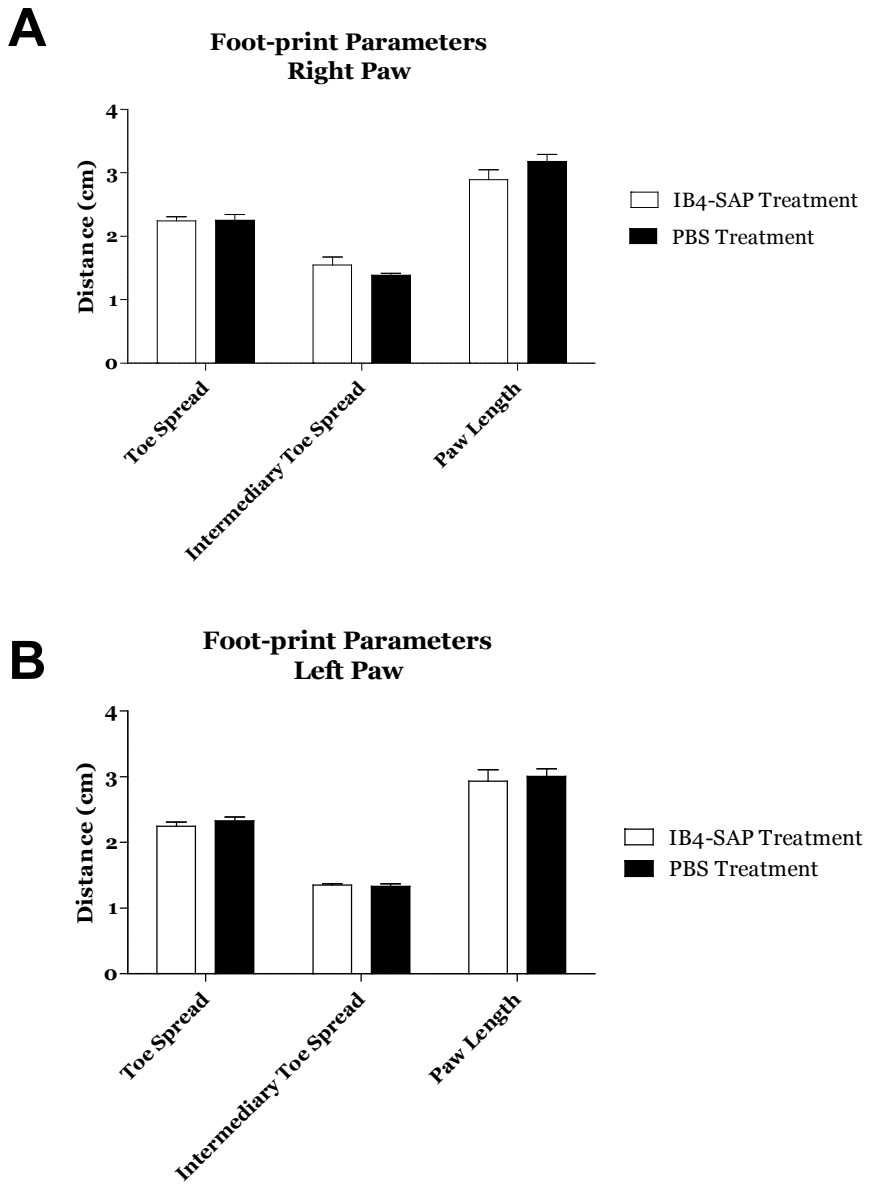


Figure 2: Analysis of foot-print parameters for the right (A) and left (B) hind paws in IB4-Saporin and PBS injected rats. Bars represent means ± SEM. Distances were measured in centimetres (cm).

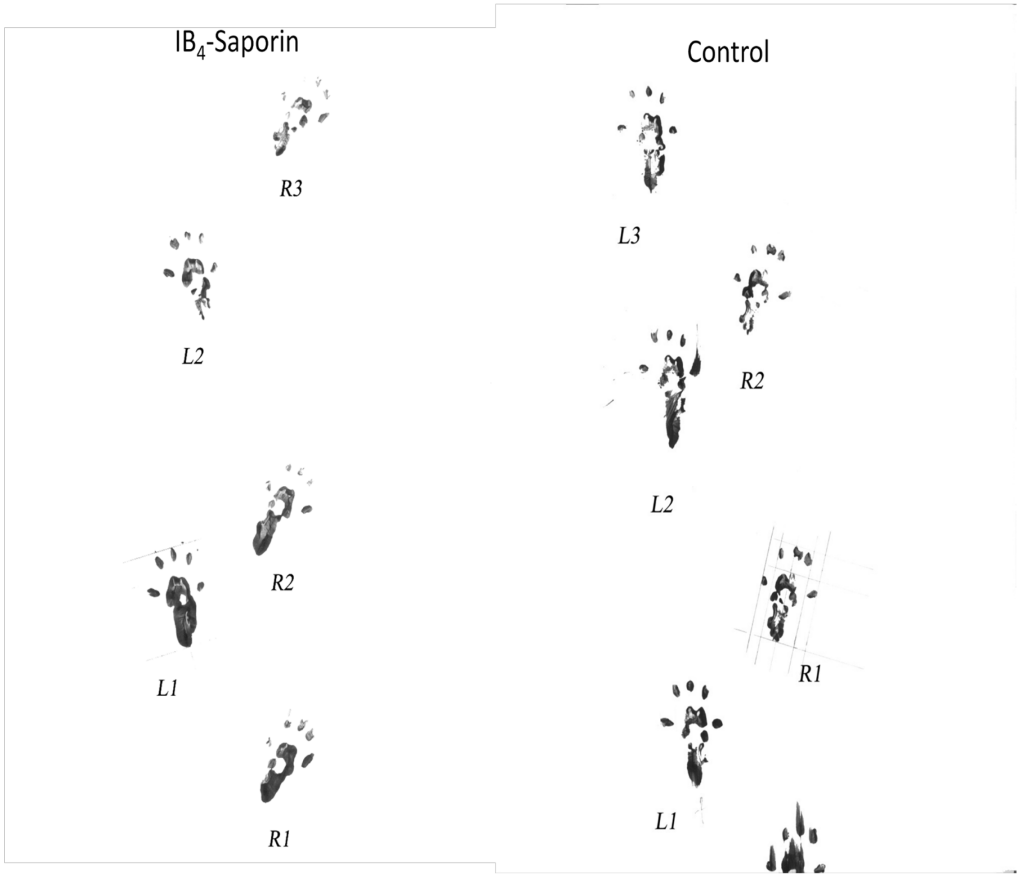


Figure 3: Walking track tracings from IB4-Saporin and PBS vehicle injected control animals.

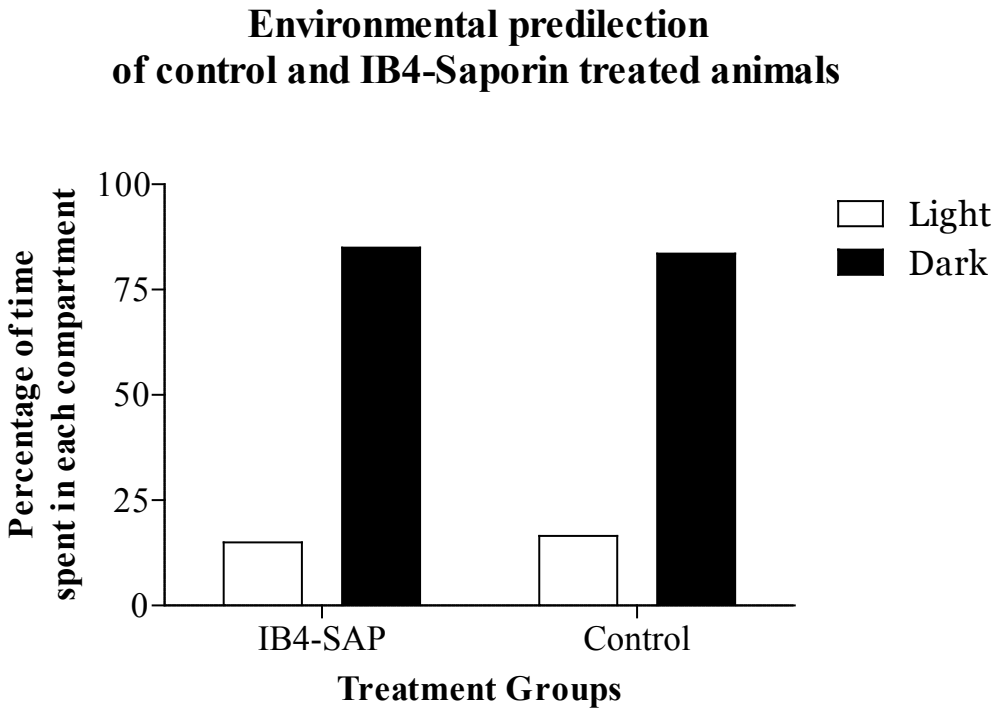


Figure 4: Evaluation of the time spent in the light or dark compartment on habituation day. Bars represent the percentage of time spent in the compartment during the two minute habituation time allotment. Statistical analysis of the absolute values demonstrates a significant preference for the dark compartment with no significant difference between lesion and control animals.

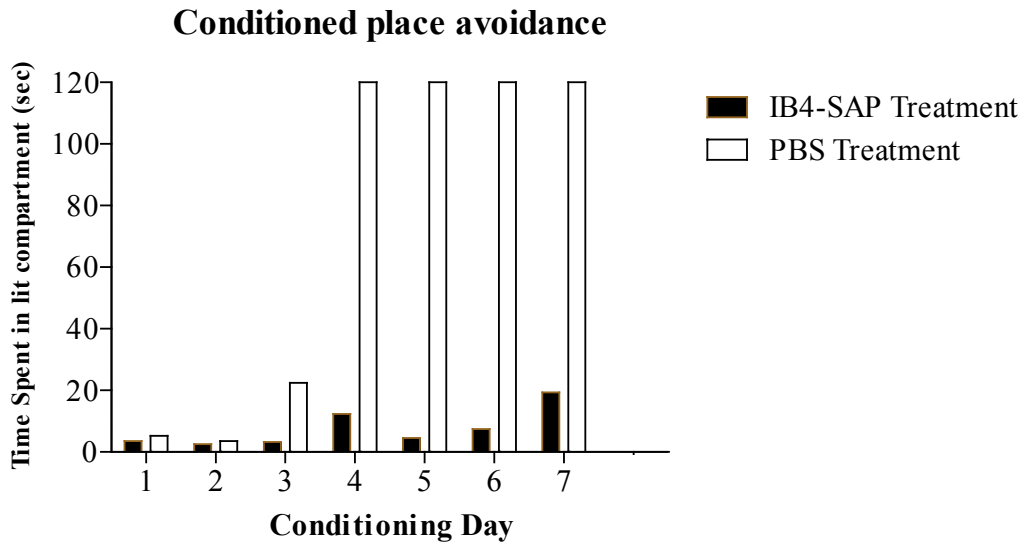


Figure 5: Evaluation of the time spent in the light or dark compartment over the course of the seven day conditioning trials. Bars represent the average time spent in the compartment during the two minute time allotment.

Chapter V
General Discussion

Section 5.1: Précis

The available literature suggests that despite their common ontological origin, diverging neurochemical and physiological properties expressed by the peptidergic and non-peptidergic subclasses of primary afferent C-fibres are highly indicative of two functionally distinct avenues of afferent input to the superficial dorsal horn, commonly referred to as a "parallel pathway" of C-fibre input to the superficial dorsal horn (Hunt, S.P., Rossi, J., 1985). At present, our understanding of the nature of nociceptive signals imparted by either C-PAF population and the post-synaptic modulation, regulation and the forward conveyance of information carried by these afferent subtypes to supraspinal centers is incomplete. Evaluating the pathological consequences of nerve injury on C-PAF morphology and neurochemistry, in addition to effects on downstream postsynaptic targets, provides crucial information pertaining to how such changes influence processes involved in the genesis and maintenance of chronic pain syndromes, bringing us a step closer to developing targeted therapeutics.

The main objective of this dissertation focused on examining the morphological, neurochemical and behavioural consequences of compromised C-PAF input to the superficial dorsal horn. Our approach was two-fold: examining the effects of a compound peripheral nerve injury of the common sciatic nerve (polyethylene cuff model of chronic constriction injury) and the more specific approach of selectively ablating the IB4-binding sub-population of C-PAFs (intra-sciatic injection of IB4-Saporin). The modified chronic constriction injury model provided the opportunity to examine the relative susceptibility of the peptidergic and non-peptidergic populations to nerve lesion. The IB4-Saporin model presented the occasion to not only examine the effects on nociceptive behaviours following the loss of a significant proportion of primary afferent input to the dorsal horn, but, in addition, offered the chance to examine the nociceptive properties of non-peptidergic afferents by evaluating the effect of their loss on postsynaptic targets as well as on their peptidergic counterpart.

Section 5.2: Technical considerations.

Several animal models of chronic constriction injury are widely used in basic research for a multitude of purposes. Their ability to induced neuropathic symptoms in laboratory animals that mimic those seen in man demonstrate their indispensability, providing the opportunity to examine the pathological consequences of compromised axon transport, immune responses to foreign bodies, behavioural manifestations, application of novel pharmacological strategies and the processes involved in the transfer from acute to chronic pain. Each neuropathy model has advantages and limitations, the latter often being the catalyst for the development of new models aimed at circumventing technical variability. The injury induced by each model is highly dependent on the materials used, the placement of the constricting apparatus and the degree of restriction created by the applied device (Xu, J., Pollock, C.H., Kajander, K.C., 1996).

The main objective of the neuropathy study (chapter 2) was to examine the effect of a constrictive injury to the sciatic nerve on neuropeptide-immunoreactivity and IB4-binding. The application of the polyethylene cuff to the common sciatic nerve intuitively induces damage to all sensory and motor fibres which comprise the nerve, thus the model used was not specific at generating a targeted lesion of C-PAFs. Ergo, the conclusions drawn by the results of this study cannot be attributed to a restrictive lesion of C-PAFs, as one must consider the effect of the lesion on myelinated afferents as well. Behavioural evaluation of nociceptive responses to low and high threshold stimuli following cuff application demonstrated abnormal sensitivities to both levels of peripherally applied stimuli. A more thorough investigation of the effects of such lesion of myelinated afferents using neurofilament markers may shed some light on the influence of cuff application on low threshold input to the spinal cord.

In the effort to better understand the nature of the relationship between the peptidergic and non-peptidergic C-PAFs, in addition to evaluating the functional role of each subpopulation in nociception, we used a cytotoxic conjugate compounds that are capable of targeting neuronal populations with minimal

collateral effects. By using such compounds we were able to circumvent the issue of collateral damage innate to constriction injury models such as the polyethylene cuff. Exploiting the natural tendency of IB4-binding induced internalization of neuron specific cell surface glycoproteins, conjugations of IB4-Saporin provided the perfect opportunity to examine the effects of a targeted elimination of a C-PAF constituent.

Administration of the IB4-Saporin was conducted by an injection into the exposed sciatic nerve, infused by pressure supplied by a syringe pump. Control experiments aimed at determining the effects of breaching the epineurium and the potential damage to nerve bundles effectuated by the needle resulted in the occurrence of a small, regional loss of IB4-binding and neuropeptide immunoreactivity restricted to the lateral border of the sciatic nerve termination territory within the superficial dorsal horn.

Furthermore, IB4-Saporin-induced lesions often presented as regions of consummate absence of IB4-binding interspersed by islands of intact IB4-binding. In addition, the extent of the lesion was dependent on the rostral-caudal location of analysis, with the largest, uninterrupted lesions located at the caudal aspect of the 5^{th} lumbar level (L5) and the entirety of the 6^{th} lumbar level (L6) as observed in both transverse and horizontal planes of sectioning. Comparative analysis of the lesions caused by the needle placement with those induced by IB4-Saporin confirmed that the latter were significantly larger than the former, however for quantitative purposes; lesions which were definitively caused by the cytotoxin were used in all analyses. When using cytotoxic conjugates, collateral damage effectuated by non-specific uptake of the compound must be taken into consideration. In the case of IB4-Saporin, the expression of the glycoprotein *Galactose*α1-*Galactose*β1-4*N-acetylglucosamine* (Galα1-3Galβ1-4GlcNAc-R) is highly restrictive to the non-peptidergic population of C-PAFs (Silverman, J.D, Kruger, L. 1990). However, there is some contention regarding the selectivity of IB4-binding as a marker for non-peptidergic afferents, as several studies suggest a moderate degree of co-expression with neuropeptides such as sP and CGRP as described in the Introduction (Price, T.J and Flores, C.M., 2007). To address the

issue regarding the potential for IB4-Saporin to unintentionally lesion peptidergic C-PAFs, we have examined neuropeptide immunoreactivity within the dorsal root ganglion and in the superficial dorsal horn with careful observation of the presence of intact peptidergic afferent terminals. The most substantiating evidence supporting the specificity of the lesion comes from the observation of the diminution in sP and CGRP immunoreactivity in the dorsal horn at two and seven weeks post lesion respectively. The differential decline in these two neuropeptides, known to be co-stored in the same synaptic vesicle is demonstrative of a compensatory mechanism in peptide synthesis, storage and release and not the loss of peptidergic sensory terminals (Tuchscher, M.M. and Seybold, V.S, 1989; Plenderleith, M.B., Haller, C.J., Snow, P.J., 1990; Ribeiro-da-Silva, A., 1995). This diminution in sP-immunoreactivity, notable at the two week time point, occurs in parallel with the degeneration of IB4-binding sensory neurons within the DRG. Thus, the decline in sP-immunoreactivity in peptidergic sensory neurons, reflecting a possible reduction in peptide levels, may be induced by signalling from moribund IB4-binding neurons. In models of spinal nerve ligation, alterations in expression profiles in uninjured neurons residing in adjacent DRGs have been observed (Fukuoka, T., Kondo, E., Dai, Y., Hashimoto, N., Noguchi, K., 2001; Obata, K., Yamanaka, H., Fukuoka, T., Yi, D., Tokunaga, A., Hashimoto, N., Yoshikawa, H., Noguchi, K., 2003). Furthermore, DRG satellite and glial cells, activated following nerve injury are known to release a number of factors which may influence protein expression in sensory neurons (Jimenez-Andrade, J.M., Peters, C.M., Mejia, N.A., Ghilardi, J.R., Kuskowski, M.A, Mantyh, P.W., 2006; Chao, T., Pham, K., Steward, O., Gupta, R., 2008). Thus, the decline in sP-immunoreactivity may be mediated by signals released from apoptotic IB4-positive neurons or activated glial profiles within the DRG. It may be possible that transient upregulation of neuropeptide levels occurred prior to the down-regulation observed. Thus, examination of sP and CGRP-immunoreactivity at several intervals post-lesion is warranted. To further ensure the specificity of the lesion, we have collected fresh tissue samples of spinal cord, nerve and dorsal root ganglion for future western blot processing. In order to gain a better understanding of the mechanisms involved in the reduction in

neuropeptide levels observed, examination of mRNA levels of sP and CGRP using *in situ* hybridization will provide valuable information pertaining to the regulation of protein expression following nerve lesion. In addition, examination of C-PAF terminals within the marginal zone and substantia gelatinosa at the ultrastructural scale will provide solid evidence of a targeted lesion restricted to the IB4-binding population of C-fibre.

Section 5.3

"Form and function are a unity, two sides of one coin. In order to enhance function, an appropriate form must exist or be created."
Ida P. Rolf, PhD (1896-1979).

The evolutionary biologist and essayist Stephen Jay Gould, in his book entitled the "*The Flamingo's Smile: Reflections in Natural History"*, discusses the salient issues regarding form and function in nature (Gould, S.J., 1985). Arguing the order in which these two properties may have developed, Gould uses the unique nature of the flamingo's beak to illustrate his point. The structure of the flamingo's beak, in relation to the beaks of other birds is upside-down. Gould questioned whether the flamingo's beak was initially structured this way, and thus the creature compensated by turning its head upside-down when feeding, or did the flamingo develop this bizarre eating habit for which evolution compensated, producing the upside-down beak (Gould, S.J., 1985)

Some may argue that form begets function (Meinertzhagen, I.A., Takemura, S.Y., Lu, Z., Huang, S., Gao, S., Ting, C.Y., Lee, C.H., 2009). This certainly appears to be the case when examining the formation of the sub-populations of C-PAFs. The developmental divergence of a ubiquitous population of small diameter sensory afferents into two neurochemically defined subtypes, each with distinct morphological and physiological properties is highly suggestive of function-based system organization.

Several studies have attempted to discern the functional role of IB4-binding, non-peptidergic afferents in the transmission of noxious stimuli to the spinal cord.

Notwithstanding a vast array of histochemical, electrophysiological and pharmacological approaches, the involvement of this neuronal population in nociception remains elusive.

The unique structural organization of the central terminals of non-peptidergic afferents into synaptic glomeruli, specifically the presence of pre-synaptic inhibitory contacts from local GABAergic interneurons, suggests that these afferents are involved in signal regulation (Ribeiro-da-Silva, A., Coimbra, A., 1982; Ribeiro-da-Silva, A., Pignatelli, D., Coimbra. A., 1985; Todd AJ, Sullivan AC., 1990; Todd, A.J., Russell, G., Spike, R.C. 1992). Quantitative analysis of lamina II synaptic glomeruli and the neurochemical identity of axonal and dendritic synaptic neighbours confirmed the presence of GABA in pre-synaptic dendrites and axons (Todd, AJ, 1988; Ribeiro-da-Silva, A., 2004). Further studies suggest that islet cells, characterized by their fusiform somata and profuse dendritic arbours oriented in the rostral-caudal plane are the likely source of the presynaptic inhibitory dendrites (Gobel, S., 1975; Barber, R.P., Vaughn, J.E., Saito, K., McLaughlin, B.J., Roberts, E. 1978; Cervero, F., Iggo, A., 1980; Bennett, G.J., Abdelmoumene, M., Hayashi, H., Dubner, R., 1980; Todd, A.J., Lewis, S.G. 1986; Todd, AJ, 1988; Todd, A.J., Lochhead, V., 1990).

The presence of presynaptic inhibitory connections with the central bouton of type Ia synaptic glomeruli is highly suggestive of an anatomical system of signal regulation or modulation. However, it may be hypothesized based upon the neuroanatomical evidence supporting a role of nociceptive signal modulation and regulation, that neurons within lamina II are responsible for maintaining signal intensity within an acceptable range. A study published in the *Journal of Pharmacology and Experimental Therapeutics* (Zhou, H.Y., Zhang, H.M., Chen, S.R. and Pan, H.L., 2008) demonstrated that following the administration of capsaicin, increased primary afferent activity in lamina II resulted in the potentiation of glycine-mediated inhibitory transmission evinced by the reduction of high intensity EPSCs (Zhou et al., 2008). The loss of such inhibitory tone may result in a shift towards enhanced excitation. In a study conducted by the same group, bath application of capsaicin on lamina II neurons produced a differential effect on spontaneous IPSCs; nearly half of the neurons evaluated exhibited a

decrease in sIPSCs whereas approximately 35% of examined neurons displayed an increase in sIPSC frequency (Zhou, H.Y, Zhang, H.M., Chen, S.R. and Pan, H.L., 2007). Antagonists of ionotropic and metabotropic glutamate receptors suggested the involvement of capsaicin-mediated release of glutamate from primary afferent terminals (Zhou et al., 2007).

Neurons within lamina II exhibit profuse, rostral-caudally oriented dendritic arbours with minimal branching in the dorsal-ventral plane (Bennett, G.J., Abdelmoumene, M., Hayashi, H., Dubner, R., 1980; Todd, A.J., Lewis, S.G., 1986; Todd A.J., 1988). However a number of studies have demonstrated that neurons located in laminae ventral to the SG (e.g. in lamina III) issue dorsally directed dendrites which engage, in a pre- and post-synaptic fashion, the central varicosity of type Ia synaptic glomeruli (Todd, A.J., Lewis, S.G. 1986; Todd, A.J. 1988). Wide dynamic range (WDR) neurons located in lamina V of the deep dorsal horn issue dorsally directed dendrites which penetrate the ventral border of lamina II and have been proposed to establish contact with primary afferent boutons within this region (Woolf, C.J., King, A.E., 1987). As electrophysiological studies have shown, WDR neurons resident to lamina V respond to C-PAF input indicative of either a direct or indirect connection with C-fibre afferents (Woolf, C.J., King, A.E., 1987). Morphological studies conducted by Grudt, T.J and Perl, E.R. (2002) in the hamster spinal cord proposed that islet cells which establish communication with primary afferent boutons engage central (glutamatergic) cells (Grudt, T.J., Perl, E.R., 2002). A rudimentary outline of the synaptic circuitry of lamina II (Lu,Y.,Perl, E.R. 2003) suggested that islet cells interact with central cells of the SG which establish synaptic contacts with vertical neurons located in the outer region of LII (Lu,Y.,Perl, E.R., 2003)**.** Despite neuroanatomical evidence confirming connections between lamina II neurons and neurons resident to the deep dorsal horn as well as those localized to lamina I (Light, A.R., Kavookjian, A.M., 1988; Lu, Y., Perl, E.R., 2005), the cellular identity of these connections have yet to be determined.

In a recent retrograde tracing study using adenoviral vector containing a fusion protein inserted between green fluorescence protein (GFP) and the non-toxic fragment C of tetanus toxin (TTC). Injection of the GFP-TTC fusion protein into the parabrachial nucleus labelled spino-parabrachial lamina I neurons in the marginal zone which displayed ventrally directed dendrites penetrating the lamina I/II border (Cordero-Erausquin, M, Allard, S., Dolique, T., Bachand, K., Ribeiro-da-Silva, A., De Koninck, Y., in press). Of particular interest was the trans-synaptic transfer of the GFP-TTC vector to pre-synaptic stalked cells located in LII and the ventral border of LI (Codero-Erausquin, M., et al., submitted). The results of this study demonstrate that some LI spino-parabrachial neurons are in direct communication with LII neurons, being postsynaptic to them.

Recent morphological data collected in our lab shows the presence of a few IB4-binding afferent terminals in close appostion to NK1-receptor expressing neurons within LI (Saeed A, and Ribeiro-da-Silva, manuscript in preparation). These data advocate a more direct line of communication between the peptidergic-LI pathway and the non-peptidergic-LII pathway: a connection with profound implications on signalling within the dorsal horn given the preponderance of NK1-receptor expressing neurons projecting to supraspinal targets. Furthermore, as the data in this thesis has shown, compensatory changes induced in the peptidergic-LI pathway following the selective ablation of IB4-binding afferents may be the consequence of this interaction.

To fully understand the value of the information conveyed by non-peptidergic afferents, their postsynaptic connections in the superficial and deep dorsal horn and their interrelations with their peptidergic C-PAF counterpart, we have examined the effect of compromised C-PAF input to the superficial dorsal horn in an animal model of peripheral nerve injury. Furthermore, we have evaluated the effects of the selective loss of non-peptidergic C-fibres on nociceptive behaviours as well as on neurochemical markers for pre- and postsynaptic neuronal populations.

Section 5.4

"Think simple, as my old master used to say, meaning reduce the whole of its parts into the simplest terms, getting back to first principles."
Frank Lloyd Wright (1867-1959).

The peripheral nerve lesion study was aimed at examining the time-related effects of a cuff-induced constriction neuropathy on the peptidergic and non-peptidergic C-PAFs using neurochemical markers for each neuronal population. The results of our study, focused on IB4-binding and CGRP-immunoreactivity in the superficial dorsal horn over the course of 3 weeks post-lesion induction at the L4-L5 lumbar spinal cord region. Observations of IB4-binding PAF terminals in lamina II revealed the transient loss of IB4-binding resultant from the degeneration of Type Ia synaptic glomeruli (Bailey, AL and Ribeiro-da-Silva, A., 2006). In addition, we evaluated paw withdrawal thresholds in these animals to peripherally applied noxious heat and innocuous mechanical and cold stimuli. Specifically, paw withdrawal latencies to noxious heat stimuli were significantly reduced in neuropathic animals throughout the 3 week time course, observable by the 5th post-operative day. Sensitivity to innocuous cold stimuli (acetone spray) was also significantly altered in neuropathic animals; neuropathic rats displayed heightened sensitivity to the application of acetone to the plantar surface of the neuropathic paw. Furthermore, application of acetone to the uninjured paw elicited a withdrawal response in the neuropathic paw denoted as a crossed withdrawal reflex, a phenomenon hypothesized to be caused by sensitized afferents which cross the midline of the spinal cord (Sotgiu, M.L., Brambilla, M., Valente, M., Biella, G.E., 2004). Paw withdrawal thresholds to innocuous and noxious mechanical stimulation (von Frey filaments) were significantly reduced in neuropathic animals. At low threshold stimulation (4 grams of force), neuropathic animal demonstrated a significant increase in the percentage of withdrawal events by the 10th post-operative day, remaining high throughout the course of the 3 week time period. Similarly, responses to 8 and 15 grams of force

were significantly increased by the 7th and 10th day post-lesion respectively and persisted throughout the remainder of the experimental time period.

Probative analysis of CGRP-immunoreactivity in the initial 3 weeks following cuff application revealed an absence in any change in detectable density of labelled varicosities within the superficial spinal dorsal horn (L4-L5) (Bailey, A.L., Ribeiro-da-Silva, A.., 2006). However, during the same time interval, preliminary data indicate a decline in sP-immunoreactivity at the L5-L6 spinal level (data not shown). Ultrastructural examination of glomerular and non-glomerular peptidergic boutons at the L4-L5 lumbar level showed no apparent signs of central terminal atrophy (i.e. synaptic dissolution, clustered synaptic vesicles, vacuolated mitochondria) (Bailey, A.L., Ribeiro-da-Silva, A.., 2006). Thus the differential expression of these key neuropeptides was not the corollary of the degenerative decay of peptidergic PAF boutons or the over-active release of peptide-containing synaptic vesicles, but speciously caused by injury-prompted changes in peptide synthesis and storage in dense core vesicles. As sP and CGRP are colocalized within the same synaptic vesicles (Plenderleith, M.B., Haller, C.J., Snow, P.J. 1990; Ribeiro-da-Silva A., 1995), over-active release of endocytotic pools of neuropeptides in central terminal varicosities would presumably result in the diminution of appreciable levels of both peptides simultaneously

5.4.1 The regenerative capacity of primary afferent C-fibers.

Early studies which examined the effects of peripheral nerve injury on thiamine monophosphatase reactivity (TMPase) and FRAP enzymatic activity demonstrated a transitory loss of FRAP reactivity in the dorsal horn following a crush injury to the sciatic nerve (Csillik B, Knyihár-Csillik E., 1981; Knyihár-Csillik E, Csillik B., 1981; Tenser, 1985; Knyihár-Csillik E, Kreutzberg GW, Csillik B., 1989). Both enzyme markers for afferent terminals located in the SG were significantly depleted within days of the initial injury followed by a restoration in reactivity occurring within several weeks (Csillik, B., et al., 1982). Repeated crushing of the sciatic nerve following the restoration of TMPase or

FRAP reactivity resulted in a similar loss of either marker within the area of replenished activity suggesting that the observed histochemical restoration likely occurred in the indigenous TMPase/FRAP population and did not reflect ectopic sprouting from adjacent unimpaired axon terminals (Csillik B, Knyihár-Csillik E., 1981; Csillik, B. Knyihár-Csillik, E and Tajti, J., 1982). Observations of the temporo-spatial patterns of enzyme depletion and restoration revealed a caudal-rostral and medial-lateral pattern of regenesis. Similar to the results reported by Knyihár-Csillik, E and Csillik, B., IB4-binding in the SG recovered following an initial period of substantial depletion (Bailey, A.L., Ribeiro-da-Silva, A., 2006).

The regenerative capacity of injured peripheral nerves has been examined at length (Kemp SW, Walsh SK, Midha R., 2008). Several studies have examined the regenerative capacity of damaged peripheral nerves in the presence of exogenous growth factors (Csillik, B. Schwab, M.E and Thoenen, H., 1985; Bennett, D.L., Koltzenburg, M., Priestley, J.V., Shelton, D.L., McMahon, S.B., 1998; Lykissas, M.G., Batistatou, A.K., Charalabopoulos, K.A., Beris, A.E., 2007). The significant degenerative atrophy of CI_a glomerular boutons observed following the application of the polyethylene cuff and their subsequent restoration suggests that these afferents are capable of post-injury regeneration.

Cell death within the dorsal root ganglion has been reported following peripheral nerve injury (crush, transection) (Groves, M.J., Schänzer, A., Simpson, A.J., An, S.F., Kuo, L.T., Scaravilli, F., 2003; Chew, D.J., Leinster, V.H., Sakthithasan, M., Robson, L.G., Carlstedt, T., Shortland, P.J., 2008). Following nerve injury, growth factors such as NGF and GDNF are altered (Nagano, M., Sakai, A., Takahashi, N., Umino, M., Yoshioka, K., Suzuki, H., 2003). Examination of GDNF levels in the L4 and L5 DRG in the days following CCI showed a substantial decrease within 7 days after injury, remaining low until for an additional 7 days (Nagano, M., Sakai, A., Takahashi, N., Umino, M., Yoshioka, K., Suzuki, H., 2003). Similarly, C-Ret expression was markedly reduced in the ipsilateral L4 and L5 DRGs at both 7 and 14 days post-CCI (Nagano, M., Sakai, A., Takahashi, N., Umino, M., Yoshioka, K., Suzuki, H., 2003). Application of GDNF has been shown to prevent the loss of IB4-binding central terminals in the superficial dorsal horn and prevent the increase in pain

sensitivities when supplied intrathecally in the CCI model of neuropathic pain (Bennett, D.L., Koltzenburg, M., Priestley, J.V., Shelton, D.L., McMahon, S.B., 1998; Nagano, M., Sakai, A., Takahashi, N., Umino, M., Yoshioka, K., Suzuki, H., 2003). This decline in GDNF and its respective signalling molecules may contribute to the degeneration of the central terminals of IB4-binding, GDNF-dependent C-PAFs noted in our study and observed by others (Krekoski, C.A., Parhad, I.M., Clark, A.W., 1996; Kashiba, H., Hyon, B., Senba, E., 1998)

Inhibition of retrograde axoplasmic transport of NGF induced by vinpocetine, a derivative of vincamine (a vinca alkaloid) produced marked transganglionic degenerative atrophy of the central terminals of primary afferent fibres in the superficial dorsal horn (Knyihar-Csillik, E., Vecsei, L., Mihaly, A., Fenyo, R., Farkas, I., Krisztin-Peva, B., Csillik B., 2007; Csillik, B., Mihály, A., Krisztin-Péva, B., Farkas, I., Knyihár-Csillik, E., 2008). The results of these studies demonstrate the impact of compromised axon transport on the viability of primary afferent terminals and the effect of trophic factor deprivation mediated by the impairment of axoplasmic transport.

The mechanisms underlying afferent regeneration have been studied *in vitro and in vivo*. Growth factor support supplied by NGF for the peptidergic subclass of C-PAFs and GDNF for the non-peptidergic respectively and their ability to promote neurite outgrowth have been investigated (Tucker, B.A., Rahimtula, M., Mearow, K.M., 2006; Leclere, P.G., Norman, E., Groutsi, F., Coffin, R., Mayer, U., Pizzey, J., Tonge, D., 2007). According to the data reported by Tucker and colleagues (2006), Laminin, a significant component of the extracellular matrix is sufficient at eliciting neurite outgrowth in peptidergic (CGRP-expressing) DRG neurons in the absence of NGF yet is unable to induce the same process in IB4-binding neurons which required further support from GDNF (Tucker, B.A., Rahimtula, M., Mearow, K.M., 2006). Similar observations by Leclere and colleagues (2007) suggested that the regenerative capability of IB4-binding neurons may be heavily dependent on GDNF. Delayed upregulation in GDNF and C-Ret in the DRG, initiated in late periods following nerve injury, in addition to the increased levels of the neurotrophin in Schwann cells located at the site of

injury may provide adequate growth factor support permitting regenesis to occur within the superficial dorsal horn. The early upregulation of GDNF receptor components following peripheral nerve injury is thought to pre-condition GDNF-receptor expressing neurons to the regenerative properties of the neurotrophin, increasing neurite outgrowth of cultured (injured) DRG neurons in response to exogenously applied GDNF (Mills, C.D., Allchorne, A.J., Griffin, R.S., Woolf, C.J, Costigan, M., 2007).

Studies investigating the neurochemical classification of apoptotic neurons in the DRG following peripheral nerve injury are subject to injury-induced phenotypic shifts in neurotransmitter and neuropeptide expression, resulting in aberrant estimations of the characterization of apoptotic populations. Thus a clear profile of the susceptibility of certain classes of sensory neurons to cell death induced by axon trauma is still under investigation. It is possible that particular neuronal populations are more likely to undergo degeneration due to deprivation of neurotrophic support caused by impaired axon transport and nerve injury mediated changes in growth factor levels as well as their receptors (Csillik, B., Mihály, A., Krisztin-Péva, B., Farkas, I., Knyihár-Csillik, E., 2008). Point in fact, complete nerve transection resulted in the differential alteration in the levels of TRKA and GFRα receptor mRNA in the ipsilateral DRG, producing a marked increase in GFRα with no change in TRKA gene expression (Kashiba et al., 1998).

5.4.2 The differential effect of the type of peripheral nerve injury on non-peptidergic C-fibre terminal recovery

The restoration of histochemical enzyme markers occurred within several weeks following the initial injury (Csillik, B. Knyihár-Csillik, E and Tajti, J., 1982). In these studies, the nerve injury model consisted of crushing the nerve with jeweller's forceps (Csillik B, Knyihár-Csillik E., 1981; Knyihár-Csillik, E., Török, A., 1989). As different methods of peripheral nerve injury induce varying degrees of axon damage, the duration of the loss and the onset and extent of the restoration of IB4-binding may differ significantly from one model to another due to the number and degree of axon damage inflicted (Mazzer, P.Y., Barbieri ,C.H.,

Mazzer, N., Fazan, V.P., 2008). Morphometric analyses of the extent of axon damage induced by peripheral nerve trauma and the degree of the pursuant central terminal degeneration indicate that the severity of the disruption in axon transport plays an important role in the extent of central terminal atrophy and regeneration (Csillik, B., Knyihár, E., Jójárt, I., Elshiekh, A.A., Pór, I., 1978; Csillik, B., Mihály, A., Krisztin-Péva, B., Farkas, I., Knyihár-Csillik, E., 2008). Ligation or crush injury of a peripheral nerve resulted in a localized trauma to axons (Mosconi T, Kruger L, 1996; Mazzer, P.Y., Barbieri, C.H., Mazzer, N., Fazan, V.P., 2008) which included localized demyelination, Wallerian degeneration and compromised axon transport. As previously mentioned, axon degeneration may be further enhanced by the deprivation of trophic factors such as NGF resultant of compromised axon transport (Koike, T., 2008). Examination of IB4-binding following nerve crush or transection demonstrated that notwithstanding a similar time course for the initial decline in lectin binding, restoration was a protracted event following transection ,taking up to 8 months to reach near baseline levels compared to that of crush (Molander, C., Wang, H.F., Rivero-Melián, C., Grant, G., 1996).

Compression, ligation or crush of a peripheral nerve produces a regionalized trauma to the underlying axons up to several millimetres in length. Such compression or constrictive injuries produce varying degrees of impairment (Mazzer, P.Y., Barbieri ,C.H., Mazzer, N., Fazan, V.P., 2008). Unmarred fibres have been shown to undergo regenerative sprouting, presumably caused by the production of trophic factors synthesized and released by damaged Schwann cells (Obata, K., Yamanaka, H., Fukuoka, T., Y,i D., Tokunaga, A., Hashimoto, N., Yoshikawa, H., Noguchi, K, 2003). Retrograde axonal transport, albeit hindered, may permit the flow of target-derived trophic factor support demonstrated by the observed accumulation of GDNF at the distal region of the constricted nerve (Holstege, J.C., Jongen, J.L., Kennis, J.H., van Rooyen-Boot, A.A., Vecht, C.J., 1998; Ohta, K., Inokuchi, T., Gen, E., Chang, J., 2001). Moreover, application of the restrictive devices, such as synthetic polymer tubing (polyethylene, silastic) or sutures composed of natural (gut) or manmade (nylon) materials promote immunological responses which may contribute to the pathological processes

observed in these models (Maves, T.J., Pechman, P.S., Gebhart, G.F., Meller, S.T., 1993). In contrast, transection of a peripheral nerve results in a localized insult to all axon fibres within the affected nerve. Axon transport from peripheral target tissues is no longer feasible which may account for the delayed upregulation of growth factors, as peripherally derived trophic factors are unable to bridge the gap.

Section 5.5: The Selective ablation of IB4-binding afferents.

The intra-sciatic injection of IB4-Saporin resulted in a time-dependent loss of IB4-binding neurons in the DRG as well as their central terminals within the superficial dorsal horn. By the 14^{th}post-operative day, IB4-binding and $P2X_3$-immunoreactivity within the SG territory corresponding to the central terminals of sciatic nerve were absent. This loss in IB4-positive terminals was still observed at 7 weeks post-injection and is presumed to be permanent. Examination of the withdrawal thresholds to a variety of acute innocuous and noxious stimuli (heat, cold and mechanical) revealed an absence of an effect on acute pain behaviours. Furthermore, evaluation of nociceptive behaviours following acute inflammation induced by an intraplantar administration of complete Freund's adjuvant also demonstrated the lack of an effect on enhanced sensitivities caused by the adjuvant administration. Similarly, intraplantar formalin treatment behaviours were unaffected by IB4-SAP treatment. However, IB4-SAP treatment effectively blocked capsaicin-induced heat hyperalgesia at both 30 and 60 minutes following intraplantar capsaicin.

Fourteen days following IB4-SAP administration, sP-immunoreactivity was markedly reduced in lesion areas of the superficial dorsal horn accompanied by an augmentation in NK1 receptor immunoreactivity in lamina I. Presumably the decline in available sP within the synaptic milieu is responsible for the up-regulation of its receptor in an effort to maintain signalling. Proposed mechanisms for such low levels of sP within lamina I of the dorsal horn included 1) non-specific lesioning of peptidergic primary sensory neurons, 2) increased release of

synaptic pools of neuropeptides within central terminal varicosities or 3) compensatory changes in synthesis and storage of the tachykinin. Examination of CGRP-immunoreactive DRG neurons at the 14 day time point excluded the first possibility as levels of CGRP-immunoreactive neurons were abundant and comparable to contralateral and sham controls. As previously mentioned, both neuropeptides are co-stored in large dense core vesicles located within the en passant boutons of peptidergic afferents. Non-specific lesioning of peptidergic sensory neurons would presumably produce a concomitant decline in both neuropeptides eliminating the possibility that collateral damage could account for the diminutive levels of sP. Furthermore, an over-active synaptic release of endocytotic pools of dense core vesicles laden with both sP and CGRP would ostensibly produce a concomitant decrease in detectable levels of both peptides within the dorsal horn. Three weeks following IB4-SAP treatment, CGRP-immunoreactivity remained comparable to control levels. However, at 7 weeks post-injection, CGRP-immunoreactivity markedly declined further emphasising the differential effect of IB4-SAP on neuropeptide expression.

Examination of sP and CGRP synthesis, storage and release in an adjuvant model of inflammation demonstrated a differential decline in sP and CGRP immunoreactivity levels in the spinal cord as determined by radioimmunoassay (RIA) (Galeazza, M.T., Garry, M.G., Yost., H.J., Strait, K.A., Hargreaves, K.M., Seybold, V.S., 1995). Specifically, levels of sP declined in the early stages of inflammation (6 hours post-CFA and remained low until the 8^{th} day following CFA treatment. In contrast, levels of CGRP declined 2 days after the induction of inflammation, declining further by the 6^{th} day, followed by a resurgence in CGRP levels beyond control levels by day 8 Galeazza, M.T., Garry, M.G., Yost., H.J., Strait, K.A., Hargreaves, K.M., Seybold, V.S., 1995). Furthermore, quantification of neuronal (DRG) expression of pre-protachykinin and pre-proCGRP mRNA levels demonstrated that mRNA levels increased in the DRG prior to the restoration in immunoreactivity levels in the spinal cord. Analysis of peptide release in the spinal cord by RIA of spinal cord superfusate revealed that basal levels of both neuropeptides remained unchanged over the 8 day examination period following CFA treatment, however stimulated release by administration of

capsaicin revealed that the quantity of sP released from afferent terminals in the spinal cord were reduced at 6 hours and 8 days whilst the amount of CGRP released by capsaicin increased at day 4 (Galeazza, M.T., Garry, M.G., Yost., H.J., Strait, K.A., Hargreaves, K.M., Seybold, V.S., 1995). These data support the notion that the synthesis and storage of sP and CGRP are differentially regulated in the setting of inflammation.

The expression levels of the genes encoding for sP and CGRP expression, preprotachykin-A (ppt-A) and α-CGRP respectively have been examined following peripheral nerve injury and in the setting of inflammation using *in situ* hybridization techniques (Henken, D.B., Battisti, W.P., Chesselet, M.F., Murray, M., Tessler, A., 1990; Marchand, J.E., Wurm, W.H., Kato, T., Kream, R.M., 1994; Ma,W., Bisby, M.A., 1998; Bulling, D.G., Kelly, D., Bond, S., McQueen, D.S., Seckl, J.R., 2001). Chronic constriction injury produced time-dependent changes in pptA mRNA levels; ppt-A mRNA expression in the DRG increased in the days immediately following nerve injury, yet declined at later time points (Marchand, J.E., Wurm, W.H., Kato, T., Kream, R.M., 1994). In contrast, sciatic nerve transection produced a significant (70%) decrease in ppt-A mRNA expression by the 3rd post-operative day, with an 80% over all decline in expression noted at two weeks (Henken, D.B., Battisti, W.P., Chesselet, M.F., Murray, M., Tessler, A., 1990). Interestingly, the decline in sP-expression did not match temporally with the drop in mRNA, peaking at a 50% loss in expression levels at the two week time point. Six months following transection, peptide and messenger RNA levels returned to control levels (Henken, D.B., Battisti, W.P., Chesselet, M.F., Murray, M., Tessler, A., 1990). In contrast, partial nerve injury or CCI did not affect either mRNA or sP peptide expression levels in injured DRG, however a marked increase in sP-expression was noted in spared DRGs following the induction of either model (Ma,W., Bisby, M.A., 1998).

Chronic adjuvant-induced inflammation of the tibio-tarsal joint produced profound and immediate increases in both ppt-A and α-CGRP mRNA levels (Bulling, D.G., Kelly, D., Bond, S., McQueen, D.S., Seckl, J.R., 2001). Moreover, the proportion of small diameter neurons in the ipsilateral DRGs expressing both

neuropeptide genes increased significantly indicating an induction in gene expression in a novel pool of small diameter neurons (Bulling, D.G., Kelly, D., Bond, S., McQueen, D.S., Seckl, J.R., 2001). Given the pro-inflammatory and vasodilatory properties of sP and CGRP respectively, such dramatic increases in their expression levels in the setting of chronic joint inflammation is expected.

These studies further exemplify how different models of chronic neuropathy differentially influence the expression of neuropeptides. Furthermore, they demonstrate the effects of nerve injury on gene expression.

5.5.1 Compensatory changes in the peptidergic C-Fibre subpopulation following IB4-Saporin treatment.

The underlying mechanisms responsible for the alterations in sP and NK1-immunoreactivity following the loss of IB4-binding afferents are speculative. The compensatory changes in neuropeptide expression levels and of their receptors (NK1-receptor) occurring in the absence of non-peptidergic sensory input to the superficial dorsal horn are suggestive of an interrelationship between the peptidergic and non-peptidergic C-fibre subpopulations.

Further characterization in our laboratory of the morphological classification of lamina I neurons which demonstrated enhanced NK1 receptor immunoreactivity in the IB4-Saporin model revealed that both multipolar and fusiform neurons demonstrated enhanced immunoreactivity, however, pyramidal neurons did not, maintaining their phenotype as NK1-negative in most cells (Saeed, A.W., et al., manuscript in preparation). This over-expression of the sP receptor in response to nerve injury is not uncommon (Allen, A.L., Cortright, D.N., McCarson, K.E., 2003; Taylor, B.K., McCarson, K.E., 2004). Specifically, in a mouse model of nerve injury (partial sciatic nerve ligation), mRNA levels for the NK1-receptor were significantly enhanced in the dorsal horn ipsilateral to the nerve injury (Taylor, B.K., McCarson, K.E., 2004).

The presence of increased numbers of cell surface receptors may not necessarily imply an increase in signalling capacity. As the NK1-receptor is of the G-protein coupled receptor family (GPCR), the functionality of these receptors may be verified using $GTP\gamma S^{35}$ assays. Such studies have been conducted in

inflammatory models of persistent pain where up-regulated NK1-receptors are frequently observed. Intraplantar formalin produced a significant increase in NK1-receptor mRNA in the dorsal horn within hours following administration, lasting several days (Winter, M.K., McCarson, K.E., 2005). Over the course of several hours to several days, the number of available cell surface NK1 receptors waxed and waned, eventually returning to baseline levels. Despite the marked increase in NK1-receptor mRNA, $GTP\gamma S^{35}$ assays demonstrated a decrease in the functionality of cell surface receptors over the course of the study. The early decline in receptor expression and functionality may be attributed to formalin-induced internalization of the NK1-receptor, further suggesting that internalized receptors are not coupled to their G-protein signalling molecules. The persistent paucity of functionally coupled receptors, notwithstanding the increased (2-fold) levels of NK1-receptor mRNA raises a salient issue regarding receptor up-regulation; increased numbers of available membrane bound receptors does not necessarily imply an increase in functional signalling. However, authors note that despite the decline in G-protein coupled receptors, membrane-bound NK1-receptors exhibited an increase in affinity for sP agonist suggesting that compensatory mechanisms in receptor availability, functionality and sensitivity, exemplifying natural homeostatic approaches to maintain baseline signalling properties in the setting of chronic inflammation (Winter, M.K., McCarson, K.E., 2005).

This study demonstrates the extent of compensatory alterations in several aspects of nociceptive signalling that may occur in the setting of acute or chronic pain syndromes. The decline in sP-immunoreactivity following IB4-SAP treatment combined with the increased expression of NK1-receptors on lamina I neurons are indicative of a system of combined primary afferent input to the dorsal horn and postsynaptic spinal cord neurons working to normalize aberrant neuroanatomical changes such that nociceptive processing can resume with as little disruption as possible.

5.5.2 The consequence of compromised IB4-binding C-fibres input to the superficial dorsal horn on postsynaptic neurons: implications on inhibitory transmission in the spinal dorsal horn.

Examination of IB4-binding neurons within the L5 and L6 DRG confirmed the loss of non-peptidergic sensory neurons following IB4-SAP treatment. The loss of a significant proportion of C-PAF afferent would presumably culminate in marked abnormalities in nociceptive signalling and would lead to altered behavioural responses to peripherally applied noxious stimuli, however, behavioural analysis of IB4-SAP treated animals demonstrated that in the absence of IB4-binding afferent input to the dorsal horn, nociceptive behaviours to a wide variety of acute stimuli did not differ from control animals.

The degeneration of the primary afferent bouton following IB4-SAP treatment would presumably produce profound effects on postsynaptic targets. Indeed, deafferentation has been shown to result in what has been termed "trans-synaptic degenerative atrophy" which may lead to cell death induced by the loss of afferent input to postsynaptic neurons (Sugimoto T, Gobel S., 1984; Sugimoto, T., et al, 1984, 1985, 1987a, 1987b; Castro-Lopes, J.M., Tavares, I., Coimbra, A., 1993; Whiteside, G.T., Munglani, R., 2001). In previous studies, chronic constriction injury produced significant, bilateral decreases in GABA and GAD-immunoreactivity within the superficial dorsal horn within days following injury (Eaton MJ, Plunkett JA, Karmally S, Martinez MA, Montanez K., 1998); confirming a previous study which reported bilateral decreases in both GABA and GAD-immunoreactivity in the same model of peripheral nerve injury (Ibuki, T., Hama, A.T., Wang, X.T., Pappas, G.D., Sagen, J., 1997).

In a model of peripheral nerve injury (CCI), apoptotic cells were observed in the dorsal horn 8 and 14 days after injury (Whiteside, G.T., Munglani, R., 2001). Evaluation of inhibitory postsynaptic currents (IPSCs) in three models of nerve injury (complete sciatic nerve transection (SNT), CCI and spared nerve injury (SNI)), demonstrated a significant, model-related reduction in several properties of PAF-evoked IPSCs, including magnitude, duration and frequency (Moore, K.A., Kohno, T., Karchewski, L.A., Scholz, J., Baba, H., Woolf, C.J., 2002). TUNEL assays confirmed the presence of apoptotic cells in the superficial dorsal

horn in addition to marked decline in GAD- immunoreactivity of the 65 kilobase isoform of the GABA synthetic enzyme (GAD_{65}) (Moore, K.A., Kohno, T., Karchewski, L.A., Scholz, J., Baba, H., Woolf, C.J., 2002). Examination of the neurochemical properties of the IPSCs generated by PAF stimulation revealed that following partial sciatic nerve injury, compound GABA and glycine-mediated IPSCs observed in naïve animals converted to primarily glycinergic IPSCs, a phenomenon demonstrated to be the result of the loss of $GABA_A$ receptor mediated signalling in lamina II postsynaptic neurons (Moore, K.A., Kohno, T., Karchewski, L.A., Scholz, J., Baba, H., Woolf, C.J., 2002). Notwithstanding the number of reported cases of cell death within the superficial dorsal horn, published data to the contrary has raised some doubt as to whether such cell death occurs. Using stereological counting methods to determine whether there is a significant loss of dorsal horn neurons within the spinal cord following CCI, the number of neurons labelled with the nuclear marker NeuN (a neuronal nuclei specific label) in the ipsilateral spinal dorsal horn were not significantly different compared to contralateral counts, nor when compared to sham operated animals (Polgár, E., Gray, S., Riddell, J.S., Todd, A.J., 2004).

The mechanisms underlying injury-induced neuronal death are speculative. However studies have confirmed the involvement of the inhibitory neurotransmitter glycine. Administration of the glycine receptor antagonist strychnine has been shown to enhance trans-synaptic degenerative atrophy of cells in the medullary spinal cord following transection of the rat inferior alveolar nerve whereas application of the $GABA_A$-receptor antagonists, bicuculline or picrotoxin did not produce the same effect (Sugimoto, T., et al., 1985, 1989).

It has been hypothesized that enhanced excitatory signalling by damaged primary afferent terminals may induce excitotoxic cell death in postsynaptic neurons mediated by augmented release of afferent-derived glutamate. Several studies have documented altered inhibitory signalling in animal models of chronic neuropathy and have noted substantial changes in excitatory and inhibitory tone, resulting in a shift towards enhanced excitation (Scholz, J., Broom, D.C., Youn, D.H., et al., 2005). Specifically, neuronal loss observed in laminas I-III in three animal models of nerve injury (SNI, CCI and spinal nerve ligation (SNL) was the

result of ongoing glutamatergic activity in damaged primary afferent terminals which resulted in activation of caspase-mediated neuronal degeneration (Scholz, J., Broom, D.C., Youn, D.H., et al., 2005). Further investigation revealed that a decline the number GABAergic neurons coincided with a significant decrease in IPSCs in lamina II (Scholz, J., Broom, D.C., Youn, D.H., et al., 2005). Ergo, the observed decrease in GAD-immunoreactivity in the spinal cords of IB4-Saporin treated animals may be indicative of a loss of inhibitory (islet) cells in the SG. The loss of primary afferent input to lamina II cells may induce a degenerative atrophy of postsynaptic SG neurons. Trans-synaptic degenerative atrophy of inhibitory neurons within the SG tasked with the tonic inhibition of local interneurons may produce a state of hyper-excitation. The phenomenon of disinhibition in the dorsal horn pursuant to the induction of a chronic pain state has attracted great interest, hypothesized to play a crucial role in the establishment of central sensitization (Traub, R.J. 1997).

Section 5.6. *Sensory discrimination or affective-motivational? Exploring the effects of IB4-Saporin treatment on associative learning in a condition place avoidance paradigm.*

As defined by the IASP, pain is "An unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" (IASP Subcommittee on Taxonomy, 1979). Nociceptive information is transmitted from the spinal cord to sensory and affective cortical centres via several supraspinal pathways (Newman HM, Stevens RT, Apkarian AV., 1996; Price, D.D, 2000, 2002; Willis, WD. Jr., 2007). The lateral and medial spinothalamic tracts, derived from the superficial and deep dorsal horn respectively comprise part of the lateral pain pathway ascribed the task of transducing pain-related information to somatosensory nuclei in the thalamus and ultimately the cortex (Bowsher, D. et al., 1957; Melzack, R and Casey, M.L., 1968; Trevino and Carstens, 1973, 1975; Carstens, E., Trevino, D.L., 1978; Willis, W.D., Kenshalo, D.R. Jr, Leonard, R.B., 1979; Giesler, G.J Jr, Menétrey,

D., Basbaum, A.I.., 1979; Lima, D and Coimbra, A., 1980; Burstein et al., 1990; Craig, A. D and Dostrovsky, J.O., 1999; Hunt, S.B. and Mantyh, P., 2001) (For review, see Lima D., 2007).

The medial pain pathway, derived from the deep dorsal horn, is responsible for transmitting information through several thalamic nuclei to affective cortical regions such as the insula, the anterior cingulate cortex (ACC) and the amygdala (Melzack, R and Casey, M.L., 1968; Ohara, P.T., Vit, J.P., Jasmin, L., 2005). These pathways are essential for the processing of encountered noxious stimuli into a sensory and emotional experience, producing the appropriate behavioural and motivational responses required to minimize the encounter.

NK1-receptor expressing neurons, located in lamina I, contribute significantly to the lateral spinothalamic tract, in addition to supraspinal projections to brainstem nuclei such as the lateral PBN, which in turn issue projections to limbic regions such as the amygdala (spino-parabrachial-amygdaloid pathway (Bernard, J.F and Besson, J.M., 1990).

Primary afferent input to the superficial dorsal horn and the synaptic connections established within, comprise the first relay in the nociceptive pathway from the periphery to the central nervous system. Notwithstanding their nociceptive phenotype, the role of these afferents in the processing of pain-related information lies not merely in their ability to detect noxious stimuli, but to where and how this information is imparted. Thus, the value of the information carried by non-peptidergic C-PAFs may lay hidden in their synaptic relationships within the SG. Despite the preponderance of interneurons within the SG, retrograde labelling from the cervical spinal cord as well as some brainstem regions are suggestive of direct supraspinal projections (Giesler, G.J. Jr,. Cannon, J.T., Urca, G., Liebeskind, J.C., 1978; Lima, D., Coimbra, A. 1991). However, the synaptic associations of SG neurons with dendrites derived from the deep dorsal horn advocates for a more circuitous route**.** Such an indirect pathway to limbic regions (amygdala, hypothalamus) has been proposed by Braz and colleagues (2005). In a transgenic mouse model using a cre-lox P system to induce WGA expression in a subset of IB4-binding neurons (IB4+ve/$Na_V1.8$+ve), the authors demonstrated

that IB4-binding afferents engaged neurons within the SG which communicated with deep dorsal horn neurons (via lamina III and IV) located in lamina V; these deep dorsal horn neurons ultimately projected to the amygdala and other limbic centres such as the bed nucleus of the stria terminalis (BNST) (Braz, J.M., Nassar, M.A., Wood, J.N., Basbaum, A.I., 2005).

The amygdala has been implicated in several aspects of nociceptive processing (Neugebauer, V., Li, W., Bird, G.C., Han, J.S., 2004). Specifically, the amygdala has been shown to mediate pain-induced fear and anxiety (Neugebauer, V., Li, W., Bird, G.C., Han, J.S., 2004). Furthermore, lesions of the amygdala produce significant deficits in conditioned place avoidance paradigms; behavioural models designed to asses associative learning in response to a painful stimulus paired with a non-threatening environment (Tanimoto S, Nakagawa T, Yamauchi Y, Minami M, Satoh M.,2003; Gao, Y.J., Ren, W.H., Zhang, Y.Q., Zhao, Z.Q., 2004). In addition to the amygdala, BNST, a region of the forebrain has been shown to play a role in the processing of negative affect such as stress, fear, anxiety and aversion. Recent lesion studies of the BNST have provided evidence of an involvement in avoidance learning in response to intraplantar formalin or intraperitoneal acetic acid (Deyama, S., Nakagawa, T., Kaneko, S., Uehara, T. and Minami, M., 2007).

The implications of the transgenic tracing study of Braz and colleagues (2005) are profound, suggesting that non-peptidergic afferent input may impart incoming nociceptive information to affective regions of the brain independent of the lamina I spinothalamic or spino-amygdaloid-parabrachial pathway. In an effort to determine whether such pathway exists in the rat, we evaluated bilaterally injected IB4-SAP treated animals in a conditioned place avoidance paradigm to investigate whether the loss of IB4-binding afferent input to the superficial dorsal horn disrupted communication to the amygdala as suggested by the circuitry proposed by Braz and colleagues (2005). Indeed, bilateral IB4-SAP treatment produced a profound deficit in avoidance learning to a noxious thermal stimulus with no detectable difference in sensory withdrawal latencies in response to the heat (52°C).

Based upon these results, it is difficult to discern whether the effects of IB4-Saporin treatment were mediated by the loss of non-peptidergic C-fibre input to the amygdala or the BNST as indicated by the pathway proposed by the tracing study as both regions have been shown to play an important role in condition avoidance in response to a noxious stimulus. Lesion studies of the BNST and the amygdala have resulted in the attenuation of conditioned avoidance behaviours (Deyama, S., Nakagawa, T., Kaneko, S., Uehara, T., Minami, M, 2008). However, it is likely that the loss of non-peptidergic C-fibre input to spinal cord neurons projecting to both cortical regions are responsible for the profound nature of the deficit in avoidance learning observed in IB4-Saporin treated animals.

The results of our study (chapter IV) lend substantial support to the pathway proposed by Braz and colleagues (2005), the implications of which suggest a significant reappraisal of the conventional tenet that non-peptidergic afferents are involved in the sensory discriminative aspect of pain. Moreover, the results of this study illustrate the importance in understanding the postsynaptic relationships established between primary afferent terminals and neurons within the dorsal horn on the transmission of nociceptive information.

5.6.1 A parallel pathway of primary afferent C-fibre input to the superficial dorsal horn: The relationship between peptidergic and non-peptidergic afferents explored through the evaluation of substance P-Saporin

It has been proposed that C-PAFs comprise two parallel pathways of conveying primary afferent information to the spinal dorsal horn (Hunt, S.P., Rossi, J., 1985). It is clear from the data obtained in our IB4-Saporin study that information transmitted from the periphery by peptidergic and non-peptidergic C-PAFs to LI and II respectively is closely linked; IB4-Saporin treatment produced an increase in NK1-immunoreactivity with a concomitant decrease in sP-immunoreactivity later accompanied by a similar decline in CGRP levels.

We have shown that the loss of IB4-binding primary afferent input to the superficial dorsal horn has affected not only neurons within their immediate synaptic proximity, but peptidergic afferents and their synaptic neighbours as

well. Moreover, the effect of IB4-Saporin treatment on associative learning has provided information pertaining to how information received within the SG is transferred and processed. Given the abundance of neuronal projections to supraspinal discriminatory and affective brain regions, one would expect a profound effect on both these aspects of pain processing following the loss of NK1-expressing neurons resident to lamina I of the spinal cord. Examining the effects of such a lesion may provide further information regarding the inter-relationship of peptidergic and non-peptidergic afferent communication within the superficial dorsal horn.

Prior to exploring the reported data regarding the consequence of sP-Saporin treatment on animal behaviour and physiology, it is important to consider the nature of the lesion induced by the neurotoxic conjugate in relation to the lesion produced by IB4-Saporin. Intrathecal sP-Saporin administration results in the selective ablation of NK1-expressing neurons localized to the superficial dorsal horn whereas the intra-sciatic injection of IB4-Saporin culminates in the loss of primary afferent fibres which supply the superficial dorsal horn with peripherally encountered noxious stimuli. It is therefore important to maintain prudence when comparing the effects of the two neurotoxin conjugates as the targeted neuronal populations play different roles in nociceptive transmission. However, despite the difference in the functional nature of the two neuronal populations, the compensatory changes in neuropeptide expression and more importantly the observed increase in NK1-receptor immunoreactivity in IB4-Saporin model is highly suggestive of an important relationship between afferent input to lamina II and nociceptive transmission in lamina I.

The previous studies which have examined the effects of the selective lesion of NK1-receptor expressing neurons and utilized Substance P conjugated to Saporin reported a 55% loss of NK1-expressing neurons (Vierck,C.J. Jr, Kline, R.H, Wiley, R.G., 2003). A recent study conducted by Wiley and colleagues (2007) using [$Sar^{9}(O2)^{11}$]-substance P-saporin (ssP-Sap), an analog of sP possessing high specificity for the NK1 receptor and greater stability (catabolized slowly) (Wiley,

R.G and Lappi, D.A , 1997, 1999; Wiley, R.G., Kline, R.H. 4th, Vierck, C.J. Jr., 2007; Wiley, R.G, 2008). Intrathecal administration of ssP-Sap resulted in an 81% loss of NK1-receptor expressing neurons demonstrating that the efficacy of ssP-Sap is seven times greater than sP-Sap (Wiley, R.G., Kline, R.H. 4th, Vierck, C.J. Jr., 2007).

NK1-receptor expression is not ubiquitously representative of all lamina I projection neurons populations; retrograde labelling studies conducted from the thalamus and the parabrachial nucleus suggest that small minority of pyramidal cells express the NK1-receptor (Yu, X.H., Ribeiro-da-Silva, A., De Koninck, Y. 2005). NK1 receptor expression has been observed in deeper laminae of the dorsal horn (Todd, A.J., McGill, M.M., Shehab, S.A, 2000). Neurons located in laminas III and IV which express the sP-receptor have been shown to contribute significantly to the lateral reticular nucleus, the lateral parabrachial nucleus and to some extent, the dorsal region of the caudal medulla (Todd, A.J., McGill, M.M., Shehab, S.A, 2000).

As pyramidal neurons contribute to supraspinal projections (9% to the thalamus, 22.7% to the lateral parabrachial nucleus) (Yu, X.H., Ribeiro-da-Silva, A., De Koninck, Y. 2005; Almarestani, L. Waters, SM. Krause, JE. Bennett, GJ. Ribeiro-da-Silva, A., 2007), their likely immunity to sP-Saporin in naïve animals may have profound impact on several aspects of nociception. Case in point; in an animal model of adjuvant-induced inflammation, pyramidal cells were observed, in a time-dependent manner, to ectopically express the NK1-receptor indicative of an inflammation-mediated phenotypic switch in their neurochemical profile (Almarestani, L. Waters, SM. Krause, JE. Bennett, GJ. Ribeiro-da-Silva, A., 2009). Thus, pyramidal neurons expressing novel NK1-receptors might escape the lethal effects of intrathecally administered sP-Saporin preceding the onset of inflammation. As LI pyramidal cells are classified as non-nociceptive neurons responsive to low (cool) temperatures **(**Han Z-S, Zhang E-T, Craig AD., 1998; Doyle, C.A and Hunt, SP. 1999)**,** the phenotypic shift observed in the adjuvant model of inflammation represents a shift in functionality (Almarestani, L., et al., submitted).

Lamina I, NK1-receptor expressing neurons receive input from sP-containing peptidergic C-PAFs (Todd, A.J., Puskár, Z., Spike, R.C., Hughes, C., Watt, C., Forrest, L., 2002). Examination of sP-immunoreactivity, in addition to other neurochemical markers following the administration of sP-Saporin revealed an absence in any change in immunoreactive levels for sP and CGRP. Given the significant nature of the loss of NK1-expressing neurons within the superficial dorsal horn, one would expect a marked response in sP-containing afferents, presumably a compensatory increase in sP-expression. Investigation of the effects of the toxin on IB4-binding or $P2X_3$-immunoreactivity has yet to be conducted. The loss of LI nociceptive processing is likely to produce profound neuroplastic changes in the spinal cord in addition to alterations in primary afferent neurochemistry. However, until such work is done, the effect of sP-Saporin on these constituents of the peripheral and central nervous system designated for nociception is merely speculative.

Several sP-Saporin studies have documented alterations in nociceptive processing (Khasabov, S.G., Rogers, S.D., Ghilardi, J.R., Peters, C.M., Mantyh, P.W., Simone, D.A, 2002, Suzuki, R., Morcuende, S., Webber, M., Hunt, S.P., Dickenson, A.H., 2002). In animals treated with intrathecal sP-Saporin, WDR and high threshold neurons located in the deep and superficial dorsal horn respectively did not display wind-up in response to suprathreshold mechanical stimuli. Deep dorsal horn neurons also exhibited smaller receptive fields, with a reduction in intensity coding for thermal and mechanical stimuli (Suzuki, R., Morcuende, S., Webber, M., Hunt, S.P., Dickenson, A.H., 2002). These data demonstrate the involvement of LI NK1-expressing neurons in the development of wind-up and central sensitization, key processes underlying the transition from acute to persistent pain (Melzack, R., Coderre, T.J., Katz, J., Vaccarino, A.L., 2001).

Analysis of nocifensive behaviours in ssP-Saporin treated animals have revealed an absence of effect on paw withdrawal reflexes to noxious heat when examined 4 weeks following treatment (Vierck,C.J. Jr, Kline, R.H, Wiley, R.G., 2003; Wiley, R.G., Kline, R.H. 4th, Vierck, C.J. Jr., 2007). However, strain and gender variations have been reported within the first 3 weeks post intrathecal ssP-

Saporin; Long Evans strain, female rats exhibited a significant reduction in withdrawal latencies and the duration of ensuing licking/guarding behaviour when placed on a 44ºC hotplate (Wiley, R.G., Kline, R.H. 4th, Vierck, C.J. Jr., 2007). The recovery in paw withdrawal responses to noxious thermal stimuli noted in the 4^{th} week following intrathecal ssP-Saporin was hypothesized to be the result of neuroplastic changes compensating for the loss of LI neurons (Wiley, R.G., Kline, R.H. 4th, Vierck, C.J. Jr., 2007). The author's proffer IB4-binding C-PAFs as a plausible source of compensatory changes; suggesting that in the absence of sP-mediated nociceptive transmission in the superficial dorsal horn, non-peptidergic afferents may adapt in an effort to handle the flow incoming information (Wiley, R.G., Kline, R.H. 4th, Vierck, C.J. Jr., 2007). As IB4-Saporin treatment resulted in an augmentation in NK1-receptor immunoreactivity, it is not without reason to postulate that in the absence of NK1-receptor expressing neurons, non-peptidergic afferents may exhibit neurochemical alterations in an effort to compensate for their loss. In terms of their ability to compensate for the reduction in paw withdrawal thresholds to noxious thermal stimuli, one must keep in mind that non-peptidergic C-PAFs terminals are located within the SG, a region of the superficial dorsal horn that differs considerably in regards to neuroanatomical design from lamina I. As intrathecal ssP-Saporin did not result in the extirpation of NK1-receptor expressing neurons in the deep dorsal horn (Wiley, R.G., Kline, R.H. 4th, Vierck, C.J. Jr., 2007), connections established between dorsally directed dendrites issued from deep dorsal horn projection neurons with LII neurons and possibly with non-peptidergic varicosities (Ritz, L.A., Greenspan, J.D., 1985; Sedivec, M.J., Capowski, J.J., Mendell, L.M., 1986; Woolf, C.J., King, A.E., 1987; Todd, A.J., 1989; Naim M, Spike RC, Watt C, Shehab SA, Todd AJ., 1997) may be augmented following ssP-Saporin treatment, facilitating the recovery of paw withdrawal responses.

Co-localization studies have confirmed the presence of the TRPV1 receptor in IB4-binding, $P2X_3$-receptor expressing C-PAFs (Guo, A., Vulchanova, L., Wang, J., Li, X., Elde, R., 1999; Guo, A., Simone, D.A., Stone, L.S., Fairbanks, C.A., Wang, J., Elde, R., 2001; Aoki, Y., Ohtori, S., Takahashi, K., Ino, H., Douya, H., Ozawa, T., Saito, T., Moriya, H., 2005). TRPV1-receptor positive (VR1) primary

afferent fibres have been shown to establish synaptic contacts with glutamatergic, NK1-receptor expressing neurons in lamina I of the dorsal horn (Hwang, S.J., Burette, A., Valtschanoff, J.G, 2003; Hwang, S.J., Burette, A., Rustioni, A., Valtschanoff, J.G., 2004). Retrograde labelling from the lateral parabrachial nucleus revealed that NK1-receptor positive neurons which received monosynaptic input from primary afferents displaying TRPV1-receptors projected to this region of the brainstem (Valtschanoff JG, Rustioni A, Guo A, Hwang SJ., 2001; Hwang, S.J., Burette, A., Valtschanoff, J.G., 2003). It is therefore possible that IB4-binding/TRPV1-positive C-PAFs establish synaptic contacts with deep dorsal horn neurons projecting to brainstem and cortical regions and that in the absence of NK1-expressing LI neurons, the recovery of heat-evoked paw withdrawal behaviours noted in the 4th weeks after ssP-Saporin treatment is re-routed through the non-peptidergic C-PAF pathway.

Section 5.7: Future directions

"Somewhere, something incredible is waiting to be known".

Dr. Carl Sagan (1934-1996)

The results of the IB4-Saporin study have important implications on our current approach to understanding the functional significance of primary afferent input to the dorsal horn.

Several avenues of investigation may be pursued based upon the findings reported herein. A number of practical experiments have been previously mentioned, such as quantification of the neurochemical changes following IB4-SAP treatment. Specifically, the observed time-related changes in neuropeptide immunoreactivity pursuant to the loss of IB4-binding C-fibre input to the dorsal horn is highly indicative of compensatory changes in nociceptive signalling. The mechanisms underlying these changes may be more thoroughly understood by evaluating peptide synthesis and expression using quantitative measures such as *in situ* hybridization and western-blot techniques respectively. Furthermore, the delayed decline in CGRP-IR observed 7 weeks following IB4-SAP treatment is indicative of protracted compensatory changes. These long-term changes may produce

significant morphological, neurochemical and behavioural effects such as primary afferent sprouting in the SG and disinhibition. Thus, an examination of these events between the 2 and 7 weeks time interval examined in our study, in addition to a thorough examination of these properties after the 7 week time point may reveal delayed compensatory changes which may provide a more detailed understanding of the consequence of primary sensory fibre deafferentation on behaviour, morphology and nociceptive signalling in the superficial dorsal horn. Moreover, a detailed examination of the neurochemical and morphological consequence of cuff-induced peripheral nerve injury aimed at examining changes in sP, NK1 and GAD-immunoreactivity over a 10 week time course may provide information regarding how peptidergic afferents and post-synaptic targets in the dorsal horn are affected by cuff application.

The results of the study have indicated the importance of the postsynaptic connections established between primary afferents and spinal cord neurons. There are several avenues of investigation that may be pursued based upon the data collected thus far. It is important that we conduct a thorough examination of the pathology of the selective loss of IB4-binding afferents; in light of the evidence of compensatory changes in the superficial dorsal horn, evaluation of the peripheral receptive field of the sciatic nerve (glabrous skin) is essential for assessing possible compensatory mechanisms in innervation. Furthermore, we have observed changes in sP, CGRP, GAD and NK1 immunoreactivity following IB4-Saporin treatment. These results warrant a closer examination. Specifically, quantification of peptide, receptor and enzyme levels, a detailed time course extending beyond the seven week time point (which may reveal delayed behavioural changes) in addition to in situ hybridization to examine the changes in mRNA levels in the DRG are potential avenues of investigation. At present, we have examined the effects of IB4-Saporin treatment on acute nociceptive behaviours. As the central terminals of IB4-binding afferents engage inhibitory interneurons tasked with the pre-synaptic inhibition of incoming nociceptive information, it may be of value to examine the effects of IB4-Saporin treatment in an animal model of chronic neuropathy.

Finally, we have compared and contrasted some of the effects of the intrathecal administration of (s)sP-Saporin with the effects of IB4-Saporin, however, an animal model of a selective lesion of peptidergic C-PAFs has yet to be designed.

5.7.1 Peripheral innervation

Non-peptidergic innervation of the glabrous skin of the rat hind paw consists of sub-epidermal axon bundles located at the junction between the dermis and epidermis (Petruska, J.C., Streit, W.J., Johnson, R.D, 1997). Single, IB4-binding axons exiting these bundles course through the dermis, penetrating the dermal-epidermal border and terminating in the epidermis (Petruska, J.C., Streit, W.J., Johnson, R.D, 1997).

The intraplantar administration of the $P2X_3$-receptor agonist αβ-Methyl-ATP in IB4-Saporin treated animals resulted in a sizeable attenuation in the duration of hind paw lifting observed over the course of a ten minute evaluation period, indicating a loss of peripheral $P2X_3$-expressing non-peptidergic terminals in the plantar surface of the hind paw. By virtue of the spread of the agonist into territories of the plantar surface innervated by the saphenous nerve, a complete elimination of agonist-induced pain could not be achieved.

The loss of IB4-binding afferent terminals in the skin may have significant consequences on sensory and autonomic innervation of the denervated territory. Peripheral mononeuropathies have demonstrated time-dependent sprouting of both sensory and autonomic fibres in both glabrous and hairy skin (Ruocco I, Cuello A.C. and Ribeiro-Da-Silva, A., 2000; Ramien, M., Ruocco. I., Cuello, A.C., St-Louis, M., and Ribeiro-Da-Silva, A., 2004; Grelik, C., Bennett, G.J. and Ribeiro-da-Silva A., 2005; Grelik, C., Allard, S. and Ribeiro-da-Silva A., 2005; Yen, L.D., Bennett, G.J. and Ribeiro-da-Silva, A., 2006).

The autonomic nervous system has been implicated as a component of complex regional pain syndrome (CRPS) (Gibbs GF, Drummond PD, Finch PM, Phillips JK, 2008). Pervading autonomic fibres in areas of the dermis and epidermis, in addition to increased sensory fibre density may play an important role in the establishment of chronic pain. The loss of non-peptidergic sensory fibres

following IB4-Saporin treatment may lead to sprouting of autonomic and sensory afferents. Such sprouting may be indicative of further compensatory mechanisms established pursuant to the loss of a sizeable population of sensory afferents.

5.7.3 Evaluation of the role of IB4-binding primary afferents in the genesis and maintenance of chronic neuropathic pain.

Intrathecal administration of $P2X_3/P2X_{2/3}$ receptor antagonists produced an attenuation of nociceptive behaviours in animal models of peripheral nerve injury (McGaraughty, S., Wismer, C.T., Zhu, C.Z., et al., 2003). In animal models of peripheral nerve injury (partial sciatic nerve ligation) and adjuvant-induced inflammation, antisense knock-down of the $P2X_3$-receptor prevented the development of mechanical allodynia and reversed hyperalgesia when administered subsequent to the induction of chronic pain (Barclay, J., Patel, S., Dorn, G., et al., 2002). These studies suggest that $P2X_3$-receptor expressing C-fibres are involved in neuropathic and inflammatory pain. $P2X_3$-receptor antagonists significantly inhibited C-PAFs evoked responses in CCI animals, whereas antagonist treatment had no effect on C-PAF responses in control animals (Sharp, C., Reeve, A.J., Collins, S.D., et al., 2006).

The exclusivity of $P2X_3$-receptor expression, combined with the results of these studies suggests a role for IB4-binding afferents in chronic pain. The relationship between non-peptidergic terminals with inhibitory interneurons and the implications of their presynaptic regulatory control, it's possible that in the setting of chronic pain, the loss of IB4-binding C-fibres induced by IB4-Saporin treatment may produce a rightward shift in stimulus-evoked response, resulting in a decrease in allodynia and hyperalgesia, in response to applied innocuous and nocuous stimuli.

Several animal models of neuropathic pain may be used (CCI, PNL, SNL), however, due to the invasive nature of the intraneural injection of IB4-Saporin, animal models that require surgical methodologies would be undesirable as it would subject the sciatic nerve to repeated exposures and post-operative healing. A more suitable method would be a systemic model of chronic neuropathic pain.

Intravenous injections of certain pharmacological therapeutics for cancer and HIV produce treatment-limiting neuropathies (Peltier AC, Russell JW.,2002; Windebank AJ, Grisold W., 2008)

There are several advantages for using such a model: several of the medications produce pathological evidence of neuropathy within several days, limiting the duration of the experimental protocol. Moreover, IB4-Saporin treatment can be administered before or after the induction of neuropathy, thus providing the opportunity to examine the effects of the loss of IB4-binding afferents on the genesis and maintenance of chronic pain.

A recent publication in the *Journal of Pain* (Joseph, E., Chen, X., Boegen, O. and Levine, J., 2008) reported the effects of intrathecal IB4-Saporin treatment in a rat model of oxaliplatin-induced peripheral neuropathy. Adminstration of oxaliplatin, a platinum derivative used for the treatment of colorectal cancer, results in a rapid onset peripheral neuropathy marked by mechanical and thermal allodynia and hyperalgesia (Joseph, E., Chen, X., Boegen, O. and Levine, J., 2008). Anti-oxidants such as acetyl-L-carnine, α-lipoic acid and vitamin C attenuated oxaliplatin-induced allodynia and hyperalgesia, however, IB4-Saporin treatment resulted in the inhibition of the development of oxaliplatin hyperalgesia suggesting that IB4-binding afferents are responsible for oxidative-stress-induced pathology noted in oxaliplatin neuropathies (Joseph, E., Chen, X., Boegen, O. and Levine, J., 2008).

Given the rapid onset of neuropathy behaviours in oxaliplatin neuropathy, the rationale for evaluating the effects of IB4-Saporin treatment in this model of cancer-medication induced neuropathy was based on available data that suggest that oxaliplatin toxicity is mediated by it's action on sodium channels (Joseph, E., Chen, X., Boegen, O. and Levine, J., 2008). The authors proposed that based upon literature suggesting that IB4-binding afferents differentially express particular sodium channels ($Na_{v1.9}$) (Fang, X., Djhouri, S., McMullan, S, Berry, C., Waxman, S.G and Lawson, S.N, 2002), that oxaliplatin-induced oxidative stress is mediated by non-peptidergic sensory fibres.

Given the strong implications of this study, a more thorough examination of the effects of IB4-Saporin treatment on oxaliplatin-induced neuropathy is warranted.

Several key control experiments were lacking in this study, specifically, an IB4-Saporin control group, immunocytochemical analysis of IB4-binding and $Na_{v1.9}$ labelling in oxaliplatin treated animals, and the examination of the effect of IB4-Saporin treatment given subsequent to establishment of neuropathy to determine whether the absence of IB4-binding afferents resulted in the attenuation or reversal of hyperalgesia.

As several models of drug-induced neuropathies are in current use in basic research, and that their pathological mechanisms differ significantly, it would be of interest to examine the effects of the selective loss of IB4-binding C-fibres in different models.

Section 5.8: Conclusions

Advancements in science have brought our concepts of pain and its value out of the dark ages and into the light of the modern era. The ultimate objective of scientific investigations of diseases and disorders lies in the quest to design and manufacture therapeutic strategies and pharmacological treatments. This may only be achieved when the pathological processes and key players (receptors, channels, signalling molecules and neuronal populations) involved in the development and evolution of a particular condition are fully understood.

Dualism in pain is a reoccurring phenomenon; the mind and the body, emotion and sensation. The dualistic nature of pain is represented in many of the anatomical structures ascribe the task of conveying and processing peripherally encountered painful stimuli exemplified by the parallel nature of primary afferent C-fibre input to the superficial dorsal horn.

The progressive divergence of a homogeneous pool of peptide-expressing, small diameter sensory neurons initiated during embryogenesis and persisting into the early post-natal period, marked by a dramatic phenotypic shift in growth factor subsistence is exemplary of function-based system organization, resulting in the differentiation of a non-peptidergic sub-population of primary afferent C-fibres.

The nociceptive nature of non-peptidergic C-fibres is not the subject of debate. The expression of ion channels such as the TRPV1 and $P2X_3$ receptors are solid evidence for their role in the transmission of nociceptive information from the periphery to the central nervous system. Pharmacological blockade, transgenic manipulation of pain-related receptors or neurotransmitters and *in vivo* electrophysiological recordings further consolidated their participation in pain transmission. Yet despite our current understanding of their nociceptive phenotype, we have yet to determine the nature and value of the information they carry.

Animal models of peripheral nerve injury are an indispensable tool, permitting the evaluation of the relative effect of axon trauma on different afferent populations and the affect this may have on their postsynaptic targets. Despite their common origin, several physiological and neuroanatomical properties of non-peptidergic and peptidergic afferents suggest they may respond to nerve injury differently. For example, in a recent publication in the journal *Neuroscience* by Matsuka et al (2007), IB4-positive trigeminal ganglion neurons, when compared to IB4-negative neurons, exhibited swift endocytosis (Matsuka, Y., Edmonds, B., Mitrirattanakul, S., Schweizer, F.E., Spigelman, I., 2007). The authors additionally suggest that IB4-binding TG neurons refresh their supply of endocytotic vesicle more rapidly than non-IB4-binding neurons; a deduction derived from the smaller decline in membrane capacitance elicited by repetitive stimulation of IB4-positive and negative neurons (Matsuka, Y., Edmonds, B., Mitrirattanakul, S., Schweizer, F.E., Spigelman, I., 2007).

In order to achieve a more thorough understanding of the effect of nerve injury on these two subpopulations, we have examined time-related changes in neurochemical markers for both C-PAFs in addition to observing their structural integrity at the ultrastructural level. The results of our neuropathy study have shown that chronic constriction injury of the common sciatic nerve resulted in the selective, transient degeneration in IB4-binding C-PAFs as indicated by the ultrastructural analysis of the central terminals of both peptidergic and non-peptidergic afferents. These results are indicative of a population-specific

differential susceptibility to axon trauma, presumably mediated by growth factor support. Furthermore, the transient nature of the terminal degeneration of non-peptidergic C-PAFs raises important questions regarding the regenerative capacity of this neuronal subgroup. As the cuff model produced a compound nerve injury, it was not possible to ascribe certain behavioural changes to a particular morphological or neurochemical event.

The selective ablation of IB4-binding, non-peptidergic afferents by the intraneural injection of IB4-Saporin provided an excellent opportunity to explore the nature of the information transduced by these afferents, but also the effect of their loss on several aspects of nociception (behavioural and neuropathological). IB4-Saporin treatment did not precipitate a deficit in paw withdrawal responses to innocuous and nocuous mechanical and thermal stimuli. Furthermore, the loss of IB4-binding C-fibres had no effect on adjuvant-induced hyperalgesia and allodynia. Similarly, IB4-Saporin treatment did not affect nociceptive behaviours in all phases of the formalin test. However, in a model of noxious heat-provoked conditioned place avoidance, the loss of IB4-binding afferent input to the SG resulted in a deficit in avoidance learning lending support to the hypothesis that postsynaptic neurons in the SG communicate with projection neurons in the deep dorsal horn in contact with affective-motivational regions of the brain.

Collated, the behavioural outcome of the cytotoxin study demonstrated that despite their nociceptive profile, the loss of IB4-binding C-PAF terminals in the SG did not influence sensory discrimination, but rather affective-emotional behaviour in response to acute painful stimuli. These results substantiate our theory that the postsynaptic relationships established by non-peptidergic varicosities hold valuable information regarding their functional relevance in nociceptive transmission.

Examination of neurochemical markers for the peptidergic C-fibre population and their postsynaptic contacts within lamina I of the dorsal horn revealed that sP and CGRP immunoreactivity were differentially affected by the loss of IB4-binding afferent input; sP-immunoreactivity declined within the first 2 weeks following lesion formation however CGRP-immunoreactivity remained

unchanged for several weeks, manifesting a decline in levels only by the 7th week post-operative. These results are likely caused by a differential affect on neuropeptide synthesis and storage. IB4-Saporin treatment resulted in a marked increase in NK1-receptor immunoreactivity perceptible at the 2 week time point following lesioning persisting until the end of the observation period at 7 weeks. The differential decline in neuropeptide levels and the increase in NK1-receptor expression are indicative of compensatory mechanisms engaged pursuant to the permanent loss of IB4-binding terminals in the dorsal horn.

The unique architectural design of non-peptidergic varicosities in the SG, specifically their postsynaptic relationship with local inhibitory interneurons makes them an auspicious contender for regulating incoming nociceptive signals. Examination of GAD-immunoreactivity demonstrated an appreciable decline in expression levels.

In summation, we have shown that peptidergic and non-peptidergic C-PAFs indeed comprise a parallel pathway of afferent input to the dorsal horn; lesions of the non-peptidergic neuronal population produced compensatory changes in neurochemical signalling mediated by the peptidergic subsidiary. In addition, we have shown the putative value in understanding the postsynaptic circuitry engaged by these afferents as, despite their nociceptive phenotype, the loss of the non-peptidergic C-fibre population, similar to the loss of NK1-expressing lamina I neurons as shown in sP-SAP studies, was ineffective at eliciting any remarkable deficit in acute pain behaviours yet yielded a profound deficiency in avoidance learning in IB4-Saporin treated animals; a manifestation of a putative connection between non-peptidergic terminals and neurons located in the deep dorsal horn projecting to the amygdala.

Each gear, spring or lever comprising the inner workings of a mechanical watch are essential components of a larger system; should they fail, or go missing, the system itself fails and the watch will no longer keep time. Similarly, each neuron, receptor and synapse comprising the neuroanatomical and physiological system attributed to the processing of pain has their essential roles. On its own, the function of the gear may not be clear, but when placed within the system, we can better understand its purpose.

Section 5.9: Original Contributions

1. *Evaluation of the time-dependent changes in IB4-binding and CGRP-immunoreactivity in the superficial dorsal horn following cuff-induced peripheral nerve lesion.*

The application of a fixed diameter polyethylene cuff on the sciatic nerve produced a significant transitory decline in IB4-binding in lamina II of the 4th and 5th lumber region of the superficial dorsal horn. This decline, perceptible by the 5th post-operative day and peaking on the 10th day following cuff application was followed by a period of marked restoration in IB4-binding notable on the 15th day which was comparable to control levels by day 21 post-lesion. At the ultrastructural scale, this loss in IB4-binding was attributed the marked presence of degenerated core boutons which form the central element of type Ia synaptic glomeruli.

Examination of CGRP-IR profiles in the marginal zone of the superficial dorsal horn revealed that cuff application did not produce any significant changes in CGRP-IR over the time course examined.

2. *Qualitative assessment of the crossed-withdrawal reflex in response to cold stimuli in the cuff model of peripheral nerve lesion.*

Examination of nociceptive behaviours in response to peripherally applied noxious and innocuous stimuli in nerve lesioned animals revealed the presence of a cross-withdrawal reflex in response to innocuous cold stimuli (acetone). Specifically, application of a light spray of acetone to the contralateral (uninjured) paw produced a marked withdrawal response in the ipsilateral paw. The mechanisms underlying this phenomenon are not well understood, however, this is the first occasion where this reflex has been reported in this model of peripheral nerve injury.

3. *Thorough evaluation of the behavioural consequences pursuant to the loss of IB4-binding C-PAFs following the intraneural injection of IB4-SAP into the sciatic nerve.*

Evaluation of nociceptive behaviours in response to a number of peripherally applied noxious and innocuous stimuli demonstrated that in the absence of IB4-binding C-PAFs, response to innocuous and noxious thermal (cold and heat respectively) stimuli remained unaltered compared to control animals over the course of the 2 week period immediately following IB4-SAP injection into the sciatic nerve. We further characterized behavioural responses to a variety of noxious chemical substances such as formalin, capsaicin, complete Freund's adjuvant and αβ-Met-ATP. Attenuated paw lifting in IB4-SAP animals induced by the intraplantar application of- αβ-Met-ATP confirmed the loss of peripheral $P2X_3$ receptors in the sciatic dermatome of the plantar surface of the hind paw. Heat hyperalgesia in response to intraplantar CFA was unaffected by IB4-SAP treatment as was formalin-induced pain. However, heat hyperalgesia produced by intraplantar capsaicin was blocked in IB4-SAP treated animals lending support to published electrophysiology data which suggests that TRPV1 receptors expressed by peptidergic and non-peptidergic DRG neurons express different sensitivities to capsaicin (see chapter 3).

4. *Time course examination of the consequence of the loss of IB4-binding C-PAFs on sP, CGRP, NK1 and GAD immunoreactivity*.

Examination of neurochemical markers for the peptidergic C-fibre population and their postsynaptic contacts within lamina I of the dorsal horn, in addition to markers for inhibitory interneurons in the SG revealed that sP and CGRP immunoreactivity were differentially affected by the loss of IB4-binding afferent input; sP-immunoreactivity declined within the first 2 weeks following lesion formation however CGRP-immunoreactivity remained unchanged for several weeks, manifesting a decline in levels only by the 7^{th} week post-operative. This is the first detailed examination of the effects of IB4-SAP treatment on neurochemical markers for primary afferent fibers and their post-synaptic neighbours.

5. *Evaluation of the effect of IB4-SAP lesions on associative learning in a condition place avoidance paradigm. These results substantiate our theory that the postsynaptic relationships established by non-peptidergic varicosities hold valuable information regarding their functional relevance in nociceptive transmission.*

IB4-SAP treatment resulted in a profound deficit in associative learning in a conditioned place avoidance paradigm. Specifically, animals which received bilateral intrasciatic nerve injections of IB4-SAP exhibited a marked disability in associating an environmental cue (dark compartment) with a noxious stimulus (heat). These results impact our current understanding of how peripherally encountered noxious information is transmitted through the dorsal horn to supraspinal nociceptive centers, suggesting a role for the SG and thus non-peptidergic C-PAFs as well in conveying incoming information to affective-motivational regions of the brain which process nociceptive information.

Furthermore, we demonstrated that the contracture of the injected hind paw associated with IB4-SAP treatment had no effect on gait as animals treated bilaterally with intra-sciatic injections of the neurotoxin displayed normal foot print parameters. Combined with our RotaRod data, we can say with some certainty that the contractures exhibited by IB4-SAP treated animals do not interfere with motor function.

Bibliography

A

Afrah, A.W., Stiller, C.O., Olgart, L., Brodin, E., Gustafsson, H. (2001). Involvement of spinal NMDA receptors in capsaicin induced in vivo release of sP in the rat dorsal horn. *Neuroscience letters. 316: 83-86*

Akkina SK, Patterson CL, Wright DE, (2001). GDNF rescues nonpeptidergic unmyelinated primary afferents in streptozotocin-treated diabetic mice. *Experimental Neurology, 167: 173-182*.

Al-Khater, K.M., Kerr, R., Todd, A.J. (2008). A quantitative study of spinothalamic neurons in laminae I, III, and IV in lumbar and cervical segments of the rat spinal cord. *Journal of Comparative Neurology, 511(1):1-18.*

Allen, A.L., Cortright, D.N., McCarson, K.E. (2003). Formalin- or adjuvant-induced peripheral inflammation increases neurokinin-1 receptor gene expression in the mouse. *Brain Research, 24;961(1):147-152.*

Almarestani, L, (2008). Changes in the spinal cord and peripheral innervation in an animal model of arthritis. Doctoral Dissertation, McGill University, Montreal, Canada.

Almarestani, L. Waters, SM. Krause, JE. Bennett, GJ. Ribeiro-da-Silva, A. (2007). Morphological characterization of spinal cord dorsal horn lamina I neurons projecting to the parabrachial nucleus in the rat. *Journal of Comparative Neurology, 504(3):287-297.*

Almarestani L, Longo G, Ribeiro-da-Silva A. (2008). Autonomic fiber sprouting in the skin in chronic inflammation. *Molecular Pain 4:56.*

Almarestani L, Fitzcharles MA, Bennett GJ, Ribeiro-da-Silva A. (2009). Characterization of the complete Freund's adjuvant animal model of localized arthritis. *Arthritis and Rheumatism Submitted.*

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

Almeida TF, Roizenblatt S and Tufik S. (2004). Afferent pain pathways: a neuroanatomical review. *Brain Research Reviews 1000 40-56*

Alvarez FJ, Rodrigo J, Jessell TM, Dodd J, Priestley JV. (1989). Ultrastructure of primary afferent fibres and terminals expressing alpha-galactose extended oligosaccharides in the spinal cord and brainstem of the rat. *Journal of Neurocytology, 18(5):631-45.*

Alvarez, F.J., Morris, H.R., Priestley, J.V. (1991). Sub-populations of smaller diameter trigeminal primary afferent neurons defined by expression of calcitonin gene-

related peptide and the cell surface oligosaccharide recognized by monoclonal antibody LA4. *Journal of Neurocytolology, 20(9):716-731.*

Alvarez, F.J., Fyffe, R.E. (2000). Nociceptors for the 21st century. *Current Review of Pain 4: 451-458.*

Alvarez, F.J, Villalba, R.M., Zerda, R., Schneider, S.P. (2004).Vesicular glutamate transporters in the spinal cord, with special reference to sensory primary afferent synapses. *Journal of Comparative Neurology. 472(3):257-280.*

Ambalavanar, R., Moritani, M., Haines, A., Hilton, T., Dessem, D. (2003).Chemical phenotypes of muscle and cutaneous afferent neurons in the rat trigeminal ganglion. *Journal of Comparative Neurology*, *460(2):167-79*.

Anand P, (2004). Neurotrophic factors and their receptors in human sensory neuropathies. *Progress in Brain Research, 146: 477-492.*

Anderson, R.T., and Anderson, S.T., (1994). Pain: Beyond all paradoxes. In The Puzzle of Pain. (pp. 121-138). Australia: G&B Arts International Limited

Andrew, D., Krout, K.E., Craig, A.D. (2003). Differentiation of lamina I spinomedullary and spinothalamic neurons in the cat. *Journal of Comparative Neurology, 458(3):257-271.*

Aoki, Y., Ohtori, S., Takahashi, K., Ino, H., Douya, H., Ozawa, T., Saito, T., Moriya, H. (2005). Expression and Co-expression of VR1, CGRP and IB4-binding
glycoprotein in the dorsal root ganglion neurons in rats: differences between the disc afferent and the cutaneous afferents. *Spine 30(13): 1496-1500*.

Attal, N., Jazat, F., Kayser,V., Guilbaud, G, (1990). Further evidence for 'pain-related' behaviours in a model of unilateral peripheral mononeuropathy. *Pain 41: 235-251.*

Attal, N., Filliatreau, G., Perrot, S., Jazat, F., Di, G.L., Guilbaud G. (1994). Behavioural pain-related disorders and contribution of the saphenous nerve in crush and chronic constriction injury of the rat sciatic nerve. *Pain 59: 301-312.*

Ataka T, Kumamoto E, Shimoji K, Yoshimura M. (2000). Baclofen inhibits more effectively C-afferent than Adelta-afferent glutamatergic transmission in substantia gelatinosa neurons of adult rat spinal cord slices. *Pain, 86(3):273-82.*

Averill, S., McMahon, S.B., Clary, D.O., Reichardt, L.F., Priestley, J.V. (1995). Immunocytochemical localization of trkA receptors in chemically identified subgroups of adult rat sensory neurons. *European journal of Neuroscience 7(7): 1484-1494*

B

Baba H, Goldstein PA, Okamoto M, Kohno T, Ataka T, Yoshimura M, Shimoji K. (2000). Norepinephrine facilitates inhibitory transmission in substantia gelatinosa of adult rat spinal cord (part 2): effects on somatodendritic sites of GABAergic neurons. *Anesthesiology, 92(2):485-492.*

Baba, H, Shimoji, K, Yoshimura, M. (2000). Norepinephrine facilitates inhibitory transmission in substantia gelatinosa of adult rat spinal cord (part 1): effects on axon terminals of GABAergic and glycinergic neurons. *Anesthesiology, 92(2):473-484.*

Baba, H. Yoshimura, M. Nishi, S. Shimoji, K. (1994). Synaptic responses of substantia gelatinosa neurones to dorsal column stimulation in rat spinal cord in vitro. *Journal of Physiology, 478 (Pt 1):87-99.*

Bai, L., Xu, H., Collins, J.F., Ghishan, F.K. (2001). Molecular and functional analysis of a novel neuronal vesicular glutamate transporter. *Journal of Biology and Chemistry, 276(39):36764-36769.*

Bailey, AL and Ribeiro-da-Silva, A. (2006). Transient loss of terminals from non-peptidergic nociceptive fibers in the substantia gelatinosa of spinal cord following chronic constriction injury of the sciatic nerve. *Neuroscience, 38(2):675-690.*

Barber, R.P., Vaughn, J.E., Saito, K., McLaughlin, B.J., Roberts, E. (1978). GABAergic terminals are presynaptic to primary afferent terminals in the substantia gelatinosa of the rat spinal cord. *Brain Research, 141(1):35-55.*

Barber RP, Vaughn JE, Roberts E. (1982). The cytoarchitecture of GABAergic neurons in rat spinal cord. *Brain Research, 238(2):305-328.*

Barclay, J., Patel, S., Dorn, G., Wotherspoon, G., Moffatt, S., Eunson, .L, Abdel'al, S., Natt, F., Hall, J., Winter, J., Bevan, S., Wishart, W., Fox, A., Ganju, P. (2002). Functional downregulation of $P2X_3$ receptor subunit in rat sensory neurons reveals a significant role in chronic neuropathic and inflammatory pain. *Journal of Neuroscience, 22(18):8139-8147.*

Bardoni, R., Torsney, C., Tong, C.K., Prandini, M., MacDermott, A.B. (2004). Presynaptic NMDA receptors modulate glutamate release from primary sensory neurons in rat spinal cord dorsal horn. *Journal of Neuroscience, 24(11):2774-27781.*

Baudet, C., Mikaels, A., Westphal, H., Johansen, J., Johansen, T.E., Ernfors, P. (2000). Positive and negative interactions of GDNF, NTN and ART in developing sensory neuron subpopulations, and their collaboration with neurotrophins. *Development, 127(20):4335-4344.*

Beal, JA. and Cooper, MH. (1978). The neurons in the gelatinosal complex (Laminae II and III) of the monkey (Macaca mulatta): a Golgi study. *Journal of Comparative Neurology, 179(1):89-121.*

Beal, J. A. (1983). Identification of presumptive long axon neurons in the substantia gelatinosa of the rat lumbosacral spinal cord: a Golgi study. *Neuroscience Letters, 41, 9-14.*

Bellocchio, E.E., Reimer, R.J., Fremeau, R.T. Jr., Edwards, R.H. (2000). Uptake of glutamate into synaptic vesicles by an inorganic phosphate transporter. *Science, 289(5481):957-960*

Bennett, D.L., Averill, S., Clary, D.O., Priestley, J.V., McMahon, S.B. (1996). Postnatal changes in the expression of the trkA high-affinity NGF receptor in primary sensory neurons. *European Journal of Neuroscience, 8: 2204-2208.*

Bennett, D.L., Dmietrieva, N., Priestley, J.V., Clary, D., McMahon, S.B. (1996). TrkA, CGRP and IB_4 expression in retrogradely labelled cutaneous and visceral primary sensory neurones in the rat. *Neuroscience Letters, 206(1):33-36.*

Bennett, D.L., Koltzenburg ,M., Priestley, J.V., Shelton, D.L., McMahon, S.B. (1998).
Endogenous nerve growth factor regulates the sensitivity of nociceptors in the adult rat. *European Journal of Neuroscience, 10(4):1282-91.*

Bennett, D.L., Michael, G.J.. Ramachandran, N., Munson, J.B.. Averill, S., Yan, Q., McMahon, S.B., Priestley, J.V. (1998). A distinct subgroup of small DRG cells express GDNF receptor components and GDNF is protective for these neurons after nerve injury.
Journal of Neuroscience, 18(8):3059-3072.

Bennett, G.J., Hayashi, H., Abdelmoumene, M., Dubner, R. (1979). Physiological properties of stalked cells of the substantia gelatinosa intracellularly stained with horseradish peroxidase. *Brain Research, 164:285-9*.

Bennett, G.J., Abdelmoumene, M., Hayashi, H., Dubner, R. (1980). Physiology and morphology of substantia gelatinosa neurons intracellularly stained with horseradish peroxidase. *Journal of Comparative Neurology, 194(4):809-827.*

Bennett GJ, Xie YK (1988). A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. *Pain, 33(1):87-107*

Benini A, DeLeo JA. (1999). René Descartes' physiology of pain. *Spine. 24(20):2115-2119.*

Benoliel R, Tanaka M, Caudle RM, Iadarola MJ. (2000). Co-localization of N-methyl-D-aspartate receptors and substance P (neurokinin-1) receptors in rat spinal cord. *Neuroscience Letters, 291(2):61-4.*

Bergman, E., Carlsson, K., Liljeborg, A., Manders, E., Hökfelt, T., Ulfhake, B. (1999). Neuropeptides, nitric oxide synathase and GAP 43 in B_4-binding and RT97 immunoreactive primary sensory neurons: normal distribution pattern and changes after peripheral nerve transaction and ageing. *Brain Research 832: 63-83*

Bernard, J.F., Besson, J.M. (1990).The spino(trigemino)pontoamygdaloid pathway: electrophysiological evidence for an involvement in pain processes. *Journal of Neurophysiology, 63(3):473-490.*

Besse, D, Lombard, M.C., Zajac, J.M., Roques, B.P., Besson, J.M. (1990). Pre- and postsynaptic distribution of mu, delta and kappa opioid receptors in the superficial layers of the cervical dorsal horn of the rat spinal cord. *Brain Research, 521(1-2):15-22.*

Bicknell, H.R. Jr., Beal, J.A. (1984). Axonal and dendritic development of substantia gelatinosa neurons in the lumbosacral spinal cord of the rat. *Journal of Comparative Neurology, 226(4):508-522.*

Bjurholm, A., Kreicbergs, A., Ahmed, M., Schultzberg, M. (1990). Noradrenergic and peptidergic nerves in the synovial membrane of the Sprague-Dawley rat. *Arthritis and Rheumatology, 33(6):859-865.*

Bleazard, L., Hill, R.G., Morris, R. (1994). The correlation between the distribution of the NK1 receptor and the actions of tachykinin agonists in the dorsal horn of the rat indicates that substance P does not have a functional role on substantia Gelatinosa (LaminaII) neurons. *The Journal of Neuroscience,14(12): 7655-7664*

Bogen, O., Dreger, M., Gillen, C., Schröder, W., Hucho, F. (2005). Identification of versican as an Isolectin B_4-binding glycoprotein from mammalian spinal cord tissue. *The FEBS Journal, 272: 1090-1102.*

Bohlhalter, S., Weinmann, O., Mohler, H., Fritschy, J-M. (1996). Laminar Compartmentalization of GABA,-Receptor Subtypes in the Spinal Cord: An lmmunohistochemical Study. *The Journal of Neuroscience, 16(1):283-297*

Bora, F.W. Jr., Osterman, A.L. (1982). Compression neuropathy. *Clinical Orthopedics and Related Research, (163):20-32.*

Bourgeais, L., Gauriau, C., Monconduit, L., Villanueva, L., Bernard, J.F. (2003). Dendritic domains of nociceptive-responsive parabrachial neurons match terminal fields of lamina I neurons in the rat. *Journal of Comparative Neurology, 464(2):238-256.*

Bowery, N.G., Hudson, A.L., Price, G.W. (1987). $GABA_A$ and $GABA_B$ receptor site distribution in the rat central nervous system. *Neuroscience, 20(2):365-383.*

Bowsher, D. (1957). Termination of the central pain pathway in man: the conscious appreciation of pain. *Brain, 80(4):606-622.*

Bradbury EJ, Burnstock G, McMahon SB. (1998).The expression of P2X3 purinoreceptors in sensory neurons: effects of axotomy and glial-derived neurotrophic factor. *Molecular and Cellular Neuroscience, 12(4-5):256-268.*

Braz, J.M., Nassar, M.A., Wood, J.N., Basbaum, A.I. (2005). Parallel "pain" pathways arise from subpopulations of primary afferent nociceptor. *Neuron, 47(6):787-93.*

Bromovsky P.R. (2005). Neuropeptides, sensory neurons and pain modulation**.** Karolinska Institute, Laresics AB, Sweden.

Brown, A.G. (1982). The dorsal horn of the spinal cord. *Quarterly Journal of Experimental Physiology, 67(2):193-212.*

Brown, J.L., Liu, H., Maggio, J.E., Vigna, S.R., Mantyh, P.W., Basbaum, A.I. (1995). Morphological characterization of substance P receptor-immunoreactive neurons in the rat spinal cord and trigeminal nucleus caudalis. *Journal of Comparative Neurology, 356(3):327-344.*

Bulling, D.G., Kelly, D., Bond, S., McQueen, D.S., Seckl, J.R. (2001). Adjuvant-induced joint inflammation causes very rapid transcription of beta-preprotachykinin and alpha-CGRP genes in innervating sensory ganglia. *Journal of Neurochemistry, 77(2):372-382.*

Burnstock G. (2007). Physiology and pathophysiology of purinergic neurotransmission. *Physiology Review, 87(2):659-797*

Burnstock G, Wood JN. (1996). Purinergic receptors: their role in nociception and primary afferent neurotransmission. *Current Opinion in Neurobiology, 6(4):526-532 (Review).*

Burry, R.W., Lah, J.J., Hayes, D.M, (1992). GAP-43 distribution is correlated with development of growth cones and presynaptic terminals. *Journal of Neurocytology, 21: 413-425.*

Burstein, R., Dado, R.J., Cliffer, K.D., Giesler, G.J. Jr. (1991). Physiological characterization of spinohypothalamic tract neurons in the lumbar enlargement of rats. *Journal of Neurophysiology., 66(1):261-284.*

Burstein R, Potrebic S. (1993). Retrograde labeling of neurons in the spinal cord that project directly to the amygdala or the orbital cortex in the rat. *Journal of Comparative Neurology, 335(4):469-885.*

C

Cajal, R. (1909). Histologie du système nerveux de l'homme et des vertébrés. Volume 1. Translated by Azoulay, L., Paris.

Calixto, J.B., Beirith, A., Ferreira, J., Santos, A.R., Filho, V.C., Yunes, R.A. (2000). Naturally occurring antinociceptive substances from plants. *Phytotherapy Research, (6):401-18*.

Cameron, A.A., Cliffer, K.D., Dougherty, P.M., Garrison, C.J., Willis, W.D., Carlton, S.M. (1997). Time course of degenerative and regenerative changes in the dorsal horn in a rat model of peripheral neuropathy. *Journal of Comparative Neurology, 379: 428-442.*

Carlton, S.M., Zhou, S., Coggeshall, R.E. (1996). Localization and activation of substance P receptors in unmyelinated axons of rat glabrous skin. *Brain Research, 734(1-2):103-8.*

Carstens, E., Trevino, D.L. (1978). Laminar origins of spinothalamic projections in the cat as determined by the retrograde transport of horseradish peroxidase. *Journal of Comparative Neurology,182(1):161-165*.

Castro, A.R., Morgado, C., Lima, D., Tavares, I. (2006). Differential expression of NK1 and $GABA_B$ receptors in spinal neurones projecting to antinociceptive or pronociceptive medullary centres. *Brain Research Bulletin*, 69(3*):266-275.*

Castro, A.R., Pinto, M., Lima, D., Tavares, I. (2004). Nociceptive spinal neurons expressing NK1 and $GABA_B$ receptors are located in lamina I. *Brain Research, 1003(1-2):77-85.*

Castro-Lopes, J.M., Coimbra, A., Grant, G., Arvidsson, J. (1990). Ultrastructural changes of the central scalloped (C1) primary afferent endings of synaptic glomeruli in the substantia gelatinosa Rolandi of the rat after peripheral neurotomy. *Journal of Neurocytology, 19(3):329-337.*

Castro-Lopes, J.M., Tavares, I., Coimbra, A. (1993). GABA decreases in the spinal cord dorsal horn after peripheral neurectomy. *Brain Research,620(2):287-291.*

Caterina, M.J., Schumacher, M.A., Tominaga, M., Rosen, T.A., Levine, J.D., Julius, D. (1997). The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature, 389(6653):816-824.*

Caterina, M.J., Leffler, A., Malmberg, A.B., Martin, W.J., Trafton, J., Petersen-Zeitz, K.R., Koltzenburg, M., Basbaum, A.I., Julius, D. (2000). Impaired

nociception and pain sensation in mice lacking the capsaicin receptor. *Science, 288(5464):306-313.*

Cauna, N. (1973). The free penicllate nerve endings of the human hairy skin. *Journal of Anatomy, 115: 277-288*

Cavanaugh, D.J., Lee, H., Lo, L., Shields, S.D,, Zylka, M.J., Basbaum, A.I., Anderson, D.J. (2009). Distinct subsets of unmyelinated primary sensory fibers mediate behavioral responses to noxious thermal and mechanical stimuli. *Proceedings of the National Acadamy of Science U S A (PNAS), 106(22):9075-9080*.

Cechetto, D.F., Standaert, D.G., Saper, C.B. (1985).Spinal and trigeminal dorsal horn projections to the parabrachial nucleus in the rat. *Journal of Comparative Neurology, 240(2):153-160.*

Cervero, F., Iggo, A., Molony, V. (1979). Segmental and intersegmental organization of neurones in the Substantia Gelatinosa Rolandi of the cat's spinal cord. *Quarterly Journal of Experimental Physiology and cognitive medical science, 64(4):315-326.*

Cervero, F., Iggo, A. (1980). The substantia gelatinosa of the spinal cord: a critical review. *Brain, 3(4):717-772*.

Cervero, F., Molony, V., Iggo, A. (1977). Extracellular and intracellular recordings from neurones in the substantia gelatinosa Rolandi. *Brain Research, 136(3):565-569.*

Chao, T., Pham, K., Steward, O., Gupta, R. (2008). Chronic nerve compression injury induces a phenotypic switch of neurons within the dorsal root ganglia. *Journal of Comparative Neurology, 506(2):180-193.*

Chen, Y., Li, G.W., Wang, .C, Gu, Y., Huang, L.Y. (2005). Mechanisms underlying enhanced P2X receptor-mediated responses in the neuropathic pain state. *Pain, 119(1-3):38-48*

Chéry, N., De Koninck, Y (1999). Junctional versus extrajunctional glycine and GABA(A) receptor-mediated IPSCs in identified lamina I neurons of the adult rat spinal cord. *Journal of Neuroscience, 19(17):7342-7355.*

Chéry, N., De Koninck, Y. (2000). GABA (B) receptors are the first target of released GABA at lamina I inhibitory synapses in the adult rat spinal cord. *Journal of Neurophysiology, 84(2):1006-1011.*

Chesler, E.J., Wilson, S.G., Lariviere, W.R., Rodriguez-Zas, S.L., Mogil, J.S. (2002). Identification and ranking of genetic and laboratory environment factors

influencing a behavioral trait, thermal nociception, via computational analysis of a large data archive. *Neuroscience Biobehavior Reviews, 26: 907-923.*

Cheunsuang, O., Maxwell, D., Morris, R. (2002). Spinal lamina I neurones that express neurokinin 1 receptors: II. Electrophysiological characteristics, responses to primary afferent stimulation and effects of a selective mu-opioid receptor agonist. *Neuroscience, 111(2):423-434.*

Chew, D.J., Leinster, V.H., Sakthithasan, M., Robson, L.G., Carlstedt, T., Shortland, P.J. (2008). Cell death after dorsal root injury. *Neuroscience Letters, 433(3):231-234*.

Christensen, B.N., Perl, E.R. (1970). Spinal neurons specifically excited bynoxious or thermal stimuli: marginal zone of the dorsal horn. *Journal of Neurophysiology, 33:293–307.*

Coakley, S., (2007). Palliative or Intensification? Pain and Christian Contemplation in the Spirtuality of the Sixteenth-Century Carmelites. In Coakely, S. and Shelemay, K.K (Eds.), Pain and its transformations. (pp. 77-101). Cambridge, Massachusetts. Harvard University Press.

Coderre, T.J., Xanthos, D.N., Francis, L., Bennett, G.J. (2004). Chronic post-ischemia pain (CPIP): a novel animal model of complex regional pain syndrome-type I (CRPS-I; reflex sympathetic dystrophy) produced by prolonged hindpaw ischemia and reperfusion in the rat. *Pain, 112(1-2):94-105*

Coggeshall, R.E., Lekan, H.A., White, F.A., Woolf, C.J. (2001). A-fiber sensory input induces neuronal cell death in the dorsal horn of the adult rat spinal cord. *Journal of Comparative Neurology, 435(3):276-82.*

Coimbra, A., Magalhâes, M.M., Sodré-Borges, B.P. (1970). Ultrastructural localization of acid phosphatase in synaptic terminals of the rat substantia gelatinosa Rolandi. *Brain Research, 22(1):142-146.*

Coimbra, A., Sodré-Borges, B.P., Magalhães, M.M. (1974). The substantia gelatinosa Rolandi of the rat. Fine structure, cytochemistry (acid phosphatase) and changes after dorsal root section. *Journal of Neurocytology, (2):199-217.*

Coimbra, A., Ribeiro-da-Silva, A., Pignatelli, D. (1984). Effects of dorsal rhizotomy on the several types of primary afferent terminals in laminae I-III of the rat spinal cord. An electron microscope study. *Anatomical Embryology (Berlin), 170(3):279-287.*

Coimbra A, Ribeiro-da-Silva A, Pignatelli D. (1986). Rexed's laminae and the acid phosphatase (FRAP)-band in the superficial dorsal horn of the neonatal rat spinal cord *Neuroscience Letters, 71(2):131-136.*

Colmant, H.J. (1959). Activity fluctuations of acid phosphatase in the spinal cord and spinal ganglia of rats after section of the sciatic nerve. *Archiv für psychiatrie und nervenkrankheiten vereinigt mit zeitschrift für die gesamte neurologie und psychiatrie 199(1):60-71 (Article in German)*

Craig, A.D, Bushnell, M.C. (1994). The thermal grill illusion: unmasking the burn of cold pain. *Science, 265(5169):252-255.*

Craig, A.D., Kniffki, K.D. (1985). Spinothalamic lumbosacral lamina I cells responsive to skin and muscle stimulation in the cat. *Journal of Physiology*, *365:197-221*

Craig AD, Dostrovsky JO. (1991). Thermoreceptive lamina I trigeminothalamic neurons project to the nucleus submedius in the cat. Experimental Brain Research, 85(2):470-474.

Cronin, J.N., Bradbury, EJ., Lidierth, M. (2004). Laminar distribution of GABAA- and glycine-receptor mediated tonic inhibition in the dorsal horn of the rat lumbar spinal cord: effects of picrotoxin and strychnine on expression of Fos-like immunoreactivity. *Pain, 112(1-2):1561-63.*

Cruccu, G., Romaniello, A., Amantini, A., Lombardi, M., Innocenti, P., Manfredi, M. (1999). Assessment of trigeminal small-fiber function: brain and reflex responses evoked by CO2-laser stimulation. *Muscle and Nerve, 22(4):508-516.*

Cruz, F., Lima, D., Coimbra, A. (1993). Periterminal synaptic organization of primary afferents in laminae I and IIo of the rat spinal cord, as shown after anterograde HRP labelling. *.Journal of Neurocytology, 22(3):191-204.*

Cruz, F. Lima, D. Zieglgansberger,W. Coimbra, A. (1991). Fine structure and synaptic architecture of HRP-labelled primary afferent terminations in lamina IIi of the rat dorsal horn. *Journal of Comparative Neurology, 305(1):3-16.*

Csillik, B., Knyihár, E., Jójárt, I., Elshiekh, A.A., Pór, I. (1978).
Perineural microtubule inhibitors induce degenerative atrophy of central nociceptive terminals in the Rolando substance. *Research Communications in Chemical Pathology and Pharmacology, 21(3):467-484.*

Csillik B, Knyihár-Csillik E. (1981). Regenerative synaptoneogenesis in the Mammalian spinal cord: dynamics of synaptochemical restoration in the Rolando Substance after transganglionic degenerative atrophy. *Journal of Neural Transmission, 52(4):303-317.*

Csillik, B. Knyihár-Csillik, E and Tajti, J. (1982). Blockade of retrograde axoplasmic transport induces transganglionic degenerative atrophy of central terminals of primary nociceptive neurons. *Acta Biologica Academiae Scientarium Hungaricae. 33(2-3):149-156.*

Csillik, B. Schwab, ME and Thoenen, H. (1985). Transganglionic regulation of central terminals of dorsal root ganglion cells by nerve growth factor (NGF). *Brain Research, 331(1):11-15.*

Csillik B, Mihály A, Krisztin-Péva B, Farkas I, Knyihár-Csillik E. (2008). Mitigation of nociception via transganglionic degenerative atrophy: possible mechanism of vinpocetine-induced blockade of retrograde axoplasmic transport. *Annals of Anatomy, 190(2):140-145.*

Cuello, A.C., Del Fiacco, M., Paxinos, G. (1978). The central and peripheral ends of the substance P-containing sensory neurones in the rat trigeminal system. *Brain Research, 152(3):499-500.*

Cuello, AC. Ribeiro-da-Silva, A. Ma, W. De Koninck, Y and Henry, JL. (1993). Organization of substance P primary sensory neurons: ultrastructural and physiological correlates. *Regulatory Peptide, 46(1-2):155-64.*

D

De Biasi S, and Rustioni A. (1988). Glutamate and substance P coexist in primary afferent terminals in the superficial laminae of spinal cord. *Proceeds National Academy of Science U S A (PNAS), 85(20):7820-4.*

De Biasi S, and Rustioni A. (1990). Ultrastructural immunocytochemical localization of excitatory amino acids in the somatosensory system. *Journal of Histochemistry and Cytochememistry,38(12):1745-1754*

Descartes, R. (1664). L'homme de Rene Descartes (Treatise of Man). Translated by Hall, ST.. Harvard University Press, Copyright 1972.

Decosterd, I., Woolf, CJ. (2000). Spared nerve injury: an animal model of persistent peripheral neuropathic pain. *Pain, 87(2):149-158.*

Devor, M., Claman, D. (1980). Mapping and plasticity of acid phosphatase afferents in the rat dorsal horn. *Brain Research, 190(1):17-28.*

Deyama, S., Nakagawa, T., Kaneko, S., Uehara, T., Minami, M. (2008). Involvement of the bed nucleus of the stria terminalis in the negative affective component of visceral and somatic pain in rats. *Behavior Brain Research, 176(2):367-371.*

Fang, X., Djouhri, L., Black, J.A., Dib-Hajj, S.D., Waxman, S.G., Lawson, S.N. (2002). The presence and role of the tetrodotoxin-resistant sodium channel Na(v)1.9 (NaN) in nociceptive primary afferent neurons. *Journal of Neuroscience, 22(17):7425-7433.*

Djouhri, L., Wrigley, D., Thut, P.D., Gold, M.S. (2004). Spinal nerve injury increases the percentage of cold-responsive DRG neurons. *NeuroReport, 15: 457-460.*

Djouhri, L and Lawson ,SN. (2004). A-beta-fiber nociceptive primary afferent neurons: a review of incidence and properties in relation to other afferent A-fiber neurons in mammals. *Brain Research, Brain Research Reviews, 46(2):131-45.*

Dodd, J., Jessell, T.M. (1985). Lactoseries carbohydrates specify subsets of dorsal root ganglion neurons projecting to the superficial dorsal horn of rat spinal cord. *Journal of Neuroscience, 5(12):3278-3294.*

Dostrovsky, J.O., Craig, A.D. (1996). Cooling-specific spinothalamic neurons in the monkey. *Journal of Neurophysiology, 76(6):3656-65.*

Dougherty, K.J., Sawchuk, M.A., Hochman, S. (2005). Properties of mouse spinal lamina I GABAergic interneurons. *Journal of Neurophysiology, 94(5):3221-3227.*

Dowdall, T., Robinson, I., Meert, T.F. (2005). Comparison of five different rat models of peripheral nerve injury. *Pharmacology, Biochemistry and Behaviour, 80(1):93-108.*

Doyle, C.A and Hunt, SP. (1999). A role for spinal lamina I neurokinin-1-positive neurons in cold thermoreception in the rat. *Neuroscience, 91(2):723-732.*

Duby, G. (1994). Physical Pain in the Middle Ages. In The Puzzle of Pain. (pp. 71-81). Australia: G&B Arts International Limited.

E

Eaton, M.J., Plunkett, J.A., Karmally, S., Martinez, M.A., Montanez, K. (1998). Changes in GAD- and GABA- immunoreactivity in the spinal dorsal horn after peripheral nerve injury and promotion of recovery by lumbar transplant of immortalized serotonergic precursors. *Journal of Chemical Neuroanatomy, 16(1):57-72*

.

England, J.D. (1999). Entrapment neuropathies. *Current Opinion in Neurology, 12(5):597-602.*

Engelman, H. S., Allen, T. B. & MacDermott, A. B. (1999).The distribution of neurons expressing calcium permeableAMPA receptors in the superficial laminae of the spinal cord dorsal horn. *Journal of Neuroscience, 19; 2081–2089.*

F

Fang, X., Djouhri, L., Black, J.A., Dib-Hajj, S.D., Waxman, S.G., Lawson, S.N. (2002). The presence and role of the tetrodotoxin-resistant sodium channel Na(v)1.9 (NaN) in nociceptive primary afferent neurons. *Journal of Neuroscience, 22(17):7425-7433.*

Fang, X., Djouhri, L., McMullan, S., Berry, C., Waxman, S.G., Okuse, K., Lawson, S.N. (2006). I ntense isolectin-B4 binding in rat dorsal root ganglion neurons distinguishes C-fiber nociceptors with broad action potentials and high Nav1.9 expression. *Journal of Neuroscience*, *26(27):7281-7292.*

Ferrington, D.G., Sorkin, L.S., Willis, W.D. Jr. (1986). Responses of spinothalamic tract cells in the cat cervical spinal cord to innocuous and graded noxious stimuli.
Somatosensory Research, 3(4):339-358.

Fitzgerald, M. (1981). A study of the cutaneous afferent input to substantia gelatinosa. *Neuroscience, 6(11):2229-2237.*

Fitzgerald, M and MacDermott, A., (2005). The development of pain systems In Hunt, S.P and Koltzenburg, M (Eds.). The Neurobiology of pain, p207-238. Oxford University press

Fricker, B., Muller, A., René, F. (2008). Evaluation tools and animal models of peripheral neuropathies. *Neurodegenerative Disorders, 5(2):72-108.*

Fyffe, R.E., Per,l E.R. (1984). Is ATP a central synaptic mediator for certain primary afferent fibers from mammalian skin? *Proceeds National Academy of Science U SA (PNAS) 81(21):6890-6893.*

Fremeau, R.T. Jr, Troyer, M.D., Pahner, I., Nygaard, G.O., Tran, C.H., Reimer, R.J., Bellocchio, E.E., Fortin, D., Storm-Mathisen, J., Edwards, R.H. (2001). The expression of vesicular glutamate transporters defines two classes of excitatory synapse. *Neuron, 31(2):247-260.*

Fukuoka T, Kondo E, Dai Y, Hashimoto N, Noguchi K. (2001). Brain-derived neurotrophic factor increases in the uninjured dorsal root ganglion neurons in selective spinal nerve ligation model. *Journal of Neuroscience, 21(13):4891-4900.*

Fullmer, J.M, Riedl, M., Williams, F.G., Sandrin, M., Elde, R. (2007). Enzymes that synthesize the IB_4 epitope are not sufficient to impart IB_4 binding in dorsal root ganglia of rat. *Journal of Neurology, 501(1):70-82.*

Furuyama, T., Kiyama, H., Sato, K., Park, H.T., Maeno, H., Takagi, H., Tohyama, M. (1993). Region specific expression of subunits of iontotropic glutamate

receptors (AMAP-type, KA-type and NMDA receptors) in the rat spinal cord with special reference to nociceptrion. *Molecular Brain Research, 18, 141-151.*

G

Galeazza, M.T., Garry, M.G., Yost., H.J., Strait, K.A., Hargreaves, K.M., Seybold, V.S. (1995).
Plasticity in the synthesis and storage of substance P and calcitonin gene-related peptide in primary afferent neurons during peripheral inflammation. *Neuroscience, 66(2):443-458.*

Gao, Y.J., Ren, W.H., Zhang, Y.Q., Zhao, Z.Q. (2004). Contributions of the anterior cingulate cortex and amygdala to pain- and fear-conditioned place avoidance in rats. *Pain, 110(1-2):343-353.*

Garland, A.M, Grady, EF, Lovett M, Vigna SR, Frucht MM, Krause, JE, and Bunnett NW (1996). Mechanisms of desensitization and resensitization of G protein-coupled neurokinin1 and neurokinin2 receptors. *Molecular Pharmacology, 49: 438-446.*

Gao YJ, Ren WH, Zhang YQ, Zhao ZQ. (2004). Contributions of the anterior cingulate cortex and amygdala to pain- and fear-conditioned place avoidance in rats. *Pain, 110(1-2):343-353.*

Gauriau C, Bernard JF. (2002). Pain pathways and parabrachial circuits in the rat. *Experimental Physiology, 87(2):251-258 (Review).*

Gerke, MB and Plenderleith, MB. (2004). Ultrastructural analysis of the central terminals of primary sensory neurones labelled by transganglionic transport of bandeiraea simplicifolia I-isolectin B_4. *Neuroscience, 127(1):165-175.*

Gerke, MB and Plenderleith, MB. (2001). Binding sites for the plant lectin Bandeiraea simplicifolia I-isolectin B(4) are expressed by nociceptive primary sensory neurones. *Brain Research, 911(1):10110-10114.*

Gerke, MB and Plenderleith, MB. (2002). Distribution of binding sites for the plant lectin Ulex europaeus agglutinin I on primary sensory neurones in seven different mammalian species. *Histochemical Journal ,34(1-2):79-84.*

Gerke, M.B., Plenderleith, M.B. (2004). Analysis of the unmyelinated primary sensory neurone projection through the dorsal columns of the rat spinal cord using transganglionic transport of the plant lectin Bandeiraea simplicifolia I-isolectin B_4. *Journal of Neurological Science, 221(1-2):69-77.*

Gibbs, G.F., Drummond, P.D., Finch, P.M., Phillips, J.K. (2008). Unravelling the pathophysiology of complex regional pain syndrome: focus on sympathetically maintained pain. *Clinical and Experimental Pharmacology and Physiology, 35(7):717-724 (Review).*

Giesler, G.J. Jr,. Cannon, J.T., Urca, G., Liebeskind, J.C. (1978). Long ascending projections from substantia gelatinosa Rolandi and the subjacent dorsal horn in the rat. *Science, 202(4371):984-986.*

Giesler, G.J Jr, Menétrey, D., Basbaum, A.I. (1979). Differential origins of spinothalamic tract projections to medial and lateral thalamus in the rat. *Journal of Comparative Neurology 184(1):107-126.*

Giesler, G.J. Jr, Yezierski, R.P., Gerhart, K.D., Willis, W.D. (1981a). Spinothalamic tract neurons that project to medial and/or lateral thalamic nuclei: evidence for a physiologically novel population of spinal cord neurons. *Journal of Neurophysiology, 46(6):1285-1308.*

Giesler, G.J Jr, Spie,l H.R., Willis, W.D. (1981b).Organization of spinothalamic tract axons within the rat spinal cord. *Journal of Comparative Neurology, 195(2):243-252.*

Gobel S. (1975). Golgi studies in the substantia gelatinosa neurons in the spinal trigeminal nucleus. *Journal of Comparative Neurology, 162(3):397-415*.

Gobel S. (1978). Golgi studies of the neurons in layer II of the dorsal horn of the medulla (trigeminal nucleus caudalis). *Journal of Comparative Neurology, 180(2):395-413.*

Gobel S, Falls, W.M. (1979). Anatomical observations of horseradish peroxidase-filled terminal primary axonal arborizations in layer II of the substantia gelatinosa of Rolando. *Brain Research, 175(2):335-340.*

Gobel S, Falls WM, Bennett GJ, Abdelmoumene M, Hayashi H and Humphrey E. (1980).
An EM analysis of the synaptic connections of horseradish peroxidase-filled stalked cells and islet cells in the substantia gelatinosa of adult cat spinal cord. *Journal of Comparative Neurology*, *194(4):781-807.*

Gobel S, Falls, W.M, Humphrey, E. (1981). Morphology and synaptic connections of ultrafine primary axons in lamina I of the spinal dorsal horn: candidates for the terminal axonal arbors of primary neurons with unmyelinated (C) axons. *Journal of Neuroscience, 1(10):1163-1179.*

Gold, M.S., Shuster, M.J., Levine, J.D. (1996). Characterization of six voltage-gated K+ currents in adult rat sensory neurons. *Journal of Neurophysiology,75(6):2629-2646.*

Gómez, L.O. (2007). Pain and the Suffering Consciousness: The Alleviation of Suffering in Buddhist Discourse. In Coakely, S. and Shelemay, K.K (Eds.), Pain and its transformations. (pp. 101-122). Cambridge, Massachusetts. Harvard University Press.

Gould, S.J., (1985). The Flamingo's Smile: Reflections in Natural History. W.W Norton and Company, New York.

Govrin-Lippmann R, Devor M. (1978). Ongoing activity in severed nerves: source and variation with time. *Brain Research, 159(2):406-410.*

Gras, C., Herzog, E., Bellenchi, G.C., Bernard, V., Ravassard, P., Pohl, M., Gasnier, B., Giros, B., El Mestikawy, S. (2002). A third vesicular glutamate transporter expressed by cholinergic and serotoninergic neurons. *Journal of Neuroscience, 22(13):5442-5451.*

Grelik C, Allard S, Ribeiro-da-Silva A. (2005). Changes in nociceptive sensory innervation in the epidermis of the rat lower lip skin in a model of neuropathic pain. *Neuroscience Letters, 389(3):140-5.*

Grelik C, Bennett GJ, Ribeiro-da-Silva A. (2005). Autonomic fibre sprouting and changes in nociceptive sensory innervation in the rat lower lip skin following chronic constriction injury. *European Journal of Neuroscience, 21(9):2475-87.*

Groves, M.J., Ng, Y.W., Ciardi, A., Scaravilli, F. (1996). Sciatic nerve injury in the adult rat: comparison of effects on oligosaccharide, CGRP and GAP43 immunoreactivity in primary afferents following two types of trauma. *Journal of Neurocytology, 25(3):219-231.*

Groves, M.J., Schänzer, A., Simpson, A.J., An, S.F., Kuo, L.T., Scaravilli, F. (2003). Profile of adult rat sensory neuron loss, apoptosis and replacement after sciatic nerve crush. *Journal of Neurocytology, 32(2):113-122.*

Grudt, T.J., Perl, E.R. (2002). Correlations between neuronal morphology and electrophysiological features in the rodent superficial dorsal horn. *Journal of Physiology, 540(Part 1):189-207.*

Guo, A., Vulchanova, L., Wang, J., Li, X., Elde, R. (1999). Immunocytochemical localization of the vanilloid receptor 1 (VR1): relationship to neuropeptides, the $P2X_3$ purinoceptor and IB_4 binding sites. *European Journal of Neuroscience, 11(3):946-958.*

Guo, A., Simone, D.A., Stone, L.S., Fairbanks, C.A., Wang, J., Elde, R. (2001). Developmental shift of vanilloid receptor 1 (VR1) terminals into deeper regions of the superficial dorsal horn: correlation with a shift from TrkA to Ret expression by dorsal root ganglion neurons. *European Journal of Neuroscience*, *14(2):293-304.*

Guseva, D and Chelyshev Y. (2006). The plasticity of the DRG neurons belonging to different subpopulations after dorsal rhizotomy. *Cellular and Molecular Neurobiology, 26(7-8):1225-34.*

H

Hammond, D.L., Ackerman, L., Holdsworth, R., Elzey, B. (2004). Effects of spinal nerve ligation on immunohistochemically identified neurons in the L4 and L5 dorsal root ganglia of the rat. *Journal of Comparative Neurology, 475(4):575-589.*

Han, Z-S., Zhang, E-T., Craig, A.D. (1998) Nociceptive and thermoreceptive lamina I neurons are anatomically distinct. *Nature Neuroscience 1, 218–225.*

Hanz, S., Fainzilber, M. (2006). Retrograde signaling in injured nerve--the axon reaction revisited. *Journal of Neurochemistry, 99(1):13-19 (Review).*

Health Canada (2002). Canada's aging population. Minister of Public Works and Government Services, Canada Cat.H39-608/2002E.

Henken, D.B., Battisti, W.P., Chesselet, M.F., Murray, M., Tessler, A. (1990). Expression of beta-preprotachykinin mRNA and tachykinins in rat dorsal root ganglion cells following peripheral or central axotomy. *Neuroscience, 39(3):733-742.*

Henry, J.L. (1976). Effects of substance P on functionally identified units in cat spinal cord. *Brain Research, 114(3):439-451.*

Henley, J.M., Jenkins, R., Hunt, S.P. (1993). Localisation of glutamate receptor binding sites and mRNAs to the dorsal horn of the rat spinal cord. *Neuropharmacology, 32(1):37-41.*

Herzberg, U., Eliav, E., Dorsey, J.M., Gracely, R.H., Kopin, I.J. (1997). NGF involvement in pain induced by chronic constriction injury of the rat sciatic nerve. *NeuroReport, 8: 1613-1618.*

Hill, E.L., Elde, R. (1991). Distribution of CGRP-, VIP-, D beta H-, SP-, and NPY-immunoreactive nerves in the periosteum of the rat. *Cell and Tissue Research., 264(3):469-480.*

Hökfelt, T., Broberger, C., Xu, Z.Q., Sergeyev, V., Ubink, R., Diez, M. (2000). Neuropeptides--an overview. *Neuropharmacology, 39(8):1337-1356. Review*

Holstege, J.C., Jongen, J.L., Kennis, J.H., van Rooyen-Boot, A.A., Vecht, C.J. (1998). Immunocytochemical localization of GDNF in primary afferents of the lumbar dorsal horn. *Neuroreport, 9(12):2893-2897.*

Honda, T., Takahashi, M., Sugiura, Y., (1999). Colocalization of the glial cell-line derive neurotrophic factor and its functional receptor c-RET in a subpopulation of rat dorsal root ganglion neurons. *Neuroscience letters, 275; 45-48*

Hubscher, C.H., Petruska, J.C., Rau, K.K., Johnson, R.D. (2001). Co-expression of P2X receptor subunits on rat nodose neurons that bind the isolectin GS-I-B_4. *Neuroreport, 12(13):2995-2997.*

Hucho TB, Dina OA, Levine JD. (2005). Epac mediates a cAMP-to-PKC signaling in inflammatory pain: an isolectin B_4(+) neuron-specific mechanism. *Journal of Neuroscience, 25(26):6119-6126.*

Hunt, S.P., Rossi, J. (1985). Peptide and non-peptide containing unmyelinated primary afferents: the parallel processing of nociceptive information. *Philosophical Transactions of the Royal Society **B**: Biological, 308, 283-289*

Hunt, S.P., Mantyh, P.W. (2001).The molecular dynamics of pain control. *Nature Review Neuroscience, 2(2):83-91*

Hwang, S.J., Burette, A., Valtschanoff, J.G. (2003). VR1-positive primary afferents contact NK1-positive spinoparabrachial neurons. *Journal of Comparative Neurology, 460(2):255-265.*

Hwang, S.J., Burette, A., Rustioni, A., Valtschanoff, J.G. (2004). Vanilloid receptor VR1-positive primary afferents are glutamatergic and contact spinal neurons that co-express neurokinin receptor NK1 and glutamate receptors. *Journal of Neurocytology, 33(3):321-9.*

Subcommittee on Taxonomy of International Association for the Study of Pain (1994) Part III: Pain Terms, A Current List with Definitions and Notes on Usage. In Merskey, H. and Bogduk, N. (Eds.), *Classification of Chronic Pain, Second Edition (pp. 209-214). International Association for the Study of Pain (IASP) Press, Seattle.*

I

Ibuki, T., Hama, A.T., Wang, X.T., Pappas, G.D., Sagen, J. (1997). Loss of GABA-immunoreactivity in the spinal dorsal horn of rats with peripheral nerve injury and promotion of recovery by adrenal medullary grafts. *Neuroscience, 76(3):845-858.*

IJkema-Paassen, J., Jansen, K., Gramsbergen, A., Meek, M.F. (2004). Transection of peripheral nerves, bridging strategies and effect evaluation. *Biomaterials, 25(9):1583-1592.*

Indo, Y. (2002). Genetics of congenital insensitivity to pain with anhidrosis (CIPA) or hereditary sensory and autonomic neuropathy type IV. Clinical, biological and molecular aspects of mutations in TRKA(NTRK1) gene encoding the receptor tyrosine kinase for nerve growth factor. *Clinical Autonomic Research, 12 Suppl 1:I20-32.*

Ivanavicius, S.P., Blake, D.R., Chessell, I.P., Mapp, P.I. (2004). Isolection B_4 binding neurons are not present in the rat knee joint. *Neuroscience 128:555-560.*

Iwasaki A, Inoue K, Hukuda S. (1995). Distribution of neuropeptide-containing nerve fibers in the synovium and adjacent bone of the rat knee joint. *Clinical and Experimental Rheumatology, 13(2):173-178.*

Iyadomi, M., Iyadomi, I., Kumamoto, E., Tomokuni, K., Yoshimura, M. (2000). Presynaptic inhibition by baclofen of miniature EPSCs and IPSCs in substantia gelatinosa neurons of the adult rat spinal dorsal horn. *Pain, 85(3):385-393.*

J

Jancsó, G., Hökfelt, T., Lundberg, J.M., Kiraly, E., Halász, N., Nilsson, G., Terenius, L., Rehfeld, J., Steinbusch, H., Verhofstad, A., Elde, R., Said, S., Brown, M. (1981). Immunocytochemical studies of the effect of capsaicin on spinal and meduallary peptide and monamine neurons using antisera to substance P, gastrin/CCK, somatostatin, VIP, Enkephalin, neurotensin and 5-hydroxytryptamine. *Neurocytology, 10: 963-980.*

Jahr, C.E., Jessell, T.M. (1983). ATP excites a subpopulation of rat dorsal horn neurones. *Nature, 304(5928):730-733.*

Jarvis MF, Burgard EC, McGaraughty S, Honore P, Lynch K, Brennan TJ, Subieta A, Van Biesen T, Cartmell J, Bianchi B, Niforatos W, Kage K, Yu H, Mikusa J, Wismer CT, Zhu CZ, Chu K, Lee CH, Stewart AO, Polakowski J, Cox BF, Kowaluk E, Williams M, Sullivan J, Faltynek C. (2002). A-317491, a novel potent and selective non-nucleotide antagonist of P2X3 and P2X2/3 receptors, reduces chronic inflammatory and neuropathic pain in the rat.
Proceedings of the Natational Academy of Science U S A;99(26):17179-17184.

Jasmin, L., Burkey, A.R., Card, J.P., Basbaum, A.I. (1997). Transneuronal labeling of a nociceptive pathway, the spino-(trigemino-)parabrachio-amygdaloid, in the rat. *Journal of Neuroscience, 17(10):3751-3765.*

Jessell, T.M, Tsunoo, A., Kanazawa, I., Otsuka, M. (1979). Substance P: depletion in the dorsal horn of rat spinal cord after section of the peripheral processes of primary sensory neurons. *Brain Research, 168(2):247-259.*

Jessell, T.M., Iversen, L.L., Cuello, A.C. (1978). Capsaicin-induced depletion of substance P from primary sensory neurones. *Brain Research, 152(1):183-188.*

Jessell, T.M., Dodd, J. (1985). Structure and expression of differentiation antigens on functional subclasses of primary sensory neurons. *Philosophical Transactions of the Royal Society of London, Series B, 308(1136):271-281.*

Jeftinija, S., Jeftinija, K., Liu, F., Skilling, S.R., Smullin, D.H., Larson, A.A. (1991). Excitatory amino acids are released from rat primary afferent neurons in vitro. *Neuroscience Letters, 125(2):191-4.*

Joseph, E., Chen, X., Boegen, O. and Levine, J. (2008). Oxaliplatin acts on IB_4-positive nociceptors to induce an oxidative stress-dependent acute painful peripheral neuropathy. *The Journal of Pain, 9(5):463-472.*

Jimenez-Andrade, J.M., Peters, C.M., Mejia, N.A., Ghilardi, J.R., Kuskowski, M.A, Mantyh, P.W. (2006). Sensory neurons and their supporting cells located in the trigeminal, thoracic and lumbar ganglia differentially express markers of injury following intravenous administration of paclitaxel in the rat. *Neuroscience Letters, 405(1-2):62-67.*

Ju, G., Hökfelt, T., Brodin, E., Fahrenkrug, J., Fischer, J.A., Frey, P., Elde, R.P, Brown, J.C. (1987). Primary sensory neurons of the rat showing calcitonin gene-related peptide immunoreactivity and their relation to substance P-, somatostatin-, galanin-, vasoactive intestinal polypeptide- and cholecystokinin-immunoreactive ganglion cells.*Cell and Tissue Research, 247(2):417-31.*

Julius, D. and Basbaum, A. I. (2001) .Molecular mechanisms of nociception. *Nature 413 (6852):203-210.*

K

Kage, K., Niforatos, W., Zhu, C.Z., Lynch, K.J., Honore, P., Jarvis, M.F. (2002). Alteration of dorsal root ganglion P2X3 receptor expression and function following spinal nerve ligation in the rat. *Experimental Brain Research, 147(4):511-9.*

Kai-Kai MA. (1989).Cytochemistry of the trigeminal and dorsal root ganglia and spinal cord of the rat. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 93(1):183-193.*

Kajander, K.C., Xu, J. (1995). Quantitative evaluation of calcitonin gene-related peptide and substance P levels in rat spinal cord following peripheral nerve injury. *Neuroscience Letters, 186(2-3):184-188.*

Kajander, K.C., Bennett, G.J. (1992). Onset of a painful peripheral neuropathy in rat: a partial and differential deafferentation and spontaneous discharge in A beta and A delta primary afferent neurons. *Journal of Neurophysiology, 68(3):734-744.*

Kanai, Y, Hara, T and Imai, A, (2005). Participation of the spinal TRPV1 receptors in formalin-evoked pain transduction: a study using a selective TRPV1 antagonist, iodo-resiniferotoxin. *Journal of Pharmacy and Pharmacology, 58: 489-493.*

Kangrga, I., Jiang, M.C., Randić, M. (1991).Actions of (-)-baclofen on rat dorsal horn neurons. *Brain Research, 562(2):265-75*.

Kashiba, H., Hyon, B., Senba, E.(1998). Glial cell line-derived neurotrophic factor and nerve growth factor receptor mRNAs are expressed in distinct subgroups of dorsal root ganglion neurons and are differentially regulated by peripheral axotomy in the rat. *Neuroscience Letters, 252(2):107-110.*

Kashiba, H., Uchida, Y., Senba, E. (2003). Distribution and colocalization of NGF and GDNF family ligand receptor mRNAs in dorsal root and nodose ganglion neurons of adult rats. *Molecular Brain research, 110: 52-62.*

Kashiba, H., Uchida, Y., Senba, E. (2001). Differences in binding by Isolectin B4 to trkA and c-ret mRNA-expressing neurons in rat sensory ganglia. *Molecular Brain Research, 95: 18-26.*

Kato G, Furue H, Katafuchi T, Yasaka T, Iwamoto Y, Yoshimura M. (2004). Electrophysiological mapping of the nociceptive inputs to the substantia gelatinosa in rat horizontal spinal cord slices. *Journal of Physiology, 560(Pt 1):303-315.*

Kawasaki, Y., Kumamoto, E., Furue, H., Yoshimura, M. (2003). Alpha 2 adrenoceptor-mediated presynaptic inhibition of primary afferent glutamatergic transmission in rat substantia gelatinosa neurons. *Anesthesiology, 98(3):68268-9.*

Kemp, S.W., Walsh, S.K., Midha, R. (2008). Growth factor and stem cell enhanced conduits in peripheral nerve regeneration and repair. *Neurology Research, 30(10):1030-1038.*

Khasabov, S.G., Rogers, S.D., Ghilardi, J.R., Peters, C.M., Mantyh, P.W., Simone, D.A. (2002). Spinal neurons that possess the substance P receptor are required for the development of central sensitization. *Journal of Neuroscience, 22(20):9086-9098.*

Kitchener, P.D., Wilson, P., Snow, P.J. (1993). Selective labelling of primary sensory afferent terminals in lamina II of the dorsal horn by injection of Bandeiraea simplicifolia isolectin B4 into peripheral nerves. *Neuroscience, 54(2):545-551.*

King AE**,** Lopez-Garcia JA**.**.(1993). Excitatory amino acid receptor-mediated neurotransmission from cutaneous afferents in rat dorsal horn in vitro. *Journal of Physiology, 472:443-457.*

Kim SH, Chung JM. (1992). An experimental model for peripheral neuropathy produced by segmental spinal nerve ligation in the rat. *Pain, 50(3):355-363.*

Kirkeby S, Moe D (2001). Binding of Griffonia simplicifolia 1 isolectin B_4 (GS1 B_4) to alpha-galactose antigens. *Immunology and Cell Biology, 79(2):121-127*.

Kirkeby S, Moe D. (2002). Lectin interactions with alpha-galactosylated xenoantigens. *Xenotransplantation, 9(4):260-267.*

Kitao Y, Robertson B, Kudo M, Grant G. (2002). Proliferation patterns of dorsal root ganglion neurons of cutaneous, muscle and visceral nerves in the rat. *Journal of Neurocytology, 31(8-9):765-776.*

Knyihár E, Gerebtzoff, M.A. (1973). Extra-lysosomal localization of acid phosphatase in the spinal cord of the rat. *Experimental Brain Research, 18(4):383-395*.

Knyihár E, László I, Tornyos, S. (1974). Fine structure and fluoride resistant acid phosphatase activity of electron dense sinusoid terminals in the substantia gelatinosa Rolandi of the rat after dorsal root transection. *Experimental Brain Research, 19(5):529-544.*

Knyihár, E., Csillik, B. (1976). Representation of cutaneous afferents by fluoride-resistant acid phosphatase (FRAP)-active terminals in the rat substantia gelatinosa rolandi. *Acta Neurologica Scandinavica, 53(3):217-225.*

Knyihár-Csillik E, Csillik B. (1981). FRAP: histochemistry of the primary nociceptive neuron. *Progress Histochemistry and Cytochemistry, 14(1):1-137.*

Knyihár-Csillik, E., Rakic, P., Csillik, B. (1985). Fine structure of growth cones in the upper dorsal horn of the adult primate spinal cord in the course of reactive synapto-neogenesis. *Cell and Tissue Research, 239: 633-641.*

Knyihár-Csillik E, Kreutzberg GW, Csillik B. (1989). Enzyme translocation in the course of regeneration of central primary afferent terminals in the substantia gelatinosa of the adult rodent spinal cord. *Journal of Neuroscience Research, 22(1):74-82.*

Knyihár-Csillik E, Rakic P, Csillik B. (1989). Transneuronal degeneration in the Rolando substance of the primate spinal cord evoked by axotomy-induced transganglionic degenerative atrophy of central primary sensory terminals. *Cell and Tissue Research, 258(3):515-525.*

Knyihár-Csillik, E., Török, A. (1989). Cytochemical restoration in the upper dorsal horn after transganglionic degenerative atrophy: temporospatial and fine structural correlates. *Neuroscience, 33(1):75-91.*

Knyihár-Csillik, E, Vecsei L, Mihaly A, Fenyo R, Farkas I, Krisztin-Peva B, Csillik B. (2007). Effect of vinpocetine on retrograde axoplasmic transport. *Annals of Anatomy, 189(1):39-45*

Koerber, H.R., Druzinsky, R.E., Mendell, L.M. (1988) Properties of somata of spinal dorsal root ganglion cells differ according to peripheral receptor innervated. *Journal of Neurophysiology, 60:1584 –1596*

Koltzenburg, M. (1999).The changing sensitivity in the life of the nociceptor. *Pain. Supplemental, 6:S93-102*

Krekoski, C.A., Parhad, I.M., Clark, A.W. (1996). Attenuation and recovery of nerve growth factor receptor mRNA in dorsal root ganglion neurons following axotomy. *Journal of Neuroscience Research, 43(1):1-11.*

Kruger, L., Perl, E.R., Sedivec, M.J., (1981). Fine structure of myelinated mechanical nociceptor endings in cat hairy skin. *Journal of Comparative Neurology, 198: 137-154.*

Kruger, L., Silverman, J.D., Mantyh, P.W., Sternini, C., Brecha, N.C. (1989). Peripheral patterns of calcitonin-gene-related peptide general somatic sensory innervation: cutaneous and deep terminations. *Journal of Comparative Neurology, 280(2):291-302.*

Kumazawa, T and Perl, ER. (1977). Primate cutaneous receptors with unmyelinated (C) fibres and their projection to the substantia gelatinosa. *Journal of Physiology (Paris), 73(3):287-304.*

Kumazawa, T and Perl, ER. (1978). Excitation of marginal and substantia gelatinosa neurons in the primate spinal cord: indications of their place in dorsal horn functional organization. *Journal of Comparative Neurology, 177(3):417-434.*

Kuniyoshi, K., Ohtori, S., Ochiai, N., Murata, R., Matsudo, T., Yamada, T., Ochiai, S.S., Moriya, H., Takahashi, K. (2007). Characteristics of sensory DRG neurons innervating the wrist joints in rats. *European journal of Pain., 11(3):323-328.*

L

Labrakakis C, Tong CK, Weissman T, Torsney C, MacDermott AB. (2003). Localization and function of ATP and GABAA receptors expressed by nociceptors and other postnatal sensory neurons in rat. *Journal of Physiology, 549(Pt 1):131-142.*

Landry M, Bouali-Benazzouz R, El Mestikawy S, Ravassard P, Nagy F. (2004). Expression of vesicular glutamate transporters in rat lumbar spinal cord, with a note on dorsal root ganglia. *Journal of Comparative Neurology, 468(3):380-394.*

Lao, L and Marvizón, JC. (2005). GABA(A) receptor facilitation of neurokinin release from primary afferent terminals in the rat spinal cord. *Neuroscience, 130(4):1013-1027.*

Lawson S.N., Nickels S.M. (1980). The use of morphometric techniques to analyse the effect of neonatal capsaicin treatment on rat dorsal root ganglia and dorsal roots.. *Proceedings of the. Physiology. Society. Abstract,. 19P*

Lawson, S.N., Perry, M.J., Prabhakar, E., McCarthy, P.W. (1993). Primary sensory neurones: neurofilament, neuropeptides, and conduction velocity. *Brain Research Bulletin, 30(3-4):239-243.*

Lawson, S.N. (2002). Phenotype and function of somatic primary afferent nociceptive neurones with C-, Adelta- or Aalpha/beta-fibres. *Experimental Physiology, 87(2):239-244.*

Leclere, P.G., Norman, E., Groutsi, F., Coffin, R., Mayer, U., Pizzey, J., Tonge, D. (2007). Impaired axonal regeneration by isolectin B4-binding dorsal root ganglion neurons in vitro. *Journal of Neuroscience, 27(5):1190-1199.*

Lee, C.J., Bardoni, R., Tong, C.K., Engelman, H.S., Joseph, D.J., Magherini, P.C., MacDermott, A.B. (2002). Functional expression of AMPA receptors on central terminals of rat dorsal root ganglion neurons and presynaptic inhibition of glutamate release. *Neuron 35:135-146.*

Lee, S.E., Shen, H., Taglialatela, G., Chung, J.M., Chung, K. (1998). Expression of nerve growth factor in the dorsal root ganglion after peripheral nerve injury. *Brain Research, 796: 99-106.*

Lee, S.E and Kim., J-H. (2007). Involvement of substance P and calcitonin gene-related peptide in development and maintenance of neuropathic pain from spinal nerve injury model of rat. *Neuroscience Research., 58:245-249.*

Leem, J.W., Willis, W.D., Chung, J.M. (1993). Cutaneous sensory receptors in the rat foot. *Journal of Neurophysiology, 69(5):1684-1699.*

Leffler, A. Mönter, B and Koltzenburg, M. (2006). The role of the capsaicin receptor TRPV1 and acid-sensing ion channels (ASICS) in proton sensitivity of subpopulations of primary nociceptive neurons in rats and mice. *Neuroscience, 139(2):699-709.*

Lescar, J. Loris, R. Mitchell, E. Gautier, C. Chazalet, V. Cox, V. Wyns, L, Pe'rez, S. Breton, C and Imberty, A. (2002). Isolectins I-A and I-B of *Griffonia*

(*Bandeiraea*) *simplicifolia*. Crystal structure of metal-free GS-IB4 and molecular basis for metal binding and monosaccharide specifity. *The Journal of Biological Chemistry, 277 (8), 6608-6614*

Li, D.P., Chen, S.R., Pan, Y.Z., Levey, A.I., Pan, H.L. (2002). Role of presynaptic muscarinic and GABA(B) receptors in spinal glutamate release and cholinergic analgesia in rats. *Journal of Physiology, 543(Pt 3):807-818.*

Li, H., Wu, L., Li, Y.Q. (2003). Opioid peptides modulate the response of neurons of the superficial laminae of the rat spinal dorsal horn to GABA. *Biochemistry and Biophysiological Research Communication, 307(3):730-736.*

Li L, Zhou XF.(2001). Pericellular Griffonia simplicifolia I isolectin B_4-binding ring structures in the dorsal root ganglia following peripheral nerve injury in rats. *Journal of Comparative Neurology, 439(3):259-274.*

Li, J., Perl, E.R. (1995). ATP modulation of synaptic transmission in the spinal substantia gelatinosa. *Journal of Neuroscience, 15(5 Pt 1):3357-3365.*

Li, J.L., Xiong, K.H., Dong, Y.L., Fujiyama, F., Kaneko, T., Mizuno, N. (2003). Vesicular glutamate transporters, VGluT1 and VGluT2, in the trigeminal ganglion neurons of the rat, with special reference to co-expression. *Journal of Comparative Neurology, 463(2):212-220.*

Li, H., Wu, L., Li, Y.Q. (2003). Opioid peptides modulate the response of neurons of the superficial laminae of the rat spinal dorsal horn to GABA. *Biochemical and Biophysical Research Communications, 307(3):730-736.*

Lidierth, M. (2006). Local and diffuse mechanisms of primary afferent depolarization and pre-synaptic inhibition in the rat spinal cord. *Journal of physiology 576: 309-327.*

Light, A.R., Perl, E.R. (1979). Reexamination of the dorsal root projection to the spinal dorsal horn including observations on the differential termination of coarse and fine fibers. *Journal of Comparative Neurology, 186(2):117-131.*

Light, A.R., Trevino, D.L., Perl, E.R. (1979). Morphological features of functionally defined neurons in the marginal zone and substantia gelatinosa of the spinal dorsal horn. *Journal of Comparative Neurology, 186:151–172.*

Light, A.R., Kavookjian, A.M. (1988). Morphology and ultrastructure of physiologically identified substantia gelatinosa (lamina II) neurons with axons that terminate in deeper dorsal horn laminae (III-V). *Journal of Comparative Neurology, 267(2):172-189.*

Lima, D., Coimbra, A. (1983). The neuronal population of the marginal zone (lamina I) of the rat spinal cord. A study based on reconstructions of serially sectioned cells. *Anatomy and Embryology (Berlin), 167(2):273-288.*

Lima, D., Coimbra, A. (1986). A Golgi study of the neuronal population of the marginal zone (lamina I) of the rat spinal cord. *Journal of Comparative Neurology, 244(1):53-71.*

Lima, D., Coimbra, A. (1988). The spinothalamic system of the rat: structural types of retrogradely labelled neurons in the marginal zone (lamina I). *Neuroscience, 27(1):215-230.*

Lima, D., Coimbra, A. (1989). Morphological types of spinomesencephalic neurons in the marginal zone (lamina I) of the rat spinal cord, as shown after retrograde labelling with cholera toxin subunit B. *Journal of Comparative Neurology, 279(2):327-339. Erratum in: Journal of Comparative Neurology (1989) 286(4):542.*

Lima, D., Coimbra, A. (1991).Neurons in the substantia gelatinosa rolandi (lamina II) project to the caudal ventrolateral reticular formation of the medulla oblongata in the rat. *Neuroscience Letters, 132(1):16-18.*

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

Lima, D., Avelino, A., Coimbra, A. (1993). Morphological characterization of marginal (lamina I) neurons immunoreactive for substance P, enkephalin, dynorphin and gamma-aminobutyric acid in the rat spinal cord. *Journal of Chemical Neuroanatomy, 6(1):43-52.*

Lima, D. (2007). Ascending Pathways: Anatomy and PhysiologyIn Bushnell, M.C and Basbaum, A.I. (Eds.), Pain, p 477-518. New York, NY: Academic Press.

Lindenlaub, T., Sommer, C. (2000). Partial sciatic nerve transection as a model of neuropathic pain: a qualitative and quantitative neuropathological study. *Pain, 89: 97-106*.

Littlewood, N.K., Todd, A.J., Spike. R.C., Watt. C., Shehab. S.A. (1995).The types of neuron in spinal dorsal horn which possess neurokinin-1 receptors. *Neuroscience, 66(3):597-608.*

Liu, H., Wang, H., Sheng, M., Jan, L.Y., Jan, Y.N., Basbaum, A.I. (1994). Evidence for pre-synaptic NMDA autoreceptors in the spinal cord dorsal horn. *Proceedings of the National Academy of Sciences (PNAS USA), 91: 8383-8387.*

Liu, H., Mantyh, P.W., Basbaum, A.I. (1997). NMDA receptor regulation of sP release from primary afferent nociceptors. *Nature 386: 721-724.*

Liu, H., Brown, J.L., Jasmin, L., Maggio, J.E., Vigna, S.R., Mantyh, P.W., Basbaum, A.I. (1994). Synaptic relationship between substance P and the substance P receptor: light and electron microscopic characterization of the

mismatch between neuropeptides and their receptors. *Proceedings of the National Academy of Sciences (PNAS USA), 91(3):1009-1013.*

Liu, M., Willmott, N.J., Michael, G.J., Priestley, J.V. (2004). Differential pH and capsaicin responses of Griffonia simplicifolia IB_4 (IB_4)-positive and IB_4-negative small sensory neurons. *Neuroscience, 127(3):659-672.*

Lopez-Garcia, J.A, King, A,E. (1994). Membrane properties of physiologically classified rat dorsal horn neurons in vitro: correlation with cutaneous sensory afferent input. *European Journal of Neuroscience, 6(6):998-1007*.

Lu, C.R., Hwang, S.J., Phend, K.D., Rustioni, A., Valtschanoff, J.G. (2003). Primary afferent terminals that express presynaptic NR1 in rats are mainly from myelinated, mechanosensitive fibres. *Journal of Comparative Neurology, 460: 191-202.*

Lu, J., Zhou, X.F., Rush, R.A. (2001). Small primary sensory neurons innervating epidermis and viscera display differential phenotype in the adult rat. *Neuroscience Research, 41(4):355-363.*

Lu,Y.,Perl, E.R. (2005). Modular organization of excitatory circuits between neurons of the spinal superficial dorsal horn (Lamina I and II). *The Journal of Neuroscience 25(15): 3900-3907.*

Lu,Y.,Perl, E.R. (2003). A specific inhibitory pathway between substantia Gelatinosa neurons receiving direct c-fiber input. *The Journal of neuroscience 23(25): 8752-8758.*

Lu Y, Perl ER. (2005). Modular organization of excitatory circuits between neurons of the spinal superficial dorsal horn (laminae I and II). *Journal of Neuroscience, 25(15):3900-3907.*

Luccarini, P., Childeric, A., Gaydier, A.M., Voisin, D., Dallel, R. (2006). The orofacial formalin test in the mouse: a behavioral model for studying physiology and modulation of trigeminal nociception. *Journal of Pain, 7(12):908-914.*

Lucifora, S., Willcockson, H.H., Lu, C.R., Darstein, M., Phend, K.D., Valtschanoff, J.G., Rustioni, A. (2006). Presynaptic low-and high affinity kianate receptors in nociceptive spinal afferents. *Pain 120: 97-105.*

Luo, W., Wickramasinghe, S.R., Savitt, J.M., Griffin, J.W., Dawson, T.M,, Ginty, D.D. (2007). A hierarchical NGF signalling cascade controls Ret-dependent and Ret-independent events during development of non-peptidergic DRG neurons. *Neuron, 54(5):739-754.*

Lynn, B., Carpenter, S.E. (1982). Primary afferent units from the hairy skin of the rat hind limb. *Brain Research, 238(1):29-43.*

Lykissas, M.G., Batistatou, A.K., Charalabopoulos, K.A., Beris, A.E. (2007). The role of neurotrophins in axonal growth, guidance, and regeneration. *Current Neurovascular Research, 4(2):143-51 (Review).*

M

Ma,W., Bisby, M.A. (1998). Increase of preprotachykinin mRNA and substance P immunoreactivity in spared dorsal root ganglion neurons following partial sciatic nerve injury. *European Journal of Neuroscience, 10(7):2388-2399.*

Malcangio, M. Bowery, N.G. (1993). $GABA_B$ but not $GABA_A$ receptor activation inhibits electrically evoked sP-LI release from rat spinal cord in vitro. *The journal of pharmacology and experimental therapeutics 266 (3): 1490-1496.*

Mantyh, P.W., Rogers, S.D., Honore, P., Allen, B.J., Ghilardi, J.R., Li, J., Daughters, R.S., Lappi D.A., Wiley, R.G, Simone. D.A. (1997). Inhibition of hyperalgesia by ablation of lamina I spinal neurons expressing the substance P receptor. *Science, 278(5336):275-279.*

Mantyh, P.W. (2002). Neurobiology of substance P and the NK1 receptor. *Journal of Clinical Psychiatry.;63 Suppl 11:6-10.*

Marchand, J.E., Wurm, W.H., Kato, T., Kream, R.M. (1994). Altered tachykinin expression by dorsal root ganglion neurons in a rat model of neuropathic pain. *Pain, 58(2):219-231*

Marmigère, F., Ernfors, P. (2007). Specification and connectivity of neuronal subtypes in the sensory lineage. *Nature Reviews Neuroscience, 8(2):114-27 (Review).*

Marshall, G.E., Shehab, S.A., Spike, R.C., Todd, A.J. (1996). Neurokinin-1 receptors on lumbar spinothalamic neurons in the rat. *Neuroscience, 72(1):255-263.*

Martindale, J, Bland-Ward, P.A, Chessell, I.P., (2001). Inhibition of C-fibre mediated sensory transmission in the rat following intraplantar formalin. *Neuroscience Letters, 316(1):33-36.*

Martin-Schild, S., Gerall, A.A., Kastin, A.J., Zadina, J.E. (1998). Endomorphin-2 is an endogenous opioid in primary sensory afferent fibers. *Peptides, 19(10):1783-1789.*

Marvizón, J.C., Martínez, V., Grady, E.F., Bunnett, N.W., Mayer, E.A. (1997). Neurokinin 1 receptor internalization in spinal cord slices induced by dorsal root stimulation os mediated by NMDA receptors. *Journal of Neuroscience 17: 8129-8136.*

Matsuka, Y., Edmonds, B., Mitrirattanakul, S., Schweizer, F.E., Spigelman, I. (2007). Two types of neurotransmitter release patterns in isolectin B_4-positive and negative trigeminal ganglion neurons. *Neuroscience, 144(2):665-674.*

Mattson, M.P. (2003). Excitotoxic and excitoprotective mechanisms: abundant targets for the prevention and treatment of neurodegenerative disorders. *Neuromolecular Medicine, 3(2):65-94 (Review).*

Maves, T.J., Pechman, P.S., Gebhart, G.F., Meller, S.T. (1993).
Possible chemical contribution from chromic gut sutures produces disorders of pain sensation like those seen in man. *Pain, 54(1):57-69. Erratum in: Pain (1993) 55(1):131-134*

Maxwell, D.J and Réthelyi, M. (1987). Ultrastructure and synaptic connections of cutaneous afferent fibres in the spinal cord. *Trends in Neuroscience. 10:117-123.*

Maxwell, D.J., Belle, M.D., Cheunsuang. O., Stewart. A., Morris. R. (2007). Morphology of inhibitory and excitatory interneurons in superficial laminae of the rat dorsal horn. *Journal of Physiology, 584(Pt 2):521-533.*

Mazzer, P.Y., Barbieri ,C.H., Mazzer, N., Fazan, V.P. (2008). Morphologic and morphometric evaluation of experimental acute crush injuries of the sciatic nerve of rats. *Journal of Neuroscience Methods, 173(2):249-258.*

McCall, W.D., Tanner, K.D., Levine, J.D. (1996). Formalin induces biphasic activity in C-fibers in the rat. Neuroscience Letters, 208(1):45-48.

McCarthy, P.W., Lawson, S.N. (1989). Cell type and conduction velocity of rat primary sensory neurons with substance P-like immunoreactivity. *Neuroscience, 28(3):745-753.*

McCarthy, P.W., Lawson, S.N. (1990). Cell type and conduction velocity of rat primary sensory neurons with calcitonin gene-related peptide-like immunoreactivity. *Neuroscience, 34(3):623-632.*

McLeod, A.L., Krause, J.E., Cuello, A.C., Ribeiro-da-Silva, A. (1998).
Preferential synaptic relationships between substance P-immunoreactive boutons and neurokinin 1 receptor sites in the rat spinal cord. *Proceedings of the National Academy of Sciences (PNAS USA), 95(26):15775-15780.*

McGaraughty, S., Jarvis, M.F. (2005). Antinociceptive properties of a non-nucleotide P2X3/P2X2/3 receptor antagonist. *Drug News Perspectives, 18(8):501-507 (Review).*

McGaraughty, S., Wismer, C.T., Zhu, C.Z., Mikusa, J., Honore, P., Chu, K.L., Lee, C.H., Faltynek, C.R., Jarvis, M.F. (2003). Effects of A-317491, a novel and selective $P2X_3/P2X_{2/3}$ receptor antagonist, on neuropathic, inflammatory and

chemogenic nociception following intrathecal and intraplantar administration. *British Journal of Pharmacology, 140(8):1381-1388.*

Meinertzhagen, I.A., Takemura, S.Y., Lu, Z., Huang, S., Gao, S., Ting, C.Y., Lee, C.H. (2009). From Form to Function: the Ways to Know a Neuron. *Journal of Neurogenetics, 1-10. (E-publication ahead of print).*

Melnick, I.V., Santos, S.F., Safronov, B.V. (2004a). Mechanism of spike frequency adaptation in substantia gelatinosa neurones of rat. *Journal of Physiology, 559(Pt 2):383-395.*

Melnick, I.V., Santos, S.F., Szokol. K., Szûcs, P., Safronov, B.V. (2004b). Ionic basis of tonic firing in spinal substantia gelatinosa neurons of rat. *Journal of Neurophysiology, 91(2):646-655.*

Meisner JG, Reid AR, Sawynok J. (2008). Adrenergic regulation of P2X3 and TRPV1 receptors: differential effects of spared nerve injury. *Neuroscience Letters, 444(2):172-175.*

Melzack, R., Casey, K.L. (1968) Sensory, motivational, and central control determinants of pain. In: Kenshalo DR (ed) The skin, senses. CC Thomas, Springfield, pp 423–439.

Melzack, R., Coderre, T.J., Katz, J., Vaccarino, A.L. (2001). Central neuroplasticity and pathological pain. *Annals of the New York Academy of Sciences, 933:157-174 (Review).*

Merighi, A., Polak, J.M., Theodosis, D.T. (1991). Ultrastructural visualization of glutamate and aspartate immunoreactivities in the rat dorsal horn, with special reference to the co-localization of glutamate, substance P and calcitonin-gene related peptide. *Neuroscience, 40(1):67-80.*

Merskey, H and Bogduk N. (Eds.) (1994). Internationa Association of the Study of Pain (IASP). Classification of Chronic Pain, (2nd ed.). Part III: Pain Terms, A Current List with Definitions and Notes on Usage. IASP Task Force on Taxonomy, IASP Press, Seattle, 209-214.

Millan, M.J., (1999). The induction of pain: An integrative review. *Progress in Neurobiology, 57, 1-164.*

Miller, F.D., Speelman, A., Mathew, T.C., Fabian, J., Chang, E., Pozniak, C., Toma, J.G. (1994). Nerve growth factor derived from terminals selectively increases the ratio of p75 to trkA NGF receptors on mature sympathetic neurons. *Developmental Biology, 161(1):206-217.*

Mills, C.D., Allchorne, A.J., Griffin, R.S., Woolf, C.J, Costigan, M. (2007). GDNF selectively promotes regeneration of injury-primed sensory neurons in the lesioned spinal cord. *Molecular and Cellular Neuroscience, 36(2):185-194.*

Molander, C., Ygge, J., Dalsgaard, C.J. (1987). Substance P-, somatostatin- and calcitonin gene-related peptide-like immunoreactivity and fluoride resistant acid phosphatase-activity in relation to retrogradely labeled cutaneous, muscular and visceral primary sensory neurons in the rat. *Neuroscience Letters, 74(1):37-42.*

Molander, C., Wang, H.F., Rivero-Melián, C., Grant, G. (1996). Early decline and late restoration of spinal cord binding and transganglionic transport of isolectin B_4 from *Griffonia simplicifolia* I after peripheral nerve transection or crush. *Restorative Neurology and Neuroscience, 10: 123-133.*

Molliver, D.C., Radeke, M.J., Feinstein, S.C., Snider, W.D. (1995). Presence or absence of TrkA protein distinguishes subsets of small sensory neurons with unique cytochemical characteristics and dorsal horn projections. *Journal of Comparative Neurology, 361(3):404-416.*

Molliver, D.C., Wright, D.E., Leitner, M.L., Parsadanian, A.S., Doster, K., Wen, D., Yan, Q., Snider, W.D. (1997). IB_4-binding DRG neurons switch from NGF to GDNF dependence in early postnatal life. *Neuron, 19(4):849-861.*

Molliver, D.C, Snider, W.D. (1997). Nerve growth factor receptor TrkA is down-regulated during postnatal development by a subset of dorsal root ganglion neurons. *Journal of Comparative Neurology, 381(4):428-438.*

Moore, K.A., Kohno, T., Karchewski, L.A., Scholz, J., Baba, H., Woolf, C.J. (2002) Partial peripheral nerve injury promotes a selective loss of GABAergic inhibition in the superficial dorsal horn of the spinal cord. *Journal of Neuroscience, 22(15):6724-6731.*

Mosconi T, Kruger L. (1996). Fixed-diameter polyethylene cuffs applied to the rat sciatic nerve induce a painful neuropathy: ultrastructural morphometric analysis of axonal alterations. *Pain, 64(1):37-57.*

Munger, B.L, Ide, C. (1988). The structure and function of cutaneous sensory receptors. *Archives in Histology and Cytology, 51(1):1-34 (Review).*

Murinson, B.B., Hoffman, P.N., Banihashemi, M.R., Meyer, R.A., Griffin, J.W. (2005)
C-fiber (Remak) bundles contain both isolectin B4-binding and calcitonin gene-related peptide-positive axons. *Journal of Comparative Neurology*, *484(4):392-402.*

Murphy, L.A., Goldstein, I.J. (1977). Five a-D-galactopyranosil-binding isolectins from bandeireae simplicifolia seeds. *Journal of biological chemistry. 252: 4749-4742.*

N

Naim, M., Spike, R.C., Watt, C., Shehab, S.A., Todd, A.J. (1997). Cells in laminae III and IV of the rat spinal cord that possess the neurokinin-1 receptor and have dorsally directed dendrites receive a major synaptic input from tachykinin-containing primary afferents. *Journal of Neuroscience, 17(14):5536-5548.*

Nagano, M., Sakai, A., Takahashi, N., Umino, M., Yoshioka, K., Suzuki, H. (2003). Decreased expression of glial cell line-derived neurotrophic factor signaling in rat models of neuropathic pain. *British Journal of Pharmacology, 140(7):1252-60. Erratum in: British Journal of Pharmacology, (2004). 141(7):1235.*

Nagy, J.I., Hunt, S.P. (1982). Fluoride-resistant acid phosphatase-containing neurones in dorsal root ganglia are separate from those containing substance P or somatostatin. *Neuroscience, 7(1):89-97.*

Nagy, J.I., Hunt, S.P., Iversen, L.L., Emson, P.C. (1981). Biochemical and anatomical observations on the degeneration of peptide-containing primary afferent neurons after neonatal capsaicin. *Neuroscience, 6 :1923-1934.*

Nagy, J.I., Vincent, S.R., Staines, W.A., Fibiger, H.C., Reisine, T.D., Yamamura, H.I. (1980). Neurotoxic action of capsaicin on spinal substance P neurons. *Brain Research 186: 435-444.*

Nagy, J.I., Iversen, L.L., Goedert, M., Chapman, D., Hunt, S.P. (1983) Dose-dependent effects of capsaicin in primary sensory neurons in the neonatal rat. *Journal of Neuroscience, 3: 399-406.*

Nahin, R.L., Humphrey, E., Hylden, J.L. (1991). Evidence for calcitonin gene-related peptide contacts on a population of lamina I projection neurons. *Journal of Chemical Neuroanatomy, 4(2):123129.*

Nakamura, S., Atsuta, Y. (2004). Electrophysiological study on primary afferent properties of a chronic constriction nerve injury model in spinal rats. *Journal of Orthopedic Science, 9(4):386-391.*

Nakagawa, F., Schulte, B.A., Spicer, S.S. (1986). Lectin cytochemical evaluation of somatosensory neurons and their peripheral and central processes in rat and man. *Cell and Tissue research. 245: 579-589.*

Nakagawa, H., Hiura, A. (2006). Capsaicin, transient receptor potential (TRP) protein subfamilies and the particular relationship between capsaicin receptors and small primary sensory neurons. *Anatomical Science International,81(3):135-155 (Review).*

Nakanishi, S. (1991). Mammalian tachykinin receptors. *Annual Review Neuroscience, 14:123-136 (Review).*

Natali, J-P., (1994). Pain: Beyond all paradoxes. In The Puzzle of Pain. (pp. 3-15). Australia: G&B Arts International Limited

Neugebauer, V., Li, W., Bird, G.C., Han, J.S. (2004). The amygdala and persistent pain. *Neuroscientist, 10(3):221-234 (Review).*

Neubert, J.K., Widmer, C.G., Malphurs, W., Rossi, H.L., Vierck, C.J. Jr., Caudle, R.M. (2005). Use of a novel thermal operant behavioral assay for characterization of orofacial pain sensitivity. *Pain, 116(3):386-395.*

Newman HM, Stevens RT, Apkarian AV. (1996). Direct spinal projections to limbic and striatal areas: anterograde transport studies from the upper cervical spinal cord and the cervical enlargement in squirrel monkey and rat. *Journal of Comparative Neurology, 365(4):640-658*

O

O'Brien, C, Woolf, C.J., Fitzgerald, M., Lindsay, R.M., Molander, C. (1989). Differences in the chemical expression of rat primary afferent neurons which innervate skin, muscle or joint. *Neuroscience, 32(2):493-502.*

Obata, K., Yamanaka, H., Fukuoka, T., Y,i D., Tokunaga, A., Hashimoto, N., Yoshikawa, H., Noguchi, K. (2003). Contribution of injured and uninjured dorsal root ganglion neurons to pain behavior and the changes in gene expression following chronic constriction injury of the sciatic nerve in rats. *Pain, 101(1-2):65-77.*

Oh, E.J., Yoon, Y.W., Lee, S.E., Hong, S.K. (2000). Changes in nerve growth factor levels in dorsal root ganglia and spinal nerves in a rat neuropathic pain model. *Experimental Brain Research, 130: 93-99.*

Ohara, P.T., Vit, J.P., Jasmin, L. (2005). Cortical modulation of pain. *Cellular and Molecular Life Science, 62(1):44-52 (Review).*

Ohta, K., Inokuchi, T., Gen, E., Chang, J. (2001). Ultrastructural study of anterograde transport of glial cell line-derived neurotrophic factor from dorsal root ganglion neurons of rats towards the nerve terminal. *Cells Tissues Organs, 169(4):410-421.*

Okazawa, M., Inoue, W., Hori, A., Hosokawa, H., Matsumura, K., Kobayashi, S. (2004). Noxious heat receptors present in cold-sensory cells in rats. *Neuroscience Letters, 359: 33-36.*

Oliveira, A.L., Hydling, F., Olsson, E., Shi, T., Edwards, R.H., Fujiyama, F., Kaneko, T., Hökfelt, T., Cullheim, S., Meister, B. (2003). Cellular localization of three vesicular glutamate transporter mRNAs and proteins in rat spinal cord and dorsal root ganglia. *Synapse, 50(2):117-129.*

Orozco, O.E., Walus, L., Sah, D.W.Y., Pepinsky, B.R., Sanicola, M. (2001). GFR alpha 3 is expressed predominantly in nociceptive sensory neurons. *European Journal of Neuroscience ,13(11): 2177-2182.*

P

Palermo, N.N., Brown, H.K., Smith, D.L. (1981). Selective neurotoxic action of capsaicin on glomerular C-type terminals in rat substantia Gelatinosa. *Brain Research, 208: 506-510.*

Papka, R.E. (1990). Some nerve endings in the rat pelvic paracervical autonomic ganglia and varicosities in the uterus contain calcitonin gene-related peptide and originate from dorsal root ganglia. *Neuroscience. 39(2):459-457.*

Pan YZ, Pan HL. (2004). Primary afferent stimulation differentially potentiates excitatory and inhibitory inputs to spinal lamina II outer and inner neurons. *Journal of Neurophysiology, 91(6):2413-2421.*

Pedersen, S.F, Owsianik, G., Nilius, B. (2005). TRP channels: an overview. *Cell Calcium, 38(3-4):233-252. (Review).*

Perl, E.R. (2007). Ideas about pain, a historical view. *Nature Review Neuroscience, 8(1):71-80. (Review).*

Perry, M.J., Lawson, S.N. (1998). Differences in expression of oligosaccharides, neuropeptides, carbonic anhydrase and neurofilament in rat primary afferent neurons retrogradely labelled via skin, muscle or visceral nerves. *Neuroscience, 85(1):293-310*

Pelissier T, Pajot J, Dallel R. (2002). The orofacial capsaicin test in rats: effects of different capsaicin concentrations and morphine. *Pain, 96(1-2):81-87.*

Peltier, A.C., Russell, J.W. (2002). Recent advances in drug-induced neuropathies. *Current Opinion in Neurology, 15(5):633-638 (Review).*

Petrenko, A.B, Shimoji, K. (2001). A possible role for glutamate receptor-mediated excitotoxicity in chronic pain. *Journal of Anesthesiology, 15(1):39-48.*

Petruska, J.C., Streit, W.J., Johnson, R.D. (1997). Localization of unmyelinated axons in rat skin and mucocutaneous tissue utilizing the isolectin GS-I-B_4. *Somatosensory and Motor Research, 14(1):17-26.*

Petruska, J.C., Cooper, B.Y., Gu, J.G., Rau, K.K., Johnson, R.D. (2000). Distribution of $P2X_1$, $P2X_2$, and $P2X_3$ receptor subunits in rat primary afferents: relation to population markers and specific cell types. *Journal of Chemical Neuroanatomy, 20(2):141-162.*

Petruska, J.C., Napaporn, J., Johnson, R.D., Gu, J.G., Cooper, B.Y. (2002). Chemical responsiveness and histochemical phenotype of electrophysiologically classified cells of the adult rat dorsal root ganglion. *Neuroscience, 115(1):15-30.*

Pignatelli, D., Ribeiro-da-Silva, A., Coimbra, A. (1989) .Postnatal maturation of primary afferent terminations in the substantia gelatinosa of the rat spinal cord. An electron microscopic study. *Brain Research, 491(1):33-44.*

Pitcher, G.M., Ritchie, J., Henry, J.L. (1999). Nerve constriction in the rat: model of neuropathic, surgical and central pain. *Pain; 83(1):37-46.*

Plenderleith, M.B., Snow, P.J. (1990). The effect of peripheral nerve section on lectin binding in the superficial dorsal horn of the rat spinal cord. *Brain Research, 507: 146-150.*

Plenderleith, M.B., Haller, C.J., Snow, P.J. (1990). Peptide coexistence in axon terminals within the superficial dorsal horn of the rat spinal cord. *Synapse, 6(4):344-350*

Plenderleith, M.B., Wright, L.L., Snow, P.J. (1992). Expression of lectin binding in the superficial dorsal horn of the rat spinal cord during pre- and postnatal development. *Developmental Brain Research, 68: 103-109.*

Plenderleith, M.B., Snow, P.J. (1993). The plant lectin Bandeiraea simplicifolia I-B4 identifies a subpopulation of small diameter primary sensory neruones wich innervate the skin in the rat. *Neuroscience letters 159: 17-20.*

Pohl, M., Benoliel, J.J., Bourgoin, S., Lombard, M.C., Mauborgne, A., Taquet, H., Carayon, A., Besson, J.M., Cesselin, F., Hamon, M. (1990). Regional distribution of calcitonin gene-related peptide-, substance P-, cholecystokinin-, Met5-enkephalin-, and dynorphin A (1-8)-like materials in the spinal cord and dorsal root ganglia of adult rats: effects of dorsal rhizotomy and neonatal capsaicin. *Journal of Neurochemistry, 55(4):1122-1130.*

Polgár, E., Hughes, D.I., Riddell, J.S., Maxwell, D.J., Puskár, Z., Todd, A.J. (2003). Selective loss of spinal GABAergic or glycinergic neurons is not necessary for development of thermal hyperalgesia in the chronic constriction injury model of neuropathic pain. *Pain, 104(1-2):229-239.*

Polgár, E., Gray, S., Riddell, J.S., Todd, A.J. (2004). Lack of evidence for significant neuronal loss in laminae I-III of the spinal dorsal horn of the rat in the chronic constriction injury model. *Pain, 111(1-2):144-150.*

Porter, R. (1994). Pain: Beyond all paradoxes. In The Puzzle of Pain. (pp. 98-119). Australia: G&B Arts International Limited

Prescott SA, De Koninck Y. (2002). Four cell types with distinctive membrane properties and morphologies in lamina I of the spinal dorsal horn of the adult rat. *Journal of Physiology, 15;539(Pt 3):817-836.*

Price, D.D., Hayashi, H., Dubner, R., Ruda, M.A. (1979). Functional relationships between neurons of marginal and substantia gelatinosa layers of primate dorsal horn. *Journal of Neurophysiology, 42(6):1590-1608.*

Price, D.D. (2000). Psychological and neural mechanisms of the affective dimension of pain. *Science, 288(5472):1769-1772 (Review).*

Price, D.D. (2002). Central neural mechanisms that interrelate sensory and affective dimensions of pain. *Molecular Intervention, 2(6):392-403, 339 (Review).*

Price, G.W., Wilkin, G.P., Turnbull, M.J., Bowery, N.G. (1984). Are baclofen-sensitive GABAB receptors present on primary afferent terminals of the spinal cord? *Nature, 307(5946):71-74.*

Price, T.J and Flores, C.M. (2007). Critical evaluation of the colocalization between CGRP, sP, TRPV1 and IB_4 binding in primary afferent neurons in the rat and mouse. *Journal of Pain, 8(3):263-272.*

Priestley, J.V., Michaels, G.J., Averill, S., Liu, M., Willmott, N. (2002). Regulation of nociceptive neurons by nerve growtyh factor and glial derived neurotrophic factor. *Canadian Journal of Pharmacology 80, 495-505.*

Puskár, Z., Polgár, E., Todd, A.J. (2001). A population of large lamina I projection neurons with selective inhibitory input in rat spinal cord. *Neuroscience, 102(1):167-176.*

Q

Quartara L, Maggi CA. (1997). The tachykinin NK1 receptor. Part I: ligands and mechanisms of cellular activation. Neuropeptides, 31(6):537-563 (Review).

Quartara L, Maggi CA. (1998). The tachykinin NK1 receptor. Part II: Distribution and pathophysiological roles. Neuropeptides, 32(1):1-49 (Review).

R

Rahman, W., Sikander, S., Suzuki ,R., Hunt, S.P., Dickenson, A.H. (2007). Superficial NK1 expressing spinal dorsal horn neurones modulate inhibitory neurotransmission mediated by spinal GABA (A) receptors. *Neuroscience Letters, 419(3):278-283.*

Ramien,M., Ruocco, I., Cuello, A.C., St-Louis, M., Ribeiro-da-Silva, A. (2004). Parasympathetic nerve fibers invade the upper dermis following sensory denervation of the rat lower lip skin. *Journal of Comparative Neurology, 469(1):83-95.*

Reid, G. (2005). ThermoTRP channels and cold sensing: what are they really up to? *Pflügers Archive, 451: 250-263.*

Ren, K. (1999). An improved method for assessing mechanical allodynia in the rat. *Physiology and Behavior, 67(5):711-716.*

Réthelyi, M., Szentágothai, J. (1969).The large synaptic complexes of the substantia gelatinosa. *Experimental Brain Research, 7(3):258-274.*

Rethelyi, M. (1981). The modular construction of the neuropil in the substantia gelatinosa of the cat's spinal cord: A computer aided analysis of Golgi specimens. *Acta Morphologica Acadamiae Scientium Hungaricae, 29(1):1-18.*

Ribeiro-da-Silva, A., Coimbra, A. (1980). Neuronal uptake of $^{[3H]}$GABA and $^{[3H]}$glycine in laminae I-III (substantia gelatinosa Rolandi) of the rat spinal cord. An autoradiographic study. *Brain Research***,** *188(2):449-464.*

Ribeiro-da-Silva, A., Coimbra, A (1981). Effects of dorsal rhizotomy on the two types of synaptic glomeruli in lamina II-II of the rat spinal cord. *Neuroscience Letters Supplemental, 10 S407*

Ribeiro-da-Silva, A., Coimbra, A. (1982). Two types of synaptic glomeruli and their distribution in laminae I-III of the rat spinal cord. *Journal of Comparative Neurology, 209 :176-186.*

Ribeiro-da-Silva, A., Coimbra, A. (1984). Capsaicin causes selective damage to type I synaptic glomeruli in rat substantia gelatinosa *Brain Research*, *290(2):380-383.*

Ribeiro-da-Silva, A., Pignatelli, D., Coimbra. A. (1985). Synaptic architecture of glomeruli in superficial dorsal horn of rat spinal cord, as shown in serial reconstructions. *Journal of Neurocytology, 14(2):203-220.*

Ribeiro-da-Silva, A., Castro-Lopes, J.M., Coimbra, A. (1986). Distribution of glomeruli with fluoride-resistant acid phosphatase (FRAP)-containing terminals in the substantia gelatinosa of the rat. *Brain Research, 377(2):323-329.*

Ribeiro-da-Silva A, Tagari P, Cuello AC. (1989). Morphological characterization of substance P-like immunoreactive glomeruli in the superficial dorsal horn of the rat spinal cord and trigeminal subnucleus caudalis: a quantitative study. *Journal of Comparative Neurology, 281(4):497-515.*

Ribeiro-da-Silva, A., Kenigsberg, R.L., Cuello, A.C. (1991 A). Light and electron microscopic distribution of nerve growth factor receptor-like immunoreactivity in the skin of the rat lower lip. *Neuroscience , 43(2-3):631-646*.

Ribeiro-da-Silva, A, Pioro, E.P., Cuello, A.C. (1991 B) Substance P- and enkephalin-like immunoreactivities are colocalized in certain neurons of the substantia gelatinosa of the rat spinal cord: an ultrastructural double-labeling study. *Journal of Neuroscience, 11(4):1068-1080.*

Ribeiro-da-Silva, A., Priestley, J.V., Cuello, A.C. (1993). Pre-embedding ultrastructural immunocytochemistry. In: Cuello, A. C. (Ed.), Immunohistochemistry II. John Wiley & Sons, Chichester, pp. 181-227.

Ribeiro-da-Silva, A. (1995a). Ultrastructural features of the colocalization of calcitonin gene related peptide with substance P or somatostatin in the dorsal horn of the spinal cord. *Canadian Journal of Physiology and Pharmacology, 73(7):940-944.*

Ribeiro-da-Silva, A., Cuello, A.C. (1995b). Organization of peptidergic neurons in the dorsal horn of the spinal cord: anatomical and functional correlates. *Progress in Brain Research, 104:41-59 (Review).*

Ribeiro-da-Silva, A., Hökfelt, T. (2000 b). Neuroanatomical localisation of Substance P in the CNS and sensory neurons. *Neuropeptides, 34(5):256-271 (Review).*

Ribeiro-da-Silva, A. (2004). The Substantia Gelatinosa of the rat spinal cord. In G. Paxinos (Ed): The Rat Nervous System, 3rd edition. Sydney: Academic Press.

Ribeiro-da-Silva, A and De Koninck, Y. (2008). Morphological and neurochemical organization of the spinal dorsal horn. In Bushnell, M.C and Basbaum, A.I. (Eds.), Pain, p 279-310. New York, NY: Academic Press.

Rigaud M, Gemes G, Barabas ME, Chernoff DI, Abram SE, Stucky CL, Hogan QH.(2008). Species and strain differences in rodent sciatic nerve anatomy: implications for studies of neuropathic pain. *Pain, 136(1-2):188-201.*

Riley, R.C., Trafton, J.A., Chi, S.I., Basbaum, A.I. (2001). Presynaptic regulation of spinal cord tachykinin signaling via $GABA_B$ but not $GABA_A$ receptor activation. *Neuroscience 103(3): 725-737.*

Rind, H.B., Von Bartheld, C.S. (2002). Anterograde axonal transport of internalized GDNF in sensory and motor neurons. *NeuroReport, 13: 659-664.*

Ritz, L.A., Greenspan, J.D. (1985). Morphological features of lamina V neurons receiving nociceptive input in cat sacrocaudal spinal cord. *Journal Comparative Neurology, 238(4):440-452.*

Rossi, H.L., Vierck, C.J. Jr., Caudle, R.M., Neubert. J.K. (2006). Characterization of cold sensitivity and thermal preference using an operant orofacial assay. *Molecular Pain, 2:37*.

Rudomin, P., Schmidt, R.F. (1999). Presynaptic inhibition in the vertebrate spinal cord revisited. *Experimental Brain Research, 129(1):1-37. (Review).*

Ruocco, I., Cuello, A.C., Ribeiro-da-Silva, A. (2000). Peripheral nerve injury leads to the establishment of a novel pattern of sympathetic fibre innervation in the rat skin. *Journal of Comparative Neurology, 422(2):287-296.*

Ruocco, I., Cuello, A.C., Shigemoto, R., Ribeiro-da-Silva, A. (2001). Sympathectomies lead to transient substance P-immunoreactive sensory fibre plasticity in the rat skin. *Neuroscience, 108(1):157-166.*

Ruocco, I., Cuello, A.C., Shigemoto, R., Ribeiro-da-Silva, A. (2001). Light and electron microscopic study of the distribution of substance P-immunoreactive fibers and neurokinin-1 receptors in the skin of the rat lower lip. *Journal of Comparative Neurology, 432(4):466-480.*

Ruocco, I., Cuello, A.C., Parent, A., Ribeiro-da-Silva, A. (2002). Skin blood vessels are simultaneously innervated by sensory, sympathetic, and parasympathetic fibers. *Journal of Comparative Neurology, 448(4):323-336.*

Rusin, K.I., Ryu, P.D., Randic, M. (1992). Modulation of excitatory amino acid responses in rat dorsal horn neurons by tachykinins. *Journal of Neurophysiology, 68(1):265-286.*

Rusin, K.I, Jiang, M.C., Cerne, R., Randic, M. (1993). Interactions between excitatory amino acids and tachykinins in the rat spinal dorsal horn. *Brain Research Bulletin, 30(3-4):329-338.*

S

Saeed, A.W. and Ribeiro-da-Silva. Manuscript in preparation.

Sah, D.W.Y., Ossipov, M., Porreca, F. (2003). Neurotrophic factors as novel therapeutics for neuropathic pain. *Nature Reviews Drug Discovery, 2:460-472*.

Santos, S.F., Rebelo, S., Derkach, V.A., Safronov, B.V. (2007). Excitatory interneurons dominate sensory processing in the substantia gelatinosa of the rat. *Journal of Physiology, 581:241-254.*

Sawynok, J., Liu, X.J. (2003). Adenosine in the spinal cord and periphery: release and regulation of pain. *Progress in Neurobiology, 69(5):313-340 (Review).*

Seagrove, L.C., Suzuki, R., Dickenson, A.H. (2004). Electrophysiological characterizations of rat lamina I dorsal horn neurons and the involvement of excitatory amino acid receptors. *Pain 108: 76-87.*

Sedivec, M.J., Capowski, J.J., Mendell, L.M. (1986). Morphology of HRP-injected spinocervical tract neurons: effect of dorsal rhizotomy. *Journal of Neuroscience, 6(3):661-672.*

Sewards TV, Sewards MA. (2002).The medial pain system: neural representations of the motivational aspect of pain. *Brain Research Bulletin, 59(3):163-180 (Review).*

Seybold, V.S., McCarson, K.E., Mermelstein, P.G., Groth, R.D., Abrahams, L.G. (2003).
Calcitonin gene-related peptide regulates expression of neurokinin1 receptors by rat spinal neurons. *Journal of Neuroscience, 23(5):1816-1824.*

Schneider, S.P., Perl, E.R. (1985). Selective excitation of neurons in the mammalian spinal dorsal horn by aspartate and glutamate in vitro: correlation with location and excitatory input. *Brain Research, 360(1-2):339-343.*

Schoenen, J. (1982). The dendritic organization of the human spinal cord: the dorsal horn. *Neuroscience, 7(9):2057-2087.*

Scholz, J., Broom, D.C., Youn, D.H., Mills, C.D., Kohno, T., Suter, M.R., Moore, K.A., Decosterd, I., Coggeshall, R.E., Woolf, C.J. (2005). Blocking caspase activity prevents transsynaptic neuronal apoptosis and the loss of inhibition in lamina II of the dorsal horn after peripheral nerve injury. *Journal of Neuroscience, 25(32):7317-7323.*

Schulte, G., Robertson, B., Fredholm, B.B., DeLander, G.E., Shortland, P., Molander, C. (2003). Distribution of antinociceptive adenosine A1 receptors in the spinal cord dorsal horn, and relationship to primary afferents and neuronal subpopulations. *Neuroscience, 121(4):907-916.*

Sharp CJ, Reeve AJ, Collins SD, Martindale JC, Summerfield SG, Sargent BS, Bate ST, Chessell IP. (2006). Investigation into the role of $P2X_3$ and $P2X_{2/3}$ receptors in neuropathic pain following CCI in the rat: An electrophysiological study. *British Journal of Pharmacology, 148:845-852*.

Shen, H., Chung, J.M., Chung, K. (1999). Expression of neurotrophin mRNAs in the dorsal root ganglion after spinal nerve injury. *Molecular Brain Research, 64: 186-192*.

Shields, S.D., Eckert, W.A. III., Basbaum, A.I. (2003). Spared nerve injury model of neuropathic pain in the mouse: a behavioral and anatomic analysis. *Journal of Pain, 4: 465-470.*

Shigetada, N. (1991). Mammalian Tachykinin Receptors. *Annual Review Neuroscience 14:123-136.*

Shir Y, Seltzer Z. (1990). A-fibers mediate mechanical hyperesthesia and allodynia and C-fibers mediate thermal hyperalgesia in a new model of causalgiform pain disorders in rats. *Neuroscience Letters, 115(1):62-67.*

Silverman, J.D., Kruger, L. (1988a). Lectin and neuropeptide labeling of separate populations of dorsal root ganglion neurons and associated "nociceptor" thin axons in rat testis and cornea whole-mount preparations. *Somatosensory Research, 5(3):259-267*.

Silverman, J.D., Kruger, L. (1988b) Acid phosphatase as a selective marker for a class of small sensory ganglion cells in several mammals: spinal cord distribution, histochemical properties, and relation to fluoride-resistant acid phosphatase (FRAP) of rodents. *Somatosensory Research 5(3):219-246.*

Silverman, J.D, Kruger, L. (1990). Selective neuronal glycoconjugate expression in sensory and autonomic ganglia: relation of lectin reactivity to peptide and enzyme markers. *Journal of Neurocytology, 19(5):789-801.*

Silos-Santiago, I., Molliver, D.C., Ozaki,S., Smeyne, R.J., Fagan, A.M., Barbacid, M., Snider, W.D. (1995). Non-TrkA-expressing small DRG neurons are lost in TrkA deficient mice. *Journal of Neuroscience, 15(9):5929-5942.*

Simone, D.A., Kajander, K.C. (1997). Responses of cutaneous A-fiber nociceptors to noxious cold. *Journal of Neurophysiology, 77(4):2049-2060.*

Smeyne, R.J., Klein, R., Schnapp, A., Long, L.K., Bryant, S., Lewin, A., Lira, S.A., Barbacid, M. (1994). Severe sensory and sympathetic neuropathies in mice carrying a disrupted Trk/NGF receptor gene. *Nature., 368(6468):246-249.*

Smith, G.D., Seckl, J.R., Harmar, A.J. (1993). Distribution of neuropeptides in dorsal root ganglia of the rat; substance P, somatostatin and calcitonin gene-related peptide. *Neuroscience Letters, 153(1):5-8.*

Snider, W.D., Silos-Santiago, I. (1996). Dorsal root ganglion neurons require functional neurotrophin receptors for survival during development. *Philosophical Transactions of the Royal Society B: Biological, 351(1338):395-403 (Review).*

Sotgiu, M.L., Brambilla, M., Valente, M., Biella, G.E. (2004). Contralateral input modulates the excitability of dorsal horn neurons involved in noxious signal

processes. Potential role in neuronal sensitization. *Somatosensory and Motor Research, 21(3-4):211-215.*

Spike, R.C., Puskár, Z., Andrew, D., Todd, A.J. (2003). A quantitative and morphological study of projection neurons in lamina I of the rat lumbar spinal cord. *European Journal of Neuroscience, 18(9):2433-2448.*

Spike, R.C., Todd, A.J. (1992). Ultrastructural and immunocytochemical study of lamina II islet cells in rat spinal dorsal horn. *Journal of Comparative Neurology, 323(3):359-369.*

Statistics Canada. (2005). Pain or Discomfort that affects activities, by age group and sex, household populations aged 12 and over, Canada, province and territories every 2 years. Canadian Community Health Survey. (CCHS 2.1 and 3.1)

Streit, W.J., Schulte, B.A., Balentine, D.J., Spicer, S.S. (1985). Histochemical localization of galactose-containing glycoconjugates in sensory neurons and their processes in the central and peripheral nervous system of the rat. *Journal of histochemistry and cytochemistry. 33: 1042-1052*

Stucky, C.L., Lewin, G.R. (1999). Isolectin $B_{(4)}$-positive and -negative nociceptors are functionally distinct. *Journal of Neuroscience, 19(15):6497-6505.*

Stucky, C.L., Rossi, J., Airaksinen, M.S., Lewin, G.R. (2002). GFR alpha2/neurturin signaling regulates noxious heat transduction in Isolectin B_4-binding mouse sensory neurons. *Journal of Physiology 545.1 43-50.*

Sugimoto T, Gobel S. (1984). Dendritic changes in the spinal dorsal horn following transection of a peripheral nerve. *Brain Research, 321(2):199-208.*

Sugimoto, T., Takemura, M., Okubo, J., Sakai, A. (1985). Strychnine and L-allylglycine but not bicuculline and picrotoxin induce transsynaptic degeneration following transection of the inferior alveolar nerve in adult rats. Brain Research, 341(2):393-398.

Sugimoto, T., Takemura, M., Sakai, A., Ishimaru, M. (1986).Topical application of colchicine, vinblastine and vincristine prevents strychnine-enhanced transsynaptic degeneration in the medullary dorsal horn following transection of the inferior alveolar nerve in adult rats. *Pain, 27(1):91-100.*

Sugimoto, T., Takemura, M., Sakai, A., Ishimaru, M. (1987a). Strychnine-enhanced transsynaptic destruction of medullary dorsal horn neurones following transection of the trigeminal nerve in adult rats including evidence of involvement of the bony environment of the transection neuroma in the peripheral mechanism. *Archives of Oral Biology, 32(9):623-629.*

Sugimoto, T., Takemura, M., Sakai, A., Ishimaru, M. (1987 b). Rapid transneuronal destruction following peripheral nerve transection in the medullary

dorsal horn is enhanced by strychnine, picrotoxin and bicuculline. *Pain, 30(3):385-393.*

Sugimoto, T., Bennett, G.J., Kajander, K.C .(1989). Strychnine-enhanced transsynaptic degeneration of dorsal horn neurons in rats with an experimental painful peripheral neuropathy. *Neuroscience Letters, 98(2):139-143.*

Sugimoto, T., Bennett, G.J., Kajander, K.C. (1990). Transsynaptic degeneration in the superficial dorsal horn after sciatic nerve injury: effects of a chronic constriction injury, transection, and strychnine. *Pain, 42(2):205-213.*

Sugimoto, T., Yoshida, A., Nishijima, K., Ichikawa, H. (1995). Disruption and restoration of dorsal horn sensory map after peripheral nerve crush and regeneration. *Pain, 63(1):49-54.*

Sugimoto, T., Li, Y.L., Kishimoto, H., Fujita, M., Ichikawa, H. (2000). Compensatory projection of primary nociceptors and c-fos induction in the spinal dorsal horn following neonatal sciatic nerve lesion. *Experimental Neurology, 164: 407-414.*

Suzuki, R., Morcuende, S., Webber, M., Hunt, S.P., Dickenson, A.H. (2002). Superficial NK1-expressing neurons control spinal excitability through activation of descending pathways. *Nature Neuroscience, 5(12):1319-1326.*

Swett, J.E., Woolf, C.J. (1985). The somatotopic organization of primary afferent terminals in the superficial laminae of the dorsal horn of the rat spinal cord. *Journal of Comparative Neurology, 231(1):66-77.*

Szabó, B.K,, Török, A., Knyihár-Csillik, E., Csillik, B. (1989) Mapping the distribution of thiamine monophosphatase, fluoride-resistant acid phosphatase, and substance P in the spinal cord with a personal computer compatible program. *Acta Histochemistry, 87(2):123-9*

Szentágothai, J. (1964). Neuronal and synaptic arrangement in the substantia gelatinosa Rolandi. *Journal of Comparative Neurology, 122:219-239.*

T

Tajti, J., Fischer, J., Knyihár-Csillik, E., Csillik, B. (1988). Transganglionic regulation and fine structural localization of lectin-reactive carbohydrate epitopes in primary sensory neurons of the rat. *Histochemistry, 88(3-6):213-218.*

Takahashi, N., Nagano, M., Suzuki, H., Umino, M. (2003). Expression changes of glial cell line-derived neurotrophic factor in a rat model of neuropathic pain. *Journal of Medical and Dentistry Sciences, 50: 87-92.*

Takamori, S., Rhee, J.S., Rosenmund, C., Jahn, R. (2000). Identification of a vesicular glutamate transporter that defines a glutamatergic phenotype in neurons. *Nature, 407(6801):189-194.*

Tallent, M.K. (2008). Presynaptic inhibition of glutamate release by neuropeptides: use-dependent synaptic modification. *Results and Problems in Cell Differentiation,44:177-200 (Review).*

Tandrup, T. (2004).Unbiased estimates of number and size of rat dorsal root ganglion cells in studies of structure and cell survival. *Journal of Neurocytology, 33(2):173-92 (Review).*

Tanimoto S, Nakagawa T, Yamauchi Y, Minami M, Satoh M. (2003). Differential contributions of the basolateral and central nuclei of the amygdala in the negative affective component of chemical somatic and visceral pains in rats. *European Journal of Neuroscience, 18(8):2343-2350.*

Tarpley, J.W., Kohlet, M.G., Martin, M.J. (2004). The behavioral and neuroanatomical effects of IB_4-saporin treatment in rat models of nociceptive and neuropathic pain. *Brain Research,1029(1):65-76.*

Taylor, A.M., Peleshok, J.C., Ribeiro-da-Silva, A. (2009). Distribution of P2X(3)-immunoreactive fibers in hairy and glabrous skin of the rat. *Journal of Comparative Neurology, 514(6):555-566*

Taylor, B.K., McCarson, K.E. (2004). Neurokinin-1 receptor gene expression in the mouse dorsal horn increases with neuropathic pain. *Journal of Pain. 5(2):71-76.*

Tao, Y.X,, Li, Y.Q., Zhao, Z.Q. (2000). Synaptic interaction between GABAergic terminals and substance P receptor-positive neurons in rat spinal superficial laminae. *Acta Pharmacologica Sinica ,21(10):911-914.*

Tenser, R.B. (1985). Sequential changes of sensory neuron (fluoride-resistant) acid phosphatase in dorsal root ganglion neurons following neurectomy and rhizotomy. *Brain Research, 332(2):386-389*.

Thoenen, H., Barde, Y-A. (1980). Physiology of nerve growth factor. *Physiology Review, 60: 1284-1335.*

Todd, A.J. (1988).Electron microscope study of Golgi-stained cells in lamina II of the rat spinal dorsal horn. *Journal of Comparative Neurology, 275(1):145-157.*

Todd, A.J. (1989). Cells in laminae III and IV of rat spinal dorsal horn receive monosynaptic primary afferent input in lamina II. *Journal of Comparative Neurology, 289(4):676-686.*

Todd AJ. (1996). GABA and glycine in synaptic glomeruli of the rat spinal dorsal horn. *European Journal of Neuroscience, 8(12):2492-2498*

Todd AJ, Watt C, Spike RC, Sieghart W. (1996) Colocalization of GABA, glycine, and their receptors at synapses in the rat spinal cord. *Journal of Neuroscience, 16(3):974-982*

Todd, A.J. (2002). Anatomy of primary afferents and projection neurones in the rat spinal dorsal horn with particular emphasis on substance P and the neurokinin 1 receptor. *Experimental Physiology, 87(2):245-249.*

Todd, A.J., Hughes, D.I., Polgár, E., Nagy, G.G., Mackie, M., Ottersen, O.P., Maxwell, D.J. (2003). The expression of vesicular glutamate transporters VGLUT1 and VGLUT2 in neurochemically defined axonal populations in the rat spinal cord with emphasis on the dorsal horn. *European Journal of Neuroscience, 17(1):13-27.*

Todd, A.J., Lewis, S.G. (1986)**.** The morphology of Golgi-stained neurons in lamina II of the rat spinal cord. *Journal of Anatomy, 149:113-119.*

Todd, A.J., Lochhead, V. (1990). GABA-like immunoreactivity in type I glomeruli of rat substantia gelatinosa. *Brain Research, 514(1):171-174*

Todd, A.J., McGill, M.M., Shehab, S.A. (2000). Neurokinin 1 receptor expression by neurons in laminae I, III and IV of the rat spinal dorsal horn that project to the brainstem. *European Journal of Neuroscience, 12(2):689-700.*

Todd, A.J., McKenzie, J. (1989). GABA-immunoreactive neurons in the dorsal horn of the rat spinal cord. *Neuroscience, 31(3):799-806.*

Todd, A.J., Puskár, Z., Spike, R.C., Hughes, C., Watt, C., Forrest, L. (2002). Projection neurons in lamina I of rat spinal cord with the neurokinin 1 receptor are selectively innervated by substance p-containing afferents and respond to noxious stimulation. *The Journal of Neuroscience, 22(10):4103-4113.*

Todd, A.J., Ribeiro-da-Silva, A. (2005). Textbook: Neurobiology of Pain Chapter 3: Molecular architecture of the dorsal horn P65-95. Oxford University Press.

Todd, A.J., Russell, G., Spike, R.C. (1992). Immunocytochemical evidence that GABA and neurotensin exist in different neurons in laminae II and III of rat spinal dorsal horn. *Neuroscience, 47(3):685-691.*

Todd, A.J., Spike, R.C., Young, S., Puskár, Z. (2005). Fos induction in lamina I projection neurons in response to noxious thermal stimuli. *Neuroscience, 131(1):209-217.*

Todd AJ, Sullivan AC. (1990). Light microscope study of the coexistence of GABA-like and glycine-like immunoreactivities in the spinal cord of the rat. *Journal of Comparative Neurology, 296(3):496-505.*

Tölle, T.R., Berthele, A., Zieglgänsberger, W., Seeburg, P.H., Wisden, W. (1993). The differential expression of 16 NMDA and non-NMDA receptor subunits in the rat spinal cord and in periaqueductal gray. *The Journal of Neuroscience, 13(12):5009-5028.*

Tominaga, M., Caterina, M.J. (2004). Thermosensation and pain. *Journal of Neurobiology, 61: 3-12.*

Tong, C., Conklin, D., Clyne, B.B., Stanislaus, J.D., Eisenach, J.C.M. (2006). Uterine and cervical afferents in thoracolumbar dorsal root ganglia express transient receptor potential vanilloid type 1 channel and Calcitonin Gene–related Peptide, but not P2X3 receptor and somatostatin . *Anesthesiology, 104(4):651-657.*

Traub, R.J., Mendell, L.M.(1988). The spinal projection of individual identified A-delta- and C-fibers. *Journal of Neurophysiology, 59(1):41-55.*

Traub, R.J. (1997). Spinal modulation of the induction of central sensitization. *Brain Research, 778(1):34-42.*

Treede, R.D., Meyer, R.A., Raja, S.N., Campbell, J.N. (1992). Peripheral and central mechanisms of cutaneous hyperalgesia. *Progress in Neurobiology, 38(4):397-421 (Review).*

Treede, R.D., Kenshalo, D.R., Gracely, R.H., Jones, A.K. (1999). The cortical representation of pain. *Pain, 79(2-3):105-111 (Review).*

Trevino DL, Carstens E. (1975). Confirmation of the location of spinothalamic neurons in the cat and monkey by the retrograde transport of horseradish peroxidase. *Brain Research, 98(1):177-182.*

Tsukagoshi, M., Goris, R.C., Funakoshi, K. (2006). Differential distribution of vanilloid receptors in the primary sensory neurons projecting to the dorsal skin and muscles. *Histochemical Cell Biology, 126(3):343-352.*

Tucker, B.A., Rahimtula, M., Mearow, K.M. (2006). Laminin and growth factor receptor activation stimulates differential growth responses in subpopulations of adult DRG neurons. *European Journal of Neuroscience 24(3):676-690.*

Tuchscherer, M.M., Seybold, V.S. (1989). A quantitative study of the coexistence of peptides in varicosities within the superficial laminae of the dorsal horn of the rat spinal cord. *Journal of Neuroscience, 9(1):195-205.*

V

Valtschanoff JG, Rustioni A, Guo A, Hwang SJ. (2001). Vanilloid receptor VR1 is both presynaptic and postsynaptic in the superficial laminae of the rat dorsal horn. *Journal of Comparative Neurology, 436(2):225-235.*

Vierck,C.J. Jr, Kline, R.H, Wiley, R.G. (2003). Intrathecal substance p-saporin attenuates operant escape from nociceptive thermal stimuli. *Neuroscience, 119(1):223-232.*

Vigna, S.R., Bowden, J.J., McDonald, D,M., Fisher, J., Okamoto, A., McVey, D.C., Payan, D.G., Bunnett, N.W. (1994). Characterization of antibodies to the rat substance P (NK-1) receptor and to a chimeric substance P receptor expressed in mammalian cells. *The Journal of Neuroscience, 14(2):834-845.*

Villar, M.J., Cortes, R., Theodorsson, E., Wiesenfeld-Hallin, Z., Schalling, M., Fahrenkrug, J., Emson, P.C., Hökfelt, T. (1989). Neuropeptide expression in rat dorsal root ganglion cells and spinal cord after peripheral nerve injury with special reference to galanin. *Neuroscience, 33: 587-604.*

von Banchet, G.S., Schaible, H.G. (1999). Localization of the neurokinin 1 receptor on a subset of substance P-positive and isolectin B_4-negative dorsal root ganglion neurons of the rat. *Neuroscience Letters, 274(3):175-178.*

von Düring, M., Fricke, B. (2007).Organization of peripheral nerves in skin, musculoskeletal system and viscera. *Progress in Experimental Tumor Research, 39:30-44 (Review).*

Vulchanova, L., Olson, T.H., Stone, L.S., Riedl, M.S., Elde, R., Honda, C.N. (2001). Cytotoxic targeting of isolectin IB_4-binding sensory neurons. *Neuroscience, 108(1):143-155.*

Vulchanova, L., Riedl, M.S., Shuste, S.J., Stone, L.S., Hargreaves, K.M,, Buell, G., Surprenant, A., North, R.A., Elde, R. (1998). $P2X_3$ is expressed by DRG neurons that terminate in inner lamina II. *European Journal of Neuroscience, 10(11):3470-3478.*

Vydyanathan A, Wu ZZ, Chen SR, Pan HL. (2005). A-type voltage-gated K+ currents influence firing properties of isolectin B_4-positive but not isolectin B_4-negative primary sensory neurons. *Journal of Neurophysiology, 93(6):3401-3409.*

W

Wall, P.D. (1980).The role of substantia gelatinosa as a gate control.
Research Publications Association for Research in Nervous and Mental Disease, 58:205-231 (Review).

Wall, P.D. (1978).The properties of substantia gelatinosa units. *Journal of Physiology, 275:71P.*

Wall, P.D., Gutnick, M. (1974). Properties of afferent nerve impulses originating from a neuroma. *Nature, 248(5451):740-743.*

Wall, P.D., Waxman, S., Basbaum, A.I. (1974). Ongoing activity in peripheral nerve: injury discharge. *Experimental Neurology, 45(3):576-589.*

Wang, H.F., Shortland, P., Park, M.J., Grant, G. (1998). Retrograde and transganglionic transport of horseradish peroxidase-conjugated cholera toxin B subunit, wheatgerm agglutinin and isolectin B_4 from Griffonia simplicifolia I in primary afferent neurons innervating the rat urinary bladder. *Neuroscience, 87(1):275-288.*

Weissner, W., Winterson, B.J., Stuart-Tilley, A., Devor, M., Bove, G.M. (2006). Time course of substance P expression in dorsal root ganglia following complete spinal nerve transection. *Journal of Comparative Neurology, 497(1):78-87.*

Wells, M.R., Vaidya, U., Schwartz, J.P. (1994). Bilateral phasic increases in dorsal root ganglia nerve growth factor synthesis after unilateral sciatic nerve crush. *Experimental Brain Research, 101: 53-58.*

Whiteside, G.T., Munglani, R. (2001). Cell death in the superficial dorsal horn in a model of neuropathic pain. *Journal of Neuroscience Research.64(2):168-173.*

Whitman, S.M. (2007). Pain and suffering as viewed by the Hindu religion. *Journal of Pain, 8(8):607-613 (Review).*

Wiley, R.G. (2008). Substance P receptor-expressing dorsal horn neurons: lessons from the targeted cytotoxin, substance P-saporin. *Pain, 136(1-2):7-10.*

Wiley, R.G, Kline, IV R.H. (2000). Neuronal lesioning with axonally transported toxins.
Journal of Neuroscience Methods,103(1):73-82 (Review).

Wiley, R.G., Kline, R.H. 4th, Vierck, C.J. Jr. (2007). Anti-nociceptive effects of selectively destroying substance P receptor-expressing dorsal horn neurons using [Sar9,Met(O2)11]-substance P-saporin: behavioral and anatomical analyses. *Neuroscience,146(3):1333-1345.*

Wiley RG, Lappi DA. (2003). Targeted toxins in pain. *Advanced Drug Delivery Reviews., 55(8):1043-1054 (Review).*

Wiley, R.G., Oeltmann, T.N. (1986). Anatomically selective peripheral nerve ablation using intraneural ricin injection. *Journal Neuroscience Methods, 17(1):43-53.*

Willis, W.D. Jr. (1999). Dorsal root potentials and dorsal root reflexes: a double-edged sword. *Experimental Brain Research, 124(4):395-421 (Review).*

Willis, W.D., Leonard, R.B., Kenshalo, D.R Jr. (1978). Spinothalamic tract neurons in the substantia gelatinosa. *Science, 202(4371):986-988.*

Willis, W.D., Kenshalo, D.R. Jr, Leonard, R.B. (1979). The cells of origin of the primate spinothalamic tract. *Journal of Comparative Neurology, 188(4):543-573.*

Willis, W.D., Westlund, K.N. (1997). Neuroanatomy of the pain system and of the pathways that modulate pain. *Journal of Clinical Neurophysiology 14(1):2-31 (Review).*

Windebank, A.J., Grisold, W. (2008). Chemotherapy-induced neuropathy. *Journal of the Peripheral Nervous System, 13(1):27-46 (Review).*

Winter, M.K., McCarson, K.E. (2005). G-protein activation by neurokinin-1 receptors is dynamically regulated during persistent nociception. *Journal of Pharmacology and Experimental Therapeutics, 315(1):214-221.*

Woolf, C.J., King, A.E. (1987). Physiology and morphology of multireceptive neurons with C-afferent fiber inputs in the deep dorsal horn of the rat lumbar spinal cord. *Journal of Neurophysiology, 58(3):460-479.*

Won, R., Jung, S.J., Park, Y.G., Chung, S.S., Lee, B.H. (2004). Crossed-withdrawal reflex in a rat model of neuropathic pain: implications in neural plasticity. *Neuroscience Letters, 369: 239-244*.

Wright, D.E., Snider, W.D. (1995). Neurotrophin receptor mRNA expression defines distinct populations of neurons in rat dorsal root ganglia. *Journal of Comparative Neurology, 351(3):329-338.*

Wu, A.M., Song, S.C., Wu, J.H., Kabat, E.A. (1995). Affinity of Bandeiraea (Griffonia) simplicifolia lectin-I, isolectin B4 for Gal alpha 1-4 Gal ligand. Biochemical and *Biophysical Research Communications, 216(3):814-820.*

Wu SY, Ohtubo Y, Brailoiu GC, Dun NJ. (2003). Effects of endomorphin on substantia gelatinosa neurons in rat spinal cord slices. *British Journal of Pharmacology, 140(6):1088-1096.*

X

Xu, J., Pollock, C.H., Kajander, K.C. (1996). Chromic gut suture reduces calcitonin-gene-related peptide and substance P levels in the spinal cord following chronic constriction injury in the rat. *Pain, 64(3):503-509.*

Y

Yajiri,Y., Yoshimura, M., Okamoto, M., Takahashi, H., Higashi, H. (1997). A novel slow excitatory postsynaptic current in substantia gelatinosa neurons of the rat spinal cord in vitro. *Neuroscience, 76(3):673-688.*

Yang, K., Feng, Y., Li, Y. (2001). Baclofen inhibition of dorsal root-evoked inhibitory postsynaptic currents in substantia gelatinosa neurons of rat spinal cord slice.*Brain Research, 900(2):320-323.*

Yang, K., Fujita, T., Kumamoto, E. (2004). Adenosine inhibits GABAergic and glycinergic transmission in adult rat substantia gelatinosa neurons. *Journal of Neurophysiology, 92(5):2867-2877.*

Yen, L.D., Bennett, G.J., Ribeiro-da-Silva, A. (2006). Sympathetic sprouting and changes in nociceptive sensory innervation in the glabrous skin of the rat hind paw following partial peripheral nerve injury. *Journal of Comparative Neurology, 495(6):679-690.*

Yoon, Y.S., Hwang, I.K., Lee, I.S., Suh, J.G., Shin, J.W., Kang, T.C., Oh, Y.S., Won, M.H. (2003). Galanin-immunoreactive cells and their relation to calcitonin gene-related peptide-, substance P- and somatostatin-immunoreactive cells in rat lumbar dorsal root ganglia. *Anatomia, Histologia, Embryologia, 32(2):110-115.*

Yoshimura, M., Jessell, T. (1990). Amino acid-mediated EPSPs at primary afferent synapses with substantia gelatinosa neurones in the rat spinal cord. *Journal of Physiology, 430:315-335.*

Yoshimura, M., Nishi, S. (1993). Blind patch-clamp recordings from substantia gelatinosa neurons in adult rat spinal cord slices: pharmacological properties of synaptic currents. *Neuroscience, 53(2):519-526.*

Yoshimura, N., Seki, S., Erickson, K.A., Erickson, V.L., Hancellor, M.B., de Groat, W.C. (2003). Histological and electrical properties of rat dorsal root ganglion neurons innervating the lower urinary tract. *Journal of Neuroscience ,23(10):4355-4361.*

Yu, X.H., Zhang, E.T., Craig, A.D., Shigemoto, R., Ribeiro-da-Silva, A., De Koninck, Y. (1999). NK-1 receptor immunoreactivity in distinct morphological types of lamina I neurons of the primate spinal cord. *Journal of Neuroscience, 19(9):3545-3555.*

Yu, X.H., Ribeiro-da-Silva, A., De Koninck, Y. (2005). Morphology and neurokinin 1 receptor expression of spinothalamic lamina I neurons in the rat spinal cord. *Journal of Comparative Neurology, 491(1):56-68. Erratum in: Journal of Comparative Neurology, 493(3):475.*

Z

Zieglgänsberger, W., Puil, E.A. (1973). Actions of glutamic acid on spinal neurones. *Experimental Brain Research, 17(1):35-49.*

Zhang, E.T., Han, Z.S., Craig, A.D. (1996). Morphological classes of spinothalamic lamina I neurons in the cat. *Journal of Comparative Neurology, 367(4):537-549.*

Zhang, E.T., Craig, A.D. (1997) Morphology and distribution of spinothalamic lamina I neurons in the monkey. *Journal of Neuroscience 17:3274–3284.*

Zhang, Q., Zhao, Y., Guo, Y., Cao, D.Y., Tian, Y.L., Yao, F.R., Wang, H.S. (2006).Electrophysiological evidence for the interaction of substance P and glutamate on A-delta and C afferent fibre activity in rat hairy skin. *Clinical and Experimental Pharmacology and Physiology, 33(12):1128-1133.*

Zhang, X., Nicholas, A.P., Hökfelt, T. (1993). Ultrastructural studies on peptides in the dorsal horn of the spinal cord--I. Co-existence of galanin with other peptides in primary afferents in normal rats. *Neuroscience, 57(2):365-384.*

Zhu, C.G., Sandri, C., Akert, K. (1981). Morphological identification of axo-axonic and dendro-dendritic synapses in the rat substantia gelatinosa. Brain Research, 230(1-2):25-40.

Zhu, C.G, Sandri, C., Akert, K. (1981). Morphological identification of axo-axonic and dendro-dendritic synapses in the rat substantia gelatinosa. *Brain Research, 230(1-2):25-40*

Zhou, H.Y., Zhang, H.M., Chen, S.R., Pan, H.L. (2007). Increased nociceptive input rapidly modulates spinal GABAergic transmission through endogenously released glutamate. *Journal of Neurophysiology, 97(1):871-882.*

Zhou, H.Y., Zhang, H.M., Chen, S.R., Pan, H.L. (2008). Increased C-fiber nociceptive input potentiates inhibitory glycinergic transmission in the spinal dorsal horn. *Journal of Pharmacology and Experimental Therapeutics,324(3):1000-1010.*

Zylka, M.J., Dong, X., Southwell, A.L., Anderson, D.J. (2003). Atypical expansion in mice of the sensory neuron-specific Mrg G protein-coupled receptor family. *Proceedings of the National Academy of Sciences USA (PNAS), 100(17):10043-10048.*

Zylka, M.J., Rice, F.L., Anderson, D.J. (2005). Topographically distinct epidermal nociceptive circuits revealed by axonal tracers targeted to Mrgprd. *Neuron, 45(1):17-25.*

Zylka, M.J. (2005). Nonpeptidergic circuits feel your pain. *Neuron, 47(6):771-772*.
Zylka, M. J., Sowa, N. A.. Taylor-Blake, B Twomey, M. A. Herrala A., Voikar, V.and Vihko. P. (2008). Prostatic acid phosphatase is an ectonucleotidase and suppresses pain by generating adenosine. *Neuron 60 (1):111-122.*