U OF L RPMS ITEM
19 0135043 X

NERVES AND NEUROPEPTIDES OF SKIN: THEIR CHANGES IN DISEASE PROVIDE EVIDENCE OF THEIR INVOLVEMENT IN THE INTEGRITY OF THE SKIN ~~AND FURTHER THE UNDERSTANDING OF DISEASE PROCESSES~~

S.S. Karanth

A thesis submitted for the degree of
Doctor of Philosophy
London University
January 1990

ROYAL POSTGRADUATE MEDICAL SCHOOL
UNIVERSITY OF LONDON

ACKNOWLEDGEMENTS

I would like to thank **Professor Julia M. Polak** for her supervision, invaluable advice and encouragement through out the period of this study.

I am specially grateful to **Dr. David R. Springall** and **Dr. Max M. Levene** for fruitful discussions and advice during these years.

My gratitude also goes to **Drs. John Wharton, Susan V. Noorden, Anne Bishop, Sally Gibson, Paul Facer, Giorgio Terenghi** and **S. Kar** who have helped in many ways throughout this study.

I would like to thank **Dr. M. Parameshwarya and Dr. D. Sridaran** who had helped me in taking biopsies and **Dr. M.A. Macrae** the Resident Medical Superintendent in Nigeria, who had given kind permission with the consent of the patients attending the Leprosy Hospital.

I am specially grateful to **Dr. S. Lucas** for the classification of the leprosy specimens, **Dr. S. Dhital** for assisting me in skin transplantation experiments and **Mr. J.P. Royston** for statistical analysis.

I also wish to thank **Dr. D.M. Levy** for providing the diabetic skin specimens, **Dr. N. Kirkham** and **C.R. Darley** for Notalgia parasthetica skin biopsies, **Dr. P. Ashby** for normal skin from patients undergoing plastic surgery, **Dr. D.K. Banerjee** for providing M. Leprae treated mice and **Dr. D.J. Mirrlees** for the Streptozotocin treated rats.

I wish to thank the Surgeons of Hammersmith Hospital for providing me with the fixed human normal skin from patients undergoing plastic surgery and other surgical operations that needed skin removal.

Finally, and most important, I thank my wife **Kusuma** for her constant encouragement and good humour at all times.

I would like to dedicate this thesis to my parents and the memory of my only brother.

NERVES AND NEUROPEPTIDES OF SKIN: THEIR CHANGES IN DISEASE PROVIDE EVIDENCE OF THEIR INVOLVEMENT IN THE INTEGRITY OF THE SKIN AND FURTHER THE UNDERSTANDING OF DISEASE PROCESSES

Abstract

The aim of the study was to investigate the changes in nerves and neuropeptides of skin in experimental and disease states in order to understand their role in regulation of skin. The study is in two parts. The first is an immunocytochemical study of the cutaneous innervation and its neuropeptides in man and laboratory animals. Antisera to general neural markers and to neuropeptides were used to estimate the density and distribution of the nerves and their anatomical relationship to skin appendages. In order to delineate the peptides in the sensory and autonomic parts of the cutaneous innervation, chemical denervation experiments were carried out in rat and guinea pig using capsaicin and 6-hydroxydopamine respectively; transplanted skin flaps of hairless mice were examined to determine the time course of nerve degeneration and reinnervation and the significance of neuropeptides in these processes; the ontogeny of nerves and neuropeptides were studied in fetal and neonatal rats to investigate the temporal sequence of the appearance of nerves and neuropeptides.

The second part is a study of the cutaneous innervation and its neuropeptides in infectious (Leprosy) and metabolic (Diabetes) diseases, in which there is a loss of sensory and autonomic functions, and also in an infrequently reported condition characterised by hyperfunctional sensory nerves (Notalgia paraesthetica). In order to evaluate neural changes in the earliest stages of these disease processes, experiments were carried out employing nude mice infected with Mycobacterium leprae and rats with streptozotocin-induced diabetes.

The study has shown, 1. PGP 9.5 was found to be the best general neural marker for the innervation of skin. 2. In all of the species studied nerve fibres immunoreactive for calcitonin gene-related peptide (CGRP) and/or vasoactive intestinal polypeptide (VIP) predominated; those immunoreactive for tachykinins, neuropeptide Y and C-flanking peptide of neuropeptide Y were less abundant, suggesting a significant contribution of neuropeptides in regulation of sensory and autonomic transmission. 3. Experimental denervation showed that not all CGRP and substance P-immunoreactive sensory axons were capsaicin-sensitive at the dosage given. However, all sympathetic TH- and NPY-immunoreactive axons were totally responsive to 6-OHDA, suggesting some demarcation of cutaneous adrenergic and cholinergic sympathetic fibres. 4. The early appearance of sensory neuropeptides (CGRP and substance P) during reinnervation of skin flaps and in ontogeny might have a role in trophic, regulatory and reparative processes. 5. The early depletion of neuropeptides in leprosy and diabetic patients reflected cutaneous sensory and autonomic dysfunctions and might have a possible prognostic or diagnostic importance. These results were further supported by the experiments carried on Nude mice infected with Mycobacterium leprae, which showed depletion of calcitonin gene-related peptide and substance P in the skin and spinal cord by 6 months. Conversely, observations on 8 to 12 weeks old streptozotocin-diabetic rats showed increases of vasoactive intestinal polypeptide and calcitonin gene-related peptide in skin. 6. Increased nerves but not neuropeptides were seen in skin affected by notalgia paraesthetica. Thus, neuropeptides may play a major role in regulation of sensory and autonomic functions of skin and may be affected differently depending on the disease processes.

CONTENTS

	PAGE
TITLE PAGE	1
ACKNOWLEDGEMENTS	2
ABSTRACT	3-4
CONTENTS	5
LIST OF TABLES	6-7
LIST OF DIAGRAMS	8
LIST OF FIGURES	9-12
ABBREVIATIONS	13
CHAPTER 1 - INTRODUCTION	14-43
CHAPTER 2 - MATERIALS AND METHODS	44-63
CHAPTER 3 - GENERAL DISTRIBUTION OF NERVES AND NEUROPEPTIDES IN SKIN OF FIVE SPECIES AND THEIR ONTOGENY IN RAT	64-108
CHAPTER 4 - CHANGES IN NERVES AND NEUROPEPTIDES IN LEPROSY	109-132
CHAPTER 5 - TIME RELATED CHANGES IN CGRP AND SUBSTANCE P IMMUNOREACTIVITY IN THE SKIN AND SPINAL CORD OF LEPROUS MICE	133-147
CHAPTER 6 - DEPLETION OF CUTANEOUS NERVES AND NEUROPEPTIDES IN DIABETES MELLITUS	147-162
CHAPTER 7 - EARLY CHANGES IN PEPTIDE CONTAINING NERES IN THE SKIN OF STREPTOZOTOCIN-INDUCED DIABETIC RATS	163-175
CHAPTER 8 - REAPPEARANCE OF NERVES AND NEUROPEPTIDES IN SKIN FLAPS	176-185
CHAPTER 9 - NERVES AND NEUROPEPTIDES IN NOTALGIA PARAESTHETICA	186-196
CHAPTER 10 - SUMMARY AND CONCLUSIONS	197-200
APPENDIX	201-212
REFERENCES	213-260
PUBLICATIONS DERIVED FROM THE PRESENT STUDY	261

LIST OF TABLES

		Page
1-	Details of general neural markers	50-51
2-	Details of peptide antisera	52-53
3-	A. Source and characterisation of peptide antisera B. Source and characterisation of PGP 9.5 antisera	55-56
4-	Details of labelled secondary immunoreagents	57
5-	Reported presence of neural antigens (general and neuropeptides) in cutaneous nerves of various species	69-70
6-	Regions from which the skin specimen was taken from man	73
7-	Comparison of immunostaining for general markers and ChAT, and acetylcholinesterase histochemistry in various regions of skin	76
8-	Species similarities/differences in density of peptide immunoreactive axons	87
9-	Areas from which the skin biopsy was taken from leprosy patients	112
10-	Neurohistological analysis of non-leprosy control specimens	114
11-	Classification of the leprosy skin biopsies examined	116
12-	Neurohistological analysis of indeterminate leprosy	125
13-	Neurohistological analysis of lepromatous leprosy	126
14-	Neurohistological analysis of tuberculoid leprosy	128

15- Clinical characteristics of diabetic patients, neurophysiological and immunohistochemical findings 156 -157

16- Body weight and plasma glucose concentration for control and diabetic rats 166

17- Density of nerves and neuropeptides-immunoreactive fibres present in the paws of rats with streptozotocin-induced diabetes and controls 168

18- Clinical characteristics of Notalgia paraesthetica patients and details of immunohistochemical findings 188

19- Density of nerves and neuropeptides-immunoreactive fibres present in Notalgia paraesthetica patients 190

LIST OF DIAGRAMS

		Page
1-	Schematic representation of alternative RNA splicing pathways in the expression of the rat preprocalcitonin gene which encodes both calcitonin and CGRP	29
2-	Schematic representation of alternative RNA splicing pathways in the expression of the preprotachykinin (PPT) gene	32
3-	Schematic representation of vasoactive intestinal polypeptide (VIP) prepromolecule	35
4-	Schematic representation of human neuropeptide tyrosine (NPY) prepromolecule	38
5-	Diagramatic representation of skin showing localisation of neuropeptides in cutaneous nerves	67
6-	Schematic representation of development of cutaneous nerves and peptides in the skin of rat	96
7-	Schematic representation of origin/distribution/ visualisation of nerves and neuropeptides in the skin of mammals	99
8-	Histogram showing the proportion of total cases in each class of leprosy which showed immunoreactivity for general neural markers	117
9-	Histogram showing the proportion of total cases in each class of leprosy which showed immunoreactivity for neuropeptides	118
10-	Schematic representation of skin flaps on the dorsal region of mouse from which the tissues are taken for the study	178

LIST OF FIGURES

Chapter 1.

FIG 1.1. Amino acid sequences of tachykinin family (p 23)

FIG 1.2. Amino acid sequence of rat CGRP (p 24)

FIG 1.3. Amino acid sequences of VIP and related peptides (p 25)

FIG 1.4. Amino acid sequences of NPY and related peptides (p 26)

Chapter 2

Fig. 2.1. Kontron IBAS-2000 system configuration.

Fig. 2.2. Tranverse section of spinal cord showing the area of dorsal horn (hatched) used for quantification. The square area indicates approximately the region of the spinal cord chosen for image analysis.

Chapter 3.

FIG 3.1. Comparison of immunostaining for general markers and ChAT, and acetylcholinesterase histochemistry in epidermis and dermis of the skin(p 78)

FIG 3.2. Comparison of immunostaining for general markers and ChAT, and acetylcholinesterase histochemistry around sweat glands of skin(p 79)

FIG 3.3. Arm skin of man showing PGP 9.5-immunoreactive fibres in epidermis and dermis, around hair follicle, blood vessel, nerve bundle and sweat glands (p 80)

FIG 3.4. Arm skin of man showing NF-immunoreactive fibres in epidermis and dermis, around hair follicle, blood vessel, nerve bundle and sweat glands (p 81)

FIG 3.5. Arm skin of man showing NSE-immunoreactive fibres in epidermis and dermis, around hair follicle, blood vessel, nerve bundle and sweat glands (p 82)

FIG 3.6. Arm skin of man showing synaptophysin-immunoreactive fibres in epidermis and dermis, around hair follicle, blood vessel, nerve bundle and sweat glands (p 83)

FIG 3.7. Arm skin of man showing S-100-immunoreactive fibres in epidermis and dermis, around hair follicle, blood vessel, nerve bundle and sweat glands (p 84)

FIG 3.8. Species differences in density of SP-immunoreactive fibres in epidermis and dermis, sweat glands and blood vessels (p 88)

FIG 3.9. Species differences in density of CGRP-immunoreactive fibres in epidermis and dermis, sweat glands and blood vessels (p 89)

FIG 3.10. Species differences in density of VIP-immunoreactive fibres in epidermis and dermis, sweat glands and hair follicle (p 91)

FIG 3.11. Skin from age matched control and capsaicin treated rats showing CGRP- and SP-immnoreactive fibres (p 94)

FIG 3.12. Skin from foetal rat showing nerves and neuropeptide-immunoreactive fibres (p 97)

Chapter 4.

FIG 4.1. PGP 9.5-immunoreactive fibres in the epidermis and dermis of control and leprosy skin (p 119)

FIG 4.2. PGP 9.5-immunoreactive fibres around sweat glands of control and leprosy skin (p 120)

Fig.4.3. CGRP-immunoreactive fibres in the epidermis and dermis of control and leprosy skin (p 121)

Fig.4.4. SP-immunoreactive fibres in the epidermis and dermis of control and leprosy skin(p 122)

Fig.4.5. VIP-immunoreactive fibres around sweat glands of control and leprosy skin (p 123)

Chapter 5.

Fig.5.1. CGRP-immunoreactive fibres in the epidermis and dermis of leprosy hind paw taken after 6 months and 12 months after subcutaneous injection of *Mycobacterium leprae* into the hind foot pads (p 137).

Fig. 5.2. Substance P-immunoreactive fibres in the epidermis and dermis of leprosy hind paw taken after 6 months and 12 months after subcutaneous injection of *Mycobacterium leprae* into the hind foot pads (p 138)

Fig. 5.3. Nose skin showing reduction in CGRP- and substance P-immunoreactive fibres in the epidermis and dermis, 12 months after subcutaneous injection of *Mycobacterium leprae* into the hind foot pads (p 139).

Fig. 5.4. Dorsal horn of L4 segment of spinal cord showing reduction in CGRP- and substance P-immunoreactive fibres after 6 and 12 months of *Mycobacterium leprae* injection into the hind foot pads (p 141).

Fig. 5. 5. Motoneurones in the ventral horn of L4 segment of spinal cord showing redution after 6 months and complete absence after 12 months after injection of *Mycobacterium leprae* into the hind foot pads (p 142).

Chapter 6.

Fig. 6. 1. Control skin of calf showing PGP 9.5- and CGRP-immunoreactive fibres in the epidermis and dermis, and VIP-immunoreactive fibres around sweat glands and blood vessels (p 152).

Fig. 6.2. Diabetic skin of calf showing PGP 9.5- and CGRP-immunoreactive fibres in the epidermis and dermis, and VIP-immunoreactive fibres around sweat glands and blood vessels (p 154).

Fig. 6.3. Diabetic skin of calf showing complete absence of PGP 9.5- and CGRP-immunoreactive fibres in the epidermis and dermis, and VIP-immunoreactive fibres around sweat glands and blood vessels (p 155).

Chapter 7.

Fig. 7.1. Control skin of lip and after 12 weeks of streptozotocin treatment showing CGRP-immunoreactive fibres in the epidermis and dermis, and VIP-immunoreactive fibres around blood vessels (p 169).

Fig. 7.2. Control skin of foot pad and after 12 weeks of streptozotocin treatment showing CGRP-immunoreactive fibres in the epidermis and dermis, and VIP-immunoreactive fibres around blood vessels (p 170).

Fig. 7.3. Control lip and foot pad skin and after 12 weeks of streptozotocin treatment showing PGP 9.5-immunoreactive fibres in the epidermis and dermis, around blood vessels and sweat glands (p 171).

Chapter 8.

Fig. 8.1. Skin flap showing appearance of nerve fibres immunoreactive for PGP 9.5 in mouse skin (p 181).

Fig. 8.2. Skin flap showing nerve fibres immunoreactive for CGRP and substance P and PGP 9.5 at various times after transplantation (p 182)

Chapter 9.

Fig. 9.1. Control skin from infrascapular region showing PGP 9.5-immunoreactive fibres in epidermis and dermis and S-100-immunoreactivity in Schwann cells and dermal fibres (p 191).

Fig. 9.2. Notalgia parasthetica skin from infrascapular region showing PGP 9.5-immunoreactive fibres in epidermis and dermis, and S-100-immunoreactivity in Schwann cells and dermal fibres (p 192).

Fig. 9.3. Infrascapular skin of control and Notalgia parasthetica showing CGRP- and substance P-immunoreactive axons in the epidermis and dermis (p 193).

ABBREVIATIONS

AChE = Acetylcholinesterase
CGRP = Calcitonin gene-related peptide
ChAT = Choline acetyltransferase
CPON = C-flanking peptide of NPY
DAB = Diaminobenzidine
FITC = Fluorescine isothiocyanate
GAL = Galanin
IMF = Immunofluorescence
NF = Neurofilaments
NKA = Neurokinin A
NPY = Neuropeptide Y
PAP = Peroxidase antiperoxidase
PHI = Peptide histidine isolucine
PHM = Peptide histidine methionine
SS = Somatostatin
SP = Substance P
TH = Tyrosine hydroxylase
VIP = Vasoactive intestinal polypeptide
6-OHDA = 6-hydroxydopamine

Chapter 1

INTRODUCTION

	PAGES
1.1. Background	**15-18**
1.1.1. History	15-16
1.1.2. Skin innervation/method of study	17-18
1.2. Neuropeptides	**19-27**
1.2.1. Discovery	19-20
1.2.2. Peptides as neurotransmitters	21
1.2.3. Peptides as neuromodulators	22
1.2.4. Peptide families	22-27
1.2.5. Coexistence of putative neurotransmitters	22
1.3. Aims of study	**27-28**
1.4. Main peptides investigated	**29-39**
1.4.1. Calcitonin gene-related peptide	28-31
1.4.2. Tachykinins	31-34
1.4.3. Vasoactive intestinal peptide/ peptide histidine isoleucine	34-37
1.4.4. Neuropeptide Y and C-flanking peptide of NPY	37-39
1.4. General neural markers investigated	**39-43**
1.5.1. Protein gene product 9.5	39-40
1.5.2. Neurofilament protein	40-41
1.5.3. Glial protein S-100	41-42
1.5.4. Neuron specific enolase	42
1.5.5. Synaptophysin	42-43

1.1 Background

1.1.1. History

The doctrine of specific energy was formulated by Johannes Muller (1838) who found differences between the nervous mechanisms subserving five senses, so that the same stimulus could produce two different sensations if applied to two different kinds of nerve. Volkmann (1844) extended Muller's idea and suggested that the cutaneous nerves mediating different sensations are distinct. The existence of a sensory mosaic of tiny spots in normal skin was discovered by Blix in the year 1884, each of the spots reacting preferentially to touch, cold, warmth or pain. The spots for warm and cold were clear cut and easily distinguishable; those for touch were much more numerous, and those for pain were closely set together. It was soon accepted that the idea of modality is served by individual anatomical substrates containing modality-specific nerve fibres. Improvements in the microscopic techniques, together with the founding of the dyestuffs industry after Perkins's discovery of aniline dyes in 1856, had enormously stimulated many morphologists to take an interest in the innervation of skin and mucous membranes. This resulted in the anatomical discovery of six types of nerve endings in addition to the free nerve endings. They were, the Paccinian corpuscles, the Meissner Corpuscles, the Krause end-bulbs, Merkel discs and domes, and the Ruffini endings. Later, Max von Frey (1896) postulated the existence of an end organ or a group of end organs specialised to respond to a particular type of stimulus. This end organ was connected to a fibre which conveyed the impulses aroused by the stimulus up to the brain, where it was appropriately interpreted. He had allocated each of the four sensory modalities to one of the known anatomical end organs, thus pain was given to free nerve endings,

touch to the basket formations of free endings around hair follicles and in glabrous skin to the Meissner's corpuscles, cold to the Krause end-bulbs and warmth to the Ruffini endings. This correlation of anatomy and physiology was subsequently disputed. Goldscheidner in 1884 criticised the inclusion of pain as a modality, since the pain spots were so densely distributed that it was almost impossible to find a touch or warm spot which was not also sensitive to pain. He maintained that pain had no separate receptors but was produced in the nervous system by the summation of impulses. In 1903, the neurologist Henry Head began his famous experiment by cutting a cutaneous nerve in his own forearm. He studied carefully the progress of the return of sensation (Head, 1905; Rivers and Head, 1908) and concluded that in cutaneous sensory nerves there are two distinct sets of nerve fibres. He suggested that there were a protopathic and epicritic systems, in other words a concept of duality, in contrast to the quadruple theory of von Frey (Head, 1921). However, the overwhelming weight of scientific opinion since then has considered Head's concepts as having no neuroanatomical basis nor clinical applicability. The conflicting anatomical evidence that contradicts Head's and/or Von Frey's concept was demonstrated by Sinclair, Weddell and co workers, and Nafe. This work paved the way for the introduction of the pattern theory. To pinpoint the nerve endings which respond to the sensations of warmth and cold, small areas of skin that were sensitive to heat and cold stimuli were examined for special end organs or terminal structures. Nothing characteristic was found in any area (Nafe and Wagoner, 1937) and subsequently, Nafe (1969) concluded that specificity lies in the non-neural tissues that contain the nerve terminals and not in the nerves themselves. Thus the pattern theory postulated that every sensation results from a particular kind of pattern carried by a wholly non-specific apparatus, and that there was no stimulus-specificity at all. This suggestion was subsequently disproved by

demonstrating that there is selective specificity of the receptors (Iggo, 1977). Thus, it was concluded that regardless of types of endings the afferent nerve fibres were concluded to be essentially alike.

1.1.2 Skin innervation/method of study

The nerves and nerve endings of the skin constitute an immense complex in which the relationship between structure and function is still confused and disputed. The nerve supply of the skin comprises a sensory and an autonomic portion. The nerve fibres of these portions mingle in the cutaneous nerve plexus, which occupies the deep part of the dermis (Woolard, 1936a & b, Tamponi, 1940, Weddell, 1941, Miller et al., 1960; Winkelmann, 1960; Montagna, 1964, Sinclair, 1981).

The sensory nerve fibres are found in the epidermis and dermis of the skin as free or specialised nerve endings (receptors), and around blood vessels, sweat glands and hair follicles. They are the dendrites of neurones which have their cell bodies in the dorsal root ganglia of the spinal nerves or in the trigeminal ganglia (Lieberman, 1976; Lawson & Harper, 1985). They are represented by both non-myelinated and myelinated types of nerve fibres, the former outnumbering the myelinated types by about four to one (Ochoa & Mair, 1969). Axons of sensory neurones terminate in stereotyped pattern according to the functional properties of the fibres, in the dorsal horn (Laminae I-IV) and, in particular in lamina II of the substantia gelatinosa.

Autonomic nerve fibres are distributed around blood vessels, sweat glands and in erector pili muscles. These unmyelinated fibres are the branches of postganglionic sympathetic fibres which have cell bodies in the ganglionated sympathetic trunk, and function neurochemically as

cholinergic and adrenergic types (Dale, 1933) using acetylcholine and catecholamines respectively as their neurotransmitters (for review see Williams & Warwick, 1980). It is believed that survival and proper functioning of the skin is dependent upon an intact nerve supply. This is partly due to the trophic influences of the nerves.

A number of neurohistological techniques have been used in the past to demonstrate cutaneous nerves and sensory receptors. Nearly every new histological technique has demonstrated different kinds of sensory nerve endings never before described. Intravital or supravital methylene blue dyes and metallic impregnation were used for the preferential differentiation of nervous structures in the skin (Woolard et al., 1940; Weddell, 1941; Weddell et al., 1954 , Arthur and Shelley, 1959a; Miller et al., 1960). However, the dyes are axonal stains, and if not properly used they will also stain collagen or reticular fibres and non-nervous cells (Kiernan, 1974). The silver impregnation technique has a history going back as far as 1871 (Williams and Warwick, 1980). In spite of much research, it is a capricious technique and often fails to demonstrate small unmyelinated fibres. In addition, neither type of technique is wholly satisfactory, and each suffers from a lack of reliability. Subsequently, cholinesterase techniques were used to demonstrate the cutaneous nerves by many workers (Beckett et al., 1956; Cauna, 1960; Adams et al., 1967; Grant et al., 1967; Roman et al., 1969; Montagna and Ford, 1969; Jenkinson et al., 1970; Leela et al., 1971; Rollison and Inzidi, 1972; Orfanos and Mahrle, 1973; Cauna, 1973; Diamond and Montagna, 1976; for reviews see Winkelman and Schmitt, 1959; Cunningham and Fitzgerald, 1972; Montagna and Mcpherson, 1973; Montagna and Parakkal, 1974). The observations made in these studies are controversial, because of the non-specific cholinesterase activity. Studies on the adrenergic innervation of skin also gave contradictory result, owing to the lack of a specific histological

method for the localization of adrenergic nerves in the tissues. The osmic acid sodium iodide technique has been stated to demonstrate the adrenergic nerves selectively, but the specificity of this method was later seriously disputed. What was thought to be a specific and sensitive formaldehyde-induced fluorescence method (Eranko, 1955; Falck et al., 1962) was then employed to study the adrenergic innervation of the skin (Falck and Rorsman, 1963; Fuxe and Nilson, 1965; Jenkinson et al., 1970; Morishma, 1970; Uno and Montagna, 1975).

In the last decade, immunohistochemistry has developed into a powerful tool in neurobiology allowing the direct visualization of a variety of substances produced and contained within neurones. This coincided with the discovery of more than 40 substances, either known to or believed to be, neurotransmitters/neuromodulators, each having characteristic inhibitory and/or excitatory effects on neurones. Antisera raised against these substances have been used to visualise their presence in the tissue. In addition, several antisera have been raised against neural specific proteins, which are used for the visualisation of the cutaneous innervation.

1.2. Neuropeptides

1.2.1. Discovery

Ultrastructural observations of peripheral autonomic neurones showed that the great majority contained large, dense-core vesicles (p-type vesicles) which can be distinguished from the small vesicles present in noradrenergic and cholinergic types. Taxi in 1965 was the first to identify these in the gut and they were named "P"-type (polypeptide neurone) by Baumgarten et al., 1970 due to their resemblance to the granular vesicles found in neuroendocrine cells.

Further studies indicated the presence of morphologically different subpopulations of p-type nerves (Gabella, 1972, 1979; Cook and Burnstock, 1976). The discovery of a third type of nerve, the most abundant in the peripheral nervous system, was consistent with reports of non-adrenergic/non-cholinergic neural mechanisms in peripheral tissues (Ambache and Zar., 1971; Furness and Costa., 1973; Burnstock et al., 1979; Gabella, 1979). These findings contradicted traditional dual classification of adrenergic and cholinergic parts of the nervous system. Later, different peptide-containing nerves could be identified by the specific morphological profile of the granules which could be correlated with particular peptides by immunocytochemistry (Probert et al., 1981, 1983). Several regulatory peptides have now been recognised in neuronal cell bodies and nerve endings in brain and peripheral organs: substance P, vasoactive intestinal polypeptide (VIP), neuropeptide Y (NPY), calcitonin gene-related peptide (CGRP) and somatostatin are a few examples of the ever increasing number of peptides. For most of these peptides numerous morphological, biochemical and neurophysiological studies have indicated a distinct biological action and possible physiological role in the body.

The discovery of new peptides has been accelerated even further by new isolation methods which allow peptides to be identified before any biological activity has been attributed to them. One of these methods has evolved from recombinant DNA technology (Milner, 1982., Sutcliffe et al., 1983); the other provides a means of chemically identifying new peptides, namely a C-terminal alpha-amide (Tatemoto and Mutt, 1980; Mutt, 1983).

1.2.2 Peptide as neurotransmitters

A number of anatomical, biochemical, physiological and pharmacological criteria have now been established which putative transmitters must satisfy before acquiring neurotransmitter status (see Orrego, 1979).

Briefly, they are,

1. synthesis and storage in a neurone and transport to nerve terminals, 2) release, and 3) mimicry of the effects supposedly mediated by the naturally occurring substance at the postsynaptic receptors (Werman, 1966). Doubts were expressed in the early years that peptides could achieve this status. In particular, it was difficult to demonstrate the release of a peptide from nerves after artificial electrical stimulation. Most important of all, the lack of antagonists for peptide actions has put in doubt their neurotransmitter status (Jessel,1983; Mathison et al., 1984). However, there are several examples in the literature of peptides causing potent, fast-acting and short-duration depolarisation (Emson, 1979; Salt and Hill, 1981) or hyperpolarisation (Pittman and Siggins, 1981) of postsynaptic membranes with concomitant changes in membrane conductance. At present there is little evidence that any known neuropeptide may fullfill all these criteria, although substance P and VIP seem to conform to most of them (for review see Skrabanek and Powell, 1980, 1983a & b; Said, 1982; Porter and O'Connor, 1982). A simplification of the criteria has also been proposed, according to which a transmitter is a substance present in a nerve terminal and which, following release by stimulation, causes an effect on a postsynaptic structure (Furness et al., 1982a).

1.2.3. Peptides as neuromodulators

When the peptide in question is not a neurotransmitter in the classical sense but in some way modifies the action of true transmitters (although the terms should not be considered mutually exclusive) it is called a neuromodulator. The most convincing evidence for neuromodulation is presented by examples of peptide co-storage with "classical" neurotransmitters. It may emerge as a fundamental principle in neurobiology that a neurone stores a peptide in addition to a classical transmitter, although it contradicts the established dogma in neuropharmacology often known as "Dale's principle" (See Whittaker, 1983), that a given neurone stores and releases only one chemical transmitter at each of its terminals.

1.2.4. Peptide families

Peptides with similarities in their aminoacid sequence indicating that they may have all arisen from a common ancestor, by gene duplication and point mutation (Blundell and Humbel, 1980) are grouped as a family. The members of the family are initially synthesised as large precursors which are subsequently processed enzymatically to form biologically active products (Andrews et al., 1987 for review) (Fig.1.1.to 1.4).

1.2.5. Coexistence of putative neurotransmitters

Dale's principle postulates that each neurone contains only one transmitter substance. However, with the rapid discovery of peptides and their widespread distribution in the nervous system, it has become evident that most neurones contain more than one messenger molecule (Hökfelt et al., 1980a & b, 1987; Cuello, 1982; Chan-Palay, 1984;

Tachykinins

Substance P	R P K P Q Q F F G L M - NH2
Neurokinin A	H K T D S F V G L M - NH_2
Neurokinin B	D M H D F F V G L M - NH_2
Kassinin	D V P K S D Q F V G L M - NH_2
Physalaemin	pE A D P N K F Y G L M - NH_2
Eledoisin	pE P S K D A F Y G L M - NH_2
Eledoisin-related peptide	K F I G L M - NH_2

Fig. 1.1 Amino acid sequences of tachykinin family. A=Ala=Alanine; B=Asx=Aspartic acid or asparagine; C=Cys=Cysteine; D=Asp=Aspartic acid; E=Glu=Glutamic acid; F=Phe=Phenylalanine; G=Gly=Glycine; H=Histidine; I= Ile=Isoleucine; K=Lys=Lysine; L=Lys=Leucine; M=met=Methionine; N=Asn=Asparagine; P= Pro=proline; Q=Gln=Glutamine; R=Arg=Arginine; S=Ser=Serine; T=Thr=Threonine; V=Val=Valine; W=Trp=Tryptophan; X= - Unknown or "other"; Y= Tyr=Tyrosine; Z= Glx=Glutamic acid or glutamine (Taken from IUPAC-IUB Commission. European J. Biochem. 5 (1968) 151-153)

Rat CGRP

5 10 15 20 25 30 35

a S C N T A T C V T H R L A G L L S R S G G V V K D N F V P T N V G S E A F -NH2

ß S C N T A T C V T H R L A G L L S R S G G V V K D N F V P T N V G S K A F -NH2

Fig. 1.2 One letter notations: A=Ala=Alanine; B=Asx=Aspartic acid or asparagine; C=Cys=Cysteine; D=Asp=Aspartic acid; E=Glu=Glutamic acid; F=Phe=Phenylalanine; G=Gly=Glycine; H=Histidine; I= Ile=Isoleucine; K=Lys=Lysine; L=Lys=Leucine; M=met=Methionine; N=Asn=Asparagine; P= Pro=proline; Q=Gln=Glutamine; R=Arg=Arginine; S=Ser=Serine; T=Thr=Threonine; V=Val=Valine; W=Trp=Tryptophan; X= - Unknown or "other"; Y= Tyr=Tyrosine; Z= Glx=Glutamic acid or glutamine (Taken from IUPAC-IUB Commission. European J. Biochem. 5 (1968) 151-153)

VIP and related peptides

VIP	H S D A V F T D N Y T R L R K Q M A V K K Y L N S I L N -NH2
PHI	H A D G V F T S D F S R L G Q L S A K K Y L E S L I - NH2
Helodermin	H S D A I F T Q Q Y S K L L A K L L Q K Y L A S I L G S R T F P P - NH2
Glucagon	H S D G T F T S E L S R L R D S A R L Q R L L Q G L V - NH2
GRF	Y A D A I F T N S Y R K V L G Q S A R K L L Q D I M S R Q Q E S N Q E R G A R A R L - NH2

Fig. 1.3 A,H,B avian, human, bovine.

A=Ala=Alanine; B=Asx=Aspartic acid or asparagine; C=Cys=Cysteine; D=Asp=Aspartic acid; E=Glu=Glutamic acid; F=Phe=Phenylalanine; G=Gly=Glycine; H=Histidine; I= Ile=Isoleucine; K=Lys=Lysine; L=Lys=Leucine; M=met=Methionine; N=Asn=Asparagine; P= Pro=proline; Q=Gln=Glutamine; R=Arg=Arginine; S=Ser=Serine; T=Thr=Threonine; V=Val=Valine; W=Trp=Tryptophan; X= - Unknown or "other"; Y= Tyr=Tyrosine; Z= Glx=Glutamic acid or glutamine (Taken from IUPAC-IUB Commission. European J. Biochem. 5 (1968) 151-153)

NPY and related peptides

NPY Y P S K P D N P G E D A P E D L A R Y Y S A L R H Y I N L I T R Q R Y - NH2

CPON S S P E T L I S D L L M R E T E N V P R T R L E D P A M W - NH2

APP G P S Q P T Y P G D D A P V E D L I R F Y D N L Q Q L N V V T R H R Y - NH2

BPP A P L E P E Y P G D N A T P E Q M A Q Y A A E L R R Y I N M L T R P Y - NH2

HPP A P L E P V Y P G D N A T P E Q M A Q A A D L R R Y I N M L T R P R Y - NH2

PYY Y P A K P E A P G E D A S P E E L S R Y Y A S L R H Y L N V T R Q R Y - NH2

Fig. 1.4. Amino acid sequences of neuropeptide Y and related peptide. A=Ala=Alanine; B=Asx=Aspartic acid or asparagine; C=Cys=Cysteine; D=Asp=Aspartic acid; E=Glu=Glutamic acid; F=Phe=Phenylalanine; G=Gly=Glycine; H=Histidine; I= Ile=Isoleucine; K=Lys=Lysine; L=Lys=Leucine; M=met=Methionine; N=Asn=Asparagine; P= Pro=proline; Q=Gln=Glutamine; R=Arg=Arginine; S=Ser=Serine; T=Thr=Threonine; V=Val=Valine; W=Trp=Tryptophan; X= - Unknown or "other"; Y= Tyr=Tyrosine; Z= Glx=Glutamic acid or glutamine (Taken from IUPAC-IUB Commission. European J. Biochem. 5 (1968) 151-153)

for reviews). There is evidence that some sympathetic neurones can produce and release both noradrenaline and acetylcholine (Burn and Rand, 1965; Hill and Hendrey, 1977; Burnstock, 1978). VIP has been found to be co-localised with AChE in neurones of cat sympathetic ganglia (Lundberg et al., 1979). There are many reports on coexistence of substance P and CGRP in a subpopulation of primary sensory neurones in dorsal root ganglia (Gibson et al., 1984; Wisenfeld-Hallin et al., 1984) The presence of biologically active peptides or other putative transmitters within nerves raises the question of whether or not all of these substances act as a neurotransmitters.

1.3. Aims of study

The investigation of peptide-containing axons supplying the skin presents a challenging, fascinating and developing research field. Although the distribution of peptidergic nerves has now been partly documented, much is desired in the way of systematic study, particularly of changes occurring in various neurocutaneous diseases. The main aims of the study were therefore as follows:-

1. To describe in detail the neuroanatomical distribution and relationships of cutaneous nerves and neuropeptides in man and laboratory animals by using immunohistochemical techniques.

2. To investigate the phenotypic expression of various peptidergic nerves in response to different experimental conditions including the grafted skin.

3. To study the ontogeny of peptidergic nerves with a view to correlation with sensory and autonomic physiology.

4. To examine the potential involvement of neuropeptides in the pathogenesis of infectious (Leprosy) and metabolic (Diabetes) diseases, in which there is a loss sensory and autonomic functions, and also in an infrequently reported condition characterised by hyperfunctional sensory nerves (Notalgia paraesthetica). In order to evaluate neural changes in the earliest stages of these disease processes, experiments were carried out employing nude mice infected with Mycobacterium leprae and rats with streptozotocin-induced diabetes.

1.4. Main peptides investigated

1.4.1. Calcitonin gene-related peptide

Calcitonin gene-related peptide (CGRP) is a 37 amino acid peptide, whose existence was predicted and later demonstrated by Amara and co-workers (1982), following analysis of the human calcitonin precursor gene. Subsequently, CGRP was isolated by using recombinant DNA and molecular biology techniques (Rosenfeld et al., 1983)(Diagram 1). Tissue-dependent modification of the precursor gene and expression of the mRNA was proposed to account for the apparent distribution of CGRP mainly in neural elements and of calcitonin in thyroidal C-cells (Amara et al., 1984; Jonas et al., 1985). Two forms of CGRP have been identified in both rat (Amara et al., 1985) and man (Morris et al., 1984) and these are derived from related alpha and beta genes. Beta CGRP differs from the previously identified alpha form by one amino acid in rat and three amino acids in man.

Immunocytochemical studies revealed CGRP in a widespread neuronal system in the brain, spinal cord and peripheral tissue (Rosenfeld et al., 1983, Springall et al., 1983, Gibson et al., 1984, Rodrigo et al., 1985; Mulderry et al., 1985) as well as in the endocrine cells of the lung (Cadieux et al., 1986). CGRP immunoreactivity is abundant in the

PREPROCALCITONIN GENE

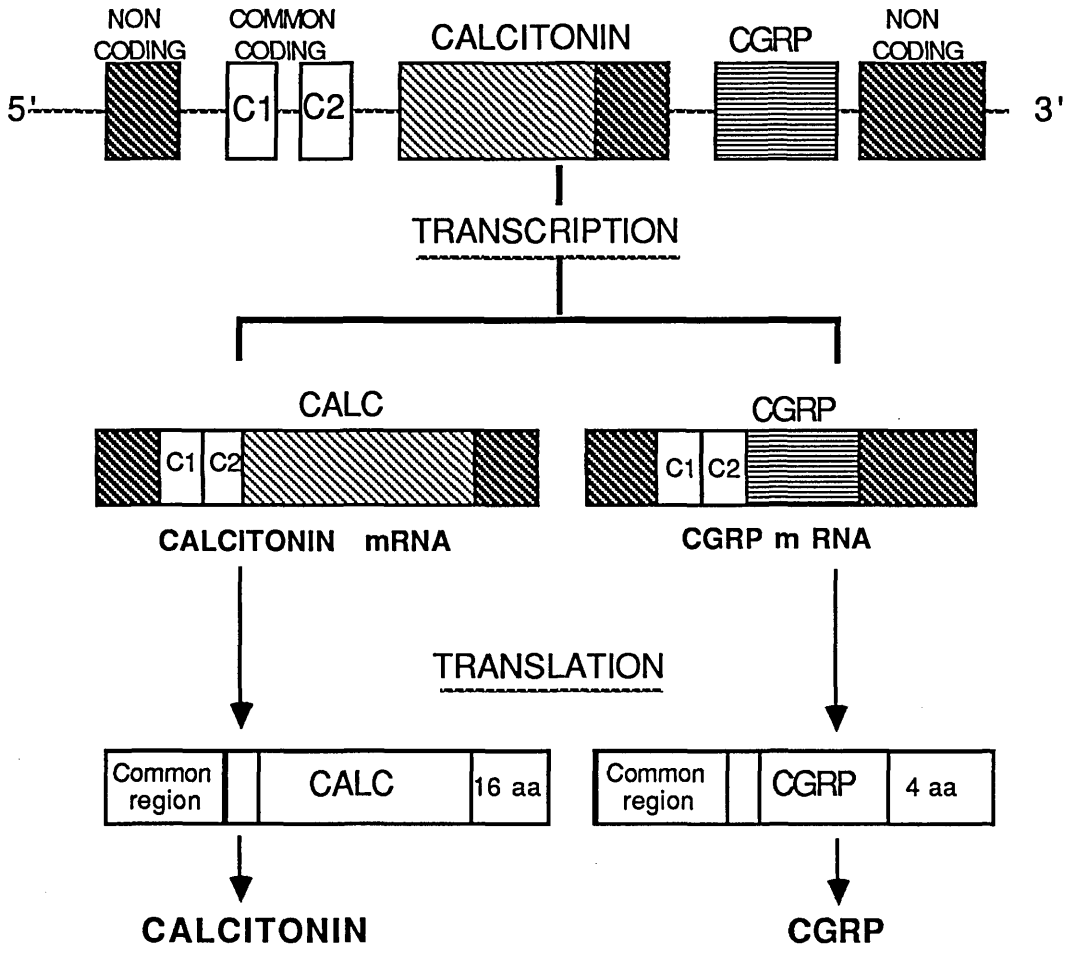


Diagram 1 - Schematic representation of alternative RNA splicing pathways in the expression of the rat prepro-calcitonin gene which encodes both calcitonin and CGRP (from data published by Rosenfeld et al., Nature 304, 129-135, 1983)

sensory nervous system, in particular in the dorsal horn of the spinal cord and in cell bodies of spinal and cranial sensory ganglia. In vitro studies showed CGRP-like immunoreactivity is released from trigeminal ganglion cells (Mason et al., 1984) and from spinal cord slices following capsaicin administration (Saria et al., 1986). Behavioral studies have shown that co-administration of CGRP and substance P potentiates the responses to noxious stimuli over and above that of substance P alone (Wiesenfeld-Hallin et al., 1984; Woolf and Wiesenfeld-Hallin, 1986).

Likewise, CGRP markedly potentiates the substance P-induced plasma extravasation in the inflammatory reaction (Brain and Williams, 1985; Gamse and Saria, 1985). Furthermore, CGRP has been demonstrated in mammalian motor end plates (Rodrigo et al., 1985; Takami et al., 1985b; Matteoli et al ., 1988) and in cranial and spinal motoneurones (Rosenfeld et al., 1983; Takami et al., 1985a; Gibson et al., 1984) suggesting a role in motor functions. CGRP is believed to play a part in the synthesis and assembly of acetylcholine receptors at the neuromuscular junctions and has been proposed as a muscle trophic factor (Fontaine et al., 1986; New and Mudge, 1986). Several immunocytochemical studies show the coexistence of CGRP immunoreactivity with substance P in sensory ganglion cells (Gibson et al., 1984; Wisenfeld-Hallin et al., 1984; Gibbins et al., 1985; Lee et al., 1985 a,b; Terenghi et al., 1985). After capsaicin treatment, both peptide immunoreactivities are depleted from the peripheral projections of sensory neurones supplying skin (Gibbins et al., 1985, Holzer et al., 1982; Norheim et al., 1985), respiratory tract (Caudieux et al., 1986) and urinary tract (Su et al., 1986), the cardiovascular system (Lundberg et al., 1985, Wharton et al., 1986) and the oesophagus, tongue and palate (Terenghi et al., 1986).

Several physiological studies indicate that CGRP exerts a potent vasodilatory action, both in vivo and in vitro, which is not inhibited by autonomic blockade (Rozsa et al., 1985; Brain et al., 1985; Uddaman et al., 1986). In addition, CGRP appears to potentiate the plasma extravasation induced by tachykinins in an inflammatory reaction (Brain & Williams. 1985; Gamse & Saria, 1985). CGRP-specific receptors have been identified in several tissues, namely, in the brain and spinal cord (Dawbarn et al., 1985; Goltzman & Mitchell, 1985), blood vessels (Sigrist et al., 1986) and adrenal gland and kidney (Goltzman & Mitchell, 1985).

1.4.2. Tachykinins

Tachykinins belong to a family of peptides which share at their carboxyl terminals a common sequence of six amino acids. It was thought until recently that substance P was the only member of this family present in mammals. The other peptides (kassinin, eledoisin, physalaemin) of this family have been found only in lower organisms. However, two new tachykinins have been isolated from porcine spinal cord and named neurokinin A, (also known as neuromedin L or substance K) and neurokinin B (also known as neurokinin beta or neuromedin K) (Kimura et al., 1983). Subsequently, two precursor mRNA's have been isolated, one of them containing the substance P sequence (apha-preprotachykinin) and the other containing the substance P sequence as well as that for neurokinin A (beta -preprotachykinin) (Nawa et al., 1983) (Diagram 2). Further studies have shown that there appears to be only one gene encoding both preprotachykinins, with alternative RNA splicing resulting in two messenger RNAs (Nawa et al., 1984). Even more recently, a third tachykinin was isolated from porcine brain and termed neuropeptide K because of its similarity to substance K or neurokinin A (Tatemoto et al., 1985). Receptor studies show that there are three types of tachykinin receptors (NK1, NK2, NK3) with preferential affinity for substance P, neurokinin A and neuropeptide K, respectively (Regoli et al., 1987).

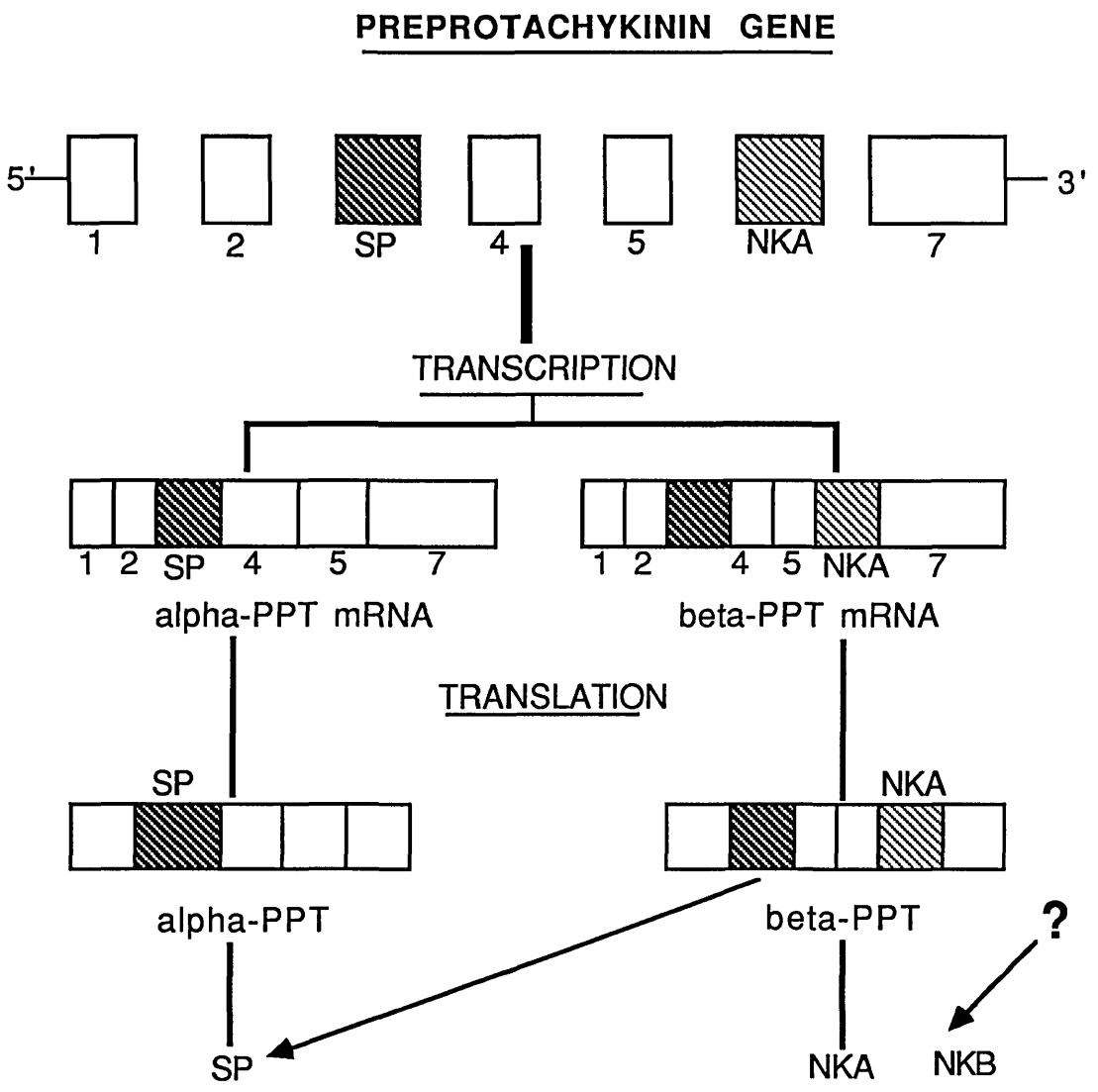


Diagram 2 - Schematic representation of alternative RNA splicing pathways in the expression of the preprotachykinin (PPT) gene. SP, substance P; NKA, Neurokinin A; NKB, Neurokinin B. The biosynthesis of neurokinin B (NKB) is not known.

Substance P was discovered by von Euler & Gaddum in 1931, when they noted the release of a substance after vagal stimulation which was capable of eliciting an atropine-resistant contraction of muscle in the gut. However, it was not until 40 years later that substance P was chemically characterised as an undecapeptide (Chang et al.,1971). It was in the previous year that its identity with Leeman's sialogogic peptide was recognised (Chang and Leeman, 1970). It is the best known peptide of the tachykinin family and has an extensive distribution in both the central and peripheral nervous systems (for reviews Hökfelt et al., 1977b; Polak & Bloom, 1981). There is strong evidence that substance P may play a role in sensory neurotransmission (Pernow, 1983; Lembeck, 1985). This was first indicated 1) by its location in primary sensory neurones of the dorsal root ganglia and in their central branches terminating in the dorsal horn (Substantia gelatinosa) of the spinal cord (Hökfelt et al., 1975b) and 2) by the marked depletion of substance P in this region after dorsal rhizotomy (Takahashi & Otsuka, 1975, Hökfelt et al., 1975a). The sensory nature of substance P-containing fibres is further supported by the finding of a substantial depletion of the peptide immunoreactivity from nerve terminals after the treatment of animals with the sensory neurotoxin capsaicin (Jessell et al., 1978; Gamse et al., 1979; Nagy et al., 1981).

In addition to its sensory role, substance P has been shown to exert a diversity of pharmacological and physiological actions. These include non-vascular smooth muscle contraction, vasodilation and hypotension (Pernow 1953, Lembeck & Zetler, 1962, Hallberg & Pernow, 1975; Edvinson et al., 1982). Substance P has also been shown to induce

antidromic vasodilatation, plasma extravasation and itching, and results in reduced pain sensitivities (for review see Lembeck and Gamse 1982, Gamse & Saria, 1985). Recently, the following evidence has implicated substance P as the neurotransmitter of pain. In the dorsal root ganglia, the subset of cells that contain substance P correspond to cell bodies of C fibres, some of which conduct nociceptive information (see Hunt, 1983). It is present in fibres and terminals and in high concentration in the dorsal horn, in the region of C fibre termination. Activation of nociceptive afferents releases substance P from the spinal cord (Yaksha et al., 1980). Analgesia can be produced by neonatal capsaicin treatment resulting in the loss of substance P immunoreactivity from primary sensory neurones and from areas of the spinal cord which receive nociceptive input (Jessell et al., 1978).

Recent studies, however, contradict many of the above suggested actions. These studies show that substance P can activate both noxious and non-noxious neurones in the dorsal horn (See Salt and Hill, 1983) and has effects on motor (Otsuka and Yangisawa, 1980) and autonomic neurones (Beckman and Henry, 1983), by depolarisation. In addition, capsaicin, which was thought to produce a selective lesion of substance P-immunoreactive dorsal root ganglion cells, actually produces a non-selective lesion of all small, dorsal root ganglion cells (Nagy et al., 1981, 1983)

1.4.3. Vasoactive intestinal polypeptide/ peptide histidine isoleucine (VIP/PHI/PHM)

Vasoactive intestinal polypeptide (VIP) was isolated from porcine upper intestinal tract tissue (Said and Mutt, 1970), and structurally characterised as being composed of 28 amino acid residues (Mutt and

VASOACTIVE INTESTINAL POLYPEPTIDE PREPROMOLECULE

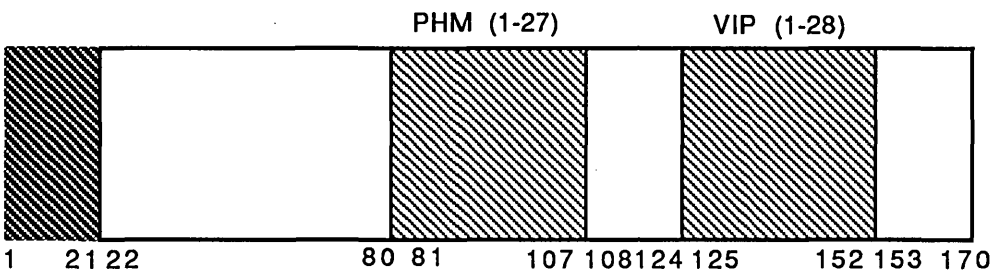


Diagram 3 - Schematic representation of vasoactive intestinal polypeptide (VIP) prepromolecule. PHM, Peptide histidine methionine (from data published by Itoh et al., 304, 547-549, 1983)

Said, 1974) (Diagram 3). More recently, another peptide, having close structural similarities to VIP, was isolated from porcine duodenum (Tatemoto and Mutt, 1981). Structurally, this peptide consisted of 27 amino acids with an amino-terminal histidine and a carboxy-terminal isoleucine and was called peptide histidine isoleucine (PHI) (Fig.1.3). Subsequently an identical substance was extracted from brain tissue (Tatemoto et al., 1983). In many tissues the equimolar concentrations of VIP and PHI immunoreactivities led to the suggestions that the peptides might be co-synthesised in the same prohormone (Christofides et al., 1982). The structure of the human form of PHI (peptide histidine methionine, PHM) suggested that they are derived from the same prepromolecule as VIP (Itoh et al., 1983). This is substantiated further by the closely parallel immunocytochemical localisation of PHI/PHM and VIP in most tissues and they are considered to be co-stored in the same neurone (Anand et al., 1984; McGregor et al., 1984; Polak and Bloom, 1984).

The first report of VIP immunoreactivity in neurones in the hypothalamus and gastrointestinal tract was by Larsson et al., 1976. Later, a detailed study was made on the distribution of VIP immunoreactivity in the central nervous system, in particular the extensive presence in the cerebral cortex (Fuxe et al., 1977). In the peripheral tissues, most VIP-immunoreactive nerves originate from postganglionic sympathetic and parasympathetic neurones or enteric neurones (Larsson et al., 1976; Lundberg et al., 1979). VIP immunoreactivity has been described in nerves of the tongue (Baeker et al., 1983), eye (Uddman et al., 1980; Loren et al., 1980), heart (Weihe et al., 1984), respiratory tract (Uddaman et al., 1978), male and female genitourinary tract (Alm et al., 1977, 1980; Larsson et al., 1977a.b; Lynch et al., 1980; Wharton et al., 1981) and exocrine glands (Bryant et al., 1976; Sundler et al., 1978; Bloom et al., 1980; Lundberg et al., 1980a).

Although most of the VIP-immunoreactive nerve fibres are generally presumed to represent postganglionic parasympathetic neurones, it is interesting to note that VIP immunoreactivity has been described in prevertebral sympathetic ganglia (Hökfelt et al., 1982) and in primary sensory neurones in spinal and vagal ganglia (Lundberg et al., 1978).

Physiologically, VIP shows several biological actions. These include the potent vasodilatory effect (Said & Mutt, 1970) and relaxation of smooth muscle in the gut (Piper et al., 1970), urogenital tract (Helme et al., 1981; Larsen et al., 1981) and respiratory tract (Piper et al., 1970). VIP has also been shown to exert a stimulatory action on both endocrine (Said, 1981) and exocrine secretion (Konturek et al., 1975; Schebalin et al., 1977). In the submandibular gland, VIP was found to be present in cholinergic neurones (Lundberg et al., 1980) and it has been suggested that acetylecholine mainly stimulates the secretory cells and VIP has predominantly a vasodilatory action. VIP is a relaxant of smooth muscle in the gut (Piper et al., 1970), genitourinary tract (Helm et al., 1981; Larson et al., 1981) and respiratory tract (Piper et al., 1970).

1.4.4 Neuropeptide tyrosine/C-terminal flanking peptide (NPY/CPON)

Neuropeptide tyrosine is a 36 aminoacid peptide isolated from porcine brain (Tatemoto, 1982). It has major sequence homologies (Fig. 1.8) with pancreatic polypeptide (PP) and peptide tyrosine tyrosine (PYY) (Tatemoto et al., 1982). NPY is widespread in both central and peripheral nervous systems (Lundberg et al., 1984a). Recent isolation and sequencing of the DNA encoding NPY has has led to the prediction of the existence of a 97 amino acid precursor peptide (Minth et al., 1984). Proteolytic processing of this precursor could yield three separate peptide products, an N-terminal signal peptide, NPY and a 30 amino acid C-terminal flanking peptide (C-PON) (Diagram 4).

NEUROPEPTIDE TYROSINE PREPROMOLECULE

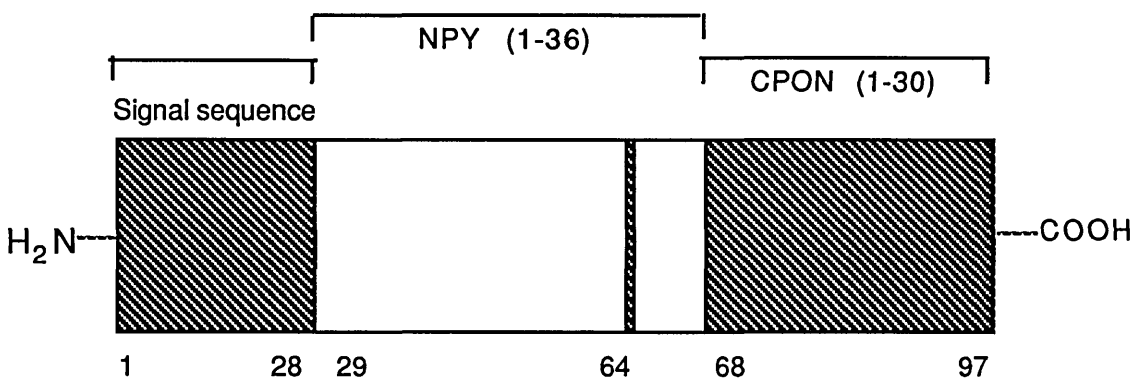


Diagram 4 - Schematic representation of human neuropeptide tyrosine (NPY) prepromolecule. C-PON, C-flanking peptide of NPY

NPY has been localised to extensive neuronal networks in the brain and several peripheral tissues (Adrian et al., 1983; Allen et al., 1983a; Polak and Bloom, 1984). In the peripheral nervous system, NPY immunoreactivity has been demonstrated in the eye (Bruun et al., 1984, heart (Gu et al., 1983b, 1984b), respiratory tract (Sheppard et al., 1984), gut (Sundler et al., 1983; Ferri et al., 1984)., adrenal medulla (Varndell et al., 1984), kidney (Ballesta et al., 1984a) and genitourinary tract (Stjernquist et al., 1983; Adrian et al., 1984). NPY has been associated with the sympathetic nervous system because of its co-localisation with adrenalin synthesising enzymes tyrosine hydroxylase (TH) and dopamine-beta- hydroxylase (DBH) in ganglion cells, and its susceptibility to 6-hydroxydopamine treatment (Lundberg et al., 1982a, 1983; Sundler et al., 1983). Administration of NPY causes several major physiological effects. These include hypotension and bradycardia (Lundberg et al., 1982c and d, 1984; Ekbald et al., 1984a). In isolated perfused heart it reduces cardiac flow and contractility (Allen et al., 1983b). NPY inhibits the electrically induced contraction of the vas deferens (Allen et al., 1982). Studies have shown the presynaptic modulatory effect of NPY on the transmitter release from non-adrenergic, cholinergic and non-adrenergic, non-cholinergic nerves (Lundberg et al., 1984b; Wahlestedt et al., 1985,1986; Pernow et al., 1986).

1.5 General neural markers investigated

1.5.1. Protein gene product 9.5

The analysis of cerebrospinal fluid and/or serum for brain-specific proteins produced after physical disruption or as a consequence of neurological disorder, provided the impetus for further research on human brain-specific proteins. While some nervous-system-specific proteins, such as S-100, neuron-specific enolase and glial fibrillary acidic protein, were already relatively well defined, the search for new human brain-specific proteins using high-resolution, two-dimensional

polyacrylamide gel electrophoresis began. This led to the identification of four new proteins. They were named according to the protein gene product (PGP) terminology of Anderson and Anderson (1979). Two of these were PGP 9.5 (9.5 referring to its elution profile during chromatography) and a modified product of PGP 9.5, another correspond to glial fibrillary acidic protein, and the remaining one had not been investigated (Thompson and Day 1988).

PGP 9.5 has a monomer molecular weight of 24,000 to 28,000 and a native molecular weight on gel filtration of 37,000 (Doran et al., 1983); the reason for this discrepancy is not clear. It is among the most abundant proteins of brain, constituting from 1 to 5% of the soluble protein. It is a cytoplasmic protein of 212 amino acids (Day and Thompson, 1987). The cloning and sequencing of the cDNA for the human form of the protein (Day and Thompson, 1987) has confirmed the previous evidence that PGP 9.5 represents a completely new neuronal and neuroendocrine marker and is unrelated to any other form of 'brain specific protein'. Recent biochemical evidence suggests that PGP 9.5 is a ubiquitin carboxyl-terminal hydrolase (Wilkinson et al 1990).

Immunohistochemistry has shown that PGP 9.5 is widely distributed in central and peripheral neurones (Thompson et al., 1983). The distribution closely parallel that of neurone-specific enolase (NSE). In the cerebral cortex and other brain regions the immunoreactivity was mainly seen in neurones. In the spinal cord and peripheral nervous system, marked staining of both myelinated and unmyelinated axons was seen. The main reason for the effective immunoreactivity may be due to the nature of PGP 9.5, which is a major component of the neuronal cytoplasm as suggested by radioimmunoassay (Thompson, 1981) and by electron microscopy (Gulbenkian et al., 1987). PGP 9.5 has been shown to be a good histological marker for the delineation of nerves and endocrine cells in other organs of the body (Gulbenkian et al., 1987; Thompson et al., 1983; Lauweryns and Van Ranst, 1988).

1.5.2 Neurofilament triplet proteins

Neurofilaments are neurone-specific filaments with a diameter of 10nm and consist of three subunit proteins. These have apparent molecular weights of 68, 150, and 200k-Daltons and are referred to as 68kD, 150kD, and 200kD neurofilament subunits. The orgin of the neurofilament polypeptides, whether they are synthesised separately or are products of a common precursor, has been a source of debate. Neurofilament subunits have been considered to be asymetrically distributed in neurones (Dahl et al., 1982; Dahl, 1983) and to undergo slow axonal transport (Hoffman and Lasek, 1975). Studies using antibodies specific to each of the polypeptides of the triplet have suggested that most nerve cells contain all three polypeptides (Shaw and Weber, 1981). However, absence of one or more neurofilament subunits in some neurones due to the phosphorylated state of these polypeptides, or as a consequence of unknown mechanisms, may account for the apparent variable expression of these proteins in neurones (Goldstein et al., 1983; Nixon et al., 1982; Sternberger and Sternberger., 1983). Several morphological observations support the hypothesis that neurofilaments are major intrinsic determinants of axonal calibre in large myelinated nerve fibres (Berthold, 1978; Hoffman et al., 1985). In these fibres neurofilaments are the most numerous cytoskeletal elements, and neurofilament number correlates closely with axonal cross-sectional area (Friede and Samorajski, 1970; Weiss and Mayr, 1971; Berthold, 1978; Hoffman et al., 1984a). However, the relationship does not appear to be closely correlated with axonal calibre in small myelinated fibres where the density may vary considerably along the length of the axon (Parhad et al., 1986) or in unmyelinated peripheral nervous system fibres, where microtubules, not neurofilaments, are the primary cytoskeletal elements (Berthold, 1978; Sasaki-Sherrington et al., 1984).

1.5.3 Protein S-100

S-100 protein is a neural-tissue-associated protein widely distributed in both central and peripheral nervous system (Moore, 1972). It is a dimer of two possible units (a and b) and has three forms: S-100 aa, S-100 ab and S-100 bb, which are dimers with a subunit composition of aa,ab and bb respectively (Isobe et al., 1978,1981, 1983). There is some suggestion that alpha subunit of S-100 is less specific for tissues of neuroectodermal origin than beta subunit of S-100 (Kato et al., 1984). It is present in the glial cells of CNS and, Schwann and satellite cells of the PNS. Although the initial work suggested that the S-100 protein was nearly completely specific to the nervous system, there is some recent evidence that it, or closely related proteins, may be expressed in some non-neural cells. Immunoreactivity for S-100 has been reported in Langerhans cells of human skin (Cocchia et al., 1981; Nakajima et al., 1982; Takahashi et al., 1984; Rowden et al., 1985; Schneider et al., 1985;), in adipocytes (Michetti et al., 1983; Haimoto et al., 1985), and in stellate cells of the pituitary gland (Girod et al., 1985). It has also been reported in the lymphoreticular system, in which the beta subunit is present in Langerhans cells, interdigitating reticulum cells, and histiocytes and the alpha subunit in monocytes, macrophages of lymph node, alveolar macrophages of lung, and some Kupffer cells of liver (Takahaski et al., 1984b), and in rat salivary glands (Molin et al., 1984).

1.5.4 Neuron specific enolase

Neuron-specific enolase (NSE), an acidic soluble isoenzyme of the glycolytic enzyme enolase with a molecular weight of 78kD, was originally isolated from rat brain (Marangos et al., 1975). It is a soluble cytoplasmic protein (Marangos and Schmechel, 1980) reported to be a good marker for the specific visualisation of both neurones and peptide-secreting endocrine cells (Schmechel et al., 1978; Bishop et al., 1982; Sheppard et al., 1983, 1984). The functional role of NSE is not yet fully understood. There is evidence that it is a glycolytic isoenzyme

(Bock and Dissin, 1975) related to neuronal development (Marangos et al., 1981). It has been suggested that staining intensity of NSE is related to the physiological state of the nerves and cells (Marangos et al., 1981; Bishop et al., 1985).

1.5.5. Synaptophysin

Synaptophysin is a major integral membrane glycoprotein characteristic of a certain type of small (30-80nm) neurosecretory vesicles with an electron microscopically translucent content, originally isolated from and identified in presynaptic vesicles of neurones by the use of monoclonal antibodies (Jahn et al., 1985; Wiedenmann and Franke, 1985). On SDS-PAGE separation the unfolded polypeptide chain appeared with molecular weights values ranging from 38,000 to 40,000, depending on the cell type (Jahn et al., 1985; Wiedenmann and Franke, 1985; Rehm et al., 1986; Widenmann et al., 1986), and it has been suggested that some of this variation may be due to different extents of glycosylation. Synaptophysin has been identified in a wide variety of normal cells and tissues (Wiedenmann and Franke, 1985; Navone et al., 1986) and neoplasms (Franke et al., 1986; Wiedenmann et al., 1986; Gould et al., 1987) and is used as a broad range diagnostic marker for neuroendocrine tumours of both neural and epithelial origin.

Chapter 2

MATERIALS AND METHODS

2.1. General 45
2.2. Tissues investigated 45
2.3. Fixation 46
2.4. Tissue preparation 46-47
2.4.1. Wax embedding and paraffin sectioning 46-47
2.4.2. Snap freezing and cryostat sectioning 47
2.3.3. Free floating thick sections 48
2.5. Immunostaining 49
2.6. Antisera 49-53
2.7. Specificity of immunostaining 54-59
2.7.1. Antisera specificity 54, 58
2.7.2. Antisera specificity controls 58
2.7.3. Method specificity 58
2.8. Conventional histology 58
2.9. Acetylcholinesterase staining 58-59
2.10. Fite stain for Mycobacterium leprae 59
2.11. Experimental procedures 59-61
2.11.1. Capsaicin treatment 59
2.11.2. 6-hydroxydopamine treatment 60
2.11.3. Transplantation of skin flap 60
2.11.4. Injection of Mycobacterium leprae into foot pads 60
2.11.5. Streptozotocin treatment 61
2.12. Semiquantitative assessment of immunostaining 61
2.13. Quantification 61-63
2.13. 1 Statistical analysis 62
2.14. Microscopy and Photography 63

2.1. General

Immunocytochemistry is used for in situ localisation of a tissue constituent by means of a specific antigen-antibody reaction using a labelled antibody which can be detected microscopically. In this study, immunocytochemistry at the light microscopic level was the main technique employed and was carried out using indirect immunofluorescence (Coons et al., 1955) (**Appendix 5a**) and the peroxidase anti-peroxidase method (**Appendix 5b**). In order to obtain an optimum immunocytochemical signal, double application of primary antisera and nascent hydrogen peroxide-diaminobenzidine (DAB)-nickel intensification (Appendix 6) methods were employed for indirect immunofluorescence and the peroxidase anti-peroxidase method, respectively. The use of neurotoxic substances such as capsaicin and 6-hydroxydopamine, which are specific for defined subpopulations of nerve types, was applied in order to establish the possible nature of the peptidergic innervation. Experimental animal models of neuropathic diseases were studied. The ensuing morphological changes were assessed by immunocytochemistry, comparing experimental and control tissues.

2. 2. Tissues investigated

The study was carried out on human tissues taken from patients undergoing surgical operations that entailed skin removal and biopsies taken from patients suffering from leprosy, diabetes and notalgia paraesthetica. The animal tissues included skin taken from adult Wistar rats (body weight 150-200 g), Dunkin Hartley guinea pigs (body weight 300-600g), mf 1 nu/nu mice (body weight 30-50g), cat and pig. In addition, to study the development, skin samples from fetal and neonatal rats were used. Skin taken from experimental animals included capsaicin-treated neonatal rats, 6-OHDA-treated adult guinea pigs and streptozotocin-treated (diabetic) rats. The skin and spinal cord of Mycobacterium-injected adult mice were investigated in order to correlate the changes in both areas. In addition, skin samples of cutaneous flap grafts were also collected to study reinnervation. Detailed account of the experiments will be given in each chapter.

2. 3. Fixation

As peptides are water-soluble antigens, they require to be "fixed", or

made insoluble, with an appropriate fixative solution before being processed for immunocytochemistry. Inadequate or uneven fixation results in loss of antigen and tissue damage, whereas overfixation leads to masking of antigenic sites. In addition, the fixation process should retain the tissue morphology, while not altering or masking the antigenic state, which has to be available for recognition by the corresponding antibodies. Fixatives commonly used introduce intra- and intermolecular cross-links and may therefore modify antigenicity, and create an antibody permeability barrier. Optimum fixation conditions vary depending on the chemical structure of the antigen, from very small peptides to large proteins/enzymes. Due to the difference in consistency and constitution of epidermis and dermis good fixation is often best achieved by rapid in situ fixation through intracardial perfusion (Palay et al., 1962) followed by post-fixation of tissues in the same solution for a certain time. Thus, a variety of different fixatives were examined to establish optimum conditions. In this study, the tissues were mainly fixed in Zamboni's fixative (Stefanini et al., 1967) (**Appendix 3C**) and p-benzoquinone solution (**Appendix 3A**). In terms of preservation of tissue and staining intensity the Zamboni's solution was found to be superior to p-benzoquinone. Fixation by perfusion was preferred when dissection took a long time, as with spinal cord. Perfusion also offered the advantage of better penetration of the fixative solution.

2. 4. Tissue preparation

Following fixation, the tissues were washed and stored in phosphate buffered saline (PBS) with 15% sucrose and 0.01% sodium azide at 4°C until further processing including embedding in wax for wax sectioning or snap freezing for cryostat sectioning.

2.4.1. Wax embedding and paraffin sectioning

This procedure was performed to facilitate the production of thin sections in order to study the morphology of tissues, particularly under disease conditions. In addition, some histochemical techniques can only be performed in wax embedded tissues (eg., Fite stain for Mycobacterium leprae). As embedding wax is not miscible with water present in the tissue and fixative, the tissue is first dehydrated by immersion in increasing concentrations of alcohol and finally in a hydrophobic solvent such as xylene before being impregnated with

molten paraffin wax at 58°C. However, the procedure is not suitable for immunohistochemical techniques on nervous tissue due to the chemicals used resulting in incomplete demonstration of neural antigens.

2. 4. 2. Snap freezing and cryostat sectioning

For cryostat sectioning the tissue blocks were washed in several changes of PBS and then stored in a solution of sucrose in PBS (15%) at 4°C to boost storage osmolarity and to act as a cryoprotectant. Tissue blocks were then rapidly frozen and then allowed to reach the cryostat temperature (-20°C). Sections of varying thickness (8 to 12μm) were mounted on pre-washed slides coated with poly-L-lysine (PLL - molecular weight 150,000-300,000, Sigma) to avoid loss of sections during processing (Huang et al., 1983).

2. 4. 3. Free-floating thick sections

The application of immunocytochemical techniques on thick sections (40μm) permits analysis of the course and inter-relationships of cutaneous innervation, in addition to providing an accurate picture of the overall distribution and density. In order to improve the quality of immunostaining, several modifications were made including increased time of treatment of the tissues with detergent (Hartman et al., 1973) for better penetration of the antibodies into the tissue, increased time of incubation with antisera and counterstaining of the preparations with pontamine sky blue (Cowen et al., 1985). These sections were placed directly in a multi-well plate or Petri dish containing PBS and used as free-floating sections for the immunostaining.

2. 5. Immunostaining

Coons et al., (1941) brought a major development to cytochemical studies by the introduction of fluorescein labelled primary antibodies for in situ identification of specific tissue antigens (direct method). This was followed by a significant improvement, with an unlabelled primary antibody identified by a labelled secondary antibody raised to the immunoglobulin of the species providing the primary antibody; this was termed the indirect method (Coons et al., 1955). This technique has the major advantage of separately identifying several specific

primary antibodies by a single labelled secondary antibody. Another major step in the identification of tissue antigens was the development of unlabelled antibody-enzyme methods. One of the most widely used is the peroxidase anti-peroxidase (PAP) method (Sternberger et al., 1970, Sternberger., 1979). This method involves use of three reagents applied to the tissues stepwise. After the primary antiserum has been applied a second unlabelled antibody, raised against the immunoglobulins of the first antiserum is applied in excess so that one of its two identical binding sites on the immunoglobulin molecule remains free. The PAP complex, which is a stable cyclic complex resulting from combination of peroxidase and antiperoxidase (raised in the donor species of the first antiserum) is then applied and attaches to the free binding site. Thus there is a high ratio of peroxidase label to primary antigen. However, both methods have advantages and disadvantages, and the use of one or the other has to be chosen according to the tissue investigated and the requirements of the immunostaining techniques selected.

To improve the quality of staining several steps were added including treatment of tissues with detergents (Hartman, 1972; Hartman et al., 1973) and alcohols and xylene (Costa et al., 1980) to aid antibody penetration of tissues by removal of tissue lipid fat. The incubation time and concentration of primary antisera was determined by experiment. In general, improvement of weak signal could be achieved by repeated application of primary antisera (Gu et al., 1983). In addition, to enhance the developed peroxidase reaction the DAB-nickel method with nascent hydrogen peroxide was used (Shu et al., 1988)

2. 6. Antisera

Most of the antisera used in this study were raised in rabbits (New Zealand white) immunised by subcutaneous injection of natural purified (e.g., Insulin and glucagon) or synthetic peptides. They were produced in collaboration with Professor. S.R. Bloom's laboratory in the Department of Medicine, Hammersmith Hospital. In order to increase the immunogenicity, peptides were coupled to a large carrier protein such as bovine serum albumin (BSA) or keyhole limpet haemocyanin (KLH) and emulsified in Freund's complete adjuvant. This large complex is more likely to stimulate the formation of antibodies than the small peptide molecule alone (Szelke, 1983). Booster injection

began three months after the primary injection but were prepared with Freund's incomplete adjuvant. This continued thereafter on a monthly basis (O'Shaughnessey et al., 1982) and the sera were taken from blood samples collected on the 7th and 10th day after each injection. Useful antibody titres were usually obtained after the third boost and did not generally improve after 7-8 boosts. The testing of antisera was carried out on a cryostat section of known 'positive' tissue. Optimum working dilutions were selected by testing several dilutions of each antiserum and by evaluating the quality of specific immunostaining against the background. Pre-absorption tests with specific antigens were used to assess the specificity of the antisera. Since Freund's complete (including Mycobacterium tuberculosis) adjuvant was used in the production of antibodies, it is almost certain that anti-mycobacterium tuberculosis antibodies would have been present at the time of the harvest of useful antipeptide antibodies. However, antibodies generated against the carrier protein do not generally interfere with immunostaining since the proteins are chosen from distant species eg., molluscan haemocyanin, to minimize any cross reactivity with any mammalian tissues. In addition, BSA is routinely used in all antiserum diluent thus removing any antibodies to the carrier protein by absorption and preventing their interference with immunostaining. The characteristics of the antisera used in this study are summarised in Tables 1 & 2

2. 7. Specificity of immunostaining

In immunocytochemistry it is important to verify the specificity of the immunostaining. A false positive staining may be the result of unwanted immunoreactions as all the various subpopulations of antibodies present in the antiserum have the opportunity to bind to antigens in the tissue. There is also the possibility that a specific antibody may cross-react with similar amino acid sequences of related or unknown peptides. These must be checked and excluded before accepting a result. When possible, immunocytochemical studies should be complemented by the biochemical isolation of the relevant antigen for the tissue being studied. The subject of specificity in immunocytochemistry has been extensively reviewed (Petrusz et al., 1976, 1977; Swaab et al., 1977; Sternberger, 1979, 1982; Larsson, 1981, 1983;

Table I

Details of general neural markers used in this study

	Antigen	Dilution	IMF	Source	Type of antibody	References
General Neuronal Markers	PGP 9.5		1:800	Ultraclone, UK	Rabbit polyclonal	1
	NF protein		1:200	Prof. D. Dhal, USA	Rabbit polyclonal	2
	Synaptophysin		1:400	Prof. R. Jahn, FRG	Rabbit polyclonal	3
	NSE		1:400	Dr. P.J. Marangos, USA	Rabbit polyclonal	4
	ChAT		1:400	Boehringer Mannheim, GmBH , FRG	Rabbit polyclonal	5
	S-100		1:800	Dr. D. Cocchia & Dr. F. Michetti, Rome	Rabbit polyclonal	6
Catecholamine Synthesising Enzyme	TH		1:400	DR. D. Kuhn, USA	Rabbit polyclonal	7

Key:

PGP 9.5	=	Protein gene product 9.5
NF	=	Neurofilament
NSE	=	Neuron specific enolase
ChAT	=	Choline acetyltransferase
IMF	=	Immunofluorescence

Refs	References
1.	Thompson et al., 1983
2.	Dahl & Bignami., 1977
3.	Jahn et al., 1985
4.	Marangos et al., 1978
5.	Furness et al., 1984 a/b
6.	Cocchia et al., 1981
7.	Thibault et al., 1981

Table 2

Details of PGP 9.5 and peptide antisera used in this study

Antigen	Carrier protein	Coupling agent	Dilution		References
			IMF	PAP	
PGP 9.5	-	-	1:800		1
CGRP	BSA	BDB	1:200	1:4000	2
Substance P	BSA	GLUT	1:100	1:5000	3
NKA (15-36)	BSA	GLUT	1:400		4
VIP	KLH	BDB	1:400		5
PHI	BSA	GLUT	1:400		6
PHM	BSA	GLUT	1:400		7
NPY	BSA	BDB	1:400		8
CPON	KLH	GLUT	1:800		9
SS-28	BSA	CDI	1:800		10

Freund's adjuvant was used during immunisation.

Key: CGRP= Calcitonin gene-related peptide; NKA= Neurokinin A; NKB= Neurokinin; VIP = Vasoactive intestinal polypeptide; PHI= Peptide histidine isoleucine; PHM=Peptide histidine methionine; NPY= Neuropeptide Y; CPON= C terminal peptide of NPY; SS-28= Somatostatin 28; BSA = Bovine serumalbumin; KLH= Keyhole limpet haemocyanin;BDB=Bis-diazobenzidene;CDI= Carbodiamide; GLUT= Glutaraldehyde;ND= Not done;

References: 1. Doran et al., 1983; 2. Mulderry et al., 1985; 3. Wharton et al., 1981; 4. Valantino et al.,1986; 5. Bishop et al., 1984; 6. Tatemoto et al., 1985; 7. Itoh et al., 1983; 8. Gulbenkian et al., 1985; 9. Gulbenkian et al., 1985; 10. Day et al., 1985

Pool et al., 1983).

2. 7. 1. Antiserum specificity

In the early, formative years of immunocytochemistry the primary antibody-antigen reaction was frequently assumed to be specific and somewhat taken for granted. The subsequent recognition of false positive results called for a more stringent approach. At present, the most generally accepted and convenient test for specificity of the primary antibody involves pre-incubation with the antigen to which it was raised and with any other possibly, cross-reacting antigen or related antigens. This is performed in solution (Liquid phase absorption) by adding an aliquot of diluted antiserum to the preweighed antigen (see below 2.7.2). Antisera of high titre obtained from hyperimmune rabbits, as used in sensitive immunocytochemical methods, generally require only very small, nanomolar quantities of antigen to inhibit the reaction. Thus, no visible or interfering precipitation is detected and the antigen antibody complex remains in the antiserum during the immunostaining. The range of decreasing concentration of different antigens can be compared to assess the degree of cross reactivity. Alternative methods to evaluate the primary antibodies involve the use of solid phase, artificial tissue-mimicking supports, such as filter paper or sepharose beads, to which specific polypeptides can be chemically fixed (Larsson 1981). By mechanical renewal of the solid support, the antibodies are removed bound to the antigen. In general, these more complex methods are used only in the event of failure of the simple pre-incubation method.

Immunostaining is always referred to as antigen-like immunoreactivity due to the indirect evidence provided by the immunocytochemical tests on the specificity of primary antibodies. Caution should be exercised for the immunolocalisation of peptides since the antigenic determinant may be as small as four or fewer amino acids and not necessarily at the same site in a molecule (Larsson, 1983). Many peptides have homologies in their aminoacid sequence and thus the possibility that a given antiserum raised against a specific peptide will cross-react with structurally related peptides must not be neglected. A number of reports have described the localisation of peptides which have subsequently proven to be quite different molecules (see Ju et al., 1986; Williams et al., 1987).

Table 3

A. SOURCE AND CHARACTERISATION OF PEPTIDE ANTIBODIES

Antigen raised to	Dilution IMF	Source	Absorption with synthetic antigen *									
			CGRP	SP	NKA	NKB	VIP	PHI	PHM	NPY	CPON	SS
CGRP (Syn. Rat)	1:200	H H	1.0	+	+	+	+	+	+	+	+	+
Substance P (Syn.Mammalian)	1:100	H H	+	1.0	±	±	+	+	+	+	+	+
NKA (15-36) (Syn.Mammalian)	1:400	K.V.	+	±	1.0	±	+	+	+	+	+	+
VIP (Porcine)	1:200	H H	+	+	+	+	1.0	+	+	+	+	+
PHI (Porcine)	1:400	H H	+	+	+	+	+	1.0	+	+	+	+
PHM (Porcine)	1:400	H H	+	+	+	+	+	+	1.0	+	+	+
NPY (Porcine)	1:400	H H	+	+	+	+	+	+	+	1.0	+	+
CPON (Syn.Human)	1:200	H H	+	+	+	+	+	+	+	+	1.0	+
SS-28 (Syn.Mammalian)	1:800	H H	+	+	+	+	+	+	+	+	+	1.0

B. SOURCE AND CHARACTERISATION OF PGP 9.5 ANTIBODIES

Antigen raised to	Dilution IMF	Source	Absorption with synthetic antigen								
			CGRP	SP	NKA	NKB	VIP	PHM	NPY	CPON	PGP 9.5 *
PGP 9.5 (Human)	1:800	Utraclone UK	+	+	+	+	+	+	+	+	1.0

Key:

CGRP= calcitonin gene-related peptide; NKA= neurokinin A; NKB= neurokinin B; VIP= vasoactive intestinal peptide; PHI= peptide histidine isoleucine; PHM = peptide histidine methionine; NPY= neuropeptide Y; CPON= C-terminal peptide of NPY; SS= somatostatin 28; ANP= atrial natriuretic peptide; HH= Hammersmith Hospital; KV= Dr. K. Valantino (USA). * = Homologous absorptions expressed as concentration of synthetic antigen required to abolish immunostaining (nmol/ml diluted antiserum); heterologous (cross) absorption at a concentration of 1nmol/ml diluted antiserum expressed as, + = no diminution of immunostaining; ± = partial diminution of immunostaining; ND= not done.

Table 4

Details of labelled secondary immunoreagents used in this study

Antibody to	Type	Donor	Reference	Dilution
Rabbit IgG FITC conjugate	Polyclonal	Sheep	Wellcome diagnostics	1:100
PAP complex	Polyclonal	Rabbit	Miles Scientific	1:100
Rabbit IgG unconjugated	Polyclonal	Goat	Miles Scientific	1:100

Key:

FITC= fluorecein isothiocyanate

PAP= peroxidase anti-peroxidase

2. 7.2. Antisera specificity controls

Rigorous specificity controls are essential to obtain reliable results. In this study liquid phase absorption/inhibition of the antiserum with homologous antigen prior to immunostaining has been used to test the antisera specificity. Adsorption or inhibition of antibodies requires their mixture with a pure and, if possible, synthetic antigen. Often only a very limited, precious supply of novel polypeptide antigen may be available therefore primary antibody should be used at the lowest concentration but should give a reliable, reproducible immunostain. In the liquid phase absorption/inhibition method the diluted primary antiserum is incubated for 16 hours at 4°C with corresponding antigen. This mixture is then applied to the tissue section as the first layer. A negative immunostaining result should be obtained if the antiserum is specific for its antigen. The antigen may be added to the diluted antiserum in a decreasing range of concentration (10nmol/ml to 0.001nmol/ml) in order to titrate the minimum amount required to abolish the immunostaining. Further characterisation of the primary antiserum can be achieved by similar incubation with related or unrelated antigens known to be present in the tissue. The degree of cross-reaction between closely related antigens can be indicated by comparison of the concentrations required to inhibit the immunostaining. With the availability of synthetic fragments of peptides, the regional specificity of antisera can be determined in the same way.

The characteristics of the antisera used in this study are summarised in **Table 3A & B & 4.** Antisera raised to substance P showed significant cross-reactivity with other tachykinins (neurokinin A and B) and vice versa. However, in this study the term substance P is used due to long standing usage, although, the nomenclature tachykinin-like immunoreactivity is more correct.

2. 7. 3. Method specificity

Method specificity includes removing the factors that cause background or false positive immunostaining. Several remedies can be used to remove or reduce such interference. Those routinely used in the present study are given below.

i) Use of primary antiserum (from hyperimmune donors) at the highest dilution giving optimum reproducible immunostaining so than any non-specifically reacting antibodies are removed by dilution.

ii) Pre-incubation of tissue sections with hydrogen peroxide to cause substrate inhibition of endogenous peroxidase sites.

2. 8. Conventional histology

To check orientation, preservation of tissue morphology and to demonstrate the localization of immunostained structures, some sections of all tissues investigated were stained with haematoxylin and eosin (**Appendix 7**). Morphological changes under pathological conditions were also determined.

2. 9. Acetylcholinesterase histochemical staining

In order to compare the enzyme histochemical method with the immunocytochemistry for the visualisation of cutaneous nerves,

acetylcholinesterase histochemistry was performed. Many variations of the original histochemical method (Koelle et al., 1949) are available; the method of choice was a modified method introduced by Tago and co-workers (1986). The staining involves a two step procedure. The first step requires a medium modified from that of Karnovsky and Roots (1964) for the AChE enzymatic reaction, to prevent overstaining. The second step is based on the procedure of Graham and Karnovsky (1966). For details of the procedure refer to the **Appendix 8.**

2.10. Fite stain for Mycobacterium leprae

Mycobacterium leprae is less acid-fast than other mycobacteria and its acid fastness is easily removed by treating it with pyridine while other mycobacteria are unaffected (Covict and Pinardi, 1972). Faraco demonstrated that by oiling the sections the acid fastness can be preserved (Faraco, 1938). Fite later modified the process of oiling the sections using a mixture of peanut oil and xylene during deparaffinization (Fite, 1967). In this study, the sections were deparaffinized by immersing in xylene and peanut oil mixture. The sections were then stained in Ziehl-Neelsen carbol fuchsin, decolourised in sulphuric acid and ethyl alcohol and counterstained with Harris' haematoxylin (**Appendix 9**).

2.11. Experimental procedures

Selective chemical denervations were performed to determine the subpopulations of nerves, using neurotoxic substances, including capsaicin (Jancso et al., 1977) and 6-hydroxydopamine. The resulting morphological changes were assessed immunocytochemically, comparing experimental and control tissues. Transplantation of skin was carried out to assess the neural and neuropeptide changes during reinnervation. To correlate the effects of leprosy on nerves in skin and spinal cord in relation to the duration of infection, nude mice were injected with Mycobacterium leprae and allowed to incubate for 6 and 12 months. To investigate possible early changes in diabetes, streptozotocin-induced diabetes of 2, 4, 8 and 12 weeks of duration in rats was used.

2.11.1. Capsaicin treatment

Capsaicin (8-methyl-N-vanillyl-6-nonenamide) is the active ingredient of chilli peppers. Neurochemical and biochemical studies have shown that capsaicin has effects on specific populations of primary sensory ganglia at virtually all cranial and spinal levels (Fitzgerald, 1983; Russel & Burchiel, 1984). Local application of capsaicin can produce analgesic effects on animals (Jancso, 1968) and decrease neurogenic inflammation involving sensory nerves (Gamse et al., 1980). Systemic administration of capsaicin in the neonatal rat, causes a selective, dose-dependent destruction of sensory neurones giving rise to unmyelinated (C) and myelinated (A delta) fibres while it does not affect efferent fibres of the autonomic nervous system (Jancso et al., 1977; Scadding, 1980; Nagy et al., 1981). In neonates (<10 days after birth) systemic administration of capsaicin induces a permanent degeneration of small dorsal root ganglion cells, in adults, the effect is reversible. Furthermore, perineural application, e.g., to the sciatic nerve of adult rats, apparently causes no degeneration of the central

terminals, but induces a longterm block of axonal transport of peptides in sensory fibres (Ainsworth et al., 1981; Gamse et al., 1982). These observations show that capsaicin-induced depletion of peptide not only enables one to establish that the peptide is sensory in origin, but also confirms the presence of the peptides in small primary sensory neurones which give rise to A alpha and C fibres.

In this study neonatal rats were treated with capsaicin (Fluka AG) in order to produce a permanent destruction of primary afferent neurones. They received either one (50mg/Kg intraperitoneally on day 2 after birth) or two (50 mg/Kg on day 2 and 3 after birth) injections of capsaicin under light ether anaesthesia. Capsaicin was emulsified in a vehicle solution (10mg/ml) containing 10% (v/v) ethanol, 10% (v/v)

Tween 80 and 80% (v/v isotonic saline. Control animals were injected with vehicle alone. The pups were weaned and sexes separated at 3 weeks of age and tissues were collected 6-11 weeks after the last injection.

2. 11. 2. 6-hydroxydopamine treatment

6-OHDA is a drug the which causes a selective degeneration of aminergic neurons (Thoenen & Tranzer, 1968; Kostrzewa & Jacobowitz, 1974) producing a similar effect to that of surgical sympathectomy. In this study 6-OHDA adminstration was carried out in order to confirm or exclude the possible localisation of the investigated peptides in sympathetic nerves. Adult guinea pigs were injected intraperitoneally with a fresh solution of 6-OHDA hydrobromide (Sigma) in saline, containing 0.1% ascorbic acid, 10 days (300mg/Kg) and 2 days (200mg/Kg) prior to sacrifice. Control animals received 2 injections of a saline solution containing 0.1% ascorbic acid over the same period. The success of the treatment was assessed by negative immunostaining for aminergic (TH positive) nerves.

2. 11. 3. Transplantation of skin flap

The operative area was washed with Savlon. Operation was performed using aseptic precautions. A rectangular flap 1cm in length, with a base of 0.5 cm on the mid dorsal region, was cut and separated from the subcutaneous tissue and panniculus carnosus and then replaced in the same position. All grafts were sutured with 6.0 silk and no protective dressings applied. Mice showing seroma or haematoma were not included in the study.

2. 11. 4. Injection of M.leprae into foot pads

Colston and Hilson in 1976 reported the enhanced growth of Mycobacterium leprae in congenitally athymic " Nude" (nu/nu) mouse, although the survival rate was very low. For the present study, the innoculation was carried out in a nude mice colony maintained at St. George's Medical College under the supervision of **Dr. D.K. Banerjee**. Nude mice received an innoculation of suspension of Mycobacterium leprae (3.6×10^6) in 0.1ml of saline into the hind foot pads. The animals were maintained successfully under specific pathogen free conditions in a germ free isolator. The temperature was maintained at 26-28°C, due to the vulnerability of mice to dehydration. Controls received no treatment, but maintained under same

conditions. Both groups were maintained for a period of 12 months. At the end of the experiment, animals were fixed by perfusion (Appendix 1).

2. 11. 5. Treatment of streptozotocin

Diabetes was induced in mature male Wistar rats by intravenous injection of streptozotocin (60mg in 0.9% saline /kg body wt) via a tail vein. The injection was carried out in ICI Pharmaceuticals Division, Alderly Park, Macclesfield, Cheshire, under the supervision of Dr. D.J. Mirrlees. Controls were given an equivalent quantity of physiological saline. All animals had free access to a standard laboratory diet and water and were weighed at weekly intervals. Diabetic rats developed glycosuria in excess of 2% within 24h of adminstration of Streptozotocin and progressively lost weight. Both groups were maintained for a period upto 3 months. At the end of the experiment animals were sacrificed by carbon dioxide euthanasia and tissues were fixed by immersion fixation. The onset of diabetes was established after 24h by the presence of polyuria and glycosuria.

2. 12. Semiquantitative assessment of immunostaining

The immunoreactive fibres present in the skin supplying epidermis, dermis, appendages and blood vessels were graded visually. The grading ranges from absence (-), very few (±), few (+), moderate (++), many (+++), and abundant (++++). Intensity of the staining was also taken into account. In any assessment at least three sections were studied and also by an observer who was unaware of the specimen group. In a comparative analyses control and test specimens were mixed in batches and a minimum of four animals in each group was studied.

2.13. Quantification

The Kontron IBAS-2000 image analysis system (Fig. 2.1) used in this study, uses a TV camera mounted on top of a microscope to scan an image which is converted to a format suitable for computer processing. The light intensity at each point in the image is converted to a number between 0 and 255 (a grey value), which represents the lower and upper limits of measurable incident light on the TV tube. Thus, O is equivalent to black and 255 to white. The stored digital image therefore consists of a square matrix of numbers, which results from sampling the image at 512 points along each of 512 scan lines (i.e., 512 x 512 = 262144 pixels). Separation of regions of staining based on differences in optical density can be done by setting two threshold values between 0 and 255 inclusive, such that all pixels in the image which have grey values within the window defined by the threshold values are highlighted. By counting the number of highlighted pixels, an estimate of the area of staining with intensities (grey values) between the two thresholds can be made. If a lower threshold is selected so as to have a density greater than the overall background staining but lower than that of the specific staining, the area measured will correspond to the area of specifically stained structures.

To quantitate the area of immunoreactivity in the dorsal horn of spinal cord (Fig. 2.2) of leprosy mice, five sections from the L4 spinal cord segments were used. The area occupied by immunoreactive fibres in

laminae I-III of both sides of the spinal cord (Fig. 2.1) was then measured. Selecting a 10x objective on the microscope an image field which contained one dorsal horn was digitised. The image was taken 20 times and averaged for image analysis (i.e., each pixel in the final image represents the mean of 20 consecutive readings for that pixel) to reduce the errors caused by TV camera noise. The background illumination was kept constant for each slide by adjusting the microscopic illumination level so that the transmitted light through an empty slide was always set to a grey value of 255. The empty slide image was also used to correct for any variation in illumination over the field. This variation was assumed to be constant, as it is a function of the microscope. The correction is achieved electronically by proportionally increasing the grey value of any pixel areas of lower illumination to a grey value of 255. During subsequent measurements of images, those corresponding pixels are also increased by the same, amount. A graticule was also used to calibrate screen measurements thereby obtaining a scaling factor for the analysis. Thus the area was measured in absolute rather than arbitrary units.

The area of immunoreactivity was separated from the unstained and non-specific background by subjective selection of a threshold grey level so as to have a optical density greater than the overall background staining but lower than that of the specific staining. That threshold level was kept constant for all the sections taken from control and treated pairs of animals. The stained area was measured in absolute units. The magnitude of differences between groups was calculated by comparing the mean stained areas for each group at 6 and 12 months.

2. 13.1. Statistical analysis

The immunoreactivity of treated and control animals was compared by the paired t test and confidence intervals were calculated. The paired t test is suitable for small samples sizes in which the differences between the paired control and M. leprae treated animals (MTM) show a normal distribution. In order to apply this procedure, the differences between control and MTM values were first calculated, followed by their mean and standard error of the mean (SEM). The t statistic is the ratio of the mean difference to its standard error. To obtain the precision of the estimate of the mean difference, 95% confidence limits were estimated (as mean difference ± 3.18 SEM where 3.18 is the appropriate factor for a sample of size 4) which give the range of values within which the true population mean difference is likely to lie.

Statistical formulae:

Let n = number of paired observations, then

1. $SD = \sqrt{\frac{\text{Sum of squared differences from mean}}{n-1}}$

2. $SEM = \frac{SD}{\sqrt{n}}$ and the t statistic is $= \frac{\text{mean difference}}{SEM}$

2. 14. Microscopy and Photography

Fluorescence preparations were screened in an Olympus AH-2 Vanox microscope with an HBO 200 WZ lamp (Olympus Optical Co. Ltd,

Honduras St. EC1). For fluorescein isothiocyanate a DM 500 dichromic mirror was used with a B-P 490 exciter filter and an AFC+ 0515 barrier filter. Preparations immunostained for transmitted light were examined using a Reichart-Jung Polyvar microscope equipped with Nomarski optics. Photographs for transmitted light microscopy were taken with Kodak, Technical Pan 2415 film, developed in Ilford Contrast FF. For immuofluorescence microscopy, Kodak Tri-X film was used and developed in Paterson Acutol. The photographs were printed on Kodak F5 or Ilford Multigrade paper and developed in Suprol (May & Baker, UK).

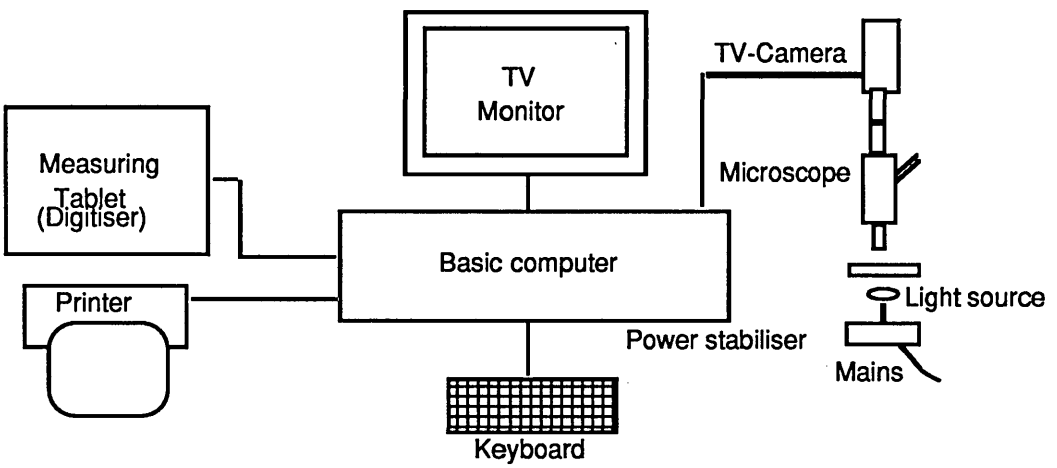


Fig. 2.1. Kontron IBAS-2000 system configuration.

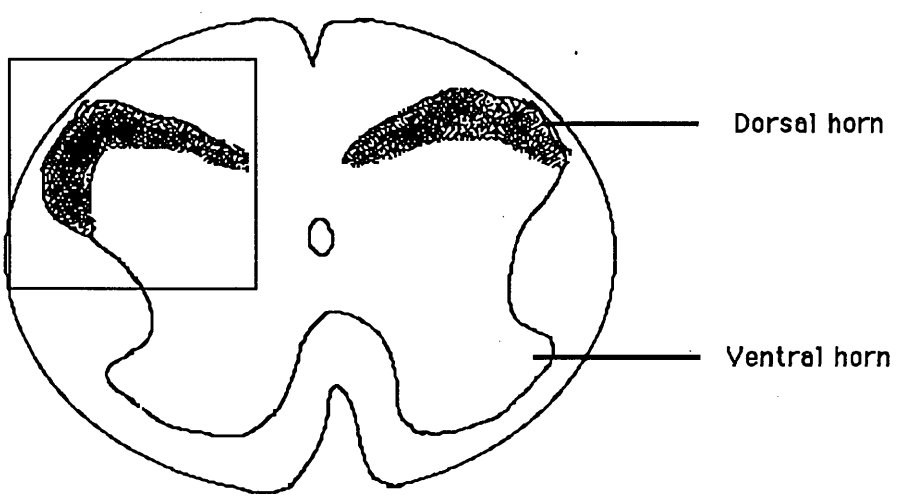


Fig. 2.2. Tranverse section of spinal cord showing the area of dorsal horn (hatched) used for quantification. The square area indicates approximately the region of the spinal cord chosen for image analysis.

Chapter 3

GENERAL DISTRIBUTION OF NERVES AND NEUROPEPTIDES IN SKIN OF FIVE MAMMALIAN SPECIES AND THEIR ONTOGENY IN RAT

3. 1. Introduction 66-71

3. 2. Experimental design 72-75

3. 2. 1 Tissues 72

3. 2. 1. 1. Adult 72

3. 2. 1. 2. Fetal and neonatal 72

3. 2. 2. Fixative 72-73

3. 2. 3. Chemical denervations 74

3. 2. 3. 1. Capsaicin treatment 74

3. 2. 3. 2. 6-hydroxydopamine (6-OHDA) treatment 74

3. 2. 4. Immunocytochemistry 74-75

3. 2. 5. Consecutive immunofluorescence and acetylcholinesterase staining 75

3. 2. 6. Quantitation 75

3.3. Results 75-98

3. 3. 1. Comparison of general neural markers 75-84

3. 3. 2. AChE staining and its comparison with immunocytochemical markers 85

3. 3. 3. Species similarities/differences 86-92

3. 3. 3. 1. Tachykinins (Substance P and Neurokinin A) 86-88

3. 3. 3. 2. Calcitonin gene-related peptide 86-89

3. 3. 3. 3. Vasoactive intestinal polypeptide and Peptide histidine isoleucine 90-91

3. 3. 3. 4. Neuropeptide Y and C-flanking peptide of neuropeptide Y 92

3. 3. 3. 5. Other peptides 92

3. 3. 4. Regional variations 92-93

3. 3. 5. Effects of capsaicin and 6-OHDA 93-94

3. 3. 6. Development of cutaneous nerves 95-98
3. 3. 7. Appearance of peptides in cutaneous nerves 95,98
3. 4. Discussion **98-108**
3. 4. 1. PGP 9.5 as a neural marker 98-101
3. 4. 2. Cholinesterase histochemistry 101-102
3. 4. 3. Species variations 103-104
3. 4. 4. Effect of anaesthesia on general markers and neuropeptides 103-104
3. 4. 5. Regional variations 105-106
3. 4. 6. Capsaicin and 6-OHDA treatment 105-107
3. 4. 7. Development of cutaneous nerves 107-108

3. 1. Introduction

Skin and its appendages evidence regional morphological specialisation as well as interspecies variability (See Montagna and Parakkal 1974). The innervation of skin is also regionally specialized and differs from species to species (Ritzius, 1892, Munger, 1971, Halata, 1975). With the advent of immunocytochemistry, considerable advances have been made in the precise localisation of neural elements in skin, using antisera to general markers and neuropeptides, which are believed to be neurotransmitters/neuromodulators, and of classical transmitter synthesising enzymes (Bloom and Polak, 1983; Dalsgaard et al., 1983; O'Shaughnessy et al., 1983; Hartschuh et al., 1984; Bjorklund et al.,1986)

To study the cutaneous innervation, the antisera to neural markers used (Dalsgaard et al., 1983; Bjorklund et al., 1986) were raised to neurofilaments, neuron-specific enolase and the Schwann cell marker, protein S-100. In addition, the antisera of choline acetyltransferase (ChAT) (Leblanc and Landis., 1986) and tyrosine hydroxylase (TH) (Bjorklund et al., 1986), which are the enzymes involved in the synthesis of acetylcholine and catecholamines respectively, were used to study the distribution of cutaneous nerves containing classical transmitters.

The peptides found or localised in sensory and autonomic components of the mammalian cutaneous innervation by immunocytochemistry and/or radioimmunoassay are summarised in diagram 5. These peptides may play an important role in regulation of cutaneous responses. Substance P is suggested to be a primary sensory neurotransmitter or neuromodulator (Lembeck, 1953) and to be involved in the transmission of nociceptive information to the central

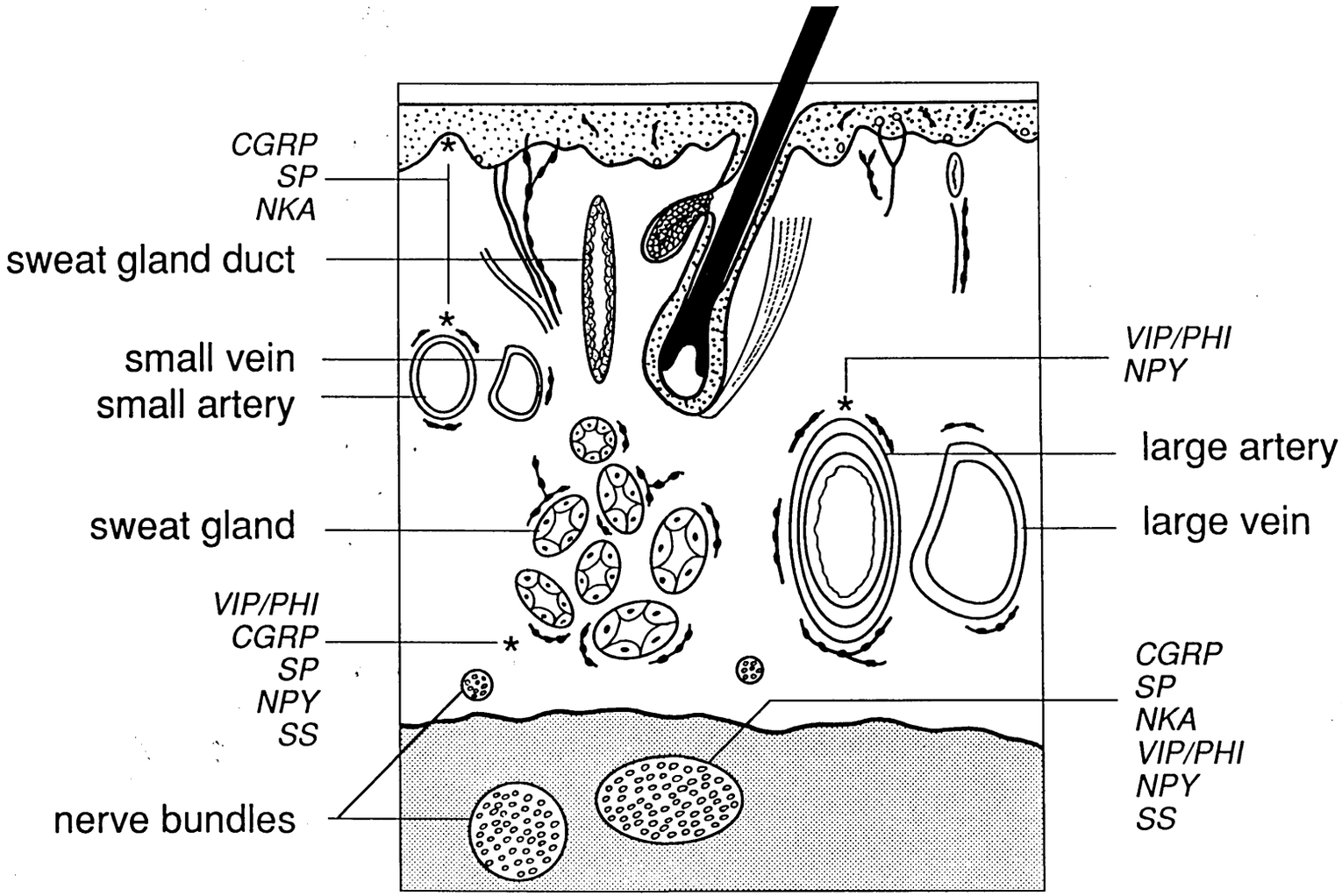


Diagram 5. Skin showing localisation of neuropeptides incutaneous nerves. SP= substance P; CGRP= calcitonin gene-related peptide; NKA= neurokinin A; VIP= vasoactive intestinal polypeptide; PHI= peptide histidine isoleucine; NPY= Neuropeptide Y; CPON= C-terminal peptide of NPY; SS= Somatostatin 28

nervous system via primary sensory neurones (Pernow, 1983). Intradermal injection of calcitonin-gene related peptide (CGRP) with either substance P or neurokinin-A produces the "triple response" (Wallengren et al., 1987). CGRP by itself increases the blood flow of skin (Brain et al., 1985). Somatostatin has an inhibitory effect on the neurogenic vasodilatation and plasma extravasation caused by substance P (Lembeck et al., 1982). Vasoactive intestinal polypeptide (VIP) is a potent vasodilator and oedema potentiator in skin (Williams, 1982; Thom et al., 1987). Neuropeptide Y (NPY) has been shown to have a vasoconstrictor effect in arteries and veins of the forearm in vivo (Lundberg et al., 1982; Pernow et al., 1986). Little is known about the action of C-flanking peptide of neuropeptide Y (CPON), atrial natriuretic peptide (ANP) and galanin on skin.

In spite of many immunocytochemical studies of the cutaneous innervation and associated peptides **(Table 5)**, the following questions remain to be answered:

1. Which of the immunocytochemical general neural markers recognise most of the neuronal elements present in skin?

2. Are there differences between species in the peptides that are present?

3. Are there differences in the regional (i.e., body area) peptidergic innervation at maturity or during development?

4. Are specific peptides related to the various appendages of skin such as hair follicles, sweat glands and blood vessels, and does the specificity vary between species?

Table 5

Reported presence of neural antigens (general and neuropeptides) in cutaneous nerves of various species

Skin areas	Pig	Cat	Man
Epidermis	SP2	SP2	NF3
	CGRP[2]	CGRP[2]	NSE[3]
	VIP1		CGRP[4]
			SS[4,7]
Dermis	SP[1, 2]	SP[1,2]	NF[3]
			NSE[3]
			SP/NKA[3]
			CGRP[3,4]
			VIP/PHI[8,10]
			NPY[5]
			GAL5 SS[6,7]
Sweat glands	VIP[1,2]	VIP[1,2]	NSE[3]
			TH[11]
			NPY[3,7,11]
			VIP[6,8]
			CGRP[6,10]
			SP[6,10]
SS[6] ANP[10]			
			GAL[10] CAT9
			ChAT[13]

Contd.....

Contd.....

Skin areas	Pig	Cat	Man
Blood vessels	VIP[1,2,8]	VIP[1,2,8]	NSE[3,6] NPY[3,7] CGRP[6] SP[4]
Hair follicles			CGRP[6] VIP[3] SS[6]
Nerve bundles			NSE[3] VIP[3] CGRP[3] SP[3,10] GAL[7,10] ANP[7]

Key: References: 1. Bloom & Polak, '83. 2. O' Shaughnessy et al., '83. 3. Bjorklund et al., '86. 4. Gibbins et al., '87. 5. Johansson et al., '88. 6. Dalsgaard et al., '83. 7. Johansson & Vaalasti, '87. 8. Hartschuh et al., '83, 9. Leblanc & Landis, '86. 10. Tainio et al., '87. 11. Tainio et al., '86. 12. Vaalasti et al., '85. 13. Leblanc and Landis, '86.

5. When do cutaneous nerves appear and when do they express their peptide phenotype?

6. Are certain peptides present, specific to either the sensory or autonomic parts of the cutaneous nervous system?

The main purpose of the present study was to identify the best general neural marker for cutaneous innervation and to carry out a systematic survey of the distribution of peptides present in the cutaneous nerves of various animals including man, and the ontogeny of nerves and peptides in the skin. In addition, the following previously unreported issues have been considered:

1. The efficiency of PGP 9.5 as a marker for the effective visualisation of various sub-populations of cutaneous nerves in all the species under study. PGP 9.5 is a novel neuron-specific protein (Jackson and Thompson, 1981; Doran et al., 1983), shown to have a widespread distribution in the central and peripheral nervous system.

2. The value of synaptophysin as a marker for the visualisation of cutaneous nerve endings in all these species. Synaptophysin is a glycoprotein of molecular weight 38 KD isolated from the membrane of pre-synaptic vesicles (Wiedemann and Frank, 1985). It can be used as a marker for visualisation of presynaptic vesicles.

3. The comparison of a modified acetylcholinesterase method (Tago et al., 1986) and immunocytochemical visualisation of nerve fibres using general markers and antisera to choline acetyl transferase (ChAT).

3. 2. Experimental design

3. 2. 1. Tissues

3.2.1.1 Adult: Skin samples from rats (Wistar; 150-300g), guinea pigs (Dunken Hartley; 300-600g), pigs and cats (n=5 for each species) were dissected from nose/snout, lip, fore- and hind-limbs, feet, dorsal and ventral regions of the abdomen, and tail region. Biopsies of human skin were taken from various regions of the body **(Table 6)** from 50 individuals with no skin diseases undergoing surgical procedures that entailed skin removal in Central Middlesex Hospital (Approved by the Ethics committee of Brent Heads Authority) and cosmetic plastic surgery operations in Hammersmith Hospital and Queen Mary's Hospital, London.

3.2.1.2 Fetal and Neonatal: Male and female Wistar rats were caged together and the day when the vaginal plug was seen was numbered as day 1. Fetal rats (n=5 of each age) were obtained by Caesarean section from these time-mated rats killed with intraperitoneal injection of sodium pentabarbitone (Euthatol, May & Baker Ltd. 40 mg/kg) on the 12th to 21st day of gestation. Birth usually occurred on gestation day 22, which was considered as postnatal day 1. Neonatal rats (n=5 of each age) were killed by the same method on the day 1, 7 and 15 after birth. Tissues were taken from the same areas as those from adults.

3. 2. 2. Fixative

All tissues were fixed in Zamboni's fluid (Zamboni and de Martino, 1967)(**Appendix 3C**) either by immersion or transcardial perfusion (**Appendix 1**) followed by immersion. Further specimens were also fixed by immersion in Bouin's fluid (**Appendix 3D**), formalin in distilled water (**Appendix 3B**) and 0.4% p-benzoquinone (**Appendix 3A**) in order to compare and select the best fixative.

Rats (neonatal and adult) and guinea pigs were perfused transcardially with Zamboni's fluid at room temperature. Skin tissues were dissected and immersed in the same fixative for 3 hours to complete the fixation. Tissues taken from pig, cat, and man were fixed by immersion in Zamboni's fluid at room temperature for 6 hours. Fetal rats were fixed by immersion in toto in the same fixative for 3 hours.

Table 6

Regions from which specimens of human skin obtained

Regions	No. of specimens
Face	5
Nose Skin	3
Ear lobe	3
Lip	5
Eye lid	1
Scapular region	4
Axilla	3
Upper arm	4
Breast	3
Abdomen	8
Thorax	5
Thigh	3
Palm	3

3. 2. 3. Chemical denervations

In order to delineate the sensory and sympathetic portions of the cutaneous innervation, neonatal rats (Wistar; 1-3g: n=10) of either sex were treated with either capsaicin or 6-hydroxydopamine.

3.2.3.1 Capsaicin treatment: Neonatal rats (n-10) were treated with capsaicin (Fluka AG) on day 2 and 3 after birth, under light ether anaesthesia. Capsaicin was emulsified (10mg/ml) in a mixture of vehicle containing 10% (v/v) ethanol, 10% (v/v) Tween 80 and 80% (v/v) isotonic saline. Control animals (n=10) received an equal volume of vehicle only. Tissues were collected from transcardially perfused animals, 6-11 weeks later.

3.2.3.2 6-hydroxydopamine (6-OHDA) treatment: Adult guinea pigs (n=5) were lightly anaesthetised with ether and injected intraperitoneally with a fresh solution of 6-OHDA hydrobromide (Sigma) in saline, containing 0.1% ascorbic acid. Tissues were collected 10 days later from transcardially perfused animals.

3. 2. 4. Immunocytochemistry

All tissues were thoroughly washed and stored in PBS (**Appendix 2**). Frozen tissue blocks were prepared and cryostat sections (10μm thick) were taken up on poly -L-lysine coated glass slides (**Appendix 4**) and allowed to dry for 60 minutes at room temperature. Sections were stained by an indirect immunofluorescence method (**Appendix 5a**) with antisera to PGP 9.5, synaptophysin, neurofilaments, NSE and tyrosine hydroxylase and ChAT for the visualisation of cutaneous nerves, and antisera to neuropeptides including two tachykinins (substance P and neurokinin A), CGRP, VIP, PHI(M), NPY/CPON, ANP, galanin and somatostatin.

3. 2. 5. Consecutive immunofluorescence and acetylcholinesterase staining

Sections stained by immunofluorescence with antibodies to PGP 9.5, synaptophysin, neurofilaments and NSE were photographed and the same sections were then used for simultaneous demonstration of AChE activity by a modified AChE method (Tago et al., 1986) (**Appendix 8**). The same areas of each section were then re-photographed for comparison with the immunofluorescence.

3. 2. 6. Quantitation

The number of immunoreactive fibres in three consecutive sections was assessed visually and also by an observer who was unaware of the specimen group. Comparison was made by blind analysis and the results expressed as absent (-), very few (+), moderate (++), many (+++) and abundant (++++). Observations were made in relation to each of the following regions and appendages; (1) epidermis, (2) dermis, (3) hair follicles, (4) sweat glands, (5) blood vessels, (6) nerve bundles in the dermis and hypodermis.

3. 3 RESULTS

3. 3. 1. Comparison of general neural markers

The comparison of immunostaining and AChE method in all the species is summarised in Table 7. Representative examples of AChE histochemistry and immunostaining in pig, and immunostaining in man are shown in Figs 3.1 to 3.7. The antiserum to PGP 9.5 showed more immunoreactive nerve fibres than did the antisera to neurofilaments, NSE, and synaptophysin, in all of the species. PGP 9.5-immunoreactive nerve fibres were seen in the epidermis (Fig. 3.1A), dermis (Fig.3.3A), around sweat glands (Figs.3.2A & 3.3D) and blood vessels (Fig.3.3C), hair follicles (Fig.3.3B) and arrector pilorum. Immunostaining was seen in nerve fascicles and individual fibres of both varicose and smooth types. In addition, immunoreactivity was seen in axons and nerve endings supplying Merkel cells and Meissner's corpuscles. In all

Table 7

SUMMARY OF THE COMPARISON OF IMMUNOSTAINING FOR GENERAL MARKERS AND ChAT, AND ACETYLCHOLINESTERASE HISTOCHEMISTRY IN PIG, GUINEA PIG, CAT, RAT AND MAN, SHOWING THE POTENTIAL OF PGP 9.5 IN IDENTIFYING NERVES IN VARIOUS REGIONS OF THE SKIN

Antigen	Epidermis	Dermis	Sweat glands	Blood vessels	Hair follicles	Nerve budles
PGP 9.5	++++	++++	++++	++++	++++	++++
NSE	+	++	+	-	++	++
NF	+	++	+	+	++	++
SYNAP	+	+	++++	+++	++	-
AChE histochemistry	++	+++	++++	+++	++	++
ChAT	-	-	-	-	-	-

Nerve fibres immunoreactive for general markers and AChE histochemistry seen in all the species are graded: absent (-), few (+), moderate (++), many (+++), abundant (++++). PGP 9.5= protein gene product 9.5; NSE= neuron-specific enolase; NF= neurofilaments; SYNAP=synaptophysin; AChE=acetylcholinesterase staining; ChAT= cholinacetyltransferase.

the species studied the immunoreactivity appeared to be both qualitatively and quantitatively suitable for the visualisation of cutaneous nerves in comparison with other neural markers.

In comparison with PGP 9.5 staining, fewer neurofilament-immunoreactive nerve fibres were seen in all of the layers and around all the appendages of the skin. The individual fibres, either varicose or smooth, were sparse in epidermis and in the papillary and reticular layer of the dermis (Figs.3.1B & 3.4A) Weak staining was observed around sweat glands (Fig.3.2B & 3.4D) However, intensely stained, large diameter immunoreactive nerve fibres, both individual and in small groups, were seen supplying the hair follicles (Fig.3.4C) and also occasionally in the papillary and reticular layer of dermis (Fig.3.4A). Blood vessels were rarely seen to be supplied by nerve fibres immunoreactive for neurofilaments (Fig.3.4C). NSE antiserum revealed more individual cutaneous nerves than did the antiserum to neurofilaments, but their density was still far less than that of nerves immunoreactive for PGP 9.5 (Fig.3.5A,B,C,D). Nerve endings supplying the Merkel cells and Meissner's corpuscles (Fig.3.5A) were immunoreactive for NSE. However, NSE-immunoreactive varicose nerves were fewer in number and the intensity of staining differed from species to species.

The antiserum to synaptophysin stained dense networks of nerve endings around sweat glands (Fig. 3.2D & 3.6D) and blood vessels (Fig.3.6C) of all sizes and the density was almost as great as that of PGP 9.5 immunoreactivity. Synaptophysin-immunoreactivity was also seen in the nerve endings and terminal axons supplying the Merkel cells, Meissner's corpuscles (Fig.3.6A) and to a lesser extent in hair follicles (Fig.3.6B). Epidermal nerve endings were few when compared with those visualised with antiserum to PGP 9.5. Axons present in

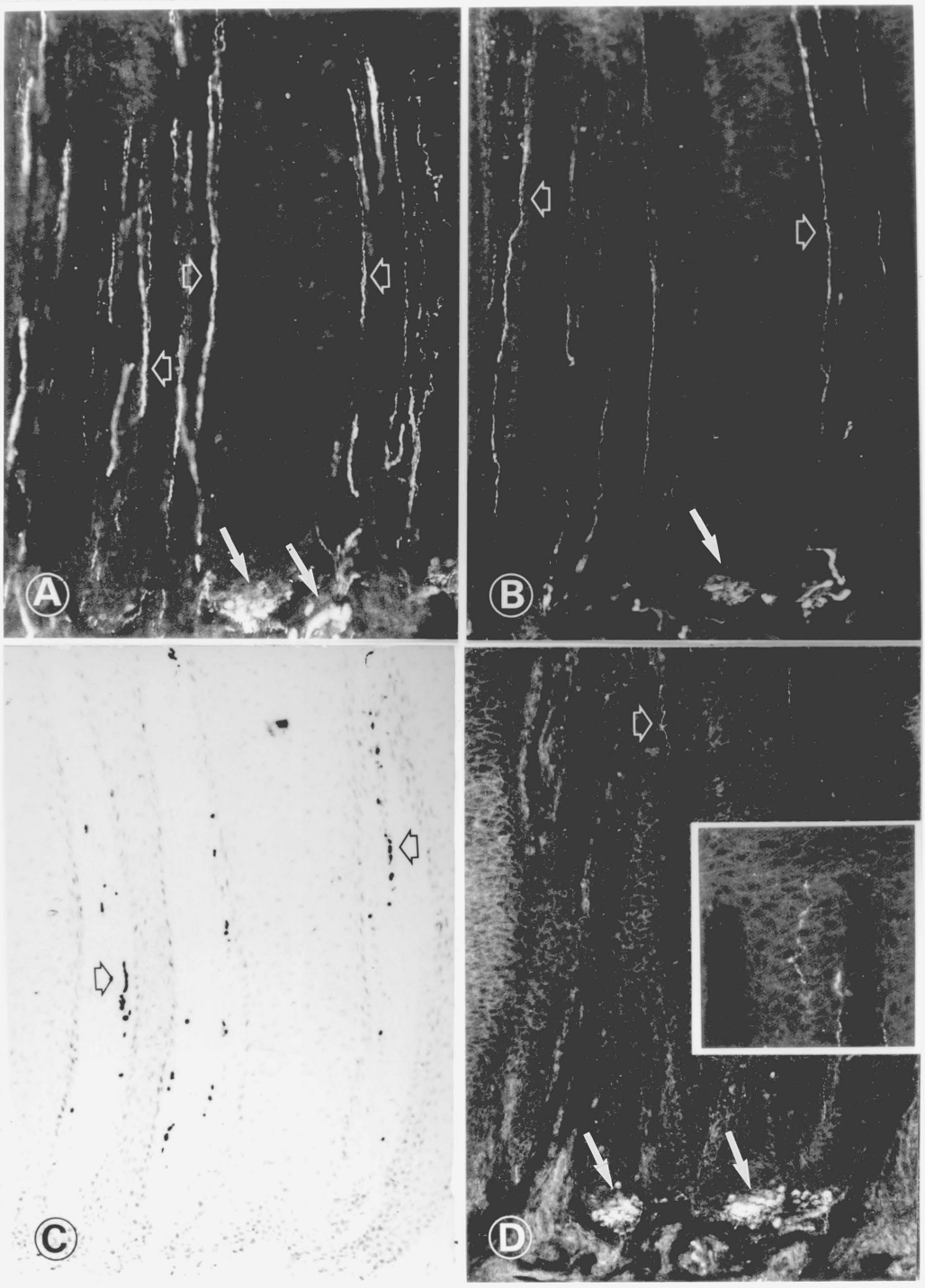


Fig. 3.1. The distribution of epidermal nerves (arrow head) in pig snout; serial sections using immunofluorescence method. (A) PGP-immunoreactive fibres; (B) NF-immunoreactive fibres; (D) Synaptophysin-immunoreactive fibres; of the skin from pig snout. (C) showing AChE positive fibres in the same section as (A). Compare the density of fibres in all the sections. Arrows indicate some of the Merkel cells immunoreactive for PGP 9.5 (A), NF (B) and Synaptophysin (D). Zamboni's fixed tissues. Cryostat sections (15µm). All magnification x140; insert x220

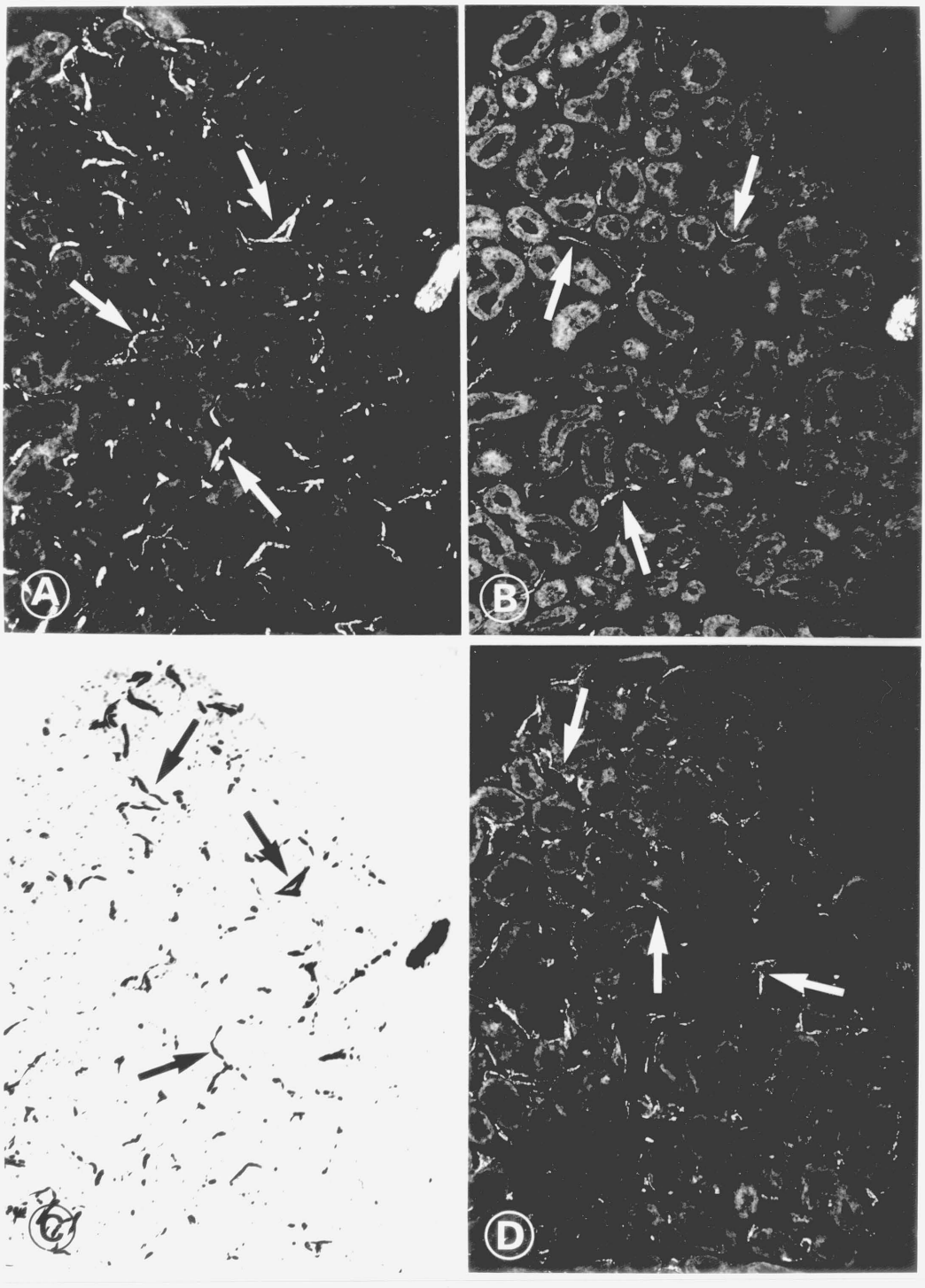


Fig. 3.2. The distribution of nerve fibres (Arrow) around sweat glands of pig in serial sections using immunofluorescence method. A) PGP-immunoreactive fibres; (B) NF-immunoreactive fibres; (D) synaptophsyin-immunoreactive fibres; (C) Showing AChE positive fibres in the same section as (A). Compare the density of fibres in all the sections. Zamboni fixed tissues. Cryostat sections (15μm). All magnification x 140

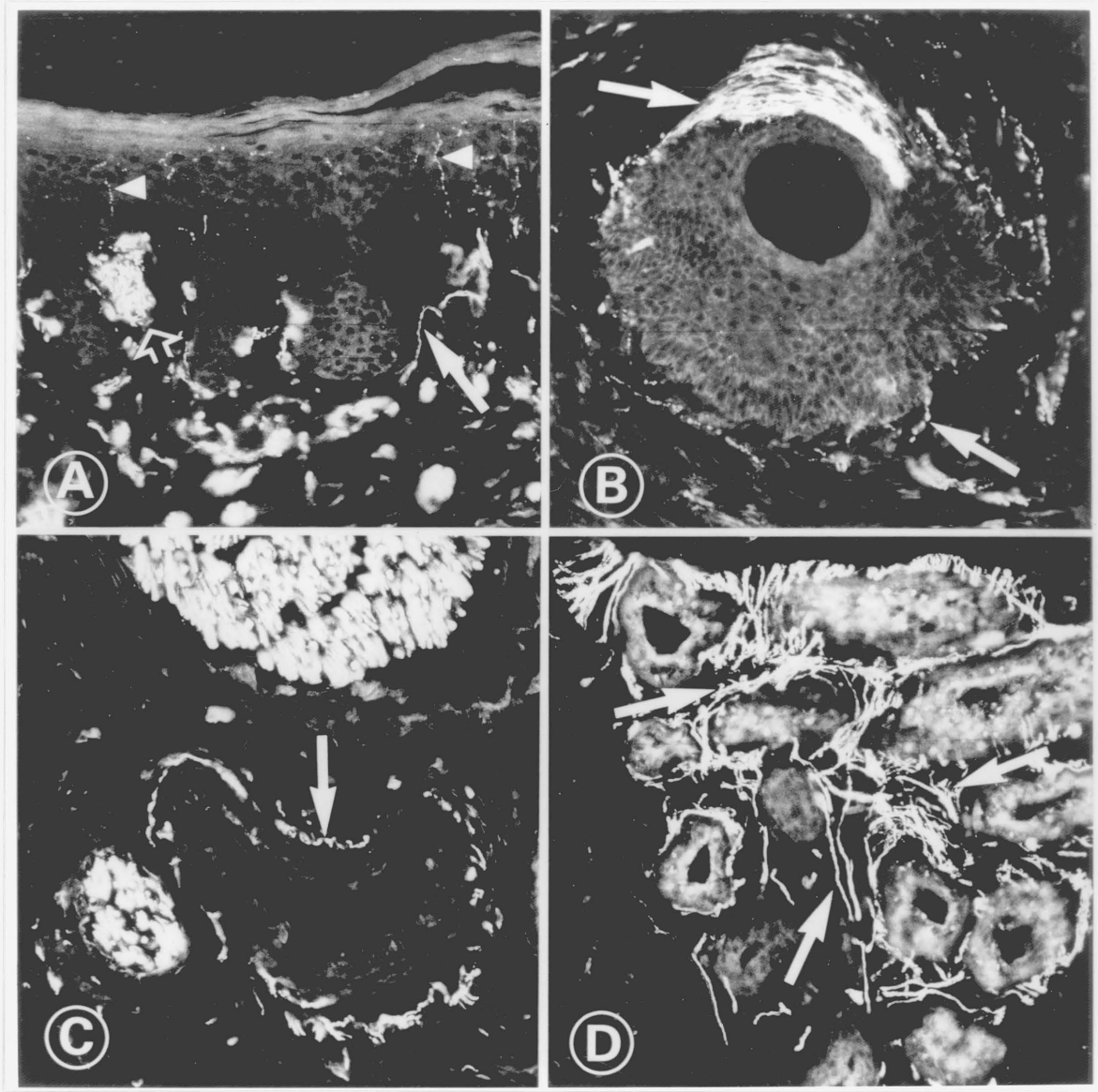


Fig. 3.3. Arm skin of man showing PGP 9.5-immunoreactive fibres in epidermis and dermis (A), around hair follicle (B),blood vessel and nerve bundle (C) and sweat glands (D). Arrow heads and arrows indicate some of the immunoreactive axons. Empty arrow indicate Meissner's corpuscles. Note the density of immunoreactive fibres. Zamboni fixed tissues. Cryostat sections (10μm).
All magnification x 250

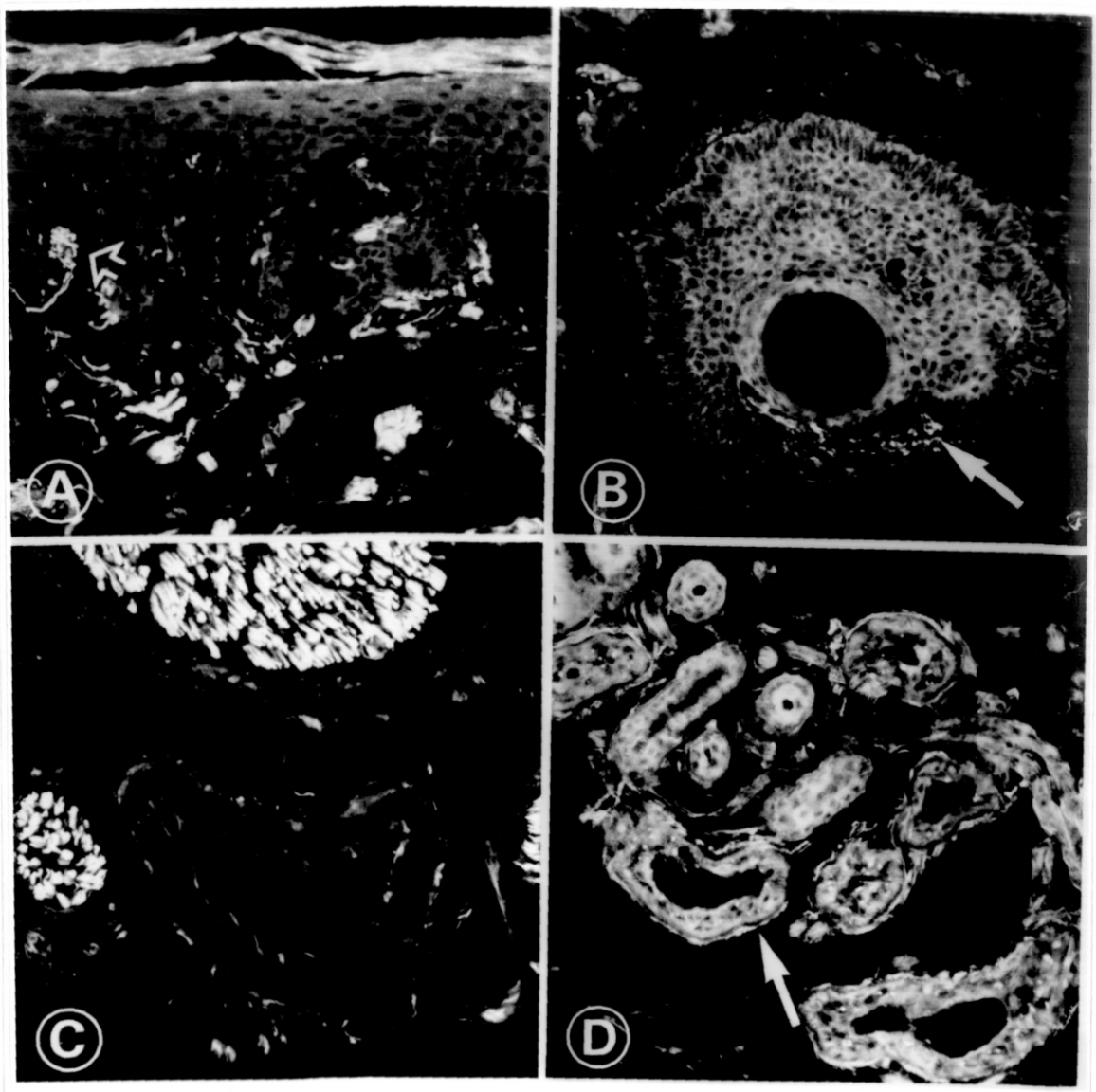


Fig. 3.4. Arm skin of man showing NF-immunoreactive fibres in epidermis and dermis (A), around hair follicle (B), blood vessel and nerve bundle (C) and sweat glands (D). Arrow heads and arrows indicate some of the immunoreactive axons. Empty arrow indicate Meissner's corpuscles. Note the density of immunoreactive fibres. Zamboni fixed tissues. Cryostat sections (10μm).
All magnification x 250

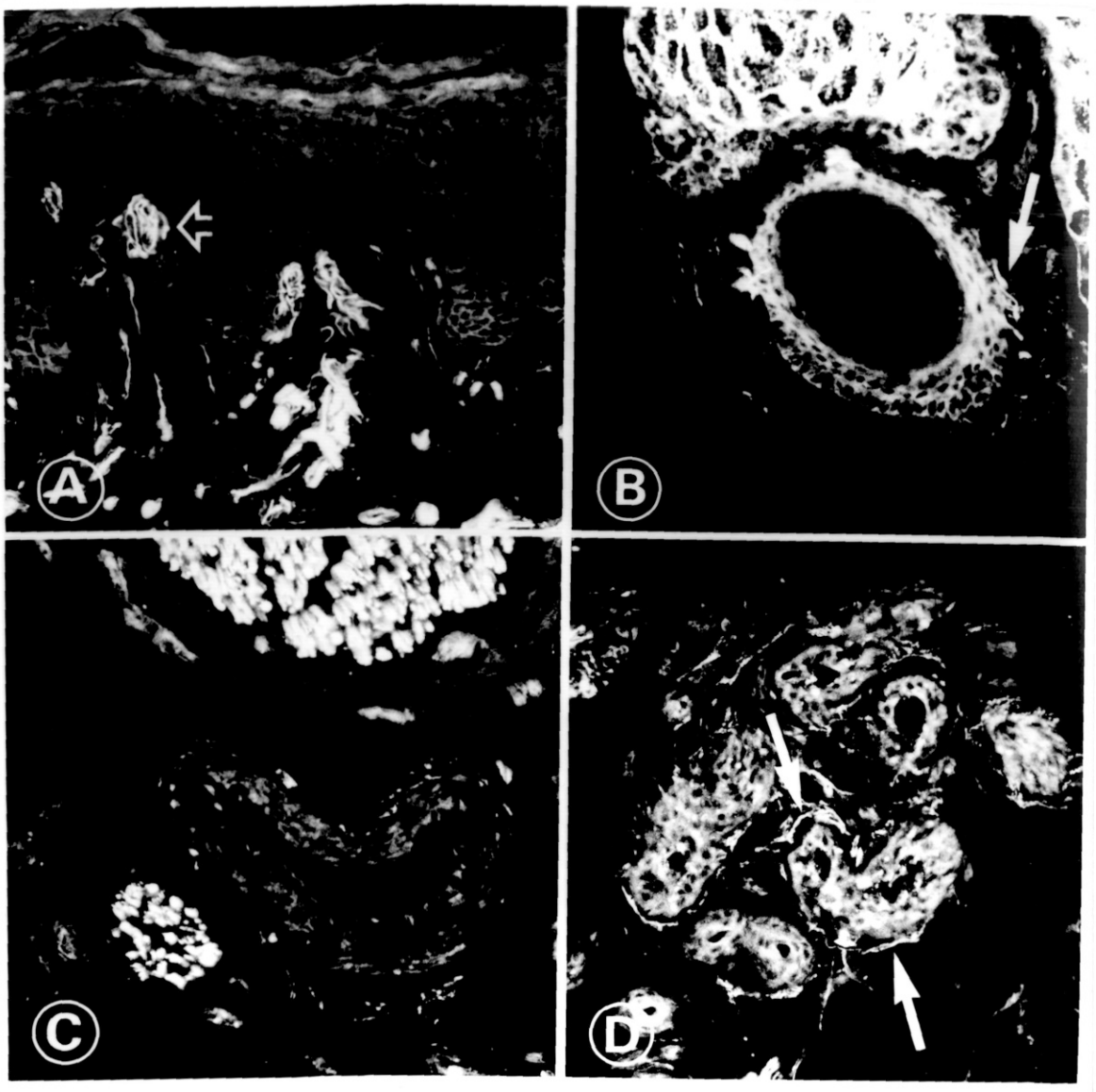


Fig. 3.5. Arm skin of man showing NSE-immunoreactive fibres in epidermis and dermis (A), around hair follicle (B), blood vessel and nerve bundle (C) and sweat glands (D). Arrow heads and arrows indicate some of the immunoreactive axons. Empty arrow indicate Meissner's corpuscles. Note the density of immunoreactive fibres. Zamboni fixed tissues. Cryostat sections (10μm).
All magnification x 250

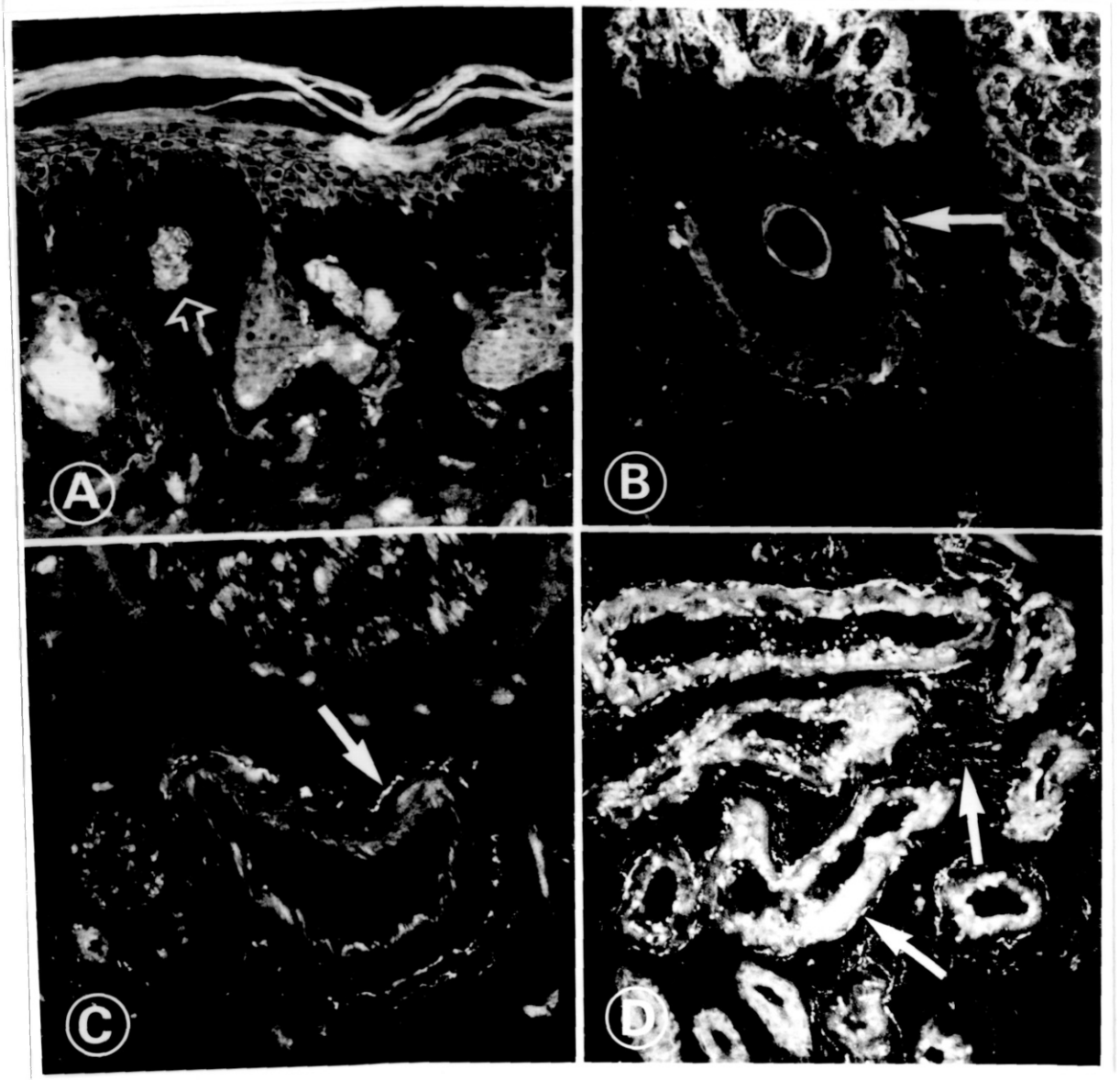


Fig. 3.6. Arm skin of man showing synaptophysin-immunoreactive fibres in epidermis and dermis (A), around hair follicle (B), blood vessel and nerve bundle (C) and sweat glands (D). Arrow heads and arrows indicate some of the immunoreactive axons. Empty arrow indicate Meissner's corpuscles. Note the density of immunoreactive fibres. Zamboni fixed tissues. Cryostat sections (10μm).
All magnification x 250

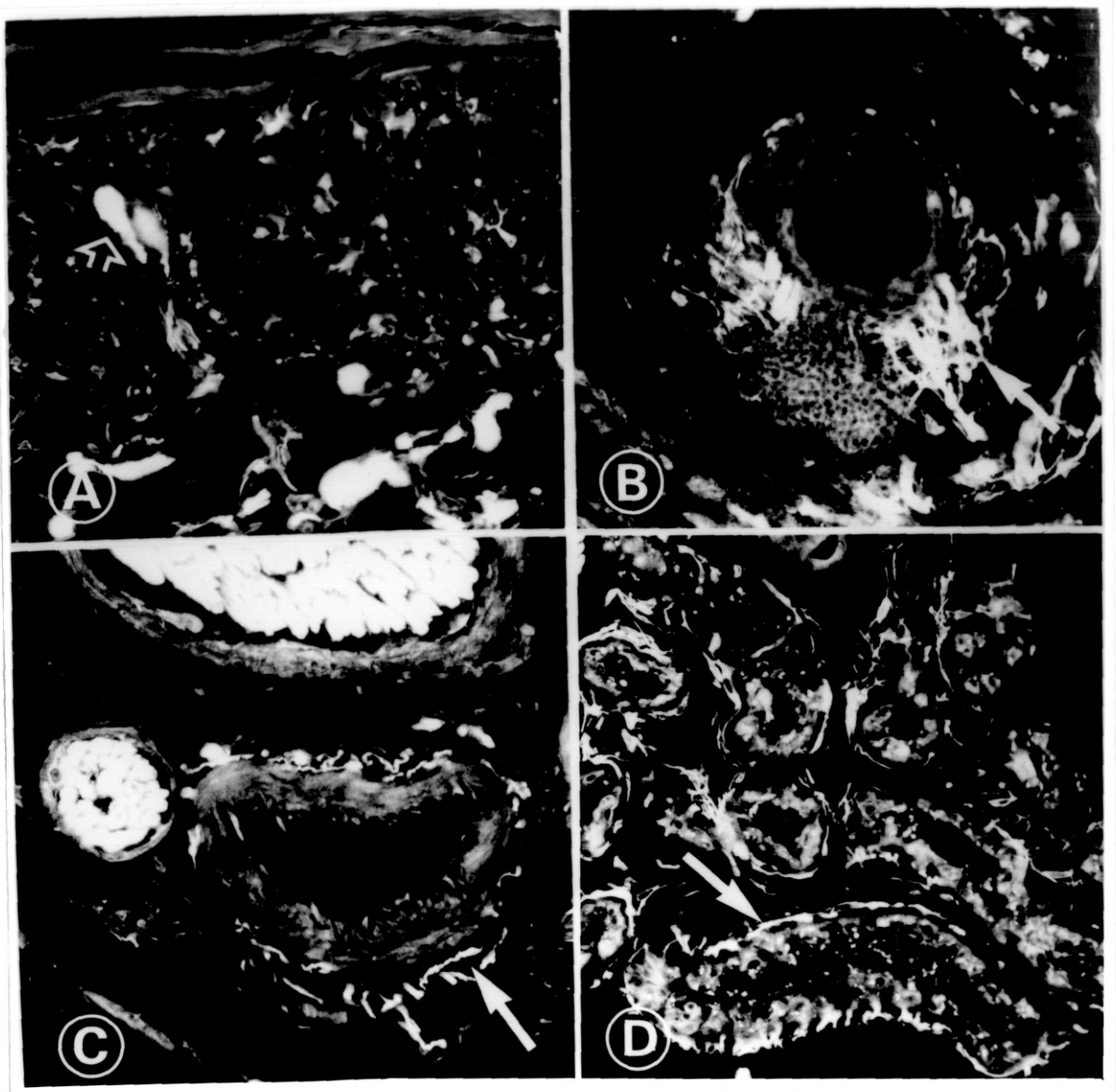


Fig. 3.7. Arm skin of man showing S-100-immunoreactive fibres in epidermis and dermis (A), around hair follicle (B), blood vessel and nerve bundle (C) and sweat glands (D). Arrow heads and arrows indicate some of the immunoreactive axons. Empty arrow indicate Meissner's corpuscles. Note the density of immunoreactive fibres. Zamboni fixed tissues. Cryostat sections (10μm).
All magnification x 250

nerve bundles were not immunoreactive for synaptophysin (Fig.3.6C). Antiserum to protein S-100 demarcated Schwann cell sheaths and/or their cell bodies around a few single or small groups of nerve fibres in the dermis (3.7A), but large numbers were seen close to the hair follicles (3.7B), sweat glands (3.7D) and nerve bundles (3.7C) in the deep dermis. However, no immunostaining was seen in the epidermis and the density of immunoreactive nerves in the dermis was comparatively less than those visualised with antiserum to PGP 9.5. In addition, human biopsy specimens taken after either general or local anaesthesia did not show significant differences in the immunoreactivity for general markers.

No immunostaining with antiserum to ChAT was obtained in any specimens, regardless of the fixation method used. However, in spinal cord, which was used as control specimen, ChAT immunostaining was seen in the cells of the intermediolateral cell column and ventral horn and in a few nerve fibres in the ventral horn.

3. 3. 2. Acetylcholinesterase staining and its comparison with immunocytochemical markers

AChE activity was seen in nerve fibres of epidermis, in hair follicles, around sweat glands (Fig.3.2C) and blood vessels, and in the nerve bundles and fine individual varicose fibres of the dermis. However, in large nerve bundles present in the deep dermis, not all the fibres were AChE-positive. When sequentially stained by immunofluoresence for general markers and AChE histochemistry, the density and distribution of AChE fibres were greater than those of nerves immunoreactive for neurofilaments and NSE but similar to those of synaptophysin-immunoreactive fibres. AChE-positive nerves (Fig.3.1C) were fewer than those immunoreactive for PGP 9.5 (Fig.3.1A) in the epidermis, and around hair follicles and large blood vessels, but nearly equivalent around sweat glands and small blood vessels. AChE activity was not seen when sections were incubated in reaction medium containing no acetylthiocholine iodide or in medium containing iso-OMPA.

3. 3. 3. Species similarities/differences

In all of the species studied, the distribution of peptidergic nerves in the various layers and appendages of the skin were similar. The differences observed between species were mainly in the density of immunoreactive peptidergic axons supplying the various areas of the skin. In addition, human biopsy specimens taken after either general or local anaesthesia did not show significant differences in the immunoreactivity for peptides. The results of immunostaining are summarised in Table 8.

3. 3. 3. 1. Tachykinins (Substance P and Neurokinin A)

The results of immunostaining with antisera to substance P and neurokinin A were grouped together under the term tachykinins due to the partial cross-reactivity of the two antisera with the heterologous antigen (Table 4). Large numbers of substance P and NKA-immunoreactive axons were seen both in the epidermis and dermis of the pig (Fig.3.8A). In cat, guinea pig and rat, nerves were seen mainly in the dermis with occasional axons in the epidermis. In man, however, immunoreactive axons were few in number and epidermal extensions were rarely seen (Fig. 3.8B). A moderate number of substance P and NKA-immunoreactive axons were seen around arteries and arterioles, mainly in the reticular layer of dermis and deep dermis in all the animals and man (Fig.3.8E &F). Sweat glands of pig (3.8C) had a sparse population of intensely immunostained substance P and NKA-immunoreactive fibres when compared with man (Fig.3.8B) where only occasional fibres were seen. These were very rare in rat and guinea pig.

3. 3. 3. 2. Calcitonin gene-related peptide

The pig and cat showed a rich supply of CGRP-immunoreactive axons in the epidermis and dermis (Fig.3.9A). In rat, guinea pig and man

Table 8

SPECIES VARIATIONS IN DENSITY OF PEPTIDE-IMMUNOREACTIVE AXONS IN VARIOUS SPECIES *

Axons and Endings IR for	Skin areas	Rat	Rat + Capsaicin	G.Pig	G.Pig + 6-OHDA	Cat	Pig	Man
SP/NKA	Epidermis	+	-	+	+	+	++	+
	Dermis	+++	+	+++	+++	+++	+++	++
	Sweat glands	-	-	-	-	+	+	+
	Blood vessels	+	+	+	+	+	++	+
	Hair follicles	-	-	-	-	-	-	-
CGRP	Epidermis	++	+	++	++	++	+++	+
	Dermis	+++	+	+++	+++	+++	++++	++
	Sweat glands	++	++	++	++	+++	+++	+
	Blood vessels	++	++	++	++	++	+++	+
	Hair follicles	-	-	-	-	-	-	-
VIP/PHI/PHM	Epidermis	-	-	-	-	-	-	-
	Dermis	-	-	+	+	+	+++	+
	Sweat glands	+++	+++	+++	+++	+++	++++	+++
	Blood vessels	++	++	++	++	++	+++	++
	Hair follicles	-	-	+	+	-	-	+
NPY/CPON	Epidermis	-	-	-	-	-	-	-
	Dermis	-	-	-	-	-	-	-
	Sweat glands	+	+	±	-	+	±	+
	Blood vessels	+	+	+	-	+	++	+
	Hair follicles	-	-	-	-	-	-	-
SS	Sweat glands	-	-	-	-	-	+++	-

Numbers of immunoreactive fibres are graded: absent (-), very few (±), few (+), moderate (++), many (+++), abundant (++++).* pig, guinea pig, cat, rat and man

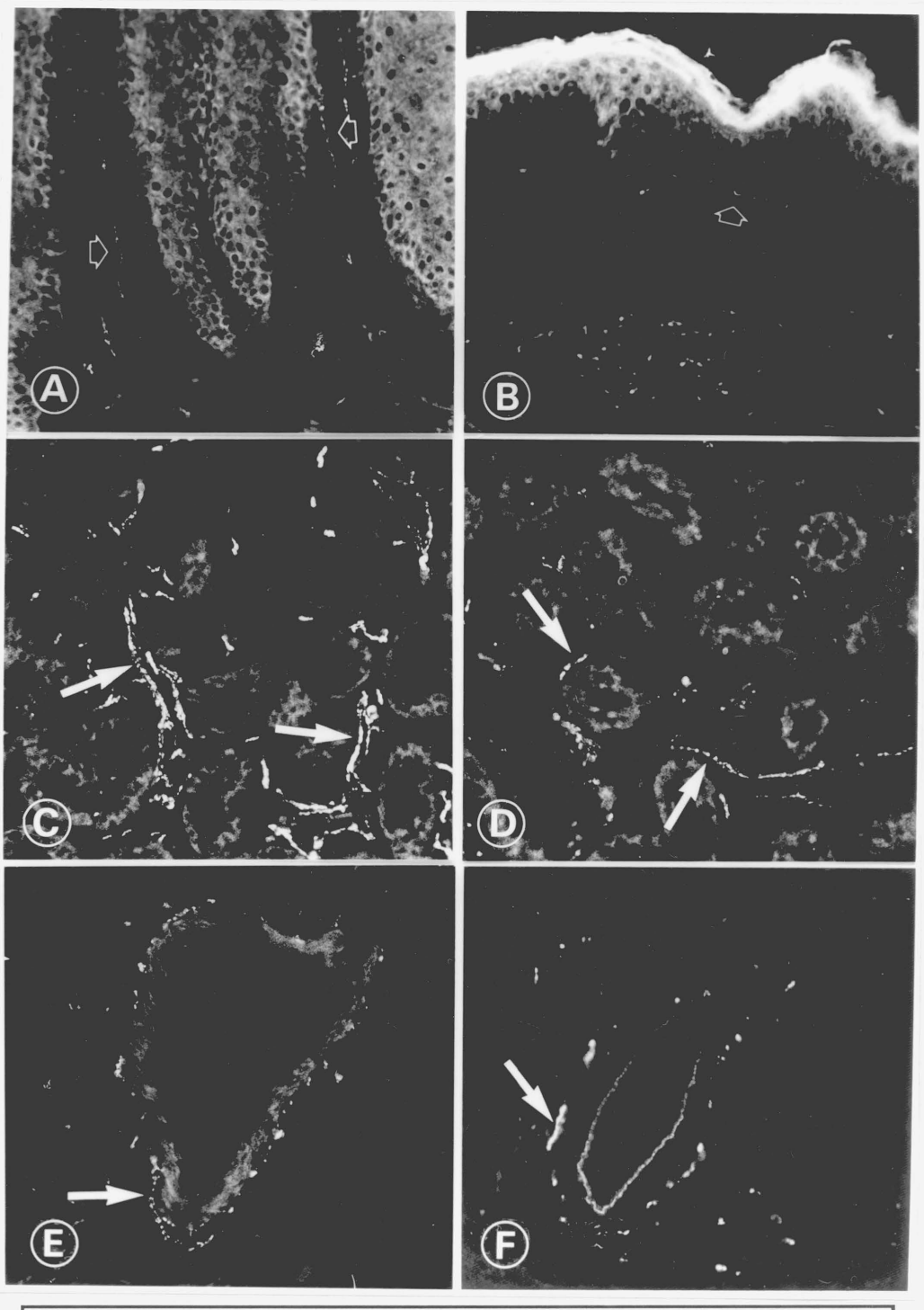


Fig. 3.8. SP-immunoreactive fibres in epidermis and dermis (Empty arrow head), sweat glands and bloodvessels (arrow head) showing species differences in density of distribution. A,B Skin of pig snout (A) showing large number of immunoreactive axons and facial skin of man (B) showing only very few dermal nerves.C,D Sweat glands of pig snout (C) showing moderate number of immnoreactive fibres and facial skin (D) of man showing occasional nerve fibres. E,F Blood vessels of pig snout (E) and facial skin (F) of man showing occasional fibres. Zamboni fixed tissues. Cryostat sections (10μm). All magnification x275

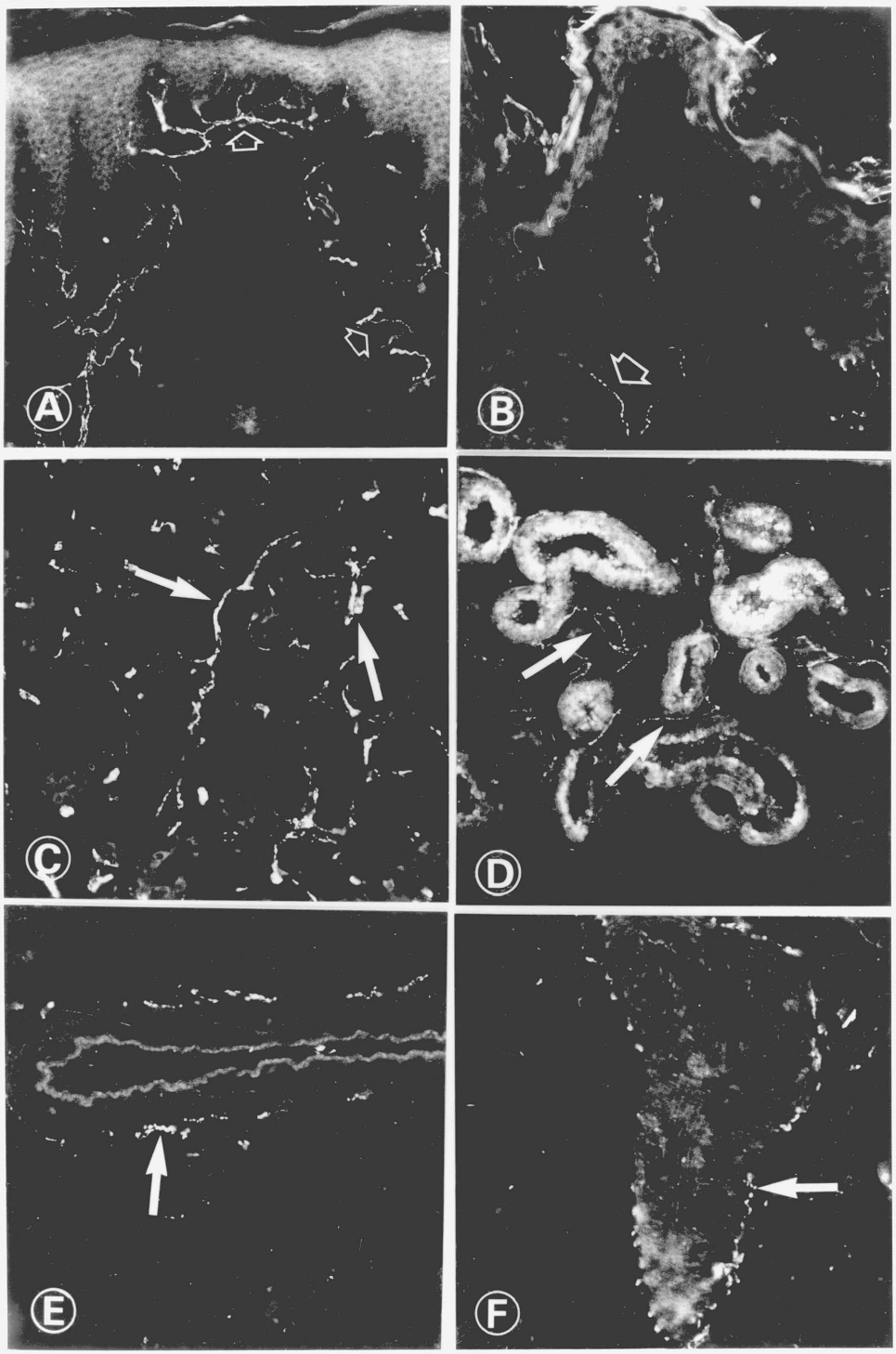


Fig. 3.9. The distribution of CGRP-immunoreactive fibres showing species differences. A,B Skin of pig snout (A) and facial skin of man (B) showing differences in density of immnoreactive fibres (Empty arrow head) in epidermis and dermis. C,D Sweat glands of pig (C) showing abundant immunoreactive fibres (arrow) and (D)of man showing sparse number of immunoreactive fibres. E,F Blood vessels of pig and man showing similar density of distribution of immunoreactive fibres (arrow). Zamboni fixed tissues. Cryostat sections (10μm). All magnification X 290

(3.9B), CGRP-immunoreactive axons were seen in moderate numbers in the dermis and infrequently in epidermis. However, in both layers CGRP-immunoreactive axons formed the largest population in comparison with SP and NKA-, and VIP-immunoreactive nerves. In the dermis, the fibres showing very fine symmetrical varicosities ran either parallel or perpendicular to the basal layer of the epidermis, mainly as individual fibres but sometimes in small bundles. A moderate number of CGRP-immunoreactive fibres supplied the blood vessels in all species (Fig.3.9E & F). CGRP-immunoreactive nerve fibres supplying sweat glands were numerous in pig and cat (Fig.3.9C), fewer in guinea pig and rat, and rare in man (Fig.3.9D). The fibres in the epidermis, dermis and around sweat glands and blood vessels were of varicose type when compared with those in nerve bundles which were smooth and of fine calibre.

3. 3. 3. 3. Vasoactive intestinal polypeptide and Peptide histidine isoleucine

In general distribution of VIP- and PHI-immunoreactive nerve fibres was identical with some exceptions, and exceeded those immunoreactive for NPY and CPON. They were seen around sweat glands and blood vessels, and in the dermis. Species variations were not pronounced in respect of VIP-immunoreactive axons supplying sweat glands but were preset in the distribution of fibres in the dermis, hair follicles and dermal blood vessels. In pig, large numbers of immunoreactive axons were seen close to the epidermis (Fig.3.10A), and occasionally in the epidermis, whereas in cat, guinea pig and man, only moderate numbers of axons were seen. Rat showed a complete absence of immnoreactive axons in the dermis (Fig.3.10B). In all the animals, dermal vessels in the papillary and reticular layer rarely received VIP- and PHI-immunoreactive axons, although large numbers of axons were seen supplying the vessels of the deep dermis

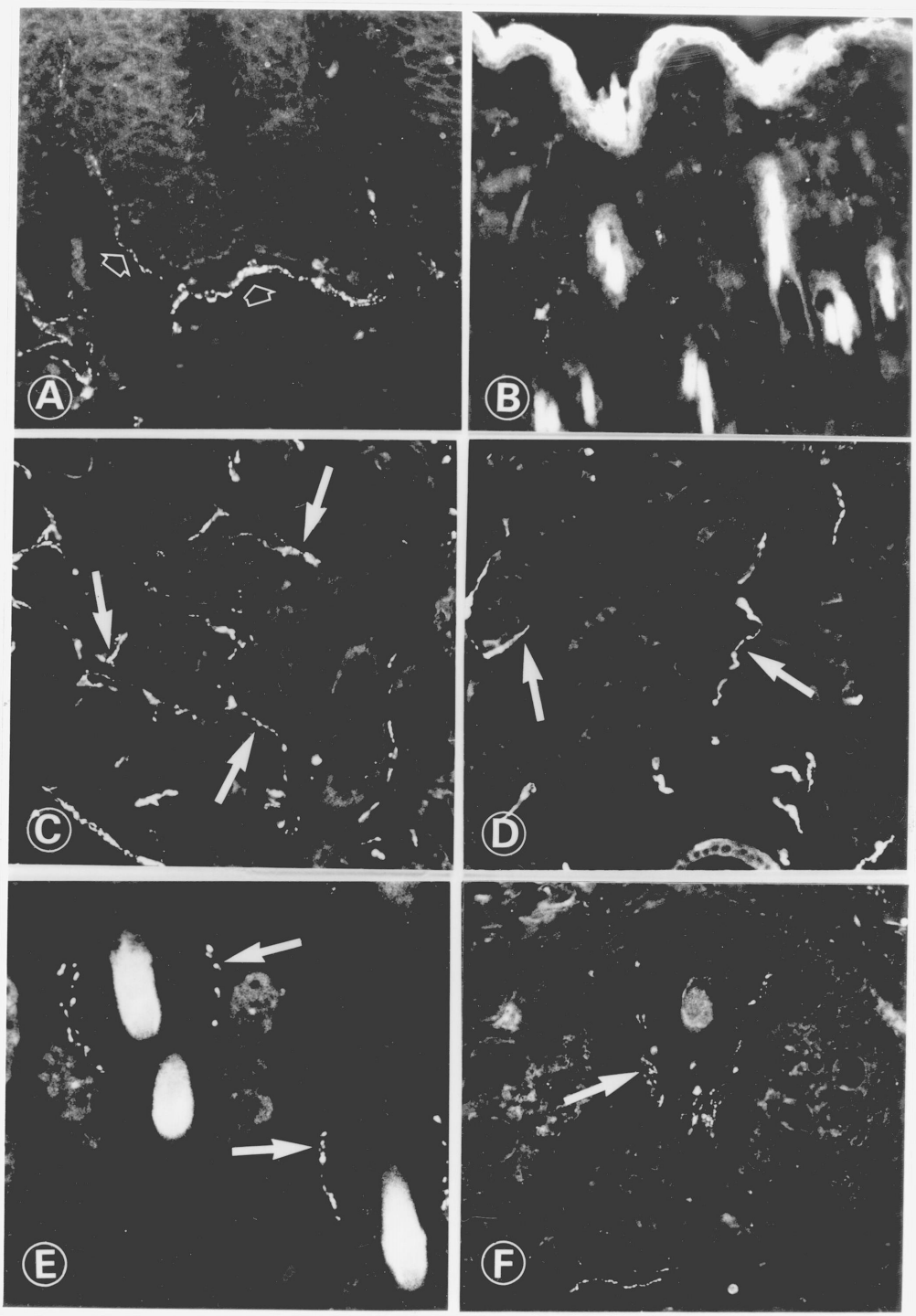


Fig. 3.10. VIP-immunoreactive fibres in epidermis and dermis, sweat glands and hair follicle showing species differences. A,B. Epidermis and dermis of (A) pig snout showing subepidermal and dermal fibres (empty arrow head); (B) rat facial region showing complete absence. C,D. Sweat glands of (C) pig and (D) guinea pig showing similar density of immunoreactive nerve fibres (arrow) distribution. E,F. Immunoreactive fibres (arrow) around hair follicles of (E) guinea pig and (F) man. Zamboni fixed tissues. Cryostat sections (10μm). All magnification X 290

and also vessels close to the sweat glands. Occasional VIP-immunoreactive axons were seen around the hair follicle in man and guinea pig (Fig.3.10.E-F). In pig, PHI-immunoreactive fibres were more frequent in the dermis and around blood vessels than VIP-immunoreactive fibres, and although fibres supplying the sweat glands were immunoreactive for both VIP and PHI, the PHI immunoreactivity was weak.

3. 3. 3. 4. Neuropeptide Y and C-flanking peptide of neuropeptide Y

The distribution of NPY- and CPON-immunoreactive axons was similar. They were seen mostly around large blood vessels in all the species studied where they formed an outer perivascular plexus of varicose fibres at the adventitial-medial border. The density of the perivascular plexus varied with vessel size and site as well as in different species, being most abundant in pig and moderate in guinea pig, rat, cat and man. Occasional NPY and CPON-immunoreactive fibres were seen around sweat glands in all species.

3. 3. 3. 5. OTHER PEPTIDES

Immunoreactivity for atrial natriuretic peptide (ANP) or galanin was not seen in any of the species studied. Somatostatin-immunoreactive axons were seen only in pig around sweat glands.

3. 3. 4. Regional variations

Regional differences were similar in all the species studied. The greatest density of nerve fibres was seen in the sensitive regions of the body, such as the nose/snout, lip, ear and footpads. The axons immunoreactive for CGRP were seen always in large numbers when

compared with SP and NKA-immunoreactive axons thereby constituting the largest population of peptide-immunoreactive axons in the skin (Figs.3.9A, 3.10A). Dorsal and ventral regions of the abdomen, thorax and fore-and hind-limbs showed fewer immunoreactive fibres and almost none were seen in the tail skin. Epidermal fibres were mainly seen in lip, nose, face, ear and foot pads and rarely in other regions. VIP- and PHI-immunoreactive fibres were seen mainly around sweat glands and around blood vessels, wherever they occurred, in all animals. The ducts of the glands received only a sparse number of VIP and PHI-immunoreactive nerves as did the small arteries present in the papillary and reticular layer of dermis. Dermal nerve endings immunoreactive for VIP and PHI were seen only in specialised areas including lip/snout (Fig.3.10A) and paws in all the animals except rat and in man. In man, PHM-immunoreactive axons were comparatively more frequent around eccrine glands than apocrine glands and dermal fibres were rarely seen. NPY and CPON-immunoreactive axons were abundant around blood vessels of lip, nose/snout and paws, and sparsely seen in other regions.

3. 3. 5. Effects of capsaicin and 6-OHDA

Capsaicin-treated rats showed a significant reduction (approximately 50 to 70% estimated visually) in the density of CGRP-, (Fig.3.11A & B) substance P- (Fig. 3.11C & D) and NKA-immunoreactive axons in the epidermis and dermis, and no change around sweat glands and blood vessels of the skin. In addition, no reduction was observed in axons displaying NPY and CPON or VIP and PHI immunoreactivity (Fig.3.11E & F). Treatment with 6-OHDA resulted in an almost complete loss of TH- and NPY-immunoreactive axons around the blood vessels and sweat glands (Figs 3.11G-L). However, substance P, NKA-, CGRP-, VIP and PHI-immunoreactive axons remained in the epidermis/dermis and around sweat glands, respectively, after the treatment with 6-OHDA.

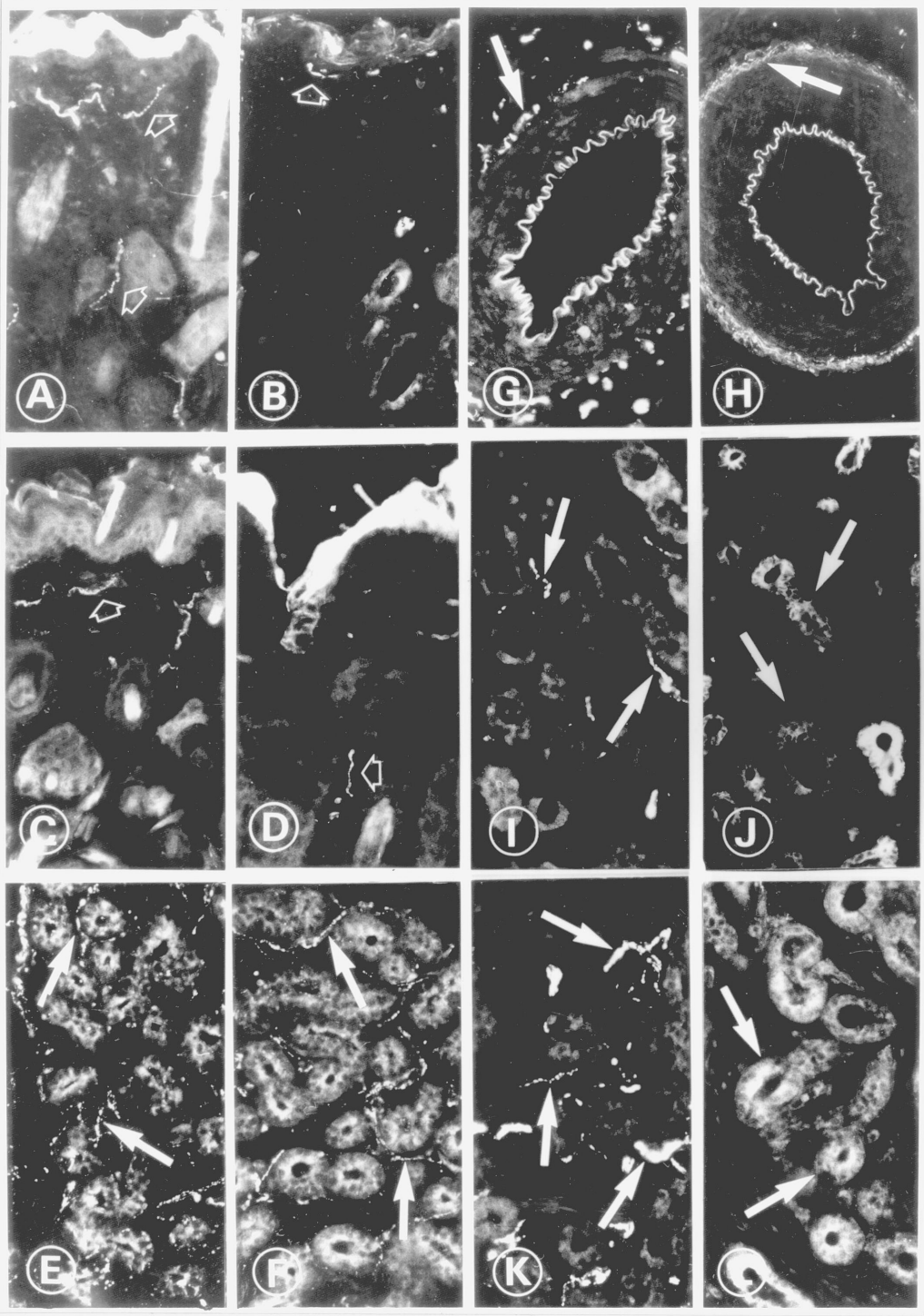


Fig. 3.11. Skin from age matched control and capsaicin treated rats (A to F) and, control and age matched 6 OHDA treated guinea pigs (G to L). A,B Epidermis and dermis showing CGRP-immunoreactive fibres (empty arrow head). Note the reduction in number of immunoreactive fibres in B. C,D Epidermis and dermis showing SP-immnoreactive fibres (empty arrow head). Note the absence of nerve fibres in D. E,F Sweat glands showing no change in the density of immunoreactive nerve fibre (arrow) distribution. G,H TH-immunoreactive fibres (arrow) around blood vessels and I,J around sweat glands. The continuous line surrounding the lumen represents the autofluorescence of internal elastic lamina. Note the absence of fibres in H and J. K,L NPY-immunoreactive fibres (arrow) around sweat glands. Note the absence of fibres in L. Zamboni fixed tissues. Cryostat sections (10μm). All magnification X 290

3. 3. 6. Development of cutaneous nerves

The results of immunostaining in developing rat are summarised in diagram 6. No PGP 9.5 immunoreactive fibres were seen in the skin of fetal rat up to and including the 15th day of development. On day 16, PGP 9.5-immunoreactive axons were seen in the region of nose and lip (Fig.3.12A) although they were absent from paws and tail (Fig.3.12B & C), and sparse in other areas including abdomen and back. In the nose, the immunoreactive axons were seen to run parallel to the surface of the skin, with occasional vertical extensions to the developing primordium of the hair follicles. The distribution of neurofilament-immunoreactive axons was similar both in density and intensity. No significant changes were seen on the 17th day of fetal development except for a slight increase in number of immunoreactive axons. On day 19, PGP 9.5- immunoreactive axons showed dichotomous branching in the dermis, particularly in the region of nose and face and fibres around developing hair follicles (Fig. 3.12D). At the same time, individual PGP 9.5-immunoreactive axons appeared in the dermis of the paws. More branches were added to the existing ones in the dermis on the 20th day in addition to the appearance of epidermal extensions. On day 21, cutaneous axons had established contacts with all the developing hair follicles (Fig.3.12E). PGP 9.5-immunoreactive axons were seen surrounding the developing sweat glands only at the end of the first week of post-natal life. More branches were added during the second week. Thereafter the distribution resembled the adult pattern.

3. 3. 7. Appearance of peptides in cutaneous nerves

The time course of appearance of peptides varied from one peptide to another. CGRP-immunoreactive axons were first seen on the 19th day of fetal life mainly in the dermis of the face, abdomen and foot pads

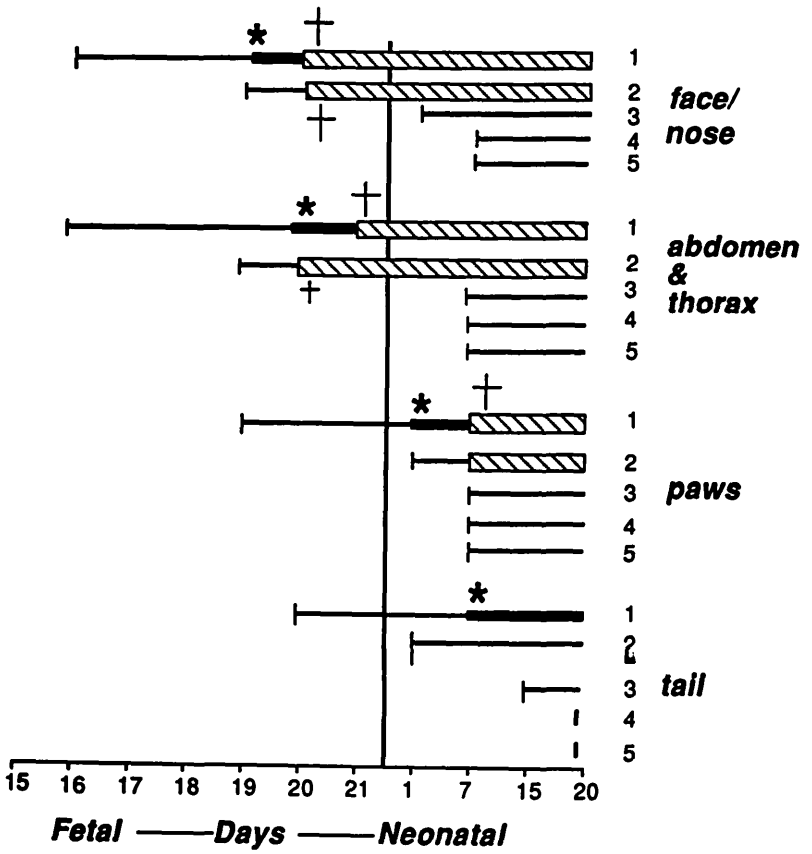


Diagram 6. Schematic representation of development of cutaneous nerves and peptides in the skin of rat.
┼ = dichotomous branching; * = epidermal fibres;
1. protein gene product 9.5; 2. calcitonin gene-related peptide; 3. substance P; 4. vasoactive intestinal polypeptide/ Peptide histidine isoleucine;
5. neuropeptide Y/ C-terminal peptide of NPY.

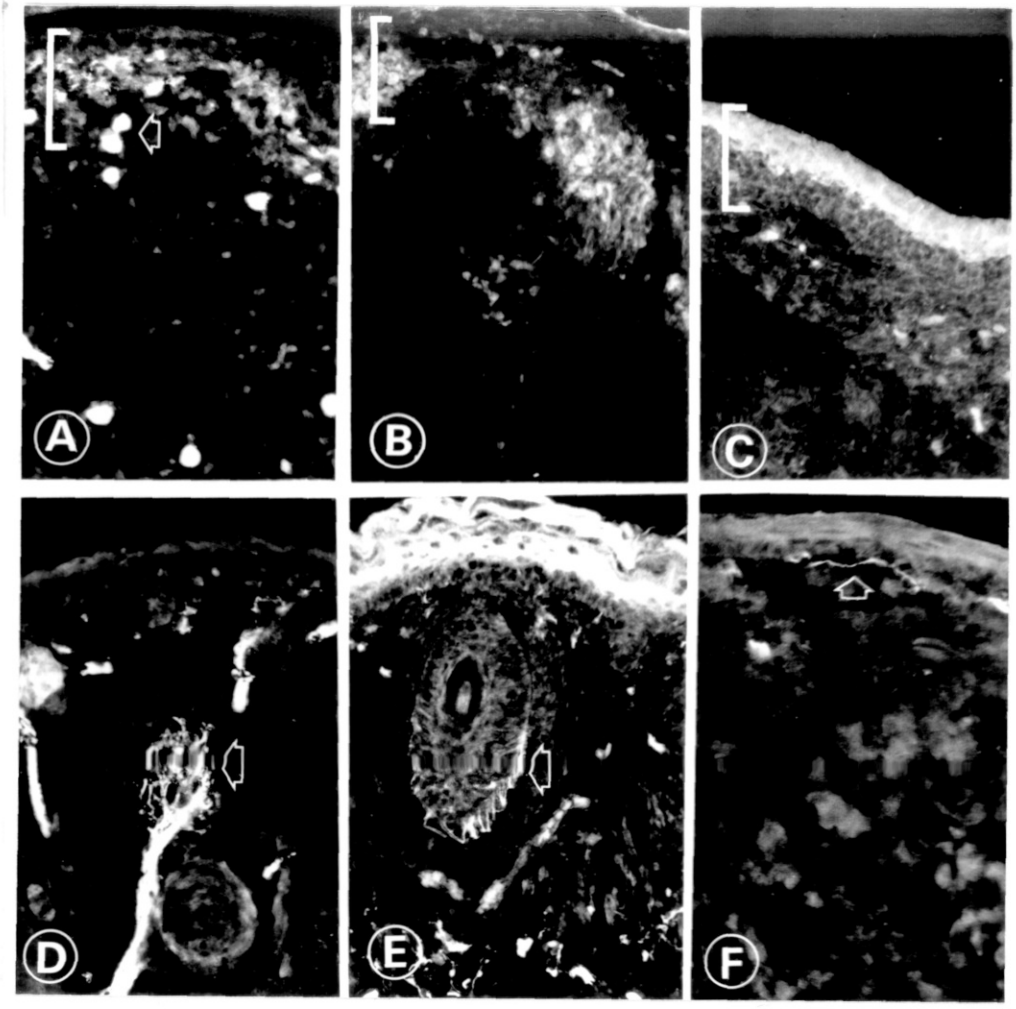


Fig. 3.12. A,B,C Skin from 16th day foetal rat showing presence of PGP-9.5 immunoreactive fibres (empty arrow head) in face (A) and absence in foot pads (B) and tail (C). D,E Skin from 19th (D) and 12th day (E) foetal rat showing PGP-immunoreactive fibres around developing hair follicle. F Skin from 19th day foetal rat showing presence of CGRP-immunoreactive fibres (empty arrow head) in the dermis of face. Zamboni 's fixed tissues. Cryostat sections (15μm). All magnification X 275

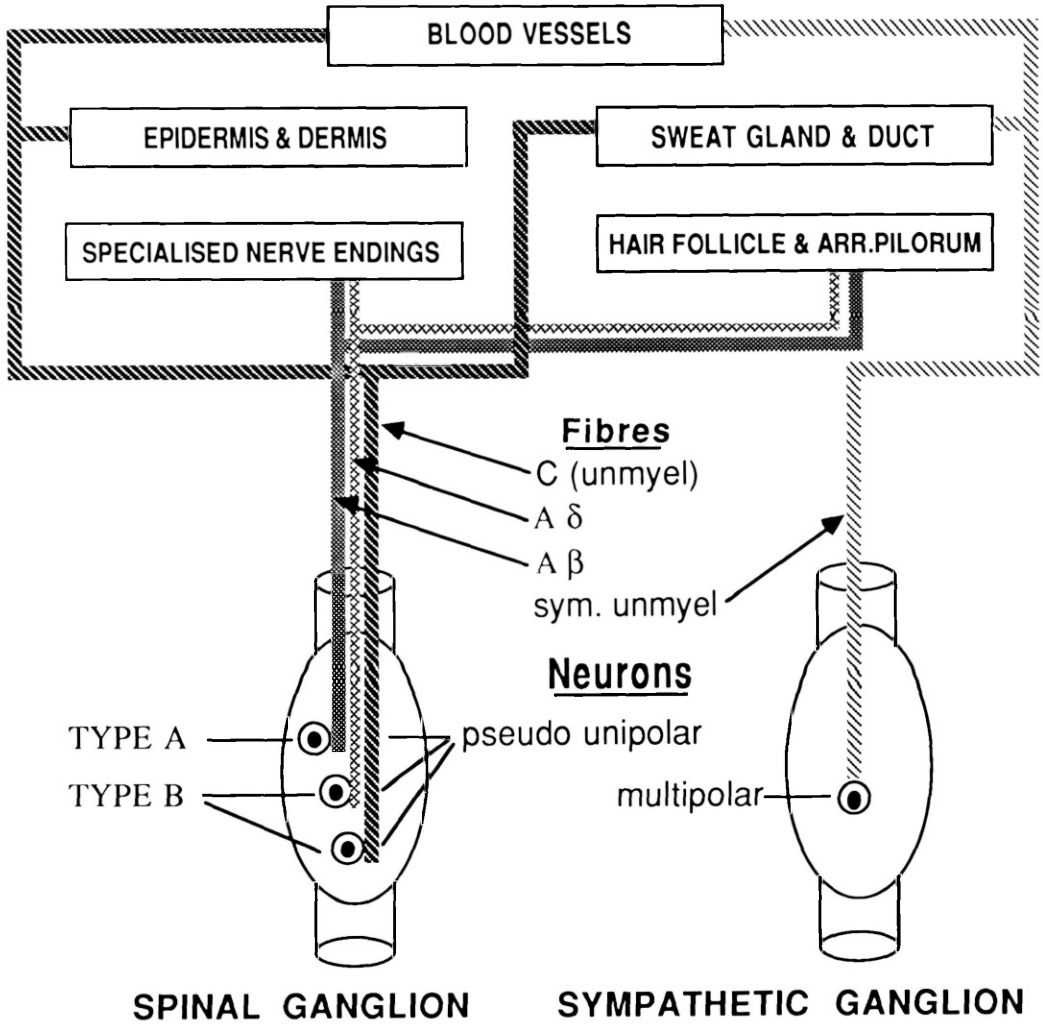
(Fig.3.12F). On the 20th day occasional immunoreactive epidermal extensions were seen. SP/NKA-immunoreactive fibres appeared only on neonatal day 1 in the region of face and footpads and were not seen in other regions of the body. VIP and NPY/CPON-immunoreactive axons were not apparent until postnatal day 7, where they were seen mainly around blood vessels and sweat glands of the foot pads.

3. 4. Discussion

The present study is a systematic and detailed examination of cutaneous innervation of non -hairy (man) and hairy mammals (pig, guinea pig, rat and cat), in order to establish a base line for further studies of skin in experimental and disease conditions. Comparison of various immunocytochemical neural markers and AChE histochemical staining provides strong evidence that antiserum to PGP 9.5 was the best overall marker for cutaneous nerves. Species and regional variations in the peptidergic nerves were mainly in their density, whereas the specific peptides in the nerves innervating epidermis, dermis, hair follicles, sweat glands and blood vessels of the skin were similar. During development, nerves immunoreactive for general markers appeared earlier than neuropeptide immunoreactivity, and sensitive regions showed a more advanced stage of development than the less sensitive regions. Schematic representation of the distribution/ visualisation of nerves and neuropeptides in all the species studied, and possible physiological correlation with the specific nerve types is shown in diagram 7.

3. 4. 1. PGP 9.5 as a neural marker

PGP 9.5 antiserum gave consistently reproducible results in all of the species examined and provided an elegant demonstration of the



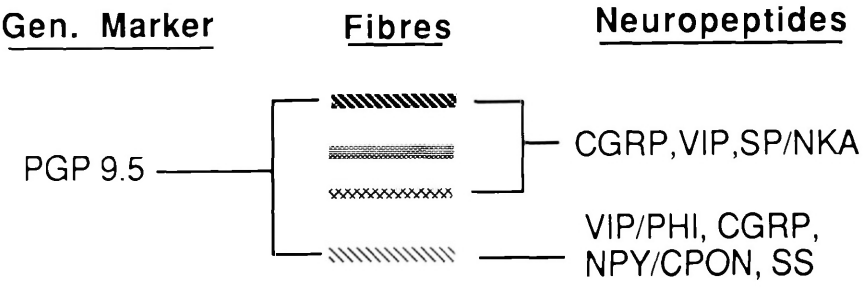


Diagram 7. Schematic representation of origin/distribution/ visualisation of nerves and neuropeptides in the skin of mammals. unmyel=unmelinated fibres; Symp= sympathetic; PGP 9.5= protein gene product 9.5; SP= substance P; CGRP= calcitonin gene-related peptide; NKA= neurokinin A; VIP= vasoactive intestinal polypeptide; PHI= peptide histidine isoleucine; NPY= neuropeptide Y; CPON= C-terminal peptide of NPY, SS= somatostatin.

patterns of nerve fibre distribution within the various layers and around the appendages of skin. Immunoreactive axons were greater in number and density, and the staining was stronger than with antisera to other neural markers such as neurofilaments and NSE. The main reason for the effective immunoreactivity may be due to the nature of PGP 9.5, which is a major component of the neuronal cytoplasm as suggested by radioimmunoassay (Thompson, 1981) and by electron microscopy (Gulbenkian et al., 1987), although its function is so far unknown. PGP 9.5 has been used as a histological marker for the delineation of nerves and endocrine cells in other organs of the body (Gulbenkian et al., 1987; Thompson et al., 1983; Lauweryns and Van Ranst, 1988). In contrast, neurofilament triplet proteins are constituents of one of the cytoplasmic organelles and NSE is an enzyme. The expression of neurofilament may depend on the axonal calibre, specific nerve subtypes and neuronal regions. Ultrastructural studies of the axoplasm of sensory fibres show that the percentage of neurofilament triplet proteins varies, being most abundant in large myelinated axons and less in fine myelinated and unmyelinated axons (Friede and Samorajski, 1970; Schlaepfer and Lynch, 1977; Shaw and Weber, 1981). Neurofilament-immunoreactivity in the dorsal root ganglia is mainly seen in the large diameter cells from which large myelinated axons arise (Anderton et al., 1983; Lawson et al., 1984). In addition, the degree of neurofilament immunostaining depends on the ability of the antiserum used to recognise all three of the triplet proteins including phosphate-dependent and -independent neurofilament proteins which influences the axonal transport along the axons (Nixon et al., 1987). Our results substantiate these observations because neurofilaments- immunoreactivity was seen mainly in nerve bundles and only occasionally in individual axons. The NSE antiserum did not recognise most of the fine varicose axons and gave staining of low intensity with much inter-species variation.

This may be due to the chemical nature of NSE, possible poor species cross-reactivity and the physiological state of the nerves (Bock and Dissin, 1975, Bishop et al., 1985; Marangos et al., 1981). The density of S-100-immunoreactive Schwann cells did not give a clear estimation of the density of nerve fibres present in the tissue, particularly in the region of superficial dermis. This may be due to the nature of one Schwann cell enveloping several unmylinated nerve fibres. In addition, the absence of immunoreactivity in the epidermis is a evidence to show that S-100-immunoreactive Schwann cells does not demarcate the nerve endings present. This may be due to the absence of Schwann cell sheath extension to the epidermal nerves. Although we do not have conclusive evidence, the density of distribution of PGP 9.5-immunoreactive nerves in all the species studied, strongly suggests the possibility of identification of all the cholinergic, adrenergic and peptidergic populations of axons. These results are consistent with a similar study on the visualisation of cardiovascular innervation (Gulbenkian et al., 1987). Synaptophysin antiserum apparently failed to show all the nerve terminals present in the skin particularly in epidermis and dermis in comparison with antiserum to PGP 9.5. However, the possibility of identification of a distinct population of nerve endings containing classical neurotransmitters exists. Light and electron microscopical examination has revealed that synaptophysin selectively labels the small synaptic vesicles and not the large dense-core vesicles (Jahn et al., 1985), supporting the idea that these vesicles are distinctly different types and belong to different pathways of neural secretion. There is evidence that the small synaptic vesicles contain classical neurotransmitters, whereas the large dense core vesicles are thought to contain peptide neurotransmitters and non-peptide molecules such as amines (Lundberg et al., 1982; Hökfelt et al., 1984; Klein et al., 1985 ; Gulbenkian et al., 1987). Therefore, the presence of synaptophysin-immunoreactive terminals in the epidermis, close to Merkel cells and hair follicles, strongly suggests that they contain

classical neurotransmitters. However, no secretion of classical neurotransmitters from sensory nerves has been documented so far. Our results are consistent with a study showing that synaptophysin immunoreactivity is present in the afferent nerve endings of skeletal muscle (De Camilli et al., 1988). The identification of fewer epidermal fibres by antiserum to synaptophysin compared with that of PGP 9.5 may suggest that only a proportion of epidermal fibres contain classical neurotransmitters.

Immunostaining of cholinergic axons using antibody to ChAT has been successful mainly in the central nervous system (Kimura et al., 1981; Eckenstein and Thoenen, 1983, Hauser et al., 1985; Ichikawa and Hirata, 1986) due to the nature of antibodies. Reports of localisation in the peripheral nervous system (Furness et al., 1984a; Furness et al., 1984b) are scanty. In a previous study, rat foot pad showed choline acetyltransferase immunoreactivity around the sweat glands (Leblanc and Landis, 1986). However, in our hands no immunostaining was seen. This could be due to the fixation procedure, immunostaining techniques or nature of the antibodies used.

3. 4. 2. Cholinesterase histochemistry

The cholinesterase method (Gomori, 1952; Koelle, 1955; Coupland and Holmes, 1957) has been used since 1950 with several modifications in the technique to demonstrate cutaneous nerves and receptors (Montagna and Becket, 1958; Montagna 1960; Winkelmann, 1960; Montagna & Parakkal, 1974). In a comparative study, species differences were observed in the response of nerve tissue to specific cholinesterase (Winkelmann and Schmit, 1959) limiting use of the technique for visualisation of all the cutaneous nerves. The large number of AChE-positive nerve fibres in sensory and autonomic areas, as shown by our study using the modified and sensitive AChE technique indicates the activity is specific to acetylcholinesterase rather than non-specific cholinesterase activity. Nonetheless, the similar distribution of AChE-positive fibres and those with PGP 9.5 or synaptophysin immunoreactivity as shown in our study is a further evidence of specificity of the AChE reaction in nerves, although such

interpretation of the AChE reaction has always been controversial due to the fact that AChE reaction is reported both in sensory and autonomic parts of cutaneous innervation and the role of acetylcholine in the sensory nerves is unknown. However, a large amount of evidence has accumulated for and against the function of acetylcholine as a sensory transmitter (Douglas and Ritchie, 1960; Koelle, 1962; Fitzgerald and Cooper, 1971; Nachmanson, 1975, Haegerstam et al., 1975).

3. 4. 3. Species variations

Considerable differences were found among the species in terms of the density of peptidergic axons. Peptide-immunoreactive axons were most abundant in pig, but moderate to sparse in other animals and in man, peptide-immunoreactive axons were comparatively few. In all the species studied CGRP- and VIP/PHI (PHM in man)-immunoreactive axons formed the largest populations of peptide-containing axons in the epidermis and dermis, and around sweat glands. However, the precise relationship between species variations and their functional correlation is not clear. Previous studies have indicated that there were species differences in relative concentrations as determined by radioimmunoassay of substance P, VIP and somatostatin in pig and cat skin (O'Shaughnessy et al., 1983). Therefore, the distribution differences seen in our study may reflect physiological differences in these species, although at this stage we are not able to correlate these findings in terms of function. Such variations may well be dependent on the thickness of the epidermis and dermis, the variety and number of cutaneous appendages, vascularity of the skin and last but not least the behavioral differences among species.

3.4.4. Effect of anaesthesia on neuropeptides

Although we do not have conclusive evidence to show whether sodium pentobarbitone affects the neuropeptides in skin of laboratory animals, immunocytochemical and radioimmunoassay analysis indicates that animals killed by ether inhalation or by an overdose of sodium pentobarbitone do not show any significant difference in the

CGRP-immunoreactivity of lung (Cadieux et al., 1986). It is also relevant that there was no obvious difference seen in the general marker and peptide immunoreactivity in the human specimens taken under local or general anaesthesia, suggesting that the effects of local anaesthetics are on receptors binding sites than neuropeptides.

3. 4. 5. Regional variations

Despite the variations seen in the density of peptidergic innervation in different regions of the body in all the species, the common feature was the presence of greater numbers of peptidergic axons, particularly in epidermis, in sensitive regions including lip and nose/snout, as compared with sparse numbers of nerve fibres in the less sensitive regions of the body. These findings agree with those of previous morphological studies showing significant differences in simple sensory corpuscles of anterior and posterior paw of the raccoon (Zollman and Winkelmann, 1962), and regional variations in peptidergic nerve distributions and peptide concentrations in pig and cat skin investigated by immunocytochemistry and radioimmunoassay (Bloom and Polak, 1983).

Sensation in the skin is mediated by afferent fibres including large myelinated fibres (A-alpha), which supply mainly the specialised end organs, small fine myelinated (A-delta) and small unmyelinated fibres (C-fibres). The understanding of skin innervation has grown step by step with improvements in investigative techniques. Now there is much immunocytochemical evidence that substance P/NKA and CGRP are present in these fine myelinated and unmyelinated fibres (Bloom and Polak, 1983, Bjorklund et al., 1986; Hökfelt et al., 1975a; Dalsgaard et al., 1985, Gibbins et al., 1985). Our results are in full agreement with these previous reports in showing SP/NKA- and CGRP-immunoreactive fibres in the epidermis, dermis, sweat glands and blood vessels. Colocalisation studies indicate the coexistence of SP and NKA as well as SP and CGRP in the cells of dorsal root ganglia and

in the nerves of skin (Gibbins et al., 1985; Gibson et al., 1984; Wiesenfeld-Hallin et al., 1984; Ju et al., 1987; Molander et al., 1987; Merighi et al., 1988). However, we do not yet know whether species differences exists in colocalisation of these peptides.

3. 4. 6. Capsaicin and 6-OHDA treatment

It has been shown by previous workers that neonatal administration of capsaicin results in an irreversible, selective, dose-dependent degeneration of the sensory ganglion cells that give rise to unmyelinated and fine myelinated fibres (Jansco et al., 1977; Nagy et al., 1981; Nagy et al., 1983; Jancso et al., 1985). Our study shows that only 50% to 70% of the axons containing SP/NKA and CGRP-immunoreactivity were depleted from epidermis and dermis, with no reduction in immunoreactivity around blood vessels and sweat glands and is in agreement with similar studies (Gibbins et al., 1985, Holzer et al., 1982; Norheim et al., 1985). This apparent partial depletion may be due to the dosage of capsaicin administered resulting in incomplete loss of immunoreactivity or due to the presence of various functional subpopulations of tachykinins- and CGRP-immunoreactive axons (Nakanishi, 1986; Amara et al., 1985; Matsuyama et al., 1986; Mulderry et al., 1988; Feher et al., 1986; Wharton et al., 1986; Su et al., 1986, Su et al., 1987). However, a similar reduction in VIP/PHI- and NPY/CPON-immunoreactive axons supplying sweat glands and blood vessels was not noticed, indicating the selective nature of the capsaicin toxicity. Nevertheless, the above results suggest either the fibres immunoreactive for SP/NKA and CGRP supplying epidermis and

dermis are partially sensory and capsaicin does destroy all sensory nerves, or that they are mostly sensory and capsaicin does not destroy all sensory nerves at the dosage given.

The administration of 6-OHDA, which destroys peripheral sympathetic nerve terminals (Gulbenkian et al., 1987; Allen et al., 1985), caused a complete depletion of TH- and NPY-immunoreactive axons seen around sweat glands and blood vessels without any reduction of VIP-immunoreactive axons, suggesting that the cholinergic and adrenergic components of the skin are distinct entities. Several previous histochemical and/or immunocytochemical studies showed the presence of sympathetic cholinergic (Leblanc and Lendis, 1986, Dale and Feldberg, 1934; Sjoqvist, 1963; Lundberg et al., 1980a) and adrenergic innervation (Uno, 1977; Lundberg et al., 1979) around sweat glands in addition to nerves containing peptides including VIP, NPY, CGRP and SP (O'Shaughnessy et al., 1983; Hartschuh et al., 1984; Hökfelt et al., 1975; Vaalasti et al., 1985; Landis and Fredieu, 1986; Gibbins et al., 1987). In skin, VIP is believed to be localised principally in cholinergic sympathetic neurons; e.g. around sweat glands (Lundberg et al., 1980) and NPY in adrenergic sympathetic neurons (Lundberg et al., 1979; Lundberg et al., 1982a; Ferri et al., 1984). However, in other organs there are exceptions to this rule showing distinct populations of non-adrenergic NPY-immunoreactive nerve fibres harbouring VIP/PHI in gut (Ekbald et al., 1984a), thyroid gland (Grunditz et al., 1988) and the cardiovascular system (Gibbins et al., 1988). VIP is shown to coexist with noradrenaline- containing nerves supplying the uterine artery (Morris et al., 1985). In addition, several previous studies showed that NPY-immunoreactive fibres were not completely abolished by treatment with 6-OHDA; e.g., perivascular nerves (Morris et al., 1985) in gut and pancreas (Su et al., 1987) due to the dual extrinsic and intrinsic nature of their origin (Sundler et al., 1983; Tanio et al.,

1986). Nonetheless, our results with 6-OHDA treatment are of relevance because they suggest that there is a clear demarcation between adrenergic and cholinergic sympathetic fibres and that NPY immunoreactive axons are purely noradrenergic in skin. To our knowledge, a clear demarcation of adrenergic and cholinergic fibres around sweat glands of skin has so far not been reported.

This study does not support previous reports of the presence of immunoreactivity for ANP, galanin and enkephalin (Tanio et al., 1987, Johansson and Vaalasti., 1987; Johansson et al., 1988) in the skin of any of the species studied. Somatostatin- immunoreactive axons were seen only in pig. This suggests that these are minor peptides, if they are present, in the skin and awaits the results of pharmacological and physiological studies to determine if they have any functional role.

3. 4. 6. Development of cutaneous nerves

The development of cutaneous nerves in rat varied from region to region. It is possible that the extension of outgrowing axons from each dorsal root ganglion may reach appropriate locations at different times. The density of fibres depended on the skin region and the presence of primordial epidermal appendages such as hair follicles and sweat glands, and blood vessels in the connective tissue. These appendages, which are the targets of developing nerves, are more frequent in lip, face and nose than trunk, tail and paws. It has been suggested that each region of skin attracts a number of nerves the density of which is regulated by nerve growth factor present in the target tissue (Letourneau, 1978; Davis et al., 1987). There was no early expression of the neuropeptides that are mainly seen in the A-delta and C-fibres except for CGRP. This substantiates reports of late physiological maturation of C fibres (Fitzgerald and Gibson, 1984; Fitzgerald, 1985)

and may be the reason for the reported experience of little pain in human neonates (Craig, 1980), assuming that the general trends of development between two species remains the same. The appearance of CGRP immunoreactivity during fetal life may indicate early neurochemical maturation of sensory neurons in comparison with autonomic neurons (Marti et al., 1987) and suggests the possible neurotrophic role in the developing tissues as suggested recently by their involvement in the differentiation of dopaminergic neurons of olfactory bulb (Dennis-Donini, 1989).

In conclusion, the results demonstrate that: 1. Antiserum to protein gene product 9.5 is the best marker for the visualisation of cutaneous nerves. 2. Regional and species differences concern only the density of peptidergic innervation, but not the specific peptides present or their distribution. 3. "Sensory" peptides appear earlier than "autonomic" peptides during development. 4. Not all the senosory axons immunoreactive for CGRP and substance P are equally capsaicin-sensitive. 5. All the sympathetic tyrosine hydroxylase- and neuropeptide Y-immunoreactive axons are totally responsive to 6-hydroxydopamine. Thus, the study shows the neurochemical, distribution and developmental differences in sensory and autonomic components of the nervous system of mammalian skin. The early maturation of sensory nerves in comparison to autonomic nerves is important and the possibility of a trophic role in skin remains to be established. The accurate identification and characterisation of cutaneous nerves has been a problem for some time preventing a better understanding of changes in cutaneous innervation; there was a need for a better histological technique. These results show that PGP 9.5 is a valuable general neural "marker" of normal innervation. The data provided should stimulate further studies of neurochemical and physiological variations of the cutaneous innervation.

Chapter 4

CHANGES IN NERVES AND NEUROPEPTIDES IN SKIN FROM LEPROSY PATIENTS

	Pages
4. 1. Introduction	**110-111**
4. 2. Experimental design	**111-113**
4.2.1. Tissues	111
4.2.2. Immunocytochemistry	113
4. 3. Results	**113-128**
4.3.1. Non leprous control specimens	113-115
4.3.2. Leprosy specimens	115-124
4.3.3. Indeterminate (early) leprosy	124-127
4.3.4. Lepromatous leprosy	126-127
4.3.5. Tuberculoid leprosy	127-128
4. 4. Discussion	**129-132**

4.1. INTRODUCTION

Since ancient times leprosy has been known for its neurological manifestations. Impaired sensations, muscle weakness and localised irregular nerve enlargements are cardinal signs of clinical leprosy. The primary focus of infection is usually a peripheral sensory nerve, resulting in impairment of sensation, followed by involvement of nerves containing both sensory and motor fibres, adding motor deficit to the early sensory loss. The final result of the disease process is disability and deformity.

Involvement of the cutaneous nerves constitutes the most unique feature of the pathological changes in leprosy (Khanolkar, 1952; Dastur, 1955). Axonal degeneration was said to be the common feature in tuberculoid leprosy (Dastur, 1972), but demyelination was described as the prominent change in lepromatous leprosy (Dastur and Razzak, 1971; Job, 1971). The causative organism is Mycobacterium leprae, which is known to have a unique characteristic of neural affinity (Weddell et al., 1963; Iyer, 1965; Job and Desikan, 1968). Reports on histopathological features of leprosy mention involvement of nerves in all stages and types of the disease. In skin, leprosy infection involves both somatic and autonomic components of the cutaneous nerves, and clinical experience shows that there is a definite pattern in the loss of cutaneous nerve function in leprosy. An early sign may be the loss of sweating followed by the loss of thermal sensation and touch (Weddell, 1977; Dabholkar et al., 1982; Boyle and Ramu, 1982) In addition, in all types of leprosy the earliest changes in peripheral nerves are seen in unmyelinated fibres followed by changes in small myelinated fibres and ultimately in the larger myelinated fibres (Shetty et al., 1978; Shetty et al., 1980). To substantiate this further, experiments have been carried out using a mouse model and have revealed a diffuse peripheral neuropathy as early as 4 months after inoculation of the bacterium (Shetty et al., 1980). These findings support the

observation that the involvement of nerves begins at a very early stage of leprosy infection.

Previous studies of cutaneous nerves in leprosy have used mainly conventional light and electron microscopic histology. Immunocytochemical techniques have been used to elucidate immunological mechanisms in the pathogenesis of the disease (Wright and Hirst, 1975; Ridley et al., 1982; Narayanan et al., 1986; Robles et al., 1985). Following the immunocytochemical localization of neuropeptides in cutaneous nerves, we have employed similar techniques in order to study the possible involvement of neuropeptides in leprosy. The specific aims of the study were to determine the pattern of cutaneous innervation in general and that of neuropeptide-containing nerves in particular in leprosy, and to correlate any abnormalities observed with the altered sensory and autonomic functions associated with the disease.

4. 2. Experimental design

4. 2. 1. Tissues

Skin biopsies were obtained in a peripheral leprosy hospital (St. Patrick's Leprosy Settlement, Mile 4 Hospital, Nigeria) from 120 suspected leprosy patients during the period between January to June of 1986, based on one of the following criteria, 1) the presence of hypopigmented or erythematous skin lesions of various types (macules, papules, nodules and plaques) with well or ill defined margins, their size and distribution with associated sensory changes 2) skin smear by slit and scrape method and 3) nerve thickening. The biopsy was performed with the patients' consent after the procedure and reasons for the biopsy has been explained to them, and with the permission of the resident Medical Superintendent, Dr. M.A. Macrae. They were taken from active lesions on the upper arm, forearm, thorax, abdomen, thigh, leg and lumbar region (Table 9). The history of the disease ranged from 6 months to several years. Normal control skin was obtained from non-leprosy patients (n=50) undergoing general surgical procedures that entailed skin removal in Central Middlesex Hospital (Approved by the Ethics committee of Brent Heads

Table 9

Area from which the skin biopsy was taken from leprous patients

	Fore Arm	Upper Arm	Leg	Thigh	Lumbar Region	Abdomen	Thorax
No. of Specimens	10	14	10	15	11	8	32

Authority) and cosmetic plastic surgery operations in Hammersmith Hospital and Queen Mary's Hospital, London. These specimens of skin were obtained from body areas comparable to those of leprosy biopsy specimens.

An elliptical incision was made through the entire skin depth and the entire biopsy was taken out with a horizontal cut parallel to the surface. Biopsies were cut into two equal portions. One portion was fixed in freshly prepared Bouin's fluid (**Appendix 3d**) for 4 hours, repeatedly washed in 30% alcohol, dehydrated, and then embedded in paraffin wax. The other was fixed in a 0.4 per cent solution of p-benzoquinone (**Appendix 3a**) in phosphate-buffered saline (**Appendix 2**).

Sections (6μm) of paraffin blocks were stained with haematoxylin and eosin (**Appendix 7**) for the subjective determination of the granulomatous fraction (the percentage of dermis occupied by the inflammatory cells), (Ridley, 1985) and by the Wade Fite method (**Appendix 8**) for the demonstration of acid fast bacilli (AFB). AFB were quantified on the standard logarithmic bacterial index (BI) scale (Ridley, 1985)

4. 2. 1. Immunocytochemistry

Biopsies from normal and leprosy cases were mixed in batches and cryostat sections (6μm) were taken upon poly-L-lysine coated glass slides (**Appendix 4**), allowed to dry for 60 min at room temperature, and then placed in PBS/Triton X-100 (**Appendix 2b**) for 30 min. Immunostaining was performed by a modification of the indirect immunofluorescence method (**Appendix 5a**) with antisera to neural antigens and neuropeptides. Staining was performed on batches of specimens, each containing samples from both groups.

Table 10

DENSITY OF NERVES AND NEUROPEPTIDE-IMMUNOREACTIVE FIBRES PRESENT IN THE SKIN OF NON-LEPROSY CONTROL SPECIMENS

Skin area	Total No.	No. of cases showing immunoreactive nerve fibres					
		PGP 9.5	NF	SP	CGRP	VIP	NPY
Epidermis	50	45	-	-	15	-	-
Dermis	50	50	50	45	50	25	-
Hypodermis	50	50	50	40	-	25	-
Hair follicle	50	35	35	-		15	-
Sweat glands	50	50	50		25	50	20
Blood vessels	50	40	10	-	30	15	45
Merkel cells	50	-	25	-		-	-

Key: PGP 9.5 = protein gene-product 9.5, NF = neurofilaments, SP = substance P, CGRP = calcitonin gene-related peptide, VIP = vasoactive intestinal polypeptide, Blank spaces (-) = None

4. 3. RESULTS

4. 3. 1. Non-leprous control specimens

The histology of these specimens showed no morphological abnormality or Wade Fite positivity. Antisera to the general neural marker PGP 9.5 and to neurofilaments were employed to reveal all the cutaneous nerves. In the control specimens, PGP 9.5-like immunoreactivity was observed in free nerve endings present in the epidermis and dermis (Fig.4.1A), around hair follicles, and sweat glands (Fig.4.2A). Immunoreactivity was also observed in nerve bundles, in relation to blood vessels and arrector pili. The distribution of neurofilament- and PGP 9.5-immunoreactive fibres was similar except that neurofilament-immunoreactive fibres were fewer in relation to blood vessels and showed only weak immunostaining around sweat glands. In addition, immunoreactivity for neurofilaments was seen in Merkel discs.

CGRP- and substance P-immunoreactive nerves were seen largely as beaded fibres running in the dermis, some apparently ending freely or in close relation with small arteries and arterioles, whereas a few fibres extended into the epidermis, reaching almost up to the stratum corneum (Figs.4.3A & 4.4A). CGRP-immunoreactive fibres were more frequent than substance P-immunoreactive fibres. Substance P-immunoreactive nerves were also found close to sweat glands and hair follicles. However, no peptide immunoreactivity was seen in the Merkel cells. The distribution patterns of VIP- and NPY-immunoreactive nerves were found to be similar, both supplying sweat glands (Fig.4.5A) and blood vessels. NPY immunoreactivity, however, was comparatively weak in intensity and was seen in fewer fibres than VIP around sweat glands. The distribution of CPON-immunoreactive fibres was similar to those showing NPY immunoreactivity both in density and in intensity. For details see **Table 10.**

Table 11

Classification of leprosy skin biopsies examined

Type*	No.	GF†		BI††					
		<10%	>10%	0	1+	2+	3+	4+	5+
Indeterminate	14	14	-	9	5	-	-	-	-
BT/TT	43	23	20	38	1	2	2	-	-
BL/LL	43	-	43	-	-	-	12	7	24

BT/TT=combined borderline tuberculoid and polar tuberculoid cases.

BL/LL=combined borderline lepromatous and polar lepromatous cases.

†GF=granulation fraction:percentage of dermis inflamed.

††BI=bacterial index: log scale bacterial density in the dermal nerves and inflammation (Ridley, 1985).

Blank spaces (-) = none

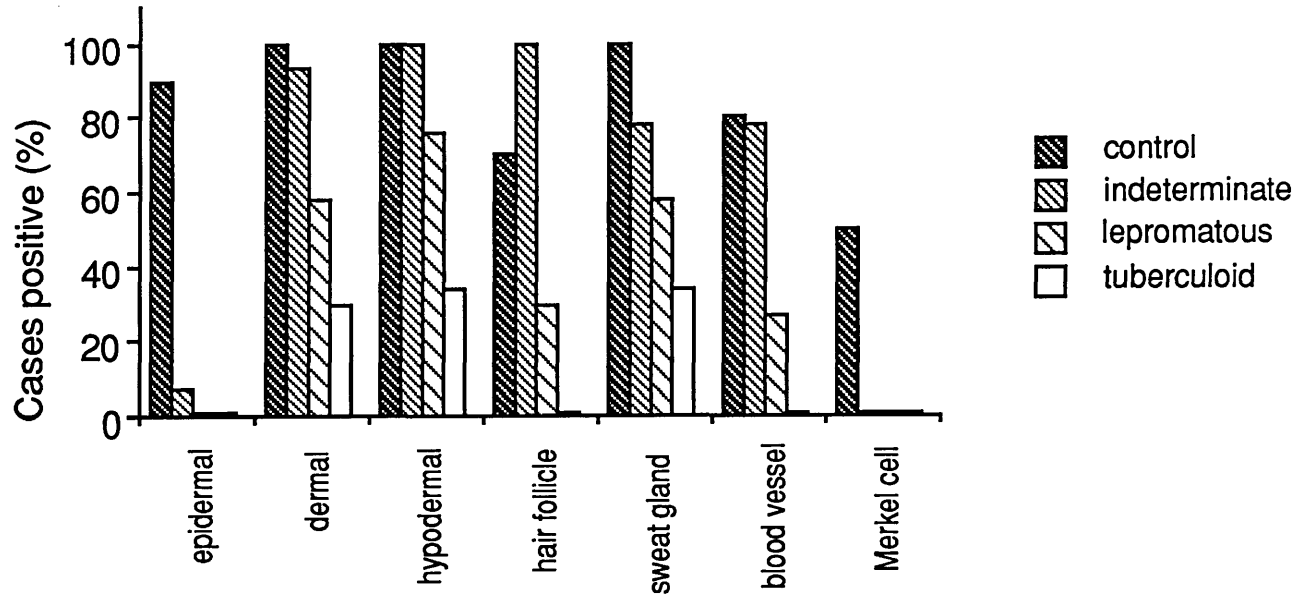


Diagram 8. Histogram showing the proportion of total cases in each class of leprosy which had immunoreactivity for PGP 9.5 or neurofilament triplet proteins in different parts of skin

Diagram 9. Histogram showing the proportion of total cases in each class of leprosy which had immunoreactivity for various neuropeptides

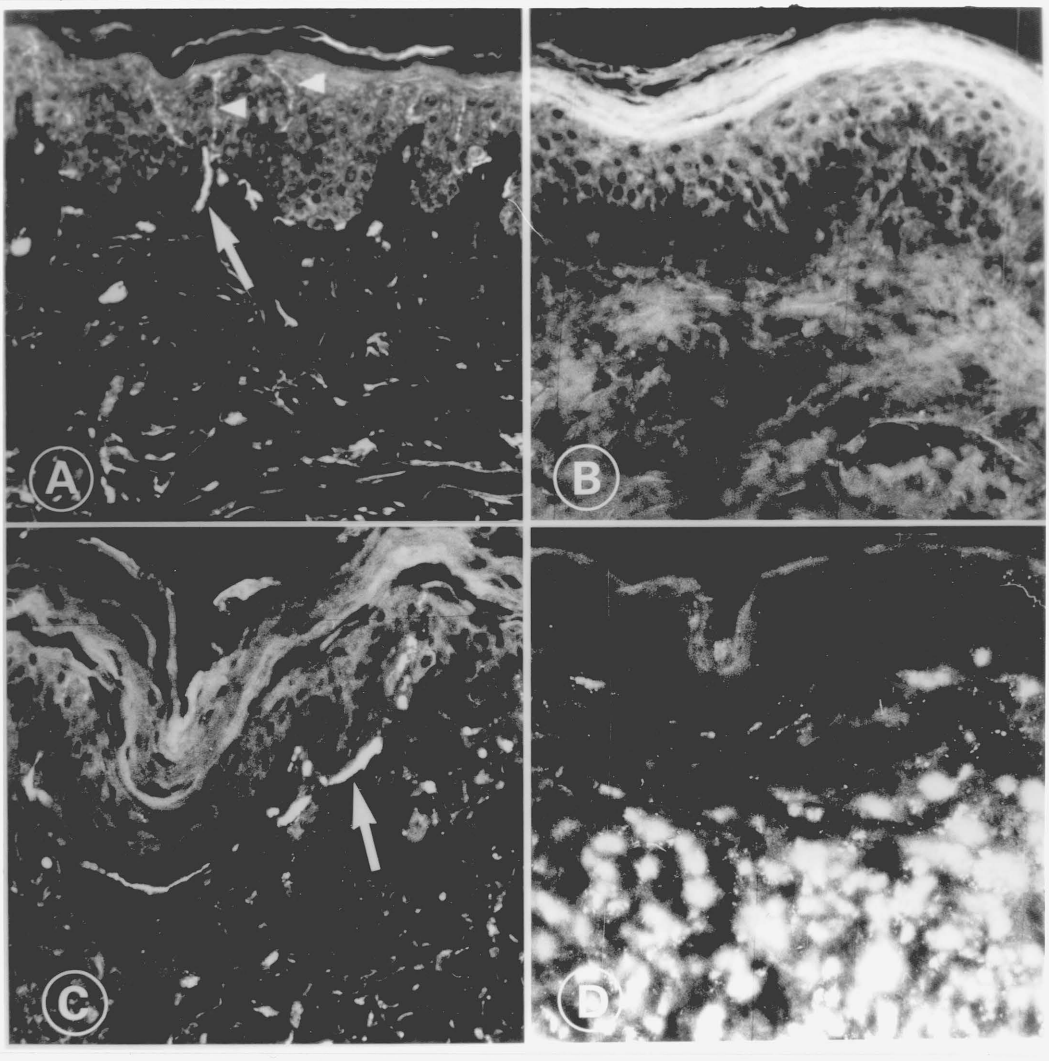


Fig. 4.1. Skin of arm showing PGP 9.5-immunoreactive fibres in the epidermis (arrowhead) and dermis (arrow). (a) Control skin showing a large number of immunoreactive fibres in the epidermis and dermis. (b) Tuberculoid leprosy specimen with complete absence of immunoreactive nerves.(c) Indeterminate leprosy. Epidermal nerves are absent and dermal fibres are reduced in number. (d) Lepromatous leprosy specimen showing a great reduction in the numbers of dermal fibres; macrophage infiltration of the dermis is present. Benzoquinone-fixed tissues, cryostat sections (6μm), indirect immunofluorescence method. All magnification x 250

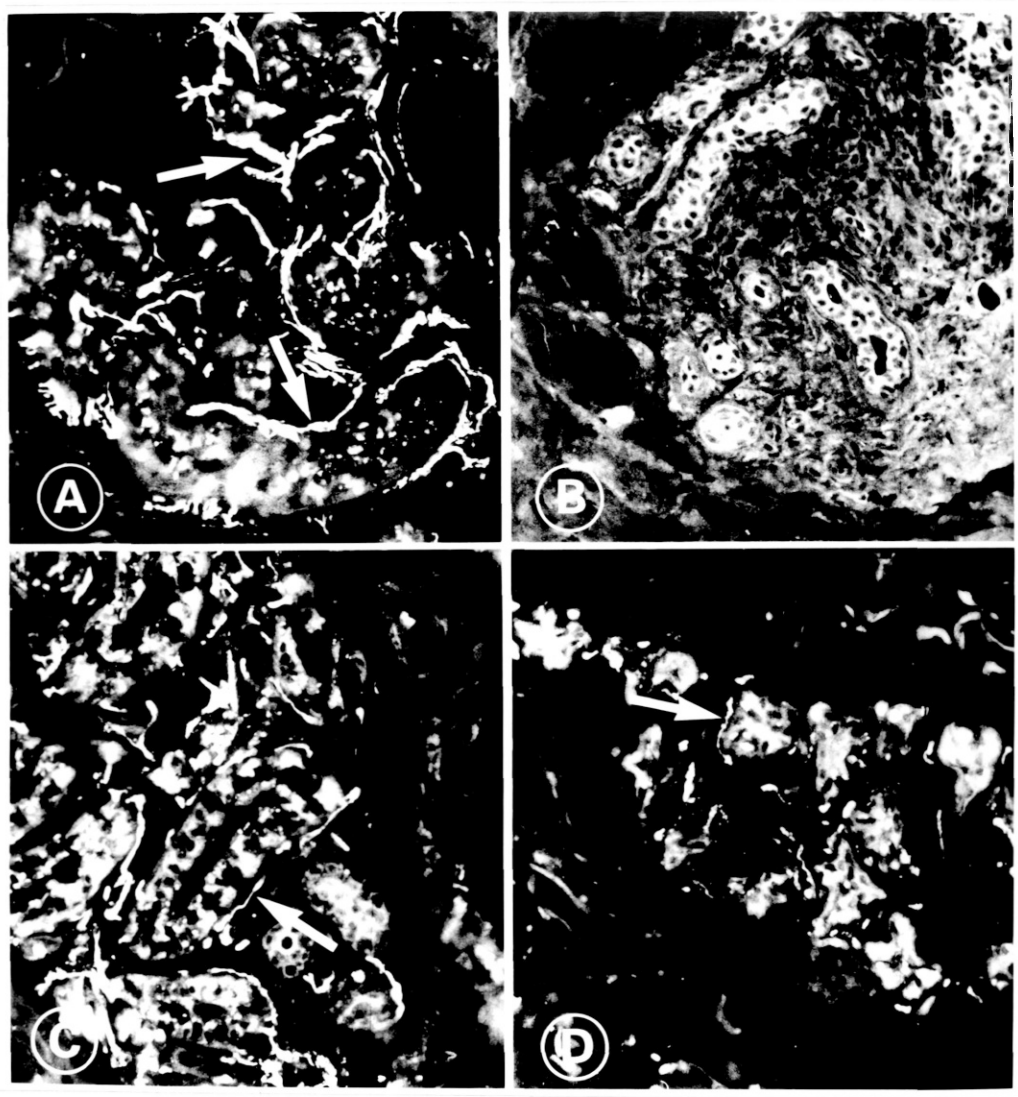


Fig.4.2. Skin of arm showing PGP 9.5-immunoreactive fibres around sweat glands. (a) Control skin showing a large number of immunoreactive fibres around sweat glands.
(b) Tuberculoid leprosy specimen with complete absence of immunoreactive nerves.(c) Indeterminate leprosy specimen showing reduced number of immunoreactive fibres.
(d) Lepromatous leprosy specimen showing a great reduction in the numbers; Benzoquinone-fixed tissues, cryostat sections (6μm), indirect immunofluorescence method.
All magnification x 250

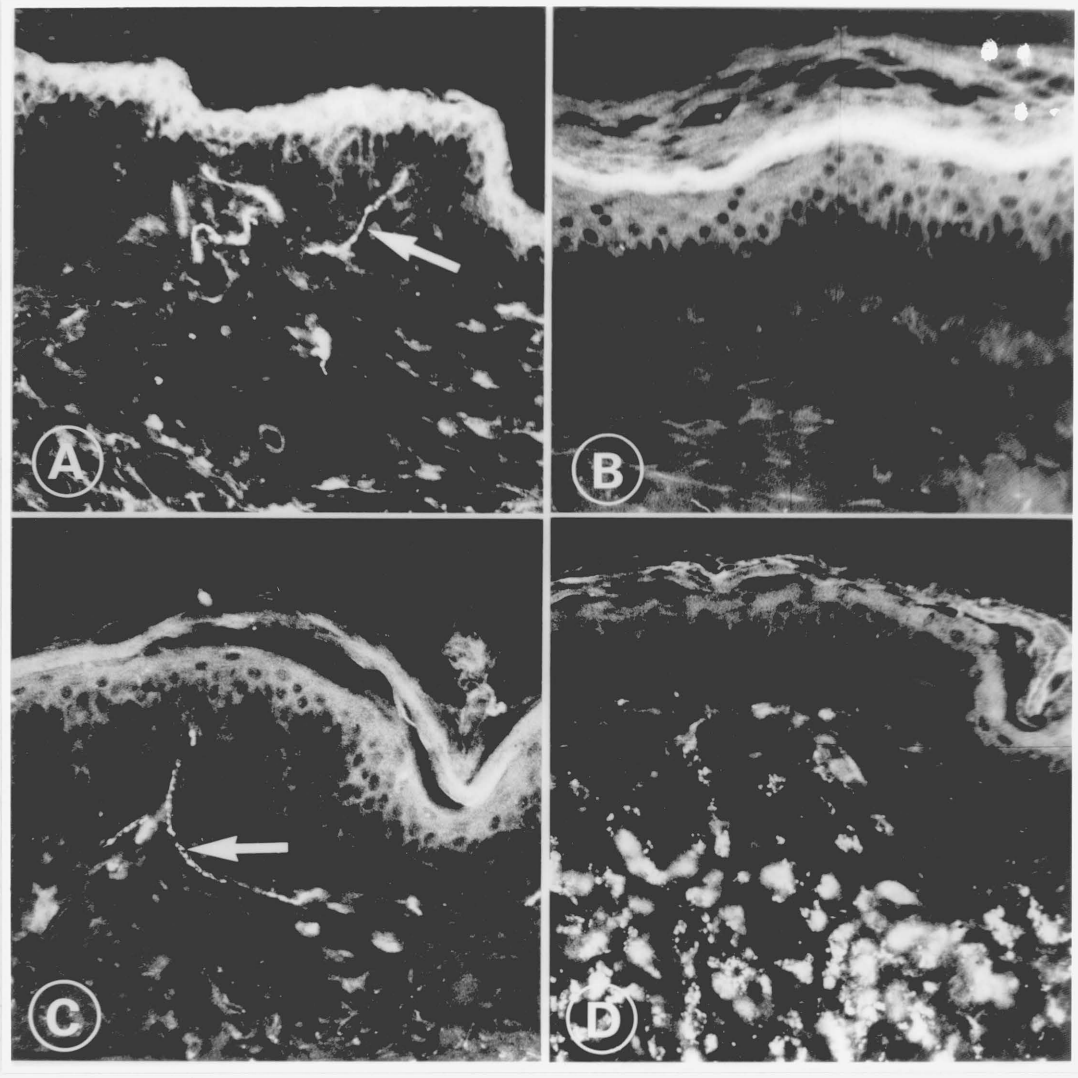


Fig. 4.3. Skin of arm showing CGRP-immunoreactive fibres (arrow). (a) Control skin showing a large number of immunoreactive fibres in the dermis. (b) Tuberculoid leprosy specimen with complete absence of immunoreactive nerves.(c) Indeterminate leprosy showing reduction in number. (d) Lepromatous leprosy specimen showing complete absence of immunoreactive fibres; Benzoquinone-fixed tissues, cryostat sections (6μm), indirect immunofluorescence method. All magnification x 250

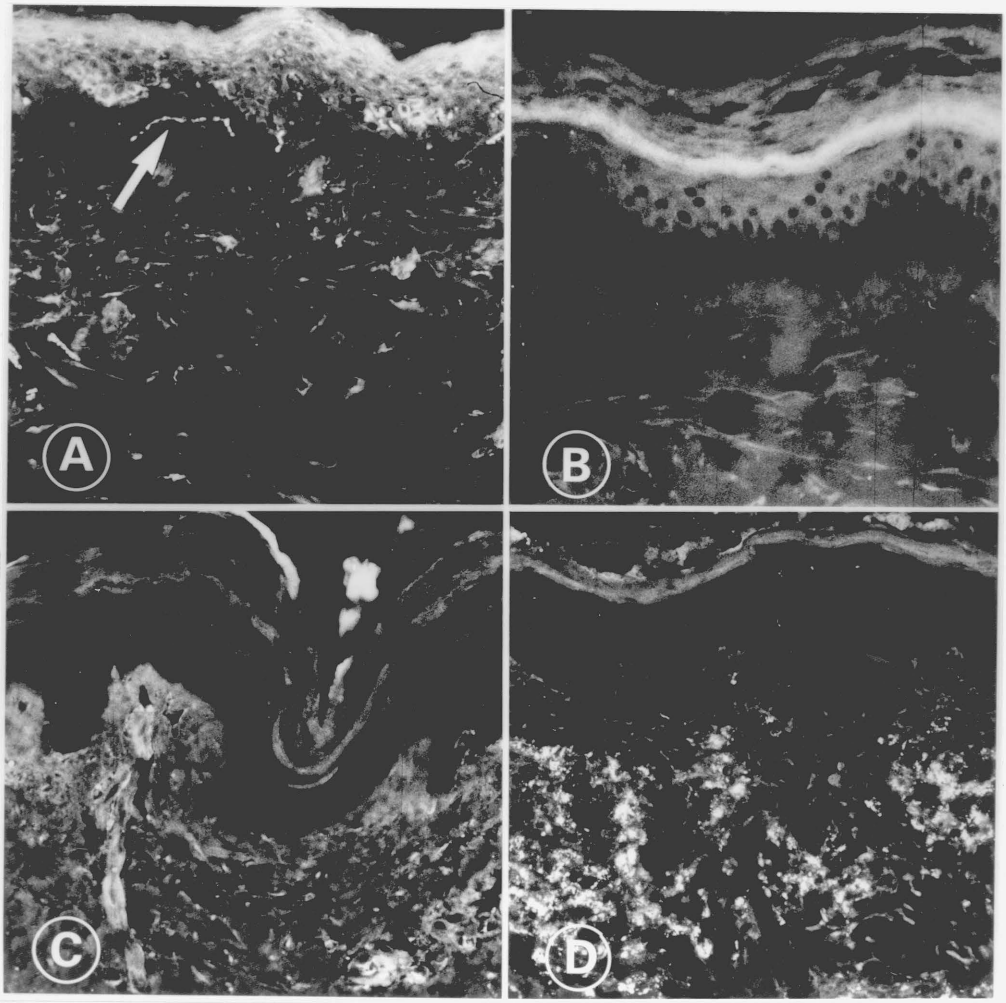


Fig. 4.4. Skin of arm showing SP-immunoreactive fibres. (a) Control skin showing a sparse number of immunoreactive fibres in the dermis. Tuberculoid leprosy (B), Indeterminate leprosy (C) and Lepromatous leprosy (d) showing a complete absence of immunoreactive fibres; Benzoquinone-fixed tissues, cryostat sections (6μm), indirect immunofluorescence method. All magnification x 250

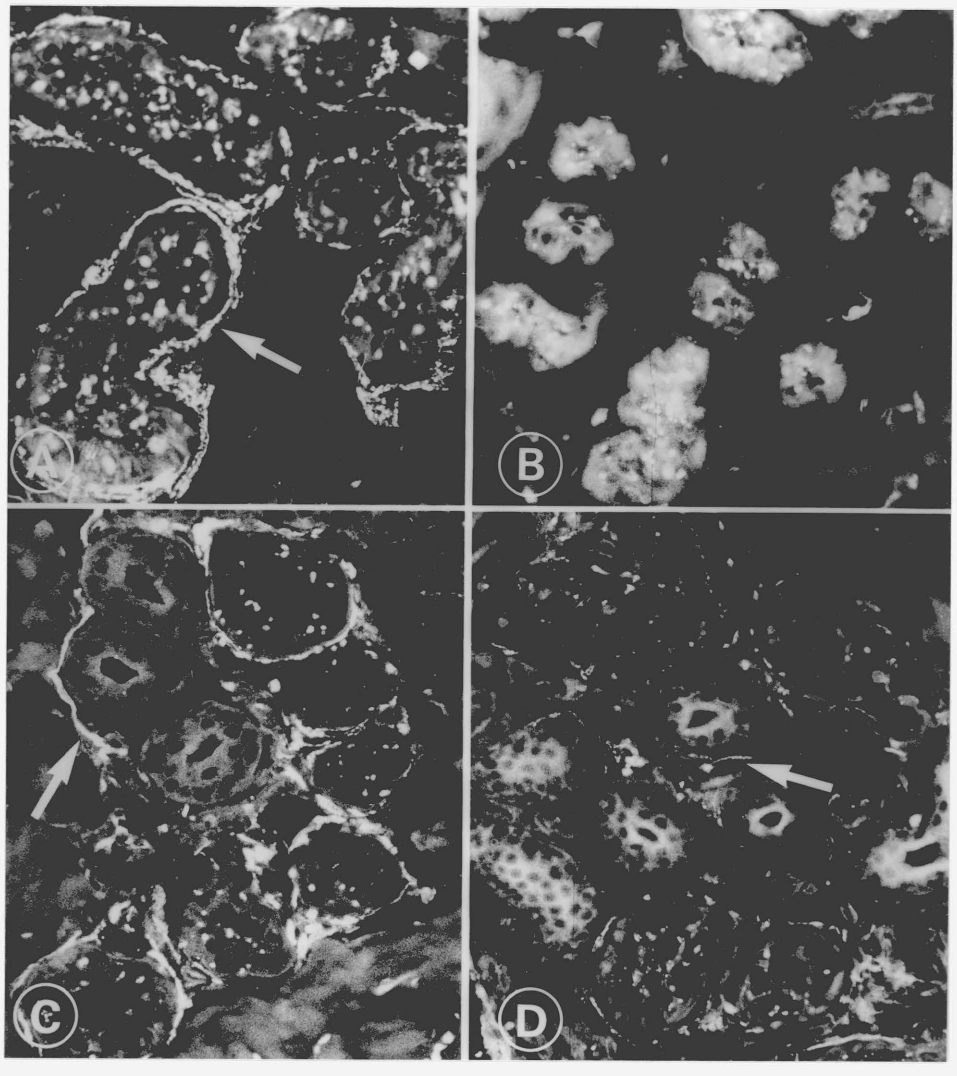


Fig.4.5. Skin of arm showing VIP-immunoreactive fibres around sweat glands. (a) Control skin showing a large number of immunoreactive fibres around sweat glands. (b) Tuberculoid leprosy specimen with complete absence of immunoreactive nerves.(c) Indeterminate leprosy specimen showing reduced number of immunoreactive fibres. (d) Lepromatous leprosy specimen showing great reduction in the numbers; Benzoquinone-fixed tissues, cryostat sections (6μm), indirect immunofluorescence method. All magnification x 320

4. 3. 2. Leprosy specimens

Of the 120 leprosy specimens histopathological analysis resulted in 100 cases of definite leprosy (e.g., intraneural granulomas, and/or acid fast bacilli in protected sites in skin), or almost certain leprosy showing dermal granulomas or non-granulomatous inflammation highly suggestive of leprosy (Fine et al., 1986). These leprosy specimens were classified according to the criteria of Ridley and Jopling (Ridley and Jopling, 1962; Ridley, 1985) (Table 11). The grouping was done in the Wolfson Tropical Pathology unit by Dr. S. Lucas. They were grouped into (1) indeterminate (early leprosy, with characteristic perineural inflammation and scanty lymphohistiocytic infiltration) but no tuberculoid granulomas in nerve or dermis and scanty or no evident AFB; (2) tuberculoid (including borderline and polar tuberculoid leprosy with epithelioid granulomas and Langhans' giant cells surrounded by lymphocytes involving nerves, and none to small numbers of AFB; and (3) lepromatous leprosy (including borderline and polar lepromatous leprosy) showing extensive infiltrate involving the entire thickness of the dermis, sparing the immediate subepidermal zone, with many AFB in macrophages and nerves. The cases of indeterminate leprosy may inevitably include some tuberculoid cases which are resolving on therapy (nonetheless, the trends in the results are not significantly affected by this). The results of immunostaining are summarised in Histograms 8 & 9.

4. 3. 3. Indeterminate (early) leprosy

The histopathology of these specimens showed 2-5 per cent granulomatous fraction. Lympho-histiocytic infiltrates around nerves, sweat glands and arrector pili were noticed frequently. Upon immunocytochemical examination a marked difference was noted when compared with controls, not only in the distribution density of nerve fibres but also in their peptide content. Only 1/14 of the biopsy specimens showed PGP 9.5-immunoreactive fibres in the epidermis, whereas in the dermis, hypodermis, sweat glands and hair follicles, PGP 9.5- or neurofilament-immunoreactive fibres were present in almost all the specimens (Figs.4.1C & 4.2C), albeit in a markedly smaller number (visually estimated as approximately 70 per cent or

Table 12

DENSITY OF NERVES AND NEUROPEPTIDES-IMMUNOREACTIVE FIBRES PRESENT IN THE SKIN OF INDETERMINATE TYPE OF LEPROSY

Skin area	Total No.	No. of cases showing immunoreactive nerve fibres					
		PGP 9.5	NF	SP	CGRP	VIP	NPY
Epidermis	14	1	-	-	-	-	-
Dermis	14	13	13	-	2	-	-
Hypodermis	14	14	14	-	-	-	-
Hair follicle	14	14	14	-	-	-	-
Sweat glands	14	11	11	-	-	-	-
Blood vessels	14	14	14	-	-	-	-
Merkel cells	14	-	-	-		-	-

Key: PGP 9.5 = protein gene-product 9.5, NF = neurofilaments, SP = substance P, CGRP = calcitonin gene-related peptide, VIP = vasoactive intestinal polypeptide, Blank spaces (-) = none

Table 13

DENSITY OF NERVES AND NEUROPEPTIDE-IMMUNOREACTIVE FIBRES PRESENT IN THE SKIN OF LEPROMATOUS LEPROSY (BL/LL)

		No. of cases showing immunoreactive nerve fibres					
Skin areas	Total No.	PGP 9.5	NF	SP	CGRP	VIP	NPY
Epidermis	43	-	-	-	-	-	-
Dermis	43	25	25	-	-	-	-
Hypodermis	43	33	33	-	-	-	-
Hair follicle	43	13	13	-	-	-	-
Sweat glands	43	25	25	-	-	-	-
Blood vessels	43	12	12	-	-	-	-
Merkel cells	43	-	-	-	-	-	-

Key: PGP 9.5 = protein gene-product 9.5, NF = neurofilaments, SP = substance P, CGRP = calcitonin gene-related peptide, VIP = vasoactive intestinal polypeptide, Blank spaces (-) = none

less) than in the controls. Immunoreactivity for peptides present in sensory and autonomic nerves was seen only in 2/14 of the specimens, where CGRP- and VIP-immunoreactive fibres (Figs.4.3C,4.4C & 4.5C) supplied dermis and sweat glands, respectively. However, substance P-immunoreactive fibres were completely absent (Table 12). Acid fast bacilli were scanty in five cases (BI = 1+) and absent in nine case. The AFB negative Indeterminate cases were diagnosed as leprosy on the basis of lymphocytic infiltration around superficial vessels, skin adenexae and around dermal nerves throughout the entire depth of the skin biopsy confirmed involvement of deep dermal nerves . Although, these are not pathognomanic of the leprosy, the perineural infiltrate was sufficiently characteristic to place them in the Indeterminate category (Table 11). All cases were seen by Dr. S. Lucas who made the histological diagnosis of leprosy.

4. 3. 4. Tuberculoid Leprosy

A great degree of neural inflammatory infiltration and nerve destruction was seen in all the 43 cases. When immunostained with antisera to PGP 9.5 or neurofilaments, only 13 cases showed nerve fibres in the dermis and around sweat glands and 15 cases showed fibres in the hypodermis surrounded by epitheloid granuloma. Partial or complete infiltration and replacement of nerve bundles was seen in the majority of cases (Fig.4.1B). In addition, granulomatous deposits surrounded all the appendages of the skin to such an extent that the morphology was completely lost (Fig. 4.2B). Neuropeptide immunoreactivity was completely absent (Figs. 4.3B, 4.4B & 4.5B) (**Table 13**). Bacterial indices were zero in 38 cases, 1+ in 1 case, 2+ in 2 cases, and 3+ in 2 cases (**Table 11**). The two BT cases with 3+ AFB were the patients who were going to downgrade to multibacillary BL.

4. 3. 5. Lepromatous leprosy

Massive macrophage infiltration was seen in the papillary and reticular layer of the dermis with many lymphocytes in the borderline lepromatous cases. When immunostained, epidermal PGP 9.5-immunoreactive nerve fibres were not seen in any of the cases examined. However, 33 cases showed PGP 9.5- or neurofilament-

Table 14

DENSITY OF NERVES AND NEUROPEPTIDE-IMMUNOREACTIVE FIBRES PRESENT IN THE SKIN OF TUBERCULOID LEPROSY (BT/TT)

Skin areas	Total No.	No. of cases showing immunoreactive nerve fibres					
		PGP 9.5	NF	SP	CGRP	VIP	NPY
Epidermis	43	-	-	-	-	-	-
Dermis	43	13	13	-	-	-	-
Hypodermis	43	15	15	-	-	-	-
Hair follicle	43	-	-	-	-	-	-
Sweat glands	43	13	13	-	-	-	-
Blood vessels	43	-	-	-	-	-	-
Merkel cells	43	-	-	-	-	-	-

Key: PGP 9.5 = protein gene-product 9.5, NF = neurofilaments, SP = substance P, CGRP = calcitonin gene-related peptide, VIP = vasoactive intestinal polypeptide, Blank spaces (-) = none

immunoreactive nerve fibres in the hypodermis, 25 cases in the dermis (Fig.4.1D), and 13 cases around hair follicles. Granuloma was brightly stained, as a distinct layer in the dermis (Fig. 4.1D), frequently surrounding the neurovascular bundles, hair follicles, and sweat glands (Fig.4.2D). The staining was not quenched by absorption of any of the primary antisera with the homologous antigen and no staining was seen when the primary antiserum was omitted. No immunoreactivity for neuropeptides was seen in any region or in association with any appendage (Fig.4.3D,4.4D & 4.5D)(**Table 14**). Bacterial indices were 3+ in 12 cases, 4+ in 7 cases, and 5+ in 24 cases (**Table 11**).

4. 4. DISCUSSION

This study has demonstrated that the cutaneous nerves in all types of leprosy showed a reduction in number as shown by decreased density of PGP 9.5-immunoreactive nerves and near complete absence of neuropeptides. The degree of reduction in number of nerves and neuropeptides was related to the type of leprosy. It is probable that the disappearance of neuropeptides may have occurred at an early stage of the infection, due to their localization mainly in unmyelinated cutaneous nerves which are the primary target in leprosy infection. This may also be due to the damage caused by the granuloma formation around the main nerve trunks thereby producing abnormal axonal transport. This is speculative and remains to be investigated. The depletion of neuropeptides seen in this study may be similar to the depletion observed after peripheral nerve transection or capsaicin treatment (Gibson et al., 1989; Kar et al., 1990). The Schwann cells of unmyelinated nerve fibres harbour Mycobacterium leprae more rapidly than myelinated fibres (Weddell and Palmer, 1963) and this may be partly responsible for their early involvement. In addition, an autoimmune reaction against neural antigens may also be partly responsible for the early involvement of these fibres in the disease process (Crawford et al., 1974; Pearson and Weddell, 1975; Shetty et al., 1985). Experimental studies have shown that the unmyelinated fibres display degenerative changes as early as 4-8 months after inoculation. These changes not only were observed at the site of inoculation, but were also seen away from it (Shetty et al., 1978; Shetty et al., 1980a;

Shetty et al., 1980b). This may well signify the diffuse vulnerability of cutaneous unmyelinated fibres in leprosy infection. The bright staining of granuloma is an interesting feature. The absorption studies showed that only the immunostaining of cutaneous nerves for PGP 9.5, NF, CGRP, substance P, VIP and NPY was quenched when treated with corresponding antigen, but not the staining of the granuloma. In addition, the incubation of the tissues with PBS instead of primary antisera did not give positive staining in the granuloma indicating that the secondary antiserum (antispecies IgG conjugated with FITC) is not binding to granuloma. This suggests that the staining is either due to residual M. Tuberculosis antibodies (due to the use of Freund's adjuvant for immunisation) in the primary antisera cross reacting with M. Leprae antigens present in the granuloma or non-specific binding to the granuloma. The epidermal and dermal nerves, and nerve endings can be made out clearly in non-leprous control specimens, where they were immunoreactive for PGP 9.5 and/or neurofilament antisera. Of the leprosy specimens, the indeterminate type showed more immunoreactive nerve fibres than other types. In tuberculoid leprosy, the immunoreactive fibres had almost disappeared in the majority of cases (15/43), showing a greater degree of neural destruction than in lepromatous leprosy, where more than half of the total number of cases (33/43) still had immunoreactive nerve fibres in all the different areas and structures of the skin examined. This observation further substantiates the established clinical finding that the impairment of sensation is most marked in tuberculoid leprosy lesions and less marked in lepromatous leprosy lesions. Sense of touch is believed to be served by nerve fibres supplying the hair follicles and Merkel disks (Winkelmann, 1959; Montagna and Ellis, 1958; Smith, 1967; Montagna, 1977; Mihara et al., 1979). These structures can be visualized in non-leprous control specimens where they are immunoreactive for PGP 9.5 and neurofilaments, respectively. However, this study does not support the reports of presence of VIP (Hartschuh et al., 1984) and CGRP (Garcia-Caballero et al., 1989) immunoreactivity in Merkel cells of man. This may be due to the different fixation conditions used in this study. In a parallel study, similar observation was made using same fixation conditions (Björklund et al., 1986). Within the leprosy spectrum, follicular endings are seen only in a few cases of indeterminate and lepromatous leprosy,

and Merkel cells appear to be absent in all types of leprosy. This observation suggests the impairment of sense of touch in all types of leprosy. The complete absence of Merkel cells may be due to the early disappearance of unmyelinated nerve fibres supplying them (Palmer, 1965; Iggo and Muir, 1969).

The most numerous sensory elements responsible for initiating pain and temperature reactions are the unmyelinated fibres (Hallin et al., 1982). The neurochemicals identified in these nerve fibres are substance P (Hökfelt et al., 1975a, Henry, 1982; Kruger et al., 1985) and neurokinin A (Dalsgaard et al., 1987) coming under the family of tachykinins (Nawa et al., 1984) and CGRP (Gibbins et al., 1987b). The putative roles of these peptides have been well documented by pharmacological and physiological studies (Jessel, 1982; Pernow, 1983; Goodman and Iverson, 1986). The nerves showing immunoreactivity to substance P and CGRP were seen in non-leprosy control specimens, whereas in leprosy immunoreactive nerve fibres were almost completely absent. It is also pertinent to recall the physiological observations in an earlier study (Dastur, 1955) where pain and temperature sensations were the first to be affected. The above observations further substantiate the early impairment of pain and temperature sensation in all the leprosy types.

Clinical experience shows that in all the leprosy skin lesions, irrespective of the type, there is evidence that the process of the triple response involves the release of substance P and CGRP (Burnstock, 1977; Hagermark et al., 1978; Jorizzo et al., 1983; Foreman, 1984; Wallengren and Hakanson, 1987; Fuller et al., 1987) Therefore it is conceivable that the diminution of triple response seen in leprosy may be due to the absence of these neurochemicals which are factors involved in neurogenic inflammation and plasma extravasation (Lembeck and Holzer, 1979; Gamse and Saria, 1985)

Clinical manifestation of anhydrosis due to the involvement of autonomic nerves has been reported by previous research workers (Sehgal, 1976; Mathur et al., 1981). Immunohistochemical studies have shown the presence of VIP and NPY in the nerves supplying sweat glands. These neurochemicals have been proposed to regulate

sweating and blood flow (Lundberg, 1981; Pernow et al., 1986) and are therefore believed to play a role in thermoregulation. PGP 9.5-immunoreactive nerve fibres were seen around sweat glands in 11/14 cases of indeterminate leprosy, 25/43 lepromatous cases, but only 13/43 tuberculoid cases. Nonetheless, VIP immunoreactivity was preserved in 2/14 of the indeterminate cases. This suggests the absence of regulatory effects on sweat glands indeterminate (i.e., tuberculoid and lepromatous) leprosy and a notable loss of these effects in early leprosy lesions.

In conclusion, immunohistochemical studies of leprosy skin biopsies showed a wide degree of variation in density and distribution of nerve fibres in different types of leprosy, as well as an almost total absence of sensory and autonomic neuropeptide immunoreactivity. The results agree well with the clinical and neurohistological changes seen in leprosy. The diffuse early degeneration of unmyelinated fibres and loss of contained neuropeptides is a novel observation. The depressed neural activity seen in leprosy may be due partly to the loss of neuropeptides and neurofilaments. The present results indicate that immunocytochemical study of neurochemicals in cutaneous nerve fibres reveals marked abnormalities even in early lesions. These results suggest the need for further studies to investigate the evolution of defects in cutaneous innervation across the immunopathological spectrum of leprosy. Furthermore, the immunocytochemical techniques may enable the more precise categorization of suspected very early leprosy lesions with obvious implications for clinical practice.

Chapter 5

TIME RELATED DECREASE OF CGRP AND SUBSTANCE P IMMUNOREACTIVITY IN THE SKIN AND SPINAL CORD OF LEPROSY MICE

5. 1. Introduction **134**

5. 2. Experimental design **135-136**

5. 2. 1. Tissues and fixation 135

5. 2. 2. Immunostaining 135

5. 2. 3. Quantification 136

5. 3. Results **137-143**

5. 3. 1. Clinical and Histological manifestation of disease 137

5. 3. 2. Skin 138-140

5. 3. 3. Spinal cord 140-143

5. 3. 4. Quantification 143

5. 4. Discussion **144-147**

5. 1. Introduction

There have been a number of light and electron microscopy studies of nerves in both human leprosy (Iyer & Desican, 1968, Job, 1971, Dastur et al., 1973, Pearson & Weddell., 1975) and in experimental animals (Weddell, Palmer & Rees., 1971, Mehta et al., 1976). In recent years, several studies have shown that somatosensory pathways from skin to the dorsal root ganglia and spinal cord involve a number of neuropeptides including substance P and calcitonin gene-related peptide (CGRP) (Hokfelt et al., 1977, Gibson et al., 1984, Ju et al., 1987, Gibbins et al., 1987b) and that these peptides are synthesised in dorsal root ganglia (Henken et al., 1988, Gibson et al., 1988b).

It is increasingly becoming evident that several neurological disorders of sensory nervous system are accompanied by changes in neuropeptide levels (Mora et al., 1989, Cummings et al., 1984, Faccini et al., 1984, Pearson et al., 1982, Kuraishi et al., 1989, Kar et al., 1989b). In the previous chapter, the study of neuropeptide changes in the skin of leprosy patients raised the following questions.

1. Do neuropeptides show changes early after infection and are these changes proportional to the duration of disease ?

2. Are the neuropeptide changes in skin localised to the site of infection, or are they seen in regions away from it?

3. Are there corresponding changes in spinal cord ?

Since a combined study involving skin and spinal cord cannot be conducted in man due to the difficulty in obtaining tissue, recourse was made to the nude mouse model.

5. 2. Experimental design

5. 2. 1 Tissues and fixation

Eight mice (MF1 nu/nu) aged 6 weeks were injected into the hind foot pad with Mycobacterium leprae as described previously (Colston & Hilson, 1976)(**Appendix 9**); an equal number of normal litter mates, matched for age, sex and body weight were kept as controls. The innoculation was carried out in a nude mice colony maintained at St. George's Medical College, under the supervision of Dr. D.K. Banerjee. Half the injected animals and controls were killed after 6 months, the remaining animals after 12 months. Mice were anaesthetised and perfused transcardially with phosphate-buffered saline (PBS) (**Appendix 2**) followed by Bouin's fluid (**Appendix 3B**). Immediately before the perfusion with Bouin's fluid, skin samples were taken from the dorsum of foot pads, abdominal region, nose, lip and ear, and fixed by immersion in Zamboni's fluid (**Appendix 3d**)for six hours. Following fixation with Bouin's fluid, C6, T6, and L4 segments of the spinal cord were dissected. All the tissues were then washed in PBS/sucrose and stored at 4°C.

5. 2. 2 Immunostaining

Control and treated specimens were mixed in batches and cryostat sections of spinal cord (20μm) and skin (10μm) were collected on poly-L-lysine coated glass slides (**Appendix 4**) and allowed to dry for 60 minutes at room temperature. Skin was immunostained by a modification of the indirect immunofluorescence method (**Appendix 5A**) and spinal cord by a modification of the peroxidase method using the glucose oxidase-DAB-nickel enhancement technique (**Appendix 5B and 6**). Sections of skin were also stained with haematoxylin and eosin (**Appendix 7**) for the determination of granuloma.

5. 2. 3. Quantification

To quantitate the area of immunoreactivity in the dorsal horn of spinal cord, five sections from the L_4 spinal cord segments were immunostained for CGRP or substance P in four treated mice and age-matched controls. Each section was then screened using the 10x objective to include the entire dorsal horn. The area occupied by immunoreactive fibres in laminae I-III of both sides of the spinal cord was then measured using the recorded image in an image analysis system (IBAS 2000, Kontron) that measures the optical density (grey level) using blue-green illumination. It is assumed that the grey level for the light passing through the empty slide represents 100% light transmission. This is connected for variation over each field using a normalising function. This particular level is kept constant wherever a measurement was made. The area of immunoreactivity was separated from the unstained and non-specific background by selecting a grey level between those of the background and the specific staining which was kept constant for all the sections taken from control and treated pairs of animals. The immunoreactivity of treated and control animals was compared, statistically using student's t-test and confidence interval analysis. The magnitude of differences between groups was calculated by comparing the mean values of optical density for each group.

5. 3. Results

5. 3. 1. Clinical and histological manifestation of disease

Skin lesions presenting in the form of nodular swellings in the hind feet were seen only in 12 month infected mice. No deformities or other clinical manifestations were seen in either 6 or 12 month infected mice. The cellular response at the dorsum of the foot in 6 months

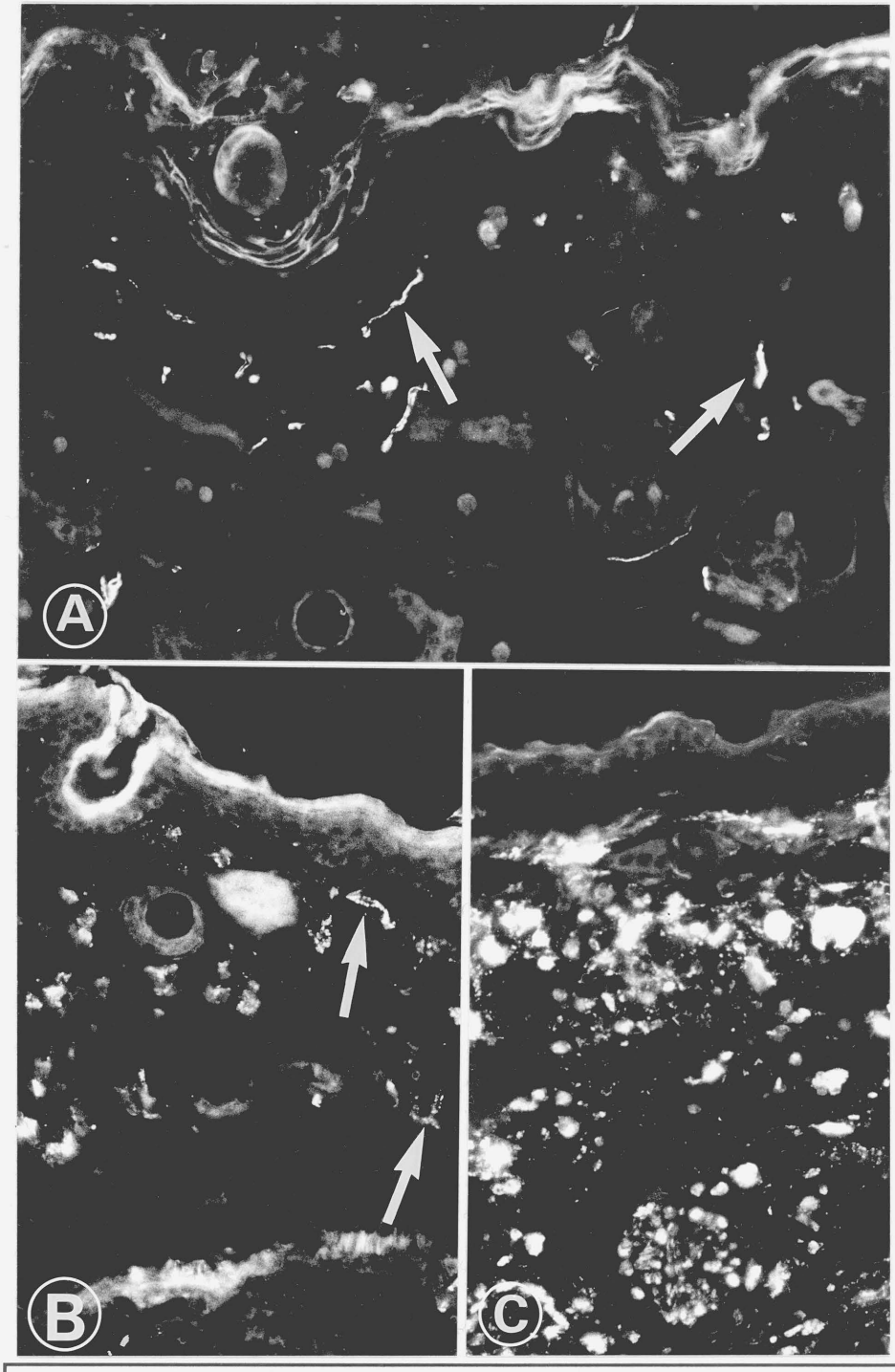


Fig. 5.1. CGRP-immunoreactive fibres in the epidermis and dermis from, (A) control skin of hind paw, (B) skin of hind paw taken 6 months and (C) 12 months after subcutaneous injection of Mycobacterium leprae into the hind foot pads. Note the disappearance of immunoreactive nerves by 12 months. Arrows indicate some immunoreactive nerves. Autofluorescence of granulomata is seen in (C). Zamboni fixed tissues.Cryostat sections (10μm). All magnification x 280

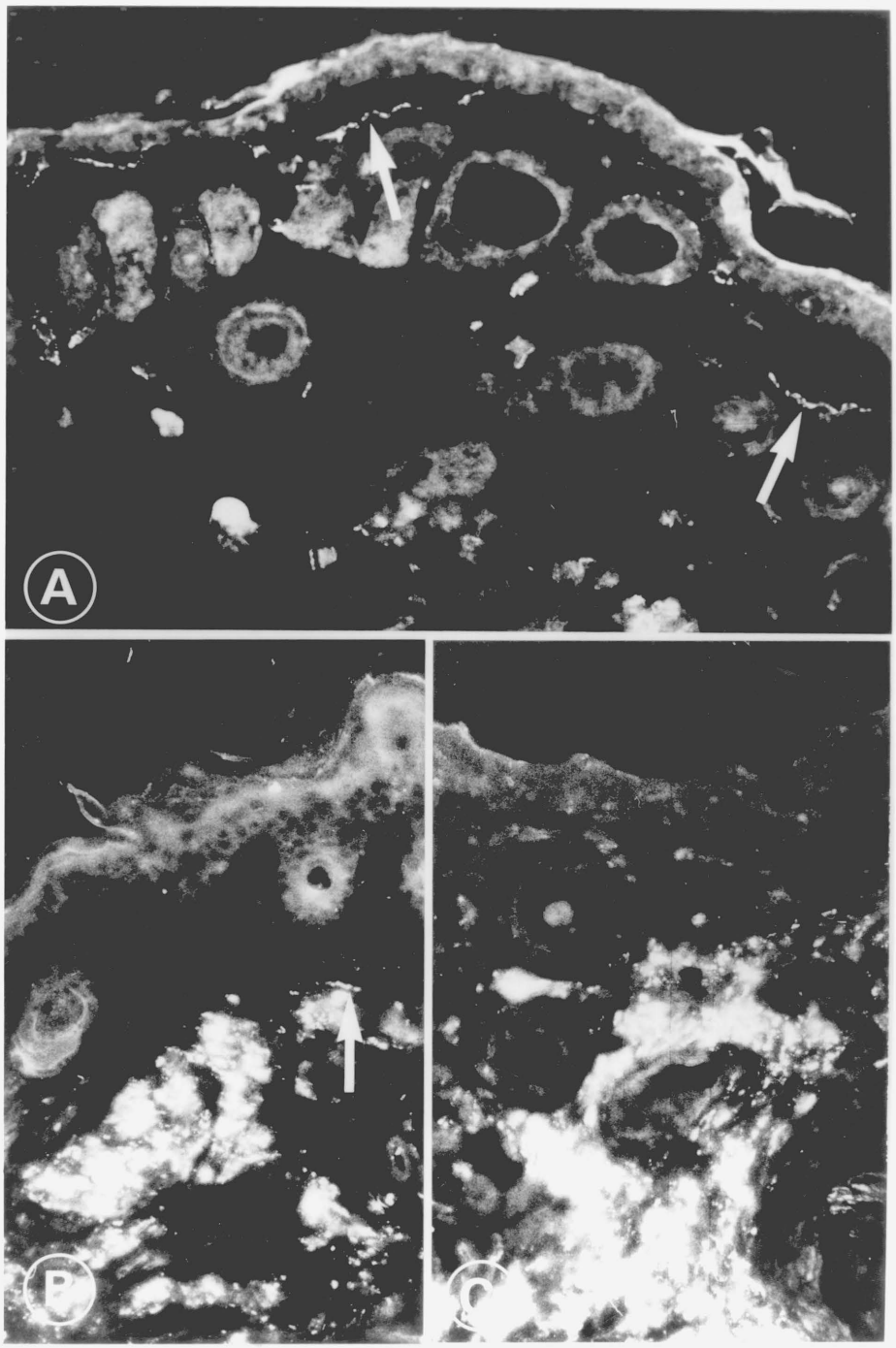


Fig. 5.2. Substance P-immunoreactive fibres in (A) epidermis and dermis from control skin of hind paw, (B) skin of hind paw taken 6 months and (C) 12 months after subcutaneous injection of Mycobacterium leprae into the hind foot pads. Note the loss of immunoreactive nerve fibres compared with control skin in Fig.1(A). Note the autofluorescence of the granuloma cells. Arrows indicate some immunoreactive nerves. Zamboni fixed tissues. Cryostat sections (10μm). All magnification x 280

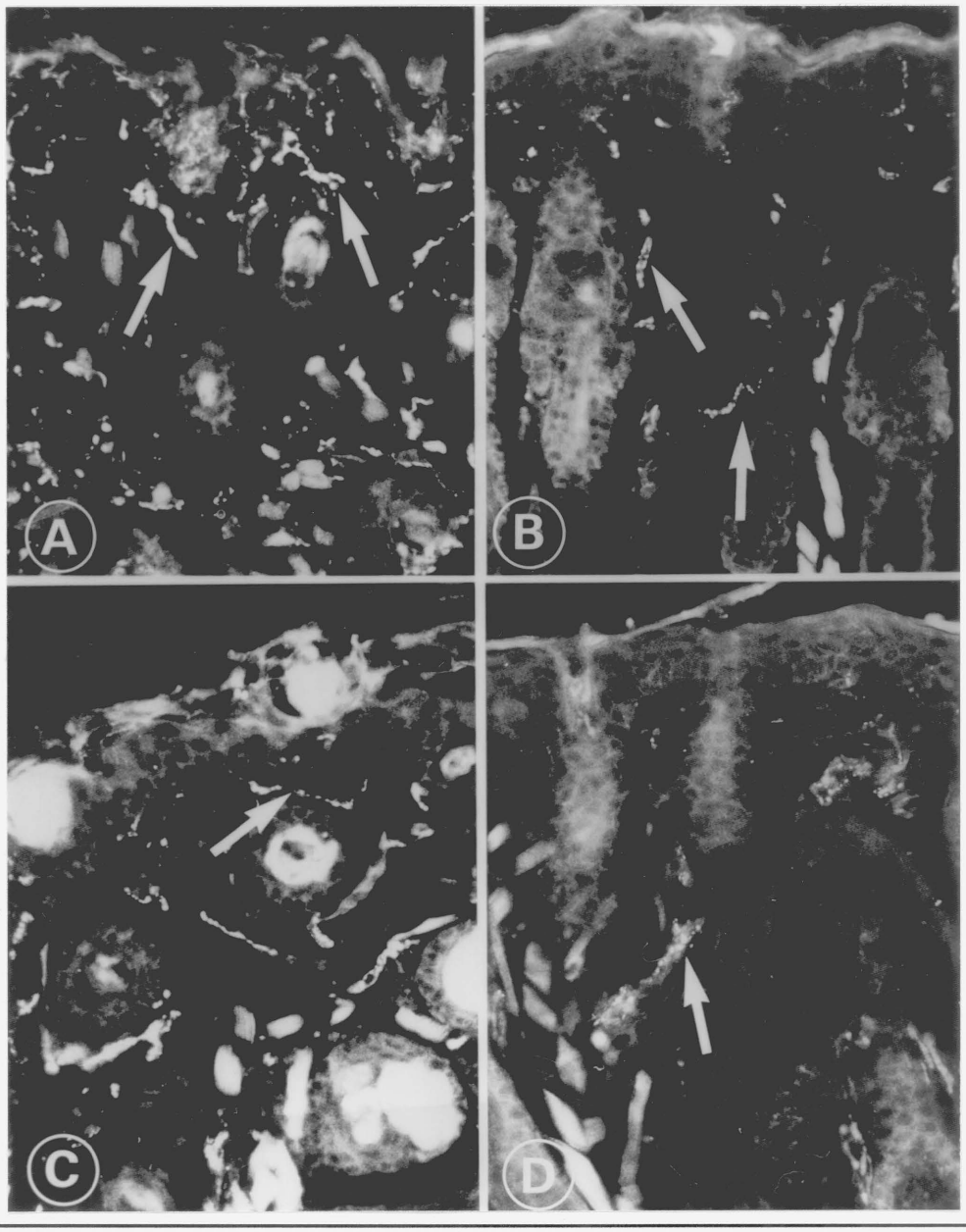


Fig. 5.3. Nose skin from control mouse showing the normal distribution of CGRP-immunoreactive fibres (A) and substance P-immunoreactive fibres (B). Nose skin showing reduction in CGRP-immunoreactive fibers (C) and substance P-immunoreactive fibres (D) 12 months after subcutaneous injection of Mycobacterium leprae into the hind foot pads. Arrows indicate some immunoreactive nerves. Zamboni fixed tissues. Cryostat sections (10μm). All magnification x 280

infected mice was minimal. After 12 months of infection, the cellular response was predominantly histiocytic, infiltrating the dermis but leaving a cell-free area below the epidermis.

5. 3. 2. Immunocytochemistry

Skin: In control animals, CGRP-immunoreactive fibres (Fig. 5.1A & 5.3A) were more frequent than those reacting with antibodies to substance P (Fig.5.2A & 5.3C), but they had similar distributions. They were seen mainly in the dermis as varicose fibres, some apparently ending freely, or seen in close relation with small arteries, arterioles, and sweat glands. A few fibres extended into the epidermis, reaching almost to the stratum corneum. Occasional small bundles of immunoreactive fibres were noted close to the epidermis.

In contrast, after 6 months of Mycobacterium leprae infection substance P (Fig. 5.2B) and CGRP-immunoreactive (Fig.5.1B) fibres were substantially reduced in epidermis and dermis, not only at the site of infection or inoculation but also in other regions of the body including forefoot pad, nose (Fig.5.3B & 5.3D), lip, ear and abdominal skin. After 12 months of infection, substance P- and CGRP-immunoreactive fibres were almost absent at the site of infection and markedly reduced in other skin regions.

5.3.3. Spinal cord

In control animals, a dense network of CGRP-immunoreactive fibres was seen in the dorsal horn of the L_4 segment (Fig. 5.4A), particularly in laminae I - III and in Lissauer's tract, but in the ventral horn sparse immunoreactive fibres were seen scattered throughout but many motoneurones were CGRP- immunoreactive (Fig.5.5A). The

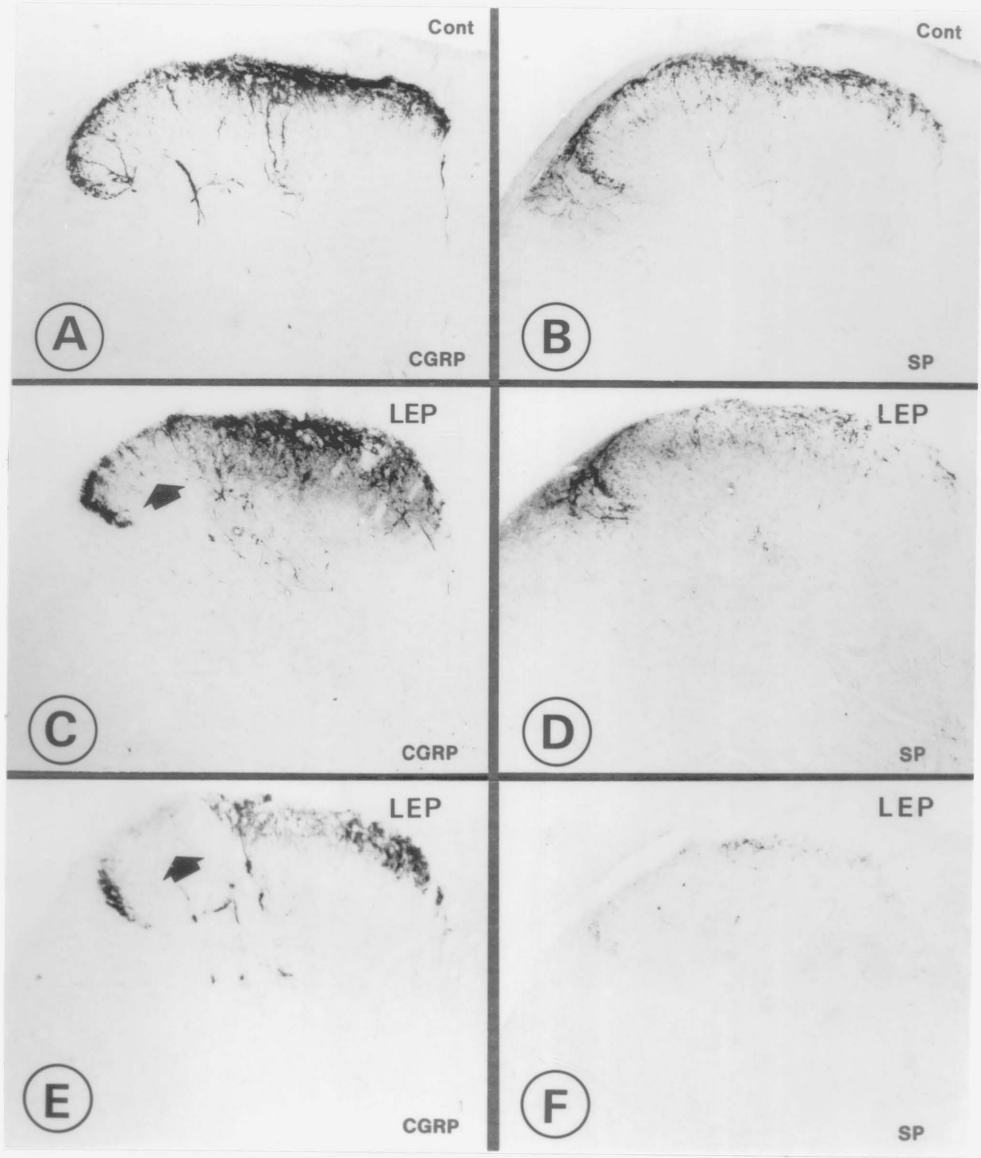


Fig. 5.4. Dorsal horn of L4 segment of spinal cord taken from control mouse showing (A) CGRP-immunoreactive fibres and (B) substance P-immunoreactive fibres. Spinal cord of mice showing reduction in nerves immunoreactive for (C) CGRP and (D) substance P after 6 months, and (E) CGRP and (F) substance P after 12 months of Mycobacterium leprae injection into the hind foot pads. Note the slight reduction in immunoreactivity in 6 months and greater reduction in 12 months old Mycobacterium leprae treated mice. Arrow indicate the loss of immunoreactivity in lateral part of dorsal horn. Bouin's fixed tissues. Cryostat sections (20μm). All magnification x 220

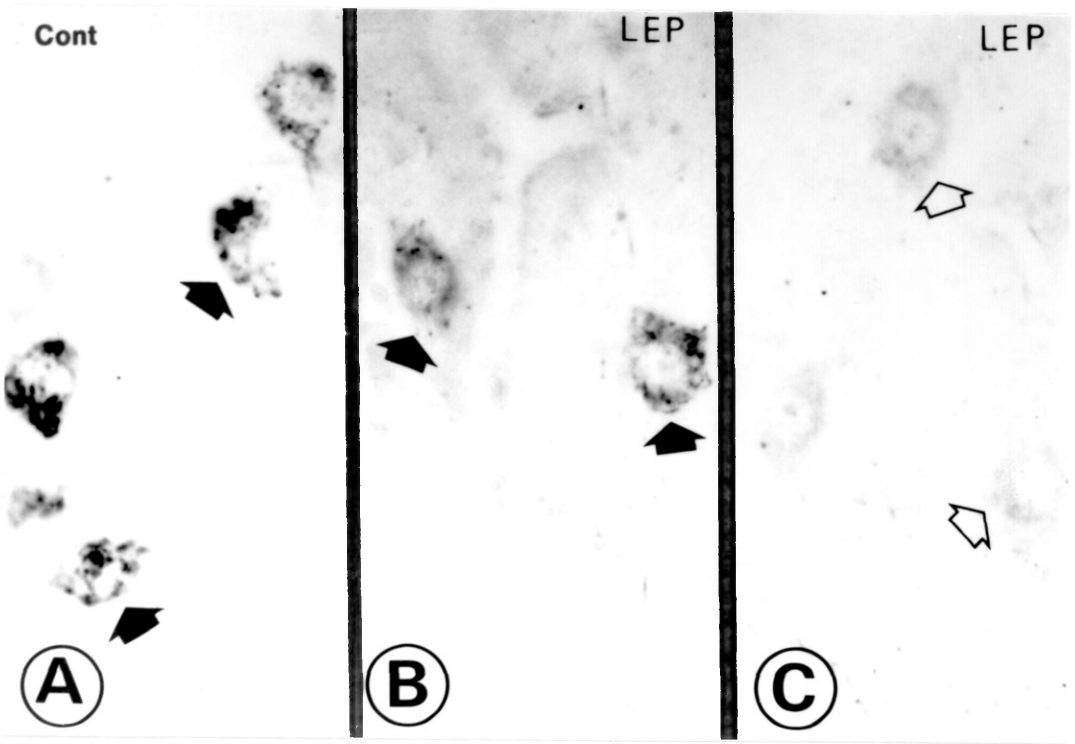


Fig. 5.5. Motoneurones in the ventral horn of L4 segment of spinal cord in control mouse (A) and in mouse 12 months after injection of Mycobacterium leprae into the hind foot pads (B). Note the presence of CGRP-immunoreactivity in (A), reduction in (B), and complete absence in (C). Bouin's fixed tissues. Cryostat sections (20μm). Arrows indicate the motoneurones. All magnification x 340

distribution of substance P was similar to that of CGRP in the dorsal horn (Fig.5.4B). In the ventral horn, there was a moderate to dense distribution of substance P-immunoreactive fibres with many seen in close apposition to motoneurones.

In mice injected with *Mycobacterium leprae*, there was a slight reduction in CGRP- and a substantial decrease of substance P-immunoreactive fibres in laminae I-III of the dorsal horn, after 6 months of infection (Figs.5.4C & D). The depletion of immunoreactivity was particularly noticeable in laminae I - II and in Lissauer's tract. In addition, very few motoneurones were immunoreactive for CGRP. After 12 months of *Mycobacterium leprae* infection the loss of CGRP- and substance P-immunoreactive fibres was substantial (Figs. 5.5B) and motoneurones immunoreactive for CGRP were completely absent (Fig.5.4E & F).

5. 3. 4. Quantification of immunoreactive nerves in the dorsal horn of spinal cord

There was a marked and statistically significant difference in the area of substance P immunoreactivity between control and treated mice. The difference ($P < 0.001$ paired t test) was 0.022 mm^2(95% confidence interval: 0.020 to 0.025mm^2) at 6 months and 0.048 mm^2 (95% confidence interval: 0.040 to 0.056 mm^2) at 12 months. Significant differences were also observed in CGRP-immunoreactive areas between control and treated mice. At 6 months the difference ($P < 0.02$) was 0.018 mm^2 (95% confidence interval: 0.0078 to 0.028 mm^2) and at 12 months the difference ($P < 0.01$) was 0.033 mm^2 (95% confidence interval 0.024 to 0.041 mm^2).

5. 4. Discussion

This study has demonstrated that the neuropeptides substance P and CGRP present in mouse skin and spinal cord, are depleted as early as 6 months of infection with *Mycobacterium leprae* and progressed with time. The decrease in the skin was seen both at the site of innoculation and far away from it (e.g., nose, lip, ear and fore foot pads). Similar changes were also noticed in the spinal cord. In addition, in the spinal cord, motoneurones showed a marked loss of CGRP immunoreactivity.

The decrease of substance P- and CGRP-immunoreactive fibres in skin corresponds to the previous observation of near absence of immunoreactivity for these peptides in the skin of leprosy patients (Chapter 4) and to several earlier morphological studies showing early changes in the structure of peripheral nerves in man (Job et al., 1975) and animals (Mehta et al.,1976; Shetty et al.,1981). A remarkable feature in the present study is the diminution of substance P and CGRP immunoreactivities in skin regions of the body remote from the site of innoculation. The general decrease may indicate the diffuse nature of leprosy. This diffuse nature is illustrated by previous histopathological studies of apparently normal skin from leprosy patients; they showed varying degrees of subepidermal infiltration, delineation of nerve bundles, and Schwann cell hyperplasia (Rea et al., 1975; Bedi et al., 1979; Sakuntala et al., 1982; Ganapathi et al., 1972). Diffuse neural damage may be attributed to the entry and spread of bacilli in nerves (Lumsden, 1964; Vaidya et al., 1970) but autoimmune mechanisms have also been suggested (Shetty et al., 1985; Wright et al., 1975). It would be of interest therefore to delineate, in fine detail, the very early changes in peptides relative to the site of infection.

In the skin, nociceptive terminals are believed to be simple 'free' endings associated with two different types of axons, one responding to strong mechanical stimuli (A-delta) (Burgess and Perl, 1967) and the other responding as polymodal nociceptors (C-unmyelinated) (Bessou and Perl, 1969). Neuropeptides identified in these nerve fibres include substance P and CGRP (Hökfelt et al., 1975; Dalsgaard et al., 1983; Gibbins et al., 1987). It has been shown in several experimental studies that capsaicin, a sensory neurotoxin, decreases CGRP- and substance P-containing primary sensory neurones and reduces the response to noxious chemical and thermal stimuli (Janscó et al., 1977; Yaksha et al., 1979; Gibson et al., 1982; Fitzgerald, 1983; Jhamandas et al., 1984; Holzer, 1988). A marked reduction in CGRP- and substance P-immunoreactive fibres in the skin at the site of innoculation, as shown in our study, suggests a loss of fibres which carry nociceptive information and therefore that there is impairment of pain perception in these animals.

The substantial depletion of substance P and CGRP in the dorsal horn of the lumbar segments of spinal cord suggests that, in addition to peripheral changes, there is a simultaneous involvement of central terminals of sensory neurones. This may be due to the destruction of peripheral sensory processes by the M. leprae. Similar results occur in animals subjected to experimental nerve lesion, dorsal rhizotomy or capsaicin treatment (Hokfelt et al., 1975; Takahashi and Otsuka., 1975; Jessell et al., 1979; Wall et al., 1981; Gibson et al 1982; Kar et al 1990) showing loss of substance P and/or CGRP in the dorsal horn of the spinal cord. However, the possibility of direct damage of the neuronal cell bodies by M. leprae still exists. This may be due to the entry of bacilli into the spinal ganglia and spinal cord, the presence of which has been reported in previous studies of man (Lumsden, 1964) and thymectomised and irradiated mice (Vaidya et al., 1970). There is considerable evidence that substance P and CGRP are involved in transmission of noxious stimuli and fibres containing these peptides terminate in laminae I, II and V of the dorsal horn of spinal cord (Kanazawa and Perl, 1977; Light and Perl, 1979) regions in which we noted loss of immunoreactivity in the infected mice. The results of this study clearly suggest that, even in the early stages of leprosy, there

is impairment of nociceptive function. The immunocytochemical changes resemble those in other mammals with diminished pain perception, including Pointer dogs with sensory neuropathy (Cummings et al., 1984), mutilated foot rats (Kar et al., 1989b) and familial dysautonomia in man (Pearson et al., 1982). In contrast, a rise in substance P and CGRP levels has been reported in cases of chronic pain in man (Williams et al., 1988), and in rats with adjuvant-induced arthritis (Kuraishi et al., 1989; Weihe et al., 1988; Kar et al., 1989a). Therefore, the present study further substantiates the hypothesis that sensory impairment is reflected by the density and distribution of sensory neuropeptides in the dorsal horn of the spinal cord as well as in the skin.

The loss of CGRP immunoreactivity from the motoneurones of injected mice was of particular interest. Several previous studies have shown the involvement of motor nerves and subsequent wasting of muscles during the later stages of leprosy (Goodwin, 1968). This is commonly attributed to lower motor neuron (spinal motoneurones) paralysis. Since motoneurone-derived CGRP is a proposed muscle trophic factor, it is possible that changes in the target tissues, the muscles, may directly influence the rate of synthesis and production of mature peptide in the motoneuronal cell body or vice versa. Loss of CGRP-immunoreactive motoneurones has been observed in a number of cases where there is muscle atrophy and/or paralysis, for e.g. in motor neurone disease in man (Gibson et al., 1988) and after spinal cord transection (Aravidsson et al., 1989). It may be suggested, as for spinal ganglia, that the neural damage in leprosy is caused by the presence of globi of bacilli (Vaidya et al., 1970). However, this study shows for the first time the reduction of motoneuronal CGRP-immunoreactivity in leprosy, which may be due to loss of target-derived neurotrophic factors (Oppenheim et al., 1988) and suggests that the diminished trophic effects and impairment of motor functions may occur during the first 6 months of infection.

In conclusion, the reduction of substance P and CGRP immunoreactivity in central and peripheral processes of sensory neurones, and the simultaneous loss of CGRP in motoneurones indicates that the neuropeptide content of sensory and motor nerves is affected at an early stage of leprosy infection, and associated with impairment of nociception and motor function. The study of human leprosy (Chapter 4) showed a marked depletion of neuropeptides in skin, but these cases were mostly of a relatively advanced nature. The present data show that the degree of change is proportional to the duration of infection and suggests that immunocytochemical examination in suspected cases of leprosy in man may be of prognostic and therapeutic importance in the management of leprosy.

Chapter 6

DEPLETION OF CUTANEOUS NERVES AND NEUROPEPTIDES IN DIABETES MELLITUS

	Page
6. 1. Introduction	149
6. 2. Experimental design	150-151
6.2.1. Tissues	150
6.2.2. Immunocytochemistry	151
6. 3. Results	151-162
6.3.1. Control specimens	151-153
6.3.2. Diabetic specimens	153-155
6.3.3. Neurophysiological tests	156-162
6. 4. Discussion	159-162

6. 1. Introduction

Peripheral neuropathy is the commonest complication of diabetes (Pirart, 1978), characterised by signs and symptoms of sensory, motor and autonomic dysfunction, although its aetiology is still poorly understood. Its response to apparently rational therapies, for example aldose reductase inhibitors (Zimmerman, 1987) and gangliosides (Gorio, 1986) is inconsistent. The judgment as to whether neuropathy is or is not present in a patient varies depending on physicians experience and understanding, and the availability of tests to evaluate the neurological deficits. This may in part be due to the indirect, predominantly neurophysiological methods currently used in diagnosis and follow-up, which have high variability (Fagius and Wahren, 1981). Also diabetic neuropathy in man is associated with reduced nerve conduction velocity (Mulder et al., 1961, Lawrence and Locke, 1961; Skillman et al., 1961) and structural changes in the nerves, which include axonal loss and segmental demyelination (Thomas and Lascelles, 1966, Chopra et al., 1969). Previous studies have shown that nerve function is more severely impaired in diabetic subjects with symptomatic neuropathy of somatosensory or autonomic nerves than in those without symptoms (Ewing et al., 1980, Young et al., 1988). Sural nerve fascicular biopsy has been useful in the study of the morphometry of diabetic nerve (Behse et al., 1975), but it is a specialised technique that currently has limited applicability. A histological method is required that more directly reflects minor and subclinical deficits of nerve function, especially in peripheral sensory and autonomic fibres. The aim of the present study is to assess whether neurological abnormalities of diabetes are reflected by concomitant changes in skin neuropeptides.

6. 2 Experimental design

6. 2. 1 Tissues

Skin biopsies were taken from 19 consecutive diabetic patients undergoing elective general surgical and orthopaedic operations in Central Middlesex Hospital. The study was approved by the Ethics Committee of Brent Heads Authority. Their clinical details are given in Table I. Their ages ranged from 20-75 years and the time since diagnosis from 0.5-34 years. Three were Type 1 (insulin-dependent), the remainder Type 2 (non-insulin-dependent). None were ketotic or hyperosmolar at the time of surgery. One patient had known chronic alcoholic liver disease; no others were known to have untreated endocrine or other disease that might affect peripheral nerve function. Patients were considered to have symptomatic neuropathy if they had any spontaneous distal paraesthesia, numbness, burning or contact discomfort. Six patients had peripheral vascular disease, as assessed by absent foot pulses; five of them were undergoing lower limb amputation and two had characteristic symptoms of long-standing painful diabetic neuropathy. Control specimens were age- and site-matched and taken from patients undergoing orthopedic or general surgical procedures. None had neurological disease, alcoholism, diabetes or other endocrine or systemic diseases known to affect peripheral nerve or vascular function.

Full-thickness skin specimens approximately 2cm x 0.3-0.5 cm were taken from the margin of the surgical incision at the time of operation, care being taken not to crush the specimens. One patient had two biopsies taken at the same time, from the abdomen and the calf. Amputation specimens were taken from the proximal viable margin, and all amputations subsequently healed uneventfully. Specimens were immediately fixed in p-benzoquinone (**Appendix 3A**) or in Zamboni's solution (**Appendix 3C**); both were then transferred to PBS/sucrose (**Appendix 2b**).

The results of two or more neurological function tests were available for seven patients. Neurophysiological examinations were carried out by Dr. D. M. Levy, according to previously published methods (Abraham et al., 1986). Thermal thresholds were measured with the Sensortek apparatus (Levy et al., 1987) and cardiac vagal autonomic function by a computerised method. Results for age-related normal subjects, or, in the case of autonomic tests, 2 or 3 were clearly abnormal by Ewing and Clarke's (1982) method.

6.2.2 Immunocytochemistry

Control and test specimens were mixed in batches and cryostat sections (6 μm) were taken upon poly-L-lysine coated glass slides (**Appendix 4**). Indirect immunofluorescence staining method (**Appendix 5A**) with antisera to general neuronal marker (PGP 9.5) and to the neuropeptides VIP, CGRP, SP and NPY was used. Three non-serial sections were used for each antiserum. Numbers of immunoreactive fibres in each specimen were assessed visually and the results graded from - (absent) to ++++ (abundant) (**Table 15**). Results were compared with those for age- and site-matched control patients.

6.3 Results

6. 3. 1. Control specimens

In all the specimens PGP 9.5 immunoreactive nerve fibres were observed in epidermis (Fig.6.1A), dermis (Fig.6.1A), around hair follicles, blood vessels, erector pilorum and sweat glands. The immunoreactivity was seen both in varicose and smooth fibres. In addition, intensely stained large and small nerve bundles were seen in the deep dermis.

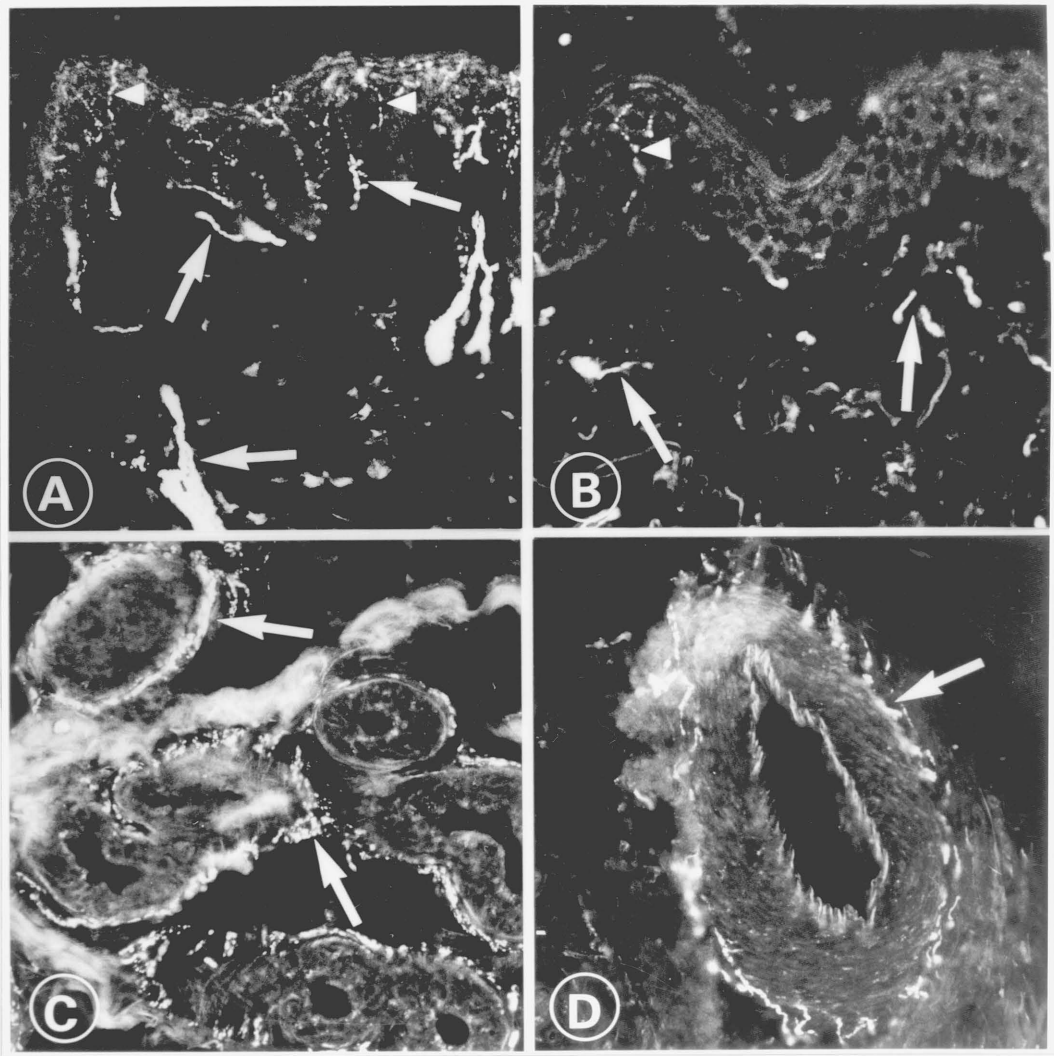


Fig. 6.1. Control skin of calf showing (A) PGP 9.5-immunoreactive fibres in the epidermis and dermis (B) CGRP-immunoreactive fibres in the epidermis and dermis. VIP-immunoreactive fibres around sweat glands (C) and blood vessels (D). Zamboni-fixed tissues, cryostat sections (10μm), indirect immunofluorescence method. All magnification x 280

Among neuropeptides, CGRP- and VIP- (Fig. 6.1C) immunoreactive axons were seen in moderate numbers, the former mainly in epidermis and dermis and the latter exclusively around sweat glands (Fig.6.1B). The distribution of substance P- and NPY-immunoreactive axons was similar to CGRP and VIP respectively, except in smaller numbers. Due to the variations in the density of PGP 9.5- and peptide-immunoreactive fibres, the control specimens were graded as ++++ for PGP, +++ for CGRP and VIP, and ++ for substance P and NPY (See **table 7.1**).

6. 3. 2. Diabetic specimens

When immunostained with antisera to PGP 9.5, only one case showed a comparable density of immunoreactive fibres to control specimens. In all the rest (19/20), the immunoreactive fibres were either markedly less (Sp. No: 1-3, 4-6, 8-13, 14,16-20) (Fig. 6.2A) or completely absent (Sp. No: 4,14,15) (Fig. 6.3A). Similar observations were made with neuropeptide immunoreactive fibres (Figs. 6.2B & 6.3B) The widespread diminution of substance P was particularly striking in specimen 9, from a 31-year-old-Type 2 (non-insulin-dependent) diabetic, diagnosed 6 months before operation, and in specimen 10, from a 20-year-old Type 1 (insulin-depedent) diabetic patient, duration 12 years. There was no SP-immunoreactivity in specimens 2 and 3, from the same patient; he had elevated thermal thresholds and abnormal cardiac autonomic function, but results of his neurophysiological tests were normal. All specimens, apart from no.7, also showed decreased CGRP-immunoreactivity, though the pattern of decreased SP-immunoreactivity did not uniformly reflect that of CGRP; for example, the two specimens taken from the same patient (nos. 2 and 3) demonstrated more CGRP-immunoreactivity in the abdominal skin specimen than in the specimen from the calf.

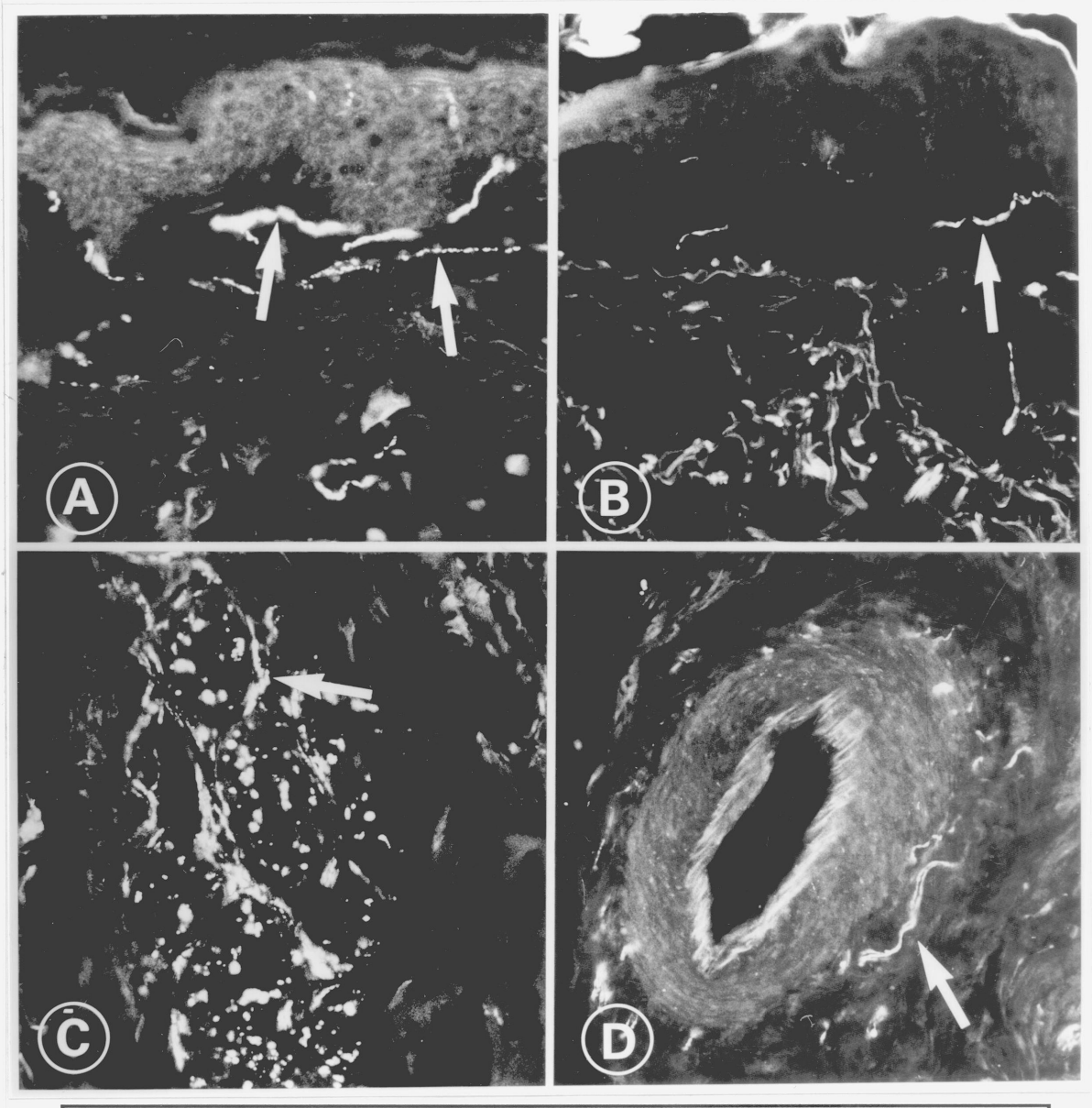


Fig. 6.2. Diabetic skin of calf showing (A) PGP 9.5-immunoreactive fibres and (B) CGRP-immunoreactive fibres in the epidermis and dermis; VIP-immunoreactive fibres around sweat glands (C) and blood vessels (D). Zamboni-fixed tissues, cryostat sections (10μm), indirect immunofluorescence method. All magnification x 280

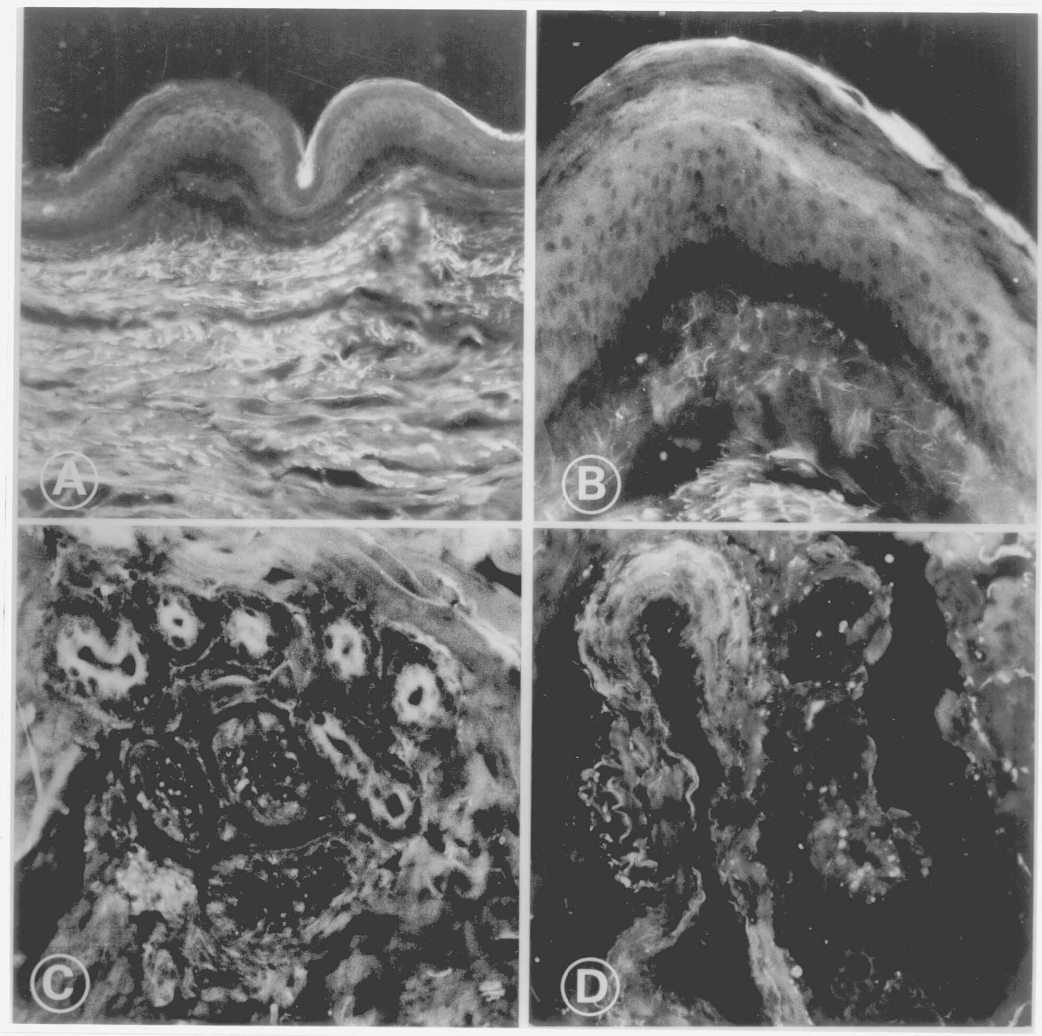


Fig. 6.3. Diabetic skin of calf showing complete absence of, PGP 9.5-immunoreactive fibres (A) and CGRP-immunoreactive fibres in the epidermis (B); VIP-immunoreactive fibres around sweat glands (C) and blood vessels (D). Zamboni-fixed tissues, cryostat sections (10μm), indirect immunofluorescence method. All magnification x 280

Table 15

Clinical characteristics of diabetic patients, neurophysiological and immunohistochemical findings

No.	Age	Type of diabetes	Duration	Symp	Neurophysiologya	Thermal thresholdb	Auto nomic functc	Skin Site	PGP 9.5	VIP	CGRP	SP	NPY
1	63	2	20	Pain	Abnor	Abnor	Abnor	Thigh	+	-	-	-	-
2	44	1	10	Yes	Norm	Abnor	Abnor	Lat. calf	++	-	+	-	+
3								Abd. wall	++	+	++	-	+
4	58	2	3	Pain	-	-	Abnor	Fore foot	-	-	-	-	-
5	58	2	6	No	-	Norm	Norm	Lat. foot	+	-	-	-	-
6	54	2	7	No	-	Norm	Norm	Iliac crest	+++	+	+	+	+
7	56	2	12	No	-	Norm	Norm	Media.wrist	++++	+++	+++	++	++
8	62	1	34	No	Normd	Norm	Norm	Upper thigh	+++	++	+	-	++
9	31	2	0.5	No	-	-	-	Lat. ankle	+++	+	+	-	-
10	20	1	12	No	-	-	-	Thigh	++	+	+	+	+
11	68	2	2	No	-	-	-	Thigh	+++	-e	++	+	+
12	59	2	5	No	-	-	-	Lat. calf	+++	+	++	+	+
13	54	2	2	No	-	-	-	Alcoholic	++	+	+	+	+
14	41	2	13	No	-	-	-Low.	leg(amp)	-	-	-	-	-

Contd.....

Contd.....

No.	Age	Type of diabetes	Duration	Symp	Neurophysiologya	Thermal thresholdb	Auto nomic functc	Skin Site	PGP 9.5	VIP	CGRP	SP	NPY
15	74	2	18	No	-	-	-Low.	leg(amp)	-	-	-	-	-
16	75	2	25	No	-	-	-Low.	leg(amp)	+++	+	+	+	+
17	58	2	10	No	-	-	-Low.	leg(qmp)	+	-	-	-	-
18	49	2	3	No	-	-	-	Dor. of hand	++	+	+	+	+
19	38	2	5	No	-	-	-	Palm of hand	++	-	-	-	-
20	65	2	17	No	-	-	-	Abdom. wall	+	-	-	-	-
Controls									++++	+++	+++	++	++

aAbnormal common peroneal motor conduction velocity (> 2SD below age-related normal value); **b**Sensortek threshold> 1.1°C; **c**Any two of the following: (1) heart rate variation with deep breathing < 11 beats per minute; (2) Valsalva ratio < 1.11; (3) 30:15 ratio < 1.04; **d**Norm peroneal conduction velocity but absent medial plantar sensory action potential; **e**No sweat glands seen in this specimen
Semi-quantitative scale of immunoreactive fibre density: absent (-), few (+), moderate (++), many (+++), abundant (++++),
PGP9.5=protein gene product 9.5, VIP=vasoactive intestinal polypeptide, CGRP=calcitonin gene-related peptide, SP=substance P, NPY=neuropeptide Y

Apart from specimen no. 7 all diabetic specimens had diminished VIP- and NPY-immunoreactivity around sweat glands (Fig.6.2C) and blood vessels (Fig.6.2D). There was a closer association between VIP and NPY levels than between SP and CGRP. They were only entirely absent in amputation specimens from patients with known peripheral vascular disease (Fig. 6.3C & D)

6. 3. 3. Neurophysiological Tests

In the seven patients with neurophysiological tests, there was a broad association between the number of abnormal tests and the immunohistochemical abnormalities (**See table 15**). However, three subjects who had normal thermal thresholds and autonomic function (nos, 5, 6 and 8) and widely differing neuropeptide immunoreactivities, though the skin sites were also different. One of these specimens (no. 8) was taken from a patient who had normal routine electrophysiological tests (common peroneal motor conduction velocity, sural sensory action potential amplitude and conduction velocity) but whose medial plantar response (the most distal sensory electrophysiological test currently available) was absent (Levy et al., 1987). Both specimens from patients with painful diabetic neuropathy (nos 1 and 4) had virtually absent immunoreactivity, though it should be noted that they both had long-standing symptoms, and also peripheral vascular disease.

6. 4. DISCUSSION

This study has demonstrated a reduction in density and distribution of immunoreactive cutaneous nerves and peptide depletion in majority of cases. The degree of reduction was related to the duration of the disease. Abnormalities were seen in all but one of twenty specimens.

Several previous studies have shown severe impairment of nerve function in diabetic patients with symptomatic neuropathy of somatosensory or autonomic nerves in contrast to those without symptoms (Ewing et al., 1980; Young et al., 1986). There have been two previous studies of dermal nerves in diabetes. Conventional argyrophilic techniques and light microscopy were used to demonstrate abnormalities of autonomic innervation of skin (Faerman et al.,1982). In this study there was an association between anhidrosis, as assessed by an abnormal sweat test, and histopathological abnormalities of small fibres in the skin, particularly those near to sweat glands. Johnson and Doll in 1984 found non-vascular perineurial cell basement membrane thickening. It is likely that these changes are relatively late, as clinical abnormalities of sudomotor function are usually found in association with clinically evident polyneuropathy; the findings are not specific to diabetes, however, since sympathectomised non-diabetic patients showed similar histological abnormalities.

In this study, the absence of immunoreactivity for both PGP 9.5 and neuropeptides reflects the structural and functional alterations in cutaneous nerves. PGP 9.5, being a major component of the neuronal cytoplasm as suggested by radioimmunoassay (Thompson et al., 1983) and electron microscopy (Gulbenkian et al., 1987), probably is a measure of the structural integrity of nerve fibres. However, the neuropeptide-immunoreactivity is more difficult to interpret, as it is a reflection of the amount of stored neuropeptids and this is influenced by synthesis, transport, release and degradation.

Whatever the known duration of diabetes, density and distribution of immunoreactive nerves in all but one specimen was abnormal compared to control specimens. The specimen with normal immunoreactivity was taken from the upper limb, where clinical and

neurophysiological evidence of neuropathy occurs late. Conversely, the known duration of diabetes in patient 9, with abnormal immunocytochemical findings in the foot, was less than 1 year.

The number of patients with neurophysiological tests was small, and further studies are required to define more precisely the relationship between immunocytochemical abnormalities and functional neurological assessments. However, the findings in the proximal (abdominal) and distal (calf) specimens taken simultaneously from the same patient with known neurophysiological abnormalities suggest there may be a proximal-distal gradient of PGP 9.5 and neuropeptide immunoreactivity similar to that shown for electrophysiology (Waxman et al., 1976).

The pattern of immunocytochemical depletion was broadly similar, with approximately 50% of specimens being devoid of immunoreactivity to each of the four neuropeptides. Within each specimen there was a similar degree of depletion. SP-immunoreactivity was most consistently reduced or absent, even in the two recently diagnosed subjects. Abnormalities of SP in diabetes are interesting, in view of the lower pain thresholds found in diabetic subjects (Morley et al., 1984) and their predisposition to abnormal pain perception. Patients with clinical and neurophysiological evidence of diabetic neuropathy also have decreased flare responses to intradermal SP (Aronin et al., 1987), suggesting receptor changes that may follow from a decrease in SP-immunoreactive nerves. Axon reflex flare responses are also reduced in patients with peripheral vascular disease (Parkhouse and Le Quesne, 1988), and our finding of virtually absent PGP 9.5 and neuropeptides in specimens from these patients would be an expected corollary. While immunocytochemical abnormalities appear from this study to be exacerbated by large-vessel ischaemia, they are unlikely to be the sole cause of them, since abnormal results were

found in specimens from areas usually unaffected by large vessel disease (eg. anterior abdominal wall, dorsum of the hand). However, this study does not shed light on the role of microvascular disease in causing PGP 9.5 and neuropeptide depletion.

Neuropeptide depletion may be a common histopathological end-point in several neuropathies and a picture similar to that in diabetes may be found in other "metabolic" neuropathies of the axonal type. This needs to be confirmed, but is suggested by the severe immunocytochemical depletion found in specimen 13, from a patient with chronic alcoholic liver disease and intermittent hepatic failure, but who had only recently been diagnosed diabetic. One of the underlying abnormalities in peripheral neuropathies may be deranged axonal transport (Jakobson et al., 1986), and this would explain the broad associations between depletion of the various neuropeptides. Decreased axonal transport is an important early abnormality in diabetes, and one which is sensitive to changes in diabetic control, being reversed with insulin treatment in experimental models. No patients in the current study were metabolically decompensated, though many had poor diabetic control. It will be important to study the effect of improved diabetic control and of therapy with drugs known to affect axonal transport (aldose reductase inhibitors and gangliosides (Martini et al., 1986). Endoneural hypoxia (Newrick et al., 1986) which has been demonstrated both in experimental and human diabetes may be another factor contributing to the picture of reduced neuropeptide immunoreactivity.

In conclusion, immunocytochemical abnormalities of PGP 9.5, a structural marker for cutaneous nerves showed a close correlation with gross neurophysiological changes. However, the more subtle decrease in neuropeptides is an interesting feature in this study as it suggests that these changes antedate neurophysiological abnormalities. This is of great significance as the changes may take place at an early stage of

diabetes when the neuropathic changes are reversible and therefore may be of therapeutic importance. The direct histological assessment of peripheral nerves in diabetes is an important aim, and currently not routinely available; while important data on the pathophysiology of diabetic neuropathy and mode of action of therapeutic agents has resulted from sural nerve biopsies (Sima et al., 1988) it is not recommended as a routine procedure (Consensus statement 1988). It cannot give information on the structure and function of the small peripheral fibres examined in this study, which are believed to play an important part in the symptoms of diabetic neuropathy (Lawrence, 1965). Skin biopsy is safe, relatively atraumatic, and simple to perform, and the use of the techniques described in this study may give further insights into the mechanism of peripheral nerve dysfunction in diabetes and its response to treatment at the histological level.

Chapter 7

EARLY CHANGES IN PEPTIDE CONTAINING NERVES IN THE SKIN OF STREPTOZOTOCIN-INDUCED DIABETIC RATS

7. 1. Introduction **164-165**

7. 2. Experimental design **165-166**

8.3.1. Tissues 165

8.3.2. Immunocytochemistry 165

7. 3. Results **167-172**

7. 4. Discussion **172-175**

7. 1. Introduction

It is well known that in patients with diabetic neuropathy there is a widespread involvement of the sensory and autonomic nervous system. These consequences range from loss of proprioception and sense of pain and temperature (Gregersen, 1968; Macbride and Mistrella, 1982; Boulton et al., 1983; Bloom et al., 1984; Cracco et al., 1984; Guy et al., 1985; Lowental and Hockaday, 1987; Dyck et al., 1985; Dyck et al., 1987), to severe burning pain and cutaneous hyperesthesia with relative preservation of propriception and kinaesthetic sense (Brown et al., 1976; Boulton et al., 1983; Heimans et al., 1986; Young et al., 1988). The autonomic dysfunctions include disturbance in sweating (Goodman, 1966; Faerman et al., 1982) and in cardiovascular (Ewing et al., 1980; Rao et al., 1983), alimentary and urinary functions (Feldman and Schiller, 1983; Atkinson and Hosking, 1983).

In leprosy, neuritis or inflammation of the nerve is the most important feature and nerve invasion is a unique characteristic of M.Leprae. The pathology of neuritis largely depends on the immune status of the patient. The features of nerve damage include the presence of M. Leprae or its antigens at cooler sites of the body, trauma, edema of the nerve and presence of granuloma and occlusive changes of intraneural pressure. However, the factors that contribute to the nerve damage of diabetes are more complex. They include metabolic abnormalities, damage to microcirculation, glycosylation of nerve proteins and ageing. There is a good evidence from animal work that through the accumulation of sorbitol and fructose in peripheral nerve there is associated myo-inositol depletion and depression of Na+/K+ ATPase activity associated with nerve dysfunction and damage. The key enzyme involved in polyol-sorbitol and tissue myo-inositol pathways is aldose reductase which is present in both autonomic and somatic nerves (Greene et al., 1987); many abnormalities of neural chemistry and function may be prevented by the administration of aldose reductase inhibitors. Impairment of nerve conduction velocity is well established in human and animal diabetic nerve and control of blood glucose generally returns this towards normal levels (Gregersen, 1968; Ward et al., 1971). Impairment of nerve conduction changes can be linked to metabolic theory, for the administration of myo-inositol or

aldose reductase inhibitors to both human and animals results in an improvement in conduction velocity (Greene et al 1984). A variety of morphological changes are described in nerves of man and animals, including axonal degeneration and loss, segmental demyelination and changes in Schwann cells, perineural cells and endoneural vessels (Johnson et al., 1986). Glycosylation of nerve proteins has been described to be the cause for alteration in the myelin-macrophage interaction and is suggested to be the initiating factor in the segmental demyelination (Vlassara et al., 1984).

In several recent experimental studies and in one human study of diabetes, defective axonal transport was shown to be the likely cause of degenerative distal neuropathies (Jakobson and Sidenius, 1980; Tomlinson et al., 1982; Tomlinson and Mayer, 1984; Jakobson et al., 1986; Robinson et al., 1987a) and was believed to occur before there was pathological change (Sidenius and Jacobsen 1987). There is much evidence that diabetic neuropathy is an axonal 'dying back' neuropathy associated with altered transport of proteins (Mayer and Tomlinson, 1983; Tomlinson and Mayer, 1984). Retrograde transport of nerve growth factor has been shown to be decreased in nerve ganglia in which axonopathy has developed (Carson et al., 1982). In both man and animals with diabetes, there is thickening of the walls, reduplication of the basement membrane of endoneurial blood vessels (Fagerberg, 1959; Powell and Myers, 1984; Sima and Robertson, 1978). There is now considerable evidence to suggest that microvascular disease plays a role in development of clinical neuropathy (Powell et al., 1985), particularly in older subjects (Malik et al., 1987).

The pathogenesis of diabetic neuropathy has been extensively studied in the streptozotocin-treated rat, a model of insulin dependent diabetes (Tomlinson et al., 1982; Lincoln et al., 1984; Belai et al., 1985; 1988; Belai and Burnstock, 1987). However, so far there has been no experimental investigation of the effects of streptozotocin-induced diabetes on the skin, but it provides an ideal body site to study the changes in both sensory and autonomic innervation.

TABLE I6

Body weight and plasma glucose concentration for control and diabetic rats

		Duration of Streptozotocin treatment			
		2 weeks	4 weeks	8 weeks	12weeks
Mean weight ± SEM (g)	Control	247 ± 7.2	320 ± 5.4	408 ± 4.2	478 ± 8.2
	Streptozotocin treated	154 ± 2.1	164 ± 2.1	176 ± 6.2	182 ± 12.5
Mean plasma glucose ± SEM (mmol/l)	Control	4.1 ± 0.6	4.2 ± 0.4	5.4 ± 0.4	4.8 ± 0.6
	Streptozotocin treated	16.8 ± 0.6	18.3 ±0.8	20.6 ±1.3	25 ± 0.7

Study of skin taken from diabetic patients (Chapter 6) showed changes in the innervation and neuropeptides, but were unable to examine the initial effects of the disease. The main aim of the present study was to demonstrate immunocytochemically the progressive effects of diabetes on skin innervation as visualised by PGP 9.5 (Chapter 3) (Doran et al., 1983; Gulbenkian et al., 1987; Dalsgaard et al., 1989) and peptide-containing population of sensory and autonomic nerves.

7. 2. Experimental design

7. 2. 1. Tissues

Male Wister rats (n=20) (weighing 150-160g) were treated with a single intraperitoneal injection (60mg in 0.9% saline/kg body wt) of streptozotocin. The onset of diabetes was established after 24h by the presence of polyuria and glycosuria. The injection was carried out in ICI Pharmaceuticals Division, Alderly Park, Macclesfield, Cheshire. under the supervision of Dr. D.J. Mirrlees. Controls (n=20) consisted of untreated animals of the same initial weight range injected with saline alone. Both control and diabetic animals were maintained under the same environmental conditions and were supplied with food and water *ad libitum*. The animals were killed by carbon dioxide euthanasia at 2, 4, 8 and 12 weeks (n = 5 each group) after the onset of diabetes. Skin samples were dissected from lip and foot pads, and immediately fixed in Zamboni's fluid **(Appendix 3C)** by immersion.

7. 2. 2. Immunocytochemistry

Control and treated specimens were mixed in batches and cryostat sections (12μm) were collected on poly-L-lysine coated slides **(Appendix 4)** and allowed to dry for 60 minutes at room temperature. Immunostaining was performed by a modified indirect immunofluorescence method **(Appendix 5a)**, using antisera to PGP 9.5, substance P, CGRP, VIP and NPY.

TABLE 17

Density of nerves and neuropeptides-immunoreactive fibres present in the lip and paws of rats with streptozotocin-induced diabetes and controls

Axons IR for	Skin areas	Controls	Duration of Streptozotocin treatment			
			2 weeks	4 weeks	8 weeks	12weeks
CGRP	Epidermis	+	+	+	+	++
	Dermis	++	++	++	++	++++
	Sweat glands*	+	+	+	+	++
	Blood vessels	+	+	+	+	++
	Hair follicles	+	+	+	+	++
VIP	Epidermis	-	-	-	-	-
	Dermis	-	-	-	-	-
	Sweat glands *	++	++	++	++	++++
	Blood vessels	++	++	++	++	++++
	Hair follicles	-	-	-	-	-
SP	Epidermis	±	±	±	±	±
	Dermis	+	+	+	+	+
	Sweat glands *	±	±	±	±	±
	Blood vessels	±	±	±	±	±
	Hair follicles	-	-	-	-	-
NPY	Epidermis	-	-	-	-	-
	Dermis	-	-	-	-	-
	Sweat glands *	±	±	±	±	±
	Blood vessels	+	+	+	+	+
	Hair follicles	-	-	-	-	-
PGP 9.5	Epidermis	++	++	++	++	+++
	Dermis	+++	+++	+++	+++	++++
	Sweat glands*	+++	+++	+++	+++	++++
	Blood vessels	++	++	++	++	+++
	Hair follicles	++	++	++	++	+++

Key: Numbers of immunoreactive fibres are graded: absent (-), very few (±), few (+), moderate (++), many(+++), abundant (++++),
* = in paws only

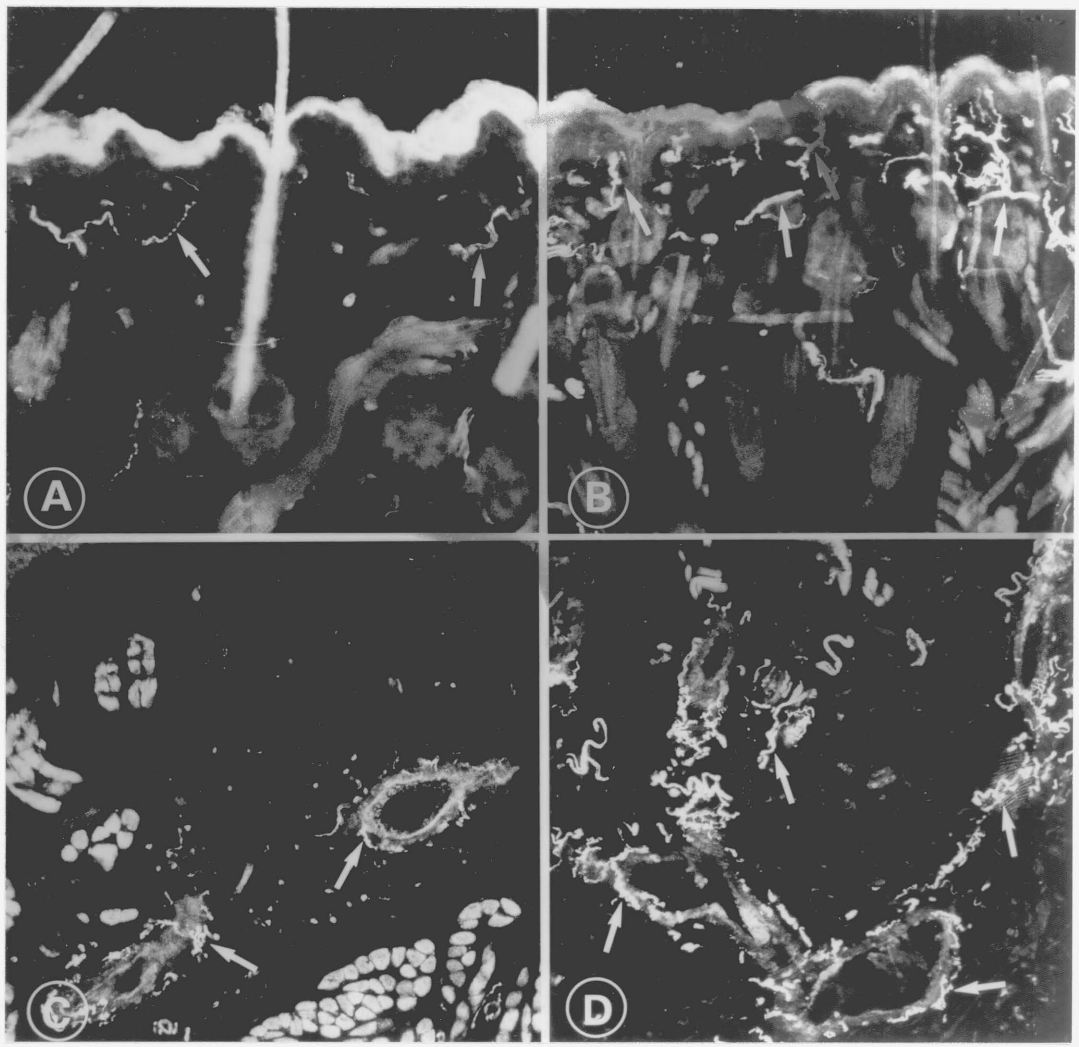


Fig. 7.1. CGRP-immunoreactive fibres in the epidermis and dermis, and VIP-immunoreactive fibres around blood vessels of the lip. Control skin of lip (A and C) and after 12 weeks of streptozotocin treatment (B and D). Note the increase in density of immunoreactive fibres in B and D. Zamboni fixed tissues. Cryostat sections (10μm). Arrows indicate some immunoreactive fibres. All magnification x 280.

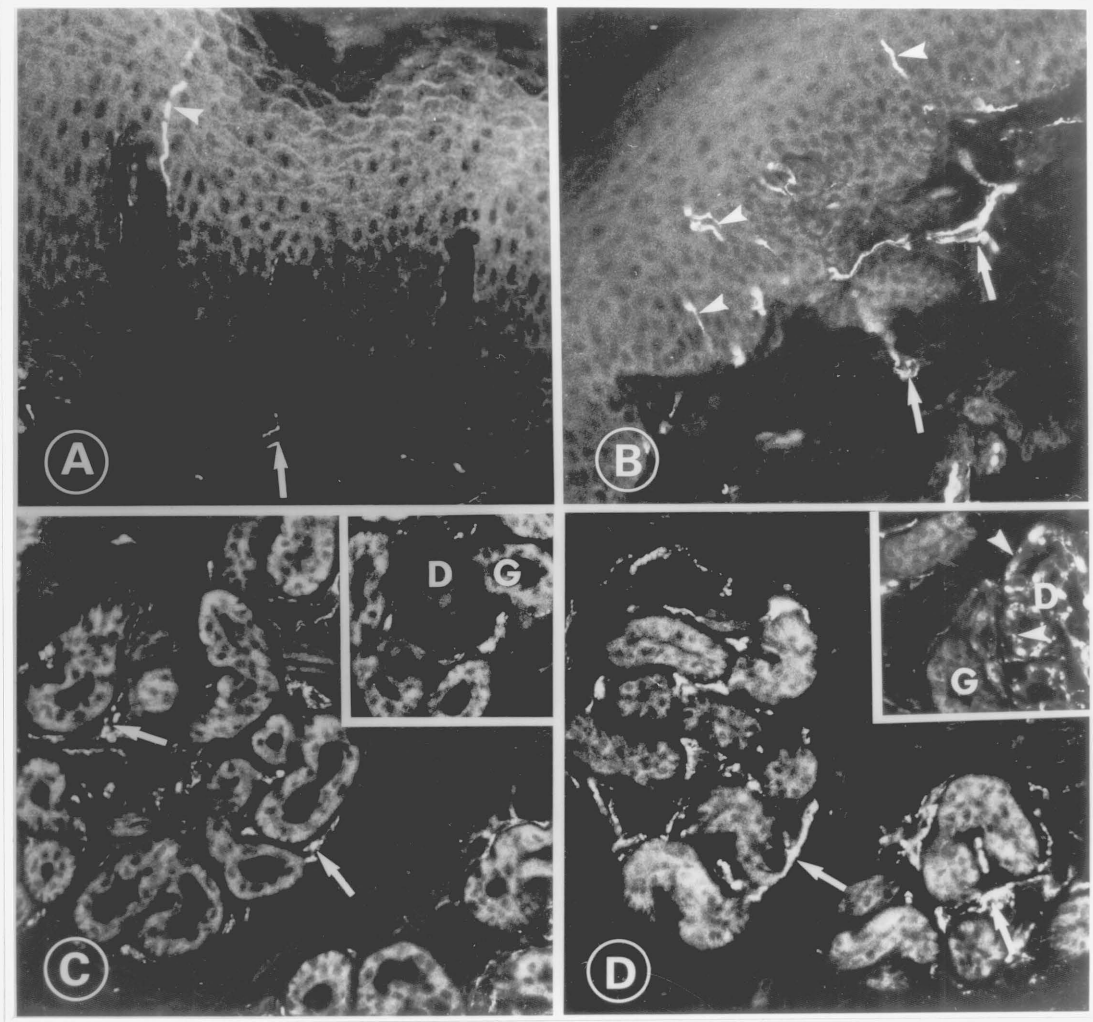


Fig. 7.2. CGRP-immunoreactive fibres in the epidermis and dermis, and VIP-immunoreactive fibres around sweat glands of the foot pad. Skin of foot pad of rat treated 12 weeks previously with vehicle solution (A and C) and after 12 weeks of streptozotocin treatment (B and D). Note the increase in density of immunoreactive fibers in B and D. Arrows indicate some immunoreactive fibres. Insert in C and D shows glands (G) and ducts (D). Note the absence of immunoreactivity around duct in insert C and presence in insert D. Zamboni fixed tissues. Cryostat sections (10μm). Arrows indicate some immunoreactive fibres. All magnification x 900.

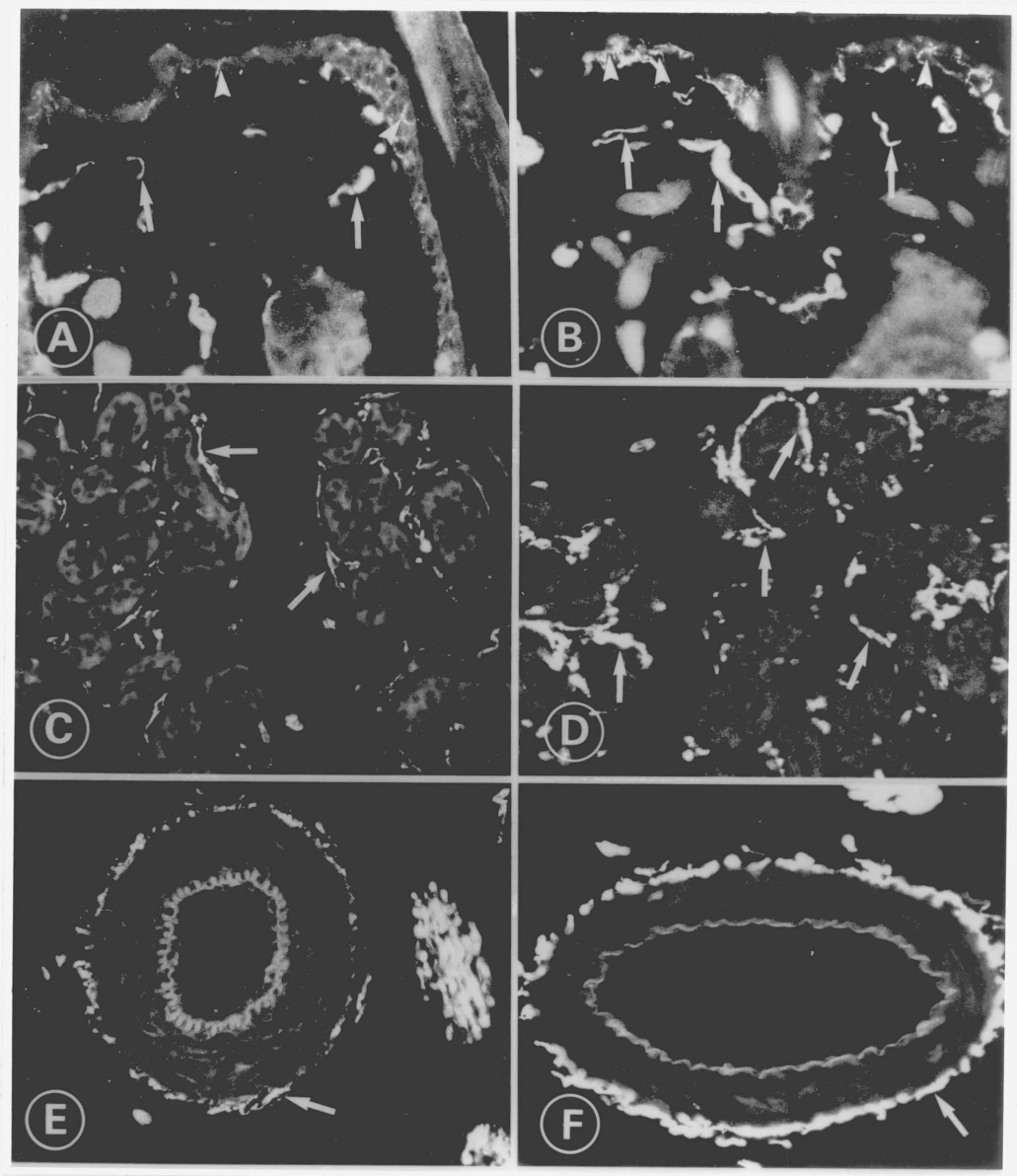


Fig. 7.3. PGP 9.5-immunoreactive fibres in the epidermis and dermis (A and B) and blood vessels (E and F) of the lip skin, and sweat glands of the foot pad (C and E). Control skin of lip (A and E) and foot pad (C), and after 12 weeks of streptozotocin treatment (B, F and D). Note the increase in density of immunoreactive fibersin B,D and F. Zamboni fixed tissues. Cryostat sections (10μm). Arrows indicate some immunoreactive fibres. All magnification x 900.

7.3. Results

7.3.1. Control animals

In both lip and foot pad, CGRP-immunoreactive axons were observed in nerve trunks and isolated or small groups of axons present in the vicinity of dermal arteries and veins. They were seen in moderate numbers in the dermis, close to hair follicles, and some axons extended into the epidermis (Figs.1A,2A), but were very sparse near the sweat glands of the foot pad. The substance P-immunoreactive axons were mainly seen in the dermis, occasionally extending into the epidermis. However, CGRP-immunoreactive axons were more numerous than substance P-immunoreactive axons in both dermis and epidermis. Axons immunoreactive for VIP were seen in moderate numbers around blood vessels (Fig.1C) in both regions and around sweat glands in the foot pad (Fig.2C). The distribution of NPY-immunoreactive axons was similar to that of VIP but they were fewer in number. In any location, axons immunoreactive for any peptide formed a small nerve subtype in the context of the whole innervation visualised by immunoreactivity for the general marker PGP 9.5 (Figs.3A,3C,3E). Some differences were observed in the relative density and distribution of peptide- and PGP 9.5-immunoreactive nerves of the skin of foot pad and lip; they were in part due to the morphological differences between the two areas. The arbitrary gradings of the immunostaining are summarised in Table 1.

7. 3. 2. Diabetic animals

No appreciable change in either the intensity of immunofluorescence, or in the relative number of different peptide- or PGP 9.5-immunoreactive nerves was observed 2, 4 and 8 weeks after the onset of diabetes. Differences were observed however, in diabetic animals 12 weeks after streptozotocin treatment. In these animals, there was a marked increase in the relative density of CGRP-, VIP- and PGP 9.5-immunoreactive fibres, associated with an increase in the intensity of immunoreactivity. In the epidermis and dermis, a large number of fibres were immunoreactive for CGRP (Figs.1B,2B). In addition, a substantial increase in nerves displaying VIP-

immunoreactivity was observed around sweat glands and ducts in the foot pad (Fig.2D), and around blood vessels in both regions (Fig.1D). The increase in immunoreactivity for CGRP and VIP was paralleled by a sharp increase in the nerve fibres immunoreactive for PGP 9.5 (Figs.3B,3D,3F). No differences were observed between control and treated rats in the relative density of substance P and NPY-immunoreactive axons in any of the groups studied. The arbitrary gradings of the immunostaining are summarised in Table 1.

7.4. DISCUSSION

In the skin of animals with streptozotocin-induced diabetes, there was a marked increase in the relative number of PGP 9.5-, CGRP- and VIP-immunoreactive nerves, between 8 and 12 weeks after treatment. No change was seen, however, in the number of substance P- and NPY-immunoreactive nerve fibres.

The increase in VIP- and CGRP-immunoreactive axons in diabetic rats is an interesting feature since they form the largest sub populations of peptide-immunoreactive axons present in the skin (Chapter 3). VIP-immunoreactive axons originate mainly from the cell bodies in the sympathetic and parasympathetic ganglia (Larsson et al., 1976) and there is evidence that VIP plays a role in vasodilatation (Said, 1982) and the modulation of sweat secretion, by acting directly on myoepithelial cells and increasing blood flow (Lundberg et al., 1982a). CGRP-immunoreactive neurones are principally sensory in nature (Cadieux et al., 1986; Su et al., 1986; Wharton et al., 1986; Terenghi et al., 1986) and their role in nociception (Dubner and Bennett 1983) has been reported. A proportion of CGRP-immunoreactive neurones are known to be co-localised with another sensory peptide, substance P (Gibson et al., 1984; Gibbins et al., 1985; Merighi et al., 1988). Similar changes in VIP and CGRP levels have been reported in initial stages of human diabetes, which may be related to functional abnormalities including hyperhydrosis, intolerance to heat and burning pain (Brown et al., 1976).

The exact mechanism which brings about the relative increase in VIP- and CGRP-immunoreactive nerves in diabetic animals is not yet clear.

The observation that it is associated with an increase in the immunostaining of PGP 9.5 suggests that the changes are confined to peptidergic cutaneous nerves. PGP 9.5 is a soluble protein component of neuronal cytoplasm (Doron et al., 1983; Gulbenkian et al., 1987) and its immunoreactivity has been utilised to visualise the general pattern of innervation supplying peripheral tissues, including skin (Dalsgaard et al., 1989), heart (Gulbenkian et al., 1987) and uterus (Lundberg et al., 1988). Previous studies have shown an increase of VIP immunoreactivity in the eye and urinary bladder of prediabetic and diabetic chinese hamsters (Diani et al.,1985) as well as in the seminal vesicles of 8 and 16 week streptozotocin-induced diabetic rats (Moss et al., 1987) and in enteric nerves (Belai et al., 1985). The increase of VIP has been claimed to contribute to the gastrointestinal (Belai et al., 1985) and vesicular dysfunction (Diani et al., 1985).

The lack of changes in immunoreactivity for substance P and NPY up to 12 weeks may be due to the very small population of nerve fibres containing these peptides in skin, whose changes cannot therefore be explored adequately by immunohistochemical methods and semiquantitative assessment. However, it seems more likely that there is no change, which confirms the previous immunohistochemical investigation in the intestine of the rat (Belai et al., 1988).

In contrast to our data, a decrease of CGRP immunoreactivity in nerves supplying ileum and proximal colon (Belai et al., 1987) and of VIP immunoreactivity in nerves supplying the penis (Crowe et al., 1983; Gu et al., 1984) has been observed. Reduction of peptide immunoreactivity has also been reported in cutaneous nerves in advanced human diabetes (Levy et al., 1989) and was associated with depletion of PGP 9.5 immunoreactivity. This was interpreted as resulting from degeneration of axons induced by diabetes. However, in the intestine of diabetic rats, the initial decrease in density of CGRP- and substance P-immunoreactive nerves was suggested to be consequent on the increase of adrenergic and VIP-immunoreactive nerves (Belai et al., 1988). A possible explanation of these differences is that in the in initial stages of diabetes, at least in experimental conditions, there is a general increase, followed by a depletion, of nerves and neuropeptides supplying different tissues. The time course of these changes, could

vary in different organs, due to a variable susceptibility of nerve fibre sub-types. If the treatment in the rat had a longer duration than 12 weeks, it is possible that a subsequent depletion of innervation and neuropeptides, similar to the results in man, would have resulted.

In conclusion, our study shows an initial increase in the relative number of PGP 9.5-, CGRP- and VIP-immunoreactive fibres in the skin of diabetic rats and is of particular interest since the easy access to skin tissue may allow the extensive investigation of peripheral nerve changes. These changes may be reflected by corresponding changes in the dorsal root ganglia, sympathetic ganglia and spinal cord. Similar changes may occur in initial stages of human diabetes and therefore invites speculation that skin biopsy screening may be valuable as clinical aid in the management of diabetes. This merits further study.

Chapter 8

REINNERVATION AND NEUROPEPTIDES IN MOUSE SKIN FLAPS

	Pages
8. 1. Introduction	177
8. 2. Experimental design	178-179
8. 3. Results	179-183
8.3.1. Control specimens	179-180
8.3.2. Grafts	180-183
8. 4. Discussion	183-185

8. 1. Introduction

Degeneration and regeneration of nerves in free grafts or skin flaps have been studied by classical methods such as silver and gold impregnation, methylene blue staining, non-specific cholinesterase reaction and formaldehyde-induced fluorescence (Davis and Kitlowski, 1934; Adeymo and Wyburn, 1957; Marvis et al., 1963; Santoni-Rugiu, 1966; Fitzgerald et al., 1967; Ridley, 1970; Orgel et al., 1972). Functional aspects of reinnervation in man have also been investigated by clinical and electrophysiological methods (Trotter and Davis, 1909; Davis, 1934, Mcgregor, 1950; Mannerfelt, 1962; Terzis, 1978); they have shown that pain perception returns first and the transplanted skin is hypersensitive to heat stimuli, with symptoms of hyperaesthesia and itching. In recent years, a number of studies have described the immunocytochemical localization of nerves and neuropeptide-immunoreactive fibres supplying epidermis, dermis and appendages of the skin (Bloom and Polak, 1983; Dalsgaard et al., 1983; Gibbins et al., 1987b). It is likely that many of these peptides play an important role in regulation of cutaneous sensation (Lembeck and Gamse, 1985; Woolf and Wisenfeld-Hallin, 1986) and blood supply (Brain and Williams, 1985) and they are believed to have trophic properties (Kjartansson et al., 1988). However, there is no reported immunocytochemical study of reinnervation, including peptidergic nerves, in skin flaps. The main purpose of the present study was to investigate, using immunocytochemistry with antisera to the general neural marker protein gene product (PGP 9.5) (Thompson et al., 1983) and neuropeptides, the sequential course of cutaneous nerve degeneration and regeneration and the distributions of nerve fibres immunoreactive for neuropeptides reinnervating the skin.

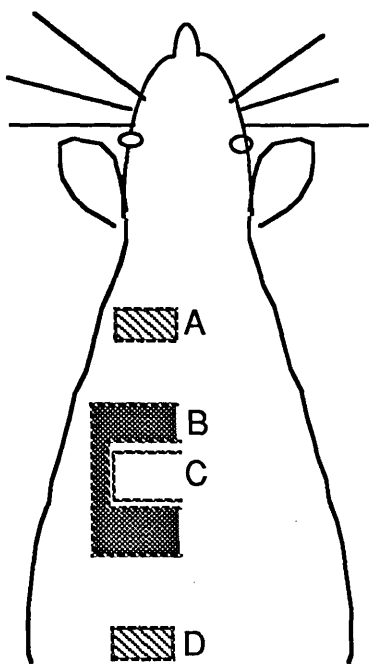


Diagram 10. Schematic representation of skin flaps on the dorsal region of mouse from which the tissues are taken for the study; A & D = control skin, B = skin adjacent to the flap, C = skin flap.

8. 2. Experimental design

Twenty seven male, hairless mice (strain MF 1, 2-3 months, 20 grams) were used. They were anaesthetised with pentobarbitone sodium (Nembutal - Abbot, 30-40mg/Kg body weight) injected intraperitoneally. The operative area was prepared and operations performed using aseptic precautions. A rectangular skin flap 1cm in length, with a base of 0.5 cm on the mid-dorsal region, was cut and separated from the subcutaneous tissue and panniculus carnosus and then replaced in the same position (Diagram 10). All flaps were sutured with 6.0 silk and no protective dressing was applied in order to monitor the healing. Mice showing seroma or haematoma (n=3) were not included in the study. Groups of 3 animals were killed at 24 and 48 hours and 5 to 7, 15, 30, 45 and 60 days post-operatively. Whole flaps were biopsied with a 2 cm portion of adjacent skin. In addition, control specimens were taken from another dermatome at the mid-dorsal level either anterior or posterior to the flap. Skin samples were fixed immediately by immersion for 6 hours at room temperature in Zamboni's fluid (**Appendix 3d**). The tissues were stored in PBS/sucrose (**Appendix 2b**). Cryostat blocks were prepared and frozen sections (12-15μm thick) were collected on poly-L-lysine coated slides (**Appendix 4**) for staining by haemotoxylin and eosin (**Appendix 7**) or by a modified indirect immunofluorescence method (**Appendix 5a**) with antisera to a general neural marker and neuropeptides.

8. 3. Results

8. 3. 1. Control specimens

Protein gene product 9.5 (PGP 9.5)-immunoreactive axons were seen in the epidermis, dermis, and around hair follicles and blood vessels

(Fig.8.1A). The immunoreactivity was intense and clearly defined, and seen in both individual fibres supplying the epidermis and dermis as well as in large nerve bundles coursing through the dermis. A subset of these axons in all areas of skin were immunoreactive for calcitonin gene-related peptide. Substance P (SP)-immunoreactive axons were seen occasionally in the epidermis and dermis, and in large nerve bundles (Fig.8.2B). Vasoactive intestinal peptide (VIP)- and neuropeptide Y (NPY)-immunoreactive axons were seen mostly around large blood vessels, where they formed perivascular plexus.

8. 3. 2. Grafts

Immunoreactivity for PGP 9.5, CGRP, substance P, VIP and NPY was not seen in the skin flaps including the central and peripheral parts and the base of the flap at 24 hours post-operatively (Fig.8.2C). This lack of neural and glial immunoreactivities was also observed in the skin surrounding the flaps up to 1 cm away in all directions. PGP 9.5- and neuropeptide-immunoreactive nerves were first observed in the surrounding skin by day 2. In contrast, PGP 9.5- immunoreactive nerves were first seen in the skin flaps by day 7, close to the pedicle and entering through the uncut base of the flap. By day 15, focal and irregular clusters of PGP 9.5-immunoreactive nerves were seen close to the body of the flap, arising from the nerves at the base of the flap; a large proportion of these nerves were immunoreactive for CGRP (Fig.8.2D) and substance P. In addition, scattered PGP 9.5- and CGRP-immunoreactive nerves were seen at the cut edges of the flap arising from nerves in the adjoining normal skin (Fig.8.1B). By day 30, PGP 9.5-immunoreactive nerve fibres originating from bundles below the flap had entered the deep surface and were coursing towards the epidermis (Fig.8.1C). In addition, occasional VIP- and NPY-immunoreactive nerves were seen close to the blood vessels. By

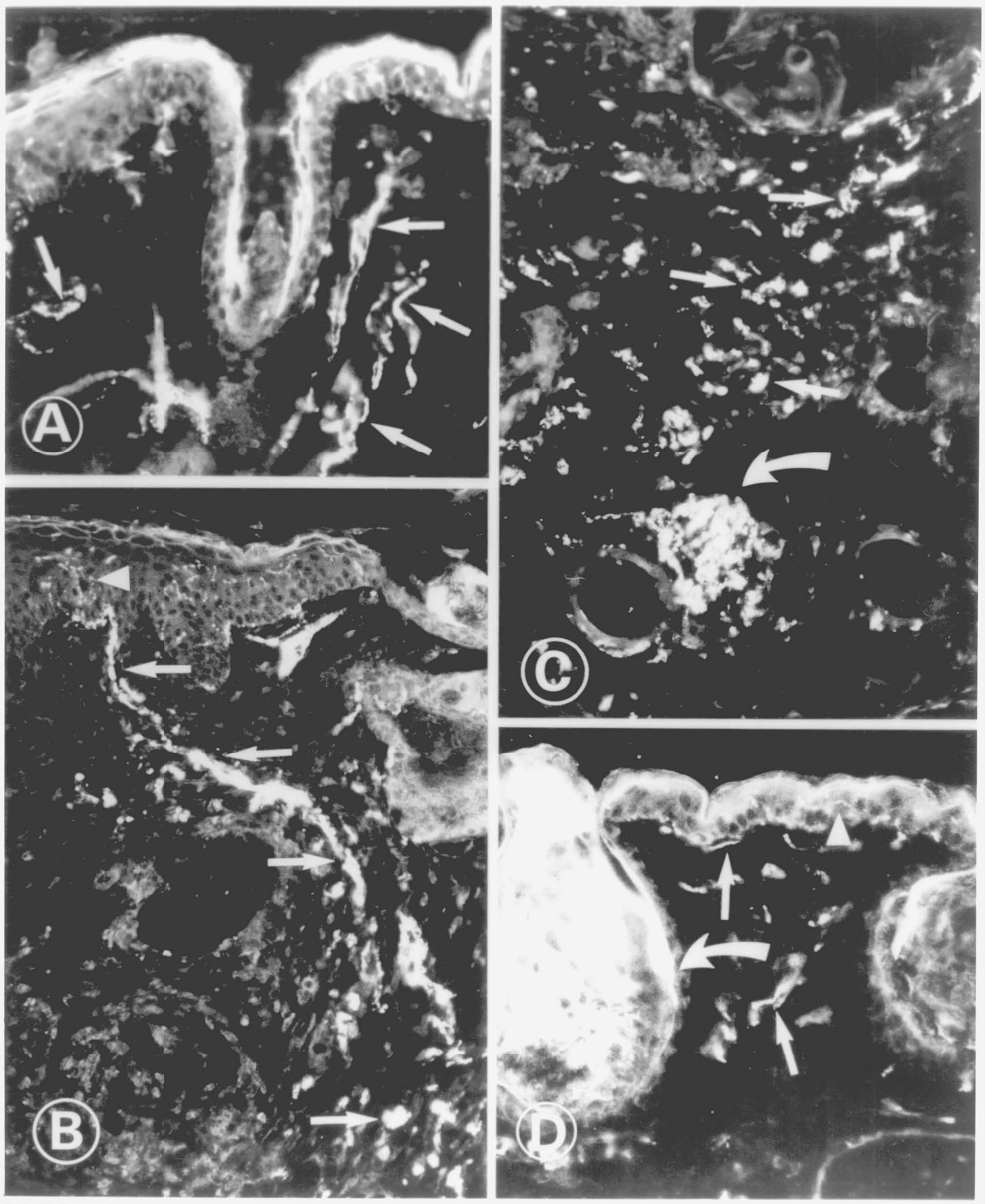


Fig. 8.1. Nerve fibres immunoreactive for PGP 9.5 in mouse skin. (A) Control dorsal skin showing PGP-9.5 immunoreactive fibres supplying epidermis and dermis. Note the density and distribution of nerve fibres. (B - D) Grafted skin. Skin flap showing appearance of irregular clusters of nerves 15 days after transplantation at the cut edge (B) and 30 days after transplantation originating at the deep nerve bundle of the graft (C), and normal distribution 45 days after transplantation (D). Note the density of nerve fibres at the early stage of reinnervation in B and C in comparison with control skin. Arrows and arrow heads indicate immunoreactivity in the dermis and epidermis, respectively; curved arrows indicate a nerve bundle in (C) and autofluorescence in hair follicle (D). All magnification x 320

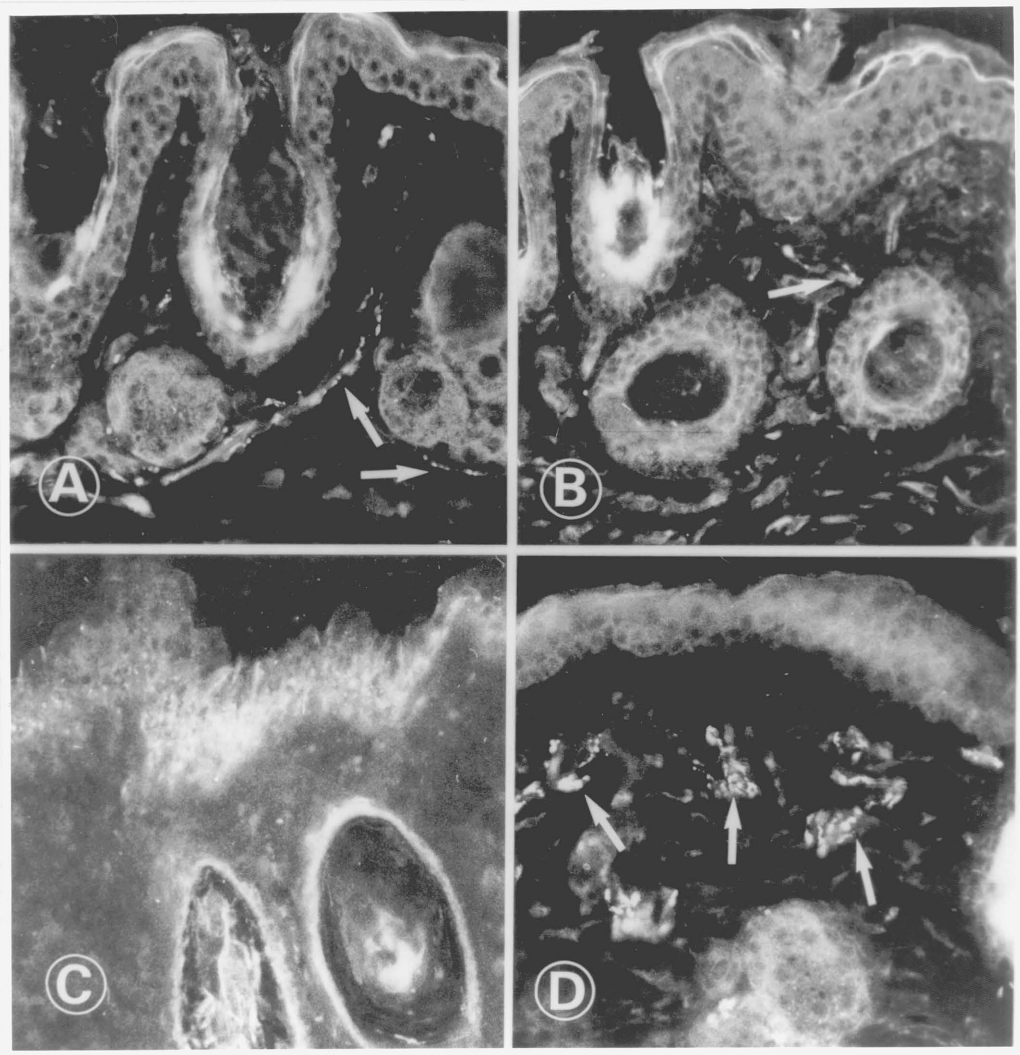


Fig. 8.2. Control dorsal skin showing nerve fibres immunoreactive for CGRP (A) and substance P (B) supplying the epidermis and dermis. Note the density and distribution of nerve fibres. (C) Skin flap showing absence of PGP 9.5 immunoreactivity 24 hours after transplantation. (D) CGRP-immunoreactive fibres arranged in focal clusters in the body of the graft 15 days after transplantation. Arrows indicate some of the immunoreactive fibres in the dermis. Zamboni fixed tissues. Cryostat sections (10μm).
All magnification x 320

day 45, nerve fibres were detected in the epidermis of the flap, and many of the focal arborisations had disappeared (Fig.8.1D). By day 60, the distributions of nerves and neuropeptides closely resembled those in the normal control skin.

8. 4. Discussion

This study has shown the time course of reinnervation in skin flaps in mice and revealed several novel observations: the disappearance of general neural marker and peptide immunoreactivity in surrounding skin, the early reappearance of general neural marker immunoreactivity in the flap and the early involvement of sensory neuropeptides in reinnervation.

The apparent disappearance of nerves in the skin surrounding the flap was shown by the loss of immunoreactivity for PGP 9.5 and neuropeptides. The loss of neuropeptides could be accounted for by functional changes in the nerves, such as temporary blockage of axonal transport or excessive release of peptides, due to surgical trauma. However, PGP 9.5 is believed to be a non-functional, cytoplasmic protein; therefore its early reappearance (day 2) together with neuropeptide immunoreactivity in nerves that are presumbly not totally destroyed suggests that it may be a sensitive indicator of neural damage.

In contrast, the disappearance of PGP 9.5 immunoreactivity in the flap by 24 hours and reappearance only at the base by day 7 suggests that the flap had become completely denervated. The complete absence of PGP 9.5-immunoreactive nerve fibres in the body of the flap may be due to the orientation of incised area, which was perpendicular to the neurovascular bundle, thereby cutting all the nerves. The results

accord with previous observations using histochemical methods showing absence of non-specific cholinesterase activity in degenerating cutaneous nerves up to the base of the flap within 2 weeks after surgery (Waris, 1978), suggesting that disappearance of immunoreactivity is due to degeneration of nerve fibres.

The sequential appearance of immunoreactivity for PGP 9.5 and peptides (substance P and CGRP) in the flap, first at the base of the flap where there is a continuity of epidermal and dermal tissues, then at the sides and apex of the flap margins and finally at its deep surface, suggests that the reinnervation follows a pattern and is systematic. However, our results appear to be in conflict with previous studies showing regenerating nerves originating either solely from the base (Fitzgerald et al., 1967) or from the margins (Waris, 1978). The focal arrangement of the hyperplastic, immunoreactive nerve fibres seen during early reinnervation may be due to extensive arborisation to enable growing immature nerves to locate their targets.

The early reappearance of CGRP- and substance P-immunoreactive fibres in greater numbers in the flap than in the normal skin is an important observation in our study. Both substance P and CGRP are involved in nociception (Henry, 1982; Woolf and Wisenfeld-Hallin, 1986). Antidromic release of substance P from sensory nerves induces vasodilation, plasma extravasation and itching (Lembeck and Gemse, 1982), effects that are potentiated by CGRP (Brain and Williams, 1985; Gamse and Saria, 1985). Such release may be the reason for hyperaesthesia and itching in transplantated skin. In addition, the initial proliferation of CGRP- and substance P-immunoreactive nerves may provide morphological evidence in support of several previous clinical studies showing that nociception (Head et al., 1905; Rivers and Head, 1908; Sharpey-Schafer, 1928) and hypersensitivity to heat stimuli

(Trotter and Davis, 1909) return early to transplanted skin. These observations suggests that CGRP and substance P have important roles in the transplanted skin.

In addition to the putative role as sensory transmitters both CGRP and substance P are known to have trophic actions that could be important in healing and repair processes in the graft. Thus for example substance P, in common with other tachykinins has the ability to promote cellular mitosis (Nilsson et al., 1985) Also CGRP has been shown to possess neurotrophic properties, being involved in the differentiation of dopaminergic neurons of olfactory bulb (Dennis-Donini, 1989) and local intravenous injection of CGRP promotes flap survival (Kjartansson and Dalsgaard, 1987) by increasing the blood flow (Kjartansson et al., 1988). Similar proliferation of substance P-immunoreactive nerve fibres was seen around regenerating blood vessels during the healing after burn injury has been reported (Kishimoto, 1984). It is of interest that during ontogeny, CGRP and substance P are the first peptides to appear in the cutaneous innervation, before VIP and NPY, indicating a possible trophic role in development (Chapter 3). This study, therefore, suggest that the neural proliferation may stimulate growth of fibroblasts in connective tissue and probably act as a sensory stimulant for the regeneration of blood vessels and nerves. Thus, the proliferation may have a trophic role in the transplanted skin akin to that in development.

In conclusion, this study shows an early, sequential pattern of reappearance of immunoreactive nerves and neuropeptides, often in apparently disorganised groups possibly as a mechanism of target searching, and the early appearance of sensory neuropeptides as in ontogeny, that have roles in vascular and neural tropism. The increase in neuropeptides suggests their possible roles in trophic, regulatory and reparative processes.

Chapter 9

INCREASE IN CUTANEOUS INNERVATION IN NOTALGIA PARAESTHETICA

9.1.Introduction **186**

9.2. Experimental design **187-189**
9.2.1. Patients 187-188
9.2.2. Biopsies 187,189

9.3. Results **189-194**
9.3.1. Histology 189
9.3.2. Immunocytochemistry 189-194

9.4. Discussion **194-196**

9. 1. INTRODUCTION

Notalgia paraesthetica is an infrequently reported condition characterised by sensory neuropathy, possibly having an hereditary component in some cases (Astvatsaturov, 1934; Weber et al., 1988), The lesion involves the posterior rami of the second through sixth intercostal nerves (Astvatsatkurov, 1934; Pleet et al., 1978). The most common symptom is pruritus in the mid-upper back, but burning, hyperalgesia and tenderness are described (Pleet et al., 1978; Massey et al., 1979; Weber et al., 1988). Secondary lichenification and/or pigmentation frequently occurs.

Since there is no information on the cutaneous innervation of skin in notalgia paraesthetica, and because changes are seen in the distribution and peptide immunoreactivity of nerves in skin from patients with other neuropathies (Chapter 4 and 6). The aim of the present chapter was to study the distribution of overall nerves and Schwann cells, using general neural marker protein gene product 9.5 (PGP 9.5) (Thompson et al., 1986), and glial marker S-100, and of peptidergic nerves by antisera to neuropeptides.

9. 2. Experimental design

9.2.1. Patients

Details of the notalgia parasthetica cases are given in Table 17

9.2.2. Biopsies

Punch biopsies were taken under local anesthesia from the affected area and unaffected contralateral side from patients attending Royal Sussex County Hospital, Brighton. Skin specimens from similar sites were obtained from patients having no neuropathy of skin disorders

Table 18

CLINICAL DETAILS OF NOTALGIA PARAESTHETICA PATIENTS

Case No	Age	Sex	Symptoms	Duration	Site	Helpful Therapy
1	68	M	Irritation Pain	12Yrs	R	Tegretol
2	54	F	Numbness Episodic irritation	1Yr	L	Topical steroid
3	72	M	Icy feel	4yrs	L	Transcutaneous stimulation
4	65	F	Irritation	3yrs	R	Topical steroid
5	66	M	Aching episodic irritation	11yrs	L	Topical steroid

undergoing surgery that needed skin removal. The tissues were fixed immediately in Zamboni's solution (**Appendix 3D**). Cryostat blocks were prepared and frozen sections (6 μm) taken up on poly-L-lysine-coated slides (**Appendix 4**), dried at room temperature for 2 hours and stained by haematoxylin and eosin (**Appendix 7**) or by a modified indirect immunofluorescence method (**Appendix 5**) with antisera to neural and glial antigens and neuropeptides. Control and treated specimens were mixed in batches before processing. The number of immunoreactive nerves was assessed visually in all specimens and graded by semiquantification (Chapter 2). The study was approved by the Ethics Committee of Brighton Health Authority.

9.3. RESULTS

9.3.1. Histology

Morphological examination of haematoxylin and eosin stained sections showed changes typical of these reported to notalgia paraesthetica. No amyloid deposits were seen in crystal violet stained preparations.

9.3.2. Immunocytochemistry

In the control specimens and the unaffected skin from the notalgia patients, the distribution and numbers of nerves were similar. Fibres and bundles immunostained with anti-PGP 9.5 serum were seen throughout the dermis, around blood vessels, sweat glands and hair follicles, running below the epidermis and occasionally penetrating into it (Fig.9.1A & B) S-100 immunoreactivity was seen in Schwann cells around the dermal nerves only, and occasionally in epidermal dendritic cells (Fig.9.1C & D). Subsets of the nerves were immunoreactive for neuropeptides. Substance P and CGRP had similar distribution although the latter predominant (Fig. 9.3). They

TABLE 19

RESULTS OF IMMUNOSTAINING OF NERVES IN SKIN

SKIN AREA	CONTROLS (N=10)						NOTALGIA PARAESTHETICA (N=5)					
	PGP9.5	S-100	Sub P	CGRP	VIP	NPY	PGP 9.5	S-100	Sub P	CGRP	VIP	NPY
Epidermis	+	-	-	-	-	-	+++	-	-	-	-	-
Dermis	++	++	+	++	-	-	+++	+++	+	++	-	-
Hypodermis	+++	++	+	++	-	-	+++	+++	+	++	-	-
Hair follicles	+++	++	+	++	-	-	++	++	-	-	-	-
Sweat glands	+++	+++	+	+	++	-	+++	+++	-	+	++	+
Blood vessels	++	++	-	-	+	++	++	++	-	-	+	++

Number of stained nerves fibres are graded: absent (-), few (+), moderate (++), many (+++),

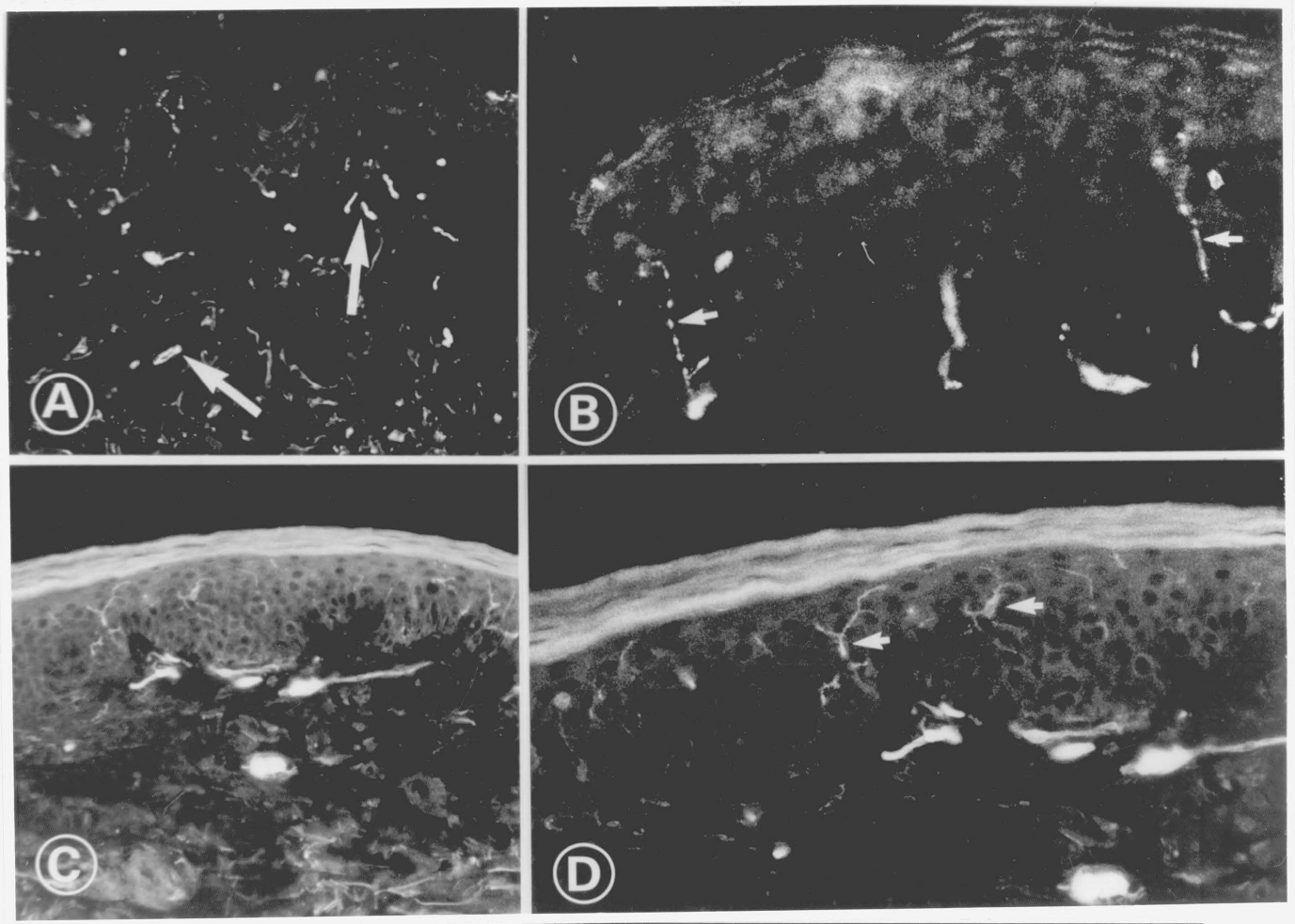


Fig. 9.1. Control skin from infrascapular region. (A & B) PGP 9.5-immunoreactive fibres in epidermis and dermis. (C & D) S-100-immunoreactivity in Schwann cells and dermal fibres. Note the density of nerve fibres and Schwann cells. Arrows indicate some of the immunoreactive fibres and Schwann cells. Magnification A = x 105, B = x 450, C = x 200, D = x 300.

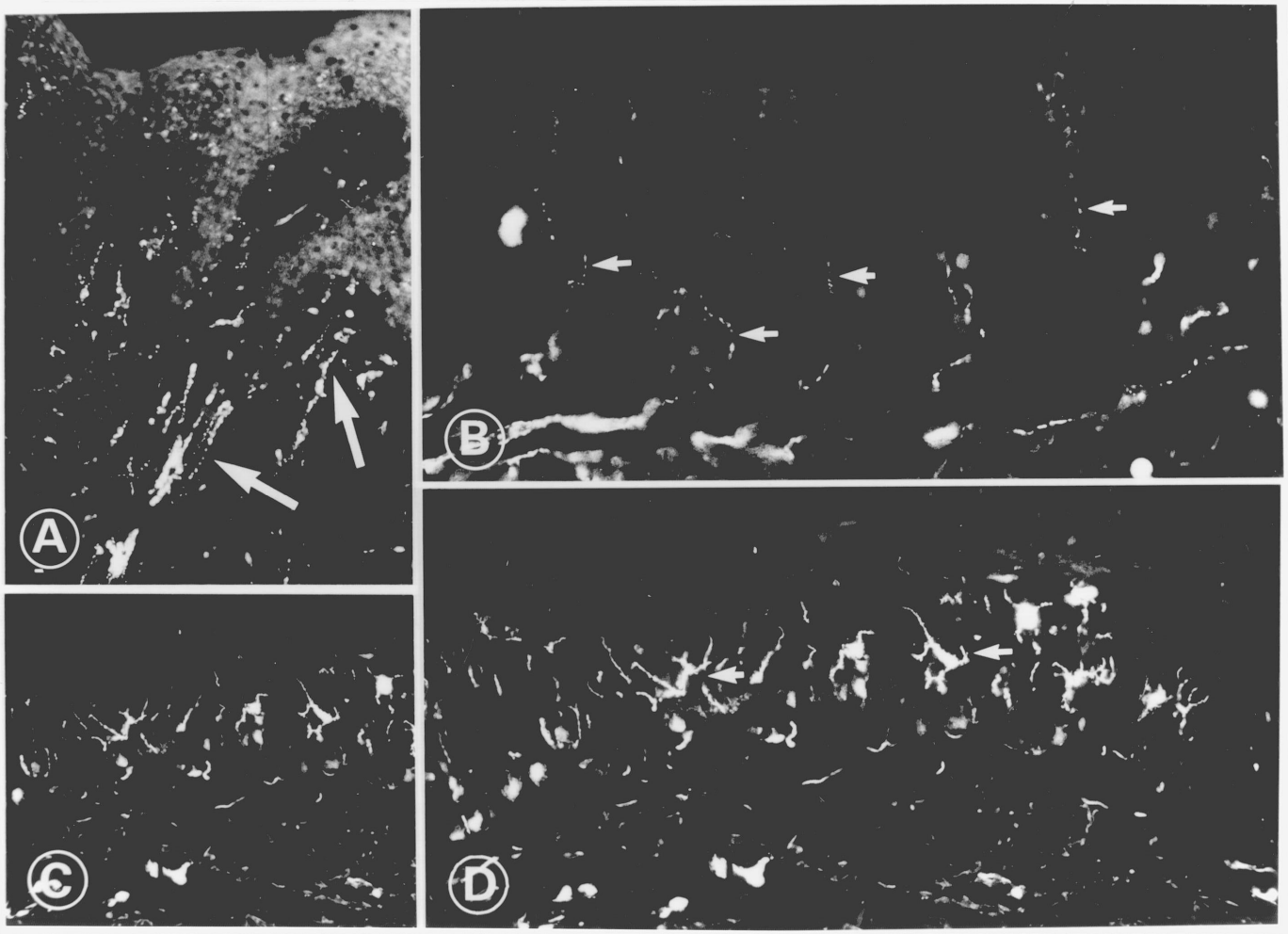


Fig. 9.2. Notalgia parasthetica skin from infrascapular region. (A & B) PGP 9.5-immunoreactive fibres in epidermis and dermis. (C & D) S-100 immunoreactive Schwann cells in the epidermis. Note the increase in epidermal fibres and Schwann cells. Arrows indicate some of the immunoreactive fibres and Schwann cells. Magnification A = x 105, B = x 450, C = x 200, D = x 300.

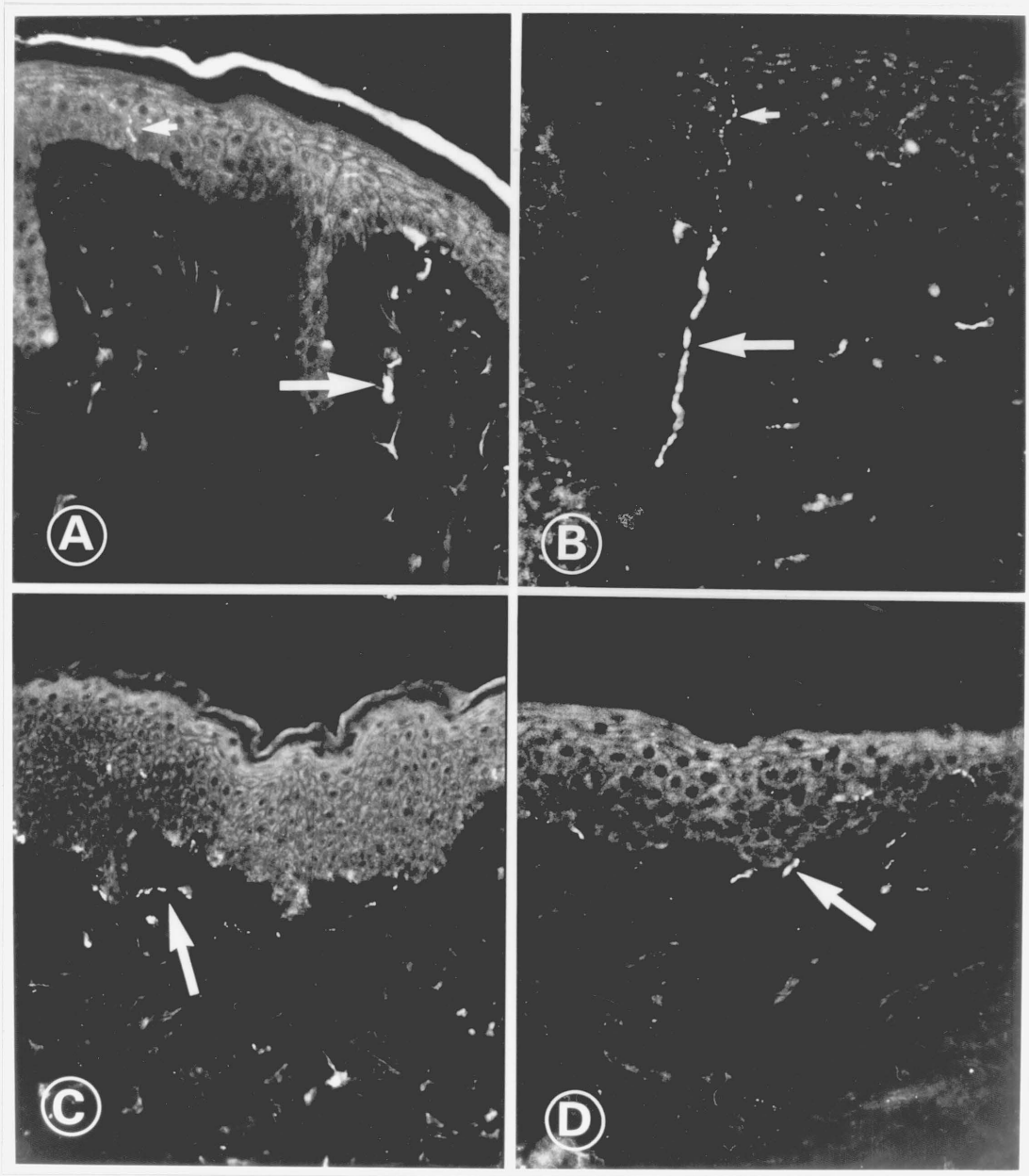


Fig. 9.3. Infrascapular skin of control (A & C) and Notalgia parasthetica (B & D) showing CGRP- and substance P-immunoreactive axons in the epidermis and dermis. No change in the density of immunoreactive fibers supplying epidermis and dermis. Arrows indicate some of the immunoreactive fibres Magnification A, B & D = x 135, C = X 105

were seen in varicose nerve fibres in the dermis and surrounding small arteries and arterioles, and occasionally around sweat glands. Scattered fibres were seen penetrating the epidermis (Fig.9.3A & C). VIP and NPY immunoreactive nerves had similar distributions, mainly in nerve around sweat glands and blood vessels.

9.3.3. Notalgia paraesthetica cases

In the affected skin area the number of PGP 9.5-immunoreactive nerve fibres (Fig.9.2A) and S-100 immunoreactive Schwann cells (Fig.9.2C) was greater than in the controls and the unaffected skin. This increase was seen in all areas of the skin, but was most noticeable (Fig.9.2B) in the PGP 9.5-immunoreactive epidermal fibres. A large number of S-100 immunoreactive dendritic cells was seen in the epidermis, particularly at the level of the stratum corneum, and around hair follicles (Fig.9.2D). No obvious changes were detected in the number of neuropeptide-immunoreactive nerves (Fig. 9.3B & D).

9. 4. DISCUSSION

This study demonstrates for the first time that in patients with pruritus and hyperalgesia - notalgia parasthetica - there is a relative increase in the number of cutaneous nerves, particularly in the epidermis, compared with controls. Furthermore, there was a dramatic increase in the number of S-100 immunoreactive dendritic cells in the affected epidermis.

The increase in the number of epidermal nerves, is of great interest because nerves in this site are believed to be sensory in nature (Björklund et al., 1986). Epidermal nerves are present in much greater number in the more sensitive areas of skin, such as lip and nose

(Chapter 3). This could explain the symptoms of notalgia parasthetica, such as pruritus and hyperalgesia, and it is notable that the increase was only seen in the affected areas of the patients and not in their skin from other areas. Therefore, it could be possible that the symptoms of pruritus and pain were not psychosomatic and might be due to the presence of large numbers of epidermal nerves, and furthermore, these nerves might be more active than normal and had a low sensory threshold. A proportion of the nerves in this site normally contained CGRP and substance P (Chapter 3), but no increase was detected in the number of peptide immunoreactive nerves. Thus, either such nerves were of a non- peptidergic type or that the peptide was depleted below the immunohistochemical detection. The presence of increased nerves also suggests that either the neural ablative procedures used therapeutically in this patient were at best only partially effective or that there is a rapid re-growth of nerves back into the skin. It is known that in animals, nerve sectioning or selective chemical denervation procedures can lead to hyperplasia of other nerve types in the denervated tissues (Kessler et al., 1983; Björklund et al., 1985; Aberdeen et al., 1988). It is possible that a similar mechanism, reportedly due to excess of nerve growth factor (Björklund et al., 1985) occurs in these cases.

The finding of large numbers of S-100-immunoreactive dendritic cells in the upper epidermis is also interesting. These could either be melanocytes or Langerhans cells, both of which are reported to be immunoreactive for S-100 (Cocchia et al., 1981). The latter seems more likely since melanocytes are usually confined to the basal layer of epidermis and some authors do not find them always to be immunoreactive for S-100 (Springall et al 1983).

This study has shown that neural changes may be evident in neuropathies having increased sensation, as shown for those involving reduced sensation such as leprosy(Chapter 4) and diabetes Chapter 6). It is concluded that the itching and painful symptoms associated with notalgia paraesthetica may be due to those neural changes and thus, at least in these cases, the symptoms are not psychosomatic.

Chapter 10

SUMMARY AND CONCLUSIONS

Immunocytochemical techniques have contributed substantially to the morphological identification of neuropeptides, the presence of which in cutaneous nerves has completely revolutionised our previous knowledge of the innervation of skin. The main focus of this morphological study was directed towards systematic screening of the nerve supply of the skin, its ontogeny and phenotypic expression in various experimental conditions and diseases effecting them. The results of the study support the hypothesis that cutaneous innervation and neuropeptides contained in it show considerable variations among species in the density of distribution and appears to play an important role in maintaining integrity and function in health and diseases. Interpretation of morphological changes and functional implications of new findings obtained during the course of this study, has been discussed in previous chapters.

There is an impressive number of different peptide-immunoreactive fibres supplying the epidermis, dermis and epidermal appendages of the mammalian skin. They exhibit both regional and species differences in the density of distribution (**Chapter 3**). Among them calcitonin gene-related peptide and/or vasoactive intestinal polypeptide are apparently the most abundant neuropeptide detected in the skin of several mammalian species including man. Those immunoreactive for tachykinins, neuropeptide Y and C-flanking peptide of neuropeptide Y were less abundant. However, in man, relatively few nerve fibres appear to display immunoreactivity for these peptides (**Chapter 3**).

Chemical denervation experiments delineated the neuropeptides present in the sensory and autonomic parts of cutaneous innervation. Capsaicin treatment destroyed approximately 70% of calcitonin gene-related peptide- and tachykinin-immunoreactive axons at the dosage given. This may also be an evidence, showing separate subsets of CGRP and tachykinin-immunoreactive sensory nerves having different thresholds of sensitivity to capsaicin, functional significance of which is yet to be determined. However, 6-OHDA treatment resulted in complete loss of neuropeptide Y- and tyrosine hydroxylase-immunoreactive axons. It is conceivable therefore that cutaneous sympathetic fibres show some demarcation between cutaneous adrenergic and cholinergic types (**Chapter 3**).

Pathological lesions often produce morphological changes from which one can deduce something about how normal tissues behave. In an attempt to throw more light on the role of peptides present in sensory and autonomic nerves during various pathological conditions resulting in peripheral neuropathy, showed that presence or absence of peptide is linked to functional status. In leprosy, an early disappearance of cutaneous neuropeptides was seen in Indeterminate, Lepromatous and Tuberculoid cases, is consistent with diminished sensitivity, in addition to suggesting that immunocytochemical study of neuropeptides may enable a more precise categorization of suspected very early leprosy lesions (**Chapter 4**). Similar depletion of neuropeptides was also noticed in skin biopsies taken from type I (insulin dependent) and type II (non-insulin dependent) diabetic patients further substantiating the role in assessment of diabetic neuropathy and may be useful in determining its response to treatment (**Chapter 7**).

In contrast to the early depletion, in notalgia parasthetica, characterised by pruritus and hyperalgesia, an increase in the number of cutaneous nerves without an increase in peptides was observed implying that there may be a population of non-peptidergic nerves involved in above changes (**Chapter 9**).

Experimental animals showed the effects of early stage of disease processes upon peptides in cutaneous nerves and the progressive changes with the advancement of disease. Skin and spinal cord of nude mice infected with Mycobacterium leprae showed depletion of neuropeptides at an early stage and the changes progressed with disease (**Chapter 5**). The loss of peptides in skin from distant sites and simultaneous depletion in motoneurones of the spinal cord is a clear indication of the functional deficit in these animals. On the other hand, an increase of vasoactive intestinal polypeptide and calcitonin gene-related peptide in skin of 8 to 12 weeks old streptozotocin-diabetic rats indicates that the changes in neuropeptides may reflect the differences in the disease process (**Chapter 7**). The increase in CGRP during regeneration of transplanted skin (**Chapter 8**) and early appearance during development (**Chapter 3**) proves further that the peptides are involved even in the normal biological processes of regeneration and development. Therefore, the functional significance of changes in distribution of neuropeptides may not necessarily be the impairment of transmission process at the nerve terminal but may also be related to tropism.

Classical studies of cutaneous innervation was always plagued by the apparent unreliability in staining technique to visualise all the nerves. This study has tested the immunostaining potential of all the available general neural markers and proved that the protein gene product 9.5 gave consistently reproducible results and precisely assess the nerve

supply of the skin in all the species examined (**Chapter 3**). PGP 9.5 was particularly useful in examining differential density of nerves during disease conditions, and thus provided an valuable marker for future investigations (**Chapters 4,6 and 7**).

The present investigations provide further insight into the significance of immunocytochemical techniques in the visualisation of cutaneous nerves, in addition to the distribution of peptide-immunoreactive fibres in normal and disease states. There is no doubt a great deal of work still needs to be done on similar lines employing latest morphological methods including *in situ* hybridisation and *in vitro* receptor binding studies in combination with neurophysiogical methods.

APPENDIX 1

Perfusion fixation

1. Guinea pigs and rats were mildly anaesthetised by inhalation of ether vapour.

2. An overdose of sodium pentobarbitone (Euthatal, May and Baker Ltd., 200mg/ml) administered by intraperitoneal injection.

3. Solutions used for perfusion were administered (20-30 ml/min) via a blunt needle placed through the left ventricle and clamped into the aortic arch. An incision in the right atrium was made in order to drain the perfusion solutions.

4. Blood was first removed by perfusion with 200ml of PBS at room temperature or at 4°C.

5. Following the perfusion technique, tissue was removed and further fixed immersion for 2 to 16 hours.

APPENDIX 2

Preparation of Buffers

a. Phosphate buffered saline (PBS) (0.01M) pH 7.2-7.4

The following compounds were dissolved in 10 litres of distilled water:

Nacl 87.9g

KH_2PO_4 2.74g

Na_2HPO_4 11.35g

b. PBS / sucrose buffer pH 7.2-7.4

15g sucrose and 0.001g sodium azide (sigma) were added to 100ml PBS.

APPENDIX 3

Preparation of Fixatives

a. Benzoquinone solution fixation

1. A fresh 0.4% (w/v) solution was prepared by dissolving purified benzoquinone (Koch-Light Laboratories Ltd) in PBS with agitation.

2. Tissues were fixed at room temperature by immersion or perfusion for 1 to 3 hours (depending on the type of tissue and size of the sample).

3. The tissues were rinsed with several changes of PBS containing 15% (w/v) sucrose and 0.01% (w/v) sodium azide at 4°C and stored in PBS/sucrose solution at 4°C.

b. Formaldehyde solution fixation

1. A 4% (w/v) solution was prepared by dissolving methanol-free paraformaldehyde (BHD Chemicals Ltd) in PBS with heating. After 15 minutes heating at 80°C, the solution was cleared by adding a few drops of 1N sodium hydroxide. The solution was then left to cool at 4°C.

2. Tissues were fixed by immersion or perfusion for 1 to 3 hours at 4°C and then rinsed with several changes of PBS/sucrose solution. Fixed tissues were then stored in a PBS/sucrose solution at 4°C.

c. Zambini's solution fixation

1. A 2% (w/v) solution was prepared by dissolving methanol-free paraformaldehyde in 0.1M phosphate buffer with heating. Solution was then left to cool. To 85ml of this solution, 15ml of saturated picric acid was added.

2. Tissues were fixed by immersion or perfusion for 9 to 18 hours at 4°C. Tissues were then washed thoroughly with PBS/sucrose solution and stored in the same buffer at 4°C.

APPENDIX 4

Slide coating

1. 1mg of Poly-L-lysine (PLL) (molecular weight: 150,000-300,000-sigma) was dissolved in 1ml of distilled water.

2. A small drop of PLL (approximately 10µl) was applied to a clean slide, and spreaded with the end of another slide, as for a blood film.

3. Coated slides may be kept up to a week.

APPENDIX 5

Immunochemical procedures

a. Indirect immunofluorescence for cryostat sections

Cryostat sections were:

1. Mounted on PLL coated slides and left to dry for 1 hour at room temperature.

2. Immersed in a solution containing 0.2% triton X-100 (Sigma Chemicals, U.K) in PBS for 30 minutes.

3. Rinsed in three changes of PBS, 5 minutes each change.

4. Incubated in primary antisera in a humid atmosphere, at 4°C for 16 to 24 hours. Antiserum was diluted in PBS containing 0.1% bovine serum albumin (BSA; Sigma) and 0.01% sodium azide (Sigma).

5. Reincubated in primary antisera in a humid atmosphere at room temparature for 3 hours.

6. Rinsed in three changes of PBS, 5 minutes each change.

7. Incubated with fluorescein isothiocyanate conjugated anti-rabbit, -rat or -mouse globulins (1:100 dilution) for 60 minutes at room temprature.

8. Rinsed in three changes of PBS, 5 minutes each change.

9. Mounted in a mixture of PBS and glycerine (1:1 v/v).

b. Peroxidase anti-peroxidase (PAP) method for cryostat sections

Cryostat sections were:

1. Mounted on PLL coated slides and left to dry for 1 hour at room temperature.

2. Immersed in a solution containing 0.2% triton X-100 in PBS for 30 minutes.

3. Immersed in a solution of 0.03% (v/v) hydrogen peroxide (Macarthys) in PBS for 30 minutes, to block endogenous peroxidase activity.

4. Rinsed in three changes of PBS, 5 minutes each change.

5. Incubated in primary antiserum in a humid atmosphere, at 4°C for 16 to 24 hours.

7. Rinsed in three changes of PBS, 5 minutes each change.

8. Incubated with goat anti-rabbit globulins (1:100 dilution) at room temperature for 30 minutes.

9. Rinsed in three changes of PBS, 5 minutes each change.

10. Incubated with rabbit PAP complex (1:300 dilution in PBS) at room temperature for 30 minutes.

11. Rinsed in three changes of PBS, 5 minutes each change.

12. Incubated 1 to 5 minutes at room temperature in a solution containing 0.025% 3,3'diaminobenzine tetra HCl (DAB; w/v) (Fluka AG) and 0.03% (v/v) hydrogen peroxide in PBS.

13. Rinsed in three changes of PBS, 5 minutes each change.

14. Lightly counterstained with haematoxylin, dehydrated, cleared and mounted in DPX (Raymond A. Lamb).

APPENDIX 6

Glucose oxidase-DAB-nickel enhancement method

The sections were processed in a similar manner as that described in the peroxidase anti-peroxidase method (see Appendix 5) up to step 11. From then on the following steps were followed:

1. Sections were incubated in **developer solution** * for 3 minutes.

2. 1% cobalt chloride (BDH, U.K) was then added to the above solution and left for 3 minutes.

3. 0.03% (v/v) hydrogen peroxide was finally added to the incubating mixture and the sections were developed for another 3 minutes.

4. Rinsed twice in PBS, 5 minutes each change.

5. Washed in running tap water for 15 minutes, dehydrated through alcohols, cleared in xylene and mounted in DPX.

*** Preparation of developer solution (Glucose oxidase-DAB-nickel solution)**

a. 2.5 g Nickel Ammonium sulphate (BDH, U.K) in 50 ml of 0.2 sodium acetate buffer (pH 6.0)

b. 50 mg DAB in 50 ml distilled water.

c. Mix the solution a and b immediately before use and then 200 mg b D-glucose oxidase (Sigma Chemicals, U.K) were added to the mixture.

APPENDIX 7

HAEMATOXYLIN AND EOSIN STAINING

1. Stain the section in Harris haematoxylin (undiluted) for 3 mins.

2. Blue sections in running tap water (pH alkaline).

3. Differentiate haematoxylin in 1% acid alcohol solution. Examine microscopically to dtermine the degree of differentiation necessary, as the time depends on the initial amount of staining.

4. Re-blue in running tap water.

5. Stain in 1% aqueous solution of eosin for 5 mins. Check the staining microscopically, and return to eosin if necessary.

6. Rinse well in running tap water.

7. Dehydrate through graded alcohols, clear and mount in DPX.

APPENDIX 8

Acetylcholinesterase Histochemistry

1. Wash sections with three changes of 0.01M maleate buffer (1.6g/dl), pH 6.0.

2. Incubate for 30 min at room temperature with a solution containing 18-36μM acetylthiocholine iodide (0.5-1.0mg/dl), 5μM K_3 Fe $(CN)_6$ (0.16/dl) 30μM CuSo4 (0.47mg/dl), and 50μM sodium citrate $2H_2O$ (1.47 mg/dl) in 0.1 M maleate buffer, pH 7.6.

3. Wash sections for 5 min at room temperature with a solution containing 0.04% DAB and 0.3% nickel ammonium sulfate in 50 mM Tris-HCL, pH 7.6.

4. Add 0.003% H_2O_2 and continue incubation for 10 min at room temperature.

5. Wash sections with 5mM Tris-HCL, pH 7.6 and mount on glass slides.

APPENDIX 9

Wade fite staining for *Mycobacterium leprae*

1. Warm section to melt wax and blot.
2. Remove wax in 1 part peanut oil, 2 parts xylene for 15 - 20 minutes. Drain.
3. Rinse quickly in xylene.
4. Blot.
5. Rinse in water.
6. Remove mercury pigment with Lugol's Iodine and 5% sodium thiosulphate for 5 min.
7. Carbol Fuchsin at room temperature for 20-30minutes.
8. Wash in tap water.
9. Rinse in acid alcohol (0.5% HCL, 70% alcohol) for 2 min.
10. Wash.
11 Counter stain with hematoxylin.
12. Wash in tap water and dry, clear and mount.

REFERENCES

Aberdeen J, Corr L, Milner P, Lincoln J and Burnstock G (1988) Increases in calcitonin gene-related peptide (CGRP) after guanethidine sympathectomy in the rat. Regul Pept 22: 385.

Abraham RR, Abraham RM and Wynn V (1986) Autonomic and electrophysiological studies in patients with signs or symptoms of diabetic neuropathy. Electroencephalogr Cli Neurophysiol 63: 223-230.

Adams CWM, Grant RT and Baylis OB (1967) Cholinesterases in peripheral nervous system. 1 Mixed motor and sensory trunks. Brain Res 5: 366-376.

Adeymo O and Wyburn GM (1957) Innervation of skin grafts. Transplantation Bulletin 4: 151-153.

Adrian TE, Allen JM, Bloom SR, Ghatei MA, Rossor MN, Roberts GW, Crow TJ, Tatemoto K and Polak JM (1983) Neuropeptide Y in the human brain. Nature 306: 584-586.

Adrian TE, Gu J, Allen JM, Tatemoto K, Polak JM and Bloom SR (1984) Neuropeptide Y in the human male genital tract. Life Sciences 35: 2643-2648.

Ainsworth A, Hall P, Wall PD, Allt GF, Lynn Mackenzie M, Gibson S and Polak JM (1981) Effects of capsaicin applied locally to adult peripheral nerve. II. Anatomy and enzyme and peptide chemistry of peripheral nerve and spinal cord. Pain 11: 379-388.

Allen JM, Polak JM, Rodrigo J, Darcy K and Bloom SR (1985) Localisation of neuropeptide Y in nerves of the rat cardiovascular system and the effect of 6-hydroxydopamine. Cardiovascular Res 19: 570-577.

Allen YS, Adrian TE, Allen JM, Tatemoto K, Crow TJ, Bloom SR and Polak JM (1983a) Neuropeptide Y distribution in the rat brain. Science 221: 877-879.

Allen JM, Bircham PMM, Edwards AV, Tatemoto K and Bloom SR (1983b) Neuropeptide Y (NPY) reduces myocardial perfusion and inhibits the force of contraction of the isolated perfused rabbit heart. Regulpeptides 6: 7-10.

Allen YS, Adrian TE, Tatemoto K, Polak JM, Hughes J and Bloom SR (1982) Two novel related peptides, neuropeptide Y (NPY) and peptide YY (PYY) inhibit the contraction of the electrically stimulated mouse vas deferens. Neuropeptides 3: 71-77.

Alm P, Aluments J, Hankanson R and Sundler F (1977) Peptidergic (vasoactive intestinal peptide) nerves in the genitourinary tract. Neurosci 2: 751-754.

Amara SG, Evans RM and Rosenfeld MG (1984) Calcitonin/Calcitonin gene-related peptide transcription unit: tissue specific expression involves selective use of alternative polyadenylation sites. Mol Cell Biol 4: 2151-2160.

Amara SG, Arriza JL, Leff SE, Swanson LW, Evans RM and Rosenfeld MG (1985) Expression in brain of a messenger RNA encoding a novel neuropeptide homologous to calcitonin gene-related peptide. Science 229: 1094-1097.

Ambache N and Zar MA (1971) Evidence against adrenergic motor transmission in the guinea-pig vas deferens. J Physiol (London) 216: 359-389.

Anand P and Bloom SR (1984) Neuropeptides are selective markers of spinal cord autonomic pathways. Trends Neurosci 7: 267-268.

Anderton B, Coakham HB, Garson JA, Harper AA, Harper EI and Lawson SN (1983) A monoclonal antibody against neurofilament protein specifically labels in large light cell population in rat dorsal root ganglia. J Physiol (Lond) 334: 97-98.

Andrews PC, Brayton K and Dixon JE (1987) Precursors to regulatory peptides: their proteolytic processing. Experientia 43: 784-790.

Aravidsson U, Cullheim S, Ulfhake B, Hökfelt T and Ternius L (1989) Altered levels of calcitonin gene-related peptide (CGRP)-like immunoreactivity of cat lumbar motoneurons after chronic spinal cord transaction. Brain Res 489: 387-391.

Aronin N, Leeman SE and Clements RS jr (1987) Diminished flare response in neuropathic diabetic patients: comparison of effects of substance P, histamine and capsaicin. Diabetes 36: 1139-1143.

Arthur RP and Shelley WB (1959) The innervation of human epidermis. J Invest Dermatol 32: 397-441.

Astvatsaturov MI (1934) Über parestehtische Neuralgien und eine besondere Form derselben - Notalgia paresthetica. Deutsche Ztschr. f. Nervenh 133: 188-196.

Atkinson MJ and Hosking DJ (1983) Gastrointestinal complications of diabetes mellitus. Clin Gastroenterol 12: 633-660.

Backman SB and Henry JL (1983) Effects of substance P and thyrotrophin-releasing hormone on sympathetic preganglionic neurons in the upper thoracic intermediolateral nucleus of the cat. Can J Physiol Pharmacol 62: 248-251.

Baeker B, Yanaihara N and Forssmann WG (1983) VIP innervation of the tongue in vertebrates, Anat Embryol 167: 173-189

Ballesta J, Polak JM, Allen JM and Bloom SR (1984) The nerves of the juxtaglomerular apparatus of man and other animals contain the potent peptide NPY. Histochem 80: 483-485.

Basbaum AI and Field HL (1984) Endogenous pain control system: brain-stem spinal pathways and endorphin circuitry. Ann Rev Neurosci 7: 309-338.

Baumgarten HG, Holstein AF and Owman C (1970) Auerbach's plexus of mammals and man: electron microscopic identification of three different types of neuronal processes in myenteric ganglia of the large intestine from rhesus monkeys, guinea-pigs and man. Z Zellforsch 106: 376-397.

Beckett EB, Bourne GH and Montagna W (1956) Histology and cytochemistry of human skin. The distribution of cholinesterase in the finger of the embryo and the adult. J Physiol 134: 202-206.

Bedi TR, Kumar B and Kaur S, (1979) Histopathological study of clinically normal appearing skin in lepromatous leprosy. Lepr India 51: 78-80.

Behse F, Buchthal F, Carlsen F and Knappeis GG (1975) Unmyelinated fibres and Schwann cells of sural nerve in neuropathy. Brain 98: 492-510.

Belai A, Lincoln J, Crowe R and Burnstock G (1985) Enteric nerves in diabetic rats: Increase in vasoactive intestinal polypeptide but not substance P. Gastroenterol 89: 967-976.

Belai A and Bunstock G (1987) Selective damage of intrinsic calcitonin gene-related peptide-like immunoreactive enteric nerve fibres in streptozotocin-induced diabetic rats. Gastroenterol 92: 730-734.

Belai A, Lincoln J, Milner P and Burnstock G (1988) Progressive changes in adrenergic, serotonergic and peptidergic nerves in proximal colon of streptozotocin-diabetic rats. Gastroenterol 95: 1234-1241.

Berthold CH (1978) Morphology of normal peripheral axons. In Waxman SG (ed): "Physiology and Pathology of Axons." New York: Raven Press, pp 3-63.

Bessou P and Perl ER (1969) Response of cutaneous sensory units with unmyelinated fibres to noxious stimuli. J Neurophysio 32: 1025-1043.

Bishop AE, Polak JM, Bloom SR and Pearse AGE (1978) A new universal technique for the immunocytochemical localisation of peptidergic innervation. J Endocrinol 77: 25-26.

Bishop AE, Ferri GL, Probert L, Bloom SR and Polak JM (1982) Peptidergic nerves. In: Polak JM and Bloom SR (eds) Structure of the gut. Glaxo Group Research Limited (Ware, UK) pp 221-237.

Bishop AE, Carlei F, Lee V, Trojanowski J, Marangos PJ, Dahl O and Polak JM (1985) Combined immunostaining of neurofilaments, neuron specific enolase, GFAP and S100: A possible means for assessing the morphological and functional status of the enteric nervous system. Histochemistry 82: 93-97.

Bjorklund H, Hokfelt T, Goldstein M, Terenius L and Olson L (1985) Appearance of the noradrenergic markers tyrosine-hydroxylase and neuropeptide-Y in cholinergic nerves of the iris following sympathectomy. J Neurosci 5: 1633-1643.

Bjorklund H, Dalsgaard CJ, Jonsson CE and Hermansson A (1986) Sensory and autonomic innervation of non-hairy and human skin. An immunohistochemical study. Cell Tissue Res 243: 51-57.

Blix M (1884) Experimantelle Beitrage zur Losung der Frage Uber die specifiche Energie der Hautnerven. Z Biol 20: 141-56.

Bloom SR and Edwards AV (1980) Vasoactive intestinal peptide in relation to atropine resistant vasodilation in the submaxillary gland of the cat. J Physiol (Lond) 300: 41-53.

Bloom SR and Polak JM (1983) Regulatory peptides in the skin. Clin Exp Dermatol 8: 3-18.

Bloom SR, Till S, Sonksen P and Smith S (1984) Use of biothesiometer to measure individual vibration threshold and their variation in 519 non diabetic subjects. Br Med J 288: 1975-1993.

Blundell TL and Humbel RE (1980) Hormone families: Pancreatic hormones and homologous growth factors. Nature (Lond) 287: 781-786.

Bock E and Dissin J (1975) Demonstration of enolase activity connected to the brain specific protein 14-3-2. Scand J Immunol 4 (suppl 12): 31-36.

Boulton AJ, Armstrong WD, Scarpello JHB and Ward JD (1983) The natural history of painful diabetic neuropathy - a four year study. Postgrad Med J 59: 556-559.

Boulton AJM, Hardisty CA, Bets RP, Worth RC, Ward JD and Duckworth T (1983) Dynamic foot pressure and other studies as diagnostic and management aids in diabetic neuropathy. Diabetes care 6: 26-33.

Boyle A and Ramu G (1982) Assessment of cutaneous autonomic nerve functions in leprosy. Lepr India 54: 518-524.

Brain SD and Williams TJ (1985) Inflammatory oedema induced synergism between calcitonin gene-related peptide (CGRP) and mediators of increased vascular permeability. Br J Pharmacol 86: 855-860.

Brain SD, Williams TJ, Tippins JR, Murrow HR and MacIntyre I (1985) Calcitonin gene-related peptide is a potent vasodilator. Nature 313: 54-56.

Brown MJ, Martin JR and Asbury AK (1976) Painful diabetic neuropathy a morphometric study. Arch Neurol 33: 164-171.

Bruun A, Ehinger B, Sundler F, Tornquist K and Uddman R (1984) Neuropeptide Y-immunoreactive neurons in the guinea pig uvea and retina. Invest Ophthalmol Vis Sci 25: 1113-1123.

Bryant MG, Polak JM, Modin I, Bloom SR, Albuquerque RH and Pearse AGE (1976) Possible dual role for vasoactive intestinal peptide as gastrointestinal hormone and neurotranamitter substance. Lancet I: 991-993.

Burgess PR and Perl ER (1967) Myelinated afferent fibres responding specifically to noxious stimulation of the skin. J Physio Lond 190: 541-562.

Burn JH and Rand MJ (1965) Acetylcholine in adrenergic transmission. Ann Rev Pharmac 5: 163-182.

Burnstock G (1977) Autonomic neuroeffector junctions-reflex vasodilation of the skin. J Invest Dermatol 69: 47-57.

Burnstock G (1978) Do some sympathetic neurones release both nor-adrenaline and acetylcholine ? Prog. Neurobiol 11: 205-222.

Burnstock G, Hökfelt T, Gershon MD, Iversen LL, Kosterlitz HW and Szurszewski JH (1979) Non-adrenergic, non-cholinergic autonomic neurotransmission mechanisms. Neurosci Res Prog Bull 17: 377-519.

Cadiex A, Springall DR, Mulderry PK, Rodrigo J, Ghatei MA, Terenghi G, Bloom SR and Polak JM (1986) Occurence, distribution and ontogeny of CGRP immunoreactivity in the rat lower respiratory tract: effect of capsaicin treatment and surgical denervations. Neuroscience

19: 605-627.

Carson KA, Sar M and Hanker SJ (1982) Immunocytochemical demonstration of nerve growth factor and histofluorescence of catecholaminergic nerves in with salivary glands of diabetic mice. Histochem J 14: 35-48.

Cauna N (1973) The free penicillate nerve endings of the human hairy skin. J Anat (London) 115: 277-288

Cauna N and Ross LL (1960) Fine structure of Meissner's touch corpuscles of human fingers. J Biophys Biochem Cytol 8: 467-482

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

Chang MM, Leeman SE and Niall HD (1971) Amino acid sequence of substance P. Nature New Biol 232: 86-87.

Chan-Palay V and Palay SL (eds.) (1984) "Coexistence of Neuroactive Substances in Neurons". John Wiley and Sons, New York.

Chopra JS, Hurwitz LJ and Montgomery DAD (1969) The pathogenesis of sural nerve changes in diabetes mellitus. Brain 92: 391-418.

Christofides ND, Yiangou Y, Blank MA, Tatemoto K, Polak JM and Bloom SR (1982) Are peptide histidine isoleucine and vasoactive intestinal polypeptide co-synthesised in the same prohormone? Lancet 11: 1398.

Cocchia D, Michetti F and Donato R (1981) Immunochemical and immunocytochemical localization of S-100 antigen in normal human skin. Nature (London) 294: 85-87.

Colston MJ and Hilson GRF (1976) Growth of Mycobacterium leprae and Mycobacterium marinum in congenitally athymic (nude) mice. Nature, London 262 (5767): 399-401.

Coming DE and Comings SN (1965) Heriditary localised pruritus. Arch. Dermatol 92: 236-237.

Consensus statement: report and recommendations of the San Antonio conference on diabetic neuropathy (1988) Diabetes Care 11: 592-597.

Convit J and Pinardi ME (1972) A simple method for differentiation of M. Leprae from other mycobacteria through routine staining techniques. Int J Lepr 40: 130-132.

Cook RD and Burnstock G (1976) The ultrastructure of Auerbach's plexus in the guinea pig. 1. Neural elements. J Neurocytol 5: 171-194.

Coons AH (1958) Fluorescent antibody methods. In: Danielli JF, (ed) General Cytochemical Methods. Academic Press, New York, pp 399-422.

Coons AH, Creech HJ and Jones RN (1941) Immunological properties of an antibody containing a fluorescent group. Proc Soc Biol Med 47: 200-202.

Coons AH, Leduc EH and Connolly JM (1955) Studies on antibody production. I. A method for the histochemical demonstration of specific antibody and its application to a study of the hyperimmune rabbit. J Exp Med 102: 49-60.

Costa M, Buffa R, Furness JB and Solcia E (1980) Immunohistochemical localization of polypeptides in peripheral autonomic nerves using whole mount preparations. Histochemistry 65: 157-165.

Coupland RE and Holmes RL (1957) The use of cholinesterase technique for the demonstration of peripheral nervous structures. Quart J Micro Sc 98:

Cowen T, Haven AJ and Burnstock G (1985) Pontamine sky blue: a counterstain for background autofluorescence in fluorescence and immunofluorescence histochemistry. Histochemistry 82: 205-208.

Cracco J, Casrells S and Mark E (1984) Spinal somatosensory evoked potentials in juvenile diabetes. Ann Neurol 15: 55-58

Craig KD (1980) Ontogeny and cultural influences on the expression of pain in man. In: Kosterlitz HW and Terenius LY (eds) Pain and Society. Verlagg Chemie GmbH, Weinheim PP 37-52.

Crawford CL, Evans DHL and Evans EM (1974) Experimental neuritis induced by sensory nerve myelin may provide a model for non-lepromatous leprosy. Nature 251: 223-225.

Crowe R, Lincoln J, Blacklong PF, Pryor JP, Lumley JSP and Burnstock G (1983) Vasoactive intestinal polypetide-like immmunoreactive nerves in diabetic penis: a comparison between streptozotocin-treated rats and man. Diabetes 32: 1075-1077.

Cuello AC (ed) (1982) "Co-transmission". MacMillan, London and Basingstoke.

Cummings JF, de Lahunta A, Simpson ST and McDonald JM (1984) Reduced substance P-like immunoreactivity in hereditory sensory neuropathy of pointer dogs. Acta Neuropathol 63: 33-40.

Cunningham FO and Fitzgerald MJT (1972) Encapsulated endings in

hairy skin. J Anat (London) 112: 93-97

Dabholkar VR and Gaitonde BB (1982) A study of autonomic functions in leprosy. Lepr India 54: 303-317.

Dahl D (1983) Immunohistochemical differeneces between neurofilaments in perikarya, dendrites and axons. Exp. Cell Res 149: 397-408.

Dahl D and Bignami A (1977) Preparation of antisera to neurofilament protein from chicken brain and human sciatic nerve. J Comp Neurol 176: 645-657.

Dahl D, Selkoe DJ, Pero RT and Bignami A (1982) Immunostaining of neurofibrillary tangles in Alzheimer's senile dementia with neurofilament antiserum. J Neurosci 2: 113-119.

Dale HH (1933) Nomenclature of fibres in the autonomic system and their effects. J Physiol 80: 10-11.

Dale HH and Feldberg (1934) The chemical transmission of secretory impulses to the sweat glands of the cat. J Physiol (Lond) 82: 121-128.

Dalsgaard CJ, Jonsson CE, Hökfelt T and Cuello AC (1983) Localisation of substance P immunoreactive nerve fibres in human digital skin. Experientia 39: 1018-1020.

Dalsgaard CJ, Bjorklund H, Jonsson CE, Hermansson A and Dahl D (1984) Distribution of neurofilament-IR nerve fibres in human digital skin. Histochemistry 81: 111-114.

Dalsgaard CJ, Haegerstrand A, Theodorsson-Norheim E, Brodin E and Hokfelt T (1985) Neurokinin A-like immunoreactivity in rat primary sensory neurons: coexistence with substance P. Histochemistry 83: 37-39.

Dalsgaard CJ, Stains W, Bjorklund H, Haegaard A and Hultgardh Nilsson A (1987) Sensory transmitter candidates and their role in periphery. Exp Brain Res Ser 16: 39-42.

Dalsgaard CJ, Rydh M and Haegerstrand A (1989) Cutaneous innervation in man visualised with protein gene-product (PGP 9.5) antibodies. Histochemistry 92: 385-389.

Dastur DK (1955) Cutaneous nerves in leprosy. The relationship between histopathology and cutaneous sensibility. Brain 78: 615-633.

Dastur DK and Razzak ZA (1971) Degeneration and regeneration in teased nerve fibres. 1. Leprous neuritis. Acta Neuropath 18: 286-298.

Dastur DK, Ramamohan Y and Shah JS (1972) Ultrastructure of nerves in tuberculoid leprosy. Neurol India Proc Suppl 1: 89-99.

Dastur DK, Ramamohan Y and Shah JS (1973) Ultrastructure of lepromatous nerves; neural pathogenesis in leprosy. Int J Lpr 41: 47-80.

Davies AM, Bandtlow C, Heumann R, Korching S, Rohrer H and Thoenen H (1987) Timing and site of nerve growth factor synthesis in developing skin in relation to innervation and expression of the receptor. Nature 326: 353-358.

Davis L (1934) The return of sensation to transplanted skin. Surg Gynec & Obst 59: 533-543.

Davis JS and Kitlowski EA (1934) Regeneration of nerves in skin grafts and skin flaps. Am J Surg 24: 501-510.

Dawbarn D, Gregory J and Emson PC (1985) Visualization of calcitonin gene-related peptide receptors in the rat brain. Eur J Pharmac 111: 407-408.

Day INM and Thompson RJ (1987) Molecular cloning of cDNA coding for human PGP 9.5 protien: A novel cytoplasmic marker for neurones and neuroendocrine cells. FEBS Lett 210:157-160.

De Camilli P, Vitadello M, Canevini MP, Zanoni R, Jahn R and Gorio A (1988) The Synaptic vesicle proteins synapsin I and synaptophysin (protein P 38) are concentrated both in efferent and afferent nerve endings of the skeletal muscle. J Neurosci 8: 1625-1631.

Dennis-Donini S (1989) Expression of dopaminergic phenotypes in the mouse olfactory bulb induced by the calcitonin gene-related peptide. Nature 339: 701-703.

Diamond RL, and Montagna W (1976) Histology and cytochemistry of human skin. The nose and lips. Arch. Dermatol 112: 1235-1244.

Di Figlia M, Aronin N and Leeman SE (1982) Light microscopic and ultrastructural localization of immunoreactive substance P in the dorsal horn of the monkey spinal cord. Neuroscience 7: 1127-1139.

Doran JF, Jackson PJ, Kynoch PAM and Thompson RJ (1983) Isolation of PGP 9.5, a new human neuron-specific protein detected by high resolution two dimensional electrophoresis. J Neurochem 40: 1542-1547.

Douglas WW and Ritchie JM (1960) The excitatory action of acetylcholine on cutaneous non-myelinated fibres. J Physiol 150: 501-504.

Dubner R and Bennett GJ (1983) Spinal and trigeminal mechanisms of nociception. Ann Rev Neurosci 6: 381-418.

Dyck PJ, Karnes JL, Daube J, O'Brein P and Service FJ (1985) Clinical

and neurotrophic criteria for the diagnosis and staging of diabetic polyneuropathy. Brain 108: 806-880.

Dyck PJ, Karnes J and O'Brien PC (1987) Diagnosis, staging and classification of diabetic neuropathy and associations with other complications. In: Dyck, Thomas, Ashbury, Winegrad, Porte (eds) Diabetic neuropathy. Saunders Philadelphia, pp 37-44.

Eckenstein F and Thoenen H (1983) Cholinergic neurons in the rat cerebral cortex demonstrated by immunohistochemical localization of choline acetyltransferase. Neurosci Lett 36: 211-215.

Edvinsson L, McCulloch J, Rosell S and Uddman R (1982) Antagonism by (D-Pro^2, D-Trp ^{7,9})-substance P of the cerebrovascular dilatation induced by substance P. Acta Physiol Scand 116: 411-416.

Ekbald E, Hakanson R and Sundler F (1984a) VIP and PHI coexist with an NPY-like peptide in intramural neurons of the small intestine. Regul Pept 10: 47-55.

Ekbald E, Edvinsson L, Wahlestedt C, Uddman R, Hakanson R and Sundler F (1984b) Neuropeptide Y co-exists and co-operated with noradrenaline in perivascular nerve fibres. Reg Peptides 8: 215-235.

Emson PC, Gilbert RFT, Lorén I, Fahrenkrug J, Sundler F and Schaffalitzky de Muckadell OB (1979) Development of vasoactive intestinal polypeptide (VIP) containing neurones in the rat brain. Brain Res 177: 437-444.

Erenko O (1955) Distribution of fluorescing islets adrenalin and noradrenalin in the adrenal meduall of the hamster. Acta endocrinol (Kbh) 18: 174-179.

Ewing DJ and Clarke BF (1982) Diagnosis and management of diabetic autonomic neuropathy. Br Med J 285: 916-918.

Ewing DJ, Cambell IW and Clarke BF (1980) Assesment of cardiovascular effects in diabetic autonomic neuropathy and prognostic implications. Ann Intern Med 92: 308-311.

Faccini E, Uzumaki H, Giovoni S, Missale C, Spano PF, Covelli V and Trabukcchi M (1984) Afferent fibres mediate the increase of met enkephalin elicited in rat spinal cord by localised pain. Pain 18: 25-31.

Faerman I, Faccio E, Calb I, Razumny J, Franco N, Dominguez A and Podesta HA (1982) Autonomic neuropathy in the skin: a histological study of the sympathetic nerve fibres in diabetic anhydrosis. Diabetologia 22: 244-250.

Fagerberg SE (1959) Diabetic Neuropathy. A clinical and histological study on the significance of vascular lesions. Acta Med Scand 164: 5-8.

Fagius J and Wahren LK (1981) Variability of sensory threshold determination in clinical use. J Neurol Sci 51: 11-27.

Falck B and Rorsman H (1963) Observations on the adrenergic innervation of the skin. Experientia 19: 205.

Flack B, Hillarp NA, Thieme G and Torp A (1962) Fluorescence of catecholamines and related compounds condensed with formaldehyde. J Histochem Cytochem 10: 348-54.

Faraco J (1938) Bacillon de Hansen e costes de paraffina. Methodo complementar para a pesquiza de bacillos de hansen em costed de material incluido em paraffina. Rev Brasil de Leprol 6: 177-180.

Feher E, Burnstock G, Varndell IM and Polak JM (1986) Calcitonin gene-related peptide-immunoreactive nerve fibres in the small intestine of the guinea pig: Electron microscopic immunocytochemistry. Cell tiss res 245: 353-358.

Feldman M and Schiller LR (1983) Disorders of gastrointestinal mobility associated with diabetes mellitus. Ann Intern Med 98: 378-384.

Ferri GL, Ali-Rachedi A, Tatemoto K, Bloom SR and Polak JM (1984) Immunocytochemical localization of neuropeptide Y-like immunoreactivity in extrinsic nor-adrenergic and intrinsic gut neurons. Front Horm Res 12: 81-84.

Fine PEM, Job CK, McDougall AC, Meyers WM and Ponnighaus JM (1986) Comparability among histopathologists in the diagnosis and classification of lesions suspected of leprosy in Malawi. Int J Lepr 54: 614-625.

Fishman HC (1986) Notalgia paresthetica. J Amer Acad Dermatol 15: 1304-1305.

Fite GL, Cambre PJ and Turner MH (1967) Procedure for demonstrating lepra bacilli in paraffin sections. Arch Path 43: 624-625.

Fithian E, Kung P, Goldstein G, Rubenfeld M, Fenoglio C and Edelson R (1981) Reactivity of Langerhans cells with hybridomo antiboby. Proc Natl Acad Sci USA 78: 2541-2544.

Fitzgerald GG and Cooper JR (1971) Actylcholine as a possible sensory mediator in rabbit corneal epithelium. Biochem Pharmacol 20: 2741-2748.

Fitzgerald M (1983) Capsaicin and sensory neurones a review. Pain 15: 109-130.

Fitzgerald M (1985) The postnatal development of cutaneous afferent fibre input and receptive field organization in the rat dorsal horn. J Physiol 364: 1-18.

Fitzgerald M and Gibson SJ (1984) The postnatal physiological and neurochemical development of peripheral sensory C fibres. Neuroscience 13: 933-944.

Fitzgerald MJT (1964) Double impregnation silver technique for nerve fibres in paraffin sections. Q J Microsc Sci 105: 359-361.

Fitzgerald MJT, Martin F and Patella FX (1967) Innervation of skin grafts. Surg Gynecol Obstet 124: 808-812.

Fontaine B, Klarsfeld A, Hökfelt T and Changeux JP (1986) Calcitonin gene-related peptide, a peptide present in spinal cord motoneurones, increases the number of acetylcholine receptors in primary cultures of chick embryo myotubes. Neurosci Lett 71: 59-65.

Foreman JC (1984) Peptides and histamine release. J Allergy Clin Immunol 74: 127-131.

Foreman JC (1987) Peptides and neurogenic inflammation. Br Med Bull 43: 386-400.

Franke WW, Grund C and Achstatter T (1986) Co-expression of cytokeratins and neurofilament proteins in a permanent cell line: Cultured rat PC 12 cells combine neuronal and epithelial features. J cell Biol 103: 1933-1943.

von Frey M (1896) Untersuchungen über die Sinnesfunctionen der menschlichen Haut: Druckempfindung und Schmerz. Abhandl sachs Ges Wiss math-phys Cl 23: 175-266.

von Frey M (1906) The distribution of afferent nerves in the skin. J Am med Ass 47: 645-648.

Friede RL and Samorajski T (1970) Axon caliber related to neurofilaments and microtubules in sciatic nerve fibres of rats and mice. Anat Rec 167: 379-388.

Fuller RW, Conradson TB, Dixon CMS, Crossman DC and Barnes PJ (1987) Sensory neuropeptides effects in human skin. Br J Pharmacol 92: 781-788.

Furness JB and Costa M (1973) The nervous release and the action of substances which affect intestinal muscle through neither adrenoceptors nor cholinoceptors. Recent developments in vertebrate smooth muscle physiology. Phil Trans R Soc 265: 123-133.

Furness JB, Papka RE, Della NG, Costa M and Eskay RL (1982) Substance P-like immunoreactivity in nerves associated with the vascular system of guinea pigs. Neuroscience 7: 447-459.

Furness JB, Costa M and Eckenstein F (1984a) Neurons localised with

acetyltransferase in the enteric nervous system. Neurosci Lett 40: 105-109.

Furness JB, Costa M and Keast JR (1984b) Choline acetyltransferase and peptide immunoreactivity of submucous neurons in the small intestine of the guinea pig. Cell Tiss Res 237: 328-336.

Fuxe K and Nilson BY (1965) Mechanoreceptors and adrenergic nerve terminal. Experientia 21: 641

Fuxe K, Hökfelt T, Said SI and Mutt V (1977) Vasoactive intestinal polypeptide and the nervous system: Immunohisochemical evidence for localization in central and peripheral neurons, particularly intracortical neurons of the cerebral cortex. Neurosci Lett 5: 241-246.

Gabella G (1979) Innervation of the gastrointestinal tract. Int Rev Cytol 59: 129-193.

Gamse R, Lembeck F and Cuello AC (1979) Substance P in the vagus nerve; immunochemical and immunohistochemical evidence for axoplasmic transport. Arch Phrmacol 306: 37-44.

Gamse R, Holzer P and Lembeck F (1980) Decrease of subsance P in primary afferent neurones and impairment of neurogenic plasma extravasation by capsaicin. Br J Pharmacol 68: 207-213.

Gamse R, Petsche U, Lembeck F and Jancso G (1982) Capsaicin applied to peripheral nerve inhibits axoplasmic transport of substance P and somatostatin. Brain Res 239: 447-462.

Gamse R and Saria A (1985) Potentiation of tachykinin induced plasma protein extravasation by calcitonin gene-related peptide. Eur J Pharmacol 114: 61-66.

Ganapathi R, Desikan KV and Iyer GGS (1972) Study of apparently normal skin in leprosy. Int J Lepr 40: 281-290.

Gibbins IL and Morris JL (1988) Coexistence of immunoreactivity to neuropeptide Y Vasoactive intestinal peptide in non-noradrenergic axons innervating guinea pig cerebral arteries after sympathectomy. Brain Research 444: 402-406.

Gibbins IL, Furness JB, Costa M, MacIntyre I, Hillyard CJ and Girgis S (1985) Colocalisation of CGRP immunoreactivity with Substance P in cutaneous, vascular and visceral sensory neurons of guinea pigs. Neurosci Lett 57: 125-130.

Gibbins IL, Furness JB and Costa M (1987a) Pathway -specific patterns of the co-existence of substance P, calcitonin gene-related peptide, cholecystochynin and dynorphin in neurons of the dorsal root ganglia of the guinea pig. Cell Tis Res 248: 417-437.

Gibbins IL, Wattchow D and Coventry B (1987b) Two immunohistochemically identified populations of calcitonin gene-related peptide (CGRP)-immunoreactive axons in human skin. Brain Res 414: 143-148.

Gibbs RC and Frank S (1969) A peculiar spotty pigmentation, report of five cases. Int J Dermatol 8: 14-16.

Gibson SJ, McGregor GP, Bloom SR, Polak JM and Wall PD (1982) Local application of capsaicin to one sciatic nerve of the adult rat induces a marked depletion in the peptide content of the lumbar dorsal horn. Neuroscience 7: 3153-3162.

Gibson SJ, Polak JM, Bloom SR, Sabate IM, Mulderry PM, Ghatei MA, McGregor GP, Morrison JFB, Kelly JS, Evans RM and Rosenfeld MG (1984) Calcitonin gene-related peptide immunoreactivity in the spinal cord of man and eight other species. J Neurosci 4: 3101-3111.

Gibson SJ and Polak JM (1986) Neurochemistry of the spinal cord. In: Polak JM, Van Noorden S (eds). Immunocytochemistry: Modern Methods and Applications (2nd ed) John Wright and Sons, Bristol, pp 360-390.

Gibson SJ, Polak JM, Katagiri T, Su H, Weller RO, Brownell DB, Holland S, Hughes JT, Kikuyama S, Ball J, Bloom SR, Steiner TJ, de Belleroche J and Clifford Rose F (1988a) A comparison of the distributions of eight peptides in spinal cord from normal controls and cases of motor neurone disease with special reference to Onuf's nucleus. Brain Res 474: 255-278.

Gibson SJ, Polak JM, Giaid A, Hamid QA, Kar S, Jones PM, Denny P, Legon S, Amara SG, Craig RK, Bloom SR, Penketh RJA, Rodek C, Ibrahim NBN and Dawson A (1988b) Calcitonin gene-related peptide messenger RNA is expressed in sensory neurons of the dorsal root ganglia and also in spinal motoneurons in man and rat. Neurosci Lett 91: 283-288.

Girod C and Durand N (1985) Immunocytochemical localization of S-100 protein in intestinal cells of the monkey *Macaca irus* pineal gland. Neurosci Lett 57: 181-184

Goldscheider A (1884) Die specifische Energie der Temperaturnerven. Mh prakt Derm 3: 198-208.

Goldstein ME, Sternberger LA and Sternberger NH (1983) Microheterogeneity ("neurotypy") of neurofilament proteins. Proc Natl Acad Sci USA 80: 3101-3105

Goltzman D and Mitchell J (1985) Interaction of calcitonin gene-related peptide at receptor sites in target tissues. Science 227: 1343-1345.

Gomori G (1952) Microscopic Histochemistry. Principles and practice.

University of Chicago press, Chicago pp 273.

Goodman J (1966) Diabetic anhydrosis. Am J Med 41: 831-835.

Goodman EC and Iversen EC (1986) Calcitonin gene-related peptide: a novel peptide. Life Sci 38: 2169-2178.

Goodwin CS (1968) The use of the voluntary test in leprosy neuritis. Lepr Rev 39: 209-220.

Gorio A (1986) Ganglioside enhancement of neuronal differentiation, plasticity and repair. CRC Cr Rev Clin Neurobiol 2: 241-296.

Gould VE, Wiedenmann B, Lee I, Schwechheirmer K, Dockhorn-Dworniczak B, Radosevich JA, Moll R and Franke WW (1987) Synaptophysin expression in neuroendocrine neoplasms as determined by immunocytochemistry. Am J Pathol 126: 243-247.

Graham RC and Karnovsky MJ (1966) The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: ultrastructural cytochemistry by a new technique J Histochem Cytochem 14: 291-302.

Grant RT, Bayliss OB and Adams CW (1967) Cholinesterases in peripheral nervous system. II The motor, sensory and sympathetic nerves in the rabbit ear perichondrium and cat cremaster muscle. Brain Res 6: 457-474.

Greene DA, Lattimer SA and Sima AAF (1987) Sorbitol phosphorinositides and sodium potassium ATPase in the pathogenesis of diabetic complications. N Engl J Med 316: 599-606.

Greene DA and Lattimer SA (1984) Action of Sorbinil in diabetic peripheral nerve. Relationship of polyol (sorbitol) pathway inhibition to a myo-inositol mediated defect in sodium-potassium ATPase activity. Diabetes 33: 712-716.

Gregersen G (1968) Variations in motor conduction velocity produced by acute changes of the metabolic state in diabetic patients. Diabetolgia 4: 273-277.

Gregersen G (1968) Vibratory perception threshold and motor conduction velocity in diabetes and non-diabetics. Acta Med Scand 183: 61-65.

Grunditz T, Ekman R, Hakenson R, Sundler F and Uddman R (1988) Neuropeptide Y and vasoactive intestinal peptide coexist in rat thyroid nerve fibres emanating from the thyroid ganglion. Regul Pept 23: 193-208.

Gu J, Islam KK and Polak JM (1983a) Repeated application of first layer antiserum improves immunostaining, a modification of indirect

immunofluorescence procedure. Histochem J 15: 475-483.

Gu J, Polak JM, Adrian TE, Allen JM, Tatemoto K and Bloom SR (1983b) Neuropeptide tyrosine (NPY)- a major cardiac neuropeptide. Lancet 1: 1008-1010.

Gu J, Lazarides M, Pryor JP, Blank MA, Polak JM, Morgan R, Marangos PJ and Bloom SR (1984) Decrease of vasoactive intestinal polypeptide (VIP) in the penises from impotent men. Lancet II: 315-317.

Gu J, Polak JM, Allen JM, Huang WM, Sheppard MN, Tatemoto K and Bloom SR (1984b) High concentrations of a novel neuropeptide Y, in the innervation of mouse and rat heart. J Histochem Cytochem 32: 1237-1243.

Gulbenkian S, Wharton J and Polak JM (1987) The visualisation of cardiovascular innervation in the guinea pig using an antiserum to protein gene product 9.5 (PGP 9.5). J Auton Nerv Syst 18: 235-247.

Guy RJC, Clark PN and Watkins PJ (1985) Evaluation of thermal and vibration sensation in diabetic neuopathy. Diabetologia 131-137.

Haegerstam G, Olgart L and Edwall L (1975) The excitatory action of acetylcholine on intra dental sensory units. Acta Physiol Scand 93: 113-118.

Hagermark O, Hökfelt T and Pernow B (1978) Flare and itch induced by substance P in human skin. J Invest Dermatol 71: 233-235.

Haimoto H, Kato K, Sujuki F and Nagura H (1985) The ultrasturctural changes of S-100 protein localization during lipolysis in adipocytes. An immunoelectron-microscopic study. Am J Pathol 121: 185-189.

Halata Z (1975) The mechanoreceptors of the mammalian skin. Ultrastructure and morphological classification. Advances in Anatomy, 50: 1-77.

Hallberg D and Pernow B (1975) Effect of substance P on various vascular beds in dog. Acta Physiol Scand 93: 277-285.

Hallin RH, Torebjork HE and Wiesenfeld Z (1982) Nociceptors and warm receptors innervation by C fibres in human skin. J Neurol Neurosurg Psychiatry 45: 313-319.

Harmann PA, Chung K, Briner RP, Westlund KN and Carlton SM (1988). Calcitonin gene-related peptide (CGRP) in human spinal cord: a light and electron microscopic analysis. J Comp Neurol 269: 371-380.

Hartman BK (1973) Immunofluorescence of dopamine-ß-hydroxylase. Application of improved methodology to the localization of the

peripheral and central noradrenergic nervous system. J Histochem Cytochem 21: 312-317.

Hartman BK, Zide D and Adenfriend SA (1972) The use of dopamine-ß-hydroxylase as a marker for the central noradrenergic nervous system in the rat brain. Proc Natl Acad Sci USA 69: 2722-2726.

Hartschuh W, Reinecke M, Weihe E and Yanaihara N (1984) VIP-immunoreactivity in the skin of various mammals: Immunohistochemical, radioimmunological and experimental evidence for a dual localization in cutaneous nerves and merkel cells. Peptides 5: 239-245.

Hauser CR, Crawford GD, Salvaterra PM and Vaughn JE (1985) Immunocytochemical localization of choline acetyltransferase in rat cerabral cortex: a study of cholinergic neurons and synapses. J Comp Neurol 234: 17-34.

Head H (1905) The afferent nervous system from a new aspect. Brain 28: 99-116.

Head H (1921) Release of function in the nervous system. Proc R Soc B 92 184-208.

Head H and Campbell AW (1900) The pathology of herpes zoster and its bearing on sensory localization. Brain 23: 353-523.

Head H, Rivers WHR and Sherren J (1905) The afferent nervous system from a new aspect. Brain 28: 99-105.

Helm G, Ottensen B, Fahrenkrug J, Larsen JJ, Owman CH, Sjoberg NO, Stolberg B, Sundler F and Walles B (1981) Vasoactive intestinal polypeptide (VIP) in the human female reproductive tract: distribution and motor effect. Biol Reprod 25: 227-234.

Henken DB, Tessler A, Chesselet MF, Hudson A, Baldino Jr F and Murray M. (1988) *In situ* hybridizationof mRNA for B-preprotachykinin and preprosomatostatin in adult rat dorsal root ganglia: comparison with immunocytochemical localization. J Neurocytol, 1988; **17:** 671-781

Henry JL (1982) Relation of substance P to pain transmission: neurophysiological evidence: In: Porter R, Conner MO (eds) Substance P in the nervous system. CIBA Foundation Symposium. Vol.91. Pitman. London, pp 206-224.

Heimans JJ, Bertelsmann FW and Van Rooy JCGM (1986) Large and small nerve fibre function in painful diabetic neuropathy. J Neurol Sci 74: 1-9.

Hill CE and Hendry IA (1977) Development of neurones synthesizing noradrenaline and acetylcholine in the superior cervical ganglion of

the rat in vivo and in vitro. Neuroscience 2: 741-749.

Hoffman PN and Lasek R (1975) The slow component of axonal transport. Identification of major structural polypeptides of the axon and their generality among mammalian neurons. J Cell Biol 66: 351-366

Hoffman PN, Griffin JW and Price DL (1984a) Control of axonal caliber by neurofilament transport. J cell Biol 99: 705-714.

Hoffman PN, Griffin JW and Price DL (1984b) Neurofilament transport in axonal regeneration: Implications for the control of axonal caliber. In Elam JS, Cancalon P (eds) "Advances in Neurochemistry, Axonal Transport in Neuronal Growth and Regeneration". Plenum Press. New York, pp 243-260.

Hoffman PN, Thompson GW, Griffin JW and Price DL (1985) Changes in neurofilament transport coincide temporally with alterations in the caliber of axons in regenerating motor fibres. J Cell Biol 101: 1332-1340.

Hökfelt T, Kellerth JO, Nilsson G and Pernow B (1975a) Experimental immunohistochemical studies on the localization and distribution of substance P in cat primary sensory neurons. Brain Res 100: 235-252.

Hökfelt T, Kellerth JO, Nilsson G and Pernow B (1975b) Substance P: localisation in the central nervous system and in some primary sensory neurons. Science 190: 889-890.

Hökfelt T, Johansson O, Kellerth JO, Ljungdahl A, Nilsson G, Nygards A and Pernow B (1977a) Immunohistochemical distribution of substance P. In: Von Euler US and Pernow B (eds) Substance P, Nobel Symposium 37. Raven Press, New York, pp 117-145.

Hökfelt T, Ljungdahl A, Terenius JW and Price DL (1977b) Immunohistochemical analysis of peptide pathways possibly related to pain and analgesia: enkephalin and substance P. Proc Natn Acad Sci USA 74: 3081-3085.

Hökfelt T, Johansson O, Ljungdhal A, Lundberg JM and Schultzberg M (1980a) Peptidergic neurons. Nature (Lond) 284: 515-521.

Hökfelt T, Lundberg JM, Schultzberg M, Johansson O, Skirboll L, Anggard A, Fredholm B, Hamberger B, Pernow B, Rehfeld J and Goldstein M (1980b) Cellular localization of peptides in neural structures. Proc Roy Soc Lond B 210: 63-77.

Hökfelt T, Schultzberg M, Lundberg JM, Fuxe K, Mutt V, Fahrenkrug J and Said SI (1982) Distribution of vasoactive polypeptide in the central and peripheral nervous system as revealed by immunocytochemistry. In: Said SI (ed) Vasoactive intestinal peptide. Raven press, New york, pp 65-90.

Hökfelt T, Millhorn D, Seroogy K, Tsuruo Y, Ceccatelli S, Lindh B,

Meister B, Melander T, Schalling M, Bartfai T and Terenius T (1987) Coexistence of peptides with classical neurotransmitters. Experientia 43: 768-780.

Holzer P (1988). Local effector functions of capsaicin-sensitive sensory nerve endings: Involvement of tachykinins, calcitonin gene-related peptide and other neuropeptides. Neuroscience. 24: 739-768.

Holzer P, Bucsics A and Lembeck F (1982) Distribution of capsaicin sensitive nerve fibres containing immunoreactive substance P in cutaneous and visceral tissues of the rat. Neurosci Lett 31: 253-257.

Huang WM, Gibson SJ, Facer P, Gu J and Polak JM (1983) Improved section adhesion for immunocytochemistry using high molecular weight polymers of L-lysine as a slide coating. Histochemistry 77: 275-279.

Hunt SP (1983) Cytochemistry of the spinal cord. In Emson PC (ed) Chemical Neuroanatomy. New York, Raven Press, pp 53-84.

Ichikawa T and Hirata Y (1986) Organization of choline acetyltransferase containing structures in the forebrain of the rat. J Neurosci 6: 282-292.

Iggo A (1977) Cutaneous and subcutaneous sense organs. Br med Bull 33: 97-102.

Iggo A and Muir AR (1969) The structure and function of a slowly adapting touch corpuscle in hairy skin. J Physiol 200: 763-796.

Isobe T and Okuyama T (1981) The amino acid sequence of the alpha subunit in bovine brain S-100a protein. Eur J Biochem 116: 79-86.

Isobe T, Tsugita A and Okuyama T (1978) The aminoacid sequence of the subunit structure of bovine brain S-100 protein (PAP I-b protein). J Neurochem 30: 921-923.

Isobe T, Ishioka N, Masuda T, Takahashi Y, Ganno S and Okuyama T (1983) A rapid separation of S-100 subunits by high performance liquid chromatography. Biochem Int 6: 419-426.

Itoh N, Obata K, Yanaihara N and Okamoto H (1983) Human preprovasoactive intestinal polypeptide contains a novel PHI-27-like peptide, PHM-27. Nature (Lond) 304: 547-549.

Iyer CGS (1965) Predilection of Mycobacterium leprae for nerves. Int J Lepr 33: 634-645.

Iyer CGS and Desikan KV (1968) Nerve involvement in leprosy; pathogenesis and significance. Neurol (India) 16: 89-92

Jackson P and Thompson RJ (1981) The demonstration of new human

brain-specific proteins by high resolution two dimensional polycrylamide gel electrophoresis. J Neurol Sci 49: 429-438.

Jahn R, Schiebler W, Ouimet C and Greengard P (1985) A 38000-dalton membrane protein (P 38) present in synaptic vesicles. Proc Natl Acad Sci 82: 4137-4141.

Jakobsen J and Sidenius P (1980) Decreased axonal transport of structural proteins in streptozotocin diabetic rats. J Clin Invest 66: 292-297.

Jakobsen J, Sidenius P and Braendgaard H (1986) A proposal for a classification of neuropathies according to their axonal transport abnormalities. J Neurol Neurosurg Psychol 49: 986-990.

Jancso G (1968) Desensitization with capsaicin and related acylamides as a tool for studying the function of pain receptors In: Armstrog D and Pardo EG (eds) Pharmacology of pain (RKS limited edition) Pergmon press, Oxford, pp 33-55

Jancso G, Kiraly E and Jansco-Gabor A (1977) Pharmacologically induced selective degeneration of chemosensitive primary sensory neurons. Nature 270: 741-743.

Jancso G, Kiraly E, Joo F, Such G, and Aniko Nagy (1985) Selective degeneration by capsaicin of a subpopulation of primary sensory neurons in the adult rat. Neurosci Lett 59: 209-214.

Jenkinson DM, Thomson GE, Kenny JD and Pearson JM (1970) Histochemical studies on mast cells in cattle skin. Histochem J 2: 419-424.

Jessell TM (1982) Fifty years of substance P. Nature (London) 295: 551-553.

Jessell TM (1983) Substance P in the nervous system. In: Iversen SD and Snyder SH (eds) Handbook of Psycopharmacology. Vol. 16. Plenum Press, New York and London, pp 1-106.

Jessell TM, Iversen LL and Cuello AC (1978) Capsaicin induced depletion of substance P from primary sensory neurons. Brain Res 152: 183-188.

Jhamandas K, Yaksh TL, Harty G, Szolcsanyi J and Go VLW (1984) Action of intrathecal capsaicin and its structural analogues in the content and release of spinal substance P; Selectivity of action and relationship to analgesia. Brain Res 306: 215-222.

Job CK (1971) Pathology of peripheral nerve lesions in lepromatous leprosy. Int J Lepr 39: 251-268.

Job CK and Desikan KV (1968) Pathological changes and their

distribution in peripheral nerves in lepromatous leprosy. Int J Lepr 36: 257-270.

Job CK and Verghese R (1975) Schwann cell changes in lepromatous leprosy. Indian J Med Res 63: 897-901

Johansson O (1986) A detailed account of NPY immunoreactive nerves and cells of human skin. Comparison with VIP, substance P and PHI containing structures. Acta Physiol Scand 128: 147-153.

Johansson O and Vaalasti A (1987) Immunohistochemical evidence for the presence of somatostatin-containing sensory nerve fibres in the human skin. Neurosci Lett 73: 225-230.

Johansson O, Vaalasti A, Tainio H and Ljungberg A (1988) Immunohistochemical evidence of galanin in sensory nerves of human digital skin. Acta Physiol Scand 132: 261-263.

Johnson PC and Doll SC (1984) Dermal nerves in human diabetic subjects. Diabetes 33: 244-250

Jorizzo JL, Coults AA, Eady RAJ and Greaves MW (1983) Vascular responses of human skin to injection of substance P and mechanism of action. Eur J Pharmacol 87: 67-76.

Ju G, Hökfelt T, Fischer JA, Frey P, Rehfeld JF and Dockray GJ (1986) Does cholecystokinin-like immunoreactivity in rat primary sensory neurons represent calcitonin gene-related peptide? Neurosci Lett 68: 305-310.

Ju G, Hökfelt T, Brodin E, Fahrenkrug J, Fischer JA, Frey P, Elde RP and Brown JC (1987) Primary sensory neurones of the rat showing CGRP-immunoreactivity and their relation to substance P-, somatostatin-, galanin-, vasoactive intestinal polypeptide-, and cholecystokinin-immunoreactive ganglion cells. Cell Tiss Res 247: 417-431.

Kabayashi H, Hashimoto K, Uchida S, Sakuma J, Takami K, Tohyama M, Izumi F and Yoshida H (1987) Calcitonin gene-related peptide stimulates adenylate cyclase activity in rat striated muscle. Experientia 43: 314-316.

Kanazawa T and Perl ER (1977) Primate cutaneous sensory units with unmyelinated (C) afferent fibres. J Neurophysiol 40: 1325-1338

Kar S, Gibson SJ and Polak JM (1989) Origins and projections of peptide immunoreactive nerves in the male genitofemoral nerve. Brain Res (in press)

Kar S, Gibson SJ, Jura WGZO, Rees RG, Brewerton DA and Polak JM (1989a) Chronic pain, inflammation and muscle dysfunction induce marked neuropeptide changes in the spinal cord. J Pathol; **158:** 356A

Kar S, Gibson SJ, Scaravilli F, Jacobs JM, Aber VR and Polak JM (1989b) Loss of calcitonin gene-related peptide (CGRP) and tachykinin immunoreactive sensory neurones is accompanied by an increase of enkephalin immunoreactivity in the dorsal horn of the mutilated foot rat. Cell Tis Res 255: 417-431.

Karnovsky MJ and Roots L (1964) A direct coloring thiocholine method for cholinesterase. J Histochem Cytochem 12: 219-221.

Kato K and Kimura S (1985) S-100AA protein is mainly located in the heart and striated muscles. Biochim Biophys Acta 842: 146-150

Kato T, Ito J, Ishikawa K, Mizutani K, Tanaka R, Wakabayashi S, Horiuchi I, Kato K and Kano-Tanaka K (1984) The absence of differentiation-promoting response of astroglioma cells to glia maturation factor. Brain Res 301: 83-89.

Kennady W, Sakuta M, Sutherland D and Goetz FC (1984) Quantitation of the sweating deficiency in diabetes mellitus. Ann Neurol 15: 482-488.

Kessler JA, Bell WO and Black IB (1983) Interactions between the sympathetic and sensory innervation of the iris. J Neurosci 3: 1301-1307.

Khanolkar VR (1952) Studies in the histology of early lesions in leprosy. Lepr India 24: 62-77.

Kiernan JA (1974) Effects of metabolic inhibitors on vital staining with methylene blue. Histochemistry 40: 51-57.

Kimura H, McGreer PL, Peng JH and McGeer EG (1981) The central cholinergic system studied by immunohistochemistry in the rat. J Comp Neurol 200: 151-158.

Kimura S, Okada M, Sugita Y, Kanazawa T and Munekata E (1983) Novel neuropeptides, neurokinin and isolated from porcine spinal cord. Proc Japan Acad 59B: 101-104.

Kishimoto S (1984) The regeneration of substance P containing nerve fibres in the process of burn wound healing in the guinea pig skin. J Invest Dermatol 83: 219-223.

Kjartansson J and Dalsgaard CJ (1987) Calcitonin gene-related peptide increases survival of a musculocutaneous critical flap in the rat. Eur J Pharmacol 142: 355-358.

Kjartansson J, Lundberg T, Samuelson UE, Dalsgaard CJ and Heden P (1988) Calcitonin gene-related peptide (CGRP) and transcutaneous electrical nerve stimulation (TENS) increase cutaneous blood flow in a musculocutaneous flap in the rat. Acta Physiol Scand 134: 89-94.

Klein RL, Lager Crantz H and Zimmerman H (eds) (1985) Neurotransmitter vesicles. Academic Press pp 1-384.

Koelle GB (1955) The histochemical identification of acetylcholinesterase in cholinergic, adrenergic and sensory neurons. J Pharmacol and Exper Therap 114: 167-184

Koelle GB (1962) A new general concept of the neurohumoral functions of acetylecholine and acetylecholinesterase. J Pharmacy Pharmacol 14: 65-90.

Koelle GB and Friedenwald JS (1949) A histochemical method for localizing cholinesterase activity. Proc Soc Exp Biol Med 70: 617-622.

Konturek SJ, Thor P, Dembinski A and Krol R (1975) Comparison of secretin and vasoactive intestinal peptide on pancreatic secretion in dogs. Gastroenterology 68: 1527-1535.

Kostrzewa RM and Jacobowitz DM (1974) Pharmacological actions of 6-hydroxydopamine. Pharmaco Rev 26: 199-288.

Kruger L, Sampogna SL, Rodin BE, Clague J, Brecha N and Yeh Y (1985) Thin fibre cutaneous innervation and its intraepidermal contribution studied by labelling methods and neurotoxin treatment in rats. Somatosens Res 2: 335-356.

Kumazawa T and Perl ER (1977) Primate cutaneous sensory units with unmyelinated (C) afferent fibres. J Neurophysiol 40: 1325-1338.

Kuraishi Y, Nanayama T, Ohno H, Fuji N, Otala A, Yajima H and Satoh M (1989) Calcitonin gene-related peptide increases in the dorsal root ganglia of adjuvant arthritic rat. Peptides 10: 447-452.

Landis SC and Fredieu JR (1986) Coexistence of calcitonin gene-related peptide and vasoactive intestinal peptide in cholinergic sympathetic innervation of rat sweat glands. Brain Res 377: 177-181.

Larsson L-I (1977) Ultrastructural localisation of a new neuronal peptide (VIP). Histochem 54: 174-176.

Larsson L-I (1981) Peptide Immunocytochemistry. Prog Histochem Cytochem 13: 1-85.

Larsson L-I (1981) A novel immunocytochemical model system for specificity and sensititvity screening of antisera against multiple antigens. J Histochem Cytochem 29: 408-410.

Larsson L-I (1983) Methods for immunocytochemistry of neurohormonal peptides. In: Bjorklund A and Hökfelt T (eds) Handbook of Chemical Neuroanatomy. Elsevier Biomedical Press, Amsterdam, New York and London pp. 147-209.

Larsson L-I, Fahrenkrug J, Schaffalitzky de Muchadell O, Sundler F, Hakanson R and Rehfeld JF (1976) Localization of vasoactive intestinal polypeptide (VIP) to central and peripheral neurons. Proc Natl Acad Sci USA 73: 3197-3200.

Larsson L-I, Fahrenkrug J and Schaffalitzky de Mushadell OB (1977) Vasoactive intestinal polypeptide occurs in nerves of the female genitourinary tract. Science 197: 1374-1375.

Laufer R and Changeux JP (1987) Calcitonin gene-related peptide elevates cyclic AMP levels in chick skeletal muscle: Possible neurotrophic role for a coexisting neuronal messenger. EMBO J 6: 901-906.

Lauweryns JM and Van Ranst L (1988) Protein gene product 9.5 expression in the lungs of human and other mammals; immunocytochemical detection in the neuroepithelial bodies, neuroendocrine cells and neurons. Neurosci Lett 85: 311-316.

Lawrence RD (1965) The diabetic life: a concise practical manual, 17th edn Churchill, London, P 157.

Lawrence DG and Locke S (1961) Motor nerve conduction velocity in diabetes. Arch Neurol (Chic) 5: 483-489.

Lawson SN and Harper AA (1985) Cell types in rat dorsal root ganglia: morphological immunohistochemical and electrophysiological analyses. In: Rowe M and Willis WD Jr. Development, Organization and Processing in Somatosensory Pathways. Alan R Liss Inc New York PP 97-103.

Lawson SN, Harper AA, Harper EI, Garson JA and Anderton BH (1984) A monoclonal antibody against neurofilament protein specifically labels a subpopulation of rat sensory neurones. J Comp Neurol 228: 263-272.

Leblanc G and Landis S (1986) Development of choline acetyltransferase (CAT) in the sympathetic innervation of rat sweat glands. J Neurosci 6: 260-265.

Lee TJF, Saito AS and Berezin I (1984) Vasoactive intestinal polypeptide like substance: the potential transmitter for cerebral vasodilation. Science 224: 898-901.

Lee Y, Kawai Y, Shiosaka S, Takami K, Kiyama H, Hillyard CJ, Girgis S, MacIntrye I, Emson PC and Tohyama M (1985a) Coexistence of calcitonin gene-related peptide and substance P-like peptide in single cells of the tringeminal ganglion of the rat: Immunohistochemical analysis. Brain Res 330: 194-196.

Lee Y, Takami K, Girgis S, Hillyard CJ, MacIntyre I, Emson PC and Tohyama M (1985b) Distribution of calcitonin gene-related peptide in

the rat peripheral nervous system with reference to its coexistence with substance P. Neurosci 15: 1227-1327.

Leela K, Kanagasuntheram R and Ahmed MM (1971) Innervation of the nasopharynx in macaca fascicularis. J Anat 110: 49-56.

Leeman SE and Gamse R (1981) Substance P in sensory neurones. Trends. Pharmacol 2: 119-121.

Leibsohn E, Hoeneke H and Mao P (1979) Puzzling posterior pigmented pruritus patches. Cutis 23: 71-73

Lembeck F (1953) Zur Frage der Zentralen Ubertragung affrener Impulse III. Mitteilung Das Vorkommen und die bedeutung der Substanz P in den dorsalen Wurzeln des Ruckenmarks. Naunyn-Schmiedebergs Arch Pharmacol 219: 197-213.

Lembeck F (1985) Substance P and sensory neurones. In: Jordon CC and Oehme P (eds) Substance p metabolism and biological actions. Taylor and Francis, London. pp 137-151.

Lembeck F and Gamse R (1982) Substance P in peripheral sensory processes. In: Porter R and O'Connor M (eds) Substance P in the Nervous system. Ciba Foundation Symposium, Vol 91. Pitman, London pp 35-54

Lembeck F and Holzer P (1979) Substance P as neurogenic mediator of antidromic vasodilation and neurogenic plasma extravasation. Arch Pharmacol 310: 175-183.

Lembeck F and Zetler G (1962) Substance p: a plypeptide of possible physiological significance especially within the nervous system. Int Rev Neurobiol 4: 159-215.

Letournean PC (1978) Chemotactic response of nerve fibre elongation to nerve growth factor. Developmental Biology 66: 183-196.

Levi-Montakini R and Calissano P (1979) The nerve growth factor. Sci Ameri 240: 44-53.

Levy DM, Abraham RR and Abraham RM (1987) Small- and large-fibre involvement in early diabetic neuropathy: a study with the medial plantar response and sensory thresholds. Diabetes care 10: 441-447.

Levy DM, Karanth SS, Springall DR and Polak JM (1989) Depletion of cutaneous nerves and neuropeptides in diabetes mellitus: an immunocytochemical study. Diabetologia 32: 427-433.

Lieberman AR (1976) Sensory ganglia. In: The peripheral Nerve. (ed) Landon DN. Chapman and Hill, London pp 188-278.

Light AR and Perl ER (1979) Re-examination of the dorsal root projection to the spinal dorsal horn including observations on the differential terminations of course and fine fibres. J Comp Neurol 186:133-150.

Lincoln J, Crockett M, Haven AJ and Burnstock G (1984) Rat bladder in early stages of streptozotocin-induced diabetes: adrenergic and cholinergic innervation. Diabetologia 26: 81-87.

Low PA, Caskey PE, Tuck RR, Fealey RD and Dyck PJ (1983) Quantitative sudomotor axon reflex test in normal and neuropathic subjects. Ann Neurol 14: 573-580.

Lumsden CE (1964) In: Cochrane RG and Davey TF (eds). Leprosy in theory and practice 2nd ed. Wright Bristol 205-220.

Lundberg JM (1981) Evidence for the existence of VIP and acetylcholine neurons in cat exocrine glands. Morphological, anatomical and functional studies. Acta Physiol Scand 126: 239-249.

Lundberg JM, Hökfelt T, Nilsson G, Terenius L, Rehfeld J, Elde R and Said S (1978) Peptide neurons in vagus, splanchnic and sciatic nerves. Acta Physiol Scand 104: 499-501.

Lundberg JM, Hökfelt T, Schultzberg M and Uvnas-Wallenstein K (1979) Occurance of VIP-like immunoreactivity in certain cholinergic neurons of the cat: Evidence from combined immunocytochemistry and acetylcholinesterase staining. Neuroscience 4: 1539-1559.

Lundberg JM, Anggard A, Fahrenkrug J, Hökfelt T and Mutt V (1980a) Vasoactive intestinal polypeptide in cholinergic neurons of exocrine glands. Functional significance of coexisting transmitter for vasodilatation and secretion. Proc Nat Acad Sci USA 77: 1651-1655.

Lundberg JM, Hökfelt T, Anggard A, Uvnas-Wallensten K, Brimijoin S, Brodin E and Fahrenkrug J (1980b) Peripheral peptide neurons: Distribution, axonal transport and some aspects on possible function. In: Costa E and Trabucchi M (eds) Neural Peptides and Neuronal Communication. Raven Press, New York, pp 25-36.

Lundberg JM, Hedlund B, Anggard A, Fahrenkrug J, Hökfelt T, Tatemoto K and Barfai T (1982a) Costorage of peptide and classical transmitters in neurons, Systemic Role of regulatory peptides: Symposia Medica Hoechst 18, Stuttgart York, Schattauer Verlag, pp 93-119

Lundberg JM, Terenius L, Hokfelt T, Marling CR, Tatemoto K, Mutt V, Polak J, Bloom SR, Goldstein M (1982b) Neuropeptide Y (NPY)-like immunoreactivity in peripheral noradrenergic neurons and effects of NPY on sympathetic functions. Acta Physiol Scand 116: 477-480.

Lundberg JM, Terenius L, Hokfelt T and Goldstein M (1983) High levels of neuropeptide Y (NPY) in peripheral noradrenergic neurons in various mammals including man. Neurosci Lett 42: 167-172.

Lundberg JM, Terenius L, Hökfelt T and Tatemoto K (1984a) Comparative immunohistochemical and biochemical analysis of pancreatic polypeptide-like peptides with special references to the presence of neuropeptide Y in central and peripheral neurons. J Neurosci 9: 2376-2386.

Lundberg JM, Hua X-Y and Franco-Cereceda A (1984b) Effects of neuropeptide Y (NPY) on mechanical activity and neurotransmission in the heart, vas deferens and urinary bladder of the guinea pig. Acta Physiol Scand 121; 325-332.

Lundberg JM, Franco-Cereceda AF, Hua X, Hokfelt T and Fischer JA (1985) Co-existence of substance P and calcitonin gene-related peptide-like immunoreactivities in sensory nerves in relation to cardiovascular and bronchoconstrictor effects of capsaicin. Eur. J Pharmacol 108: 315-319.

Lundberg LM, Alm P, Wharton J and Polak JM (1988) Protein gene product 9.5 (PGP 9.5). A new neuronal marker visualizing the whole uterine innervation and pregnancy-induced and developmental changes in the guinea pig. Histochemistry 90: 9-17.

Lynch EM, Wharton J, Bryant MG, Bloom SR, Polak JM and Elder MG (1980a) The differential distribution of vasoactive intestinal polypeptide in the normal female genital tract. Histochem 67: 167-177.

Malik RA, Newrick PG and Sharma AK et al (1987) Nerve microangiopathy in human diabetic neuropathy: Relationship between capilliary abnormalities and the severity of neuropathy. Diabetolgia 30: 554A.

Mannerfelt L (1962) Evaluation of functional sensation of skin grafts in the hand area. Brit J Plast Surgy 15: 136-153.

Marangos PJ, Zomzely Neurath C, Luk DCM and York C (1975) Isolation and characterization of the nervous system specific protein 14-3-2 from rat brain. Purification, subunit composition and comparison to the breef protein. J Biol Chem 250: 1884-1891.

Marangos PJ, Zis AP, Clark RL and Goodwin FK (1978) Neuronal, non-neuronal and hybrid forms of enolase in brain: Structural, immunological and functional comparisons. Brain Res 150, 117-133.

Marangos PJ and Schmechel D (1980) The neurobiology of the brain enolases. Eassays Neurochem Neuropharmacol 4: 211-247.

Marangos PJ, Schmechel DE and Oertel WH (1981) Neurone specific

enolase: a functional marker for the diffuse endocrine system. In: Bloom SR and Polak JM (eds) Gut Hormone (2nd Ed). Churchill Livingstone, Edinburgh, pp 101-106.

Marini P, Vitadello M, Bianchi R, Triban C and Gorio A (1986) Impaired axonal transport of acetylcholinesterase in the sciatic nerve of alloxon-diabetic rats: effects of ganglioside treatment. Diabetologia 29: 254-258.

Marti E, Gibson SJ, Polak JM, Facer P, Springall DR, Van Answegen G, Aitchinson M and Koltzenberg M (1987) Ontogeny of peptide- and amine-containing neurones in motor, sensory and autonomic regions of rat and human spinal cord, dorsal root ganglia and rat skin. J Comp Neurol 266: 332-359.

Marvis F, Jurkovic I, Kohirt P and Suchanek A (1963) Re-innervation of free and flap skin grafts. Acta Chir Plast (Praha) 5: 54-62.

Mason RT, Peterfreund RA, Sawchenko PE, Corrigan AZ, Rivier JE and Vale WW (1984) Release of the predicted calcitonin gene-related peptide from caltured rat trigeminal ganglion cells. Nature 308: 653-655.

Massey EW and Pleet AB (1979) Notalgia paresthetica. J Amer Med Assoc 241: 1464

Massey EW and Pleet AB (1979) Localised pruritus-notalgia paresthetica. Arch Dermatol 115: 982-983.

Mathison R, Stofer P, Barja F and Immer M (1984) Development of a new tachykinin antagonist. Experientia 40: 86-88.

Mathur NK, Pasrieha JS, Dharampal and Naunihal Singh (1981) Comparison of cutaneous and somatic nervous functions in the lesions of leprosy. Int J Lepr 39: 146-150.

Matskuyama T, Wanaka H, Yoneda S, Kimura K, Kamada T, MacIntyre I, Emson P and Tohyama M (1986) Two distinct calcitonin-gene related peptide containing peripheral nervous systems: distribution and quantitative differences between the iris and cerebral artery with special reference to substance P. Brain Res 373: 2052-212.

Matsumura H, Sakurada T, Hara A, Sakurada S and Kisara K (1985) Characterization of the hyperalgesic effect induced by intrathecal injection of substance P. Neuropharmacol 24: 421-426.

Matteolli M, Haimann C, Torri-Tarelli F, Polak JM, Cecceralli B and De Camilli P (1988) Differential effect of alpha-latrotoxin on exocytosis from small synaptic vesicles and from CGRP-containing large dense core vesicles at the frog neuromuscular junction. Proc Natl Acad Sci USA 8: 7366-7379.

Mayer JH and Tomlinson DR (1983) Prevention of defects of axonal transport and nerve conduction velocity by oral administration of myo-inositol or an aldose reductase inhibitor in streptozotocin-diabetic rats. Diabetolgia 25: 433-438.

McBride P and Mistrella CM (1982) Light touch thresholds in diabetic patients. Diabetic Care 5: 311-315.

McGregor GP, Gibson SJ, Sabate IM, Blank MA, Christofides ND, Wall PD, Polak JM and Bloom SR (1984) Effect of peripheral nerve section and nerve crush on spinal cord neuropeptides in the rat: increased VIP and PHI in the dorsal horn. Neurosci 13: 207-216.

McGregor IA (1950) Regeneration of sympathetic activity in grafted skin as evidenced by sweating. Brit J Plast Surg 3: 12-18.

Mehta LN, Shetty VP and Antia NH (1976) Study of early nerve lesions in mice infected with M. Leprae. Lepr Ind 180: 26-49.

Melzack R and Wall PD (1965) Pain mechanisms: a new theory. Science N Y 150: 971-979.

Merighi A, Polak JM, Gibson SJ, Gulbenkian S, Valantino KL and Peirone SM (1988) Ultrastructural studies on calcitonin gene-related peptide-, tachykinins- and somatostatin-immunoreactive neurones in the rat dorsal root ganglia: Evidence for the colocalization of difference peptides in single secretory granules. Cell Tiss Res 254: 101-109.

Michetti F, Dell'Anna E, Tiberio G and Cocchia D (1983) Immunochemical and immunocytochemical study of S-100 protein in rat adipocytes. Brain Res 262: 352-356.

Mihara M, Hashimoto K, Ueda K and Kumakiri M (1979) The specialized junctions between Merkel cell and neurite: an electron microscopic study. J Invest Dermatol 75: 325-334.

Miletic V and Tan H (1988) Iontophoetic application of calcitonin gene-related peptide produces a slow and prolonged exitation of neurons in the cat lumbar dorsal horn. Brain Res 446: 169-172.

Millan MJ (1986) Multiple opioid system and pain. Pain 27: 303-347.

Miller MR, Ralston HJ and Kasahasa M (1958) The pattern of cutaneous innervation of the human hand. Am J Anat 102: 183-218.

Miller MR, Ralston HJ and Kasahara M (1960) The pattern of cutaneous innervation of the human hand, foot, breast. In: Montagna W (ed) Advances in biology of skin: Cutaneous innervation, Vol. 1. Pergmon press, Newyork, pp 1-47.

Milner RJ (1982) Recombinant DNA strategies and techniques. Trends in Neurosci 5: 297-299.

Minth CD, Bloom SR, Polak JM and Dixon JE (1984) Cloning characterization, and DNA sequence of human cDNA encoding neuropeptide tyrosine. Proc Natn Acad Sci USA 81: 4577-4581.

Molander C, Ygge J and Dalsgaard CJ (1987) Substance P- somatostatin- and calcitonin gene-related peptide-like immunorectivity and fluoride resistant acid phosphatase-activity in relation to retrogradely labeled cutaneous, muscular and visceral primary sensory neurons in the rat. Neuroscience Lett 74: 37-42.

Molin SO, Rosengren L, Haglid K, Baudier J and Hamberger A (1984) Differential localization of "brain-specific" S-100 and its subunits in rat salivary glands. J Histochem Cytochem 32: 805-814.

Montagna W (1960) Cholinesterase in the cutaneous nerves of man. In: Montagna W (ed) Advances in Biology of Skin, Vol.1 Pergamon Press, Oxford, pp 74-87.

Montagna W (1977) Morphology of cutaneous sensory receptors. J Invest Dermatol 73: 325-334.

Montagna W and Becket EB. (1958) Cholinesterase and alpha esterase in the lip of the cat. Acta Anat 32: 256-261

Montagna W and Ellis RA (1958) The vascularity and innervation of human hair follicles. The Biology of Hair Growth. New York: Academic Press 219-227.

Montagna W and Ford D (1969) Histology and cytochemistry of human skin. The eyelid. Arch Dermatol 100: 256-261.

Montagna W and MacPherson E (1973) Similarities in cutaneous nerve receptors. Arch Dermatol 107: 383-385.

Montagna W and Parakkal PF (1974) Cutaneous innervation. in; The structure and function of the skin (3rd edition). Academic press, New York and London, pp 157-171.

Montagna W, Yun J, Ore B, Formisano V and Ri P (1964) Histology and cytochemistry of human skin cholinesterase-containing nerves in face. J Invest Dermat 90: 526-529.

Moore BW (1972) Chemistry and biology of two proteins, S-100 and 14-3-2, specific to the nervous system. Int Rev Neurobiol 15: 215-225.

Mora M, Marchi M, Gibson SJ, Polak JM and Cornelio F (1989) Calcitonin gene-related peptide immunoreactivity at the human neuromuscular junction. Brain Res (in press)

Morishima T (1970) Histochemical studies on the monoaminergic nerves in the skin chiefly by means of fluorescence method of Falck

and Hillarp. Acta Dermatovener (Stockholm) 50: 329-337.

Morley GK, Mooradian AD, Levine AS and Morley JE (1984) Mechanism of pain in diabetic peripheral neuropathy: effect of glucose on pain perception in humans. Am J Med 77: 79-82.

Morris HR, Panico M, Etienne T, Tippins J, Girgis SI and MacIntyre I (1984) Isolation and characterisation of human calcitonin gene-related peptide. Nature 308: 746-748.

Morris JL, Gibbins IL, Furness JB, Costa M and Murphy R (1985) Co-localisation of neuropeptide Y, vasoactive intestinal peptide and dynorphin in nor-adrenergic axons of the guinea pig uterine artery. Neurosci Lett 62: 31-37.

Morris JL, Murphy R, Furness JB and Costa M (1986) Partial depletion of neuropeptide Y from noradrenergic perivascular and cardiac axons by 6-hydroxydopamine and reserpine. Reg Peptides 13: 147-162.

Moss HE, Crowe R and Burnstock G (1987) The seminal vesicle in eight and 16 week streptozotocin-induced diabetic rats: Adrenergic, cholinergic and peptidergic innervation. J Urol 138: 1273-1278.

Mulder DW, Lambert EH, Bastron JA, and Sprague RG (1961) The neuropathies associated with diabetes mellitus: a clinical and electromyographic study of 103 unselected diabetic patients. Neurology 11: 275-284.

Mulderry PK, Ghatei MA, Allen KM, Rosenfeld MG, Polak JM and Bloom SR (1985) Calcitonin gene-related peptide in cardiovascular tissues of the rat. Neuroscience 12: 947-954.

Mulderry PK, Ghatei MA, Spokes RA, Jones PM, Pierson AM, Hamid QA, Kanse S, Amara SG, Burrin JM, Legon S, Polak JM and Bloom SR (1988) Differential expression of æ-CGRP and ß-CGRP by primary sensory neurons and enteric autonomic neurones of the rat. Neurosci 25: 195-205.

Muller J (1838) Handbuch der Physiologie des Menschen. 2nd ed Coblenz.

Munger BL (1971) Patterns of organisation of periphral sensory receptors. In: Lowenstein WR (ed) Handbook of Sensory Physiology, Vol 1. Springer-Verlag, New York. pp 523-556.

Mutt V (1983) New approaches to the indentification and isolation of hormonal polypeptides. Trends in Neurosci 7: 357-360.

Mutt V and Said SI (1974) Structure of porcine vasoactive intestinal octacospeptide, the amino acid sequence, use of kallikrein in its determination. Eur J Biochem 42: 581-589.

Nachmanson D (1975) Sequence of energy transformations. In: Nachmanson D (ed) Chemical and Molecular Basis of Nerve Activity. Academic Press, New York, pp 69-99.

Nagy JI and Hunt SP (1982) Fluoride-resistant acid phosphatase-containing neurones in dorsal root ganglia are seperate from those containing substance P or somatostatin. Neuroscience 7: 89-91.

Nagy JI and Senba E (1985) Neural relations of cremaster motoneurones, spinal cord systems and the genitofemoral nerve in the rat. Brain Res Bul 15: 609-627.

Nagy JI, Hunt SP, Iverson LL and Emson PC (1981) Biochemical and anatomical observations on the degeneration of peptide-containing primary afferent neurones after neonatal capsaicin. Neuroscience: 6: 1923-1934.

Nagy JI, Iversen LL, Goedert M, Chapman D and Hunt SP (1983) Dose dependent effects of capsaicin on primary sensory neurones in the neonatal rat. J Neurosci 3: 399-406.

New HV and Mudge AW (1986) Calcitonin gene-related peptide regulates muscle acetylcholine receptor synthesis. Nature (Lond) 323, 809-811.

Nakajima T, Watanabe S, Sato Y, Shimosato Y, Motoi M and Lennert K (1982) S-100 protein in langerhans cells, interdigitating reticulum cells and histiocytosis X cells. Gann 73: 429-432.

Nakanishi S (1986) Structure and regulation of the preprotachykinin gene. TINS 1: 41-44.

Narayanan RB, Giridhar BK, Ravinder KL and Sengupta U (1986) In vitro studies on dermal granulomas of human leprosy: characterization of cells using monoclonal antibodies. Int J Lepr 54: 268-272.

Navone F, Jahn R, Digioia G, Stukenbrok H, Greengard P and De Camilli P (1986) Protein P 38: an integral membrane protein specific for small vesicles of neurons and neuroendocrine cells. J Cell Biol 103, 2511-2527.

Nawa H, Hirose T, Takashima H, Inyami S and Nakanishi S (1983) Nucleotide sequences of cloned cDNAs for two types of bovine brain substance P precusors. Nature 306: 32-36.

Nawa H, Katani H and Nakanishi S (1984) Tissue specific generation of two preprotachykinin mRNA's from one gene by alternative RNA splicing. Nature (London) 312: 729-734.

New HV and Mudge AW (1986) Calcitonin gene-related peptide regulates muscle acetylcholine receptor synthesis. Nature 323: 809-811.

Newrick PG, Wilson AJ, Jakubowski J, Boulton AJM and Ward JD (1986) Sural nerve oxygen tension in diabetes. Br Med J 293: 1053-1054.

Nilsson JAM, Von Euler AM and Dalsgaard CJ (1985) Stimulation of connective tissue cell growth by substance P and substance K. Nature 315: 61-63.

Nixon RA, Brown BA and Marotta CA (1982) Post translational modification of neurofilament protein during axoplasmic transport: implications for regional speicalization of CNS axons. J Cell Biol 94: 150-158.

Nixon RA, Lewis SE and Marotta CA (1987) Post translational modification of neurofilament proteins by phosphate during axoplasmic transport in retinal ganglion cell. J Neurosci 7: 1145-1158.

Norheim ET, Hua X, Brodin E and Lundberg JM (1985) Capsaicin treatment decreases tissue levels of neurokinin A-like immunoreactivity in the guinea pig. Acta Phys Scand 124: 129-131.

Ohhashi T and Jacobowitz DM (1988) Effects of calcitonin gene-related peptide on neuromuscular transmission in the isolated rat diaphragm. Peptides 9: 613-617.

Ohlen A, Lindborn L, Hökfelt T, Hedgevist P and Staines W (1987) In: JL Henry J.L. Couture R, Cuello AC, Pelletier G, Quirion R, Regoli D (eds). Substance P and neurokinins. Springler-Verleg PP 192-194.

Oku R, Satoh M, Fujii N, Otaka AM, Yajima H and Takagi H (1987). Calcitonin gene-related peptide promotes mechanical nociception by potentiating release of substance P from the spinal dorsal horn in rat. Brain Res. 403: 350-354.

Oppenheim RW, Haverkamp LJ, Prevette D, McManaman JL and Appel SH (1988) Reduction of naturally occuring motoneuron death in vivo by a target derived neurotrophic factors. Science 240: 919-922.

Orfanos CE and Mahrle G (1973) Ultrastructure and cytochemistry of human cuteneous nerves, with special reference to the ultrastructural localization of specific and non-specific cholinesterases in human skin. J Invest Dermatol 61: 108-120.

Orgel M, Agnayo A and Williams HB (1972) Sensory nerve regeneration: an experimental study of skin grafts in the rabbit. J Anat 111: 121-126.

Orrego F (1979) Criteria for the identification of central neurotransmitters and their application to studies with some nerve tissue preparations in vitro. Neuroscience 4: 1037-1057.

O,Shaughnessy DJ (1982) Antibodies. In: Bloom SR & Long RG (eds) Radioimmunoassay of gut regulatory peptides. Sauders WB & Co London pp 11-20.

O'Shaughnessy DJ, McGregor GP, Ghatei MA, Blank MA, Springall DR, Gu J, Polak JM and Bloom SR (1983) Distribution of bombesin, somatostatin, substance-P and vasoactive intestinal polypeptide in feline and porcine skin. Life Sci 32: 2827-2836.

Otsuka M and Yanagisawa M (1980) The effects of substance P and baclofen on motor neurones of isolated spinal cord of the newborn rat. J Exp Biol 89: 201-214.

Otsuka M, Konishi S, Yanagisawa M, Tsunoo A and Akagi H (1982) Role of substance P as a sensory transmitter in spinal cord and sympathetic ganglia. In: Porter R and O' Connor M (eds) Substance P in the nervous system, Pitman London, PP 13-30.

Palmer P (1965) Ultrastructural alterations of Merkel cells following denervation. Anat Rec 151: 391-397.

Palay SL, McGee-Ruseel SM, Gordon S and Grille MA (1962) Fixation of neural tissues for electron microscopy by perfusion with solutions of osmium tetroxide. J cell Biol 12: 385-410.

Parhad IM, GRiffin JW, Hoffman PN and Koves JF (1986) Selective interruption of axonal transport of neurofilament proteins in the visual system by beta, beta'-iminodipropionitrile(IDPN) intoxication. Brain Res 363: 315-324.

Parkhouse N and Le Quesne PM (1988) Quantitative objective assessment of peripheral nociceptive C fibre function. J Neurol Neurosurg Psychol 51: 28-34.

Pearson JMH and Weddell AGM (1975) Perineural changes in untreated leprosy. Lepr Rev 46: 51-67.

Pearson J, Pytel BA, Grover-Johnson N, Axelrod F and Domcis J (1978) Quantitative studies of dorsal root ganglia and neuropathethologic observations on spinal cords in familial dysautonomia. J Neurol Sci 35: 77-92.

Percey MF, Einsphor F, Dorbry PTK, Schroeder LA and Holllister RP (1980) Morphine does not antagonize the substance P mediated excitation of dorsal horn neurons. Brain Res 186: 421-434.

Pearson J, Brandeis L and Cuello AC (1982) Depletion of substance P-containing axons in the substantia gelatinosa of patients with diminished pain sensitivity. Nature 295: 61-63.

Pernow B (1953) Studies on substance P. Purification, occurrence and biological actions. Acta Physiol Scand 105 (Suppl.) 29: 1-90.

Pernow B (1983) Substance P. Pharmacol Rev 35: 85-141.

Pernow J, Saria A and Lundberg JM (1986) Mechanisms underlying pre- and post-functional effects of neuropeptide Y in sympathetic vascular control. Acta Physiol Scand 126: 239-249.

Peterfreund RA and Vale WW (1986) Local anesthetic inhibit veratridine-induced secretion of calcitonin gene-related peptide (CGRP) from cultared rat trigeminal ganglion cells. Brain Res 380: 159-161.

Petrusz P, Sar M, Ordonneau P and Di Meo P (1976) Specificity in immunocytochemical staining. J Histochem Cytochem 24: 1110-1112.

Petrusz P, Sar M, Ordonneau P and Di Meo P (1977) Reply to letter of Swaab. J Histochem Cytochem 25: 388-390 "Can specificity ever be proved in immunocytochemical staining?" J Histochem Cytochem 25: 390-391.

Piercey MF, Einsphor F, Dobry PTK, Schroeder LA and Hollister RP (1980) Morphine does not antanonize the substance P mediated excitation of dorsal horn neurons. Brain Res 186: 421-434.

Piper PJ, Said SI and Vane JR (1970) Effects on smooth muscle preparations of unidentified vasoactive peptides from intestine and lung. Nature 225: 1144-1146.

Pirart J (1978) Diabetes mellitus and its degenerative complications: a prospective study of 4400 patients observed between 1947 and 1973. Diabetic care 1: 168-188.

Pittman QJ and Siggins GR (1981) Somatostatin hyperpolarizes hippocampal pyramidal cells in vitro. Brain Res 221: 402-408.

Pleet Ab and Maseey EW (1978) Notalgia paresthetica. Neurology 28: 1310-1312.

Polak JM and Bloom SR (1981) The peripheral substance P-ergic system. Peptides 2 (suppl.2) 133-148.

Polak JM and Bloom SR (1984) Regulatory peptides-the distribution of two newly discovered peptides: PHI and NPY. Peptides 5: 79-89.

Polak JM, Gu J, Mina S and Bloom SR (1981) Vipergic nerves in the penis. Lancet II: 217-219.

Pool CW, Buijs RM, Swaab DF, Boer GJ and Van Leewen FW (1983) On the way to a specific immunocytochemical localization. In: Cuello, A.C (ed) Immunohistochemistry. John Wiley and Sons. Chichester, pp 1-46.

Porter R and O'Connor M (eds) (1982) Substance P in the nervous system - Ciba Symposium, Pitman, London.

Powell HC and Myers RR (1984) Axonopathy and microangiopathy in chronic alloxan diabetes. Acta Neuropathol 65: 128.

Powell HC, Rosoff J and Myers RR (1985) Microangiopathy in human diabetic neuropathy. Acta Neuropath 68: 295-305.

Probert L, De Mey J and Polak JM (1981) Distinct sub-populations of enteric p-type neurones contain substance P and vasoactive intestinal polypeptide. Nature 294: 470-471.

Probert L, De Mey J and Polak JM (1983) Ultrastructural localization of four different neuropeptides within separate populations of p-type nerves in the guinea pig colon. Gastroenterology 85: 1094-1104.

Rao VS, Vadlamudi V and McNeil JH (1983) Effects of alloxan and streptozotocin induced diabetes on isolated rat heart responsiveness to carbachol. J Pharmocol Exp Ther 225: 410-415.

Rea TH, Gottlib B and Levan NE (1975) Apparently normal skin in lepromatous leprosy. Arch Dermatol 111: 1571-1578.

Regoli D, Drapeau G, Dion S and D'Orleans-Juste P (1987) Pharmacological receptors for substance P and Neurokinins. Life Sci 40: 109-117.

Retzius G (1892) Ueber die Nervendigungen in den Haaren. Biol Untersuch N.F., 4: 45-48.

Ridley A (1969) Silver staining of nerve endings in human digital glabrous skin. J Anat 104: 41-48.

Ridley DS (1985) Skin Biopsy in Leprosy. 2nd ed. CIBA-GEIGY, Basel

Ridley DS and Jopling WH (1962) A classification of leprosy for research purposes. Lepr Rev 33:119.

Ridley MJ, Russell FD and Ridley DS (1982) An immunoperoxidase study of immunological factors in skin lesions across the spectrum of leprosy. Int J Lepr 50: 11-19.

Ridley A (1970) A biopsy study of the innervation of forearm skin grafted to the fingertip. Brain 93: 547-554.

Rioux F, Bachelard H, Martel JC and St Pierre S (1986) The

vasoconstrictor effect of neuropeptide Y and related peptides in the guinea pig isolated heart. Peptides 7: 27-31.

Rivers WHR and Head H (1908) A human experiment in nerve division. Brain 31: 323-450.

Robels CL, Mendoza AG, Suarez GP and Arreguir SM (1985) Use of human skin to demonstrate antinuclear antibodies in lepromatous leprosy patients. Int J Lepr 53: 373-377.

Robertson DM and Winkelmann RK (1970) A whole mount cholinesterase technique for demonstrating corneal nerves: Observations in the albino rabbit. Invest Opthalmol 9: 710-715.

Robinson JP, Willars GB, Tomlinson DR and Keen P (1987a) Axonal transport and tissue contents of substance P in rats with long term streptozotocin diabetes effects of the aldose reductase inhibitor 'statil'. Brain Res 426: 339-348.

Robinson JP and Tomlinson DR (1987b) Fast anterograde axonal transport of chloine containing lipids in rats with experimental diabetes. J Neurol Sci 81: 93-100

Rodrigo J, Polak JM, Fernandez L, Ghatei MA, Mulderry P and Bloom SR (1985) Calcitonin gene-related peptide immunoreactive sensory and motor nerves of the rat, cat and monkey oesophagus. Gastroenterology 88: 444-451.

Rollinson DH and Inzidi MH (1972) The distribution of nerves, monoamine oxidase and cholinesterase in the skin of the camel (camelus dromedarius). Res Vet Sci 13: 304-305.

Rosenfield MG, Mermod JJ, Amara SG, Swanson LW, Sawchenko PE, Rivier J, Vale WW and Evans RM (1983) Production of a novel neuropeptide encoded by the calcitonin gene via tissue-specific RNA processing. Nature 304: 129-135.

Rowden G, Boudreau S and Higley H (1985) Langerhans cells and extraepidermal dendritic cells. An investigation in laboratory animals and man with immunomorphological methods. Scand J Immunol 21: 471-478

Ruffini A (1904-1905) Les dispositifs anatomiques de la sensibilité cutanéé mammiferes. Rev gen Hist 1: 421-538.

Rush RA (1984) Immunohistochemical localization of endogenous nerve growth factor. Nature 312: 364-367.

Russell LC and Burchiel KJ (1984) Neurophysiological effects of capsaicin. Brain Res Rev 8: 165-176.

Said SI (1981) VIP overview. In: Bloom SR and Polak JM (eds) Gut hormones (2nd edition) Churchill Livingstone, Edinburgh. pp 379-383.

Said SI (ed) (1982) Vasoactive Intestinal Polypeptide. Raven Press, New york.

Said SI and Mutt V (1970) Polypeptide with broad biological activity isolation from small intestine. Science 169: 1217-1218.

Sakuntala R, Pratap VK, Sharma NK, Dayal SS and Aggarwal SK (1982) Histological profile in apparently normal skin of leprosy patients. Lepr Ind 54: 40-47.

Salt TE and Hill RG (1983) Neurotransmitter candidates of somatosensory primary afferent fibres. Neuroscience 10: 1083-1103.

Santoni-Rugiu P (1966) An Experimental study on the reinnervation of free skin grafts and pedicle flaps. Plast Reconst Surg 38: 98-104.

Saria A, Gamse R, Petermann J, Fischer JA, Theodorsson-Norheim E and Lundberg JM (1986) Simultaneous release of several tackykinins and calcitonin gene-related peptide from rat spinal cord slices. Neurosci Lett 63: 310-314.

Sasaki-Sherrington SE, Jacobs JR and Stevens JK (1984) Intracellular control of axial shape in non-uniform neurites: A serial electron microscopic analysis of organelles and microtubules in AI and AII retinal amacrine cells. J Cell Biol 98: 1279-1290.

Scadding JW (1980) The permanent anatomical effects of neonatal capsaicin on somatosensory nerves. J Anat (London) 131: 473-484.

Schebalin M, Said SI and Makalouf GM (1977) Stimulation of insulin and glucagon secretion by vasoactive intestinal peptide. Am J Physiol 232: 197-200

Schneider SA, Fukuyama K, Macera J and Epstein WL (1985) Effect of ultraviolet B radiation on S-100 protein antigen in epidermal Langerhans cells. J Invest Dermatol 84: 146-148.

Schlaepfer WW and Lynch RG (1977) Immunoflurescence studies of neurofilaments in the rat and human peripheral nervous system. J Cell Biol 74: 241-250.

Schmechel D, Marangos PJ and Brightman M (1978) Neuron specific enolase is a molecular marker for peripheral and central neuroendocrine cells. Nature 276: 834-836.

Sehgal VN (1976) Significance of local sweat response in assessing progress of leprosy. Br J Dermatol 96: 615-618.

Shaw G and Weber K (1981) The distribution of the neurofilament triplet proteins within individual neurones. Exp Cell Res 136: 119-125.

Sharpey-Schafer E (1928) The effects of de-nervation of a cutaneous area. Q J Exp Physiol 19: 85-107.

Shepard CC (1960) The experimental disease that follows the injection of human leprosy bacilli into footpads of mice. J Expt Med 112: 445-454.

Sheppard MN, Polak JM, Allen JM and Bloom SR (1984) Neuropeptide tyrosine (NPY): a newly discovered peptide is present in mammalian respitratory tract. Thorax 39: 326-330.

Shetty VP and Antia NH (1981) Degeneration and regeneration of unmyelinated fibres in experimental leprous neuropathy. Int J Lepr 49: 324-330.

Shetty VP, Mehta LN and Antia NH (1978) Unmyelinated fibres in leprous neuritis. An ultrastructural study. Bull Electron Microsc Soc India 2: 2-5.

Shetty VP, Mehtha LN, Irani PF and Antia NH (1980a) Study of evolution of nerve damage in leprosy. Part I- Lesions of the index branch of the radial cutaneous nerve in early leprosy. Part II-Observation on the index branch of the radial cutaneous nerve in contacts of leprosy. Lepr India 52: 5-25.

Shetty VP, Vidyasagar PB and Antia NH (1980b) Study of evolution of nerve damage in leprosy. Part III-Sciatic nerve lesions in mice innoculated with M. leprae with nerve conduction velocity correlates. Lepr India 52: 26.

Shetty VP, Mistry NF and Antia NH (1985) Serum demyelinating factors and adjuvant like activity of Mycobacterium Leprae: possible causes of early nerve damage in leprosy. Lepr Rev 57: 221-227.

Shu S, Ju G and Fan L (1987) The glucose oxidase-DAB-nickel method in peroxidase histochemistry of the nervous system. Neurosci Lett 85: 169-171.

Sigrist S, Franco-Cereceda A, Muff R, Henke H, Lundberg JM and Fischer JA (1986) Specific receptor and cardiovascular effects of calcitonin gene-related peptide. Endocrinology 119: 381-389.

Sidenius P and Jakobsen J (1987) Axonal Transport in Human and Experimental Diabetes. In: Dyck PJ, Thomas PK, Asbury AK, Winegrad AI, Porte D, eds. Diabetic Neuropathy. Philadelphia: Saunders, pp 260-265.

Sima AAF and Robertson DM (1978) The perineurial and blood-nerve barriers in experimental diabetes. Acta Neuropathol 44: 189.

Sima AAF, Bril V, Nathaniel V, McEwen TAJ, Brown MB, Lattimer SA and Greene DA (1988) Regeneration and repair of myelinated fibres in sural-nerve biopsy specimens from patients with diabetic neuropathy treated with sorbinil. N Engl J Med 319: 542-548.

Sjoqvist F (1963) The correlation between the occurrence and localization of acetylcholinesterase rich cell bodies in the stellate ganglion and the outflow of cholinergic sweat secretory fibres to the foot pad of the cat. Acta Physiol Scand 57: 339-352.

Skillman TG, Johnson EW, Hamwi CJ and Driskill HJ (1961) Motor nerve conduction velocity in diabetes mellitus. Diabetes 10: 46-51.

Skrabanek P and Powell D (eds) (1980) Substance P, Volume 2, Eden Press Inc, Montreal.

Skrabanek P and Powell D (eds) (1983) Substance P, Volume 3, Eden Press Inc, Montreal.

Skrabanek P and Powell D (eds) (1983b) Substance P, Dublin 1983, Boole press, Dublin.

Smith KR (1967) The structure and function of the Haarschabe. J Comp Neurol 131:459-474.

Springall DR, Polak JM, Ghatei MA, Lackie P, Rosenfeld MG and BLoom SR (1983) Calcitonin-gene-related peptide (CGRP) in mucosal endocrine cells and in the lung innervation of rat and man. (Abstract). Regul Pepts 7: 301.

Springall DR, Gu J, Cocchia D, Michetti F, Levene A, Levene MM, Marangos PJ, Bloom SR and Polak JM (1983) The value of S-100 immunostaining as a diagnostic tool in human malignant melanomas. Virchows Arch Pathol Anat 400: 331-343.

Stefanini M, De Martino C and Zamboni L (1967) Fixation of ejaculated spermatozoa for electron microscopy. Nature 216: 173-174.

Sternberger LA (ed) (1979) Immunocytochemistry. 2nd edition. John Wiley and Sons Inc, New York.

Sternberger LA (1982) Transition of immunocytochemistry from anatomical to a biochemical science: evolution of the concept of neurotypy. Neuroscience 7: 2937-2947.

Sternberger LA and Sternberger NH (1983) Monoclonal antibodies distinguish phosphorylated and nonphosphorylated forms of neurofilaments in situ. Proc Natl Acad Sci USA 80: 6126-6130.

Sternberger LA, Hardy PJ Jr. Cuentis JJ and Meyer HG (1970) The unlabeled antibody-enzyme method of immunohistochemistry. Preparation and properties of soluble antigen-antibody complex

(horseradish peroxidase-antihorseradish peroxidase) and its use in identification of spirochetes. J Histochem Cytochem 18: 315-324.

Stjernqvist M, Emson p, Owen C, Sjoberg F, Sundler F and Tetemoto K (1983) Neuropeptide Y in the female reproductive tract of the rat. Distribution of nerve fibres and motor effects. Neurosci Lett 39: 279-284.

Su HC, Bishop AE, Power RF, Hamada Y and Polak JM (1987) Dual intrinsic and extrinsic origions of CGRP and NPY immunoreactive nerves of rat gut and pancreas. J Neurosci 7(9): 2674-2687.

Su HC, Wharton J, Polak JM, Mulderry PK, Gathei MA, Gibson SJ, Terenghi G, Morrison JFB, Ballesta J and Bloom SR (1986) CGRP immunoreactivity in afferent neurons supplying the urinary tract: combined retrograde tracing and immunocytochemistry. Neuroscience 18: 727-747.

Sundler F, Alumets J, Hakanson R, Fahrenkrug J and Schaffalitzky de Muckadell O (1978) Peptidergic (VIP) nerves in the pancreas. Histochem 55: 173-176.

Sundler FE, Moghimzaden E, Hakanson R, Ekelund M and Emson PC (1983) Nerve fibres in the gut and pancreas of the rat displaying neuropeptide-Y immunoreactivity. Intrinsic and extrinsic origin. Cell tissue Res 230: 487-493.

Sutcliffe JG, Milner RJ, Shinnick TM and Bloom FE (1983) Identifying the protein products of brain-specific genes using antibodies to chemically synthesized peptides. Cell 33: 671-682.

Swaab DF, Pool CW and Van Leeuwen FW (1977) Can specificity ever be proved in immunocytochemical staining? J Histochem Cytochem 25: 388-390.

Szelke M (1983) Raising antibodies to small peptides. In: Polak JM and Van Noorden S (eds) Immunocytochemistry - Applications in Pathology and Biology. John Wright PSG, Bristol, pp 53-68.

Tago H, Kimura H and Maeda T (1986) Visualisation of detailed acetylcholinesterase fibres and neuron staining in rat brain by a sensitive histochemical procedure. J Histochem & Cytochem 34: 1431-1438.

Tainio H, Vaalasti A and Rechardt R (1986) The distribution of sympathetic, adrenergic, tyrosine hydroxylase and neuropeptide Y-immunoreactive nerves in human axillary sweat glands. Histochemistry 85: 117-120.

Tainio H, Vaalasti A and Rechardt L (1987) The distribution of substance P-, CGRP-, galanin- and ANP-like immunoreactive nerves in human sweat glands. Histochem J 19: 375-380.

Takahashi T and Otsuka M (1975) Regional distibution of substance P in the spinal cord and nerve roots of the cat and the effect of dorsal root section. Brain Res 87: 1-11.

Takahashi K, Isobe T, Ohtsuki Y, Sonobe H, Takeda I and Akagi T (1984a) Immunohistochemical localization and distribution of S-100 proteins in the human lymphoreticular system Am J Pathol 116: 497-503.

Takahashi K, Isobe T, Ohtsuki Y, Akagi T, Sonobe H and Okuyama T (1984b) Immunohistochemical study on the distribution of alpha and beta subunits of S-100 protien in human neoplasm and normal tissues. Virchows Arch 45: 385-396

Takami K, Kawai Y, Shiosaka S, Lee Y, Girgis S, Hillyard CJ, MacIntyre I, Emson PC and Tohyama M (1985a) Immunohistochemical evidence for the coexistence of calcitonin gene-related peptide- and choline acetyltransferase-like immunoreactivity in neurones of the rat hypoglossal, facial and ambiguus nuclei. Brain Res 328: 386-389.

Takami K, Kawai Y, Uchida S, Tohyama M, Shiotani Y, Yoshida H, Emson PC, Girgis S, Hillyard CJ and MacIntyre I (1985b) Effect of calcitonin gene-related peptide on contraction of striated muscle in the mouse. Neurosci Lett 60: 227-230.

Tatemoto K (1982) Neuropeptide Y: complete amino acid sequence of the brain peptide. Proc Natl Acad Sci USA 79: 5485-5489.

Tatemoto K and Mutt V (1980) Isolation of two novel candidate hormones using a chemical method for finding naturally occurring polypeptides. Nature 285: 417-419.

Tatemoto K and Mutt V (1981) Isolation and characterisation of the intestinal peptide porcine PHI (PHI-27) a new member of the glucagon-secretin family. Proc Natl Acad Sci USA 78: 6683-6607.

Tatemoto K, Carlquist M and Mutt V (1982) Neuropeptide Y - a novel brain peptide with structural similarities to peptide YY and pancreatic polypeptide. Nature 296: 659-660.

Tatemoto K, Carlquist M, McDonald TJ and Mutt V (1983) Isolation of a brain peptide identical to the intestinal PHI (peptide HI). FEBS Lett 153: 248-252.

Tatemoto K, Jornvall H, Mcdonald TJ, Carlquist M, Go VLW, Johansson C and Mutt V (1984) Isolation and primary structure of human PHI (Peptide HI) FEBS Lett 174: 258-261.

Tatemoto K Lundberg JM, Jornvall M and Mutt V (1985) Neuropeptide K: Isolation, structure and biological activities of a novel brain tachykinin. Biochem Biophys Res Comm 128: 947-953.

Taxi J (1965) Contribution a l'etude des connexions des neurones moteurs du systeme nerveux autonomie. Ann Sci Natl Zool Biol Anim 12: 413-467.

Terenghi G, Polak JM, Ghatei MA, Mulderry PK, Butter JM, Unger WG and Bloom SR (1985) Distribution and origin of calcitonin gene-related peptide (CGRP) immunoreactivity in the sensory innervation of the mammalian eye. J Comp Neurol 233: 506-516.

Terenghi G, Polak JM, Rodrigo JM, Mulderry M and Bloom SR (1986) Calcitonin gene-related peptide immunoreacitve nerves in the tongue, epiglottis and phyrynx of the rat: Occurance, distribution and origin. Brain Res. 365:1-14.

Terzis JK (1978) Functional aspects of reinnervation of free grafts. Plast Reconstr Surg 58: 142-148.

Thibault J, Vidal D and Gros F (1981) In vitro translation of mRNA from rat phaeochromocytoma tumours, characterisation of tyrosine hydroxylase. Biochem Res Commun 99: 960-968.

Thoenen H and Tranzer JP (1968) Chemical sympathectomy by selective destruction of adrenergic nerve endings with 6-hydroxydopamine. Naunyn-Schmiedebergs Arch Pharmacol 261: 271-288.

Thom SM, Hughes AD, Goldberg P, Martin G, Schachter M and Sever PS (1987) The actions of calcitonin gene-related peptide and vasoactive intestinal peptide as vasodilators in man. Br J Clin Pharm 24: 139-144.

Thomas PK and Lascelles RG (1966) The pathology of diabetic neuropathy, Quart J Med 35: 489-509.

Thompson RJ (1981) Human nervous system specific proteins. In: Alberti KGMM. and Price CP (eds). Recent Advances in Clinical Biochemistry, vol. 2. Churchill Livingstone, Edinburgh, pp 295-307.

Thompson RJ, Doran JF, Jackson P, Dhillon AP and Rode J (1983) PGP 9.5 - A new marker for vertebrate neurons and neuroendocrine cells. Brain Res 278: 224-228.

Tomlinson DR and Mayer JH (1984) Defects of axonal transport in diabetes mellitus - a possible contribution to the aetiology of diabetes neuropathy. J Autonom Paramacol 4: 59-72.

Tomlinson DR, Gillan KRW and Smith MG (1982) Axonal transport of noradrenaline and noradrenergic transmission in rats with streptozotocin and noradrenergic transmission in rats with streptozotocin-induced diabetes. Diabetologia 22: 199-204.

Trotter W and Davies HM (1909) Experimental studies on the

innervation of the skin. J Physiol London 38: 134-246.

Uddman R, Aluments J, Densert O, Hakanson R and Sundler F (1978) Occurrence and distribution of VIP nerves in the nasal mucosa and tracheobronchial wall. Acta Otolaryngol 86: 443-448.

Uddman R, Alumets J, Ehinger B, Hakanson R, Loren I and Sundler F (1980) Vasoactive intestinal polypeptide nerves in ocular and orbital structures of the cat. Invest Ophthalmol Vis Sci 19: 878-885.

Uddman R, Edvinsson L, Ekblad E, Håkanson R and Sundler F (1986) Calcitonin gene-related peptide (CGRP): perivascular distribution and vasodilatory effects. Reg Peps 15: 1-23.

Uno H (1977) Sympathetic innervation of the sweat glands and piloerrector muscles of macaques and human beings. J Invest Dermatol 61: 112-120.

Uno H and Montagna W (1975) Catecholamine-containing nerve terminals of eccerine sweat glands of macaques. Cell Tissue Res 158: 1-13.

Vaalasti A, Tainio Hand L and Rechardt L (1985) Vasoactive intestinal polypeptide (VIP)-like immunoreactivity in the nerves of human axillary sweat glands. J Invest Dermatol 85: 246-248.

Vaidya MC, Elizabeth Palmer, Weddell G and Rees RJW (1970) A note on the presence of Mycobacterium Laprae in the central nervous system of a mouse with lepromatous leprosy. J med Microbiol, 3: 194-196.

Varndell IM, Polak JM, Allen JM, Terenghi G and Bloom SR (1984) Neuropeptide tyrosine (NPY) immunoreactivity in noradrenaline-containing cells and nerves of the mammalian adrenal gland. Endocrinology 114: 1460-1462.

Volkmann AV (1844) In: Wagner R (ed) Handworterbuch der Physiologie, Vol.2 Braunschweig pp 521-526.

Von Euler US and Gaddum JH (1931) An unidentified depressor against choline acetyltransferase. Neurochem Int 6: 163-183.

Wahlestedt C, Edvinsson L, Ekblad E and Hankanson R (1985) Neuropeptide Y potentiates noradrenaline-evoked vasoconstriction. Mode of action. J Pharmac Exp Ther 234: 735-741.

Wahlestedt C, Yanaihara N and Hakanson R (1986) Evidence for different pre- and post-junctional receptors for neuropeptide Y and related peptides. Reg peptides 13: 307-318.

Wall PD and Fitzgerald M (1981) Effects of capsaicin applied locally to adult peripheral nerve. I. Physiology of peripheral nerve and spinal

cord. Pain 11: 363-377.

Wall PD and Fitzgerald M (1982) If Substance P fails to fulfil the criteria as a sensory transmitter, what might its function be ? In: Porter R and O'Connor M (eds) Substance P in the Nervous system. CIBA Foundation Symposium 91, Pitman, London pp 249-266.

Wallengren J and Hakanson R (1987) Effects of substance P, Neurokinin A and callcitonin gene related peptide in human skin and their involvement in sensory nerve mediated response. Eur J Pharmacol 143: 267-273.

Ward JD, Barnes CG, Fisher DJ, Jessop JD and Boher WR (1971) Improvement in nerve conduction following treatment in newly diagnosed diabetics. Lancet 1: 428-430.

Waris T (1978) Reinnervation of free skin autografts in the rat, Scand. J Plast Reconstr Surg 12: 85-93.

Waris T and Rechardt L (1977) Histochemically demonstrable catecholamines and cholinesterases in nerve fibres of rat dorsal skin. Histochemistry 53: 203-210.

Waxman SG, Brill MH, Geschwind N, Sabin TD and Lettvin JY (1976) Probability of conduction deficit as related to fibre length in random-distribution models of peripheral neuropathies. J Neurol Sci 29: 39-53.

Weddell AGM (1941) The pattern of cutaneous innervation in relation to cutaneous sensibility. J Anat (Lond) 75: 346-367.

Weddell AGM (1977) Disorders of peripheral cutaneous nerves. Int J Dermatol 69: 130-135.

Weddell AGM and Palmer E (1963) The pathogenesis of leprosy: an experimental approach. Lepr Rev 34: 57-61.

Weddell AGM, Palmer E, Rees RJW and Jamison DG (1963) Experimental observations related to the histopathology of leprosy. In: Wolstenholme GEW and O'Connor M (eds) The Pathogenesis of Leprosy. Ciba Foundation Study Group No 15. J & E Churchill London, pp 3-15.

Weddell AGM, Pattie and Palmer E (1954) The morphology of peripheral nerve terminations in the skin. Quart J Microsc Sci 95: 483-501.

Weddell AGM, Palmer E and Rees RJW (1971) The fate of Mycobacterium leprae in CBA mice. J Patho 104: 77-92.

Weihe E, Reinecke M and Forssmann WG (1984) Distribution of vasoactive intestinal polypeptide-like immunoreactivity in the mammalian heart. Interrelation with neurokinin- and substance P-

like immunoreactive nerves. Cell Tiss Res 236: 527-540.

Weihe E, Nohr D, Millan MJ, Stein C, Muller S, Gramsch C and Herz A (1988) Peptide neuroanatomy of adjuvant induced arthritic inflammation in rat. Agents and actions 25: 255-259.

Weiss PA and Mayr R (1971) Orgamelles of neuroplasmic ("axonal") flow: Neurofilaments. Proc Natl Acad Sci USA 68: 846-850.

Werman R (1966) A review - criteria for indentification of a central nervous system transmitter. Comp Biochem Physiol 18: 745-766.

Wharton J, Polak JM, Bloom SR, Will JA, Broen MR and Pearse AGE (1979) Substance P-like immunoreactive nerves in the guinea pig heart. Neurosci 6: 2193-2204.

Wharton J, Polak JM, McGregor G, Bishop AE and Bloom SR (1981) The distribution of substance P-like immunoreactive nerves in the guinea pig heart. Neurosci 6: 2193-2204.

Wharton R, Gulbenkian S, Mulderry PK, Ghatei MA, McGregor GP, Bloom SR and Polak JM (1986) Capsaicin induces a depletion of calcitonin gene-related peptide (CGRP) immunoreactive nerves in the cardiovascular system of the guinea pig and rat. J Auton Nerv Syst 16: 289-309.

Wharton J, Gulbenkian S, Merighi A, Kuhn DM, Jahn R, Taylor KM and Polak JM (1988) Immunohistochemical and ultrastrucural localization of peptide containing nerves and myocardial cells in the human atrial appendage. Cell Tiss Res 254: 155-166.

White DM and Helme RD (1985) Release of substance P from peripheral nerve terminals following electrical stimulation of the sciatic nerve. Brain Res 336: 27-31.

Widenmann B and Franke WW (1985) Identification and localization of synaptophysin, an integral membrane glycoprotein of MW 38,000 characteristic of presynaptic vesicles. Cell 41: 1017- 1028

Widenmann B, Franke WW, Kuhn C and Moll R (1986) Synaptophysin: A marker for neuroendocrine cells and neoplasms. Proc Natl Acad Sci 83: 3500-3504.

Wiesenfeld-hallin Z, Hökfelt T, Lundberg JM, Forssmann WG, Reinecke M, Tschopp FA and Fischer JA (1984) Immunoreactive calcitonin gene-related peptide and substance P coexist in sensory neurons to the spinal cord and interact in spinal behavioral responses of the rat. Neurosci Lett 52: 199-204.

Williams P and Warwick R (1980) Neurology. In: Williams and Warwick R (eds) Gray's anatomy (36th edition) Churchill Livingstome, Edinburg pp 802-1227.

Williams RG, Dimaline R, Varro A, Isetta AM, Trizio D and Dockray GJ (1987) Cholecystokinin octapeptide in rat central nervous system. Immunocytochemical studies using monoclonal antibody that does not react with CGRP. Neurochem. Int. 11: 433-442.

Williams S, Wells C and Hunt S (1988) Spinal cord neuropeptides in a case of chronic pain. Lancet 8593: 1047-1048

Williams TJ (1982) Vasoactive intestinal polypeptide is more potent than prostaglandine E2 as a vasodilator and oedema potentiator in rabbit skin. Br J Pharmacol 77: 505-509.

Winkelmann RK (1959) The innervation of a hair follicle. Ann NY Acad Sci 83: 400-407.

Winkelmann RK and Schmit RW (1959). Cholinesterase in the skin of the rat, dog, cat, guinea pig and rabbit. J Invest Dermatol 33: 185-190.

Winkelmann RK (1960) Nerve endings in normal and pathological skin. Charles C. Thomas, Springfield, Illinois.

Wilkinson KD, Lee K, Deshapande S, Duerksen-Hughes P, Boss JM, Pohl J (1990) The neuron-specific protein PGP 9.5 is a ubiquitin carboxyl-terminal hydrolase. Science 246: 670-673.

Woolf C and Wisenfeld Hallin Z (1986) Substance P and calcitonin gene-related peptide synergistically modulate the gain of the nociceptive flexor withdrawal reflex in the rat. Neurosci Letts 66: 226-230.

Woollard HH (1936a) Anatomy of peripheral sensation. Br med J 2: 861-862.

Woolard HH (1936b) Intraepidermal nerve endings. J Anat (Lond) 71: 54-60.

Woolard HH, Weddell G and Harpman JA (1940) 'Observations on neurohistological basis of cutaneous pain'. J Anat (Lond) 74: 413-425

Wright DJM, Hirst RA, Waters MFR (1975) Neural auto antibodies in leprosy. Lepr Rev 46: 157-169.

Yaksh TL, Farb DH, Leeman SE and Jessell TM (1979) Intrathecal capsaicin depletes substance P in the rat spinal cord and produces prolonged thermal analgesia. Science 206: 481-483.

Yaksh TL, Jessell TM, Gamse R, Mudge AW and Leeman SE (1980) Intrathecal morphine inhibits substance P release from spinal cord in vivo. Nature 286: 155-157.

Young RJ, Ewing DJ and Clarke BF (1988) Chronic remitting painful diabetic neuropathy. Diabetes Care 11: 43-40.

Zamboni L and de Martino C (1967) Buffered picric acid formaldehyde: a new rapid fixative for electron microscopy. J Cell Biol 35: 148A.

Zallman PE and Winkelamnn RK (1962) The sensory innervation of the common North American racoon (Procyon Lotor). J Comp Neurol 119: 149-157.

Zimmerman BR (1987) Current status of aldose reductase inhibitors. Diabetes Care 10: 123-125.

Publications derived from the present study

1. S. S. Karanth, D.R. Springall, S. Lucas, D. Levy, P. Ashby, M.M. Levene and J.M. Polak Changes in nerves and neuropeptides in skin from 100 leprosy patients investigated by immunocytochemistry. J Pathol (1989) 157: 15-26.

2. S.S. Karanth, D.R. Springall, S.Kar, S.J. Gibson, J.P Royston, D.K. Banerjee and J.M. Polak. Time related decrease of substance P and CGRP in central and peripheral projections of sensory neurones in *Mycobacterium leprae* infected nude mice: a model for lepromatous leprosy in man. J Pathol (1990) 161: 335-345.

3. D.M. Levy, S.S. Karanth, D.R. Springall and J.M. Polak. Depletion of cutaneous nerves and neuropeptides in diabetes mellitus: an immunocytochemical study. Diabetologia (1989) 32: 427-433.

4. S.S. Karanth, D. R. Springall, D.M. Kuhn and J.M. Polak. Nerves and neuropeptides in skin of five mammalian species and their ontogeny in the rat. Am J Anatomy (1989) (Submitted)

5. S.S. Karanth, D.R. Springall, D.J. Mirrlees and J.M. Polak. Early increase in CGRP- and VIP-immunoreactive nerves in the skin of streptozotocin-induced diabetic rats. Histochemistry. (Submitted)

6. S.S. Karanth, S. Dhital, D.R. Springall and J.M.Polak. Reinnervation and neuropeptides in mouse skin flap grafts. J Auton Nerv Syst (Submitted)

7. D.R. Springall, S.S. Karanth, N. Kirkham, C.R. Darley and J.M. Polak. Symptoms of Notalgia paraethetica may be explained by increased dermal innervation. J Invest Dermatol (Submitted)