PARASYMPATHETIC INNERVATION OF THE RAT LOWER LIP SKIN FOLLOWING SENSORY DENERVATION

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ABSTRACT

The sympathetic division of the autonomic nervous system is known to play a role in the genesis of neuropathic pain. In the skin of the rat lower lip (hairy skin), sympathetic and parasympathetic fibres normally innervate the same blood vessels in the lower dermis but do not occur in the upper dermis. However, we have shown that sympathetic fibre migration into the upper dermis occurs following mental nerve lesions (J. Comp. Neurol. 422:287-296, 2000). As sensory denervation has a dramatic effect on sympathetic fibre innervation patterns in the rat lower lip skin, we decided to investigate the possible changes in the other autonomic fibre type in the skin – the parasympathetic fibre.

Sensory denervation of the rat lower lip was achieved by bilateral transection of the mental nerve, and animals were allowed to recover for one to eight weeks. Lower lip tissue was processed for double labelling light microscopic immunocytochemistry (ICC), using antibodies against substance P (SP), which labels a subpopulation of peptidergic sensory fibres, and against the vesicular acetycholine transporter (VAChT), used as a marker for parasympathetic fibres. In sham-operated rat, SP-immunoreactive (IR) sensory fibres were found in the epidermis and upper and lower dermal regions, whereas VAChT-IR fibres were confined to the lower dermis. Mental nerve lesions induced the gradual disappearance of SP-IR fibres from all skin layers accompanied by the progressive migration of VAChT-IR fibres into the upper dermis. Cholinergic fibre migration was evident by the second week post-surgery and the ectopic innervation of the upper dermis by these fibres persisted even at the last time point studied (8 weeks). VAChT-IR fibres were observed in the upper dermis, well above the opening of the sebaceous glands into the hair follicles. These results show that considerable changes occur in the innervation patterns of parasympathetic fibres following mental nerve lesions.

The unique cutaneous innervation of the trigeminal region – sensory, sympathetic, and parasympathetic – presents novel opportunities for close interactions between these three fibre types and may have implications for improving the existing understanding and treatment of neuropathic pain in this region.

RÉSUMÉ

Il est connu que la division sympathique du système nerveux autonome joue un rôle dans la genèse de la douleur neuropathique. La peau de la lèvre inférieure du rat représente de la peau de la variété avec poil. Elle est innervée, dans des conditions normales, par des fibres nerveuses des systèmes sympathique et parasympathique seulement dans le derme inférieur, qui sont absentes du derme supérieur. Cependant, une étude précédente de notre laboratoire a prouvé qu'il y a une invasion du derme supérieur par des fibres sympathiques après des lésions bilatérales du nerf mentonnier (J. Comp. Neurol. 422:287-296, 2000). À cause de l'effet dramatique de la dénervation sensorielle sur la distribution des fibres sympathiques, nous avons décidé d'étudier la possibilité de changements dans un autre système de fibres du système nerveux autonome – les fibres parasympathiques.

Pour obtenir une dénervation sensorielle de la peau de la lèvre inférieure du rat, nous avons coupé le nerf mentonnier des deux cotés. Les animaux ont récupéré de la chirurgie pendant une période allant d'une à huit semaines. Le tissu de la lèvre inférieure a été traité pour obtenir une coloration immunocytochimique double de microscopie optique, en utilisant des anticorps contre la substance P (SP) – qui identifie une population des fibres sensorielles peptidergiques - et contre le transporteur vésiculaire de l'acétylcholine (VAChT) – qui identifie les fibres parasympathiques. Dans les animaux utilisés comme contrôles, les fibres SPergiques ont été trouvés pénétrant l'épiderme, ainsi que dans la partie supérieure et inférieure du derme; cependant, les fibres VAChTergiques on été trouvées seulement au derme inférieur. Les lésions du nerf mentonnier ont causé la disparition graduelle des fibres SPergiques de toutes les couches de la peau, accompagnée de la migration progressive des fibres VAChTergiques dans le derme supérieur. Cette migration de fibres cholinergiques était déjà évidente à la fin de la deuxième semaine après la chirurgie; l'innervation ectopique du derme supérieur par ces fibres VAChTergiques persistait encore à la fin de huit semaines, la durée de survie la plus longue que nous avons étudiée. Ces fibres cholinergiques ont été observées dans le derme supérieur, bien au-dessus de l'ouverture des glandes sébacées dans les follicules

pileux. Ces résultats révèlent des changements considérables de l'innervation parasympathique de la peau après des lésions des nerfs mentonniers.

Les caractéristiques particulières de l'innervation du territoire du nerf trijumeau par des fibres sensorielles, sympathiques et parasympathiques, et les changements après lésions des nerfs sensoriels, conduisent à la création de proximités entre ces trois populations de fibres nerveuses favorisant les interactions fonctionnelles entre elles. La compréhension des détails de ces interactions a probablement des implications pour le traitement des situations de douleur de type neuropathique les lésions nerveuses dans cette région.

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GLOSSARY

Ach	Acetylcholine
AMPA	α-amino-3-hydroxy-5-methyl-isoxazole
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
CCI	Chronic constriction injury
CGRP	Calcitonin-gene related peptide
CNS	Central nervous system
CRPS	Complex regional pain syndrome
CTb	Cholera toxin b
DAB	3,3'-diaminobenzidine tetrachloride
DBH	Dopamine-β-hydroxylase
EM-2	Endomorphin-2
FRAP	Fluoride-resistant acid phosphatase
GDNF	Glial cell line-derived neurotrophic factor
GFL	GDNF family ligand
GFR	GDNF ligand family receptor
G-protein	Guanine nucleotide-binding protein
GSA-IB4	*Griffonia simplicifolia* isolectin B4
HRP	Horseradish peroxidase
i.m.	Intramuscular
ICC	Imunocytochemistry
IgG	Immunoglobin G
MN	Mental nerve
NA	Noradrenaline
NGF	Nerve growth factor
NGS	Normal goat serum
NK1r	Neurokinin-1 receptor
NK2	Neurokinin-2
NK3	Neurokinin-3
NKA	Neurokinin A
NKB	Neurokinin-B
NMDA	N-methyl-D-aspartate
NPY	Neuropeptide Y
NT3	Neurotrophin-3
NT3	Neurotrophin-3
NTRN	Neurturin
P2	Purinergic
$p75^{NTR}$	P75 neurotrophin receptor
PB	Phosphate buffer
PBS	Phosphate buffered saline
PBS+T	Phosphate buffered saline containing 0.2% Triton X-100
PSL	Partial sciatic nerve ligation
s.c.	Subcutaneous
SCG	Superior cervical ganglion

SNL	Spinal nerve ligation
SP	Substance P
SST	Somatostatin receptor
TNF	Tumor necrosis factor
Trk	Tropomyosin receptor kinase
v/v	Volume/volume
VAChT	Vesicular acetylcholine transporter
VIP	Vasoactive intestinal peptide

CONTRIBUTIONS OF AUTHORS

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

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Dr. A. Ribeiro-da-Silva: Principal investigator of the project. Provided intellectual influence in the manuscript. Edited manuscript.

Dr. A. C. Cuello: Investigator in the project. Edited the manuscript and provided antibodies against DβH and SP.

Dr. I. Ruocco: Investigator in the project. Provided intellectual influence and planned experiments. Performed mental nerve transections and sympathectomies.

M. St-Louis: Provided technical assistance with animal perfusions and immunocytochemistry.

M. Ramien: Investigator in the project. Performed animal perfusions, immunoctyochemistry, analysed and quantified data, and wrote the initial version of the manuscript.

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ORIGINAL CONTRIBUTION

I confirmed the initial observation by Ruocco and Ribeiro-da-Silva that cholinergic parasympathetic fibres, which normally innervate blood vessels in the lower dermis, sprout into the upper dermis after sensory denervation (achieved by transection of the mental nerve) in the skin of the rat lower lip. I provided the first evidence that VAChT-IR fibres persisted in the upper dermis even after the sensory SP-IR nerve fibres had re-innervated the territory. This novel finding suggests that in addition to the sensory-sympathetic coupling observed previously in the same model (Ruocco, Cuello et al., 2000b), parasympathetic fibres may be involved in abnormal interactions with sensory and sympathetic fibres after nerve injury.

CHAPTER ONE: GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1. PAIN AND ITS TRANDSUCTION FROM PERIPHERY TO CNS

Pain has been defined by the International Association for the Study of Pain as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage"(Merskey and Bogduk, 1994). Pain is an adaptive response; it signals tissue damage and serves a protective function to the organism. For example, the pain of burning one's hand on a hot frying pan elicits a reflex withdrawal of the affected limb to prevent further injury. However, some individuals do not have the neural circuitry required to perceive pain [congenital insensitivity to pain (McMurray, 1950)] while others lack the normal affective response to pain [congenital indifference to pain (Jewesbury, 1970) and pain asymbolia (Berthier, Starkstein et al., 1988)]. Pain insensitivity of the extremely rare hereditary sensory and autonomic neuropathy (HSAN) of type IV – only 37 cases reported in total (Schulman, Tsodikow et al., 2001) – is particularly interesting on a basic science level, because it results from mutations in the receptor for nerve growth factor, tropomyosin receptor kinase (Trk) A (Indo, Tsuruta et al., 1996). Since TrkA is required for normal development of all pain-conducting primary afferents (C- and Aδ- fibres) and sympathetic neurons, the deficit in pain perception is logical. These individuals as such are in constant peril of inflicting irreparable damage upon themselves, commonly suffering painless burns, finger and toe mutilations, and joint injuries (Schulman, Tsodikow, Einhorn, Levy, Shorer, and Hertzanu, 2001). Tears and sweat are absent and children self-mutilate as soon as they have teeth (Schulman, Tsodikow, Einhorn, Levy, Shorer, and Hertzanu, 2001). The most common fatality among the HSAN type IV population is internal organ dysfunction that remains untreated for so long that it causes toxicity and death. From this example, the importance of functional pain perception and recognition is obvious.

Another example that highlights the importance of a functional pain system is the condition of neuropathic pain (see section 1.5). While individuals lacking normal pain perception systems do not respond to tissue-damaging stimuli, in neuropathic pain states, affected individuals suffer from excruciating

and often constant stabbing, burning, and/or aching pains that seriously reduce mobility and quality of life. In some cases, normally painless stimuli become painful, such as the contact of soft fabrics on the skin, while in others, spontaneous pains shoot through the affected regions at irregular intervals. Neuropathic pain arises from abnormalities within the neurons of the pain transduction system themselves, and has both genetic and environmental causes. Though the symptoms of neuropathic pain patients are diverse and of different etiologies, depression and decreased social interaction are commonly precipitated. No effective therapeutic has yet been developed to treat neuropathic pain. Like the population affected by congenital insensitivity to pain, though at the opposite end of the spectrum, this population's lack of a functional pain perception system prevents it from living a normal life.

The pathways responsible for conveying pain-related information, or nociception, from the periphery – skin, visceral organs, muscle, etc. – to the brain centres that can process and respond to the noxious stimuli are multi-synaptic and modulated at many points. Pseudounipolar primary afferent nociceptive neurons receive noxious mechanical, thermal, or chemical stimuli at their peripheral terminals, and convey the excitation along their axons to central terminals in the superficial region of the spinal grey matter, or trigeminal subnucleus caudalis (for the head region). The region in which nociceptors terminate is called the superficial dorsal horn, and it is further subdivided into laminae I-III (ascending order from superficial to deep) based on cell size, shape, and density of packing[1] (Rexed, 1952). Primary afferent neurons synapse onto either transmission neurons that project to the higher levels of the central nervous system (CNS) or interneurons that form synapses locally with other spinal neurons. The axons of transmission neurons are carried in tracts that project to the thalamus, parabrachial nucleus, and reticular formation, which directly, or via other neurons, terminate in synapses on neurons in the ventral and medial regions of the thalamus, which in turn project to the somatosensory cortex. Pain can be pharmacologically

[1] The entire grey matter of the spinal cord is divided into laminae, or layers, based on these same criteria, in ascending order from superficial (lamina I) to deep (lamina IX) with lamina X as the region around the central canal (Molander, Xu et al., 1984; Molander, Xu et al., 1989).

modulated at several levels in the pathway. For example, local anaesthetics desensitize the peripheral endings of nociceptive fibres to prevent the initiation of noxious stimuli produced upon incision and operation, or block the transmission along the nerves, while morphine acts at synapses in the brain and spinal cord as well as at peripheral sites. In addition, there is a descending inhibition of pain transmission by projections from the brain regions to which pain information is directed. Thus the pain transduction system forms a complete circle in which all points in the pathway are interconnected.

In this general introduction, a review of the literature will be presented for each level of the pain transduction system, from the periphery to the CNS, with particular emphasis on the peripheral elements as these are the focus of the current thesis. Further, the importance of this research will be highlighted by drawing correlations to clinical conditions to which the results are relevant, namely painful peripheral cutaneous neuropathy. Given all this information, the rationale behind the current thesis will be briefly summarized.

1.2. SKIN STRUCTURE – 'THE PERIPHERY'

The human body, in all its complexity, is composed of a mere four types of tissue: epithelial, connective, muscular, and nervous (Junqueira L.C, Carneiro et al., 1992). The largest organ in the body by weight, the skin, which comprises about 16% of total body weight, has a surface area of 1.2-2.3 m^2 in adults (Montagna W.W. and Parakkal, 1974) and is made up of two of the four tissue types: epithelial and connective, if we exclude the nerves. The epidermis, the outermost layer of the skin, is an epithelial tissue of ectodermal origin, while the embryonic origin of the underlying connective tissue dermis and hypodermis is the mesodermal layer. The main functions of the skin are protection from the external environment, maintenance of the internal environment, and reception of information about both environments. While the two former roles are filled by the structural features of the skin, the last is related to the presence of the sensory nervous system. The skin protects the organism from damage due to friction,

pressure, ultraviolet radiation, extremes of temperature, and water loss, and conveys innocuous and noxious sensory stimuli to the CNS, respectively.

Upon self-examination, it is evident that there are two main types of skin that cover the body. The first is the skin of the palms and the soles of the feet, which is thick, tough, and hairless, called glabrous skin. There are characteristic sulci and ridges to it that appear starting at 13 weeks of intrauterine development and form patterns of loops, arches, and whorls that are unique to each individual. Based on this individuality, fingerprints (or dermatoglyphics) are used as a method of identification for legal and anthropological purposes (Gartner and Hiatt, 1997). The second type of skin is that which covers the rest of the body. It is softer and smoother and is covered by hair of varying thickness, hence the name hairy skin. This also is the type of skin found in the rat lower lip, which is studied in the present thesis. The difference in thickness between glabrous and hairy skin depends entirely on the epidermis – in the former epidermis averages, in the human, 400 – 600 μm, while in the latter between 75 and 150 μm thickness is typical (Gartner and Hiatt, 1997) – giving rise to the denominations thick (glabrous) and thin (hairy).

With respect for relevance for this thesis, the epidermis and hypodermis will be described without much detail in order to only orient the reader within the context of the skin, while information pertaining to the dermis will be discussed in greater depth.

1.2.1. The Epidermis

The epidermis is renewed every 15 - 30 days by intense nocturnal mitotic activity in its basal layers (Junqueira L.C, Carneiro, and Kelley, 1992; Gartner and Hiatt, 1997)}. Keratin-producing cells (keratinocytes) proliferate in the region closest to the dermis then gradually migrate towards the surface of the skin, becoming increasingly large and differentiated along the way. In the most superficial layer, dead cells are sloughed off (desquamation) (Williams, 1984). The main function of the epidermis is to provide an impermeable protective layer to the skin.

Keratinocytes in the epidermis are classified into five layers: stratum basale or germinativum, stratum spinosum, stratum granulosum, stratum lucidum, and stratum corneum (MacKenzie, 1972). The basal layer, stratum basale, consists of a single layer of rapidly dividing undifferentiated stem cells (Lavker and Sun, 1982), joined by tight cell adhesions to create a barrier against pathogen invasion and fluid loss (Allen and Potten, 1975). Stratum spinosum is characterized by keratinocytes with "studded" surfaces (Holbrook and Wolff, 1987), that maintain strong cell-cell adhesions to provide resistance to wear and abrasion. It is thicker is in areas of high abrasion and friction, and is the epidermal layer responsible for the thickness differential between glabrous, thick and hairy, thin skin (Junqueira L.C, Carneiro, and Kelley, 1992). The granular layer of thin skin is not well-defined, but granular layer cells are present in the appropriate region (Gartner and Hiatt, 1997). Notably, these cells release lipids to the extracellular space where they create a water-impermeable coating that prevents both water loss and the supply of nutrients to superficial cells. Only present in thick skin, the stratum lucidum is also called the transition zone because it divides the viable layers of the epidermis – basale, spinosum, and granulosum, which are below the impermeable granular layer – from the overlying non-viable corneal layer. In hairy skin, the stratum corneum is thin and is made up of flattened, keratinized cells without organelles and nuclei that are undergoing their final differentiation into dead cells filled with keratin (corneocytes) (Mukhtar, 1992). The superficialmost cells, horny cells or squames, are continuously shed from the surface (Junqueira L.C, Carneiro, and Kelley, 1992).

Other cell types including melanocytes, Merkel cells, and Langerhans cells are found among the epidermal layers. The first two are found in stratum basale and are involved in skin pigmentation and mechanoreception respectively. Langerhans cells (or dendritic cells) play a role in generation of an immune response to foreign bodies by binding of antigens and activation of T lymphocytes (Gartner and Hiatt, 1997). In addition, nerve cell axons and axon terminals are located in the epidermis. Mechanoreceptive nerve fibres are only found in the basal layer, where they form complexes with Merkel cells, while the non-

receptor-associated free endings of small nociceptive fibres extend to more superficial levels. Schwann cell encasements are lost when fibres enter the epidermis, therefore all epidermal fibres are unmyelinated while their parent axons may or may not be myelinated (Kruger, Perl et al., 1981).

1.2.2. The Dermis

Unlike the epidermis, which is an epithelial tissue of ectodermal origin, the dermis is composed of collagenous connective tissue that originates from the mesodermal layer. The dermis is much thicker than the epidermis, ranging in thickness from 0.6 mm in the eyelids to 3 mm on the palms and soles (Gartner and Hiatt, 1997) and provides the skin with its strength. The dermis is functionally separated from the overlying epidermis by a basal lamina that also defines the dermal-epidermal junction. Downwards epidermal projections interdigitate with upwards dermal papillae to create a strong attachment between the dermal and epidermal layers. In addition, downwards growth of epidermal derivatives such as hair follicles, sweat and sebaceous glands, that lie in the dermis further deforms the dermal-epidermal junction.

The dermis is a highly vascular tissue, containing arterioles, capillaries, venules (Winkelmann, Sheen et al., 1961), and lymph vessels in dense networks (Junqueira L.C, Carneiro, and Kelley, 1992). Such high vascularity allows temperature and blood pressure regulation and nourishment of the avascular epidermis via capillary networks around descending epidermal ridges (Junqueira L.C, Carneiro, and Kelley, 1992). The dermis also contains skin appendages of epidermal origin such as hairs, nails, sebaceous glands, eccrine and apocrine sweat glands (Junqueira L.C, Carneiro, and Kelley, 1992), and a rich supply of autonomic and sensory nerves (discussed in the section 1.3).

There are two dermal layers, designated as upper and lower in the rat, or papillary and reticular in the human, respectively, though no clear boundaries exist. Previous studies from our lab have taken the convention of classifying the dermis above the opening of the sebaceous glands as the upper dermis, while that

below the opening of the sebaceous glands is considered the lower dermis (Ruocco, Cuello et al., 2000; Ruocco, Cuello et al., 2001a).

1.2.2.1. The upper dermis or papillary layer

Composed of loose connective tissue, the thin upper dermis lies directly below the epidermis, and is mainly made up of the area of the dermal papillae (hence the name "papillary" layer). It contains the fibroblasts, macrophages, and mast cells characteristic of this type of tissue (Gartner and Hiatt, 1997). Fibroblasts are the most common cell type throughout the dermis and they play an essential role in maintaining the structural integrity of the upper dermis as they synthesize all other components of the tissue: collagen, reticular, and elastic fibres, and amorphous intercellular substance. In adults they are quiescent until damage induces their activation to replaced lost tissue (Junqueira L.C, Carneiro, and Kelley, 1992). Macrophages in the upper dermis phagocytose and destroy non-viable cells, cellular debris, and foreign microorganisms (Gartner and Hiatt, 1997). They also function as antigen-presenting cells. Mast cells are mainly concentrated around small upper dermal blood vessels (Gartner and Hiatt, 1997), and are activated by exposure of the organism to foreign antigens in immediate hypersensitivity or anaphylactic reactions (Gartner and Hiatt, 1997).

Mechanoreceptors are also found in the upper dermis, namely Meissner corpuscles and Krause endbulbs [(Gartner and Hiatt, 1997), please refer to section 1.3.1. for more detail on mechanoreception].

1.2.2.2. The lower dermis or reticular layer

Although there is no physical separation of the papillary and reticular layers of the dermis, the lower dermis displays denser networks of collagen and elastic fibres and fewer cells than the upper dermis (Gartner and Hiatt, 1997). As described previously, for the purpose of this line of research, the division of the dermis in the rat lower lip skin into upper and lower is considered to be the opening of the sebaceous glands into the hair follicles (Ruocco, Cuello, and Ribeiro-da-Silva, 2000; Ruocco, Cuello, Shigemoto, and Ribeiro-da-Silva,

2001a). Furthermore, thorough characterization of the innervation of the rat lower lip skin by Ruocco et al. (2000; 2001a; 2001b; 2002) has provided landmarks for distinguishing dermal layers – for example, autonomic fibres do not extend into the upper dermis in normal animals, thereby allowing the boundary between upper and lower dermis to be easily delimited using the appropriate immunocytochemical markers (please see section 1.3.2.3.). The lower dermis also contains mechanoreceptive Pacinian corpuscles and Ruffini endings [(Gartner and Hiatt, 1997), please refer to section 1.3.1. for more detail on mechanoreception].

1.2.3. The Hypodermis

Below the dermis is the hypodermis, also known as subcutaneous tissue or superficial fascia in gross anatomy (Junqueira L.C, Carneiro, and Kelley, 1992). It is not considered part of the skin. The hypodermis consists of loose connective tissue and contains adipocytes, components that underlie its major functions: skin mobility and energy storage, respectively (Junqueira L.C, Carneiro, and Kelley, 1992).

1.3. INNERVATION OF THE SKIN – PERIPHERAL TERMINALS OF NOCICEPTORS AND OTHER NEURONS

In addition to its role as a protective barrier from environmental influences, the skin provides a large surface area for communication with the external environment. It is densely innervated by the peripheral terminals of primary sensory neurons in order to maximize the amount of information that can be gained from this interaction. As such, the skin plays an instrumental role in the perception of tactile painful and painless stimuli; effective reception of pain stimuli at this level is the first crucial step in the perception of pain. The skin also receives autonomic innervation from the sympathetic nervous system to maintain homeostasis and for thermoregulation. The head and neck region is a territory of unique innervation in that both parasympathetic and sympathetic systems are present (Ruocco, Cuello et al., 2002).

1.3.1. Somatosensory innervation of the skin

Somatic sensation has four major modalities: discriminative touch, proprioception, temperature sense, and nociception. Discriminative touch refers to the ability to recognize shape, texture and movement of objects, proprioception relates to sensation of static position and movement of limbs and body, and nociception is the recognition of actual or potential tissue damage or chemical irritation, namely pain or itch (Gardner, Martin et al., 2000). Regardless of the modality, all somatosensory information is conveyed by dorsal root ganglion (DRG) neurons in the limbs and trunk. In the head and neck region, including the face, lips, oral cavity, conjunctiva, and dura mater, trigeminal sensory neurons convey somatosensory information. In addition to their functional similarities, DRG and trigeminal primary sensory neurons are anatomically identical pseudounipolar cells. Cell soma are housed in ganglia at the dorsal root of the spinal nerve or of the trigeminal nerve, while the single process bifurcates into two branches, one peripherally-directed to the target organ and the other centrally-directed to the spinal cord or trigeminal nuclei (Gardner, Martin, and Jessell,

2000). Considering the high degree of anatomical and functional similarity between DRG and trigeminal ganglion neurons, and the much more comprehensive literature available on the former, the findings from studies in either area are generalized to both and are hence presented without distinction. The differences between primary afferent neurons of various modalities arise from the heterogeneity of peripheral terminals, axonal diameters, and myelination status.

Touch and proprioception[2] are sensed by peripheral primary afferent terminals encapsulated by a connective tissue structure (encapsulated receptors). These are classified as Aα and Aβ afferents based on their large diameters, myelinated axons, and related high conduction velocity. They bear Trk C receptors and respond to neurotrophin-3 (NT3) (Hory-Lee, Russell et al., 1993; McMahon, Armanini et al., 1994; Lindsay, 1996). Sensitivity to touch is greatest in the glabrous skin, particularly in the fingertips, where there exist five types of mechanoreceptive terminals (Gartner and Hiatt, 1997). The most superficial, Merkel disk receptors or Merkel cell-neurite complexes, are found in the basal layer of the epidermis (see section 1.2.1.) and consist of a small epithelial cell that encapsulates the afferent terminal and responds to deformation of the epidermal ridge. The dermal papillae of the upper dermis (see section 1.2.2.1.) contain mechanoreceptors that respond to slight deformation of the epidermis – Meissner corpuscles – and that are more numerous in areas that are particularly sensitive to touch such as the lips (Gartner and Hiatt, 1997). These two mechanoreceptors confer fine mechanical sensitivity, as their relatively superficial location allows them to respond to localized deformations of the skin. Krause endbulbs, another type of encapsulated receptor, are also found in the upper dermis, but their function is unknown (Gartner and Hiatt, 1997). Encapsulated mechanoreceptors also reside in the lower dermis (see section *2.2.*). Pacinian corpuscles are activated by pressure and vibrational stimuli, while Ruffini endings respond to tensional forces (Gartner and Hiatt, 1997). These receptors sense deformation of larger skin

[2] Proprioception does not involve receptors in the skin, but rather, those in muscles and joints. It will not be considered further due to lack of relevance to the current discussion.

areas due to their distance from the skin surface. In hairy skin, such as that of the rat lower lip, the two main types of mechanoreceptors are hair receptors, which detect displacement of hairs, and field receptors that sense skin stretch and are primarily located in the joints of the finger, wrists, and elbows. Modified Merkel disk receptors, Ruffini endings and Pacinian corpuscles are also present in the dermis of hairy skin (Gardner, Martin, and Jessell, 2000).

Thermal sensation is conducted by small diameter, finely myelinated fibres with bare nerve endings – these are Aδ fibres. Thermal receptors fire action potentials at a steady low rate when the temperature of the skin is normal (34°C). Cold receptors fire at a higher frequency at temperatures less than 25°C, while warmth receptors respond maximally to temperatures greater than 45°C (Gardner, Martin, and Jessell, 2000). Each terminal has its own optimal temperature at which it is maximally excited (at other temperatures it does not fire), so it is the overall relative activity of different populations of thermal receptors that allows the organism to sense skin temperature. At temperatures greater than 50°C, or less than 5°C, thermal receptors are unresponsive and the excessive temperature change is perceived as pain, detected by nociceptors.

Nociceptive neurons (nociceptors) fall into three physiological classes: mechanical, thermal, and polymodal. Some are finely myelinated (Aδ fibres) while others are completely unmyelinated (C-fibres) – the two populations are thought to mediate the rapid, acute, sharp "first pain" and the delayed, more diffuse, duller "second pain", respectively (Basbaum and Jessell, 2003). Mechanical nociceptors represent thinly myelinated Aδ fibres and are excited by high intensity or strong tactile stimuli such as pinching or cutting the skin and non-noxious temperatures around 43°C. They are named high threshold mechanoceptors (HTM) or Aδ type II nociceptive primary afferents. These never express neuropeptides (see below). In contrast, some thermal nociceptors for noxious heat at greater than 50°C correspond to Aδ type I fibres, which also respond to noxious mechanical stimulation and express neuropeptides. The final class of nociceptors, polymodal unmyelinated C-fibre afferents, respond to

chemical, mechanical and thermal stimuli and evoke a slow burning pain. The nociceptors responsible for transducing noxious cold (below 5°C) are C-fibres.

1.3.2. Nociceptor peripheral terminals in the skin

Like other somatosensory neurons, nociceptive neuronal somata are located in the dorsal root ganglion (limbs and trunk) or trigeminal ganglion (head and neck), peripheral terminals are directed towards the skin or other target organ, and central terminals synapse on superficial spinal dorsal horn cells or trigeminal subnucleus caudalis neurons (Ribeiro-da-Silva A., 2003; Nolte, 1999; Gardner, Martin, and Jessell, 2000). The major classical transmitter in nociceptive fibres is likely glutamate with aspartate as a cotransmitter at some terminals (De Biasi S. and Rustioni A., 1988; Battaglia G. and Rustioni A., 1988; Tracey, De Biasi et al., 1991; Merighi, Polak et al., 1991). Due to bi-directional transport of proteins synthesized in the cell body to central and peripheral terminals [Dale's principle (Dale, 1935)], the nociceptive neuron performs its afferent function of conducting stimulus information to the CNS and an efferent release of the same peptides and transmitters peripherally.

C-fibre nociceptive primary afferents can be grossly subdivided into two populations, both of which are implicated in the relay of pain-related information to CNS. However, though this classification of nociceptive C-fibres into peptidergic and non-peptidergic populations provides a useful division, it is not entirely comprehensive as a small number of neurons have both peptidergic and non-peptidergic markers [(Alvarez and Fyffe, 2000); described later in section 1.3.2.1.a.] and thus evade classification. The central terminals of the nociceptive neuronal populations are found in different areas of the dorsal horn – peptidergic terminations are found in laminae I and V, non-peptidergic are found in inner lamina II, and the unclassifiable population terminates in laminae I and II (Ribeiro-da-Silva A., 2003; Bradbury, Burnstock et al., 1998).

1.3.2.1. Peptidergic nociceptors

The first population expresses neuropeptides such as substance P (SP), neurokinin-A (NKA), calcitonin gene-related peptide (CGRP), galanin, and endomorphin-2 (EM-2) (Todd and Ribeiro-da-Silva A., 2004), and relies on nerve growth factor (NGF) secreted by keratinocytes (Tron, Coughlin et al., 1990; English, Harper et al., 1994), fibroblasts (Acheson, Barker et al., 1991), and Merkel cells (Vos, Stark et al., 1991) for trophic support.

Early in development, NGF is integral to cell survival and to the development of nociceptive fibre markers, while in adulthood it is important for maintenance of the acquired peptidergic phenotype (Crowley, Spencer et al., 1994; Smeyne, Klein et al., 1994). In mice, during early development, NGF is expressed in the epidermis at very high levels, supporting its infiltration by peptidergic nociceptor projections (Davies, Bandtlow et al., 1987) – as noted previously, nociceptors are the only sensory fibre type that exists above the basal layer of the epidermis. Peptidergic fibres express receptor TrkA, the high affinity receptor for NGF (Averill, McMahon et al., 1995; Molliver, Radeke et al., 1995) that is necessary and sufficient to confer NGF responsiveness (Koizumi, Contreras et al., 1988; Loeb, Maragos et al., 1991; Ibanez, Ebendal et al., 1992), but also express the p75 neurotrophin receptor ($p75^{NTR}$)

$p75^{NTR}$ is a member of the tumor necrosis factor (TNF) superfamily (Davies, Lee et al., 1993; Lee, Davies et al., 1994) that binds NGF and other neurotrophins. Targetted $p75^{NTR}$ deletion results in decreased neuronal sensitivity to NGF (Davies, Lee, and Jaenisch, 1993; Lee, Davies, and Jaenisch, 1994); $p75^{NTR}$ expression is upregulated after nerve lesion or seizure (Roux, Colicos et al., 1999) suggesting that it may play an important role in nerve remodelling and regeneration (Bibel and Barde, 2000). Available data suggests that in cells where $p75^{NTR}$ is co-expressed with Trk receptors, $p75^{NTR}$ functionally collaborates with the Trks to either enhance responses to preferred Trk ligands, to reduce neurotrophin-mediated Trk receptor activation resulting from non-preferred ligands, or to facilitate apoptosis resulting from neurotrophin withdrawal (Barker, 1998).

Cell damage and inflammation increase the level of tissue NGF which has been proposed to induce collateral sprouting of sensory fibres bearing TrkA (Diamond, Coughlin et al., 1987) and to increase synthesis of neuropeptides, such as SP and CGRP, involved in the signalling of pain (Lindsay and Harmar, 1989; Lewin and Mendell, 1993).

1.3.2.1.a. Neuropeptides Secreted: Substance P, CGRP

SP is member of the mammalian tachykinin family, which also includes neurokinin A (NKA) and neurokinin B (NKB). SP and NKA are encoded by the preprotachykinin I gene (and consequently always colocalized in synaptic vesicles), while NKB is encoded by the preprotachykinin II gene (Nakanishi, 1987; Fong T.M., 1996). There are three neurokinin ot tachykinin receptors, NK1, NK2, and NK3, that bind to all neurokinins though they have higher relative affinities for SP, NKA, and NKB, respectively [(Fong T.M., 1996); please refer to 1.3.2.3.b. for details]. All three tachykinins are found in the CNS, but in the periphery only SP and NKA are expressed at detectable levels (Otsuka and Yoshioka, 1993). SP is an undecapeptide (Severini, Improta et al., 2002) and its distribution is restricted to peptidergic nociceptive afferent fibres in the skin (Fundin, Pfaller et al., 1997).

CGRP exists in two isoforms: α and β (Emeson R.B., 1996; Hall J.M. and Brain S.D., 1996). The former is found predominantly in sensory neurons, while the latter is primarily localized in enteric neurons (Mulderry, Ghatei et al., 1988). CGRP is synthesized in more primary afferent neurons than SP or somatostatin (Segond, Pastor et al., 2002). In the rat, CGRP is synthesized in up to 50% of the small- and medium- diameter DRG neurons (Ju, Hökfelt et al., 1987; McCarthy and Lawson, 1990) and is anterogradely transported to both peripheral (Heppelmann, Shahbazian et al., 1997) and central terminals (McNeill, Chung et al., 1988). Notably, CGRP is also found in some motor and autonomic fibres (Fundin, Pfaller, and Rice, 1997) therefore its usefulness for labelling nociceptive fibres without the use of additional markers to eliminate the non-nociceptive afferents may be limited.

Immunocytochemical studies of the distributions of SP and CGRP reveal that almost all SP-positive fibres are also CGRP-positive, however, the reverse is not true (Wiesenfeld-Hallin, Hökfelt et al., 1984; Ju, Hökfelt, Brodin, Fahrenkrug, Fischer, Frey, Elde, and Brown, 1987). A small population of uncharacterized SP-negative and CGRP-positive fibres co-express somatostatin, lectin *Griffonia simplicifolia* isolectin B4 (GSA-IB4) binding sites, and P2X3 purinergic receptors and are NGF-dependent (Alvarez and Fyffe, 2000; Priestley, Michael et al., 2002). These correspond to the population of primary afferent nociceptive fibres that cannot be classified as peptidergic or non-peptidergic (mentioned previously in section 1.3.2.1.).

Peptidergic peripheral terminals play an important role in the self-sustaining phenomenon of neurogenic inflammation. Nerve injury directly (via activation of mechanosensory fibres) and indirectly (via changes in the local chemical environment) activates nociceptors, which simultaneously convey the excitation to their central terminals and release neuropeptides peripherally (Julius and Basbaum, 2001). Importantly, SP and NKA induce plasma extravasation by increasing venous permeability which is exacerbated by the potent vasodilatory effects of CGRP on skin arterioles (Holzer, 1998). These effects are mediated by receptors on blood vessel walls and by mast cell or leukocyte activation, which leads to release of inflammatory mediators that sensitize the nociceptive terminals and lead to enhanced neuropeptide release (Baluk, 1997; Holzer, 1998). In humans, this condition is visualized by reddening of the skin due to small vessel dilatation, flare or spreading of the vasodilatation by axon reflex-mediated release of neuropeptides from other branches of the activated nociceptors, and a wheal reaction due to increased permeability of local vessels (Lewis, 1927; Baluk, 1997; Holzer, 1998). Neurogenic inflammation may play a role in the enhanced responses to painful and non-painful stimuli characteristic neutorpathic pain patients.

1.3.2.2. Non-peptidergic nociceptors

The second population of nociceptive fibres is fluoride-resistant acid phosphatase (FRAP)-positive (Coimbra, Magalhaes et al., 1970; Knyihar and Gerebtzoff, 1973; Coimbra, Sodre-Borges et al., 1974; Knyihar, Laszlo et al., 1974), expresses the purinergic receptor P2X3 (Bradbury, Burnstock, and McMahon, 1998; Snider and McMahon, 1998; Burnstock, 2000), and has GSA-IB4 binding sites (Alvarez and Fyffe, 2000). Since these fibres do not contain neuropeptides, this population is described as non-peptidergic. Non-peptidergic neurons derive trophic support from glial cell-line derived neurotrophic factor (GDNF) in adulthood. During early postnatal and embryonic development non-peptidergic fibres, like the peptidergic division, express TrkA receptors and are NGF-responsive (Silos-Santiago, Molliver et al., 1995). Subsequently, in the early postnatal period, approximately half of the all small diameter DRG cells lose the TrkA receptor (Molliver, Wright et al., 1997). These non-peptidergic fibres express GDNF family ligand receptors (GFRs) GFRα1 and GFRα2 and Ret tyrosine kinase (Bennett, Michael et al., 1998).

1.3.2.3. Receptors on Nociceptors

Nociceptive neurons express receptors on their peripheral axons and axon terminals that allow them to respond to local chemical stimuli, such as injection of capsaicin, and to modulate their responses to noxious mechanical and thermal stimuli. The majority of C-fibres are chemosensitive, a property conferred by specific types of receptors on their axons and terminals. Importantly, the existence of specific receptors also allows these neurons to respond to the neuropeptides and neurotransmitters that they or nearby neurons release, producing a local feedback system that may either positively or negatively modulate activity at nociceptors. Although the localization of receptors to sensory fibres has been the subject of many studies and reviews, thorough descriptions of the type of sensory fibres and nociceptor subtypes (i.e. peptidergic versus non-peptidergic) that express the receptors are not always available. Although a comprehensive review of all receptor types on primary afferent nociceptor terminals is beyond the scope

of this thesis, receptors relevant for interactions with autonomic fibres will be discussed. For a comprehensive review of the ligands and receptors present on the peripheral terminal of nociceptive afferents please refer to (Coggeshall and Carlton, 1997)

1.3.2.3.a. Glutamate Receptors

Ionotropic receptors are found on nociceptor terminals. The N-methyl-D-aspartate (NMDA) receptor requires NR1 and NR2 subunits to be functional (Petralia, Wang et al., 1994). The NR1 subunit is ubiquitous in DRG neurons of small and large size (cell body size correlates with axonal diameter) (Shigemoto, Ohishi et al., 1992). The NR2B subunit of the NMDA receptor is selectively and most highly expressed of the NR2 subunit in the superficial dorsal horn, suggesting a localisation to nociceptive fibres (Petralia, Wang, and Wenthold, 1994; Boyce, Wyatt et al., 1999). In the periphery, it has been localized predominantly to small diameter nociceptive fibres, where it is likely involved in an autocrine feedback loop controlling peripheral glutamate release (Ma and Hargreaves, 2000). Cutaneous unmyelinated axons are also labelled with antibodies against α-amino-3-hydroxy-5-methyl-isoxazole (AMPA) and kainate glutamate receptors (Carlton, Hargett et al., 1995), whereas only AMPA and NMDA but not kainate receptors have been found on large DRG cells (Coggeshall and Carlton, 1997). Considering its specific localization on small diameter fibres, the kainate receptor may play an important role in nociception, particularly because all nociceptor terminals release glutamate in the periphery and at spinal cord synapses.

1.3.2.3.b. Neuropeptide Receptors

Receptors for neuropeptides are found on fine-diameter peripheral fibres – notably, these include opioid receptors, neurokinin receptors, and CGRP receptors.

Antibodies against μ- and δ- opioid receptors reveal positively-stained unmyelinated skin afferents (Stein, Gramsch et al., 1990; Coggeshall, Zhou et al., 1997). Both receptors are guanine nucleotide-binding protein (G-protein) coupled

to inhibition of adenylyl cyclase and voltage-gated calcium currents presynaptically and to enhancement of potassium current postsynaptically (Twycross, 1999). Delta-opioid receptors are not normally highly expressed on the neuronal membrane, but appear rapidly after inflammatory reactions, suggesting that the control of expression is determined at the translational rather than the transcriptional level (Stein, 1993; Schafer, Imai et al., 1995; Antonijevic, Mousa et al., 1995; Cahill, Morinville et al., 2001). However, the μ-opioid receptor is expressed on the plasma membrane (Arvidsson, Riedl et al., 1995; Cheng, Liu-Chen et al., 1997). Opioid peptides are produced by immune cells that infiltrate the area of tissue damage (Carr, 1991; Hassan, Pzewlocki et al., 1992) and possibly also by fine cutaneous afferents (Weihe, Hartschuh et al., 1985; Hassan, Pzewlocki, Herz, and Stein, 1992) in inflammation, such as after tissue damage. They are further postulated to mediate local analgesic effects of peripheral opioid administration (Stein, 1993) and are possibly activated by release of EM-2 from nociceptors.

Receptors for neurokinins are also found on nociceptive peripheral axons and terminals. There are metabotropic neurokinin receptors NK1, NK2, and NK3, but the most studied is the SP receptor, NK1. In glabrous skin, 32% of fine, unmyelinated fibres express NK1 receptors (NK1r) (Carlton, Zhou et al., 1996) that are activated by SP and probably function as autoreceptors that enhance the activity of nociceptive fibres (Li and Zhao, 1998). Li and Zhao (1998) showed that NK1r activation results in calcium ion influx in approximated 90% of small diameter DRG cells, and suggested that via this mechanism activation of the NK1r on the DRG cell could lead to increased calcium-dependent release of neurotransmitter and peptides in the periphery and in the spinal cord. These studies suggest that SP and neurokinin-A act on NK1rs in an autocrine manner on peptidergic fibres and in a paracrine fashion on nearby non-peptidergic fibres with excitatory effects.

The peptidergic subpopulation of unmyelinated, peptidergic primary afferent neurons also expresses receptors for the other peptide it releases. CGRP binding sites can be activated by CGRP in an excitatory fashion. CGRP receptors

1 and 2 are G-protein coupled (Dennis, Fournier et al., 1991; Poyner, 1992; Quirion, Van Rossum et al., 1992), and are distinguished based on their different sensitivities to the antagonist $CGRP_{8-37}$ (Dennis, Fournier, Guard, St Pierre, and Quirion, 1991). Since these neurons also synthesize and release CGRP, these are autoreceptors that may sensitize primary afferent neurons and influence the release of mediators (Segond, Pastor, Biskup, Schlegel, Benndorf, and Schaible, 2002). Somatostatin receptors are also found on peripheral nociceptive afferent axons and terminals (Carlton, Du et al., 2001a). There are five types somatostatin receptor (SST) 1 to SST5, and SST2 is further divided into a and b subtypes, all of which are coupled to inhibition of adenylyl cyclase (Patel, Greenwood et al., 1995). Carlton et al. (2001a) showed that SST2a-immunoreactive fibres were present at the dermal-epidermal junction in rat glabrous skin and that injection of a somatostatin agonist, octreotide, decreased nociceptive behaviours in response to formalin and activation of C-fibres in response to thermal stimuli. Furthermore, the same group also reported that administration of the somatostatin antagonist, cyclo-somatostatin, to normal animals resulted in nociceptive behaviours and increased activity in nociceptive fibres (Carlton, Du et al., 2001b). Peripheral administration of somatostatin in humans provides effective analgesia, when injected to the knee joint, for osteo- and rheumatoid arthritis sufferers (Silveri, Morosini et al., 1994; Matucci-Cerinic, Borrelli et al., 1995), providing further support for its potential inhibitory action on nociceptive peripheral terminals. Somatostatin released from the peripheral terminals of activated nociceptors likely binds to autoreceptors as well as receptors on nearby neurons to exert an inhibitory influence on nociceptor excitability.

1.3.2.3.c. Cholinergic Receptors

Cholinergic receptors are classified as nicotinic or muscarinic, based on their preferential activation by agonists. Nicotinic receptors are ligand-gated cation channels while muscarinic receptors are coupled to G-proteins. There are two nicotinic subtypes (neuronal-type and muscle-type) that are composed of different combinations of α and β subunits. There are five muscarinic subtypes (M1-M5) which have been identified and characterized. M1, M3, and M5 are G_q/G_{11} coupled to stimulation of the phosphoinositol cascade while M2 and M4 inhibit adenylyl cyclase via G_i/G_o. Muscarinic receptors have been reported on peripheral nociceptors in many studies (Steen and Reeh, 1993; Tata, Plateroti et al., 1994; Wanke, Bianchi et al., 1994; Haberberger, Henrich et al., 1999; Bernardini, Levey et al., 1999; Bernardini, Sauer et al., 2001; Bernardini, Roza et al., 2002). Agonism of M2 cholinergic receptors on SP-positive terminals has been reported to desensitize the nociceptor to mechanical and heat stimuli, and to decrease CGRP release evoked by noxious heat (Bernardini, Roza, Sauer, Gomeza, Wess, and Reeh, 2002). In another study, M2 receptors were shown to be preferentially localized to GSA- IB4-positive small diameter neurons (Haberberger, Henrich, Couraud, and Kummer, 1999), illustrating the vagueries of receptor localisation studies as described previously. However, it can be assumed that acetycholine exerts an inhibitory effect on nociceptors via the muscarinic cholinergic subtype.

Nicotinic receptors have also been localized to DRG neurons (Boyd, Jacob et al., 1991) and to small cells in the trigeminal ganglion (Flores, DeCamp et al., 1996) where they are thought to sensitize rather than desensitize nociceptive C-fibres to heat but not mechanical stimuli (Bernardini, Sauer, Haberberger, Fischer, and Reeh, 2001). Studies have demonstrated that nicotine excites rat sensory neurons (Sucher, Cheng et al., 1990; Roberts, Stevenson et al., 1995), rabbit corneal afferent nerves (Tanelian, 1991), and rat trigeminal ganglion neurons (Liu, Chang et al., 1998) and evokes CGRP release from pulmonary tissue (Lou, Karlsson et al., 1991; Lou, Franco-Cereceda et al., 1992), trachea (Hua, Back et al., 1994; Hua, Jinno et al., 1994; Jinno, Hua et al., 1994), and cultured DRG

neurons (Franco-Cereceda, Rydh et al., 1992). Activation of C-fibre terminals is produced by administration of acetylcholine to neuronal receptive fields and is blocked by nicotinic antagonists (Tanelian, 1991; Steen and Reeh, 1993). The predominant subunit composition of nicotinic receptors on small diameter fibres in the trigeminal area is likely $\alpha4\beta2$, as concluded by deductive reasoning, since *in situ* hybridization that showed the predominant neuronal nicotinic receptor subtype expressed in trigeminal ganglion neurons, $\alpha3\beta4$, to be mainly located on large- and medium-diameter, peripherin-negative (peripherin labelling can be used as a marker for small diameter sensory neurons) cells (Flores, DeCamp, Kilo, Rogers, and Hargreaves, 1996).

Thus the two types of cholinergic receptors on small diameter afferents are M2 muscarinic, that inhibit excitation, and neuronal-type nicotinic, that facilitate activation of nociceptors.

1.3.2.3.d. Adrenergic Receptors

Adrenergic receptors have been demonstrated on sensory neurons by pharmacological studies, although their anatomical localization has not been convincingly performed. The two types of adrenergic receptor, α and β, are G-protein coupled to second messenger systems. Of particular importance in nociceptors are the $\alpha2$ and $\beta2$ subtypes. Alpha2 adrenoreceptors are coupled to inhibition of adenylate cyclase and calcium channels, while $\beta2$ adrenoreceptors are coupled to stimulation of adenylate cyclase (Rang, Dale et al., 1999). The $\alpha2$ subtype splice variants A and C ($\alpha2_A$ and $\alpha2_C$ respectively) have been detected on DRG neurons by immunocytochemistry, but only moderate increases in $\alpha2_A$ have been observed after nerve injury (Birder and Perl, 1999). The greatest increase in $\alpha2_A$ immunoreactivity occurred in medium and large diameter low threshold mechanoreceptor DRG cells (Birder and Perl, 1999). In control animals, acetylcholine has no effect on nociceptors, but after experimental nerve injury sympathetic stimulation or noradrenaline administration after mixed nerve injury leads to activation of C fibre terminals (Sato and Perl, 1991; O'Halloran and Perl, 1997). Alpha2 receptor unpregulation on nociceptive neurons after nerve injury has been suggested to play a key role in sensitization of nociceptors to

sympathetic adrenergic input, although the mechanisms by which activation of these receptors causes excitation is counterintuitive (considering their coupling to inhibition of adenylyl cyclase) and remains unknown (Perl, 1999). Based on coupling to excitatory second messenger system

Beta2 adrenoreceptors in small diameter neurons are also activated by norepinephrine (Abdulla and Smith, 1997; Caterina, Schumacher et al., 1997). Beta2 adrenoreceptor activation has been shown to decrease CGRP release from capsaicin-sensitive peptidergic neurons of the dental pulp (trigeminal region) when capsaicin and a β2 adrenoreceptor agonist are co-administered (Bowles, Flores et al., 2003). Thus the role of the β2 subtype of adrenergic receptor is primarily one of desensitization.

Like the cholinergic receptors, two types of receptor with opposite actions are possibly localised on the same primary afferent nociceptors. Overall the integrated effective of noradrenaline on injured nociceptors is excitation.

1.3.2.3.e. P2X3 purinergic receptors

Purinergic or P2 receptors are divided into 4 classes: P2X, P2Y, P2T, and P2Z (Bradbury, Burnstock, and McMahon, 1998). The purinergic receptor $P2X_3$ occurs selectively in the non-peptidergic population of nociceptive afferents (Bradbury, Burnstock, and McMahon, 1998; Guo, Vulchanova et al., 1999). It is a ligand-gated ion channel activated by adenosine triphosphate (ATP). The peripheral projections of nociceptive neurons in the skin, tongue, and tooth pulp are immunoreactive for P2X3 (Bo and Burnstock, 1994). Furthermore, P2X3 expression is upregulated followed by transport of the receptor to the primary afferent terminals in trigeminal sensory neurons after nerve injury (Eriksson, Bongenhielm et al., 1998). In the trigeminal ganglion, P2X3 receptor immunoreactivity was located in both small and large neurons and their processes (Burnstock, 2000). Although most primary afferent neurons express purinergic receptors, the specific localisation of the P2X3 receptor on non-peptidergic C afferents suggests a role for ATP in the activation of these fibres.

1.3.2.3.f. Other Receptors on Nociceptors

Nociceptor peripheral terminals express receptors for many other chemicals and neurotransmitters released by nearby neuronal and non-neuronal cells. These include receptors for neuropeptide Y (NPY), cholesystokinin, bombesin, γ-aminobutyric acid, prostaglandin E_2, histamine, angiotensin II, serotonin, bradykinin, and capsaicin. The first four ligands are released from nociceptors, while the rest come from nearby non-neuronal cells (please refer to (Carlton and Coggeshall, 1998) for a review of the topic).

1.3.2.4. Nociceptor innervation of the skin of the rat lower lip

In the skin, innervation by the SP-positive subpopulation of small fibres has been extensively characterized. These peptidergic fibres are found throughout the dermis where they are associated with blood vessels (Hokfelt, Kellerth et al., 1975; Dalsgaard, Jonsson et al., 1983; Ruocco, Cuello, Shigemoto, and Ribeiro-da-Silva, 2001a; Ruocco, Cuello, Parent, and Ribeiro-da-Silva, 2002), hair follicles (Cuello, Del Fiacco et al., 1978; Ruocco, Cuello, Shigemoto, and Ribeiro-da-Silva, 2001a), sweat glands (Hokfelt, Kellerth, Nilsson, and Pernow, 1975; Dalsgaard, Jonsson, Hokfelt, and Cuello, 1983; Tainio, Vaalasti et al., 1987), and Meissner corpuscles (Hokfelt, Kellerth, Nilsson, and Pernow, 1975; Dalsgaard, Jonsson, Hokfelt, and Cuello, 1983), and extending into the epidermis where they terminate as free nerve endings (Hokfelt, Kellerth, Nilsson, and Pernow, 1975). In the rat lower lip, SP-positive fibres innervate hair follicles, sebaceous glands, mast cells, and blood vessels and send branches into the epidermis (Ruocco, Cuello, Shigemoto, and Ribeiro-da-Silva, 2001a). The distribution observed in rat lower lip is similar to that described in human skin, although a few differences do arise – Meissner corpuscles are not innervated by SP-IR fibres; sweat glands do not exist and therefore are not innervated by SP-positive fibres (Ruocco, Cuello, Shigemoto, and Ribeiro-da-Silva, 2001a). The similarity of the innervation in this experimental model to human skin supports its use to study changes that may lead to painful clinical neuropathy.

Concerning the termination of the non-peptidergic fibres, much less is known. However, by extrapolation from studies using the pan-neuronal axonal

marker PGP9.5 and followed by subtraction of the SP-positive population, non-peptidergic innervation appears to be abundant, although it has been reported to be less associated with blood vessels (Fundin, Pfaller, and Rice, 1997; Rice, Albers et al., 1998). Preliminary data from our lab (Grelik and Ribeiro-da-Silva, unpublished observations) suggests that non-peptidergic innervation of the rat lower lip skin may be more abundant than peptidergic, and that blood vessels are highly innervated.

1.3.3. Autonomic Innervation of the Skin

The autonomic nervous system has three divisions: sympathetic, parasympathetic, and enteric. The latter innervates the gastrointestinal system. Autonomic fibres are predominantly efferent fibres, and the pathway to the periphery consists of two neurons arranged in series, named the pre- and post-ganglionic neurons (Rang, Dale, and Ritter, 1999). In structures of the body, sympathetic and parasympathetic innervation produce opposite effects (i.e. in gut smooth muscle), while in others only one system is found (i.e. in sweat glands). The major role of the autonomic nervous system is to control smooth muscle, exocrine and some endocrine secretions, rate and force of the heart, and metabolism (Rang, Dale, and Ritter, 1999) in order to adapt to new situations such as postural changes or exercise.

In most regions of the body, it is accepted that the skin is innervated by the sympathetic system. The skin of the head and neck region is unique in that it is also innervated by both sympathetic and parasympathetic systems (Ruocco, Cuello, Parent, and Ribeiro-da-Silva, 2002). The existence of these two autonomic fibre types in the skin of the head and neck suggests that the changes that occur after nerve injury in this area may be different than those that occur in other body regions. This may have implications for the management of pain after nerve injury in the trigeminal area.

1.3.3.1. Sympathetic Innervation

Traditionally, the sympathetic system is simplistically associated with the "fight-or-flight" response to stress including non-shivering thermogenesis, cutaneous vasoactivity, sweating, and piloerection (Hemingway and Price, 1968). Its preganglionic cell bodies are located in the lateral horn of the grey matter of thoracic and lumbar segments of the spinal cord, ganglia are located in the paravertebral chain, and post-ganglionic axons run in the spinal nerve to reach their peripheral destinations (Rang, Dale, and Ritter, 1999). In the skin, sympathetic fibres from the superior cervical ganglion terminate on the *arrector pili* and blood vessel smooth muscle where they release noradrenaline (NA), and on sweat glands, where they release acetylcholine (ACh) (Katzung, 2001). NA-containing synaptic vesicles colocalize ATP (approximately 4 molecules per molecule of NA) and chromogranin A (Rang, Dale, and Ritter, 1999). ATP is responsible for the rapid phase of contraction of smooth muscle (Lundberg, 1996). These noradrenergic sympathetic terminations induce vasoconstriction of skin blood vessels and piloerection via activation of α-adrenoreceptors. The sympathetic system has also been shown to produce vasodilatation after electrical stimulation of lumbar sympathetic ganglia in cat (Bell, Janig et al., 1985) and human (Lundberg, Norgren et al., 1989), and as a reflex response to increased environmental temperature in the orofacial region (see (Izumi, 1999) for a review of the neural regulation of orofacial blood flow).

Several receptors have been localized to sympathetic nerve fibres. Of relevance to the results of the current thesis in which interactions between sensory and sympathetic fibres may exist, there is evidence for P2X3 (Xiang, Bo et al., 1998), α2 adrenergic (Tracey, Cunningham et al., 1995), ionotropic glutamate (Carlton, Chung et al., 1998), and TrkA (Crowley, Spencer, Nishimura, Chen, Pitts-Meek, Armanini, Ling, MacMahon, Shelton, Levinson, and ., 1994; Smeyne, Klein, Schnapp, Long, Bryant, Lewin, Lira, and Barbacid, 1994) receptors. The potential interactions between fibre types are thoroughly examined in sections 4.5 – 4.9.

1.3.3.2. Parasympathetic Innervation

The parasympathetic system is commonly remembered by the mnemonic "rest and digest". In the visceral smooth muscle of the gut and urinary bladder and in the heart, the parasympathetic system opposes the effects of the sympathetic system. Its preganglionic axons are found in cranial nerves III, VII, IV, X, and from the *nervi erigentes* in the sacral spinal cord ganglia. Ganglia are located close to their target organs therefore postganglionic neurons have very short axons. In the head region, cranial nerve III (oculomotor) carries parasympathetic fibres to the eye, VII (facial) and IX (glossopharyngeal) carry fibres to the salicary glands and nasopharynx) (Rang, Dale, and Ritter, 1999).

Postganglionic parasympathetic fibres colocalize ACh and vasoactive intestinal peptide (VIP) (1991). VIP-positive neurons have been revealed by immunohistochemistry in the VII and IX cranial nerves and probably originate from VIP-immunoreactive perikarya in microganglia associated with the tympanic plexus, chorda tympani, lingual nerve and Vidian nerve, as well as from cells in the otic, sphenopalatine, submandibular and sublingual ganglia (Gibbins, Brayden et al., 1984). One study reported that neuropeptide Y, enkephalin, and SP are also found in cranial parasympathetic neurons innervating the facial skin in guinea pigs (Gibbins, 1990), but this finding has not been confirmed in the rat. Parasympathetic neurons have also been shown to release NGF at their peripheral termini (Hasan and Smith, 2000).

Post-ganglionic parasympathetic neurons have been shown to possess receptors that may contribute to their interactions with sympathetic and sensory fibres, and that are of relevance for the results of the present thesis. These include purinergic P2X2 and P2X4 (Smith, Hansen et al., 2001), NK2 and NK3 (Hardwick, Mawe et al., 1995), and GFR α1 and α2 (Golden, DeMaro et al., 1999) receptors. The potential interactions between fibre types are explored in sections 4.5 – 4.9.

1.3.3.3. Autonomic innervation of the skin of the rat lower lip

The innervation of skin by autonomic fibres has been extensively studied in the rat and cat. These studies consistently report that sympathetic and parasympathetic fibres exist in this region (Kaji, Shigematsu et al., 1988; Kaji, Maeda et al., 1991; Kuchiiwa, Izumi et al., 1992; Izumi and Karita, 1992; Izumi and Karita, 1993; Kuchiiwa and Kuchiiwa, 1996; Ruocco, Cuello, Parent, and Ribeiro-da-Silva, 2002). Significantly, there is a species difference in the types of fibres carried in the mental nerve – in cat, the mental nerve carries sympathetic, parasympathetic, and sensory innervation to the lower lip skin (Kuchiiwa and Kuchiiwa, 1996), while in the rat the mental branch contains only sensory fibres as only sensory innervation is lost after mental nerve transection (Ruocco, Cuello, and Ribeiro-da-Silva, 2000). Furthermore, parasympathetic innervation of the lower lip in the cat originates from otic, sphenopalatine, and submandibular ganglia (Kuchiiwa, Izumi, Karita, and Nakagawa, 1992). In the rat, the skin of the lower lip receives parasympathetic fibres from the otic ganglion (Kaji, Shigematsu, Fujita, Maeda, and Watanabe, 1988; Kaji, Maeda, and Watanabe, 1991), which itself receives preganglionic fibres via the facial nerve, and sends its postganglionic projections to the skin by way of branches of the auriculotemporal nerve (Al Hadithi and Mitchell, 1987).

In the skin of the rat lower lip, autonomic fibres are confined to the lower dermis in normal animals (Ruocco, Cuello, and Ribeiro-da-Silva, 2000). They form characteristic mesh-like sympathetic plexuses around and more linear parasympathetic associations with vessels also innervated by SP-immunoreactive fibres (Ruocco, Cuello, Shigemoto, and Ribeiro-da-Silva, 2001a). Sympathetic fibres were labelled with an antibody against dopamine-β-hydroxylase (DβH), the last enzyme in the synthesis of noradrenaline from tyrosine (Ruocco, Cuello, and Ribeiro-da-Silva, 2000; Ruocco, Cuello, Shigemoto, and Ribeiro-da-Silva, 2001a). The DβH antibody has shown to provide superior labeling of sympathetic fibres as compared to catecholamine fluorescence or tyrosine hydroxylase activity (Hartman, 1973). Parasympathetic fibres have been detected immunohistochemically by both VIP (Kaji, Shigematsu, Fujita, Maeda, and

Watanabe, 1988; Kaji, Maeda, and Watanabe, 1991) and vesicular acetylcholine transporter (VAChT) (Ruocco, Cuello, Parent, and Ribeiro-da-Silva, 2002), although the former is suggested to be a less specific and reliable marker due to its non-selective expression on other fibre types. There are no sweat glands in the rat lower lip, thus all detected cholinergic fibres are parasympathetic (Ruocco, Cuello, Shigemoto, and Ribeiro-da-Silva, 2001a).

1.4. CENTRAL MECHANISMS OF PAIN TRANSDUCTION

The dorsal horn of the spinal cord is the zone to which nociceptive primary afferent neurons send their central projections through the dorsal roots. Small diameter nociceptive afferents (Aδ or C) bifurcate and enter Lissauer's tract, where they travel up and down for one or two spinal segments before branching in the dorsal horn (Szentágothai 1964). As mentioned in section 1.3.2.1., peptidergic neuron terminations are found in laminae I and V, non-peptidergic neurons synapse in inner lamina II, and the population that expresses both peptidergic and non-peptidergic markers terminates in laminae I and II (Ribeiro-da-Silva A., 2003; Bradbury, Burnstock, and McMahon, 1998).

In the spinal cord, primary afferents can form either simple or complex synaptic arrangements with second order neurons that project to different brain and brainstem structures. In the simple type, the primary afferent terminal synapses directly on the axon or dendrite of a second order neuron. Complex arrangements called synaptic glomeruli consist of a primary afferent terminal that forms both pre- and post-synaptic contacts with multiple spinal neurons. Glomeruli have both "multiplier" and "modulatory" functions based on the described arrangement (please refer to (Ribeiro-da-Silva A., 2003) for review of synaptic glomeruli). In the rat, glomeruli are virtually absent from lamina I, and are rare in outer lamina II, but are relatively numerous in the inner part of lamina II and in lamina III. In the superifical dorsal horn of the rat spinal cord, peptidergic primary afferents generally form simple synapses in lamina I, while non-peptidergic afferents projecting to lamina II form many complex contacts (Ribeiro-da-Silva A., 2003). Therefore, peptidergic nociceptors are mostly

involved in simple, axodendritic interactions with neurons projecting to the thalamus or parabrachial nucleus whereas non-peptidergic nociceptors' terminals become the central elements of complex glomerular synaptic arrangements, which form both pre- and postsynaptic contacts (Ribeiro-da-Silva et al., 2003; Todd and Ribeiro-da-Silva, 2003). Input from nociceptive primary afferents may be modulated by intrinsic dorsal horn neurons before being relayed to projection neurons.

Noxious stimulation is conveyed from the spinal cord to the brain by different systems – one that mediates the sensory or discriminative qualities of the painful stimulation, and the other that is primarily concerned with the affective or unpleasant qualities of the pain sensation [see (Hunt and Mantyh, 2001) for review]. The spinothalamic tract, whose first neurons are located in laminae I and V of the spinal dorsal horn, projects to the ventro –basal and –posterior thalamus, which in turn projects to the cortex. This pathway is thought to be responsible for signalling the location and type of stimulation (Hunt and Mantyh, 2001). The spinoparabrachial and spinoreticular pathways, which originate from neurons in lamina I, teminate in the parabrachial nucleus and reticular formation, that in turn project to the hypothalamus and amygdala, brain structures involved in autonomic control and emotions (Hunt and Mantyh, 2001). Changes in neuronal phenotype may occur at every neuron, from primary afferent nociceptor to the final neurons synapsing on cortical, hypothalamic, and amygdala sites, in the ascending pain pathway in response to noxious peripheral stimulation (Hunt and Mantyh, 2001).

1.5. NEUROPATHIC PAIN

Under normal circumstances, pain plays a protective role in preventing the organism from sustaining tissue damage (please refer to section 1.1). However, it can also be pathological. When damage or dysfunction of the nervous system leads to pain, it is designated as neuropathic (Merskey and Bogduk, 1994), meaning that the problem is with the nerves themselves. The etiologies of nerve damage are diverse and include infections, trauma, metabolic abnormalities, chemotherapy, surgery, radiation, neurotoxins, nerve compression, inflammation, and tumor infiltration (Dworkin, 2002). Clinically, the most common neuropathic

pain syndromes are chemotherapy-induced, HIV-induced, and painful diabetic neuropathies, complex regional pain syndrome, neuropathy secondary to tumor infiltration, phantom limb pain, postherpetic neuralgia, postmastectomy pain, and trigeminal neuralgia, in no particular order (Dworkin, 2002). The most recent estimate of neuropathic pain incidence in the United States is 3.8 million or 1.5% of the population, if low back pain is included (Bennett, Michael, Ramachandran, Munson, Averill, Yan, McMahon, and Priestley, 1998). However, some have suggested that these estimates may not be entirely accurate as chronic neuropathic pain is not accurately recognized and/or reported (Dworkin, 2002). Most research has focussed on postherpetic neuralgia and painful diabetic neuropathy, but it is likely that the mechanisms involved cannot be generalized across all types of neuropathic pain.

Painful neuropathy can be subdivided into "small fibre painful neuropathy" and "small-and-large fibre painful neuropathy" based on the results of electromyographic and nerve conduction studies. The results of electrodiagnosis and nerve conduction testing are normal in small-only and abnormal in small-and-large fibre neuropathy (Mendell and Sahenk, 2003). Neuropathic pain is characterized by pain-like responses to non-painful ro innocuous stimuli (allodynia) and exaggerated responses to painful stimuli (hyperalgesia) (Merskey and Bogduk, 1994; Chong and Bajwa, 2003). Neuropathic pain sufferers with small fibre involvement most commonly describe burning, sharp pain, shooting pain, and aching in the toes and feet (Scadding, 1999; Mendell and Sahenk, 2003). While the pain-related symptoms of the two painful neuropathies may be indistinguishable, in the latter pain is accompanied by disturbances of proprioception, vibratory sensation, muscle-stretch reflexes, and muscle strength (Mendell and Sahenk, 2003). In the head region, trigeminal neuropathic pain is diagnosed on the basis of continuous dysaesthetic pain and altered sensibility in the facial skin area supplied by one or two branches of the trigeminal nerve, with or without a known nerve injury (Eide and Rabben, 1998). The most excruciating form of facial neuropathic pain is *tic douloureux*, which is characterized by stabbing pains intermittent with brief pain-free periods

(MacFarlane, Wright et al., 1997). In this region, neuropathic pain most commonly results from neurovascular compression of the trigeminal nerve as it leaves the brainstem (MacFarlane, Wright, O'Callaghan, and Benson, 1997), but may also include accidental transection of these fibres during oral procedures, such as damage to the inferior alveolar nerve during wisdom tooth extractions, or other surgical procedures performed in the region.

Currently available therapies for neuropathic pain do not effectively provide relief. The root of the problem is a lack of knowledge about the neural systems involved in maintaining an abnormal pain state, and this knowledge is especially deficient for the head and neck (trigeminal) regions. This presents a huge challenge for physicians and for patients alike, because both must experiment with multiple combinations of anticonvulsants, antidepressants, sodium channel blockers, sympathetic blockers, NMDA and NK1R antagonists, often with neglible to no therapeutic effect (please refer to (MacFarlane, Wright, O'Callaghan, and Benson, 1997) and (Chong and Bajwa, 2003) for reviews of the medical management of neuropathic pain). Some patients' neuropathic pain responds to sympathetic blocking agents or sympathectomy and it thus classified as sympathetically-maintained pain, while others are non-responders and their pain is designated sympathetically-independent (Munglani and Hill, 1999). The neuropathic pain conditions most commonly associated with sympathetically-maintained pain include acute shingles (herpes zoster), neuralgias (including postherpetic neuralgia), painful metabolic neuropathies, panthom limb pain, traumatic nerve injuries, and soft tissue injury (Munglani and Hill, 1999). Complex regional pain syndromes (CRPS) I and II are characterized by the symptoms of spontaneous burning pain, allodynia, or hyperalgesia which is disproportionate to the injury and edema or abnormalities in skin blood flow and sudomotor activity in the painful area, concluded to arise from sympathetic nervous system dysfunction (Stanton-Hicks, Janig et al., 1995). The diagnosis of CRPS is to the exclusion of other putative causes for these symptoms. CRPS II results from an indentified nerve injury, whereas CRPS I does not have a nerve injury as its precipitating event (Munglani and Hill, 1999)

1.5.1. Neural mechanisms of neuropathic pain

Although the focus of this thesis is on the peripheral anatomical changes that follow nerve injury, there are equally changes in the dorsal root ganglion and CNS that contribute to abnormal pain syndromes. Although changes in the peripheral nervous system are critical for the initiation of a pain state after nerve injury, they are often transient (Basbaum and Jessell, 2003). In contrast, the accompanying central changes are of long duration and may persist even after the peripheral situation has resolved itself. It has been suggested that in contrast to the anatomical changes observed in the periphery, central changes are primarily mediated by alterations in the neurochemistry of intrinsic spinal neurons and incoming primary afferents and ensuing supraspinal alterations (Todd and Ribeiro-da-Silva A., 2004). The peripheral and central mechanisms of neuropathic pain are reviewed in detail elsewhere (Todd and Ribeiro-da-Silva A., 2004; Ramer M.S., Thompson S.W.N. et al., 1999; Julius and Basbaum, 2001; Hunt and Mantyh, 2001). A brief overview is provided here only to provide a context in which peripheral changes can be understood as a possible contributor but not a unique determining event in the genesis of neuropathic pain states.

1.5.1.1. Peripheral nociceptor contribution to neuropathic pain

Sensitization, or lowering of the threshold for activation, of primary afferent nociceptors results in increased excitation (noxious stimulation) conveyed to the spinal cord. This may occur due to an upregulation of receptors and secreted neurotransmitters in neurons and non-neural cells, or due changes in the skin's innervation by sensory and autonomic fibres (please refer to chapter 4: discussion).

In the inflammatory pain that follows nerve or tissue injury, chemical mediators are released *en masse* from damaged tissue cells and mast cells that infiltrate the damaged area. These substances, including bradykinin, histamine, prostaglandins, leukotrienes, acetylcholine, serotonin, ATP, hydrogen ions, and SP, increase the sensitivity of mechano- and thermal nociceptors to applied

stimuli – some also are capable of actually activating nociceptors (i.e. histamine) (Julius and Basbaum, 2001; Basbaum and Jessell, 2003). Furthermore, the nociceptor itself releases SP and CGRP, resulting in increased vascular permeability and vasodilatation (refer to section on neurogenic inflammation 1.3.2.1.a.), respectively, as well as histamine release from mast cells. Ultimately, the result is additional infiltration of the injury site by inflammatory cells and tissue edema. These changes in the peripheral nociceptor result in an increased nociceptive input to spinal cord neurons that may contribute to the establishment of centrally-mediated neuropathic pain.

Upregulation of sensory neuron-specific sodium channels on nociceptive primary afferent terminals following nerve injury may contribute to the prolonged firing of these afferents in neuropathic pain states (Hunt and Mantyh, 2001). Furthermore, an increase in the number/density of nociceptive fibres innervating a given area due to sprouting, or changes in the properties of the sprouted fibres, may contribute to peripheral sensitization.

1.5.1.2. DRG involvement in neuropathic pain

Under normal circumstances, there are no sympathetic fibres in the DRG. However, after dorsal root ganglion axotomy in the lumbar region, sympathetic fibres from perivascular plexuses grow into the DRG (McLachlan et al., 1993) where they form complicated, woven arrangements ("baskets") around large neuronal somata (McLachlan, Janig et al., 1993; Chung, Kim et al., 1993; Chung, Lee et al., 1996; Ramer and Bisby, 1998; Julius and Basbaum, 2001; Basbaum and Jessell, 2003). The extent and timecourse of sympathetic fibre sprouting depends on the animal model of nerve injury (Ramer and Bisby, 1997; Lee, Yoon et al., 1998) and distance between the site of nerve injury and the DRG (Kim, Na et al., 1996). Extent of fibre sprouting is inversely correlated with distance, while sprouting occurs most in the following rank order of increasing time: spinal nerve ligation, chronic constriction injury and partial sciatic nerve ligation, complete transection (these models are discussed in section 1.6). Notably, sympathetic baskets were observed to form transiently around small diameter neurons in the

Chung spinal nerve ligation study (Chung, Lee, Yoon, and Chung, 1996) but not after complete transection (McLachlan, Janig, Devor, and Michaelis, 1993; McLachlan E.M. and Hu P., 1998). Therefore, while removal of lumbosacral sympathetic ganglia reversed pain behaviours in the Chung model, the effect might not have been observed in McLachlan's studies (complete transection).

In the end, however, sympathetic sprouting onto DRG neurons does not provide any intuitive explanation for long-term sympathetically-maintained pain because the lasting in-growth occurs on the large diameter non-pain-transducing population. The overall significance of this in-growth is uncertain as the onset, degree, and duration of anatomical change only loosely correlates with that of pain behaviours (Kim, Na et al., 1998; Ramer M.S., Thompson S.W.N., and McMahon S.B., 1999).

1.5.1.3. Central mechanisms contributing to neuropathic pain

Central sensitization is an enhancement of synaptic transmission in the spinal cord following an increase in afferent activity (Woolf, 1983; Basbaum and Jessell, 2003). Under normal conditions, modulation of incoming nociceptive stimulation occurs in the spinal cord such that not all is passed on to the brain. There are basically two schools of thought on how central sensitization occurs. One believes that central sensitization occurs due to changes in the neurochemistry of intrinsic spinal neurons, while the other supports the view that new connections are formed between primary afferent fibres and their second order spinal neurons.

Hökfelt and others support a neurochemical reorganization of the spinal, that is, expression of new and existing proteins at non-normal levels [(Hokfelt, Zhang et al., 1994; Hokfelt, Broberger et al., 2000) recently reviewed in (Todd and Ribeiro-da-Silva A., 2004)]. For example, after nerve transection normally expressed peptides SP, CGRP, and somatostatin are lost from small diameter central nociceptive terminals, while VIP is simultaneously synthesized *de novo* in these neurons and NPY begins to be produced in myelinated afferents (Todd and Ribeiro-da-Silva A., 2004). Different changes in peptide and receptor expression

occur in the spinal cord in response to inflammatory noxious stimuli (please refer to (Todd and Ribeiro-da-Silva A., 2004) for a recent review of evidence). Some researchers have proposed that central sensitization occurs via unpregulation of NMDA-type glutamate receptor activity (Basbaum and Jessell, 2003) but the exact mechanisms are still incompletely understood.

Previous studies from Woolf and colleagues (Woolf, 1983; Woolf, Shortland et al., 1992; Mannion, Doubell et al., 1996) concluded that large, myelinated fibre central projections sprout into lamina II after nerve injury. However, the rationale behind the study was disproved when it was shown that cholera toxin B (CTb), their marker for the mechanoreceptive fibres, was in fact transported to by induction of binding sites in unmyelinated non-peptidergic fibres after nerve damage thereby explaining the increase in CTb labelling in lamina II (Tong, Wang et al., 1999). Anatomical rearrangement of the sensory pathways in the spinal dorsal horn after injury has also been concluded from other studies (Okamoto, Baba et al., 2001). For example, recordings from substantia gelatinosa interneurons by Okatamoto et al. (2001), showed a dramatic increase in monosynaptic excitatory current evoked by stimulation of Aβ fibres after nerve injury, which the authors suggest supports the morphological data produced by Woolf and colleagues.

It is possible that both neurochemical and anatomical changes in the spinal dorsal horn may contribute to the phenomenon of central sensitization.

1.6. CHARACTERIZATION OF THE MENTAL NERVE TRANSECTION MODEL

The majority of neuropathic pain rat models have been developed in the trunk and limb region, including the chronic constriction injury (CCI) model (Bennett and Xie, 1988), Kruger model (Mosconi and Kruger, 1996), partial sciatic nerve ligation (PSL) model (Seltzer, Dubner et al., 1990), spinal nerve ligation model (SNL) (Kim and Chung, 1992), and complete peripheral nerve transection (Wall, Devor et al., 1979). The first three models consist of applying ligatures or fixed-diameter cuffs to constrict all or part of the peripheral nerve,

while SNL involves complete tight ligation of the L_5 and L_6 spinal nerves, and transection, as the name implies, is a cut across the nerve. CCI and Kruger models mimic compression nerve injury, as occurs clinically due to hard tumour formation and pressure on nerves, or herniation of the lumbar discs; PSL is a model of accidental nerve bruising or damage, such as from gunshot wounds; SNL simulates nerve plexus and dorsal root injury; complete nerve transection simulates the clinical situation in which accidental denervation may occur due to transection of peripheral nerves during surgery (Zimmermann, 2001).

In contrast to the traditional lower trunk and limb models, the mental nerve transection model was developed by Ruocco et al. in our lab in the skin of the rat lower lip (Ruocco, Cuello, and Ribeiro-da-Silva, 2000). The structure and innervation of the rat lower lip skin has been studied extensively (Kaji, Shigematsu, Fujita, Maeda, and Watanabe, 1988; Ribeiro-da-Silva, Kenigsberg et al., 1991; Kaji, Maeda, and Watanabe, 1991; Ruocco, Krause et al., 1997; Ruocco, Cuello et al., 1998; Ruocco, Cuello et al., 1999; Ruocco, Cuello, and Ribeiro-da-Silva, 2000; Ruocco, Ramien et al., 2001; Ruocco, Cuello, Shigemoto, and Ribeiro-da-Silva, 2001a; Ruocco, Cuello, Parent, and Ribeiro-da-Silva, 2002), and, in fact, this provides an ideal experimental model because parasympathetic, sympathetic, and sensory innervation derive from different sources (sections 1.3.1.1. and 1.3.2.3.) allowing each system to be lesioned independently. This is a major advantage over the existing models that inflict damage upon mixed sensory-motor-autonomic nerves because it allows the alterations in facial sensory fibres subsequent to their transection to be studied in isolation. Later, the findings from these studies can be compared to those from "mixed nerve" studies (i.e. sciatic nerve lesion) to better understand which phenomena are related specifically to sensory fibres, and which arise from their interaction with other fibre types. The results of such basic science studies have implications for the types of dental and facial pain that develop following accidental sectioning of peripheral sensory nerves during surgical procedures in the head region.

1.7. HYPOTHESIS, OBJECTIVES, AND RATIONALE

A previous study from our lab has shown that sympathetic fibres sprout into the upper dermis from perivascular plexuses in the lower dermis following sensory denervation of the rat lower lip (Ruocco, Cuello, and Ribeiro-da-Silva, 2000). This finding has obvious implications for the development of sympathetically-maintained pain (SMP). Although many SMP patients obtain complete relief of their painful symptoms after sympathectomy or adrenoreceptor antagonist administration (O'Halloran and Perl, 1997), a significant number respond incompletely, and many initial responders eventually become refractory to these treatments (Ramer M.S., Thompson S.W.N., and McMahon S.B., 1999). Furthermore, many neuropathic pain sufferers have sympathetically-independent pain (SIP) that does not ever respond to sympathetic blocking (Munglani and Hill, 1999). SIP, in particular, represents a major challenge to clinicians due to the growing number of affected individuals and absence of appropriate treatment. It simultaneously presents a challenge to basic scientists to improve the knowledge of factors, other than sympathetic anomaly, that may contribute to the genesis of neuropathic pain.

Based on the results of a previous study from our lab (Ruocco et al, 2000) that revealed a massive and abnormal invasion of ectopic skin regions by sympathetic fibres after sensory nerve injury and the clinical occurrence of many sympathetically-independent neuropathic pain syndromes, we foresaw the requirement to better define the non-sympathetic changes that occur following sensory denervation. Furthermore, the existence in the literature of evidence receptors for parasympathetic mediators on nociceptive peripheral terminals provided add incentive for an investigation of the changes in the other population of autonomic fibres that innervate the head and neck region implicit in this model – the parasympathetic fibre.

Based on the above, we generated the following hypothesis:

HYPOTHESIS: Sprouting of sympathetic fibres into the upper dermis after nerve injury is accompanied by parasympathetic fibre

sprouting. This may contribute to the development of sympathetically-independent pain.

The main objective of this thesis was to investigate immunocytochemically the distribution of parasympathetic fibres after sensory nerve injury, in order to confirm the working hypothesis. A secondary aim was to perform the first objective using parameters that would allow comparisons to be drawn to the results of a previous study on sympathetic sprouting after nerve injury. Finally, the results of this thesis will be tied to clinical conditions in which parasympathetic fibre changes may be involved.

CHAPTER TWO: MATERIALS AND METHODS

2.1. ANIMAL PROTOCOLS

Male Wistar rats 250-300 g in weight were used in all studies. All rodents were treated in accordance with the guidelines described in The Care and Use of Experimental Animals of the Canadian Council on Animal Care. In addition, all experimental procedures were reviewed and approved by the McGill University Animal Care Committee before the initiation of experiments.

2.2. SURGICAL PROCEDURES

2.2.1. Mental Nerve Transection

The day before performing the mental nerve transection surgery, rats were numbered and weighed, and their weights recorded for anaesthetic dosing. On the day of surgery, animals were sequentially anaesthetised with acepromazine (0.2ml/kg, s.c.), xylazine (0.25ml/kg, i.m.), and ketamine (0.5ml/kg, i.m.) injections. A ten-minute waiting period separated each injection. Sixty rats (30 for double-labelling and 30 for single-labelling immunocytochemistry (ICC)) received bilateral mental nerve (MN) transections, and 24 rats underwent sham operations. A surgical microscope (Leitz, Wetzlar, Germany) was used for the MN transection operations. Once completely asleep, as assessed by no response to pinching of the toes, rats were transferred to the operating table and placed on their backs with front paws extending to the sides and taped down. An incision was made at the midline of skin of the rat lower lip and muscles were teased apart using haemostat clamps to reveal the MN. The MN was then freed from surrounding connective tissue, and a segment of the nerve measuring 1.0-2.0 mm was removed at its exit point from the mental foramen (Ruocco et al., 2000). Incisions were then stitched up and an antibiotic cream applied. Rats were observed until they awoke from the anaesthetic then returned to the animal centre, where they were housed three per cage. Sham operations were performed in an identical manner but the MN was not cut. The animals were allowed to recover for 1 – 8 weeks before they were sacrificed for ICC studies.

2.2.2. Dual Mental Nerve Transection and Sympathectomy

Fifteen rats underwent bilateral superior cervical ganglion (SCG) removal followed by bilateral MN transection. Due to the greater relative difficult of SCG removal, this procedure was performed first. The SCG, a white fusiform organization, was located at the bifurcation of the carotid artery. It was teased away from the surrounding fascia and removed with a portion of nerve on each side. Visual confirmation of SCG completeness and attached nerves was obtained by close observation under the surgical microscope. Only animals with successful bilateral ganglionectomies were operated on to transect the MN. The operations were performed in immediate succession. Animals were then allowed to recover for a 1, 4, or 8 week period before being perfused. Lip tissue from dually-denervated animals was processed for all immunocytochemical markers described in section 2.4, namely both double- and single- labelling.

2.3. ANIMAL PERFUSIONS AND TISSUE SECTIONING

At each post-surgical time (1, 2, 3, 4, 6, and 8 weeks), 5 rats with MN transections and 4 sham-operated rats were anaesthetised with 0.4 ml/kg of Equithesin (6.5mg chloral hydrate and 3mg sodium pentobarbital in a volume of 0.3 ml, i.p., per 100g body weight) and perfused transcardially with perfusion buffer followed by for 30 minutes with a solution made up of 4% paraformaldehyde, 15% picric acid (volume/volume; v/v), and 0.1% glutaraldehyde in 0.1M phosphate buffer (PB), pH 7.4, then for a further 30 minutes with a mixture of 4% paraformaldehyde and 15% picric acid in PB. Perfusions were executed using a gravity system.

Using a scalpel blade, an incision of approximately 0.5 cm in length was made from the free edge of the lip into the hairy skin at the center of the lip. A triangular piece of tissue was then detached by cutting through the skin from the corner of the mouth to the end of the central incision. The lower lips were collected and post-fixed in the latter fixative for one hour at 4°C. Following 12 hours of cryoprotection by immersion in 30% sucrose in PB, the tissue was

trimmed, snap frozen in liquid nitrogen, thawed in 0.1M PB, placed on a tissue holder, and embedded in Tissue Tek (OCT). Sections, 50 μm-thick, were cut at –20°C on a cryostat (Reichert-Jung 2800 Frigocut N). All sections were collected in phosphate-buffered saline (PBS) containing 0.2% Triton-X 100 (+T), pH 7.4.

2.4. IMMUNOCYTOCHEMISTRY

All immunocytochemistry was performed free-floating following the protocol described by Coté et al. (Coté, 1993) with some modifications for the tissue involved. The skin is a technically challenging area for ICC studies because of its high connective tissue content, which leads to non-specific adhesion of anti-mouse IgG molecules. Modification of all lip ICC protocols to include preabsorption of secondary anti-mouse IgG antibodies with rat lip tissue successfully prevented such excessive non-specific binding (Ruocco, Cuello, and Ribeiro-da-Silva, 2000). Furthermore, the use of the alcohol-sensitive SG chromogen for double-staining studies required abbreviation of the dehydration procedure to prevent loss of labelling.

Two fifteen minute washes in PBS+T separated each step described below, unless otherwise indicated. Each labelling procedure was followed by the mounting of all sections on gelatin-subbed slides, rapid dehydration in ascending ethanols, clearing through xylene, and coverslipping with Permount (Fisher).

2.4.1. Double-labelling for Substance P and vesicular acetylcholine transporter (VAChT)

Sections were treated with 1% sodium borohydride and 5% normal goat serum (NGS) for 30 minutes each. The sections were then incubated overnight at 4°C in a PBS+T solution containing a rat bi-specific anti-SP/anti-horseradish peroxidase (HRP) monoclonal antibody (1:10) (Suresh et al., 1986) and a rabbit polyclonal serum against VAChT (1:10 000) (Gilmor, Nash et al., 1996). Both antibodies are very well characterized. The anti-SP bi-specific monoclonal antibody was raised against the C-terminal of SP and recognizes equally SP and

NKA, but does not recognize NKB in the concentrations used in immunocytochemistry (McLeod, Krause et al., 2000). As SP and NKA are derived from the same gene and are fully co-localized in the rat nervous system (Ribeiro-da-Silva, McLeod et al., 2000), the antibody is a very sensitive and reliable marker of SP-containing sensory fibres. The anti-VAChT antibody was generated against a fusion protein containing the amino acid sequence from 478-530 of the C-terminus of the VAChT molecule; its specificity has been studied by Western blot analysis and shown to be highly specific for VAChT (Gilmor, Nash, Roghani, Edwards, Yi, Hersch, and Levey, 1996). Subsequently, sections were treated for 1 hour with HRP (Sigma type VI, 5 μg/ml), then reacted with 3',3'-diaminobenzidine tetrachloride (DAB) (6mg/ml), and 1% hydrogen peroxide (H_2O_2) to reveal SP antigenic sites. The sections were next incubated for 1 hour in biotinylated goat anti-rabbit immunoglobulin G (IgG) (1:800 in 5% NGS; Vector Labs), followed by an hour incubation in avidin-biotin complex (ABC) (1:400, Vector Labs). Finally, VAChT antigenic sites were revealed using an SG chromogen labelling kit as instructed by the manufacturer (Vector Labs).

2.4.2. Double-labelling for VAChT and dopamine-β-hydroxylase (DβH)

Tissue sections were pre-treated in 50% ethanol, 1% sodium borohydride and 5% NGS prior to overnight incubation in a mouse monoclonal anti-DβH antibody (Mazzoni, Jaffe et al., 1991) (1:10) and polyclonal rabbit anti-VAChT serum. The anti-DβH antibody was generated against rat DβH and has been characterized as highly specific (Mazzoni, Jaffe, and Cuello, 1991). The sections were then incubated for 2 hours in goat anti-mouse IgG (1:50; American Qualex) preabsorbed in fixed rat lip tissue for eight hours at 4°C (Ruocco et al., 2000), and then treated for one hour with a mouse monoclonal anti-HRP antibody (Seralab, UK) (Semenenko, Bramwell et al., 1985) to which 5 μg/ml HRP had been previously added. After further washing, DβH-immunoreactivity was revealed by reacting the sections with DAB and H_2O_2. For VAChT, the immunostaining protocol was followed as described above, using SG as a chromogen.

2.4.3. Single-labelling with VAChT for quantification

Sections were pre-treated with 0.3% H_2O_2, 1% sodium borohydride and 5% NGS prior to an overnight incubation in polyclonal rabbit anti-VAChT serum (1:10000). The following day, sections were incubated in biotinylated goat anti-rabbit IgG (1:800; Vector Labs) for 1 hour. After washes in PBS+T, the sections were incubated in ABC (1:400; Vector Labs) for 1 hour. VAChT antigenic sites were then revealed using DAB and H_2O_2.

2.5. QUANTIFICATION

2.5.1. Degree and timecourse of fibre migration

In order to study the time course of fibre migration into the upper dermis, we counted the number of VAChT-IR fibres in the region between the openings of the sebaceous glands (the point of reference for dividing upper and lower dermis) and the surface of the skin at all post-surgical time points, in an approach similar to that applied in a previous publication from our laboratory (Ruocco et al., 2000). An Olympus BX51 microscope equipped with a 20x objective and connected to an MCID Elite image analysis system (Imaging Research Inc., St.Catharines, ON, Canada) via a CCD camera was used.

A box of dimensions 280 μm x 65 μm was created using the image analysis software and placed at four locations along the rat lip skin between the skin surface and the openings of the sebaceous glands as illustrated schematically in Figure 1. The first box was rotated to a 45° angle to the horizon and placed in the first area on the curved surface of the lip into which it could fit between the opening of the sebaceous glands and the skin surface. The second box was located at the first flat region of the lip skin, the third at the last possible flat surface of the skin, and the fourth equidistant between the second and third boxes. One hundred and forty-four sections were processed (six time points, four animals per time point, six sections per animal). To ensure that a representative volume of tissue was quantified per animal, sections were collected bilaterally; per animal 3 sections were from lip tissue obtained from the right side and 3 from the left side. The sections of lip collected from the right and left side were separated by at least

3 mm, as the midline portion of lip was discarded, and those from the same side separated by at least 50 μm each. As measurements were performed in 4 rectangles of 18,200 μm^2 each per section, and the sections were collected from very distinct points in lip, we sampled a considerable volume of lip tissue.

Three parameters were quantified: number of fibres crossing into the box, total number of fibres inside ths box, and total number of fibres and branches inside the box. The number of VAChT-IR fibres that crossed the bottom and right-hand corners of the boxes was counted to establish a time course of fibre appearance in the upper dermis. The total number of fibres inside the boxes was counted to measure the total number of fibres existing in the upper dermis at every time point, and the total number of fibres and branches was recorded to look at the degree of fibre branching. Statistical analysis was determined by applying a one-way analysis of variance (ANOVA) followed by two-sided Dunnett's test with post-hoc Bonferroni correction.

2.5.2. Distance migrated by sprouted fibres

The distance of VAChT-IR fibre migration at the 4-week time point was analyzed using the Olympus microscope (10x objective) and image analysis system. The slides used for this quantification were single-labelled with VAChT and were coded to ensure that the observer was unbiased by treatment status. Codes were broken only prior to statistical analysis of the data. We measured the distance between the surface of the skin and the most superficial tip of all fibres visible within the field, using a length tool in the image analysis program. Five MN-transected and five sham-operated rats were quantified using six sections per animal. An unpaired Student's t-test was performed on the average distances obtained for each rat in sham-operated and MN-operated groups to assess statistical significance.

CHAPTER THREE: RESULTS

The upper dermis corresponds to the region between the epidermis and the opening of the sebaceous glands into hair follicles. The lower dermis corresponds to the region between the opening of the sebaceous glands into hair follicles and the hypodermis. SG chromogen non-specifically lightly and diffusely labels sebaceous glands (Figures 2, 3), but this artifact is easily distinguished from the intense and highly localized SG chromogen staining of immunoreactive fibres. This observation is consistent with previous studies from our lab (Ruocco, Cuello, and Ribeiro-da-Silva, 2000; Ruocco, Cuello, Shigemoto, and Ribeiro-da-Silva, 2001a; Ruocco, Cuello et al., 2001b; Ruocco, Cuello, Parent, and Ribeiro-da-Silva, 2002).

3.1. PATTERN OF LIP INNERVATION BY SP-, DβH- and VAChT-IR FIBRES IN CONTROL ANIMALS

As seen in a previous study from our laboratory (Ruocco et al., 2000), SP-IR fibres were found throughout the dermis and epidermis of the lower lip, although fibre density was highest in the upper dermis. In the lower dermis, SP-IR fibres were associated with blood vessels and hair follicles. Some upper dermal SP-IR fibres extended their terminals into the epidermis, whereas other fibres were associated with blood vessels in the upper dermis (Figure 2A). These observations reproduced the previous results from our laboratory using the same anti-SP antibody in rat lip tissue (Ruocco et al., 2001).

DβH- and VAChT-IR fibres were confined to the lower dermis and hypodermis, where they were associated with blood vessels (Figure 2B). DβH-positive fibres formed a mesh-like network around blood vessels, studded with varicosities, while VAChT-IR fibres formed more linear associations with vessels. There was no innervation of the upper dermis by these fibres. Our present results corroborate those of previous work (Ruocco et al., 2002).

Sham-operated and naïve rats had identical patterns of fibre distribution and fibre density (not shown). This provided confirmation the surgical procedure did not cause the changes observed at the various post-MN lesion time points.

3.2. CHANGES IN SP AND VACHT IMMUNOREACTIVITY AFTER BILATERAL MN TRANSECTION

The loss of sensory SP-IR fibres after sensory denervation was very rapid and is consistent with the time course of fibre degeneration observed in our previous study (Ruocco et al., 2000). The number of SP-IR fibres in the skin of the lower lip was drastically reduced to just above detection levels at one week post-surgery (not shown). At this time point, VAChT-IR fibres were detected only in the lower dermis, as in controls. At two weeks post-surgery, very thin, weakly immunoreactive SP-IR fibres were rarely detected in all skin regions, while varicose VAChT-IR fibres had established a noticeable presence in the upper dermis (Figure 2 C, D). Three weeks post-surgery (not shown), the invasion of VAChT-IR fibres into the upper dermis had progressed significantly from the 2-week time point, accompanied by a virtually absolute disappearance of SP-IR fibres from the rat lower lip skin. VAChT-IR fibres were also observed closer to the surface of the skin than at the two-week time point, suggesting that these fibres were migrating towards the surface of the skin. By 4 weeks post-surgery, a few SP-IR fibres had reappeared in rat lower lip skin and were beginning to innervate the same structures as in sham-operated animals (Figure 2 E, F). However, they had not yet reached their normal proximity to the skin surface and were thinner and less branched than control SP-IR fibres. At this point, VAChT-IR fibre numbers were maintained in the upper dermis, however they seemed to be continuing their approach to the surface of the skin. At six and eight weeks post-surgery, SP-IR fibres grew thicker and more numerous throughout the lower lip skin, although their numbers did not attain the control level. Re-grown sensory fibres were seen to run in close proximity, and often aligned with, persisting VAChT-IR fibres in the upper dermis, regularly displaying closely apposed varicosities (Figure 3A). VAChT-IR fibres were also localized in close arrangements with sprouted noradrenergic (DβH-IR) fibres (Figure 3B). The two autonomic fibre types were frequently observed running in parallel trajectories in the upper dermis at the 6- and 8- week timepoints. We have previously shown that DβH-IR and SP-IR fibres co-exist in the upper dermis at the same post-surgical period (Ruocco et al., 2000).

A schematic illustration of the changes described in this section is provided in Figure 4.

3.3. QUANTIFICATION OF THE EXTENT OF VAChT-IR FIBRE MIGRATION INTO THE UPPER DERMIS

To examine more precisely the chronology of cholinergic fibre invasion of the upper dermis in rat lower lip skin and to allow for comparisons to be made between the time courses of parasympathetic and sympathetic fibre invasion of the upper dermis (Ruocco et al., 2000), we quantified the number of VAChT-IR fibres present in the upper dermis at all post-surgical time points. From Table 1, it can be seen that peak parasympathetic fibre migration into the upper dermis first occurred at 3 weeks post-MN surgery and the maximal number of fibres and branches held constant until the following week (4 weeks post-MN transection). By the following time point, 6 weeks post-surgery, the number of cholinergic fibres and branches had decreased significantly, while a further decrease in fibre number was again observed two weeks later. It is important to note that the number of VAChT-IR fibres in the upper dermis at eight weeks post-MN surgery was still greater than that observed in control animals. No major changes in degree of fibre branching were observed. This parameter was assessed based on the difference between the number of fibres inside the counting area and total number of fibres and branches inside the counting area.

3.4. QUANTIFICATION OF DISTANCE OF FIBRE MIGRATION TOWARDS THE SKIN SURFACE

We also evaluated the minimal distance between the surface of the skin and VAChT-IR fibres at the 4-week time point (Figure 5), in order to compare the maximal distance of cholinergic fibre migration to that of sympathetic fibres (Ruocco et al., 2000). In control animals, VAChT-IR fibres were found 381.4 $\pm$ 12.5 μm from the skin's surface while in MN-transected animals, they were significantly closer (189.2 $\pm$ 8.0 μm) ($n = 8$, $p < 0.001$, Student's t-test). Additionally, there were far more upper dermal VAChT-IR fibres in the former than in the latter sections.

3.5. FIBRE DISTRIBUTION FOLLOWING DUAL MN TRANSECTION AND SYMPATHECTOMY

As a control for sympathetic fibre ingrowth, we bilaterally transected the MN and SCG of 15 rats and allowed fibre migration to occur for 1, 4, and 8 weeks. Sympathectomy did not affect the massive VAChT-IR fibre ingrowth into the upper dermis observed at 4 weeks post-MN transection, confirming that the observed cholinergic fibres are more than likely of parasympathetic origin. In the upper dermis, sprouted cholinergic fibres were abundant. In the lower dermis, VAChT-IR fibres ran in thick bundles and had many branches (Figure 6). Similarly, sympathectomy had no effect on VAChT-IR innervation of the skin 1 or 8 weeks post-surgery (not shown).

FIGURE 1. ***Diagrammatic representation of the quantification method.*** A 280μm x 65μm box was placed at four locations along the surface of the rat lip skin as indicated. The first box (box 1) placed placed at the first amenable region on a 45° angle. The box was then rotated and moved to the first flat region of the lip (box 2), after which it was stationed at the last flat region of the lip surface (box 3). The final box was placed at a location intermediate to the second and third boxes (box 4).

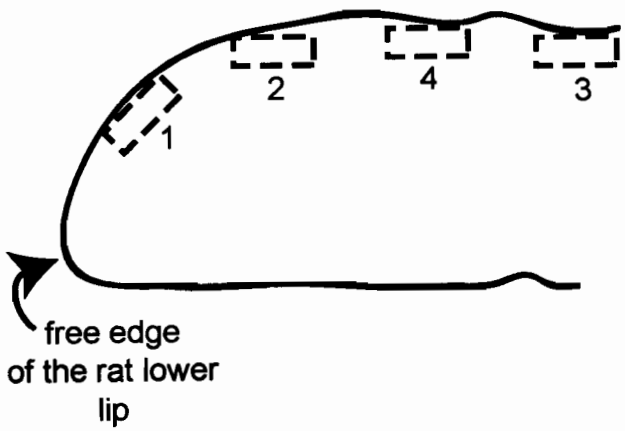

1
2
4
3
free edge
of the rat lower
lip

Figure 2. ***Changes in patterns of substance P (SP) and vesicular acetylcholine transporter (VAChT) immunoreactivity post-MN transection.*** *Control (sham-operated) lower lip(A, B).* **A.** In the upper dermis (above the opening of sebaceous glands into hair follicles), an abundant network of highly branched SP-IR fibres is apparent (arrowheads; brown). **B.** In the lower dermis, there are SP-IR (arrowheads) and VAChT-IR (arrows; blue) fibres wrapping around the same blood vessels. *Two weeks post-surgery (C, D).* **C.** In the upper dermis, no SP-IR fibres are detected and the anomalous presence of cholinergic fibres (arrows) in the upper dermis has begun; note also a cholinergic fibre wrapping around a sebaceous gland (arrow), in lower dermis. **D.** In the lower dermis, the absence of SP-IR fibres around a blood vessel is noteworthy, while it remains innervated by cholinergic (VAChT-IR) fibres (arrows). *Four weeks post-surgery (E, F).* **E.** In the upper dermis, SP-IR fibres have reappeared (arrowheads), and are in close proximity to VAChT-IR fibres (arrows). **F.** In the lower dermis, a dense network of cholinergic fibres can be seen innervating a blood vessel (arrows). S = sebaceous gland. Scale bar = 50μm.

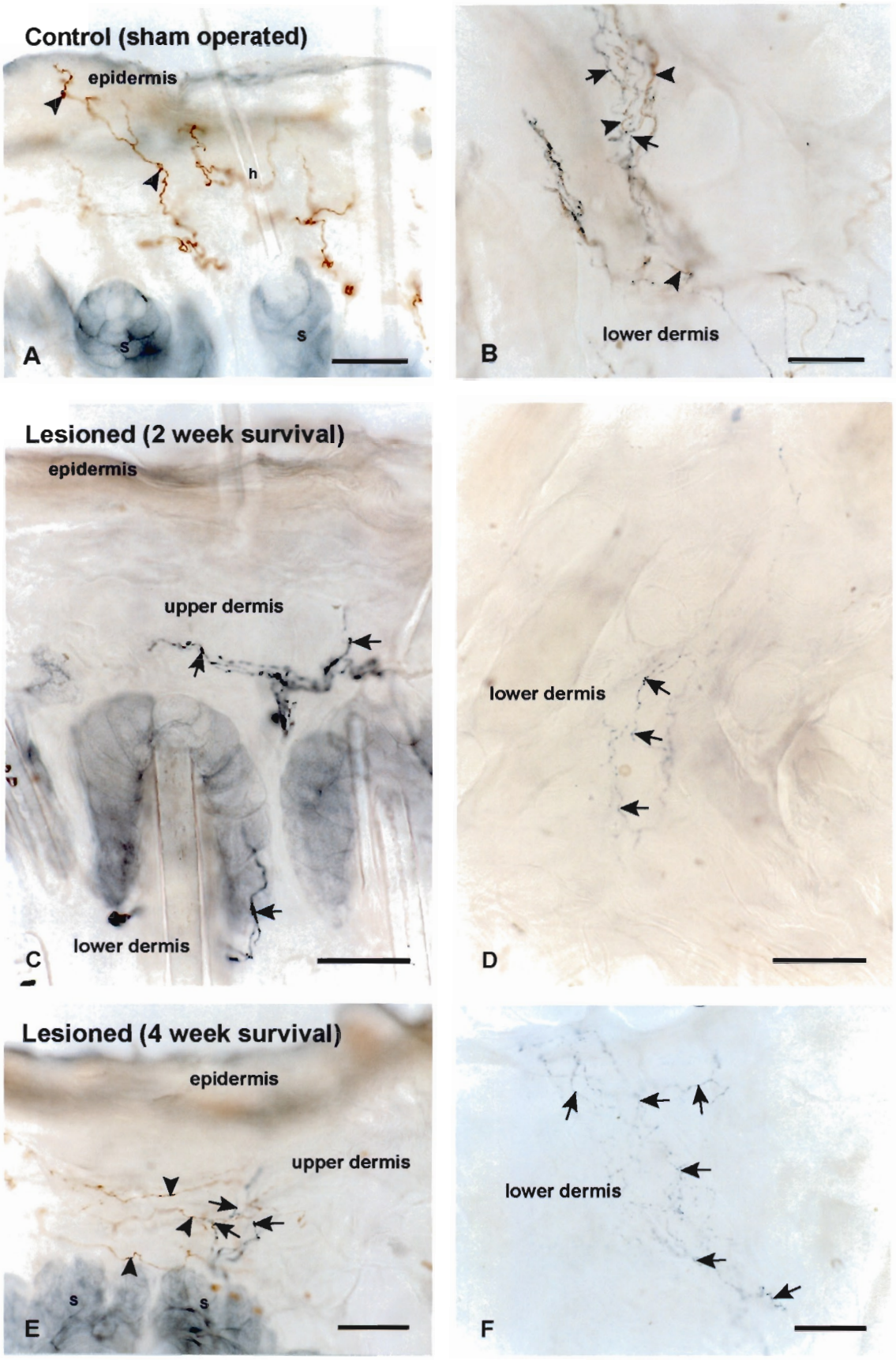

Control (sham operated)
epidermis
h
s
s
A
B
lower dermis
Lesioned (2 week survival)
epidermis
upper dermis
lower dermis
C
lower dermis
D
Lesioned (4 week survival)
epidermis
upper dermis
s
s
E
lower dermis
F

FIGURE 3. ***Persistence of anomalous autonomic fibres after sensory re-innervation eight weeks post-surgery.*** **A.** In the upper dermis, there is now a considerable network of re-grown sensory SP-IR fibres (arrowheads) and they are in close proximity to cholinergic fibres (arrows). **B.** In the upper dermis, note that sympathetic (DβH-IR; double arrows) fibres are also found in close apposition to cholinergic fibres (single arrows). h = hair; S = sebaceous glands. Scale bar = 50μm.

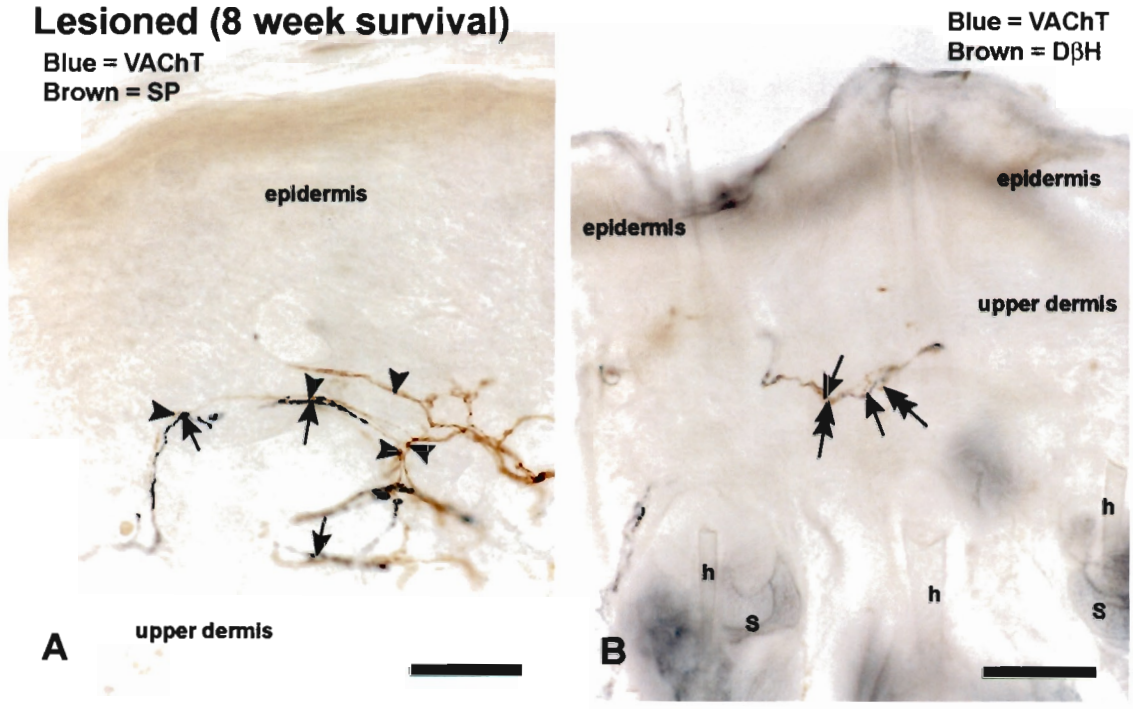
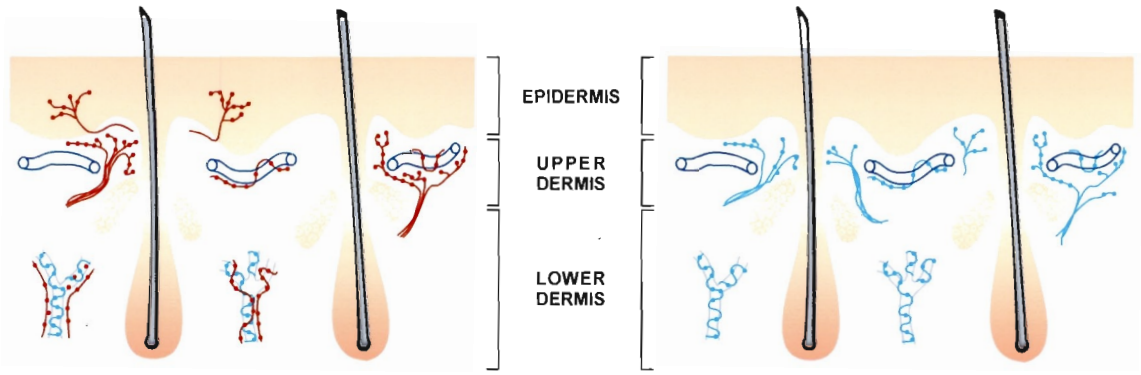

Lesioned (8 week survival)
Blue = VAChT
Brown = SP
epidermis
upper dermis
A
Blue = VAChT
Brown = DβH
epidermis
epidermis
upper dermis
h
h
h
S
S
B

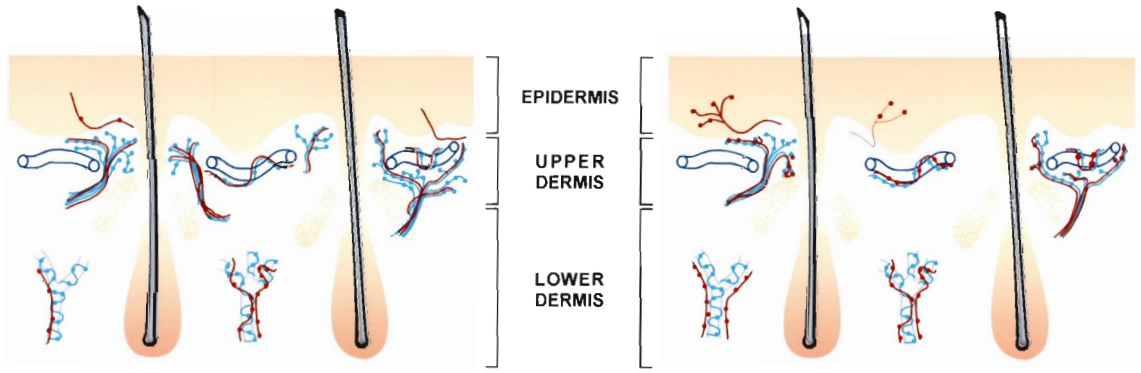
FIGURE 4. ***Schematic representation of the changes observed in the rat lower lip after sensory denervation.*** In naïve animals, SP-IR fibres (red) are found throughout the epidermis, upper and lower dermis of the lower lip, where they innervate blood vessels. Autonomic fibres are restricted to the lower dermis, where VAChT-IR (blue) fibres are found in association with blood vessels. Two weeks after MN lesion, sensory SP-IR fibres have degenerated to rare, thin fibres in the lower lip, while cholinergic fibres begin to grow into the upper dermis. At 4 weeks post-MN lesion, VAChT-IR fibres have reached their maximum density in the upper dermis, and sensory fibres have begun to regrow. These two fibre types are often found running close together in the upper dermis. This panel emphasizes that even when their migration is maximal, VAChT-IR fibres do not extend into the epidermis. Eight weeks after sensory denervation, SP-IR fibre have grown thicker and more numerous, although they do not regain their normal density. Regrown sensory fibres run in close proximity to VAChT-IR fibres in the upper dermis.

Normal



2 weeks

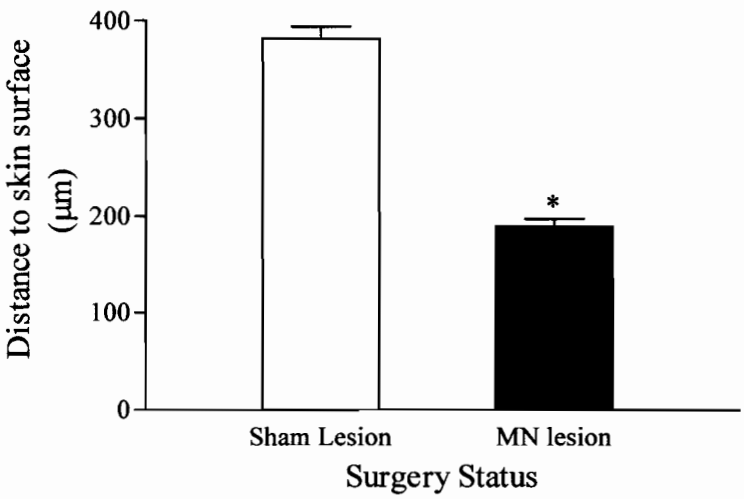
4 weeks



8 weeks

FIGURE 5. ***Quantification of the extent of VAChT-IR fibre migration into the upper dermis at 4 weeks post-MN lesion.*** By using an M5 image analysis system to quantify the distance between sprouted VAChT-IR fibres and the skin, it was seen that, at 4 weeks post-MN lesion the VAChT-IR fibres in lesioned animals (381.4 $\pm$ 12.5 μm) were significantly closer to the surface of the skin compared with the control animals (189.2 $\pm$ 8.0 μm). n = 4 for each group. $*P < 0.001$, Student's t-test.

Cholinergic Fibre Migration after MN transection



* $p < 0.001$, $n = 8$, Student's t-test

FIGURE 6. ***Persistence of parasympathetic fibres after dual sympathetic and sensory denervation.*** In the upper dermis at 4 weeks post-dual surgery, there are many sprouted VAChT-IR fibres (arrowheads) in the upper dermis, very close to the surface of the skin. In the lower dermis, note bundles of cholinergic fibres are indicated by arrows. Scale bar = 100μm.

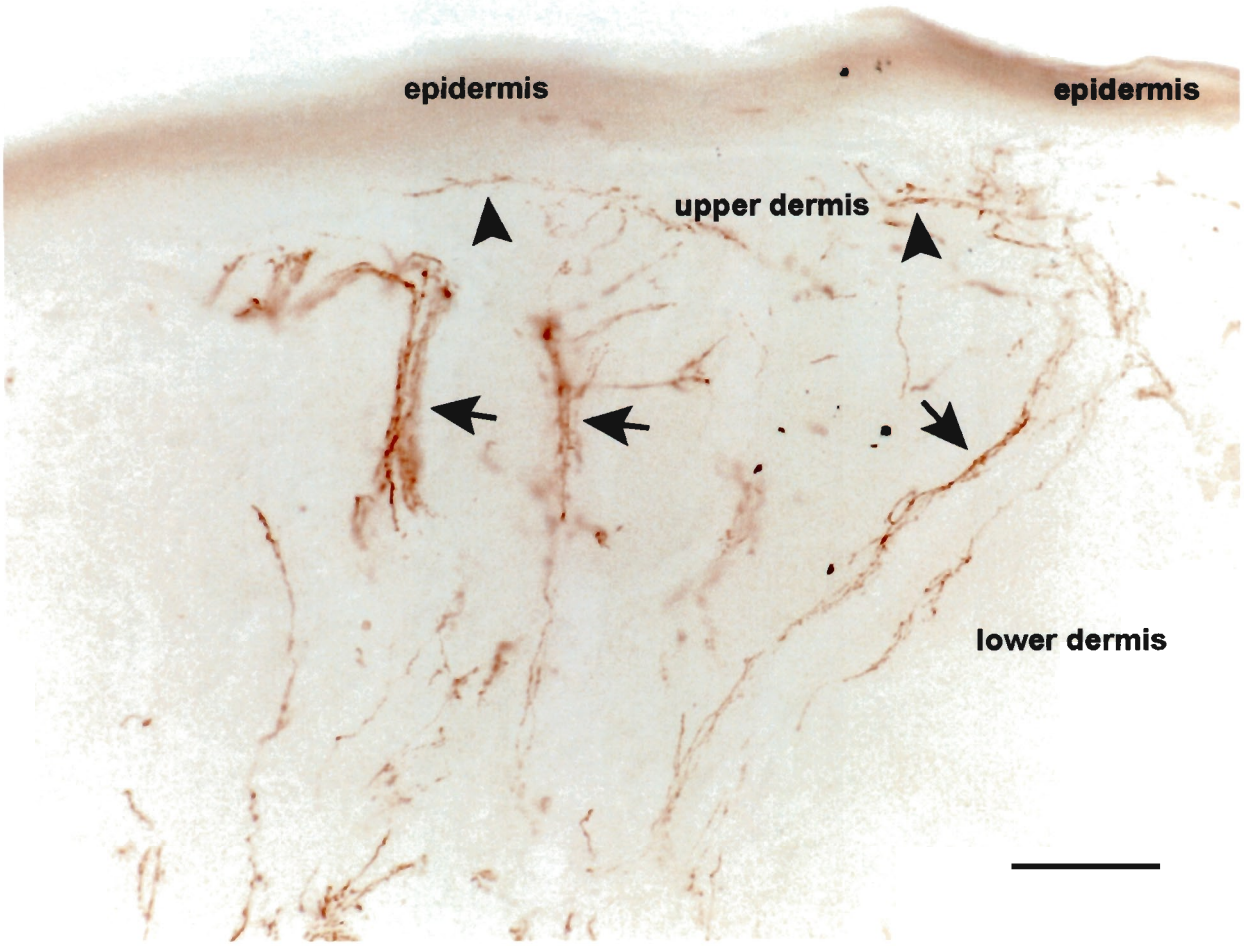

epidermis
epidermis
upper dermis
lower dermis

TABLE 1. QUANTIFICATION OF THE MIGRATION OF VESICULAR ACETYLCHOLINE TRANSPORTER IMMUNOREACTIVE FIBRES INTO THE UPPER DERMIS AT ALL POSTSURGICAL TIME POINTS[3]

Postsurgical time points	Number of fibres intersecting the counting area	Number of fibres inside the counting area	Total number of fibres & branches inside the counting area
0 weeks	0	0	0
1 week	47	32	58*
2 weeks	131	91	137**
3 weeks	172	100	208**
4 weeks	187	123	214**
6 weeks	107	71	126*
8 weeks	27	16	31

* $p < 0.05$ (one-way ANOVA followed by two-sided Dunnett's test and post-hoc Bonferroni correction)

** $p < 0.001$ (one-way ANOVA followed by two-sided Dunnett's test and post-hoc Bonferroni correction)

[3] Values represent the total number of fibres counted in 144 sections of rat lower lip (six time points, 4 rats per time point, 6 sections per animal).

CHAPTER FOUR: DISCUSSION

4.1. RELEVANCE OF THE CURRENT RESULTS

Neuropathic pain is a growing concern in today's society. It manifests following damage to peripheral nerves as a result of trauma, prevalent diseases such as diabetes or herpes zoster, cancer and AIDS, or following chemical nerve injury, as occurs with chemo- or antiretroviral therapy. Diabetes, herpes zoster, and cancer – the most common causes of neuropathic pain – are all diseases likely to affect a growing number of patients as the North American population ages (Bennett, 1998). The symptoms experienced by neuropathic pain sufferers are very severe – a number of patients experience hyperalgesia so strong that the contact of clothing is too much to bear. The aetiology of the condition is not well characterized. Some patients suffer from sympathetically-maintained pain (SMP), and can be relieved by the administration of sympathetic blocking agents or by surgical sympathectomy (O'Halloran and Perl, 1997). However, other patients experience sympathetically-independent pain (SIP) after peripheral nerve lesions, the generation of which is poorly understood. Neuropathic pain is very difficult to treat, as it is unresponsive to conventional pain treatments such as opioid analgesics and non-steroidal anti-inflammatory drugs (NSAIDs). SIP, in particular, represents a major challenge to clinicians due to the growing number of affected individuals and absence of appropriate treatment. A better understanding of the central and peripheral changes resulting from sensory denervation or nerve injury will inspire basic scientists to find new approaches to the treatment of both SIP and SMP.

Previous studies from our lab have shown that the sympathetic nervous system responds to sensory nerve injury in a drastic manner. In our lab, sympathetic fibres in the hairy skin of the head region of rats have been shown to grow from their normal location in the lower dermis into the upper dermis, where they branch extensively (Ruocco, Cuello, and Ribeiro-da-Silva, 2000). The infiltration of the upper dermis by ectopic fibres was demonstrated to persist after sensory fibres had re-innervated the region, lasting at least eight weeks (the longest time point examined in this study). Such an anatomical rearrangement of the skin's innervation is undoubtedly significant in the complicated process of

developing and sustaining sensory changes because it allows a heightened interplay to develop between sympathetic efferents and sensory fibres, which normally only interact in the lower dermis. Significantly, SP-containing sensory fibres, which are nociceptors, terminate mainly close to blood vessels in the lower dermis where their function is likely predominantly efferent vasodilatation mediated by peptide release. In the upper dermis, they not only terminate on blood vessels but also have non-blood vessel-directed termini which are likely to play a greater role in the transduction of pain. Thus, formation of sympathetic-sensory relationships in the upper dermis likely has a greater effect on nociception following nerve lesions than the close localisation of the same fibres in the lower dermis, whereas lower dermal associations are probably more relevant to modulation of blood flow, vessel dilatation, and plasma extravasation, all of which occur with inflammation and related pain. In contrast to the long-term changes maintained after sensory denervation, sympathetic denervation produced only a very transient sprouting and branching of SP-positive sensory fibres and sympathetic fibres did not regrow (Ruocco, Cuello, Shigemoto, and Ribeiro-da-Silva, 2001a). This is significant because it suggests that sensory abnormalities after sympathectomy cannot be explained by changes in the anatomy of peripheral sensory terminals. Possible explanations may lie in changes in the skin's innervation by other populations of sensory and/or nociceptive terminals, properties of the nociceptor terminals, or in central changes in pain transmission pathways. Anomalous sympathetic innervation of the DRG has been reported by other groups to ensue after sensory nerve lesion (McLachlan, Janig, Devor, and Michaelis, 1993; Chung, Kim, Na, Park, and Chung, 1993; Chung, Lee, Yoon, and Chung, 1996; Ramer, Kawaja et al., 1998; Julius and Basbaum, 2001; Basbaum and Jessell, 2003). However, several incongruencies exist between the anatomical changes observed at the DRG and behavioural correlates as described previously in this thesis (please refer to section 1.5.1.2.). Of course, it will be important to characterize the relationship between sympathetic sprouting in the skin and pain behaviours (described further in section 4.10.).

The aim of the present thesis was to look at the possibility that sensory denervation-induced sympathetic sprouting might be accompanied by similar or different changes in the parasympathetic innervation of the skin. Based on previous studies from our lab, we expected that sprouting might occur, but there was no other precedent in the literature to indicate that the parasympathetic system is affected by sensory nerve injury.

4.2. COMPARISON TO PREVIOUS DATA FROM OUR LAB: PARASYMPATHETIC VERSUS SYMPATHETIC FIBRE SPROUTING

In this study, we confirmed that sensory denervation of the rat lower lip skin changes the innervation of the upper and lower dermis by SP-IR sensory fibres and added the new observation that cholinergic fibres sprout into the upper dermis (Figure 4). Mental nerve transection caused a rapid degeneration of SP-IR sensory fibres in the skin. The time course of sensory fibre disappearance from upper and lower dermis was consistent with that discerned from a previous study (Ruocco et al., 2000). Briefly, near-absolute loss of SP-IR fibres was noted at 2 weeks post-surgery, and regrowth of SP-IR fibres to near-normal levels was not remarked until 4 weeks later – at the 6-week post-surgical time point. Sensory fibre re-innervation likely occurred by NGF-dependent collateral sprouting of spared axons (Diamond, Coughlin, Macintyre, Holmes, and Visheau, 1987; Diamond, Holmes et al., 1992) from the cervical branch of the facial nerve, the lingual branch of the trigeminal nerve, or the cervical plexus, rather than by fibre regeneration, as the MN is completely severed bilaterally in the surgery. It is noteworthy that regeneration of sensory fibres from the proximal MN stump is not possible in this model because a segment of the nerve is completely removed, and re-innervation must occur by collateral sprouting from the facial nerve. However, the new sensory fibres do not reach the density of innervation observed in controls, suggesting that some of their trophic support may be diverted to maintain other fibre types. Furthermore, since the sprouted fibres must migrate from a sensory nerve more distant from the skin than the mental nerve, it is possible that their density may return the control level at a later point than we

examined in this study. In parallel, cholinergic (VAChT-IR) fibres first appear ectopically in the upper dermis 1 week post-MN lesion and infiltrate the region maximally by 3 weeks post-lesion, before decreasing in number at 6 and 8 weeks post-surgery, though a small population persists even at the last measured time point. Although VAChT-positive fibres sprout into the upper dermis, they were not observed in the epidermis in any sections we examined. VAChT-IR fibres were confirmed to be of parasympathetic origin by dual sensory and sympathetic denervation surgeries.

4.3. MIGRATING FIBRES ARE OF PARASYMPATHETIC ORIGIN

Past studies (Kaji, Maeda, and Watanabe, 1991; Fundin, Pfaller, and Rice, 1997) have been hampered by the caveats that vasoactive intestinal peptide (VIP) and cholinesterase labelling are not restricted to the parasympathetic system, and can therefore not be used as reliable markers on their own. Anti-VAChT antibodies were applied to visualize cholinergic fibres as they are both more sensitive and more reliable for accurate visualization of cholinergic terminal fields in the peripheral nervous system than the alternative markers (Goodness, Albers et al., 1997).

In order to confirm that the migrating cholinergic fibres identified by this approach were not sympathetic, SCG ganglionectomies were combined with mental nerve transection surgery in the same animals. Lower lips were observed 4 weeks after MN-lesion, the time when maximal fibre migration was quantified. This procedure was followed to convince readers and reviewers who know that some cholinergic fibres that innervate the skin, such as those that supply the sweat glands, are of sympathetic origin. However, in the rat lower lip there are no sweat glands and consequently no cholinergic sympathetic fibres should exist. At the end of the allotted recovery time, cholinergic fibres persisted in the upper dermis. The absence of cholinergic fibre changes following sympathetic denervation of the lip proves that these fibres do not originate from the sympathetic nervous system.

The migrating fibres observed and quantified in this study were likely from a parasympathetic source, most probably the otic ganglion (see section 1.3.2.3.). However, control otic ganglion removal was not performed due to the extreme difficulty of the technique, although previous researchers have been successful in this regard. A previous study showed that otic ganglionectomy resulted in the loss of all VIP-IR fibres from the blood vessels of the lower lip in rat (Kaji, Shigematsu, Fujita, Maeda, and Watanabe, 1988), leading the authors to conclude that the otic ganglion is the source of parasympathetic innervation of the lower lip.

4.4. DIFFERENTIAL TROPHIC SUPPORT OF SENSORY, SYMPATHETIC AND PARASYMPATHETIC FIBRES

Although sprouting and migration of both sympathetic and parasympathetic fibres is observed after sensory denervation, the trophic factors responsible for the initial attraction and subsequent maintenance of these ectopic autonomic fibres are distinct, and probably derive from different sources. Sympathetic fibres have been shown to express TrkA and to rely on NGF for survival and development in knockout mice studies (Crowley, Spencer, Nishimura, Chen, Pitts-Meek, Armanini, Ling, MacMahon, Shelton, Levinson, and ., 1994; Smeyne, Klein, Schnapp, Long, Bryant, Lewin, Lira, and Barbacid, 1994) while parasympathetic neurons express the GDNF family ligand (GFL) receptors GFRα1 and GFRα2 coupled to Ret tyrosine kinase and bind neurturin (NTRN) and GDNF. Though *in vitro* experiments previously suggested the occurrence of preferential associations between GFRα1 and GDNF and GFRα2 and NTRN, recent studies have proven that NTRN and artemin (another GFL) can signal via GFRα1 while GDNF can activate the GFRα2 (Airaksinen, Titievsky et al., 1999; Rosenthal, 1999; Wang, Shih et al., 2000). These studies suggest a low specificity of the GDNF receptors, GFRα1 and α2, expressed on parasympathetic neurons for GDNF and neurturin. GDNF/Ret interactions are essential for parasympathetic neuronal precursor proliferation and development, and Enomoto et al. (2000) have suggested that GDNF acts as a chemoattractant to this end. Sources of excess NGF and GDNF in the hairy skin of the rat lower lip are not

present under normal conditions, but may become available after sensory nerve damage.

Sensory nerves are comprised of myelinated A fibres and smaller diameter unmyelinated C-fibres. Some Aδ fibres and most C-fibres represent nociceptive afferents. Transection of the MN, which results in sensory denervation of the skin in the receptive fields of the damaged fibres, results in the degeneration of all A- and C-fibres carried in the nerve, independently of being nociceptive or not. The peptidergic and non-peptidergic C-fibres, which represent the great majority of nociceptive afferents, are supported by the same trophic factors required for autonomic fibre migration and survival. Peptidergic fibres synthesize SP and CGRP and express TrkA, the high affinity NGF receptor (see section 1.3.2.1.). Non-peptidergic fibres are FRAP-positive, and express the purinergic receptor P2X3, GSA-IB4-binding sites, and the GDNF family receptor types GFRα1 and GFRα2 [(Bennett, Michael, Ramachandran, Munson, Averill, Yan, McMahon, and Priestley, 1998); see section 1.3.2.2). These nociceptive fibre types require trophic support from their target cells – the peptidergic fibres respond to NGF secreted by keratinocytes (Tron, Coughlin, Jang, Stanisz, and Sauder, 1990; English, Harper, Stayner, Wang, and Davies, 1994), fibroblasts (Acheson, Barker, Alderson, Miller, and Murphy, 1991), Merkel cells (Vos, Stark, and Pittman, 1991), and Schwann cells (Taniuchi, Clark et al., 1986), while the non-peptidergic fibres respond to GDNF (Bennett, Michael, Ramachandran, Munson, Averill, Yan, McMahon, and Priestley, 1998) synthesized by Schwann cells (Henderson, Johnson, Jr. et al., 1994), and likely also by keratinocytes. Furthermore, the mental nerve carries mechanoreceptive afferent fibres – Aα and Aβ – that have large diameters and myelinated axons. These fibres express TrkC receptors for NT3 (McMahon, Armanini, Ling, and Phillips, 1994; Wetmore and Olson, 1995; Wright and Snider, 1995; Verge, Gratto et al., 1996), a member of the NGF family which also binds with some affinity to GFRα2 (Baloh, Enomoto et al., 2000).

4.5. TROPHIC FACTOR HYPOTHESIS TO EXPLAIN AUTONOMIC FIBRE SPROUTING

Peripheral nerve transection and subsequent degeneration of fibres leaves spaces previously occupied by the fibres empty and allows growth factors produced by adjacent target cells to accumulate, as they are not taken up by the nerve fibres. As small sensory fibres degenerate, the Schwann cells lining the nerve tract increase their production of NGF and the low affinity neurotrophin receptor $p75^{NTR}$ (Taniuchi, Clark, and Johnson, Jr., 1986). Simultaneously, TrkA is temporarily down-regulated in sensory neurons (Verge, Merlio et al., 1992) after nerve damage. In a previous publication from our laboratory, it was suggested by Ruocco et al. (2000) that these hollow Schwann cell tracts, with high levels of unbound NGF, would attract sympathetic fibres and serve as guides allowing their growth into the upper dermis following nerve lesion. Keratinocyte production of NGF in the epidermis would further contribute to the establishment of a steep NGF gradient, since nociceptive sensory fibres are lost in this region as well [current results; (Ruocco et al., 2000)]. However, TrkA expressing parasympathetic fibres never extend past the dermal-epidermal junction (please refer to section 4.10 for a possible explanation). SP-IR sensory fibre re-innervation of the upper dermis did not begin until around 4 weeks post-MN transection, consistent with the initial decreased TrkA expression on these fibres noted previously (Verge, Merlio, Grondin, Ernfors, Persson, Riopelle, Hokfelt, and Richardson, 1992).

However, the focus on the putative NGF tropism of certain fibres is not a wholistic perspective on the events that may follow sensory denervation. As noted previously, mental nerve transection results in a loss of all sensory fibres – that is nociceptive peptidergic and non-peptidergic fibres and non-nociceptive fibres – and consequent local increases in the trophic factors supporting these fibres, too. Trupp et al. (1995) have shown that GDNF levels are also elevated after peripheral nerve transection and degeneration, which presumably results from the loss of non-peptidergic fibres in transection surgery. Thus created, a GDNF gradient may promote the in-growth of parasympathetic fibres. It has previously been demonstrated that GDNF is the key GFL responsible for parasympathetic

neuron migration (Enomoto, Heuckeroth et al., 2000) while other growth factors such as NTRN, ciliary neurotrophic factor, hepatocyte growth factor are more involved in promoting survival and growth of parasympathetic neurons *in vitro* and *in vivo* (Heuckeroth, Enomoto et al., 1999; Rossi, Luukko et al., 1999; Enomoto, Heuckeroth, Golden, Johnson, and Milbrandt, 2000; Davey, Hilton et al., 2000). Although data on the level of NT3 expressed after nerve injury and loss of proprioceptive fibres is lacking, supposing it is upregulated like NGF, it would provide and additional trophic support to GFR-expressing parasympathetic neurons, possibly resulting in a greater and/or more rapid infiltration of the upper dermis by these fibres.

This trophic factor-nerve tract hypothesis is supported by observations from our current and previous studies from our laboratory (Ruocco et al., 2000) showing that sympathetic, parasympathetic and sensory fibres run in close proximity in the upper dermis following the re-innervation of the area by SP-containing fibres, suggesting a tripartite sharing of the Schwann cell myelin sheath. In addition, the observation that the density and branching of sensory SP-IR peptidergic fibres do not regain their pre-lesion levels suggests that some of their trophic support is diverted to maintain sprouted sympathetic fibres (Ruocco, Cuello, and Ribeiro-da-Silva, 2000). Based on this logic, similar decreases in non-peptidergic and possibly also proprioceptive fibres would be expected following sensory re-innervation. Assuming that sensory innervation remains lower than in the control situation, there must be other factors that explain the nociceptor sensitization that ensues from nerve injury. One possibility is that the terminals begin to express, either de novo or at higher levels, receptors for the new substances released in their vicinity by sprouted autonomic fibres.

4.6. NOVEL INNERVATION PATTERNS AND THE POSSIBLE ROLE OF ADRENERGIC AND CHOLINERGIC RECEPTORS IN SENSORY FIBRE SENSITIZATION

The expansion of autonomic fibre innervation from lower-only to both upper and lower dermis provides the anatomical substrate for possible interactions

between sensory, parasympathetic, and sympathetic fibres in the region of the upper dermis after sensory fibre re-innervation (Table 2).

It has been previously postulated that sensory and sympathetic fibres interact peripherally after nerve injury via binding of NA secreted by the latter fibre type to α2-adrenergic receptors on the former (Taniuchi, Clark, and Johnson, Jr., 1986; Sato and Perl, 1991; McLachlan, Janig, Devor, and Michaelis, 1993; Devor, Janig et al., 1994). The proposed interaction could occur very easily in the skin due to the close proximity of the two fibre types observed after peripheral nerve injury (Ruocco et al., 2000). However, the localization of α2-adrenergic receptors to the peripheral ends of sensory fibres has not yet been accomplished due to lack of antibodies of sufficiently high affinity (Ribeiro-da-Silva, personal communication), although we have strong indications that they do occur (please refer to section 1.3.2.3.d.). Beta-2 adrenergic receptors have also been reported on nociceptors (Bowles, Flores, Jackson, and Hargreaves, 2003), but their role is desnsitizing, so they are likely not upregulated or involved in the increased sensitivity that develops after nerve injury.

The close apposition of parasympathetic and sensory fibres observed after MN -transection in the present study, coupled with the existence of both muscarinic and nicotinic receptors on sensory fibres (muscarinic: (Steen and Reeh, 1993; Tata, Plateroti, Cibati, Biagioni, and Augusti-Tocco, 1994; Wanke, Bianchi, Mantegazza, Guatteo, Mancinelli, and Ferroni, 1994; Haberberger, Henrich, Couraud, and Kummer, 1999; Bernardini, Levey, and Augusti-Tocco, 1999; Bernardini, Sauer, Haberberger, Fischer, and Reeh, 2001; Bernardini, Roza, Sauer, Gomeza, Wess, and Reeh, 2002); cholinergic: (Boyd, Jacob, McEachern, Caron, and Berg, 1991; Flores, DeCamp, Kilo, Rogers, and Hargreaves, 1996); please refer to section 1.3.2.3.c.), provides the anatomical basis for an additional sensory-autonomic interaction via release of acetylcholine from the former. While M2 muscarinic receptors are proposed to desensitize C-fibres to mechanical and heat stimuli (Bernardini, Roza, Sauer, Gomeza, Wess, and Reeh, 2002), nicotinic receptors are involved in excitation of nociceptor terminals after ACh administration, as the excitatory response is blocked by nicotinic antagonists

(Tanelian, 1991; Steen and Reeh, 1993). As for NA, the prevailing effect of acetylcholine is mediated by excitatory receptors, in this case neuronal nicotinic type. However, nicotinic receptor expression has not been studied in the period that follows nerve injury, and possible changes in the relative levels of muscarinic and nicotinic subtypes may modulate the response of C-fibres to acetylcholine.

Peripheral nerve lesion results in an expansion of the terminal field size of autonomic efferents, as these fibres invade the upper dermis of the rat lower lip skin, a territory normally reserved for sensory fibres. The altered pattern of innervation following sensory fibre loss in the skin presents the opportunity for novel close interactions between sensory, sympathetic, and parasympathetic fibres in a novel setting.

4.7. ROLE OF PURINERGIC RECEPTORS IN SENSORY, SYMPATHETIC, AND PARASYMPATHETIC FIBRE INTERACTIONS

Another putative interaction between sympathetic fibres and both sensory and parasympathetic fibres may occur after autonomic fibre invasion of the upper dermis via a purinergic mechanism. It is well-known that non-peptidergic, GSA-IB4-positive, small diameter afferents express P2X3 purinoreceptors (Vulchanova, Arvidsson et al., 1996; Vulchanova, Riedl et al., 1997; Vulchanova, Riedl et al., 1998; Bradbury, Burnstock, and McMahon, 1998). The peripheral projections of these neurons also express P2X3 receptors (Bo, Alavi et al., 1999). This receptor expression has been shown to be upregulated in trigeminal sensory neurons after nerve injury (Eriksson, Bongenhielm, Kidd, Matthews, and Fried, 1998). Although P2X3 receptors were initially thought to be confined to expression on sensory fibres, Xiang et al. (1998) proved immunohistochemically that a very low level of the receptor is localized to sympathetic fibres originating from the SCG. Purinergic receptors have also been identified on parasympathetic neurons. Submandibular ganglion neurons exhibit elevated P2X2 and P2X4 purinoreceptor immunoreactivities and increased responsiveness to ATP after dissociation (Smith, Hansen, Liu, and Adams, 2001), which suggests that purinergic receptors other than P2X3 are induced on the cell surface of

parasympathetic neurons after nerve injury or synaptic blockade. In the skin, the one source of ATP is its co-release with NA and NPY from sympathetic nerves (Burnstock, 1990). If all of the above suggestions are true, then the ATP released from sympathetic fibres invading the upper dermis may have paracrine actions on sensory and parasympathetic projections, with which sympathetic fibres are closely associated, in addition to autocrine actions on themselves.

4.8. OTHER RECEPTORS IN SENSORY, SYMPATHETIC, AND PARASYMPATHETIC FIBRE INTERACTIONS

The localization of various receptor types to nociceptor terminals was described in section 1.3.2.3. of this thesis. Of importance, due to the close localization of sensory fibres vis-à-vis sensory and parasympathetic fibre, is the expression of adrenergic and P2X3 and cholinergic receptors, respectively. Small sensory neurons also express inhibitory NPY receptors (Jazin, Zhang et al., 1993) that may be activated by release of NPY in sympathetic vesicles (Zukowska-Grojec and Wahlestedt, 1993). In addition, parasympathetic fibre associations with peptidergic fibres might be promoted by the release of NGF from the former in response to α2 adrenoreceptor activation, which could be mediated by further close interactions with sympathetic fibres (see below).

There is additional evidence that sensory nerve terminals could release certain molecules that might have effects on nearby autonomic fibres in the skin after nerve injury. Importantly for sensitization of peripheral nociceptive fibres, nociceptive effectors may either increase the amount of sensitizing substances released from autonomic fibres or sustain the presence of autonomic fibres in their ectopic region by an activity-dependent mechanism. For example, sympathetic neurons express ionotropic receptors that can be activated by glutamate release from excited nociceptor terminals (Carlton, Chung, Ding, and Coggeshall, 1998) and some parasympathetic neurons (of the cardiac ganglion in guinea pigs) express NK2 and NK3 tachykinin receptors and are densely apposed by SP-IR terminals (Hardwick, Mawe, and Parsons, 1995), which co-release SP and NKA. The possibility of a peptidergic-sensory input to autonomic neurons cannot be overlooked. Such input has been demonstrated in sympathetic ganglia that

innervate the gut, which receive collaterals for SP-containing sensory fibres (Matthews and Cuello, 1982; Matthews and Cuello, 1984; Matthews, Connaughton et al., 1987; Kaji, Maeda, and Watanabe, 1991; Fundin, Pfaller, and Rice, 1997). However, there is not much support in the literature for such an interaction on parasympathetic neurons or on sympathetic neurons in other parts of the body.

4.9. INTERACTIONS BETWEEN PARASYMPATHETIC AND SYMPATHETIC FIBRES

Parasympathetic-sympathetic interactions may also exist in the innervation pattern observed after peripheral nerve injury (Figure 3). Although parasympathetic and sympathetic fibres co-exist on lower dermal blood vessels in normal skin, they form different patterns of association with their targets – parasympathetic form more linear arrangements as compared to sympathetic mesh-like networks (Ruocco, Cuello, Parent, and Ribeiro-da-Silva, 2002). As such, the novel associations of parasympathetic and sympathetic fibres described in this study, likely within the nerve tracts of degenerated sensory fibres, are more intimate and therefore there exists increased potential for inter-fibre interactions.

Vanhoutte and Shepherd (1973) demonstrated the ability of acetylcholine to attenuate the response of sympathetic fibres to electrical stimulation. It was suggested that the vasodilatation observed in their animal model was due to an inhibitory presynaptic action of ACh on terminal receptors. Additionally, Hasan and Smith (2000) have demonstrated that parasympathetic neurons synthesize NGF and transport it to peripheral axons where it may promote prejunctional interactions with sympathetic neurons. The mechanism suggested by Hasan and Smith (2000) is mediated by sympathetic release of NA, which binds to $\alpha 2$ adrenergic receptors on adjacent parasympathetic neurons, and stimulates the production of NGF. NGF released in the vicinity of sympathetic nerve endings would bind TrkA and activate downstream signalling cascades to promote axoaxonal interactions between the two autonomic fibre types. The relevance of this study to our results is that parasympathetic fibres may inhibit sympathetic

noradrenaline release, and parasympathetic fibres may attract sympathetic neurons by releasing NGF in an adrenergic-mediated fashion.

4.10. FUTURE DIRECTIONS

Although the mental nerve transection model presents a system in which sensory, sympathetic, and parasympathetic systems can be easily manipulated independently, the behavioral correlate of the model's characterization has not yet been performed. Since both mechanoreceptive and nociceptive fibres are lost following mental nerve transection, a period of analgesia would be expected to develop in the days immediately after denervation. This would likely be followed by mechanical and thermal hyperalgesia, which could be verified using a modified protocols for von Frey hair testing (Chaplan, Bach et al., 1994) and radiant heat exposure [developed by D'Amour and Smith (D'Amour and Smith D, 1941)]. It is important to establish development of pain behaviours after MN transection to validate the use of this model to extrapolate useful results for clinically correlated cutaneous neuropathic pain in the trigeminal region.

Another point of interest that has yet to be explored is the innervation of the skin by unmyelinated non-peptidergic fibres. Preliminary data from our lab (Grelik and Ribeiro-da-Silva, unpublished observations) suggests non-peptidergic innervation of the rat lower lip skin may be more abundant than the peptidergic, and also occurs around blood vessels. This is particularly relevant for the study of nociception as many of these primary afferent neurons form multiple synapses in the spinal cord and have been implicated to also play a role in the establishment and maintenance of neuropathic pain. Immunocytochemistry for non-peptidergic markers has previously been attempted in our lab, but as mentioned previously, the skin is an extremely technically-challenging tissue to study and to date we have not met with much success. Once the obstacles are overcome, non-peptidergic innervation following sensory denervation should unquestionably be examined to see if it diverges from the findings for peptidergic fibres. Due to their different trophic factors, a different time course and/or pattern of re-innervation are not unlikely.

Another direction into which the current study could be taken is a thorough examination of the temporal dependence of innervation changes. For example, we know that sprouted sensory fibres are observed at 4 weeks but not 3 weeks after MN transection. It may be interesting to study very closely the intervening period in order to have a higher temporal resolution of the anatomical events leading to complete re-innervation. Of course, this will also likely be a period of great interest for behavioural testing, since the correlation between anatomical arrangement and hyperalgesia is also an important objective. Furthermore, longer timepoints after MN lesion should be investigated to determine whether or not skin innervation by nociceptive sensory fibres ever returns to control levels, and if autonomic fibres eventually disappear from the upper dermis. Based on the trophic dependency of these fibres, it is possible that gradual loss of parasympathetic and sympathetic fibres will allow non-peptidergic and peptidergic fibres to gradually re-establish themselves in the upper dermis at normal levels.

In normal skin, the only fibre type that innervates the epidermis beyond the basal layer is the nociceptor (please refer to section 1.2.1.) – peptidergic and non-peptidergic. After nerve injury, nerve tracts are left vacant, including those leading into the epidermis. With regard to our neurotrophic hypothesis to explain autonomic fibre sprouting, the reasons for which autonomic fibres do not extend their sprouted branches into the epidermis cannot be extrapolated – one possible explanation for their confinement to the dermis includes insufficient epidermal keratinocyte production of NGF after sensory denervation. This is a probable cause, since the source of NGF in the epidermis is the keratinocyte alone; Schwann cell encasements of nociceptors are often lost above the level of the dermal-epidermal junction (Kruger, Perl, and Sedivec, 1981), thus cannot contribute to a chemoattractive gradient in the epidermis. If this is true, nociceptive fibres should also be expected not to re-innervate the epidermis to control levels. Another contributing factor may be a decreased sensitivity to NGF as a migration factor in mature neurons as compared to immature neurons, the latter in which the majority of trophic dependency studies have been conducted.

Although we have not looked specifically at intraepidermal density in experimental and naïve animals, current and previous studies suggest that the epidermis is re-innervated, but as occurs for the dermis, the density of SP-IR fibres does not return to control levels (Ruocco, Cuello, and Ribeiro-da-Silva, 2000). These preliminary findings are comparable with the clinically observed strong correlation between of decreased intraepidermal small fibre density in skin biopsies from painful peripheral neuropathy patients (Periquet, Novak et al., 1999), and support the validity of this model for studying human pain conditions.

The mental nerve carries two types of sensory fibres: nociceptive and mechanoreceptive. The changes in mechanoreceptor innervation of the skin following mental nerve transection should also be investigated. The growth factor for large diameter TrkC-expressing afferents is NT3, but NT3 also binds to GFRα2 and TrkA receptors with some affinity. The implications for anatomical reorganization of the skin innervation after sensory denervation are two-fold: 1) if large diameter fibres do not eventually return to their normal number, mechanosensation may decrease relatively, and 2) this may mean that some of their trophic support is diverted to maintaining other fibre types (namely peptidergic and sensory via TrkA, since NGF and NT3 are from the same neurotrophin family). A clinical trial was initiated to investigate the possibility of administering NT3 to attenuate loss of large fibres and the resulting neuropathy caused by diabetes and by administration of cytotoxic cancer and HIV/AIDS drugs (Lindsay, 1996). Thus knowledge of the anatomical changes in mechanosensitive innervation of the skin after sensory nerve injury is crucial to understand all possible outcomes and their applications to clinical conditions.

Finally, although possibly a never-ending task, an attempt should be made to identify receptors for the substances released from parasympathetic, sympathetic, and sensory fibres during the period in which they are closely associated. This will provide substantiation of the possibilities for inter-fibre associations suggested in the previous section. Ultrastructural observation of the adjacent or contacting regions of the three fibre types could also provide insight into the type of interactions between the fibres, for example characteristic

synaptic specializations may form between some pairs of fibres but not others. As well, ultrastructural studies would allow the distance between sympathetic, parasympathetic, and sensory fibres to be quantified and may reveal closer associations between certain pairs of fibre types than between others.

TABLE 2. SUBTANCES RELEASED BY AND RECEPTORS EXPRESSED ON SENSORY, SYMPATHETIC, AND PARASYMPATHETIC FIBRES[4]

FIBRE TYPE	SUBSTANCES SECRETED	RECEPTORS EXPRESSED
Sensory	Glutamate SP, NKA (peptidergic) CGRP (peptidergic) EM-2 (non-peptidergic) Somatostatin	NMDA, AMPA, kainate NK1r (peptidergic & non-peptidergic) CGRP types 1 and 2 (peptidergic) μ- and δ- opioid SST2a P2X3 (non-peptidergic) NPY M2 cholinergic Neuronal nicotinic cholinergic α2 adrenergic β2 adrenergic TrkA (peptidergic) GFR α1 and α2 (non-peptidergic)
Sympathetic	NA ATP NPY Chromogranin A	P2X3 α2 adrenergic ionotropic glutamate TrkA
Parasympathetic	VIP Ach NGF	P2X2, P2X4 NK2, NK3 GFRα1, GFRα2

[4] Based on a review of the literature discussed in sections 1.3.2., 1.3.3., and 4.4-4.9.

CHAPTER FIVE: CONCLUSION

The rat lower lip is an ideal model for the independent manipulation of parasympathetic, sympathetic, and sensory fibres due to its separate sources of innervation, and future studies of the ultrastructure and receptor expression on sprouted autonomic fibres should provide insight into the significance of the close proximity of different fibre types. In the present study, we have shown that loss of sensory innervation of the rat lower lip skin induces an anomalous sprouting of parasympathetic fibres. Our findings expand upon those of our previous study, in which sensory nerve transection was shown to lead to sympathetic fibre invasion of the upper dermis (Ruocco, Cuello, and Ribeiro-da-Silva, 2000), and provide evidence of close proximity of sprouted sensory, sympathetic, and parasympathetic fibres. The balance between autonomic and sensory fibre types in the head region is obviously very tightly regulated as loss of one fibre type results in the rapid establishment of a novel innervation pattern. The amount of interplay between sensory, parasympathetic, and sympathetic fibres suggests an ongoing feedback loop that may be instituted after peripheral nerve injury, and may maintain the hyperalgesia and allodynia associated with neuropathic pain.

Though central sensitization also develops in neuropathic pain states, and often persists and maintains the abnormal pain after peripheral sources of pain input have resolved themselves, a thorough understanding of the peripheral changes induced by nerve injury is still paramount. If peripheral changes and rearrangements can be prevented, central sensitization, which occurs in the CNS and thus represents a much more elusive and complex therapeutic target, will not be induced and pain can be cut off at its initiation site. This project has the potential to produce results that may be critical to the understanding and eventual defeat of cutaneous neuropathic pain following nerve injury in the head region.

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McGill University
Animal Use Protocol – Research

Guidelines for completing the form are available at www.mcgill.ca/fgsr/rgo/animal/

Protocol #: 3550
Investigator #: 756
Approval End Date: Sept 30, 2003
Facility Committee: MGD

☐ Pilot ☐ New Application ☒ Renewal of Protocol # 3550 (NO SUBSTANTIAL CHANGES FROM LAST YEAR)

Title (must match the title of the funding source application): Project 1) Synaptic organization of dorsal horn modulatory circuits; Project 2) Plasticity of skin innervation following peripheral nerve lesions

1. Investigator Data:

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2. Emergency Contacts: Two people must be designated to handle emergencies.

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Name:	Manon St. Louis	**Work #:**	398-3617	**Emergency #:**	450-689-6195

3. Funding Source:

External ☒
Source (s): CIHR Operating Grant MOP 38093 and CIHR operating grant MOP 53278
Peer Reviewed: ☒ YES ☐ NO**
Status : ☒ Awarded ☐ Pending
Funding period: April 1, 2000 to March 31, 2005 and April 1, 2002 to March 31, 2005 respectively

Internal ☐
Source (s):
Peer Reviewed: ☐ YES ☐ NO**
Status: ☐ Awarded ☐ Pending
Funding period:

ACTION DATE
✓ Nov. 7 '02

** All projects that have not been peer reviewed for scientific merit by the funding source require 2 Peer Review Forms to be completed . e.g. Projects funded from industrial sources. Peer Review Forms are available at www.mcgill.ca/fgsr/rgo/animal/

Proposed Start Date of Animal Use (d/m/y): or ongoing ☒

Expected Date of Completion of Animal Use (d/m/y): or ongoing ☒

Investigator's Statement: The information in this application is exact and complete. I assure that all care and use of animals in this proposal will be in accordance with the guidelines and policies of the Canadian Council on Animal Care and those of McGill University. I shall request the Animal Care Committee's approval prior to any deviations from this protocol as approved. I understand that this approval is valid for one year and must be approved on an annual basis.

Principal Investigator: Alfredo Ribeiro-da-Silva **Date:** 20 Nov. 2002

Approval Signatures:

Chair, Facility Animal Care Committee:	Paul B S Clarke	**Date:** 12/11/2002
University Veterinarian:	K H Keith	**Date:** 11/13/02
Chair, Ethics Subcommittee(as per UACC policy):		**Date:** 10/19/02
Approved Period for Animal Use	**Beginning:** Oct. 1, 2002	**Ending:** Sept. 30, 2003

☐ This protocol has been approved with the modifications noted in Section 13.

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