Sympathetic Sprouting and Changes in Nociceptive Sensory Innervation in the Glabrous Skin of the Rat Hind Paw Following Partial Peripheral Nerve Injury

Laurene Dao-Pei Yen

Department of Pharmacology and Therapeutics

McGill University, Montreal Canada

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Abstract

Previous studies have suggested that sympathetic sprouting in the periphery may contribute to the development and persistence of sympathetically-maintained pain in animal models of neuropathic pain. The purpose of this thesis was to examine morphological changes in the cutaneous innervation in rats after chronic constriction injury (CCI) to the sciatic nerve. More specifically, this study addresses the question of whether sympathetic fibres sprout *de novo* into the upper dermis of the rat hindpaw skin after CCI of the sciatic nerve. We also determined changes in peptidergic sensory innervation following CCI.

At several periods post-injury, hind paw skin was harvested and processed using a monoclonal antibody against dopamine-β-hydroxylase to detect sympathetic fibres and a polyclonal antibody against calcitonin gene-related peptide to identify peptidergic sensory fibres. We observed migration and branching of sympathetic fibres into the upper dermis of the hind paw skin, from where they were normally absent. This migration was first detected at 2 weeks, peaked at 4 to 6 weeks and lasted for at least 20 weeks post-lesion. At 8 weeks post-lesion, there was a dramatic increase in the density of peptidergic fibres in the upper dermis. Quantification revealed that densities of peptidergic fibres 8 weeks post-lesion were significantly above levels of sham animals. Interestingly, the ectopic sympathetic fibres did not innervate blood vessels but formed a novel association and wrapped around sprouted peptidergic nociceptive fibres. Our data show a long-term sympathetic and sensory innervation change in the rat hind paw skin after the chronic constriction injury. This novel fibre arrangement after nerve lesion may play an important role in the development and persistence of sympathetically-maintained neuropathic pain after partial nerve lesions.

Résumé

Des études antérieures suggèrent que la prolifération du système nerveux sympathique dans la périphérie pourrait contribuer au développement et la persistance de la douleur maintenue par ce système dans des modèles animaux de douleur neuropathique. Le but de la présente thèse est d'examiner les changements morphologiques dans l'innervation cutanée des rats après blessure de type constriction chronique (CCI pour Chronic Constriction Injury) du nerf sciatique. De façon plus spécifique, cette étude pose la question de savoir si des fibres sympathiques prolifèrent de novo dans l'épiderme de la peau de la voûte plantaire après constriction chronique du nerf sciatique. Nous avons aussi déterminé s'il existait des changements dans l'innervation sensorielle peptidergique à la suite de cette même constriction chronique.

A différent temps post-chirurgie, la peau de la voûte plantaire a été prélevée et traitée pour l'immunocytochimie en utilisant un anticorps monoclonal dirigé contre la dopamine-β-hydroxylase afin de détecter les fibres sympathiques, et un anticorps polyclonal dirigé contre le peptide dérivé du gène de la calcitonine (calcitonine gene-related peptide, CGRP) afin d'identifier les fibres sensorielles peptidergiques. Nous avons observé la migration et le branchement des fibres sympathiques dans la peau de la voûte plantaire où elles sont normalement absentes. Cette migration a été détectée à partir de 2 semaines, avec un maximum de 4 à 6 semaines, et dure au moins 20 semaines après la lésion. Huit semaines après la lésion il y avait aussi une forte augmentation de la densité de fibres peptidergiques dans l'épiderme. Leur quantification montra que la densité de fibres peptidergiques était significativement supérieure à celles des animaux contrôles. De façon intrigante, les fibres sympathiques ectopiques

n'innervaient pas les vaisseaux sanguins mais formaient une nouvelle association en s'enveloppant autour des fibres nociceptives peptidergiques. Nos données ont donc montré un changement à long terme du système nerveux sympathique et sensoriel dans la peau de la voûte plantaire de rat après constriction chronique du nerf sciatique. Ce nouvel arrangement après une lésion pourrait jouer un rôle important dans le développement et la persistance de la douleur neuropathique maintenue par le système nerveux sympathique après la lésion partielle de nerfs périphériques.

Contributions of Authors

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

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L.D. Yen, G.J. Bennett, A. Ribeiro-da-Silva

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Responsibilities of authors and co-authors:

Dr. A. Ribeiro-da-Silva: Principal investigator of the project. Provided intellectual influence in the planning of experiments. Helped with preparation of the figures. Edited manuscript.

Dr. G.J. Bennett: Showed L.D. Yen how to perform Chronic Constriction Injury. Revised manuscript.

L.D. Yen: Investigator of project. Planned all experiments, performed all surgeries and all immunocytochemistry, observed the material with the fluorescence and confocal microscopes, carried out the quantitative analyses of the data for manuscript. Wrote initial version of manuscript.

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Chapter 1

Introduction

Pain is a complex experience that results from a multitude of environmental stimuli and, emotional and cognitive processing in the brain. The primary role of nociception, the detection of harmful or potential harmful stimuli, is to prevent tissue damage or the further deterioration of already existing damage (Wall and Melzack, 1994). Though nociception is meant to serve a protective role, it can also manifest in conditions where pain does not serve any protective role and becomes debilitating and chronic. In these chronic conditions we can include chronic inflammatory pain, neuropathic pain and cancer pain. Chronic inflammatory pain occurs in diseases such as rheumatoid arthritis. Neuropathic pain originates from a disturbance or pathological condition in the nervous system (Merskey and Bogduk, 1994; Campbell and Meyer, 2006). Cancer pain accompanies most forms of cancer and has sufficient characteristics of its own to be considered a specific type of pain, although often the pain has a strong neuropathic component(Mantyh, 2006). As this thesis focuses on an animal model of neuropathic pain, this type of pain will be examined in detail.

In neuropathic pain, nociceptive pathways may become hyperexcitable or sensitized, causing pain in response to stimuli that would not normally cause pain. Allodynia is nociception and painful sensation from normally innocuous stimuli and hyperalgesia is a hypersensitivity to mildly noxious stimuli(Campbell et al., 1989). Both allodynia and hyperalgesia occur in neuropathic states as well as in inflammatory and cancer pain. Neuropathic pain may occur in diabetes, following herpes zoster infection, nerve compression, nerve trauma or autoimmune disease. Individuals suffering from neuropathic pain experience burning pain, pain to light stroking of the skin, attacks of pain without provocation and widespread pain not explicable by an obvious cause(Campbell and Meyer, 2006) .

Just as neuropathies can be debilitating, lack of nociception can be life threatening (Rosemberg et al., 1994). Patients with a rare condition known as Hereditary Sensory and autonomic neuropathy (HSAN) have a mutation in the nerve growth factor receptor, tyrosine receptor kinase A (TrkA)(Indo et al., 1996). TrkA is required for development of the nociceptive C and Aδ fibres and sympathetic neurons (Crowley et al., 1994). As a result, these patients are unable to detect painful stimuli and suffer from self-inflicted wounds, self-mutilation, painless burns, and joint injuries (Schulman et al., 2001) Many of these individuals die due to internal organ dysfunction discovered at a terminal stage because of lack of the ability of the patient to feel pain caused by the dysfunction.

Therefore, pathologies arise at both ends of the pain spectrum, from the hyperalgesia and allodynia of neuropathic pain to the lack of nociception in HSAN. Hence, normal nociception may be a fine balance between many interacting factors.

1.1] Neuropathic Pain

The International Association for the Study of Pain has defined neuropathic pain as "any pain syndrome in which the predominating mechanism is a site of aberrant somatosensory processing in the peripheral or central nervous system" (Merskey and Bogduk, 1994). Neuropathic pain can be characterized by a lack of correlation between injury and pain (Cervero and Laird, 2003). Neuropathic pain may occur through many different mechanisms, such as a focal injury due to trauma of a particular nerve, from a more widespread disease as in diabetic neuropathy, amyotrophic lateral sclerosis, or multiple sclerosis, chemical mechanisms, viral (Herpes Zoster), autoimmune, mechanical , vascular and thermal causes. Despite various etiologies, neuropathic pain syndromes share certain clinical characteristics: spontaneous and continuous pain of burning

character; paroxysmal pain of shooting or lancinating nature; evoked pain from thermal and mechanical stimuli such as allodynia and hyperalgesia; summation after sensation and sensory loss in the painful area (Attal and Bouhassira, 1999).

1.1.1 Models of Peripheral Neuropathic Pain

Over the years, many animal models have been developed to try elucidate the peripheral mechanisms for neuropathic pain. As it is partial nerve injury that usually leads to neuropathic pain, many of the models aim to produce a partial injury. In the spinal nerve ligation model both the L5 and L6 spinal nerves or the L5 spinal nerve alone of one side of the rat are tightly ligated (Kim and Chung, 1992). This model produces long-lasting hyperalgesia to noxious heat, mechanical allodynia of the affected foot as well as behavioral signs of the presence of spontaneous pain in the affected foot. This spinal nerve ligation model manifests the symptoms of human patients with causalgia and has proved very useful as a model of neuropathic pain. The chronic constriction injury model (CCI) (Bennett and Xie, 1988) produces a peripheral mononeuropathy by the placement of loosely constrictive chromic gut ligatures around the sciatic nerve. The CCI model produces hyperalgesia in the cutaneous territory of the affected nerve as well as allodynia and signs of spontaneous pain. Interestingly, this model also produces symptoms seen in the human condition known as causalgia, now named complex regional pain syndrome II,, such as abnormalities in skin temperature (Wakisaka et al., 1991) and changes in claw growth. A variation of the CCI model consists of the application of one or several polyethylene cuffs around the sciatic nerve (Mosconi and Kruger, 1996). The partial sciatic nerve ligation model consists of a unilateral ligation of approximately half of the sciatic nerve in the upper thigh (Seltzer et al., 1990). Within a few hours after the operation, and for several months

thereafter, the rats developed signs of pain including mechanical and thermal hyperalgesia, allodynia as well as signs of spontaneous pain. The spared nerve injury is a variant of partial denervation of the sciatic nerve. In this model, a lesion of two of the three terminal branches of the sciatic nerve (tibial and common peroneal nerves) leaves the remaining sural nerve intact. The spared nerve injury model differs from other models of partial nerve injury in that the co-mingling of distal intact axons with degenerating axons is restricted, enabling behavioral testing of the non-injured skin territories adjacent to the denervated areas (Decosterd and Woolf, 2000). In a model of experimental neuroma (Lombard et al., 1979; Wall et al., 1979) the sciatic and saphenous nerves were sectioned in the upper leg in rats and mice. The animals displayed sympathetically-related autotomy which was attenuated by guanethidine. Lastly, a modified version of the CCI model in the mental nerve consists of a bilateral ligation of the mental nerve (Grelik et al., 2005b). Animals in this model showed episodes of directed grooming, indicating spontaneous pain.

Interestingly, many of these animal models were shown to have components of sympathetically maintained pain. In the spinal nerve ligation model (Kim and Chung, 1992) heat and mechanical hypersensitivity were partially reduced by surgical sympathectomy (Kim et al., 1993; Xie et al., 2001), and the hypersensitivity was rekindled following intradermal norepinephrine (NE) or α2 receptor ligands (Moon et al., 1999). An adrenoceptor antagonist in combination with a purinergic receptor antagonist reduced allodynia in the spinal nerve ligation neuropathic rats (Park et al., 2000) .This suggested that the purinergic substance ATP is co-released with NE from sympathetic nerve terminals and is involved in the maintenance of the sympathetically dependent component of pain behaviors in some neuropathic rats. In the partial

nerve ligation paradigm, sympathectomy was also shown to alleviate sensory disorders in rats following neuropathic surgery (Seltzer and Shir, 1991).

1.1.2 Peripheral Mechanisms of Neuropathic Pain

Upon injury to the skin, two types of hyperalgesia are evoked; primary and secondary (Meyer et al., 2006). Primary hyperalgesia occurs at the site of injury and is mediated by the sensitization of the primary afferent receptors, while secondary hyperalgesia occurs in the uninjured tissue around the injury. The secondary hyperalgesia is thought to be due to sensitization of the central nervous system (Torebjork et al., 1992).

Under normal physiological conditions, peripheral nociception is mediated by the activity of unmyelinated C-fibre primary afferents and high-threshold myelinated Aδ primary afferent fibres. While the mechanisms of central sensitization in hyperalgesia have been studied in detail, the peripheral mechanisms of primary afferent sensistization have not been elucidated in such detail. Lesions to these primary afferents in the peripheral nervous system may result in functional alterations of these nociceptors, contributing to neuropathic pain. There are at least four major mechanisms by which lesions in the peripheral nervous system may lead to neuropathic pain: abnormal spontaneous activity at ectopic sites, nociceptor sensitization, abnormal interaction between fibres and hypersensitivity to catecholamines (Attal and Bouhassira, 1999). In this thesis we focus on the role of peripheral catecholamines and their possible role in nociceptors sensitization.

1.1.2.3 Etiology of Neuropathic pain

Although, much in known about nociception in non-disease states, there is much to be discovered regarding the mechanisms of neuropathic pain, as the etiology is vast and can vary between different neuropathic states. Despite the amount of research done regarding neuropathic pain, the treatment is still unsatisfactory. An increased knowledge of pain-generating mechanisms and their translation into symptoms and signs may allow a dissection of the contributing mechanisms that operate in each patient. It is estimated that neuropathic pain may affect one-sixth of the population (Campbell and Meyer, 2006). If a phenotypic characterization of the neuropathic pain in an individual is combined with drugs that act on those mechanisms, it should ultimately be possible to design optimal treatments for individuals suffering from these conditions (Baron, 2006).

1.2] Nociceptive primary afferent fibres or nociceptors

1.2.1 Primary Afferents

The nociceptive pathways start in the periphery at the level of the termination of primary afferent fibres in the skin, viscerae, muscle, joints, tendons and bone. These primary afferents which convey pain-related information (nociception) are named nociceptors. Nociceptors are classified based on absence or presence of myelin (respectively C-fibres or A-delta fibres), and the modalities of stimulation that evoke a response and response characteristics (Campbell et al., 1989). Some nociceptors are stimulated by many different stimuli such as mechanical, heat, cold and chemical stimuli. These receptors are called polymodal nociceptors. However, some nociceptors only respond to certain types of stimulus such as heat and mechanical stimuli (Campbell et al., 1989).

1.2.2 C-fibre nociceptors

This class of nociceptor is non-myelinated, slowly conducting and small in diameter. The C-fibres are thought to mediate the delayed, diffuse and dull pain evoked by noxious stimuli, also known as secondary pain (Basbaum and Jessell, 2000). Histological studies of the dorsal root ganglion have revealed two separate classes of C-fibres. The peptidergic population expresses the neurotransmitter Substance P and the growth factor receptor for nerve growth factor (NGF), TrkA. The second population is labeled with the α-D-galactosyl-binding lectin IB_4, possesses fluoride resistant acid phosphatase (FRAP) activity and expresses the $P2X_3$ receptor (an ATP-gated ion channel) (Snider and McMahon, 1998; Hunt and Mantyh, 2001).

1.2.3 A-delta nociceptors

Aδ nociceptors are thought to mediate detection of rapid, acute sharp pain that is usually well localized. These fibres are thinly myelinated and have a faster conduction velocity of 1-30m/s compared to that of the C-fibres (less than 1m/s). Aside from nociception, Aδ fibres are also cooling receptors and low-threshold mechanoreceptors (Julius and Basbaum, 2001). There are two types of Aδ nociceptors, the high threshold mechanoreceptors (HTM), which do not express neuropeptides or bind IB4, and the peptidergic Aδ nociceptors, which express substance P (Ribeiro-da-Silva, 2004).

1.3|The Involvement of the Sympathetic Nervous System in Neuropathic pain

Injury to somatosensory nerves is sometimes accompanied by chronic neuropathic pain. Under physiologic conditions, primary afferent nerve endings are not stimulated by catecholamines and are functionally distinct from sympathetic efferents (Jänig et al., 1996). However, this may change in certain pain syndromes such as Complex regional pain syndrome (CRPS).

1.3.1 Complex Regional Pain Syndrome

The involvement of the sympathetic nervous system in neuropathic pain was first described by Paget in 1862 as "distressing" pain in the fingers of patients after nerve injury. In 1867, Silas Weir Mitchell described a syndrome known as *causalgia* in which patients felt a burning pain that was accompanied by "glossy skin" and increases in skin temperature (Mitchell, 1872). Since then these particular symptoms of pain, vasomotor skin changes, functional impairment and trophic changes following a musculoskeletal trauma have been categorized into syndromes and given many names including minor causalgia, posttraumatic spreading neuralgia , shoulder-hand syndrome, sympathalgia and Reflex sympathetic Dystrophy (RSD)(Bonica, 1990). The name Reflex Sympathetic dystrophy resulted from the belief that symptoms such as vasomotor changes were caused by an abnormal sympathetic tone in the given region. In 1993, these group of syndromes were renamed as Complex Regional Pain Syndrome I and II (CRPS I and II) (Merskey and Bogduk, 1994). CRPS I follows a soft-tissue injury and requires all of the aforementioned symptoms of vasomotor changes, disproportionate pain, allodynia or hyperalgesia to the inciting event in addition to the presence of an initiating noxious event or cause of immobilization. However, the inciting injury in CRPS I may be so slight that the patient

does not realize it and the the development and extent of CPRS I does not depend on the severity of the injury (Raja and Grabow, 2002; Schwartzman and Popescu, 2002; Janig and Baron, 2003). CRPS II follows a well-defined nerve lesion and the symptoms are not limited to the distribution of the lesioned nerve (Bonica, 1990).

1.3.2 Sympathetically Maintained Pain

Many studies have shown that after partial or complete peripheral nerve lesion in animal models, regenerative sprouts in the cells of the dorsal root ganglia show excitatory responses to NE or stimulation of the sympathetic nerve trunk (Devor and Janig, 1981; Habler et al., 1987). In some patients, the activity of the sympathetic innervation of the region of painful peripheral neuropathy is believed to cause, or at least to modulate, this pain. (Birklein, 2005) Such patients receive pain relief following sympathectomies or sympathetic blocks, and the pain in these cases is thus said to be "sympathetically-maintained pain" (SMP) (Roberts, 1986). Moreover, in patients who have had their pain eliminated by sympathectomy, or temporarily relieved by a sympathetic block, a transient recurrence of their pain is seen when epinephrine or NE is injected into their previously symptomatic skin (reviewed in Mailis-Gagnon and Bennett, 2004). Interventions that block the sympathetic outflow have been shown to reduce neuropathic pain in animal models (Shir and Seltzer, 1991; Kim et al., 1993; Choi et al., 1994),although the effect is controversial and not well understood (Lavand'homme et al., 1998; Moon et al., 1999; Lee et al., 1999; Ringkamp et al., 1999; Park et al., 2000; Banik et al., 2001; Lee et al., 2001; Wei et al., 2002; Lee et al., 2003). Administration of NE in neuropathic skin in humans aggravates pain and hyperalgesia (Choi and Rowbotham, 1997; Ali et al., 2000) and plasma NE levels are increased in patients with diabetic neuropathy(Tsigos et al., 1993).However, in conditions with

inflammation, peripheral injections of NE can have either an anti-nociceptive effect(Binder et al., 2004) or pronociceptive effect (Banik et al., 2001; Baik et al., 2003).

The term *Sympathetically Maintained Pain* (SMP) is often used synonymously with CRPS. However, as SMP describes pain that can be relieved by sympathectomy, it is a mechanism of pain rather than a syndrome. The issue of SMP is not black and white, as patients may have partial or full relief of pain from sympathectomy or sympathetic block. Therefore, patients with CRPS may have only a portion of their disease attributable to SMP and conversely, SMP is not necessarily limited to patients with CRPS as patients with other neuropathies may find relief from sympathetic block (Loh and Nathan, 1978).

Studies on animal models of neuropathic pain have shown a de novo α-adrenoceptor-mediated excitatory response in the regenerative sprouts of damaged axons (Wall and Gutnick, 1974b; Devor and Seltzer, 1999), a phenomenon that appears to be specific to unmyelinated nociceptive fibres (Bossut and Perl, 1995). In addition, studies in animal models have shown the sprouting of sympathetic fibres in the dorsal root ganglia (DRG) where they form baskets around neurons (Chung et al., 1993; Chung et al., 1996; Chung et al., 1997; Ramer and Bisby, 1997; Lee et al., 1998; Ramer et al., 1998a), which can be activated by sympathetic stimulation (McLachlan et al., 1993). These sprouted fibres have also been shown to display synaptic varicosities close to the DRG soma (Chung et al., 1997). This novel anatomical organization would suggest an interaction between the sensory neurons and postganglionic sympathetic fibres after nerve lesion, which may play a role in the development and maintenance of SMP (Chung et al., 1996).

However, there is a lack of data showing an unquestionable link between this sympathetic sprouting in the DRG and pain-related behaviours in these animal models (Janig and Habler, 2000). The question remains as to the location where the sympathetic nervous system interacts

with the sensory system and contributes to neuropathic pain. While the sprouted sympathetic fibres in the DRG may play a role in neuropathic pain, it is possible that the sympathetic fibres and sensory neurons interact at a much more distal site such as the skin, where noxious stimuli are detected. Injection of NE into the skin of causalgic patients, whose pain was relieved by sympathectomy or sympathetic block, can rekindle former neuropathic pain, suggesting this SMP may be caused by an interaction between NE and sensory nerve terminals in the skin (Wallin et al., 1976; Campbell et al., 1989). Similarly, when NE is injected into the skin of rats suffering from neuritis, it exacerbates mechanical hyperalgesia mediated by peripheral α_1- and α_2-adrenoceptors (Baik et al., 2003). Moreover, NE injection in the skin excites C-nociceptors following nerve lesions, whereas such injections have no effect in the normal case (Sato and Perl, 1991; Bossut and Perl, 1995).

1.4] Skin Structure

Because the studies in this thesis were performed in the skin, this organ will be described in some detail regarding its histological organization and innervation pattern. The skin is the largest organ of the body consisting of 16% of the total body weight. The skin covers the entire body surface and is continuous with mucous membranes of the eyes, lips, anus and nose and at external orifices of the urogenital system (Gartner and Hiatt, 2001). As an organ system, the skin serves many functions, namely providing a waterproof barrier to protect from desiccation, protection against physical, chemical and biological injury, absorbing ultraviolet radiation for vitamin D synthesis and protection, excretion, thermoregulation, immunologic defense for the body via Langerhans cells and lastly monitoring the external environment through various nerve endings and Merkel cells (Gartner and Hiatt, 2001).

The skin is composed of two separate layers: the superficial epidermis layer and the more basal dermis. The epidermis and dermis are closely associated by interdigitations. Epidermal ridges called epidermal papillae are separated by a basement membrane from the dermal papillae of the dermis which match the interdigitations of the former. Together these two structures form the rete apparatus (Gartner and Hiatt, 2001). Below the dermis lies a fascial sheath known as the hypodermis, which separates the skin from deeper structures. The hypodermis consists of loose connective tissue and may contain unilocular adipocytes, however is not considered part of the skin.

1.4.1 Epidermis

The thickness of skin varies over different regions of the body. In humans, the thickness of the epidermis ranges from 0.07 mm on the eyelids to 0.12 mm, with thicker portions of 0.8 to 0.14 mm, occurring on the palms and soles (Gartner and Hiatt, 2001). Accordingly, skin can be classified as either thick or thin skin, depending on the thickness of the epidermis. Glabrous or thick skin is found on the palms of hands and the soles of the feet while non-glabrous or hairy skin is located throughout the rest of the body. In glabrous skin, the thickness of the epidermis ranges from 400-600 μm and contains all five layers of the epidermis. Thick skin lacks hair follicles, *arrector pili* muscles and sebaceous glands however it contains sweat glands. The thin skin covers all other parts of the body and is much thinner, with an epidermis measuring from 75-150 μm. This type of skin possesses a thin stratum corneum, no stratum lucidum or definite stratum granulosum (see below for description of these layers in thick skin). Unlike glabrous or thick skin, thin skin is characterized by the presence of hair follicles, arrector pili muscles, and

sebaceous glands. Similarly to thick skin, the thin skin also has sweat glands (Gartner and Hiatt, 2001).

1.4.2 Origins of skin layers

The different layers of the skin are of different cell lineages. The epidermis is of ectodermal origin and consists of cells which are keratinizing, while the dermis is of mesodermal origin.

1.4.3 Cell types in epidermis

There are four types of cells present in the epidermis: keratinocytes, melanocytes, Langerhans cells and Merkel cells.

1.4.3.1Keratinocytes

Keratinocytes are the most populous cell in the epidermis and are responsible for producing keratin and make up the majority of the five different epidermal layers. These cells are derived from ectodermal origin. Keratinocytes are constantly sloughed off of the skin as they migrate upwards to form the stratum granulosum. Therefore these cells must be constantly renewed through mitotic activity. Mitotic renewal occurs in the first layer of the epidermis, the stratum germinativum. As the keratinocytes divide, they are pushed upwards and begin to differentiate and accumulate keratin filaments in the cytoplasm. This will be described in more detail in the section pertaining to the different layers of the epidermis. As a result of the differentiation of the keratinocytes, five distinct layers are formed in the epidermis, each with a different form of keratinocyte (Gartner and Hiatt, 2001).

1.4.3.2 Melanocytes

The melanocytes arise from neural crest cells and invade the skin at 3-6 months of gestation. They are the second most populous cell in the epidermis and are located between keratinocytes in the stratum basale, hair follicles and the superficial parts of the dermis. The non-keratinizing melanocytes produce melanin, a pigment which is produced in melanosome organelles and protects against ultraviolet radiation. Melanocytes have long cytoplasmic extensions which protrude into the spaces between cells in the stratum spinosum. The amino acid tyrosine is transported into melanosomes where it is converted to melanin through a series of reactions(Gartner and Hiatt, 2001). The melanosomes travel to the tips of the cell processes of the cells of the stratum spinosum and are secreted by cytocrine secretion. These melanosomes are then transported to the supranuclear region of the stratum intermedium where they form a protective barrier between the nucleus and UV rays from the sun. The number of melanocytes varies in different regions of the skin from 800 to 2300 /mm^2. The number of melanocytes in the skin is generally the same for all races. However, individuals with darker skin have higher activity of the enzyme tyrosinase in their melanocytes(Gartner and Hiatt, 2001).

1.4.3.3 Langerhans Cells

Langerhans cells, also known as dendritic cells, function as antigen presenting cells in the skin. They are derived from hematopoietic stem cells of the mononuclear phagocyte system (Gartner and Hiatt, 2001) and occur mostly in the stratum spinosum. These cells contain characteristic membrane-bound, rod-shaped Birbeck granules. The Langerhans cells participate in cutaneous immune responses and can migrate from the skin to lymph nodes once activated in order to present antigen to T and B-cells. Langerhans cells also possess complement (C3) and

other receptors that allow them to phagocytose antigens and further contribute to their primary role of antigen presentation.

1.4.3.4 Merkel Cells

Merkel cells are involved in mechanoreception and are found in the stratum basale of the skin with a large concentration in the tips of the fingers, oral mucosa and the base of hair follicles. These cells differentiate from epithelial cells in the fetal epidermis (Gartner and Hiatt, 2001). The Merkel cells have characteristic dense-cored granules in the perinuclear zone. (Gartner and Hiatt, 2001), and are in contact with thick myelinated afferent fibres, forming a Merkel-neurite complexes, which function as mechanoreceptors in the skin.

1.4.4 Layers of the Epidermis

Thick skin consists of 5 layers from deep to superficial: stratum basale, stratum spinosum, stratum granulosum, stratum lucidum, and stratum corneum. Thin or hairy skin consists of three to four layers. These distinct layers form as a result of the cytomorphosis of keratinocytes during the migration from stratum basale to stratum corneum (Gartner and Hiatt, 2001).

1.4.4.1 Stratum Basale (Stratum germinativum)

This layer is the most basal layer of the epidermis and consists of a single layer of cuboidal to columnar cells resting on a basement membrane. The cells of this layer have a basophilic cytoploasm and large nuclei. It is from this layer which the epidermis is able to regenerate via mitosis and renew layers of desquamated skin. These cells are attached to the basal lamina via hemidesmosomes. New layers of cells are pushed to the surface of the epidermis

giving rise to the next layer of the epidermis called the stratum spinosum. Renewal of epithelial cells occurs nocturnally and renewal of the entire epidermis occurs every 15-30 days, varying in different regions of the body (Junqueira and Carniero, 1992; Gartner and Hiatt, 2001).

1.4.4.2 Stratum Spinosum

This is the thickest layer of the epidermis and consists of nucleated polyhedral cells filled with bundles of intermediate filaments, also known as tonofilaments. These tonofilaments radiate from the nuclear region towards cellular processes towards cellular processes from other adjacent cells. The cellular processes attach to each other via desmosomes. These cellular processes and desmosomes give this layer a spiney or prickled look, hence the name stratum spinosum. Cells of this layer contain cytoplasmic secretory granules referred to as membrane-coating granules or lamellar granules which store glycolipids. The secretion of these granules helps provide a waterproof barrier for the skin. The cells of the stratum spinosum also have nocturnal mitotic activity and are essential for the renewal of the epidermis (Gartner and Hiatt, 2001).

The stratum basale and the stratum spinosum may be referred to collectively as the stratum Malpighi. Together, their continuous mitotic activity allows for the migration of cells towards more superficial layers of the epidermis.

1.4.4.3 Stratum Granulosum (Granular Layer)

The stratum granulosum consists of three to five layers of flattened keratinocytes with large keratohyalin granules within the cytoplasm. These granules are associated with tonofilaments which may pass through or be embedded in the granules. As the keratohyalin granules accumulate, the cells will eventually become overwhelmed and this will lead to

destruction of the nucleus and organelles. The lamellar granule is another type of granule found in this layer. The lamellar granule is small and rod-shaped, measuring 0.1-0.3 μm and contains lamellar disks. These lamellar granules collect at the cell periphery and are exocytosed to form multiple-layered membrane bilayers in the intercellular space. These granules contribute to the waterproof barrier of the skin (Gartner and Hiatt, 2001).

1.4.4.4 Stratum lucidum

The stratum lucidum lies superficially to the stratum granulosum. This layer is relatively thin and is not always obvious when viewed in bright field microscopy due to a lack of nuclei or organelles. It is a translucent layer between the stratum granulosum and stratum corneum only present in palmar and solar skin. Despite a lack of nucleus and organelles these cells do contain densely packed keratin filaments and eleidin, a transformation product of keratohyalin (Gartner and Hiatt, 2001).

1.4.4.5 Stratum corneum

This is the most superficial layer of the epidermis and consists of many layers of dead, flattened, keratinized cells. All cytoplasm is replaced by filaments of keratin embedded in amorphous matrix and the cell membrane is thickened by deposition of non-keratin material on the inner side of the cell membrane. The intercellular space is filled with material from lamellar granules. Cells closer to the surface of the skin, known as squames or horny cells, have modified desmosomes which are no longer able to hold cells together. These surface layers of the stratum corneum are dead and desquamate.

1.4.5 The Dermis (reticular and papillary layer)

The dermis lies directly below the epidermis and is separated from the latter by the basement membrane of the stratum basale, forming the dermal-epidermal junction. The dermal-epidermal junction consists of 3 components: 1) the plasma membrane of the keratinocyte at hemidesmosomes 2) the basal lamina 3) and connective tissue components, anchoring fibrils, microfibril bundles and type III collagen fibrils. The connective tissue component of the dermal-epidermal junction anchors the epidermis to the papillary layer of the dermis through association of anchoring fibrils and reticular fibrils in loops.

Unlike the epidermis, this layer is non-keratinizing and is derived from cells of mesodermal origin. The dermis is composed mainly of dense, irregular collagenous connective tissue (Gartner and Hiatt, 2001). The connective tissue consists of mostly type I collagen and elastic fibres which anchor the skin the deep hypodermis layer. The dermis is subdivided into two distinct layers in humans: the papillary layer and the reticular layer. The papillary layer is the most superficial part of the dermis which interdigitates with the epidermal papilla.

1.4.5.1 Papillary layer

The papillary layer is the superficial layer of the dermis. It interdigitates, in an uneven fashion, with the papillae of the epidermis to form dermal papillae. This layer contains types III collagen and elastic fibres. The dermis is bound to the epidermis by anchoring fibrils in the basal lamina which project into the papillary dermis. The cellular component of the papillary layer includes fibroblasts, macrophages, plasma cells and mast cells.

Small capillary loops are present in the papillary layer and extend to the dermal-epidermal junction. The role of the capillary loops is to regulated body temperature by vasoconstriction or

vasodilation. These vessels also bring nutrients to the avascular epidermis (Gartner and Hiatt, 2001).

1.4.5.2 Reticular Layer

This is the deepest layer of the skin. The reticular layer is continuous with the papillary layer, lacking a clear demarcation between the two layers. The reticular dermis contains thick bundles of type I collagen which interact with thick elastic fibres. This layer is less cellular than the papillary layer, however fibroblasts, macrophages and fat cells make up the cellular component of this layer. The space between the cells is filled with proteoglycans rich in dermatan sulfate.

The reticular layer also contains many structures derived from the epidermis such as sweat glands, sebaceous glands, and smooth muscle cells such as arrector pili. There are two types of mechanoreceptors in the reticular layer; the Pacinian Corpuscles and Ruffini corpuscles. The Pacinian corpuscles respond to pressure and vibrations while Ruffini corpuscles respond to tensile forces.

In the experiments of this thesis, the dermis was divided into upper and lower dermis. The upper dermis is delineated as tissue extending from the dermal-epidermal junction to 150 μm from the dermal-epidermal junction. The lower dermis consists of the dermis 150 μm below this boundary and continues until the hypodermis (please see Chapter 2: Materials and Methods). This subdivision was based on the virtual absence from the upper dermis of sympathetic fibres. This subdivision of upper and lower dermis is not analogous to the subdivision of papillary and reticular dermis as the upper dermis comprises both layers.

1.4.6 Hypodermis

The hypodermis, also known as the subcutis, lies deep to the dermis and is not considered part of the skin. This layer consists mainly of adipose tissue which is compartmentalized by vertical fibrous septae running from the reticular dermis to a fibrous tissue layer below the hypodermis (Young et al., 2006). The degree of compartmentalization and the thickness of the hypodermis varies throughout different regions in the body. Depending on the location in the body, the hypodermis may contain different structures, such as the lower parts of anagen hair follicles in the scalp, apocrine glands in the axilla or eccrine glands in the palms of the feet(Young et al., 2006).

1.5] Skin Innervation

1.5.1 Afferent Nervous System

Both myelinated and non-myelinated fibres comprise the afferent nervous system of the skin. The afferent system is responsible for detection of stimuli such as temperature and touch, and transmitting this information from nerve endings in the skin via the afferents to the spinal cord or brain. Sensory nerve endings may be free nerve endings such as C-fibres, or specialized encapsulated nerve endings such as Meissner's, Pacinian and Ruffini's corpuscles.

1.5.1.1Pacinian Corpuscles

The Pacinian corpuscles detect deep pressure and vibrations. They are located as small clusters or singly in the hypodermis and are especially numerous in the palms and soles. These receptors detect touch and pressure and high frequency mechanical vibrations.

1.5.1.2 Meissner's corpuscles

Meissner's corpuscles are mechanoreceptors present in the papillary layer. They contain a connective tissue capsule with non-myelinated nerve endings of Aβ fibres. These receptors are stimulated by slight deformations in the epidermis cause by light touch and low frequency vibration of 30-50Hz. There is a higher concentration of these receptors in areas of skin sensitive to *tactile* stimuli such glabrous skin of hand and feet, lips, external genitalia and nipples(Gartner and Hiatt, 2001).

1.5.1.3 Merkel disk receptors

These are more superficial mechanoreceptors, as each consists of a Merkel cell, which is located in the epidermis (see above), and the termination of a slowly-adapting afferent fibre. They respond to light steady pressure. It is thought that they play a role in the tactile discrimination of shapes, edges and rough textures and are seen mostly in the skin of the hand.

1.5.1.4 Ruffini corpuscles

Ruffini corpuscles are also located in the dermis of glabrous and hairy skin. These receptors are mechanoreceptors and are commonly found in the soles of the feet. This type of receptor detects mechanical tissue stretching (Gartner and Hiatt, 2001).

1.5.1.5 Free Nerve endings

Free nerve endings are either myelinated or non-myelinated such as A-delta fibres or C-fibres, respectively. The free nerve endings are responsible for detecting stimuli such as pain, itch or temperature. This subset of afferents is located mainly in the papillary dermis with some projections into the epidermis. Free nerve endings are also found in the perifollicular fibrous sheath of the hair follicle (Young et al., 2006).

The pain receptors or nociceptors, are terminal branches of either small non-myelinated nerve fibres (C-fibres) or thinly myelinated fibres (Aδ fibres). The free endings of these receptors contain ionic channels that respond to noxious stimuli such as heat, chemical or cold. The stimulation causes a depolarization, resulting in an action potential which will travel to the spinal cord. The non-myelinated fibres carry impulses much slower than the myelinated Aδ fibres. Correspondingly, the C-fibres are responsible for the slow, aching secondary pain whereas the faster Aδ fibres are responsible for the sharper, more immediate acting pain sensation. Both C and Aδ fibres are the peripheral processes of pseudo-unipolar neurons located in the DRG or trigeminal ganglia., with the central processes terminating in the superficial dorsal horn of the spinal cord or trigeminal subnucleus caudalis (Ribeiro-da-Silva, 2003; Grant and Robertson, 2004). The peptidergic fibres terminate in lamina I and V whereas the non-peptidergic fibres terminate in the inner lamina II (Bradbury et al., 1998; Ribeiro-da-Silva, 2003)

The C-fibres can be divided into the peptidergic or the non-peptidergic populations. The non-peptidergic C-fibres have fluoride resistant acid phosphatase (FRAP) (Coimbra et al., 1970; Knyihár et al., 1974) activity, express the purigenic $P2X_3$ receptor (Snider and McMahon, 1998)and bind the lectin IB4(Alvarez and Fyffe, 2000). For all nociceptors, glutamate is the

predominant excitatory neurotransmitter (Battaglia and Rustioni, 1988; Merighi et al., 1991). However, despite this classification there are some neurons which express both peptidergic and non-peptidergic markers (Alvarez and Fyffe, 2000). This population of sensory fibres co-localizes CGRP and somatostatin, does not respond to NGF and binds IB4 (Priestley et al., 2002).

The difference between the peptidergic and non-peptidergic populations lies in their neurotrophic factor support in the adult. Both populations require NGF during development but at some point prior to birth, one population becomes glial cell-derived neurotrophic factor (GDNF)-dependant (Molliver et al., 1997; Gavazzi et al., 1999). This growth factor dependence corresponds to the expression of certain receptors. The peptidergic population expressed the NGF high affinity receptor TrkA and the non-peptidergic fibres express GDNF receptors.

1.5.1.6 Peptidergic Nociceptors

The peptidergic population of C-fibres contains the neurotransmitters Substance P (SP), calcitonin gene related peptide (CGRP), neurokinin A (NKA), galanin and endomorphin 2 (EM-2)(Todd and Ribeiro-da-Silva, 2005) and expresses TrkA, the high-affinity tyrosine kinase receptor for Nerve growth factor (NGF)(Snider and McMahon, 1998). In the epidermis, SP immunoreactivity is seen in terminal fibre branches around blood vessels, hair follicles and sebaceous glands and in fibres in cutaneous nerves(Dalsgaard et al., 1989; Grelik et al., 2005b). The role of SP in the skin is sensory and has been shown to disappear after deafferentation (Ruocco et al., 2001). Peptidergic nerve terminals play an important role in neurogenic inflammation. Both SP and NKA release upon nerve injury induce plasma extravasation by increasing venous permeability, in addition to the potent vasodilatory effects of CGRP on skin arterioles (Holzer, 1998). Activation of SP receptors on blood vessel walls, as well as mast cell

and leukocyte activation leads to them enhanced neuropeptide release. This neuropeptide release leads to a small vessels dilatation and a spreading of this vasodilation by an axon-mediated release of neuropeptides from other activated nociceptors (Baluk, 1997; Holzer, 1998).

1.5.1.7 Non-peptidergic neurons

Representative of the name, these fibres lack expression of neuropeptides. As mentioned previously, this population possesses FRAP, expresses the purigenic $P2X_3$ receptor and bind IB4. In adulthood, these fibres are supported by GDNF, however the embryonic population of these fibres does express TrkA and responds to NGF (Silos-Santiago et al., 1995). Until recently, the study of the non-peptidergic fibres in the skin has been lacking due to difficulty in labeling this population of fibres. Studies using, a combination of antibodies against CGRP and protein gene product (PGP) 9.5 have been used to determine the expression of these non-peptidergic small diameter fibres (Rice and Rasmusson, 2000). In the skin, non-peptidergic fibres have been shown to penetrate the epidermis, where they are more numerous than peptidergic fibres (Rice and Rasmusson, 2000); they also occur in hair follicles, although the classical view is that they are not present around blood vessels. The abundance of the non-peptidergic fibres in the epidermis compared to peptidergic fibres may be due to a difference in neurotrophic support (Lu et al., 2001; Grelik et al., 2005a). Like their peptidergic counterparts, non-peptidergic fibres show a decrease in fibres length in the epidermis following nerve lesions and later, a transient hyperinnervation (Grelik et al., 2005a). The fact that these fibres show plasticity after a nerve lesion suggests they may play a role in neuropathic pain.

1.6] Efferent Nervous System

This branch of the nervous system consists of non-myelinated fibres from the sympathetic component of the nervous system as well as parasympathetic fibres. In other experimental models, parasympathetic innervation of the skin of the lower lip was determined to be limited to blood vessels in the lower dermis. After mental nerve transection, vesicular acetylcholine transporter immunoreactive (VAChT-IR) fibres were observed in the upper dermis, well above the opening of the sebaceous glands into the hair follicles (Ramien et al., 2004). We found that in the glabrous skin of the rat hindpaw, parasympathetic innervation was lacking and immunoreactivity for VAChT was limited to fibres of sympathetic origin at sweat glands (Yen and Ribeiro-da-Silva, unpublished observations). In this thesis, the parasympathetic innervation of the rat skin will not be discussed further.

Sympathetic nerves are typically found to innervate skin appendages such as eccrine sweat glands and arrector pili muscles as well as blood vessel smooth muscle (Katzung, 2001; Young et al., 2006). The distribution of this population of nerves corresponds to the sympathetic "fight or flight "response which is characterized by sweating, piloerection and cutaneous vasoactivity. In the skin, the sympathetic terminals may release NE to the α-1 receptors on blood vessel walls and arrector pili or acetycholine onto sweat glands (Katzung, 2001). In rats, sympathetic nerves were found to be in the lower dermis of the skin of the lower lip, mostly in association with blood vessels (Ruocco et al., 2000; Grelik et al., 2005b). There is a characteristic sympathetic innervation of blood vessels in the lower dermis as a mesh-like arrangement around the vessel wall (Ruocco et al., 1999; Ruocco et al., 2000), whereas the SP-containing fibres travel along the vessel wall and show longer segments between the varicosities (Ruocco et al., 2000).

We have also shown similar results in the lower dermis of the glabrous skin of the rat hindpaw - see chapter 3 (Yen et al., 2006). These efferent nerves supply blood vessels in the skin and are responsible for the control of vessel diameter and blood flow. The sympathetic nerve fibres most likely reach their destination in the skin through the main cutaneous nerves or through the main arteries supplying the skin (Baron and Maier, 1996; Janig and Habler, 2000).

The cell bodies of the sympathetic neurons innervating the cutaneous vasculature are located in the ganglia of the paravertebral sympathetic chain or in the superior cervical ganglia for sympathetic nerves innervating the skin of the face (Gibbins, 1997; Ruocco et al., 2001). About 50% of these sympathetic neurons in the ganglia are located in vasoconstrictor pathways, although at present the exact proportion of neurons that project to the cutaneous arteries is unknown.

1.6.1 Neurotransmitters of sympathetic neurons and pain modulation

As previously mentioned, one of the most significant neurotransmitters released from sympathetic neurons is NE. The involvement of NE in pain modulation has been extensively studied in animal models as well as human patients (See section *3] "The Involvement of the Sympathetic Nervous System in Neuropathic pain"*). NE modulates pain mainly through α-adrenoceptors whereas β-adrenoceptors mediate epinephrine-induced modulation of pain (Pertovaara, 2006).

Despite the focus on NE, there are other neurotransmitters of importance that may contribute to pain modulation. Sympathetic neurons also release ATP, acetylcholine (in fibres innervating sweat glands), neuropeptides (Burnstock, 2000) and prostaglandins (Gonzales et al., 1991). All of these neurotransmitters and modulators have been shown to increase excitability of

nociceptors, be it cutaneous afferent excitability by NE and acetylcholine (Akoev, 1980) or SP in postganglionic neurons (Levine et al., 1993) or prostaglandins (Birrell et al., 1991). The release of these neurotransmitters from sympathetic neurons may play different roles in pain modulation. In humans, guanethidine, a drug which depletes NE stores of sympathetic neurons, has been shown to attenuate pain in rheumatoid arthritis (Levine et al., 1986).

Another neuropeptide that is correlated with the sympathetic modulation of pain is Neuropeptide Y (NPY). NPY is found in cutaneous sympathetic neurons of rats as well as in humans (Gibbins et al., 1985). NPY was co-localized with galanin in vasoconstrictor neurons (Lindh et al., 1989), and with somatostatin in sympathetic neurons (Lundberg et al., 1991). NPY has been shown to potentiate the effects of NE, especially in injured nerves in the rat (Frisen et al., 1992; Edvinsson et al., 1994) and administration of NPY to the rat paw with neuropathic pain will increase the mechanical and thermal hyperalgesia and this effect is attenuated by sympathectomy (Tracey et al., 1995).

Therefore, the contribution of sympathetic fibres to pain modulation in the periphery is complex and extends beyond simply NE and adrenoceptors. There are many factors released by this population of fibres whose functions in nociception have yet to be determined.

1.7] Circulation of the skin

The circulation of the skin serves several purposes including nutritional supply of skin and appendages, increased blood flow to increase heat loss in hot conditions and decreased blood flow to preserve heat in cold conditions. Arteries of the skin are located in the hypodermis and branch upwards to form two plexuses of anastomosing vessels. The deeper cutaneous plexus is located at the junction of the subcutis and dermis and the more superficial subpapillary plexus in located at the junction between the papillary and reticular dermis (Young et al., 2006). The cutaneous plexus supplies the fatty tissue in the hypodermis, the deeper aspect of the dermis, capillary networks around hair follicles, sweat glands and deep sebaceous glands. The subpapillary plexus supplies the upper dermis and capillary networks around superficial appendages. The subpapillary plexus also supplies one capillary loop for each dermal papilla, connecting to the venous drainage with a pattern corresponding to the arterial supply.

As mentioned previously, one of the functions of the circulation in the skin is to control the body temperature. This occurs mainly in the numerous arteriovenous shunts in the dermis. These shunts play an important role in thermoregulation by controlling blood flow to different parts of the dermis. Lymphatic drainage in the skin is largely associated with the microcirculation and forms plexuses corresponding to the plexuses of the vascular system(Young et al., 2006).

1.8] Rationale for Study

Our laboratory has previously shown sprouting of sympathetic and parasympathetic fibres in the skin of the rat lower lip following both mental nerve (MN) transection or CCI up to eight

and sixteen weeks post-lesion (Ruocco et al., 2000; Grelik et al., 2005b). The sympathetic fibres migrated into the upper dermis of the lower lip, a region from which they are normally absent. In the MN ligation model, the incidence of grooming directed towards the site of denervation was found to be significantly increased relative to grooming seen in sham-operated animals, and this increase was correlated with the time course of sympathetic sprouting (Grelik et al., 2005b). This evidence, in addition to that from previous studies, strongly supports the occurrence of peripheral mechanisms for SMP at the level of the sympathetic and nociceptive terminals in the skin. Moreover, unlike what is seen after sciatic nerve injury, there was no sympathetic sprouting seen in the trigeminal ganglia after MN injury (Grelik et al., 2005b). There is also evidence that transection of the rat's trigeminal nerve does not give rise to the spontaneous ectopic discharge of sensory neurons that reliably follows sciatic nerve injury (Tal and Devor, 1992). It is thus possible that fundamental differences in pathophysiology exist for the pain states seen after lesions to the trigeminal and sciatic nerves (Bennett, 2004).

Our aim for this thesis was to investigate the changes in sympathetic innervation in the skin of the rat hind paw following a partial sciatic nerve lesion. We used the CCI model (Bennett and Xie, 1988), which produces a partial nerve lesion that leaves some sensory fibres intact. This particular model was suitable for our study as standardized pain behaviour testing is performed on the plantar surface of the hind paw skin.

Chapter 2

Methods and Results

Materials and Methods

Male Sprague Dawley rats weighing 175-200g (Charles River) were used in all studies. The guidelines in The Care and Use of Experimental Animals of the Canadian Council on Animal Care, and the guidelines of the International Association for the Study of Pain (Zimmermann, 1983) were followed and all experimental procedures were approved by the Facility Animal Care Committee of the Faculty of Medicine, McGill University.

2.1 Surgical Procedures

Animals were anaesthetized using isoflurane. Seventy two rats underwent ipsilateral CCI of the common sciatic nerve (Bennett and Xie, 1988). A group of 42 rats was used as sham-operated controls and another group of 42 rats served as unoperated age-matched controls. Both sham and lesion surgeries were performed with the aid of a surgical microscope (Leitz, Wetzlar, Germany). The right sciatic nerve was exposed and freed of surrounding muscle and fascia. Four loosely constrictive chromic gut ligatures (4.0; Ethicon) were placed around the sciatic nerve at mid-thigh level in an area spanning 7 mm in length. The incision was then closed using absorbable sutures (Vicryl; Ethicon) and animals were allowed to recover for up to 20 weeks. In sham-operated animals, surgeries were performed as described above with the exception of the application of chromic gut sutures.

2.2 Animal perfusions and histological processing

At the post-surgery times of 2, 4, 6, 8, 12, 16 and 20 weeks, rats were anaesthetized with a lethal dose of 0.4mg/kg of Equithesin and perfused transcardially with 4% paraformaldehyde, 15 %

picric acid (v/v) and 0.1% glutaraldehyde in 0.1M phosphate buffer (pH 7.4) for 30 minutes followed by another 30 minute perfusion of 4% paraformaldehyde and 15 % picric acid in 0.1M phosphate buffer. The hind paw skin was collected and post-fixed in the latter fixative for 1 hour, then cryoprotected in 30% sucrose overnight. The skin specimens were taken from the glabrous skin located between the tori, where mechanical allodynia testing is commonly performed. Special care was taken to avoid the base of the tori because the innervation of this region is atypically dense. Tissue was then embedded in an optimum cutting temperature medium (OCT) compound (TissueTek). Fifty μm-thick sections were cut at -20°C on a cryostat (Leica) and placed in phosphate buffered saline (PBS).

2.3 Immunofluorescence

All sections used for quantification were stained for a single signal to prevent any bleed-through when performing confocal microscopy and to maximize the accuracy of the analysis.

2.3.1 Single labelling for quantification

Rat hind paw skin sections were pretreated with 50% ethanol for 30 minutes and washed for 30 minutes in phosphate buffered saline plus 0.2% Triton X-100 (PBS+T). The sections were then treated with 1% sodium borohydride in PBS followed by a 60 minute wash in PBS. Tissues were incubated in 10% normal goat serum (NGS: Sigma) to prevent non-specific binding of secondary antibodies. The paw skin sections were then incubated at 4°C for 48 hours with one of the following primary antibodies: a) mouse monoclonal anti-dopamine-β-hydroxylase (clone DBH 41; spent tissue culture supernatant diluted 1:5; (Mazzoni et al., 1991); Medicorp, Montreal, QC, Canada; gift of Dr. A. Claudio Cuello),or b) a rabbit polyclonal anti-CGRP (1:2000; product

number C8198, lot 013K4842 Sigma). The anti-dopamine-β-hydroxylase (DBH) antibody was raised against rat purified DBH and its specificity was assessed using Western blots, ELISA and immunocytochemistry; it was found that clone DBH 41, the one we used, is specific for rat DBH exclusively, not producing any immunostaining in mouse, human, rabbit, bovine, guinea pig or cat tissues (Mazzoni et al., 1991). The anti-CGRP antibody was raised against rat CGRP and does not cross-react with any other peptide except human CGRP and rat and human β-CGRP (data supplied by Sigma). Staining was completely abolished when the antiserum was pre-absorbed with rat CGRP. After replacing the primary antibodies with pre-immune serum of the same species in which the antibodies were generated, no staining was observed except for unspecific sticking of the secondary antibody conjugates to the corneal layer of the epidermis. The use of the anti-DBH antibody did not produce any staining in skin from the mouse and monkey (data not shown). Primary antibodies were diluted in PBS+T. After 48 hours of incubation, the sections were washed for 30 minutes in PBS+T and then incubated in one of two secondary antibodies for 2 hrs at room temperature: a) a highly cross-adsorbed Alexa Fluor 488 conjugated goat anti-rabbit IgG (1:400, Molecular Probes) for sections stained for CGRP, or b) a highly cross-adsorbed goat anti-mouse IgG conjugated to Alexa Fluor 596 (1:800, Molecular Probes) for sections stained for DBH. Sections were then washed for 20 minutes with PBS and mounted on gelatine-coated slides, allowed to dry overnight and cover-slipped using Aquapolymount (Polysciences).

2.3.2 Double labelling for co-localization studies

Double-labelled sections were pretreated identically to single-labelled sections and subsequently incubated for 48 hours with a mixture of anti-CGRP and anti-DBH antibodies at 4°C. After 48

hours, the sections were washed for 30 minutes with PBS+T and then placed in a solution containing a mixture of Alexa Fluor 488 conjugated goat anti-rabbit IgG (1:400, Molecular Probes) and highly cross-adsorbed goat anti-mouse IgG conjugated to Alexa Fluor 596 (1:800, Molecular Probes) and 10% NGS, for 2 hrs at room temperature. Sections were then washed for 20 minutes with PBS and mounted and cover-slipped as described above.

2.4 Quantification

Six experimental animals were used at each time point for quantification of sensory and autonomic fibres and compared to 6 sham-operated animals. Non-operated animals were initially included in the quantification, but as no significant differences were found between sham-operated and naive animals, only data from sham-operated animals is shown as controls. For each animal, a total of 10 sections were used in order to ensure representative sampling.

Autonomic fibre density was determined by counting the number of DBH-IR fibres in the upper dermis. The upper dermis was defined as the area spanning 150 μm below the dermal-epidermal junction. This area was determined by what was typically observed in control and sham-operated animals as the area of dermis from which autonomic fibres were normally absent. The value for the total area analysed was calculated by multiplying the total length of the section by the thickness of the upper dermis (150 μm). The counts were performed using a Zeiss Axioplan 2 Imaging fluorescence microscope, equipped with a high resolution colour digital camera and connected to a computer with Zeiss Axiovision 4.1 Software (Zeiss, Canada). The mean number of fibres in the upper dermis per unit area was tested for significance using the Mann-Whitney test with Bonferroni correction. Statistical significance was accepted at $p < 0.05$.

In addition to alterations in autonomic fibre density, changes with respect to the distance of the sprouted fibres from the dermal-epidermal junction were assessed in sham-operated and lesioned animals for each time point. Six random fields were taken from each section using the 40x objective of the Zeiss Axioplan 2 Imaging fluorescence microscope, resulting in a total of 60 images per animal. The distance in micrometers from the most superficial tips of the fibres to the dermal epidermal junction was measured directly on the digital images using the Zeiss Axiovision 4.1 Software. Fibres penetrating the epidermis were given a value of 0 μm for distance from the epidermis. The results were tested for significance by comparing the mean distances of the fibres from the dermal-epidermal junction for each time period using a one-way ANOVA. Pairwise comparisons were made using the Dunnett *post hoc* test.

Changes in CGRP-immunoreactive (IR) innervation in the hind paw skin were determined through analysis of images taken using a Zeiss LSM 510 confocal microscope equipped with Argon and Helium Neon lasers and a 40X water immersion objective. A single optical section measuring 1.5 μm in thickness was used for each field. Four randomly chosen fields in the upper dermis were captured in each section and used for quantification. Images were exported in TIFF format to the MCID Elite image analysis system (Imaging and Research, St. Catherines,ON, Canada) and total fibre length per unit area was determined. One-way ANOVA was used to test for significance for the mean length of fibres in the upper dermis between all time points and pairwise comparisons were assessed using the Dunnett *post hoc* test.

Chapter 3

Results

3.1 Patterns of sympathetic and peptidergic sensory innervation in sham-operated and control animals

No significant differences were found in the innervation patterns of sensory and sympathetic fibres between naïve and sham-operated animals. Therefore, sham-operated animals were used as the control group in quantification.

In sham-operated animals, CGRP-IR (peptidergic) sensory fibres were observed throughout the hind paw skin, appearing to be most dense in the upper dermis. These fibres had a mesh-like innervation pattern with relatively long distances between varicosities (Figure 1A) and occurred as single fibres rather than large nerve bundles. However, in the deeper layers of the dermis, larger bundles of CGRP-IR fibres were seen. CGRP-IR fibres most often terminated at or close to the dermal-epidermal junction (Figure 1A) but were occasionally seen to penetrate the epidermis.

In control and sham-operated animals, DBH-IR fibres (sympathetic) were restricted to the lower dermis (Figure 1B), with the rare occurrence of a single fibre seen in the upper dermis in each of only two of the animals. Sympathetic fibres were largely associated with blood vessels in a mesh-like arrangement around the vessel wall in the lower dermis. In comparison to the sensory fibres, the sympathetic fibres had a higher number of varicosities that were separated by shorter axonal segments (Figure 1B). This population of fibres was observed as single axons rather than large fibre bundles. These findings are similar to previous work from our lab showing DBH-IR innervation to be found only in the lower dermis of the lower lip skin in control rats (Ruocco et al., 2000; Grelik et al., 2005b).

3.2 Changes in CGRP immunoreactivity in upper dermis after CCI

The total mean length of CGRP-IR fibres per unit area ($0.04mm^2$) in the upper dermis of sham animals was 205.8± 41.9 μm. Two weeks post-CCI, there was a significant decrease in peptidergic sensory innervation in the upper dermis of these animals (57.6 ± 19.3 μm) (Figure 2) . Extremely sparse and patchy innervation of these CGRP-IR fibres remained. This was expected as the CCI of the sciatic nerve causes a partial lesion of A-delta and C-fibres (Munger et al., 1992). At the 4 and 6 weeks post-lesion time-points, total length of CGRP-IR fibres per unit area had changed to levels above (271.4 ± 25.9 μm and 277.8 ±35.1 μm respectively) but not significantly different from what was seen in sham-operated animals (Figure 2). Interestingly, there was sprouting of CGRP-IR fibres to a level significantly above sham levels at 8 weeks post-lesion ($p<0.01$, 437.3 ± 47.3 μm) (Figures 2). These sprouted fibres were thicker in diameter and were in thicker bundles as well. As in sham-operated animals, these fibres penetrated into the epidermis of the hind paw skin on occasion (Figure 3A). The distribution pattern of these sprouted fibres was similar to that seen in sham-operated animals, with the notable exception of an increase in number of fibres (Figure 3B). Values of CGRP-IR fibre length per unit area remained significantly elevated above sham levels until the last time point examined of 20 weeks (378.9± 41.5). The values at 12, 16 and 20 weeks did not significantly differ from the 8 week time point, indicating that fibre density values reached a plateau (Figure 2).

3.3 Changes in DBH immunoreactivity after CCI

3.3.1 Sympathetic fibre density in upper dermis

One of the main aims of this study was to determine if CCI would result in migration of sympathetic fibres to regions of the dermis in which they do not normally occur. Two weeks

post-lesion was the first time-point at which sprouted DBH-IR fibres were detected in the upper dermis of lesioned rats (Figure 4A). The values were expressed as number of fibres per mm^2. The levels of sympathetic fibres at the early time point of 2 weeks post-lesion ($8.4 \pm 0.9/mm^2$) were found to be significantly different from sham-operated levels ($p<0.05$, $0.2 \pm 0.11/ mm^2$) (Figure 5). The number of fibres in the upper dermis continued to increase at 4 weeks post-lesion ($p<0.01$, $28.7 \pm 3.2/mm^2$) and remained constant until the last observed time point of 20 weeks ($p<0.01$, $39.7 \pm 9.5/mm^2$) (Figure 5). These migrated DBH-IR fibres appeared to contain more varicosities than what was observed in sham-operated animals (Figures 4A-D). These varicosities also appeared to have shorter inter-varicose axons when compared to sham-operated animals. Although fibre number levels appeared to be similar from 4 until 20 weeks post-lesion, it is noteworthy that the patterns of innervation at these time points were not the same. At 2 and 4 weeks post-lesion, the sprouting was patchy and not consistent throughout the upper dermis, appearing very robust in some areas and absent in others. At the longer time points such as 12,16 and 20 weeks (Figure 6A-C), sprouted fibres were consistently visible in all areas of the upper dermis. In some instances, these sympathetic fibres were observed to penetrate the epidermis in lesioned animals (Figure 6C). There was no distinct correlation between time after lesion and fibres penetrating the epidermis, although these intra-epidermal fibres did seem to occur more often in animals with more robust sprouting.

3.3.2 Migration of sympathetic fibres to surface of skin after CCI

Distance of sympathetic fibre migration was measured in addition to sympathetic fibre density in the upper dermis. The aim of this portion of the study was to determine if there was a distinctive pattern of migration throughout the time periods after nerve injury. The distance was

measured from the dermal-epidermal junction to the closest tip of the sprouted fibre. This was repeated for every fibre in the field of the pictures used for quantification.

The mean distance of sympathetic fibres from the epidermis in sham-operated animals was 360.01 ± 15.63 μm (Figure 7). Two weeks after CCI lesion, the distance had decreased to a mean of 186.32 ± 18.27 μm. By 4 weeks post-lesion the mean distance of sympathetic fibres from the epidermis was 61.31 ± 2.60 μm. As with the other parameters measured in this study, this mean at the four week time point remained more or less constant until the 20 week post-lesion time point (67.35 ± 19.13 μm) (Figure 7). At 20 weeks there was more variability between animals in terms of distance of sprouting, as shown by the larger SEM values.

3.3.3 Association of CGRP- and DBH-IR fibres after CCI

In addition to the changes seen in the patterns of CGRP and DBH-IR fibre innervation after injury, we also observed a unique association of these two distinct populations of fibres after CCI. In many instances, the sprouted sympathetic fibres wrapped around the peptidergic sensory fibres in a winding fashion (Figure 4A-D and Figure 6A-C). As there was never a complete loss of peptidergic sensory innervation, this association could already be seen in the earliest observed time point of 2 weeks after lesion (Figure 4A). This phenomenon continued to be present and became very common as the sympathetic fibre sprouting progressed at later time points (Figure 6A-C). In sham and control animals, peptidergic sensory and sympathetic fibres were seen in close proximity especially around blood vessels, however, they did not follow the same pattern of innervation nor were they associated so close to one another as in the CCI animals.

In control animals, the peptidergic fibres were seen throughout the dermis as isolated fibres and fibre bundles (Figure 1A). Sympathetic fibres were seen in the lower dermis and

mostly around blood vessels in a mesh-like pattern. Peptidergic sensory fibres were also seen in the wall of blood vessels but travelled in a pattern perpendicular to the mesh-like sympathetic fibres, i.e., the sensory fibres ran parallel to the length of the vessel rather than around it (Figure 1B). The sprouted fibres were found to wind around the sensory fibres and followed the nerve tracts exactly (i.e. they were mostly parallel to each other), suggesting the fibres may be following the same trophic signal. This post-lesion association would create an ideal environment for the interaction or exchange of signals between the two populations of fibres.

Figure 1. DBH and CGRP IR-fibres in the upper and lower dermis of control (sham-operated) animals. A: Note the presence of CGRP-IR fibres (in green) throughout the upper dermis. Note the absence of DBH-IR fibres (sympathetic) in the upper dermis. **B:** In the lower dermis, CGRP-IR fibres travel parallel to blood vessels while DBH-IR (sympathetic fibres) form a mesh-like pattern around blood vessels. DBH-IR fibres are mostly associated with blood vessels in the lower dermis. The stratum corneum was apparently labeled for CGRP and DBH, but this was a result of non-specific tissue labeling of the fluorochrome-conjugated secondary antibodies as it was still present when the primary antibodies were omitted.

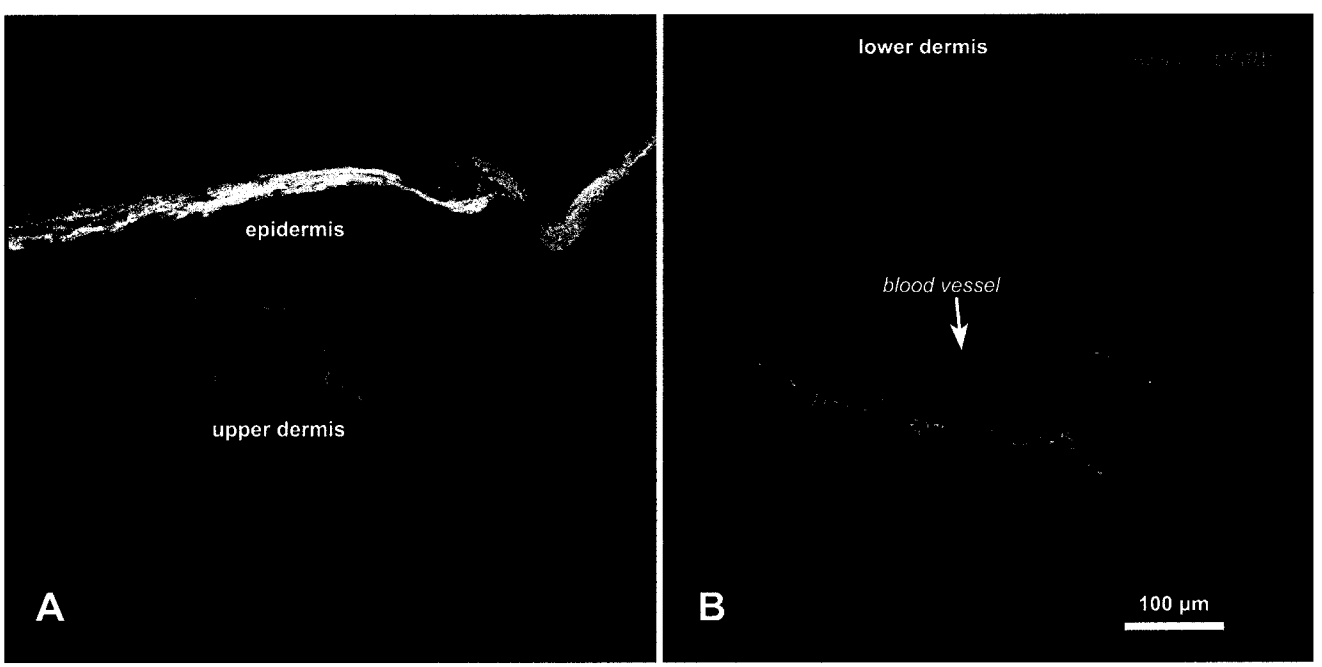

epidermis
upper dermis
A
lower dermis
blood vessel
B
100 µm

Figure 2. Total length of CGRP-IR fibres (±SEM) in the upper dermis, per 0.0345 mm^2, in sham-operated and CCI rats. At 2 weeks post-CCI, the length per unit area of CGRP-IR fibres decreased significantly from sham-operated levels. By 4 and 6 weeks post-CCI, length of CGRP-IR fibres had reached values above but not significantly different from sham-operated levels. At 8 weeks post-CCI, the density of fibres had significantly increased above sham-operated levels. The total length per unit area of CGRP-IR fibres remained significantly above sham-operated levels until the last observed time-point of 20 weeks. Comparisons were carried out with one-way ANOVA followed by Dunnett post hoc comparisons. $n=6$ * = $p<0.05$, ** = $p<0.01$.

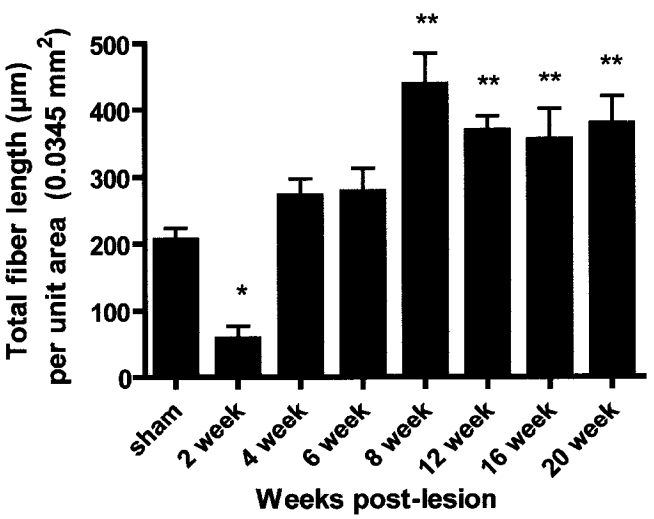

Total fiber length (µm)
per unit area (0.0345 mm²)
500
400
300
200
100
0
**
*
**
**
**
sham
2 week
4 week
6 week
8 week
12 week
16 week
20 week
Weeks post-lesion

Figure 3. CGRP-IR sensory innervation at 8 weeks post-surgery in sham-operated and CCI animals. A: In sham-operated animals, CGRP-IR (peptidergic sensory) fibres in green, were numerous in the upper dermis, occasionally penetrating the epidermis. **B:** Interestingly, at 8 weeks post-CCI, the CGRP-IR sensory innervation was considerably denser than in sham-operated animals. Note that these fibres tended to be thicker and branched more than in sham-operated animals. CGRP-IR fibres in CCI animals also travelled in larger bundles than what was typical of control and sham-operated animals. These increased fibre levels were sustained until the last observed time point of 20 weeks post-CCI.

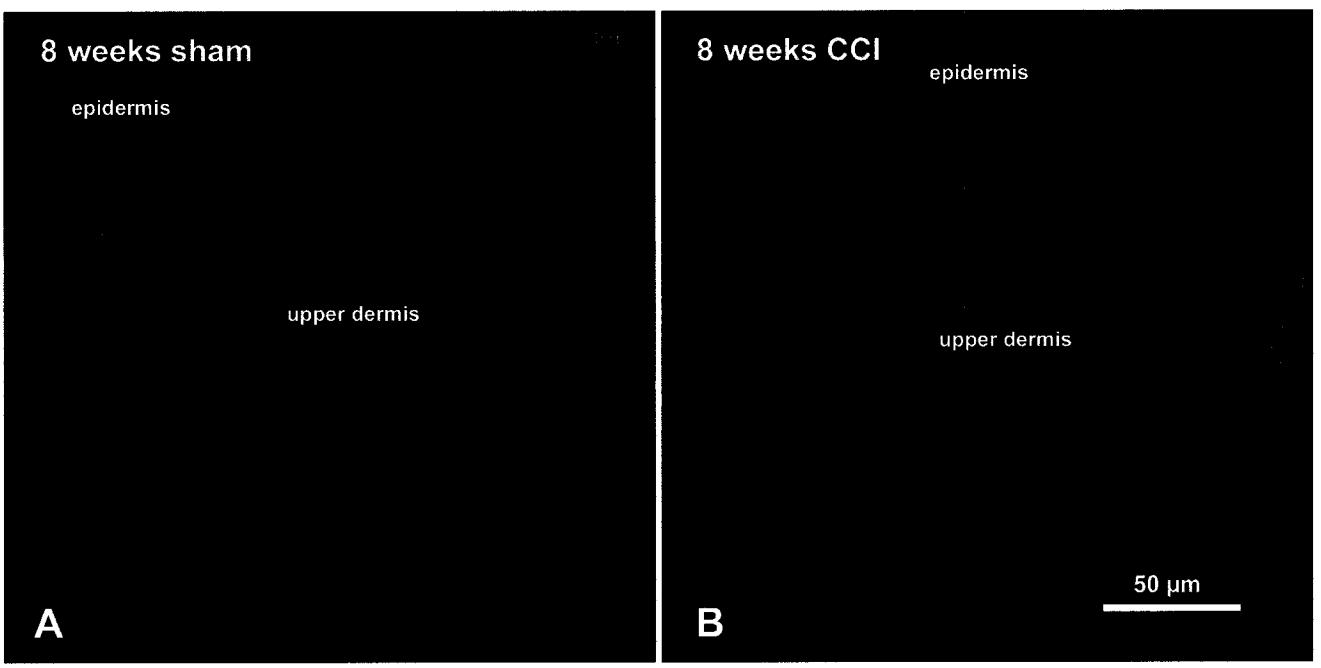

8 weeks sham
epidermis
upper dermis
A
8 weeks CCI
epidermis
upper dermis
50 µm
B

Figure 4. Pattern of CGRP- and DBH-IR fibre innervation 2, 4, 6, and 8 weeks after CCI. **A**: Note the presence of sprouted DBH-IR fibres in the upper dermis as early as 2 weeks post-CCI. At this time point, these novel fibres were sparse but at levels still significantly higher than in sham-operated animals. Note a DBH-IR fibre (in red) wrapping around the remaining CGRP-IR fibres (in green) in the upper dermis. No DBH-IR fibres were seen to penetrate the epidermis at this time point. **B:** At 4 weeks post-lesion, sprouted DBH-IR fibres were more numerous and more sensory-sympathetic associations were observed as CGRP-IR fibres started to regenerate. **C and D:** At 6 and 8 weeks post-CCI, respectively, there was a very robust sprouting of DBH-IR and CGRP-IR fibres. At 8 weeks post-CCI, CGRP-IR fibres were thicker and travelled in thicker bundles. These regenerated CGRP-IR fibres often wrapped around DBH-IR fibres. The stratum corneum was apparently labeled for CGRP, but this was a result of non-specific tissue labeling of the Alexa Fluor 488 conjugated goat anti-rabbit IgG as it was present when the primary antibody was omitted.

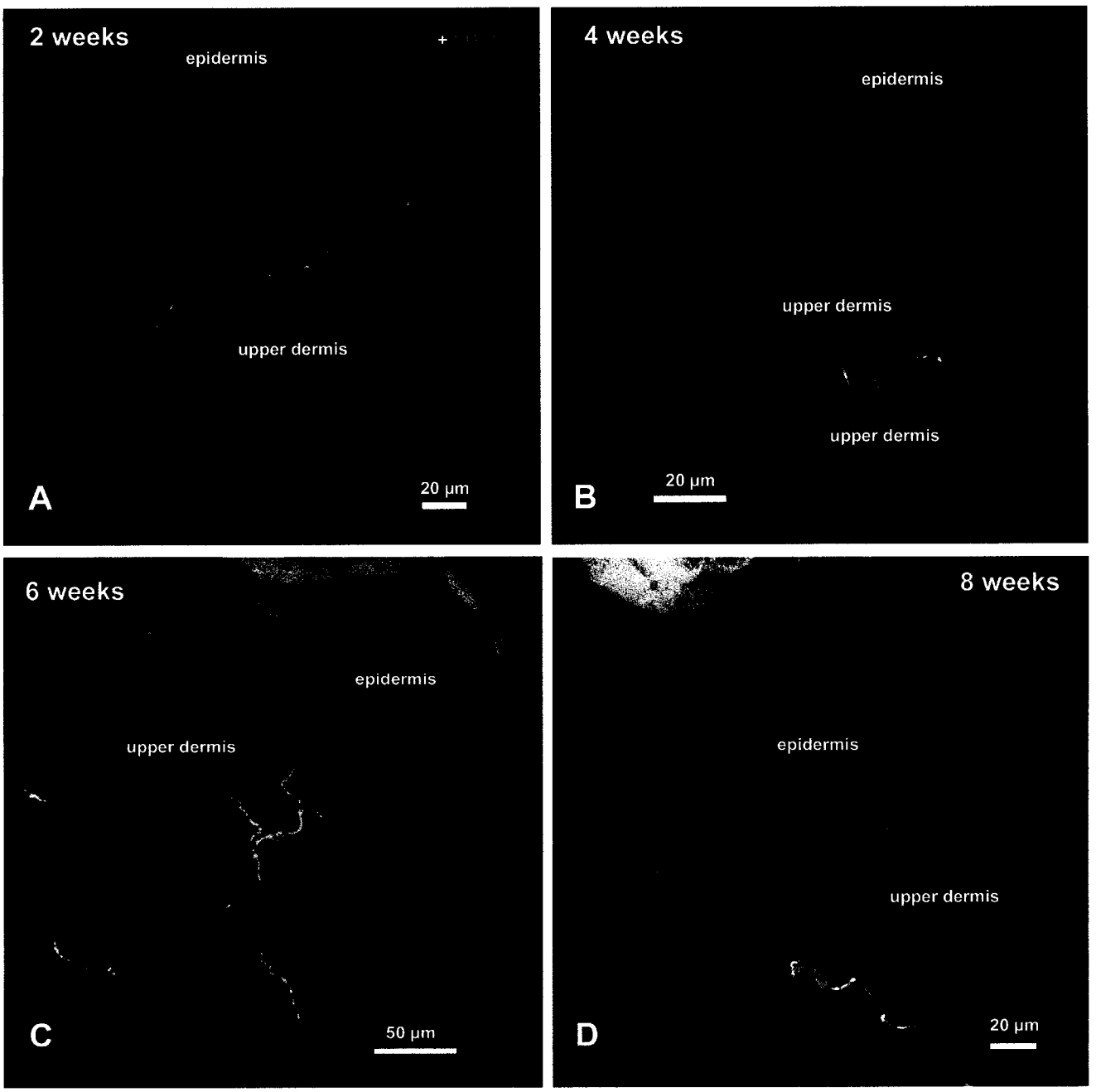

2 weeks
epidermis
upper dermis
A
20 µm
4 weeks
epidermis
upper dermis
upper dermis
B
20 µm
6 weeks
epidermis
upper dermis
C
50 µm
8 weeks
epidermis
upper dermis
D
20 µm

Figure 5. Average number of DBH-IR fibres in the upper dermis in sham-operated animals and CCI animals per mm^2. Sympathetic fibres were very rarely found in the upper dermis of the glabrous hind-paw skin in sham-operated rats. Levels significantly increased at 2 weeks post-CCI. The number of DBH-IR fibres in the upper dermis continued to increase at 4 weeks, remaining significantly higher than in sham-operated rats. DBH-IR fibre densities remained at peak levels until the last observed time point of 20 weeks. Mann-Whitney test with Bonferroni correction, n=6, ** = $p<0.01$.

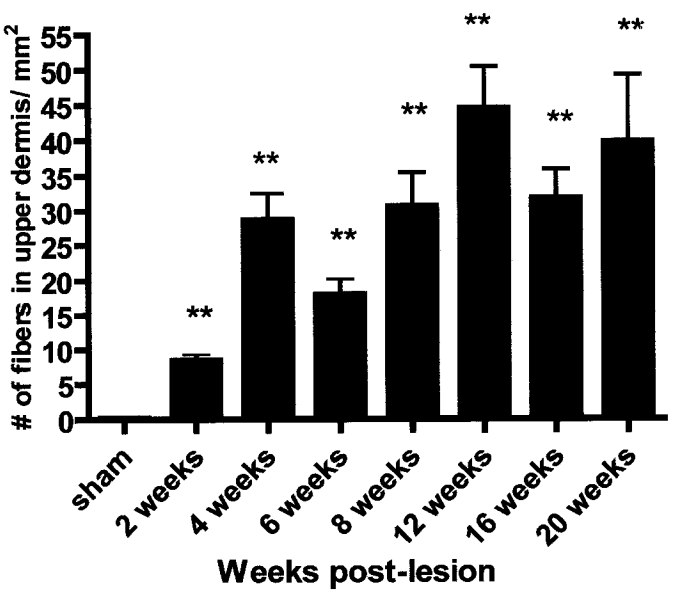

# of fibers in upper dermis/ mm2
55
50
45
40
35
30
25
20
15
10
5
0
**
**
**
**
**
**
**
sham
2 weeks
4 weeks
6 weeks
8 weeks
12 weeks
16 weeks
20 weeks
Weeks post-lesion

Figure 6. Pattern of DBH-IR and CGRP-IR fibre innervation at 12, 16 and 20 weeks post-CCI. At 12-20 weeks post-CCI, DBH-IR sprouted fibres remained numerous in the upper dermis and occurred with a more homogenous distribution compared to the patchy pattern seen at earlier time points. Many intra-epidermal DBH-IR (arrows) and CGRP-IR fibres were observed. Many large CGRP-IR fibre bundles were seen to wrap around sprouted DBH-IR fibres. The levels of sprouted DBH-IR and CGRP-IR were maintained at peak levels from 8-20 weeks. The stratum corneum was apparently labeled for CGRP, but this was a result of non-specific tissue labeling with the Alexa Fluor 488 conjugated goat anti-rabbit IgG as it was present when the primary antibody was omitted.

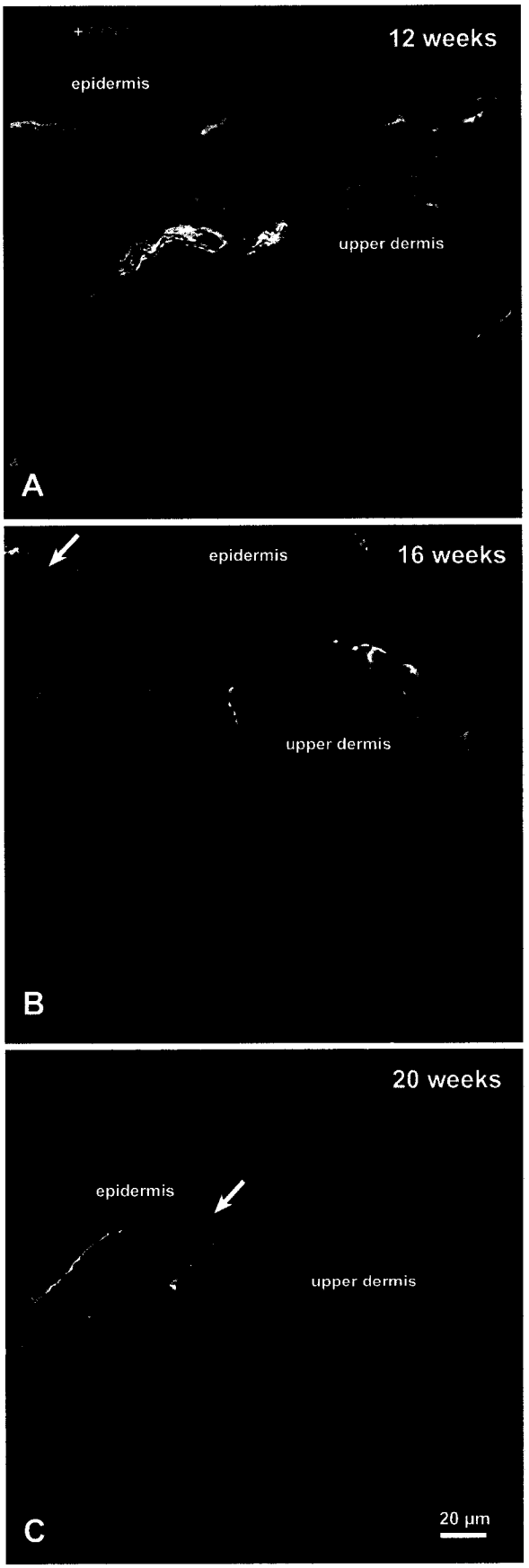
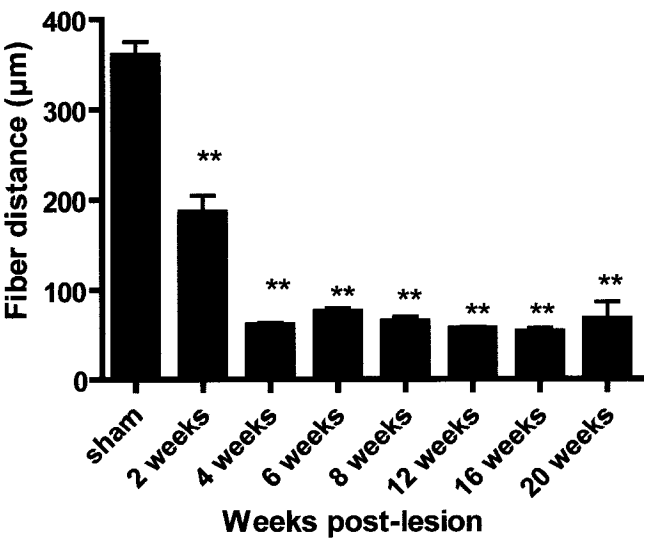

12 weeks
epidermis
upper dermis
A
epidermis
16 weeks
upper dermis
B
20 weeks
epidermis
upper dermis
C
20 µm

Figure 7. Average distances of DBH-IR fibres from dermal-epidermal junction in glabrous skin of hindpaw in sham-operated and lesioned animals (in μm). Distances of DBH-IR fibres from the dermal-epidermal junction were significantly decreased compared to sham levels starting at 2 weeks post-lesion. Average distances decreased further at 4 weeks post-CCI and remained at significant levels until the last observed time point of 20 weeks post-CCI. One way ANOVA followed by Dunnett post hoc comparisons (n=6). ** = $p<0.01$.


400
300
200
100
0
Fiber distance (µm)
**
**
**
**
**
**
**
sham
2 weeks
4 weeks
6 weeks
8 weeks
12 weeks
16 weeks
20 weeks
Weeks post-lesion

Chapter 4

General Discussion

4.1 Sympathetic fibre sprouting into the upper dermis after CCI

In this study, we have shown that following a CCI of the sciatic nerve, there is significant sprouting of both sympathetic and peptidergic sensory fibres in the upper dermis of the plantar surface of the rat hind paw skin (Figures 2 and 5). Sympathetic fibres are normally absent from the upper dermis and, before this study, had never been shown to migrate in non-hairy skin after neuropathic nerve injury. The sympathetic sprouting became significantly different from sham levels as soon as two weeks post-lesion. This is a surprising observation, as some of the sympathetic fibres travel in the sciatic nerve (Small et al., 1996) and are therefore likely to have been damaged by the lesion. The fact that we did not detect any decrease in the sympathetic innervation would indicate that such a decrease might be masked by a very robust sprouting already significant at 2 weeks and/or that the number of sympathetic fibres traveling in the sciatic nerve is minor compared to that traveling with blood vessels. Sprouting peaked at 4 and 6 weeks and remained constant until our last time point of 20 weeks post-lesion. Overall, we observed a steady sprouting and decrease in fibre distance from the epidermis that remained constant until the last time point (Figure 7).

Previous reports have focused on sympathetic sprouting around DRG neurons following spinal nerve ligation and CCI (Chung et al., 1993; Chung et al., 1996; Ramer and Bisby, 1997). In some cases, the sprouting around DRG neurons was consistent with neuropathic behaviour however, while in other models neuropathic behaviour was present without significant sprouting after nerve injury (Lee et al., 1998). These inconsistencies in sympathetic sprouting around the DRG indicate the need for further investigations into the role of sprouting in neuropathic behaviour. A possibility is that sympathetic sprouting does contribute to neuropathic pain in

these models, but at a more peripheral location. In this thesis we show findings which represent the first report on a more distal location of the sympathetic sprouting following CCI of the sciatic nerve, as we demonstrate it in the glabrous skin of the plantar surface of the hindpaw.

In comparison to a previous investigation from our laboratory in which sprouting in the hairy skin of the lower lip was studied following a bilateral CCI of the MN (Grelik et al., 2005b), the model used in the present study leads to a more robust and permanent sprouting. In the previous study, it was observed that sympathetic sprouting in the upper dermis peaked at four and six weeks post-lesion. These sprouted sympathetic fibres persisted until 16 weeks at which time they decreased in numbers to finally return to control levels at 24 weeks (Grelik et al., 2005b). Our data show a later onset of sympathetic sprouting, as in the previous study sprouting was detected at 1 week post-lesion, and we did not observe ectopic sympathetic fibres until 2 weeks. One major difference between the two models is that in the MN model no sympathetic fibres were lesioned, as they do not travel in the MN of the rat (Grelik et al., 2005b), however they do travel in the sciatic nerve(Small et al., 1996). Therefore, if some sprouted fibres originate from collaterals of those travelling along blood vessels, others are likely regenerated fibres and these have to regrow along the sciatic for a rather long distance (Gloster and Diamond, 1992).

Lastly, in comparison to the MN injury, CCI sympathetic sprouting in the hind paw was more robust. Grelik et al. (2005b) observed an average of 10 fibres per mm^2 at 4 weeks post-lesion while we observed an average 28.6 sprouted sympathetic fibres per mm^2. These differences between models may be attributed to the different characteristics of the two injured nerves. In the rat, the MN is almost exclusively sensory, while the sciatic contains both sensory and sympathetic fibres, as well as motor fibres.

4.2 Role of adrenergic sensitivity in neuropathic pain after CCI

It is probable that the sympathetic nervous system plays a role in some types of neuropathic pain (Roberts, 1986; Bonica, 1990; Ren et al., 2005), however the exact mechanism or location of interaction with the sensory system has yet to be revealed. Several lines of evidence suggest that peripheral adrenergic receptors are involved in SMP (Davis et al., 1991). At some point after nerve injury, primary afferent cell-bodies develop *de novo* adrenergic agonist sensitivity at the level of the dorsal root ganglia (Michaelis et al., 1996), as do the nociceptors in the skin (Sato and Perl, 1991; Gold et al., 1994; Bossut and Perl, 1995), which may result from the expression of α-adrenoceptors in primary afferent neurons (Birder and Perl, 1999). In the case of neuromas, there is evidence of the development of α-, but not β-, adrenergic sensitivity(Wall and Gutnick, 1974a; Devor and Janig, 1981).

Studies of peripheral manipulation with adrenergic antagonists and agonists also support the role of peripheral adrenergic receptors in pain (Treede et al., 1991). Topical application of the α_2 adrenergic receptor agonist, clonidine, relieves hyperalgesia in SMP patients (Davis et al., 1991). Blockade of adrenergic receptors with phentolamine (Raja et al., 1991; Treede et al., 1991), phenoxybenzamine or prasozin have also shown efficacy in alleviating SMP (Abram and Lightfoot, 1981; Ghostine et al., 1984), while the β-adrenergic receptor antagonist, propanolol, does not show as much efficacy in SMP (Scadding et al., 1982; Raja et al., 1991). Peripheral application of NE can cause exacerbation of the hyperalgesia from complete Freund's adjuvant-induced neuritis (Baik et al., 2003), while intradermal injection of NE or α-adrenergic agonists can rekindle pain and hyperalgesia in patients and animals which had been relieved by sympathectomy or sympathetic block (Wallin et al., 1976; Xie et al., 1995; Moon et al., 1999).Results from Ali et al. (1999), suggest it is the uninjured C-fibres that develop α-adrenergic

sensitivity and hyperactivity after nerve injury. As small diameter sensory fibres did sprout in the upper dermis in our model, and occur in higher number than in controls, if adrenergic receptors are upregulated in these fibres as well, we might have a possible explanation for the increased adrenergic sensitivity observed following nerve lesion in some models. Further studies are needed to confirm this hypothesis.

Although the sympathetic fibre sprouting in DRG may play a role in SMP, there is considerable evidence suggesting that pain, especially cutaneous pain, is caused by a mechanism at the skin level. Our anatomical evidence of sprouted sympathetic fibres after nerve injury in close proximity to sensory fibres would support a role of cutaneous sympathetic fibres in SMP. In addition to the sprouting of these fibres, our evidence for a close association of sprouted sympathetic fibres to the remaining and the regenerated peptidergic sensory fibres suggests the sympathetic and sensory terminals as the initiation site of SMP, as it provides an ideal environment for the release of NE from the sympathetic fibres to act on sensory terminal axons. Many studies have shown behavioural evidence that this may be the case in both rats and humans. In rats, cutaneous injections of NE can exacerbate neuritis-induced hyperalgesia (Baik et al., 2003), and rekindle mechanical allodynia in sympathectomized neuropathic rats (Xie et al., 1995). In addition, NE injected into skin of patients with Complex Regional Pain Syndromes of types I (reflex sympathetic dystrophy or CRPS-I) and II (causalgia or CRPS-II) causes a response of prolonged burning pain and allodynia, i.e., the rekindling phenomenon (Wallin et al., 1976; Torebjork et al., 1995; Ali et al., 2000). It is difficult to see how this phenomenon would be centrally mediated or mediated by sympathetic sprouts in the DRG when the evoked response is caused by an injection of α-adrenergic agonists to the skin (Mailis-Gagnon and Bennett, 2004).

4.3 Sensory fibre sprouting in upper dermis after CCI

We have also shown a significant increase in the peptidergic sensory fibre innervation in the upper dermis of the rat hind paw after CCI (see Figure 3). There was an initial decrease in the peptidergic sensory fibre innervation followed by a recovery (see Figure 2). Lindenlaub and Sommer (2002), observed an almost complete loss of CGRP-IR fibres in the epidermis 7 and 30 weeks after CCI, followed by a partial late recovery at 53 days post-lesion. This is very different from what we observed. In contrast, our observations concur with those of Lin et al. (2001), where only partial denervation of epidermal fibres resulted from CCI and was correlated with thermal hyperalgesia. Surprisingly, we found that the peptidergic sensory fibre innervation density increased to a level significantly higher than that of sham-operated animals. It is worth noting that sprouted peptidergic sensory fibres in the upper dermis at and after the 8 week time point were more numerous (Figure 3) and in thicker bundles than in sham-operated animals. As these fibres are more numerous and have likely developed adrenergic sensitivity (Korenman and Devor, 1981; Scadding, 1981; Sato and Perl, 1991; Baik et al., 2003), after the nerve lesion, this could be a possible mechanism for hyperalgesia and sensitivity to sympathetic input.

This increase in sensory innervation has been seen previously in the rat in the skin of the lower lip after CCI of the MN and correlated with isolated grooming episodes directed to the area of nerve injury (Grelik et al., 2005b). However, the sprouting in the lower lip skin was transient, whereas the sciatic nerve lesion resulted in chronic sprouting in the hindpaw skin for at least 20 weeks. Conversely, sensory sprouting appeared more robust in the MN CCI; at 8 weeks post-CCI we observed a peak of 437.3 ± 47.3 μm of CGRP-IR fibre length per 0.04 mm^2, with an overall average change from sham of 231.5 μm per 0.04 mm^2, while Grelik et al. (2005b) observed a

peak in CGRP-IR fibre length four weeks post-lesion with an average value of 1436.9±45.7 μm per 0.02 mm^2 and an overall change of 714.7 μm per 0.02 mm^2 from sham levels.

Increased numbers of unmyelinated sensory fibres have also been observed in sections of sciatic nerves after application of a fixed diameter, polyethylene cuff to the sciatic nerve (Mosconi and Kruger, 1996), a model different but comparable to the one we used here. Hence, it is possible that this sensory hyperinnervation after sciatic nerve injury may not be limited to the dermis and may play a role in neuropathic pain. The contribution of these sensory fibres to hyperalgesia may be time-dependent as sprouting was shown to peak and then decrease over time (Mosconi and Kruger, 1996; Grelik et al., 2005b). Unlike the unmyelinated sprouting in the distal sciatic nerve (Mosconi and Kruger, 1996), the sprouting in our model was seen at the level of the terminal receptive fields and, therefore, may be more significant with respect to hyperalgesia.

4.4 Interaction between sprouted sympathetic fibres and sensory fibres

The appearance of sprouted sympathetic fibres and their morphological interaction with sensory fibres from the early time period of 2 weeks post-lesion onwards (Figure 4A), strongly suggests there may be a sympathetic contribution to the hyperalgesia in these rats. The close proximity and similarities in innervation patterns provide an ideal environment for the sympathetic fibres to release factors which will stimulate the newly adrenergic-sensitive sensory fibres. It has been previously suggested that a coupling exists between sensory and sympathetic fibres after nerve lesion (Habler et al., 2000). The close interaction we see in our data would support a mechanism of coupling such as exchange of neurotransmitters between the two populations of fibres. However, not all sprouted sympathetic fibres were associated with

peptidergic sensory fibres, suggesting that close proximity may not be the only level at which these fibres contribute to hyperalgesia.

4.5 Source of sympathetic and sensory sprouting

The source of this sprouting is unclear as there are reports showing both collateral sympathetic sprouting and regenerative sprouting after nerve injury (Gloster and Diamond, 1992). Collateral sprouting is from undamaged, intact axons of neighbouring regions while regenerative sprouting is from damaged axons of the injured nerve. Additionally, collateral sprouting was found to be dependent on growth factors derived from the target tissue , namely NGF, while regenerative sprouting was NGF-independent (Gloster and Diamond, 1995; Ro et al., 1998). The source of sympathetic sprouting into the upper dermis in this model is unknown as sympathetic fibres in the rat hindlimb travel in both the sciatic nerve as well as along blood vessels. Further studies of the presence of sympathetic axons in the sciatic nerve after CCI may give an indication of the likelihood of regenerative versus collateral sprouting in this model. The sprouting may be collateral at earlier time points, such as two weeks, and may be regenerative at later time points. This time-dependence of sprouting source may also translate to NGF-dependent or independent sprouting. In other models of sympathetic sprouting in the skin, such as the complete bilateral transection of the MN (Ruocco et al., 2000), it is safe to say that the source was collateral sprouting because the MN contains only sensory neurons.

The mechanism and source of sensory sprouting may be similar to what was previously mentioned for regenerative and collateral sympathetic sprouting (Mearow, 1998). As the CCI directly lesions sensory axons, the sprouting may be both regenerative from the sciatic nerve and collateral from the non-lesioned, sural nerve territory of the paw. The types of sensory sprouting

may be time, injury as well as growth-factor dependent. Previous studies in animals and humans have shown sprouting of collateral fibres from adjacent cutaneous sensory axons into denervated areas for a limited distance after peripheral nerve injury (Devor et al., 1979; Jackson and Diamond, 1984; Inbal et al., 1987). This sprouting was shown to be regulated by NGF (Ro et al., 1996).

4.6 Mechanism of sensory and sympathetic sprouting in the periphery: the role of neurotrophic factors

Neurotrophic factors are necessary for neuronal survival, neuronal growth during development of the nervous system and the maintenance of structural and functional integrity and plasticity of the adult nervous system during injury or disease (Sah et al., 2003). Many neurotrophic factors such as NGF, neurotrophin-3 (NT3), brain-derived neurotrophic factor (BDNF) and GDNF have been implicated in neuropathic pain as well as in sympathetic sprouting. Further research is needed to determine the exact roles of these neurotrophic factors in neuropathic pain and the effect they have on sympathetic involvement in neuropathic pain.

4.6.1 Nerve growth factor

Numerous studies have shown a link between NGF and neuropathic pain (Ro et al., 1996; Ro et al., 1999). NGF and its high affinity tyrosine kinase A (trkA) receptor may play the main roles in signaling the migration of sympathetic fibres as well as sprouting of sensory fibres into the upper dermis of the hindpaw as they have already been shown to regulate sensory sprouting in the rat hairy skin (Diamond et al., 1992). Local or systemic injections of NGF into the skin of

the hindpaw induce hyperalgesia (Amann et al., 1996) and this hyperalgesia can be blocked by the receptor neutralizing antibody trkA-IgG (McMahon et al., 1995).

Sensory and sympathetic fibres take up NGF and retrogradely transport it via a trkA receptor-controlled mechanism to the cell body (Anand, 2004) where it plays a key role in the survival of these neurons (Levi-Montalcini, 1987). NGF is not normally expressed in significant amounts in non-neuronal cells, however, after nerve lesion there is a substantial increase in NGF mRNA and nerve growth receptors in macrophages, fibroblasts and Schwann cells, proximal to the lesion site in the nerve and along the distal segment (Taniuchi et al., 1986; Ramer and Bisby, 1997; Zhou et al., 1999). The same is seen in other areas affected by the nerve trauma such as the skin: NGF mRNA expression increases in the distal nerve pathways and non-nerve associated cells, such as cells in the basal epidermal layer after denervation (Mearow et al., 1993). This NGF upregulation is necessary for collateral sprouting of sensory neurons in the thoracic skin of the rat (Mearow and Kril, 1995) and collateral sprouting from neighbouring intact axons of denervated rat skin (Mearow et al., 1993; Ro et al., 1998).

Mice with an over-expression of NGF driven by a GFAP promoter show increased sympathetic sprouting in the DRG and enhanced ipsilateral responses to thermal and mechanical stimulation compared to wild-type mice (Ramer et al., 1998a). Transgenic mice over-expressing NGF in the skin have sympathetic basket-like projections to trkA expressing and NGF-IR sensory neurons similar to those previously seen in chronic models of pain (Walsh and Kawaja, 1998; Davis et al., 1998).

Conversely, it was found that antisera specific to NGF reduced the formation of sympathetic sprouting around the DRG after spinal nerve ligation. NGF anti-sera was also reported to attenuate allodynia(Zhou et al., 1999; Deng et al., 2000b). Previous studies found

upregulation of the trkA receptor on peptidergic sensory fibres in the lower lip skin after CCI of the MN (Grelik et al., 2005b). It is possible that the sensory and sympathetic fibres have an increased response to NGF released from surrounding tissues due to this trkA upregulation. As sensory fibres degenerate after nerve injury in the upper dermis of both the lip skin and the paw skin, they may be responding and migrating towards the increase in NGF expression (Ruocco et al., 2000). In this sense, NGF would be acting as a chemo-attractant signal, triggering the sprouting (Grelik et al., 2005b). Sympathetic fibres have been shown to synthesize and secrete NGF protein (Hasan et al., 2003), so it is possible that sympathetic neurons upregulate NGF secretion after injury. This excess NGF may act in an autocrine manner to cause sympathetic sprouting in addition to sensitizing nearby primary afferents.

As NGF has proved to be involved in sympathetic sprouting and neuropathic behaviour in other models of neuropathic pain, it is possible that it plays a similar role in the sympathetic sprouting in the rat hindpaw following CCI as well as in the associated neuropathic behaviour.

4.6.2 Neurotrophin-3

Like NGF, NT-3 is able to induce *in vitro* neurite outgrowth from sympathetic neurons (Belliveau et al., 1997). NT-3 also plays a role in the differentiation, development and survival of sympathetic neurons in early development (Zhou and Rush, 1995). Anti-NT3 antibodies when injected either systemically or locally reduced sympathetic sprouting around the DRG after nerve lesions. Many similarities can be drawn in the modulatory affects of NGF and NT3 on sympathetic growth and sprouting, including their action on the TrkA receptor (Belliveau et al., 1997). NT3 has also been shown to attenuate allodynia after nerve lesion (Deng et al., 2000b). NT3 is traditionally the growth factor for large-diameter TrkC expressing afferents but also binds

TrkA. In the environment of a nerve lesion such as the CCI where both large diameter and small diameter neurons are damaged, the environment and trophic factor support may be altered and NT3 may play a role in sympathetic sprouting and neuropathic pain that it would not play under normal physiological conditions.

4.6.3 Brain-derived neurotrophic factor

BDNF is expressed mostly within small neurons and medium-sized sensory neurons which also express CGRP and TrkA (Michael et al., 1997). This molecule is among the main neurotrophic factors implicated in neuropathic pain. BDNF is yet another growth factor that induces sprouting of sympathetic axons in intact DRG and after nerve lesion(Deng et al., 2000a). Antisera for BDNF have been shown to attenuate sympathetic sprouting in the DRG, but to a lesser extent that NGF and NT3 antisera. However, antisera for BDNF also attenuates allodynia following peripheral nerve lesion (Deng et al., 2000b)

It is interesting to observe that so many neurotrophic factors have similar effects on sympathetic sprouting and neuropathic pain. There are many redundancies and connections between the pathways and it is not doubtful that many of these pathways converge and modulate one another. The previously mentioned example of NT3 and NGF acting through TrkA is an example. Additionally, exogenous NGF administration was shown to increase BDNF levels in TrkA positive DRG neurons (Michael et al., 1997). It is possible that these neurotrophic factors act in concert to contribute to sympathetic sprouting and neuropathic pain.

4.6.4 Other growth factors

Other factors have been shown to be involved in sympathetic sprouting: chemokines such as leukemia inhibitory factor (LIF) and Il-6 have been shown to influence sympathetic sprouting after nerve injury in animal models of neuropathic pain (Bolin et al., 1995; Kurek et al., 1996; Thompson and Majithia, 1998; Ramer et al., 1998b).

4.7 Future Directions

The results of this thesis make a small contribution to the field of research in sympathetically-maintained pain. The results show a sprouting of sympathetic fibres after partial peripheral nerve injury as well as sprouting and hyperinnervation of nociceptive peptidergic sensory fibres. The close association of sprouting sympathetic fibres and regenerated sensory fibres suggests a peripheral role of the sympathetic nervous system in neuropathic pain. However, much research is left to be done for this model as we have only shown anatomical association and not a behavioural or molecular association between neuropathic pain and the peripheral sympathetic nervous system. Time-course studies correlating the pain-related behaviour changes with the trophic factor expression changes and the fibre sprouting should be carried out. Changes in trophic factor receptor expression over time should also be done.

4.7.1 Behavioural link to sympathetic and sensory sprouting

Numerous studies have explored the link between sympathetic sprouting and neuropathic pain behaviour in animal models of pain. Most of these studies looked at sprouting in the DRG after a spinal nerve injury or in transgenic animal models (see section 3.2). Sprouting in the

hindpaw skin after partial nerve injury is a newly discovered phenomenon and therefore a behavioural correlation has yet to be done. Local injections of factors relased from sympathetic fibres and evoked and spontaneous pain studies could be used to determine if the regenerated sensory fibres have become sensitive to molecules released by sympathetic fibres such as NE or ATP. At the same time a chemical sympathectomy in animals using guanethidine or 6-hydroxydopamine (Wei et al., 2002) could serve as a control by eliminating a source or peripheral release of sympathetic factors . An α-adrenoceptor blocker such as phentolamine or yohimbine (Hord et al., 2001) could be used to see if blockage of these receptors decreased neuropathic behaviour (Kinnman et al., 1997). Ideally, in order to show a peripheral mechanism for sympathetically maintained pain, sympathetically evoked pain behaviour would appear around the same times as sprouting and fluctuate in parallel with sprouting levels. This may prove to be difficult as it is unknown if sympathetically maintained pain is entirely an effect of the central or peripheral nervous system or of both. Other studies have shown relief of neuropathic behaviour four days after CCI using α1 and α2 adrenergic antagonists(Hord et al., 2001). Our preliminary studies have shown a correlation in spontaneous pain behaviour following NE injection in the paw skin in rats after CCI for up to 20 weeks (Yen, Bennett and Ribeiro-da-Silva unpublished data). In addition to testing adrenergic sensitivity, immunohistochemistry should be done to detect any upregulation of adrenergic receptors on nociceptive fibres in the hindpaw skin in the area of peptidergic fibres sprouting and sympathetic sprouting.

4.7.2 NGF and molecular mimicry on behaviour and sprouting

The next logical step in this project will be to determine the mechanism behind this sprouting of sympathetic fibres in the trigeminal territory as well as in the area of the hindpaw. It has been shown that the collateral sprouting of mature,undammaged sympathetic axons is dependent on the availability of NGF (Gloster and Diamond, 1992). Recent data from our lab has also shown a qualitative increase in the NGF receptor, trkA, in the dermis of the lower lip after CCI of the mental nerve (Grelik et al., 2005b). These results suggest that NGF may be acting as a chemo-attractant signal, triggering the sprouting of these sympathetic fibres. The quantification of changes in NGF and its receptor trkA in the dermis after CCI could be performed through Western blotting. Assuming that these sprouted sympathetic fibres play a major role in the genesis of neuropathic pain, modulation of this abnormal fibre sprouting with trkA antagonists as well as anti-NGF antibodies would help determine the signalling required for sprouting. These molecules could be injected in the skin for varying periods of time following nerve injury to observe the effects of sprouting of sensory and autonomic fibres. Modulation of this sprouting by antagonists could help to determine the involvement of these tropic factors in the development of pain. The administration of these NGF and trkA antagonists may also lead to an effect on sensory neurons to prevent neuropathic pain and neuropathic behaviour linked to upregulated NGF and trkA. A modulation of both sympathetic sprouting and sympathetically maintained neuropathic pain behaviour by anti-NGF and trkA receptor modulators would prove a peripheral mechanism for sympathetically maintained pain in the CCI model.

4.7.3 Correlation to human disease

Lastly, a correlation of the data from this animal model to human pathology and disease is very important in order for this area of pain research to flourish and translate to novel drug therapies and approaches to pain management. Compared to animal studies, fewer studies have been done on changes in human skin after nerve injury. Nonetheless, studies of human cutaneous innervation will prove useful. Different studies have shown cutaneous innervation changes in patients diagnosed with CRPS and other types of neuropathic pain (Drummond et al., 1996; Oaklander, 2001). Unfortunately, many of these studies have been done in a very small number of individuals as it is difficult to obtain skin samples from patients.

Recently, changes in innervation of human skin have been reported which would support a hypothesis of sympathetic involvement in neuropathic pain (Albrecht et al., 2006). Changes in human CRPS skin may include a decrease in CGRP innervation in the upper dermis and dermis, as well as aberrant CGRP innervation of the hair follicles (Albrecht et al., 2006). Of particular interest is an upregulation of α2C adrenoceptors which are normally associated with inhibition of neuronal activity. This inhibitory receptor may be upregulated to compensate for an increase in neuronal firing where a sensitivity to sympathetic activity after nerve injury has occurred (Sato and Perl, 1991; Drummond et al., 1996). CRPS skin also shows aberrant DBH and NPY expression in sweat glands as well as in superficial pre-capillary arterioles. These results are not as drastic as the changes seen in the hindpaw skin of the CCI animal model in this thesis, but these results do suggest the mechanism of sympathetically maintained pain in humans may be similar to that seen in animal models.

4.8 Conclusion

Our results show an increase in sympathetic fibres and an initial decrease followed by hyperinnervation of peptidergic sensory fibres after CCI, in the rat hindpaw skin. The sprouting of sympathetic fibres supports the evidence of sympathetic sprouting found in previous studies of neuropathic nerve injury in both the DRG and other peripheral sites. We document for the first time sprouting of sympathetic fibres in the glabrous skin of the hindpaw after nerve lesion. This sprouting may play a role in SMP as application of adrenergic agonists to this site has been previously shown to elicit pain. We have also shown a delayed increase in the density of peptidergic sensory innervation in the upper dermis, the same location in which the sympathetic sprouting occurred.

Though further research is necessary to understand the mechanism behind sympathetically maintained pain, this thesis supports a possible involvement of sympathetic sprouting in the rat hindpaw skin after nerve injury in SMP through a peripheral mechanism.

Chapter 5

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Works Cited

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Appendix A

Laurene Dao-Pei Yen, Ms

To...

Cc...

Bcc...

Subject: FW: FW: Permission Request: 0471559792

Attachments:

From: BJohns@wiley.com [mailto:BJohns@wiley.com]
Sent: Mon 02/07/2007 11:20 AM
To: Alfredo Ribeiro-da-Silva, Dr.
Cc: Laurene Dao-Pei Yen, Ms
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