PERIVASCULAR INNERVATION OF CEREBRAL ARTERIES AND VASA NERVORUM: CHANGES IN DEVELOPMENT AND DISEASE

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ABSTRACT

Using fluorescence histochemical, and chemical assays, this thesis aims to study the perivascular innervation of cerebral arteries and that of nerve-trunk vasculature in both cranial and peripheral nerves.

The first part of the thesis concerns the innervation of blood vessels within nerve trunks (vasa nervorum). A novel whole-mount preparation for the visualization of these vessels is described. Perivascular nerves supplying the vasa nervorum of various nerves in different species have been shown to contain noradrenaline, 5-hydroxytryptamine, substance P, vasoactive intestinal polypeptide, neuropeptide Y and calcitonin gene-related peptide. This is followed by a study of the changes in innervation seen in experimental and human diabetic nerves. Eight weeks following induction of diabetes, there was an increase in the density of NA-containing fibres innervating the vasa nervorum in the sciatic, vagus and paravertebral sympathetic nerve trunks. This was in marked contrast to a lack of NA-containing fibres within the optic nerve sheaths in the same animals. A lack of NA-containing perivascular nerves supplying vasa nervorum in a limited number of diabetic human sural nerve biopsies is also described.

The second part of the thesis examines the innervation of the major cerebral arteries during development and hypertension. Both these studies were confined to rat models. Following an initial post-natal increase in the density of innervation, it is shown that in the aging rat there is a decrease in the number of nerves containing

vasoconstrictor neurotransmitter substances and an increase in the those containing vasodilator neurotransmitter substances. In the spontaneously hypertensive rat, there is an increased density of perivascular nerves containing NA and NPY at four weeks of age. The possibility that such an increase in innervation, before the onset of marked hypertension and medial hypertrophy, may be important in the development and maintenance of hypertension is discussed.

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PREFACE

The aim of this thesis is to study the perivascular innervation of two distinct circulatory beds supplying nervous tissue, namely that of vasa nervorum supplying blood to nerve trunks and of the major cerebral arteries. The work carried out in this thesis was completed in 1986 and most of the experimental results were published at that time. Due to University regulations while I became a full time student of medicine at another university, and subsequent clinical commitments including my surgical examinations, I have been unable to submit this work until now. Although the experimental chapters presented here are in the form when they were published ten years ago, I am deeply aware of the discussions being outdated. As such I have updated all the relevant discussions, not only as 'Chapter Updates' at the end of each experimental chapter, but also in the final section of General Discussion.

As this thesis concerns two separate vascular beds and therefore two sections, I have for reasons of clarity, included introductory chapters to each section in addition to the main General introduction at the beginning.

The first part of the thesis addresses the perivascular innervation of vasa nervorum in various peripheral nerves, the autonomic sympathetic chain and in the optic and vagus cranial nerves in normal and diseased tissue. The presence of classical and putative neurotransmitter substances within nerve varicosities along the normal vasa nervorum is demonstrated in the rabbit and guinea-pig using histochemical and immunohistochemical techniques. An assessment of the developmental changes in the pattern of this innervation is made in the rabbit. This is followed by looking at the effect of diabetes predominantly in an experimental model. For this, the streptozotocin-induced diabetic rat model was used. This demonstrated a marked increase in the density of adrenergic perivascular nerve

induction of diabetes. This contrasted with a lack of adrenergic perivascular fibres in the optic nerve over the same time course. This finding was substantiated by measurements of noradrenaline using high-performance liquid chromatography with electrochemical detection. In a limited number of human sural nerve biopsies taken from diabetic patients, there is a lack of perivascular adrenergic innervation.

The second part of the thesis looks at the innervation of the major cerebral arteries of the rat in normal development and in the hypertensive state. The pattern and density of normal perivascular innervation by nerve fibres containing noradrenaline (NA), neuropeptide Y (NPY), vasoactive intestinal polypeptide (VIP, calcitonin gene-related peptide (CGRP), and substance P (SP), is assessed from birth through to 27 months of age. This demonstrated that with age there is an increase in the expression of neuropeptidergic vasodilator but not vasoconstrictor nerves supplying the cerebral arteries in the rat. The cerebrovascular innervation by fibres containing NA and NPY is also studies in hypertension. For this, the spontaneously hypertensive rat (SHR) model was used, and perivascular innervation was determined from 4 to 12 weeks of age. Not only did this study show an overall greater innervation pattern for both NA and NPY in the SHR animals, but more importantly highlighted a differential time course for the appearance of NA and NPY during development.

These findings are discussed with special emphasis on the plasticity of perivascular innervation, on the interplay between different nerve types, and their relevance to normal growth and disease processes.

CHAPTER 1

THE AUTONOMIC NERVOUS SYSTEM AND VASCULAR INNERVATION: GENERAL INTRODUCTION

1.1 HISTORICAL INTRODUCTION.

The earliest reference to the autonomic nervous system is attributed to the second century Greek physician Galen (AD 129 -200) who described the paravertebral sympathetic chain including the superior and inferior cervical and the semilunar ganglia. His descriptions, however, like the anatomical diagrams of Vesalius (1543) made some fourteen centuries later, were erroneous in assuming that the sympathetic and vagal trunks were a single structure arising from inside the cranium. This was finally corrected by Francois-Pourfour du Petit (1727), who also made the original observation of pupillary paralysis following cervical sympathectomy. In 1664, Willis introduced the important physiological notion of distinct voluntary and involuntary movements, the latter of which he considered as being initiated in the cerebellum. While the classical works of William Harvey (1628), describing a continuous circulatory system comprising the heart, arteries and veins, challenged the Galenic viewpoint of the cardiovascular system in which blood was assumed to ebb and flow, it was Whytt (1765) who initiated a similar parting from traditional thinking with regard to the nervous system. He claimed that "sympathy" presupposes feeling, which consequently must be dependent on nerves arising from the brain and spinal cord, and not via the flow of animal spirits through an anastomosing network of nerve tubes.

At the turn of the 19th century, biologists embarked on a more critical and scientific analysis of the way in which the human body interacted with environmental stimuli. Xavier Bichat (1771-1802) observed emotional responses to the environment in the circulatory, respiratory, and digestive systems and on the functioning of secretary glands. He, however, made a mistake in thinking of sympathetic ganglia as independent nerve centres where integration took place between somatic ("1a vie animale") and visceral ("la vie organique") functions.

The development and improvement of the microscope led to more precise anatomical descriptions of the structure of sympathetic ganglia (Ehrenberg, 1833; Valenti, 1836) and notably of the unmyelinated nerve fibres arising from them (Remak, 1838). Remak (1854) additionally described the white and gray communicating rami.

Samuel Luca's (1810) observation that branches from nerve trunks project to periarterial connective tissue and the vascular musculature, was the first description of an anatomical relationship between the nervous system and the circulation. Further studies by Goering (1834), quoted by Abraham (1969), clearly established the presence of perivascular nerve fibres. The extent to which these nerves penetrated into the vascular wall and whether or not they innervated the muscle layer was to remain until recently the central controversy over this anatomical relationship.

Physiological evidence to support a nervous influence upon the circulation came from studies by Val 'ter in the 1840s (quoted by Grigor'eva, 1962) who demonstrated the vasoconstrictor action of sympathetic nerves, and soon afterwards by Bernard and Brown-Sequard who independently in 1852 observed vasoconstrictor responses following sympathetic nerve stimulation. He went on to discover the presence of vasodilator fibres after eliciting vasodilatation of vessels supplying the submaxillary gland by stimulation of the chorda tympani (Bernard, 1878). The nervous control of the cardiovascular system provided Bernard with a mechanism to explain temperature regulation and a neurogenic basis for the homeostasis of tissue metabolism. He considered the stability of the internal environment of the organism, which he referred to as the "*milieu interieure*" to be "*...the primary condition for the freedom and independence of existence; the mechanism which allows for this is that which ensures in the milieu interieure the maintenance of all conditions necessary for life of the elements. ...These are the*

same conditions as are necessary for life in simple organisms; but in the perfected animal , whose existence is independent, the nervous system is called upon to regulate the harmony which exists between all these conditions".

Based on these new anatomical and physiological studies, Bernard was able to reject theory of ganglionic independence, and proposed that all sympathetic reflex activity was mediated through the spinal cord. His work on the submaxillary gland also gave the first indication for the presence of a dual antagonistic division of the autonomic nervous system. This idea was subsequently followed and developed by Gaskell and Langley.

Gaskell (1886) described the three outflows of finely myelinated small calibre nerve fibres from the bulbar, thoracolumbar and sacral levels, which connected peripheral ganglia to the neuraxis. He subsequently suggested the term "*involuntary nervous system*" (Gaskell, 1916) to describe the system of efferent nerve fibres situated outside the central nervous system, and which projected to involuntary structures.

Based on the action of nicotine on sympathetic ganglia, Langley (1893) described "preganglionic" and "postganglionic" neurones and went on to introduce the term "autonomic nervous system". The discovery of adrenaline by Oliver and Schafer (1895), and the finding that its action mimicked the response to thoracolumbar stimulation (Langley, 1901; Eliot, 1905) led Langley to distinguish this part of the spinal cord outflow (sympathetic) as separate from those arising from tectal and bulbosacral levels. Noradrenaline, which was found to mimic the actions of sympathetic nerves more accurately (Dale, 1914), was finally established as the neurotransmitter of the mammalian sympathetic nervous system by Von Euler in 1946.

The similarity in responses elicited by drugs such as pilocarpine, and those produced by tectal and bulbosacral stimulation led Langley to group this set of

autonomic nerves into the "parasympathetic" system (Langley, 1905). Chemical neurotransmission in the parasympathetic division was further advanced by the use of muscarine-like substances by Dixon in 1907 (quoted by Koelle, 1970) and the discovery of acetylcholine by Dale in 1914. In view of the lack of proof for a central connection of the myenteric and submucous plexuses, Langley had also introduced the term "enteric nervous system" and considered it to be separate from the autonomic nervous system. He additionally introduced the concept of receptors by suggesting that effector cells possessed excitatory and inhibitory "receptive substances".

New and improved techniques in fluorescence histochemistry (particularly immunohistochemistry), electron microscopy, electrophysiology and pharmacology has since led to a wealth of discoveries that have profoundly reshaped our understanding of the autonomic nervous system. The dramatic rise in the number of putative neurotransmitter substances which show a marked overlap in their localisation and function, represent a wide spectrum of physiological roles. These findings add newer concepts and further complexity to autonomic neuroeffector mechanisms and now demand a reappraisal of our definitions for subclassing neurohumoral agents into neurotransmitters, neuromodulators and trophic factors.

1.2 STRUCTURE OF THE VASCULAR NEUROEFFECTOR JUNCTION

Unlike the classical arrangement of the neuroeffector junction in skeletal muscle and that in ganglia, the model of the autonomic vascular neuroeffector junction based on that proposed by Burnstock and Iwayama (1971), is characterised by an extensive terminal branching of the autonomic nerve forming a two-dimensional perivascular 'autonomic ground plexus' confined to the adventitial-medial border (Hillarp, 1946; Burnstock, 1970). In this manner the whole muscle bundle and not merely a single smooth muscle cell becomes the effector. A special feature of this extensive terminal apparatus is the presence of varicose regions which are free of Schwann cell covering. The pearl-like varicosities (1-2 μm in diameter) of which there are a few hundred per millimetre in mammalian adrenergic terminals (Burnstock and Costa, 1975; Gabella, 1976) are separated by narrow inter-varicose regions (0.1-0.3 μm).

Neurohumoral substances stored in vesicles or granules within the varicosities are released 'en passant' during the conduction of nerve impulses and interact with receptors on the postjunctional membrane of the smooth muscle cells of the outer coat of the tunica media. The eventual change in membrane potential leads to the electrotonic spread of activity via specialised regions of low-resistance pathways termed 'nexuses' or 'gap-junctions' which interconnect and couple smooth muscle cells (Burnstock et al., 1980).

Although thickenings are sometimes shown in the prejunctional varicosity membrane, postjunctional specialisations as seen in skeletal neuromuscular junctions (Katz, 1966) and ganglionic synapses (Skok, 1973) are never seen in vascular neuroeffector junctions. The vascular nerve muscle junctional cleft is a variable feature ranging from 50-100 nm in some densely innervated small muscular arteries to as much as 2 μm in certain large elastic arteries (Burnstock, 1975;

Burnstock et al., 1980). In contrast to arteries, it appears that cleft width is independent of vessel diameter in veins (Rowen and Bevan, 1982). In densely innervated tissues like the vas deferens and iris this distance can be as little as 20 nm.

Although the structure of the autonomic ground plexus and the wide variation in cleft width makes it difficult to calculate the percentage of active varicosities during a single nerve impulse, the asymmetrical geometry suggests that the vascular neuroeffector junction is open to both pre- and postjunctional modulatory influences by both nerve-mediated, locally produced and circulating neurohumoral substances (for excellent reviews see Burnstock 1986a, 1987; Milner and Burnstock, 1995).

1.3 PATTERN OF PERIVASCULAR INNERVATION

Classical silver impregnation and methylene blue staining techniques (Woollard, 1937; Grigor'eva, 1962; Abraham, 1969) were limited in their specificity to basic descriptions of the presence of perivascular nerve fibres. It was not until improvements in microscope design and the development of more selective staining methods that an accurate assessment could be made regarding the pattern and distribution of perivascular nerves. The introduction of formaldehyde-induced fluorescence histochemistry (Falck et al., 1962) allowed for the first time the visualisation of a specific set of nerves, namely those containing biogenic amines. More recent modifications of this fluorescence histochemical technique, improvements in electron microscopy and the use of immunohistochemical methods have allowed further and more specific differentiation of perivascular nerve types. Our detailed knowledge of perivascular neuronal geometry, largely based on sympathetic NA-containing nerves, allows us to make certain generalisations about the pattern and distribution of vascular innervation.

The density of innervation is inversely related to the size of the vessel such that it is sparse in large elastic arteries and increases in density to reach a peak at the level of small arteries and large arterioles. Thickenings at sites of arterial branching known as 'intimal cushions' are also densely innervated as are some precapillary sphincters. Indeed, the degree of innervation appears to be dependent on the amount of resistance that a particular type of vessel represents in a given vascular bed. It is therefore appropriate that the small precapillary vessels which can account for upto 80% of total peripheral resistance and which represent the largest influence on local blood flow are the most densely innervated vascular structures. Similarly, pharmacological studies have shown that the neuronal NA uptake capacity and arterial diameter are also inversely related (Su et al., 1977). Nexuses are more

frequent (Bevan and Su, 1973) and the mechanism of electrotonic spread of excitation is more prominent (Burnstock and Prosser, 1960; Bevan and Ljung, 1974) in smaller arteries.

Although there is still much controversy about the innervation of capillaries, some studies suggest that at least in some areas these vessels may be innervated. There is evidence for sympathetic and non-sympathetic innervation of feline hypothalamic capillaries based on ultrastructural and light microscopical studies respectively (Rennels and Nelson, 1975; Rennels et al., 1979, 1983). Acetylcholinesterase (Ach)-positive axons have been shown to be in contact with cerebral capillaries in the turtle (Iijima, 1977), bullfrog (Tagawa et al., 1979) and the lamprey (Iijima and Wasano, 1980). There is also physiological evidence for innervation of brain microvessels by serotonin-containing fibres (Reinhard et al., 1979; Itakura et al., 1985). Substance P (SP)-, cholecystokinin (CCK)-, enkephalin (Enk)-, and neurotensin (NT)-containing nerve fibres have also been shown immunohistochemically to be closely associated with cerebral microvessels (Chan-Palay, 1977; Hendry et al., 1983; Kapadia and De Lanerolle, 1984). The immunohistochemical findings of α smooth muscle actin in pericytes and autoradiographic demonstration of microvascular adrenoceptors (Hakura et al ., 1985; Kobayashi et al., 1985) suggest the possibility of extrinsic neural regulation of capillary function with regard not only to microcirculatory haemodynamics but also to transcapillary transport processes. Although the nerve-muscle junctional cleft in these capillaries may be less than the neuromuscular distance in the intestine where the smooth muscle cells are considered to be influenced by transmitter release, the basis of morphological proximity alone can not be considered to imply the presence of synapses and confer functional transmission. While there is increasing evidence for the innervation of capillaries, it must be noted that at the level of the microcirculation, circulating and locally-produced agents probably play a greater

role than do perivascular nerves in the control of circulatory mechanisms (Burnstock and Griffith, 1983; Burnstock, 1990f, 1993; Burnstock and Ralevic, 1994; Ralevic and Burnstock, 1993, 1996).

The presence of α_2-adrenoceptors on endothelial cells (Cocks and Angus, 1983; Miller and Vanhoutte, 1985) has added further complexity to the mechanisms involved in vascular reactivity (Vanhoutte and Miller, 1989). While the α_2-adrenoceptors on the vascular smooth muscle elicit vasoconstriction upon activation, these same receptors on the endothelium lead to release of endothelium-dependent relaxing factor and thus cause an inhibition of the constrictor response. This endothelium-mediated inhibitory effect is more pronounced on α_2-adrenoceptor rather than α_1-adrenoceptor function, most likely due to the greater α_1 receptor pool (Flavahan and Vanhoutte, 1988). When administered intraluminally, α_2-adrenoceptor agonists actually cause endothelial-dependent vasodilatation in dog and rat perfused vessels (Matsuda et al., 1985).

The venous system, which in man can contain up to 70% of the blood volume, serves as an adaptable reservoir under the influence of perivascular sympathetic nerves. Although veins in general are less innervated, their basic innervation pattern is similar to that described for arteries. Thus large veins are sparsely innervated while some medium-sized muscular veins receive a fairly rich innervation. Collecting venules and small veins are sparsely innervated if at all (Furness, 1973). Veins which are exposed to high hydrostatic pressure loads are characterised by thickening of the smooth muscle media and dense sympathetic innervation. Veins located at or above the level of the heart, where capacitance effects are small, receive a sparse innervation. An exception to this generalisation is the dense innervation of intracranial veins (Edvinsson et al., 1971), a feature

possibly related to the importance of cerebral blood volume in regulating intracranial pressure.

The perivascular nerve fibres which make up the neural plexus around the media are not to be mistaken with larger paravascular nerves running along the outer adventitia of the vessel to innervate both vascular and non-vascular structures more distally. The presence of ganglion cells within the vessel wall as observed in feline aorta and coronary arteries (Grigor'eva, 1962), rabbit portal vein (Burnstock et al ., 1979), canine cerebral arteries (Borudulya and Pletchkova, 1976) and skeletal muscle arterioles (Honig and Frierson, 1976), appears to be a relatively rare occurrence.

1.4 NEUROMODULATION AND CO-TRANSMISSION.

Extensive comparative studies on the evolution of the autonomic nervous system (Burnstock, 1969) and the evidence for co-existence of biologically active substances in some invertebrate nerves (Brownstein et al., 1974; Cottrell, 1976) led Burnstock to question the validity of the principal that one nerve releases only one transmitter (Burnstock, 1976). The possibility that some nerves store and release more than one transmitter has now received abundant experimental support which strongly indicates that the majority of nerve fibres utilise several neuroactive substances, some with neurotransmitter roles while others may function as neuromodulators or trophic factors (Burnstock, 1980a, 1982a, 1983, Burnstock and Ralevic, 1996)). However for a substance to be referred to as a cotransmitter, it is necessary to show that it has a postjunctional action via its own specific receptor.

Neuromodulators are substances which influence neurotransmission either by action on receptors on the prejunctional varicosity membrane to alter transmitter release or by action on postjunctional receptors to affect the degree and time course of transmitter action on the effector cell. Neuromodulators can be any of a variety of substances from, circulating neurohormones, locally released agents (prostanoids, bradykinin, histamine and endothelin) and neurotransmitters from neighbouring nerve fibres (Burnstock, 1990a). There are a number of possible interactions through which cotransmitters and neuromodulators can effect neurotransmission.

1. Autoinhibition: The ability of a transmitter to modify its own release, in addition to its postjunctional effects. This interaction can in turn, effect the release of any cotransmitter.
2. Cross-talk: Whereby a neuromodulator acts on closely juxtaposed terminals.

3. Synergism: Each of two transmitters, either cotransmitters or from different nerve terminals have the same postjunctional effect so that there is a reinforcement of their individual effect.
4. Opposite actions: This is a rarer interaction where a transmitter can have opposite actions in different effector cells, or that the response may depend on the tone of the effector cell.
5. Prolongation of effect: Neuromodulators may act on degredative enzymes, for example, peptidases responsible for the removal of neuropeptides from the junctional clefts, to prolong the time course of their effect (Burnstock, 1987).
6. Trophic effects: A neurotransmitter may affect the expression of another transmitter or receptor within a population of neurons (eg. ganglia) at the level of gene transcription (Fontaine et al., 1986; New and Mudge, 1986).

The inhibition of transmitter release by NA, ACh and ATP via action on prejunctional α_2-adrenoceptors, muscarinic and P_2-purinoreceptors respectively are all examples of autoinhibition. The concept of 'cross talk' between different nerve types as seen in the inhibitory actions of ACh on responses to sympathetic nerve stimulation via prejunctional muscarinic receptors (Story et al., 1975) and that of NA-induced reduction of ACh release from parasympathetic nerves (Paton and Vizi, 1969) is supported by both pharmacological studies (Vanhoutte, 1974; Su, 1978) and by the close anatomical apposition of sympathetic and parasympathetic nerve varicosities which are often enclosed within the same Schwann cell sheath (Burnstock and Costa, 1975). Studies have also shown that opioid receptors on sympathetic nerve terminals of rabbit ear artery may serve to modulate NA release by presynaptic action (Fukuda et al ., 1985).

The demonstration of several vesicle types in ultrastructural examination of nerve profiles and recent pharmacological findings are consistent with the multitransmitter concept. However, further electron microscopical studies using specific cytochemical methods for labelling transmitters and related enzymes are needed before vesicle types can be identified as being characteristic of particular transmitter substances.

There is now increasing evidence not only for the co-existence of established transmitters with several peptides, but also for peptide/peptide interactions and co-transmission in perivascular nerves. For example the localisation of CGRP- as well as dynorphin-Ll in sensory and sympathetic perivascular nerves and ganglia (Rosenfeld et al., 1983; Gibbins et al., 1985; Lundberg et al., 1985; Mulderry et al., 1985) and the inhibition of SP release from primary sensory fibres by opiates and somatostatin (Lembeck et al., 1982), support the concept that many peptides serve as neuromodulators of various neurotransmitters in different types of perivascular nerves which are involved in regulating diverse aspects of cardiovascular function. It must be noted that the immunohistochemical evidence per se should not be necessarily interpreted as evidence for cotransmission.

1.5 PERIVASCULAR NERVE TYPES

In the past decade it has become clear from the overwhelming evidence of cotransmission and neuromodulation that we can no longer refer to autonomic nerves as 'adrenergic', 'cholinergic', 'purinergic', 'aminergic', 'peptidergic' or even 'non-adrenergic non-cholinergic'. In view of the observations of a remarkable pattern for colocalization of various transmitter substances, it is more appropriate to refer to these nerves as sympathetic, parasympathetic and sensory-motor nerves. This has greatly simplified the nomenclature of autonomic nerves. Additionally there are neurotransmitters in intrinsic neurons.

1.5.1 Sympathetic Nerves

Modifications of the Falck-Hillarp technique (Ajelis et al., 1979; Falck and Owman, 1965; Lindvall and Bjorklund, 1974; De La Torre and Surgeon, 1976; Furness et al., 1977) and more recently the use of specific antibodies against enzymes of catecholamine synthesis (Geffen et al., 1969) have led to a detailed knowledge of the neuronal geometry of NA-containing perivascular nerves. These nerves are the most prolific nerve type and represent the main neurogenic elements in the homeostatic regulation of vascular resistance.

Sympathetic nerves largely mediate vasoconstriction by the release of noradrenaline and its subsequent activation of adrenoceptors. Once limited to α- and β-receptor types (Ahlqvist, 1948) they have since been subdivided into α_1/α_2 (Langer, 1974) and β_1/β_2 (Lands et al., 1967) on the basis of pharmacological studies. Postjunctional α_1- and α_2-receptors are responsible for the pressor effects on vascular smooth muscle while prejunctional α_2-receptors are involved in the inhibition of noradrenaline release (McGrath, 1982, 1983).

While α-adrenoceptor-mediated pressor effects represent the main physiological role of the adrenergic system, vasodilatation upon activation of β-receptors has also been reported. Such vasodilatory responses to noradrenaline after α-receptor blockade has established the presence of β-adrenoceptors in cerebral, digital (Rose and Moulds, 1979), pulmonary (Boe et al., 1980), coronary, facial (Pegram et al., 1976; Mellander et al., 1982; Winquist and Bevan, 1981), skeletal (Lundvall and Jarhult, 1976) and hepatic circulations (Greenway and Lawson, 1966).

Studies on rat thoracic aorta (Allan et al., 1983), porcine and canine coronary, mesenteric and renal arteries (Cocks and Angus, 1983) and rabbit and cat cerebral arteries (Verecchia et al., 1985) show that NA can cause vasodilatation after α_1 - and β-adrenoceptor blockade, by the release of 'endothelium-derived relaxing factor' (EDRF) via activation of α_2-receptors.

A third type of adrenergic receptor, namely the γ-receptor, has been proposed to account for the pressor responses to electrical stimulation after α-receptor blockade (Hirst and Neild, 1980). However, due to a lack of specific receptor antagonists, the high concentration of noradrenaline needed to activate them, the persistence of prazosin-resistant responses after depletion of NA by reserpine, and the compelling evidence in support of release of adenosine 5'-triphosphate (ATP) as a co-transmitter with NA during sympathetic nerve stimulation (Sneddon and Burnstock, 1984a; Burnstock, 1985c), the γ-receptor theory still remains controversial (Sneddon and Burnstock, 1984b).

Perivascular NA-containing nerves originate from cell bodies located in pre- and paravertebral ganglia of the sympathetic nervous system. In addition to this peripheral source, cerebral blood vessels also receive NA-containing nerve fibres of central origin from the locus coeruleus and related cell groups in the lower brain stem (De La Torre and Surgeon, 1976).

Changes in perivascular NA-containing innervation have been reported in alcoholism (Low et al., 1975), amyloidosis (Rubenstein et al., 1983), orthostatic hypotension (Shy-Drager syndrome) (Bannister et al., 1981), subarachnoid haemorrhage (Lobata et al., 1980), diabetes (Scott et al., 1984) and hypertension (Lee and Saito, 1984a).

A review of sympathetic nerves is no longer complete without mentioning ATP and NPY. There is much evidence for cotransmitter roles for NA and ATP in the sympathetic nervous system (Su et al., 1971; Nakanishi and Takeda, 1973; Westfall et al., 1978; Burnstock, 1990b, 1996c). Although many of the early studies were made on the vas deferens (Swedin, 1971; Stjarne, 1989), many studies since have looked at the sympathetic cotransmission of NA and ATP in various vascular beds which include, rat tail artery , rabbit ear artery, dog basilar artery, mesenteric artery from a number of species, rabbit pulmonary artery, guinea-pig and rabbit saphenous artery, and in the rabbit hepatic artery (reviewed in Burnstock and Ralevic, 1994). Rat mesenteric resistance vessels, circulation of skeletal muscle, arterial resistance vessels of cat intestine, rat kidney, and the dog skin all have been demonstrated to show sympathetic cotransmission involving NA and ATP (Burnstock, 1990a,b; Burnstock and Ralevic, 1994). The exact proportion of ATP and NA that is released upon sympathetic stimulation, seems to vary for different tissues and species. This is illustrated by a substantial ATP component in the rabbit saphenous and mesenteric arteries (Burnstock and Warland, 1987; Ishikawa, 1985), in contrast to a small ATP component in the rabbit ear artery (Kennedy et al., 1986). In the guinea-pig sub-mucosal arterioles the functional effects of sympathetic nerves seem to be exclusively provided by ATP, while NA acts as a prejunctional neuromodulator (Evans and Surprenant, 1992). The degree to which each of these substances contributes to the neurogenic contraction appears to depend on stimulatory parameters: such that short bursts (1 sec) at low frequencies (2-5 Hz)

favours a purinergic component and longer periods of stimulation (30 sec or more) favour the adrenergic component (Kennedy et al., 1986).

NPY is also stored and released from most sympathetic nerves, including those supplying the heart and blood vessels (Lundberg et al., 1985b; Lundberg and Hokfelt, 1986). Ultrastructural studies on rat vas deferens and human atrial appendage have demonstrated a preferential localisation of NPY with NA and ATP in large dense-cored vesicles, while the small dense-cored vesicles, which are the main storage sites for NA and ATP, contained no NPY (Stjarne et al., 1986; Wharton and Gulbenkian, 1987). A preferential release of NPY is seen at high frequency intermittent bursts of sympathetic stimulation (Lundberg et al., 1986). In most blood vessels, NPY does not appear to act as a geniune cotransmitter, due to its little direct effect postjunctionally (Pernow et al., 1986). Its main role seems to be that of a neuromodulator by causing prejunctional inhibition of NA and ATP release (Wahlestedt et al., 1986; Ellis and Burnstock, 1990), and postjunctional potentiation of the effects of NA and ATP (Glover, 1985; Saville et al., 1990). There is evidence that the mode of neuromodulation effected by NPY is dependent on the width of the junctional cleft. A narrow cleft (20 nm eg. vas deferens) favors prejunctional modulation of cotransmitter release and widening the cleft to those present in most blood vessels (100 - 500 nm) and large elastic arteries (1000 - 2000 nm) increasingly favors a postjunctional modulatory role (Burnstock, 1990a).

A study of cutaneous blood vessels in guinea-pigs has demonstrated a different chemical coding for sympathetic nerves depending on the size of the blood vessel; in precapillary arterioles sympathetic nerves contain dynorphin (DYN) instead of NPY as a cotransmitter with NA; in smaller arteries, DYN, NPY and NA are all present; and in larger arteries NA and NPY only are present (Gibbins and Morris, 1990).

Sympathetic Cerebrovascular Innervation

Cerebral vessels are supplied with a rich plexus of perivascular sympathetic nerves. These include nerves containing NPY. The colocalization of NA and NPY has been demonstrated in these cerebrovascular nerves and relevant sympathetic ganglia (Everitt et al., 1984; Edvinsson et al., 1988b). Denervation and neural tracing studies have shown that these nerves originate mainly from the ipsilateral superior cervical and stellate ganglia with some bilateral projections (Edvinsson et al., 1972). There is some evidence for additional fibres originating from the locus coeruleus and related cell groups in the lower brain stem which project to small pial and intraparenchymal vessels (Edvinsson et al., 1973). It is presumed that these latter fibres may have an important role in blood-brain barrier functions, possibly via direct influence on vascular permeability.

NA, when applied directly, released by tyramine, or upon sympathetic stimulation, leads to α-adrenoceptor-mediated vasoconstriction of cerebral vessels (Edvinsson and Owman, 1974; Kuschinsky and Wahl, 1976).

Pial veins appear to be more responsive than pial arterioles to NA (Edvinsson et al., 1982a; Ulrich et al., 1982; Skarby et al., 1985). There is also a species variation in the type of postjunctional adrenoceptor present, such that in cat and pig the receptors are predominantly of α_2 type, whilst in rat, dog and man, they are of the α_1 type (Edvinsson, 1982).

NPY-mediated vasoconstriction of cerebral vessels which is similar in magnitude to that elicited by NA but is more sustained (is not altered by adrenoceptor antagonists nor by 5-HT blockers (Edvinsson et al., 1983b, 1984b). Its effects are however attenuated by calcium antagonists (Edvinsson, 1985).

The lack of significant effects cerebrovascular tone following denervation studies, and given the dense cerebrovascular innervation it supplies, the sympathetic nerves do not appear to play an important role in the regulation of resting cerebral

blood flow (Edvinsson and MacKenzie, 1976; Edvinsson et al., 1978b). It does however appear to play a role in extending the upper limit of the pressure autoregulation curve (Bill and Linder, 1976; MacKenzie et al., 1979b), and in regulating cerebral capacitance. Studies involving denervation and stimulation of these sympathetic nerves have shown these sympathetic nerves to alter cerebral blood volume, intracranial pressure and cerebro-spinal fluid collection (see Edvinsson and MacKenzie 1977b).

1.5.2 Parasympathetic Nerves

The distribution of perivascular ACh-containing nerves in the cardiovascular system is less extensive than that of sympathetic nerves. Unlike their constrictor role in poikilotherms, postganglionic parasympathetic nerves in mammals are largely vasodilatory. With the exception of pulmonary arteries of certain species (Daly et al., 1966), some veins (Grab et al., 1929) and human umbilical vessels, the presence of parasympathetic constrictor nerves is rare in mammals.

Histochemical and physiological studies have shown that ACh-mediated vasomotor control of parasympathetic origin is present in the brain, heart, kidney, bladder, salivary gland, tongue, and external genitalia (Burnstock, 1969, 1980b) while sympathetic cholinergic vasodilator fibres (Uvnas, 1966), once thought to be limited to a functional role in skeletal muscle) are also found in skin (Brody and Shaffer, 1970), uterus (Ryan et al., 1974) and possibly in the renal vasculature (McKenna and Angelakos, 1968) in certain species. In skeletal muscle, these

vasodilatory sympathetic fibres are involved in reflex changes in muscle blood flow during behavioral reactions to emotional stress (Abrahams and Hilton, 1958) and are not activated by postural changes or exercise (Roddie et al., 1957; Blair et al., 1961).

Histochemical demonstration of ACh-containing nerves has so far relied on the localisation of acetylcholinesterase (AChE), the degradatory enzyme of acetylcholine (Koelle and Friedenwald, 1949; Karnovsky and Roots, 1964). Although these nerves are very rich in AChE, this method is no longer accepted as being specific. Nevertheless, using the AChE stain, these parasympathetic nerves have been associated with both adrenergic and SP-containing nerves (Burnstock and Robinson, 1967; Eranko and Eranko, 1971; Chubb et al., 1980). There is also anatomical evidence for the enclosure of both NA- and ACh-containing nerve varicosities within the same Schwann cell sheath (Barajas and Wang, 1975; Burnstock and Costa, 1975). It has been claimed that ACh-containing nerves innervating blood vessels supplying skeletal muscle originate from sympathetic chain ganglia (Bulbring and Burn, 1935) and those of the uterine artery originate from the paracervical ganglia (Bell, 1974). Intracranial perivascular AChE-positive nerves which are not affected by superior cervical ganglionectomy (Edvinsson et al., 1972b; Kobayashi et al., 1981) are thought to originate from the sphenopalatine and otic ganglia (Hara et al., 1985), and parasympathetic nerve fibres innervating coronary vessels appear to originate from intrinsic cardiac ganglia (Osborne and Silva, 1970).

The development of a more specific method relying on the localisation of the enzyme choline acetyltransferase (ChAT) (Kasa et al., 1970) has so far proved largely inadequate for the demonstration of autonomic cholinergic neurons (Burnstock, 1979). An improvement in this method involves immunohistochemical use of specific antibodies raised against ChAT (Peng et al., 1981; Eckenstein and Thoenen, 1982) or choline storage vesicles (Dahlstrom et al., 1981).

Similar to the coexistence of ATP and NPY in sympathetic neurons, the parasympathetic system also exhibits colocalization of transmitter substances. The substance most widely associated with ACh is VIP. This was initially established by the experiments on cat exocrine salivary gland (Lundberg, 1981). These studies showed that VIP and ACh were stored in separate vesicles in the same nerve terminal. Both substances are coreleased upon transmural nerve stimulation, but the nature of transmitter is influenced by the stimulatory parameters, such that low-frequency stimulation primarily released ACh to elicit a degree of vasodilatation of the blood vessels within the salivary gland. At high-frequency stimulation the release of VIP was favoured. While VIP did not have a direct effect on the acinar cells, once released, it acts as a neuromodulator to significantly increase the postjunctional effects of ACh, as well as to increase the release of ACh by action on prejunctional receptors.

VIP and choline acetyltransferase (ChAT) have been colocalized in many perivascular nerves (Hara et al., 1985; Leblanc et al., 1987). Peptide histidine isoleucine (PHI) which is derived from prepro-VIP (Pernow et al., 1986), has a similar distribution as VIP and has been found to be colocalized with VIP/ChAT in parasympathetic perivascular nerves or parasympathetic ganglia which have vascular targets. The colocalization of VIP/ChAT with NPY (Leblanc et al., 1987; Leblanc and Landis, 1988), CGRP (Mione et al., 1990) and nitric oxide (NO: Bredt and Snyder, 1992) in nerves supplying some vascular targets has added further complexity to parasympathetic cotransmission.

Parasympathetic Cerebrovascular Innervation

A possible role for parasympathetic nerves in the regulation of cerebrovascular function was put forward more than 60 years ago (Chorobski and Penfield, 1932; Cobb and Finesinger, 1932). The presence of a substantial AChE-containing cerebrovascular nerve plexus was however only described two decades ago (Edvinsson et al., 1972b). In reference to the parasympathetic nerve supply to cerebral vessels, as explained before, we must discuss both Ach and VIP. There is now extensive evidence for the presence of VIP-LI nerves innervating cerebral vessels of many species (Larsson et al., 1976a; Edvinsson et al., 1980; Kobayashi et al., 1983; Matsuyama et al., 1983; Gibbins et al., 1984a,b; Suzuki et al., 1988a) including man (Edvinsson and Ekman, 1984). These VIP-LI nerves have also been shown to innervate intraparenchymal cerebral vessels (Eckenstein and Baughman, 1984; Chedotal et al., 1994). The origin of both Ach and VIP-containing fibres has now become clearer (see Dauphin and MacKenzie, 1995 for a detailed review of the parasympathetic innervation of cerebral vessels). VIP-containing fibres seem to originate from four sources: the sphenopalatine ganglion which contributes roughly a third of the VIP-LI innervation (Hara et al., 1985,1989; Brayden and Conway, 1988); the otic ganglion (Walters et al., 1986; Suzuki et al., 1988a); mini ganglion of the internal carotid artery (Suzuki et al., 1988); and from local intraparenchymal cortical VIP-containing neurons (Loren et al., 1979; Eckenstein and Baughman, 1984; Zhang et al., 1991; Chedotal et al., 1994).

Recent studies have clarified the position with regard to the colocalization of VIP and Ach. This coexistence was initially based on the basis of AChE histochemistry (Hara et al., 1985). However, the use of more selective markers have now revealed that apart from a fraction of the nerve population, VIP/AChE-containing fibres supplying cerebral arteries and pial vessels seem to be different from those containing ChAT-LI (Saito et al., 1985; Miao and Lee, 1990,1991).

There is also varying degree of Ach and VIP colocalization within cortical neurons which are thought to contribute a central element to the Ach/VIP-containing cerebrovascular innervation (Eckenstein and Baughman, 1984; Chedotal et al., 1994).

Both ACh and VIP cause significant vasodilatation of cerebral vessels including those of man (Edvinsson et al., 1977; Larsson et al., 1976a,b; Suzuki et al., 1984; Edvinsson and McCulloch, 1985; Jansen et al., 1992). The ACh effects are mediated via muscarinic receptors (Edvinsson et al., 1977) and those of VIP via specific VIP receptor (Suzuki et al., 1984). While the interaction of ACh with endothelial cells appears to be important for the vasodilatation of cerebral and systemic vessels (Furchgott and Zawadzki, 1980; Edvinsson et al., 1985), the vasodilatory actions of VIP in the cerebral circulation seems to be independent of endothelial mechanisms as evidenced by the persistence of its action following removal of the endothelial layer (Duckles and Said, 1982a;Edvinsson et al., 1985).

Similar to the sympathetic system, denervation studies of the parasympathetic supply to cerebral vessels have failed to demonstrate any significant change in the resting CBF (Branston et al., 1995; Suzuki et al., 1990). This raises the possibility that the parasympathetic innervation of cerebral vasculature may have a trophic role, or come into play during altered physiology such as in cerebral ischaemia or hypoxia (Koketsu et al., 1992).

1.5.3 Sensory-motor nerves

It has become clear that some autonomic nerves subserve a dual sensory-efferent function, whereby the same neurons which are sensitive to an external stimulus are also able to release a transmitter substance which then has postjunctional effect (Maggi and Meli, 1988). Two potent vasodilator substances, SP and CGRP, appear to be the principle transmitters of primary afferent nerves, and are known to coexist not only within the same perivascular nerve terminal (Gibbins et al., 1985; Lee et al., 1985a,b;Lundberg et al., 1985a; Uddman et al., 1985a), but also the same large granular vesicles (Gulbenkian et al., 1986; Wharton and Gulbenkian, 1987).

Capsaicin-sensitive sensory neurons have also been shown to contain other substances including neurokinin A, somatostatin and VIP (Maggi and Meli, 1988). CCK/CGRP/DYN/SP are also contained within unmyelinated perivascular nerves supplying guinea-pig cutaneous arterioles (Gibbins et al., 1987). There is additionally some evidence that ATP may also be a cotransmitter with SP and CGRP in sensory nerve terminals (Burnstock, 1990a, 1996c).

SP and CGRP have, in addition to their direct effects, pre- and postjunctional neuromodulatory interactions. CGRP is known to produce a long-lasting vasodilatation, but when injected together with SP into human skin, the vasodilatory action is only transient. This mechanism is thought to be secondary to the action of proteases released from mast cells as a consequence of SP-induced activation (Brain and Williams, 1988). CGRP is known to enhance the tachykinin-induced plasma extravasation in rat and rabbit skin (Gamse and Saria, 1985). Prejunctional regulation of sensory-motor nerve-mediated vasodilatation of the rat mesenteric arterial circulation has been demonstrated by adenosine (Rubino et al.,

1992,1993), opioid peptides (Li and Duckles, 1991; Ralevic et al., 1994), NPY (Li and Duckles, 1991; Kawasaki et al., 1991) and NA (Kawasaki et al., 1990a).

The precise nature of neurotransmitter coding, and the role each cotransmitting substance plays in neurovascular transmission within these sensory-motor nerves have yet to be elucidated (see Rubino and Burnstock, 1996 for an excellent review of sensory-motor neurotransmission in cardiovascular function).

1.5.4 Intramural nerves

The autonomic nervous system contains many intrinsic neurons (heart, lung, bladder and alimentary tract) whose identity has been demonstrated by extrinsic denervation studies and tissue culture techniques (Hassal et al., 1989; Springhall et al., 1990). While much is known about the intrinsic neurons in the myenteric and submucous plexuses of the alimentary tract (Furness and Costa, 1987), there is less understanding with regard to intrinsic cardiac neurons. Tissue culture studies have shown that intrinsic neurons from guinea-pig atria exhibit variable proportion of immunofluorescence for NPY and 5-HT (Hassal and Burnstock, 1984,1986), and that some of these neurons in situ project to form contact with small-resistance coronary vessels (Corr et al., 1990). It is probable that these two substances, which on their own have potent vasoconstrictor activity, may have synergistic action in perivascular neurotransmission. More recently, these intrinsic cardiac neurons from the guinea-pig heart have been shown to exhibit immunoreactivity for NOS (Hassal et al., 1992). The precise distribution of NO within neurones supplying projection to blood vessels, and its role in the complex interactions between transmitter and neuromodulator substances in the autonomic nervous system have yet to be determined.

Intrinsic neurons in other tissues are also known to supply perivascular nerves to local blood vessels. There is evidence for this in the cerebral circulation

(Burnstock and Griffith, 1983),and in the mesenteric and gut vessels (se Furness and Costa, 1987).

1.5.5 Purinergic Transmission

The potent vasoactive effects of purine nucleotides and nucleosides on the vasculature had been noted as early as 1929 (Drury and Szent-Györgyi, 1929). Investigations to identify the transmitter substance in non-sympathetic non-parasympathetic nerves supplying the gut and bladder led to the 'purinergic nerve hypothesis' (Burnstock, 1972) whereby ATP or a related purine nucleotide was proposed as the transmitter for these nerves. Since then, both vasodilator and vasoconstrictor effects of purine compounds, mediated via purinoceptors, have been reported extensively in the vasculature (Burnstock, 1980b).

Analogous to the subdivision of adrenergic, cholinergic and histaminergic receptors, purinoceptors have been classified into P_1 and P_2 (Burnstock, 1978, also see Table 1.1) mainly on the basis of selective actions of agonists and antagonists. P_1-purinoceptors have been further subdivided into A_1/R_i and A_2/R_a (Van Calker et al., 1979; Londos et al., 1980). The A_2 purinoceptor has subsequently been further divided into high- and low-affinity subtypes, A_{2a} and A_{2b} (Bruns, 1980; Daly et al., 1983; Jarvis etal., 1989; Stehle et al., 1992). Furthermore, two other subtypes, A_3 (Ribeiro and Sebastiao, 1986) and A_4 (Zhou et al., 1992; Cornfield et al., 1992) have also been suggested. The P_2- purinoceptor has also been classified, initially into P_{2x} and P_{2y} subtypes (Burnstock and Kennedy, 1985), and more recently the P_{2z}, P_{2t} (Gordon, 1986) as well as P_{2u} (O'Connor et al., 1991) and P_{2D} (Pintor and Miras-Portugal, 1993) subtypes have also been described.

The distribution of both P_1- and P_2-purinoceptors has been demonstrated in many vascular beds (Burnstock and Brown, 1981; Su, 1981). P_1-receptors, which are more widely distributed than P_2-receptors, mediate vasodilatation. These

receptors are also located on perivascular nerve terminals where they are involved in prejunctional inhibition of transmitter release (De Mey et al., 1979). P2-receptors are located exclusively postjunctionally and mediate both pressor and depressor responses (Kennedy et al., 1985), P2x-receptors mediating vasoconstriction and P2y-receptors mediating vasodilatation. Particularly in the microcirculation, purine-mediated vasodilatation appears to be mediated via the activation of P_{2y}-receptors located on the endothelial cells with the subsequent release of EDRF (Furchgott, 1981).

ATP-containing nerve fibres can be demonstrated by a fluorescence histochemical method using quinacrine or mepacrine binding. Quinacrine has been shown to bind strongly to ATP (Olson et al., 1976; Da Prada et al. 1978; Burnstock et al., 1979). The role of ATP as a cotransmitter in sympathetic and perhaps in sensory-motor nerves has been discussed in previous sections. It is becoming increasingly clear that purines have a far wider role in neuroregulatory mechanisms than was originally envisaged (Burnstock, 1985c; Burnstock and Kennedy, 1986).

There is mounting evidence for the involvement of purinergic mechanisms in the regulation of cerebrovascular function. Increased levels of purine nucleosides have been collected from brain tissue and cerebrospinal fluid during hyperaemia and hypoxia. The levels being high enough to activate both endothelial P_2-receptors and the P_1-receptors on the vascular smooth muscle to cause vasodilatation (Rubio et al., 1975; Winn et al., 1979; Berne et al., 1974). Both ATP and adenosine mediate marked relaxation of pial but not extracranial arteries (Forrester et al., 1979). ATP is 10 - 100 times more potent than adenosine in causing alterations of CBF at concentrations comparable to those released from electrically stimulated brain slices (Pull and McIlwain, 1972; Forrester et al., 1979). This action of purines on cerebral vessels does not in itself imply that they are released from nerves as transmitters. There is however abundant evidence from other studies that ATP is released from

sympathetic nerves (see Hoyle, 1994). The coexistense of ATP and NA in perivascular nerve fibres has now been demonstrated in a number of vessels including those of the cerebral circulation (Su, 1975; Head et al., 1977; Katsuragi and Su, 1980, 1982; Muramatsu et al., 1981; Levitt and Westfall, 1982; Sneddon and Burnstock, 1984b; Kugelgen and Starke, 1985). Autoradiographic studies using tritiated adenosine, which is taken up by a subpopulation of central neurons, has shown labelled nerve processes around cerebral blood vessels (Schubert and Kreutzberg, 1976). Histochemical studies using quinacrine (Crowe and Burnstock, 1984) and antibodies to adenosine deaminase and adenosine (Nagy et al., 1984; Braas et al., 1986) have revealed a population of neurons in some brain regions. While these studies suggest a neuronal release of purines, other important sources of ATP with regard to cerebral neurovascular transmission, are the endothelial cells (Burnstock and Kennedy, 1986) and platelets (Holmsen, 1985). The possible involvement of ATP in both the prodromal and the headache phase of migraine has been postulated (Burnstock, 1981a; Alafaci et al., 1988). For extensive reviews on purinergic mechanisms, including their role in the cerebral circulation, see Burnstock, 1993,1995,1996a,b).

1.5.6 Serotoninergic Transmission

Originally discovered as a vasoactive substance in blood serum (Rapport et al., 1948), serotonin (5-hydroxytryptamine; 5-HT), is a leading candidate for a non-adrenergic, non-cholinergic neurotransmitter in autonomic nerves. Autoradiographic (Chan-Palay, 1976, 1977; Amenta et al., 1985a), fluorescent histochemical, immunohistochemical (see Table 1.2), and pharmacological studies provide strong support for serotonin as a perivascular autonomic neurotransmitter.

Depending on the vascular bed and the concentration, serotonin can mediate both vasoconstriction and vasodilatation (Page, 1954; Vanhoutte et al., 1984). Serotonin induces vasoconstriction in most isolated large blood vessels, and it is particularly potent in the cerebral and coronary vasculatures.

Serotonin-induced vasoconstriction may take place by activation of postjunctional vascular 5-HT receptors, either by interaction with postjunctional α-adrenoceptors (Apperley et al ., 1976; Black et al., 1981) or by enhancement of pressor responses to other vasoconstrictor agonists (Van Neuten et al ., 1984) and sympathetic nerve stimulation (Moritoko and Su, 1981). The existence of two serotonin receptor types was proposed by Gaddum and Picare11i (1957). 5-HT responses blocked by morphine were suggested to be mediated by M-receptors, and those blocked by dibenzyline were mediated by D-receptors. A further class of serotonin receptors (5-HT_1/ 5-HT_2) were later described in the brain (Peroutka and Snyder, 1979). More recently, the use of molecular cloning technology has revealed a larger group of serotonin receptors. At present, seven distinct subfamilies the serotonin receptor (5-HT_{1-7}) are recognized, with the 5-HT-1, 5-HT-2 and 5-HT-5 each having five, three and two subtypes respectively. The remaining four subfamilies each have only one subtype to date (see Baez et al., 1995;Hamel, 1999). Pharmacological studies provide evidence that both 5-HT_1 and 5-HT_2-receptors mediate vasoconstriction (Cohen et al., 1981; Blackshear et al., 1985) including that of temporal arteries in man (Verheggen et al., 1998). Immunohistochemical and pharmacological studies have demonstrated the presence of 5-HT_{1B} receptors both in the smooth muscle and on the endothelium od human umbilical (Schoeffter et al., 1995) and cerebral vessels (Nilsson et al., 1999).

Vasodilatory actions of serotonin, which are particularly marked during elevated sympathetic tone, are mainly mediated via inhibition of adrenergic neurotransmission. This is achieved either by prejunctional inhibition via α_2-

adrenoceptors (Humphrey et al., 1983) of NA release or by activation of vascular β - adrenoceptors (Edvinsson et al., 1978a). Other inhibitory mechanisms of serotonin may involve the release of vasodilator substances such as vasoactive intestinal polypeptide, (Eklund et al., 1980) stimulation of prostaglandin production (Coughlin et al., 1981) or the activation of endothelial-dependent relaxations (Cocks and Angus, 1983).

Although serotonin has been shown to have vasomotor actions on various vascular beds, perivascular serotoninergic nerve fibres, with the exception of brain stem and spinal vessels (Di Carlo, 1977, 1981, 1984a, 1984b; Lamotte et al., 1982), human digital and mesenteric vessels (Griffith et al., 1982; Griffith and Burnstock, 1983a,b; Arneklo-Nobin and Owman, 1985; Cowen et al., 1986) have only been consistently localized in cerebral vessels (see Table 1.2).

Although we have numerous examples of the presence of 5-HT in perivascular nerve varicosities both in cerebral and systemic vessels, more recent evidence suggests that it is a 'false transmitter', in that it is taken up and stored for release as opposed to being and stored (Jackowski and Burnstock, 1989). If this is the case then the uptake mechanism seems to be influenced by denervation. Both 5-HT and DBH, which have been shown immunohistochemically to colocalize in cerebrovascular nerves, are lost following surgical sympathectomy (Chang and Owman, 1988). Thus it appears that only a subpopulation of cerebrovascular nerves, in this case sympathetic nerves, seem able to take up and store 5-HT. In contrast, there is considerable evidence to suggest that small pial vessels and intraparenchymal microvessels receive true 5-HT-containing nerve fibres originating from neurons in the raphe nuclei (Bonvento et al., 1994).

1.5.7 Peptidergic nerves

The presence of similar peptides throughout the animal kingdom, from man to primitive organisms such as protozoa and urochordates shows a remarkable preservation of these chemical substances during evolution. From possibly being simple mediators of cell-to-cell communication, the role of peptides seems to have evolved to that of hormones, neurotransmitters, neuromodulators and tissue factors.

The rapid advances in increasingly specific immunohistochemical methods and techniques for the synthesis and characterisation of peptides has revealed a widespread distribution of these substances in exocrine, endocrine and paracrine tissues. Notable is the demonstration of many peptidergic perivascular nerve types which strongly suggest that they have an important role in the control of cardiovascular function. This section reviews some of the major neuropeptides implicated in neurovascular control. Where appropriate, I have omitted from discussing coexistence of these substances in sympathetic and parasympathetic nerves as this has been mentioned above. There continues to be an emphasis on the relevance of these substances to neurovascular transmission in the cerebral circulation.

Tachykinins

The tachykinins represent a family of peptides which share a marked degree of C-terminal sequence homology and consist of substance P (SP), neurokinin A and neurokinin B (NKA and NKB). Substance P, the first 'brain-gut' peptide originally described by von Euler and Gaddum (1931) and later characterised as an undecapeptide (Chang et al., 1971), is now widely accepted as a

neurotransmitter of primary sensory neurons (Hokfelt et al., 1977, 1981) and has been demonstrated immunohistochemically in perivascular nerves of many vascular beds (see Table 1.3) As such they have been implicated in blood flow regulation and vascular pain (Moskowitz, 1984).

Perivascular SP-containing nerve fibres which are capsaicin sensitive (Duckles et al., 1982b; Papka et al., 1981; Barja et al., 1983) and unaffected by sympathectomy (Edvinsson et al., 1983c; Norregaard and Moskowitz, 1985), most probably originate from the sensory trigeminal (Liu-Chen et al., 1983; Yamamoto et al., 1983) and spinal ganglia (Barja and Mathison, 1984; Lundberg et al., 1985a).

The pattern of perivascular SP-containing nerve fibres shows an opposite trend to that of those containing vasoactive intestinal polypeptide (VIP) and noradrenaline (Furness et al., 1984a) such that larger arteries and veins have a greater density of innervation than smaller ones.

SP has potent vasodilatory (Hallberg and Pernow, 1975; Burcher et al., 1977; Edvinsson et al., 1981) as well as vasoconstrictor (Berube et al., 1978; Hellstrand and Jarhult, 1980) actions in many blood vessels. SP is reported to be involved in reflex vasodilatation (Burnstock, 1977; Lembeck and Hoizer, 1979; Gazelius and Olgart, 1980). Receptor blockers to SP, namely [D-Pro_7, D-Phe_7, Trp_9]SP and [D-Pro_7, D-$Trp_{7,9}$]SP, have been shown to block SP-induced and neurogenic vasodilation in the dental pulp, oral mucosa (Rossel et al., 1981), rat hindlimb (Lembeck et al., 1982), and SP-induced relaxation of cerebral arteries (Edvinsson and Uddman, 1982). On the other hand, chemical and ganglionic lesions and the lack of correlation between the density of innervation and the vascular responses to SP tend to support the concept that perivascular SP-LI fibres have a purely sensory function and do not mediate direct vasomotor influences. Studies in the rat show that the superior mesenteric artery which receives a dense SP-LI perivascular plexus is unresponsive to exogenous SP, while the carotid artery

which lacks SP innervation exhibits a dose-dependent relaxation (Barja et al., 1983). The direct action of SP on vascular smooth muscle receptors has also been challenged by studies showing SP-induced relaxation to be endothelium dependent (Hardebo et al., 1985; Zawadzki et al., 1981). These studies and the suggestion that SP fibres may serve to signal peripheral nerve damage (Wall and Fitzgerald., 1982), indicate that perivascular SP-LI nerve fibres do not play a role in direct short-term autoregulation of blood flow but that they may have an important influence on the regulation of vascular tone in response to noxious stimuli or during pathological conditions of the vasculature.

Both SP and NKA have been colocalized in capsaicin-sensitive (Duckles and Buck, 1982) cerebrovascular nerve fibres and the trigeminal ganglion (Edvinsson et al., 1987). Selective lesioning of the trigeminal ganglion results in the disappearance of SP-LI nerve fibres (Liu-Chen et al., 1983; Uddman et al., 1985). The origin of these fibres from the trigeminal ganglion has been supported by retrograde tracer studies (Yamamoto et al., 1983; Mayberg et al., 1984).

Both these substances induce concentration-dependent vasodilatation of cerebral vessels and these effects are blocked by tachykinin receptor antagonists (Edvinsson and Jansen, 1987). These vasodilatory responses of SP and NKA are endothelium-sensitive as shown by the disappearance of their effect after removal of the vascular endothelium (Edvinsson et al.,1985b; Edvinsson and Jansen, 1987). Further support for the interaction between sensory perivascular nerves and the endothelium in brain vessels has been shown by Milner et al., 1995).

Vasoactive intestinal polypeptide (VIP)

VIP, a 28-amino acid residue peptide structurally related to secretin, glucagon and gastric inhibitory peptide, which was first isolated from the porcine duodenum, is a potent vasodilator (Said and Mutt., 1970). Originally considered a classic gut hormone, VIP-like immunoreactivity (VIP-LI) has since been shown to be widely distributed in the body and especially in association with the cardiovascular system (see Table 1.4).

The pattern of perivascular VIP-LI nerve fibres closely resembles that of adrenergic and cholinergic systems (Furness et al., 1984b;Uddman et al., 1981a). VIP-LI fibres are more abundant in the cerebral than in peripheral blood vessels (Duckles and Said., 1982a; Larsson et al., 1976a; Uddman et al., 1981a) and for this reason most pharmacological and physiological studies on VlPergic responses have been carried out in the cerebral vasculature. These studies are described in more detail in Chapter 6.

Unlike the depressor actions of SP, ATP, acetylcholine (ACh) and bradykinin, VIP-induced responses are independent of an intact endothelium (Lee et al., 1984b; Hardebo et al., 1985). The failure of β-adrenoceptor, cholinergic and histamine H_2 blockers to antagonise responses to VIP (Lundberg et al., 1982) and a study showing a dose-dependent and reversible inhibition of these responses by an antibody to VIP (Brayden and Bevan, 1985) suggest that there are specific VIP receptors. Autoradiographic studies have localized VIP receptors in vascular smooth muscle in both human and guinea-pig lung (Barnes and Carstairs, 1985).

There is a marked reduction of VIP-containing perivascular nerve fibres in diabetic penile tissue in rat and man (Crowe et al., 1983), and in the cerebral circulation of diabetic rats (Lagnado et al., 1987). Conversely, elevated levels of VIP have been reported in perivascular nerves innervating the choroid in prediabetic and diabetic Chinese hamsters (Diani et al., 1985). Furthermore, the ability of VIP

to mimic many of the metabolic and cardiovascular symptoms of endotoxin shock (Freund et al., 1981) strongly implicates this peptide in vascular pathology. While we can confer an important role for VIP in the cardiovascular system, an understanding of its precise physiological function and any therapeutic advantages of manipulating VlPergic system will be evident with the use specific antagonists.

The role of VIP in cerebrovascular nerves has been discussed above under the section for parasympathetic nerves.

Neuropeptide Y (NPY)

The isolation of the 36-amino acid peptide NPY from porcine brain (Tatemoto et al., 1982) has added yet another substance to the growing list of putative non-adrenergic, non-cholinergic, peptidergic neurotransmitters. NPY, which is similar to pancreatic polypeptide (PP) and which shows a close sequence homology with avian PP, is present in a wide range of tissues as evidenced by immunohistochemical studies (Allen et al., 1985). Since the original studies showing NPY-induced vasoconstriction in the submandibular gland (Lundberg and Tatemoto, 1982) and NPY-like immunoreactivity and its action in cerebral vasculature (Edvinsson et al., 1983b), the localisation and vasomotor actions of NPY have been widely reported in the cardiovascular system (see Table 1.5).

The potent, calcium-dependent (Edvinsson et al., 1983b; Dahlof et al., 1985a), pressor action of NPY, both in vivo and in vitro, is resistant to combined α- and β-adrenergic blockade as well as to the serotonin antagonist ketanserin (Edvinsson et al., 1983a, 1984a; Corder et al., 1985b; Dahlof et al., 1985b). These pressor responses are potentiated by reserpine and markedly reduced by the calcium channel blocker nifedipine (Mabe et al., 1985).

Physiological studies have demonstrated NPY-induced reduction of cerebral (Allen et al., 1984a; Tuor et al., 1985) and splenic (Lundberg et al., 1985b)

blood flow, strongly implicating this peptide in the maintenance of vasomotor tone in normal and pathological conditions.

The distribution of perivascular NPY-containing nerve fibres is similar to that of adrenergic fibres and there is strong evidence that NPY and NA co-exist in the autonomic innervation to many vessels (Lundberg et al., 1982; Ekblad et al., 1984; Zhang et al., 1984; Glover et al., 1985; Sternini and Brecha, 1985; Uddman et al., 1985b). In the heart, NPY is reported to inhibit sympathetic neurotransmission via a mechanism on NA release, causing coronary vasoconstriction (Franco-Cereceda et al., 1985). There is also evidence for the coexistence of NPY with VIP and dynorphin II in the guinea-pig uterine artery (Morris et al., 1985). The actions of NPY in the perivascular nerves supplying cerebral vessels has been discussed above under the section for sympathetic nerves.

Calcitonin gene-related peptide (CGRP).

CGRP, is a 37 amino acid residue peptide which has been immunohistochemically localised in both the central and peripheral nervous systems, is the main product generated from calcitonin gene expression in non-thyroidal tissue (Amara et al., 1982; Rosenfeld et al., 1983). Perivascular CGRP-containing nerve fibres are distributed extensively in the cardiovascular system (see Table 1.6). The vascular innervation pattern of CGRP clearly resembles that of SP, and the two peptides have been shown to co-exist in peri- and nonperivascular tissues (Gibbins et al., 1985; Lee et al., 1985a; Lundberg et al., 1985a; Takami et al., 1985; Uddman et al., 1985a).

Systemic administration of CGRP results in a marked dose-dependent vasodilatation and a prolonged increase in heart rate in the rat, while

intracerebroventricular administration results in vasoconstriction, and increased plasma noradrenaline and adrenaline levels (Fisher et al., 1983; Franco-Cereceda et al., 1987) The mechanism by which CGRP acts in the central nervous system to modulate sympathetic and adrenomedullary activity remains unclear.

CGRP is a leading candidate for a non-adrenergic, non-cholinergic, peptidergic neurotransmitter in the neuronal systems in both central and peripheral nervous systems sub-serving both motor and sensory functions. These studies and the evidence that circulating CGRP comes from perivascular nerves (Zaidi et al., 1985), strongly implicate CGRP in the regulation of blood flow and in the processing of sensory information during vascular injury. The potentiating action of CGRP on histamine and bradykinin-induced oedema (Brain et al., 1985) and its central actions in inhibiting gastric acid secretion and in raising plasma noradrenaline levels (Tache et al., 1984), indicate that CGRP may also be involved in neurogenic inflammation and that it may also be an extracellular modulator.

The loss of perivascular CGRP-containing nerve fibres in guinea-pigs treated with capsaicin suggest that these nerves originate from sensory ganglia (Gibbins et al., 1985; Lundberg et al., 1985a; Terenghi et al., 1986a). CGRP-LI has been shown in the vagal and trigeminal ganglia, within the brain stem, and in co-existence with ChAT-LI in motor neurons of the facial, hypoglossal and ambiguous nuclei (Rosenfeld et al., 1983; Rodrigo et al., 1985; Takami et al., 1985). CGRP-LI is also present in enteric neurons (Clague et al., 1985; Gibbins et al., 1985) and there is evidence that some positively stained varicose fibres follow blood vessels in the submucosa. These intrinsic gut CGRP-containing fibres are resistant to capsaicin treatment. It would appear that CGRP-containing perivascular nerve fibres may have a sympathetic as well as sensory, motor and enteric origins (Gibbins et al., 1985; Terenghi et al., 1986b).

Using immunohistochemical techniques, CGRP has been abundantly localized in perivascular nerves supplying the cerebral vasculature (see table 1.5) and in the trigeminal ganglion from where it is shown to be released (Uddman et al., 1985a; Lee et al., 1985a; Rosenfeld et al, 1983; Mason et al., 1984). These studies have also demonstrated its colocalization with SP and NKA within cerebrovascular nerve varicosities as well as in the trigeminal ganglion.

CGRP is the most potent of all vasodilator neuropeptides so far identified in the perivascular nerve plexus of cerebral vessels, as evidenced by its effects in both in vitro and in situ studies (McCulloch et al., 1986). This relaxation is independent of the vascular endothelium (Hanko et al., 1985). CGRP may act as an emergency neurogenic system capable of an immediate and local reflex response in conditions where excessive cerebrovascular constriction occurs, such as in subarachnoid haemorrhage and migraine (McCulloch et al., 1986)

Other peptides.

A number of other peptides, besides those described above have also been demonstrated immunohistochemically, mainly along cerebrovascular nerves (see Table 1.7), albeit with a more limited distribution, and shown to induce vasomotor response.

1.5.8 Nitric Oxide Transmission

There is increasing evidence for nitric oxide (NO) having an important role in neurovascular transmission by virtue of its presence and release both from endothelial cells and autonomic nerves (see Lincoln et al., 1994 for a review). The first evidence of its importance in vascular neuroeffector mechanisms came from the pioneering work by Furchgott and Zawadski (1980) which demonstrated that the

acetylcholine-mediated relaxation of vascular smooth muscle was infact dependent on an intact endothelium. Since then, many other substances were found to act via endothelial receptors causing the release of endothelial-derived relaxing factor (EDRF). Several years later, two groups identified EDRF to be NO (Furchgott et al., 1987; Ignarro et al., 1987).

In the vascular system , mounting evidence points to the possibility that NO mediates its actions on smooth muscle via a nitrergic transmission as well as via endothelium-mediated interactions.

Numerous studies have now demonstrated the presence of NADPH-diaphorase activity and NOS-immunoreactivity not only in a subpopulation of intrinsic cardiac neurons (Hassal et al., 1992) but also localized in perivascular nerves supplying a number of different blood vessels including the cerebral vessels (Nozaki et al., 1993). In many instances it is shown to be colocalized with VIP (Kumner et al., 1992). NOS (neuronal isoform, type I)-immunoreactivity has also been demonstrated ultrastructurally in a population of perivascular nerves supplying the rat basilar artery (Loesch et al., 1994). Recently, there have been more examples of the localization of NO within cerebrovascular nerves. Ultrastructural studies on rat basilar arteries has shown the presence of two isoforms of NOS (types I and III) by demonstrating both neuronal and endothelial NOS-immunoreactivity (Loech and Burnstock, 1996). Barroso et al., (1996) have demonstrated the presence of NOS-immunoreactive perivascular fibres supplying the major cerebral arteries of guinea-pigs. They also showed that NOS is localized in nerves distinct from those expressing SP and TH; that NOS-immonureactivity is localized AChE-containing nerves; and that NADPH-diaphorase activity was seen in VIP-immunoreactive cells. Ultrastructurally, NOS activity is present in perivascular nerve varicosities. This study confirms earlier findings of the presence of NOS-immunoreactivity in the sphenopalatine ganglion where a population of neurons express VIP (Nozaki et al.,

1993). Some NOS-immunoreactive neurones were also demonstrated in the trigeminal ganglion, and sectioning the postganglionic fibres from both these ganglia causes a marked reduction (more marked in the spenopalatine ganglion) in the perivascular expression of NOS-immunoreactivity (Nozaki et al., 1993; Iadecola et al., 1993). There is some evidence for some NOS-immunoreactive fibres supplying the cerebral vessels to originate from central neurons (Poeggel et al., 1992; Regidor et al., 1993). Thus, central neurons expressing NOS may be able to regulate their own blood supply locally.

A developmental study on the ultrastructural distribution of the neuronal isoform of NOS in pulmonary vasculature of rats, suggests that NOS and endothelial cells play a role in the local modulation of vascular tone throughout their lifespan (Loech and Burnstock, 1996).

TABLE 1.1 PURINOCEPTOR SUBTYPES

P_1- Purinoceptors

	Distribution	Transduction Mechanism
A_1	Brain, Testis, kidney adipose tissue, heart	$\downarrow$cAMP, $\downarrow Ca^{2+}$, $\uparrow K^+$
A_{2A}	Brain	$\uparrow$cAMP
A_{2B}	Widespread, High in GIT	$\uparrow$cAMP
A_3	Testis. Widespread in some species	$\downarrow$cAMP
A_4		$\uparrow K^+$

P_2- Purinoceptors

	Distribution	Transduction Mechanism
P_{2X}	Ganglia, Smooth muscle brain, heart, spleen	Selective ion channel
P_{2Y}	Widespread	G-protein-coupled
P_{2Z}	Mast cells, macrophages	Non-selective pore
P_{2T}	Platelets, megakaryocytes	G-protein-coupled, $\downarrow$cAMP
$P_{2U(n)}$	Widespread	G-protein-coupled
P_{2D}	Chromaffin granules, brain	G-protein-coupled

From Burnstock, 1995

TABLE 1.2 Immunohistochemical and fluorescent histochemical demonstration of 5-HT-containing perivascular nerves

Tissue	Species	References
Brain	Man, monkey, cat, rabbit lamprey, guinea-pig, rat gerbil	1 - 11
Spinal cord	Monkey, cat, rat	12 - 14
Gastrointestinal Tract	Man, lamprey	9, 16,17
Limb vessels	Man	18

(1) Alafaci et al., 1986a; (2) Baumgarten, 1972; (3) Burnstock and Griffith, 1983; (4) Cummings and Felten, 1979; (5) Di Carlo, 1977; (6) Di Carlo, 1981; (7) Edvinsson et al., 1983a; (8) Felten and Crutcher, 1979; (9) Griffith and Burnstock, 1983b; (10) Iijima and Wasano, 1980; (11) Itakura et al., 1985; (12) Di Carlo, 1984a; (13) Di Carlo, 1984b; (14) Lamotte et al., 1982; (15) Griffith and Burnstock, 1983a; (16) Honma, 1970; (17) Arneklo-Nobin and Owman, 1985.

TABLE 1.3 Localisation of SP-immunoreactive perivascular nerves

Tissue	Species	References
Brain	Man, monkey, cat, rat guinea-pig, hamster	1 - 12
Heart and Systemic vessels	Man, monkey, dog, cat chicken, rat, lizard, frog, fish	7, 13 - 21
Respiratory tract	Man, cat, guinea-pig, rat	18, 22 - 28
Gastrointestinal tract	Man, cat, guinea-pig, rat chicken	13, 25, 30 -33
Hepatobiliary system	Rat, guinea-pig	13, 34 - 37
Spleen	Rat, guinea-pig	7, 13, 36
Kidney and Urogenital tract	Man, pig, dog, cat, rat, rabbit opossum, mouse	7, 13, 15, 3845
Skeletal Muscle	Rat, guinea-pig	7, 13, 15,36,41
Endocrine glands	Rat	46
Exocrine glands	Rat	47
Ear	Guinea-pig	48
Eye	Monkey, cat, rabbit, rat guinea-pig	49 - 51

(1) Alafaci et al., 1986b; (2) Chan-Palay, 1977; (3) Edvinsson and Uddman, 1982; (4) Edvinsson et al., 1981; (5) Edvinsson et al., 1983c; (6) Edvinsson et al., 1985a; (7) Furness et al., 1982; (8) Itakura et al., 1984; (9) Liu-Chen et al., 1983; (10) Matsuyama et al., 1984; (11) Simons and Ruskell, 1988; (12) Yamamoto et al., 1983; (13) Barja et al., 1983; (14) Dalsgaard et al., 1986; (15) Dhall et al., 1986; (16) Gibbins et al., 1988; (17) Helke et el., 1980; (18) Hokfelt et al., 1977; (19) Lundberg et al., 1985a; (20) Reinecke et al., 1980; (21) Wharton et al., 1981; (22) Baluk and Gabella, 1989; (23) Lundberg et al., 1980b; (24) Lundberg and Saria, 1982; (25) Polak and Bloom, 1980; (26) Saria et al., 1985; (27) Springall et al., 1988; (28) Sundler et al., 1977a; (29) Costa et al., 1980; (30) Costa et al., 1981; (31) Jessen et al., 1980 (32) Saffrey et al., 1982; (33) Shultzberg et al., 1980; (34) Barja and Mathison, 1982; (35) Barja and Mathison, 1984; (36) Galligan et al., 1988; (37) Goheler et al., 1988; (38) Alm et al., 1978; (39) Ferguson and Bell, 1985; (40) Forssman et al., 1982; (41) Furness et al., 1984a; (42) Mattiason et al., 1985; (43) Papka et al., 1985; (44) Stjernquist et al., 1983; (45) Wharton et al., 1981; (46) Hedge et al., 1984; (47) Ekstrom et al., 1988; (48) Uddman et al., 1982; (49) Cole et al., 1983; (50) Stone et al., 1982; (51) Terenghi et al., 1982.

TABLE 1.4 Localization of VIP-immunoreactive perivascular nerves

Tissue	Species	References
Brain	Monkey, cow, pig, dog, cat, rabbit hamster, gerbil, rat, guinea-pig, mouse	1 - 10
Heart and Systemic vessels	Cat, guinea-pig, rat	11 - 15
Respiratory tract	Man, dog, cat, rat	12, 16 - 21
Gastrointestinal tract	Man, monkey, pig, dog, cat, rat, mouse, chicken, frog, fish guinea-pig	11, 22 - 29
Hepatobiliary system	Man, pig, cat, rat, guinea-pig	30 - 33
Kidney and Urogenital tract	Man, monkey, pig, dog, cat, rabbit guinea-pig, rat, mouse ,chicken, frog, fish	11, 34 - 46
Skeletal Muscle	Cat, rat, guinea-pig	11, 47, 48
Endocrine glands	Cat, rat	49 - 52
Exocrine glands	Pig, cat, rat	53 - 54
Ear	Cat, guinea-pig	55, 56
Eye	Cat, guinea-pig, hamster, rat	36, 57, 58

(1) Alafaci et al., 1986b; (2) Edvinsson et al., 1980; (3) Edvinsson et al., 1982aa; (4) Edvinsson et al., 1985a; (5) Gibbins et al., 1984; (6) Hara et al., 1985; (7) Kobayashi et al., 1983; (8) Lindvall et al., 1978; (9) Matsuyama et al., 1983; (10) Lee et al., 1984; (11) Dhall et al., 1986; (12) Lundberg et al., 1980a; (13) Weihe, 1991; (14) Weihe et al., 1984; (15) Gibbins et al 1988; (16) Uddman and Sundler, 1979; (17) Uddman et al., 1978a; (18) Springall et al., 1988; (19) Geppetti et al., 1988; (20) Uddman et al., 1980b; (21) Dey et al., 1981; (22) Buchan et al., 1981; (23) Holmgren et al., 1982; (24) Jessen et al., 1980; (25) Larsson et al., 1976; (26) Reinecke et al., 1981; (27) Saffrey et al., 1982; (28) Schultzberg et al., 1980; (29) Uddman et al., 1978b; (30) Barja and Mathison, 1982; (31) Hellstrand et al., 1985; (32) Jarhult et al., 1982; (33) Sundler et al., 1977a; (34) Alm et al., 1980; (35) Crowe et al., 1983; (36) Diani et al., 1985; (37) Forssman et al., 1982; (38) Gu et al., 1983; (39) Larsson et al., 1977a; (40) Larsson et al., 1977b; (41) Mattiason et al., 1985; (42) Morris et al., 1985; (43) Papka et al., 1985; (44) Sternquist et al., 1983; (45) Strom et al., 1981; (46) Vaalasti et al., 1980; (47) Azanza and Gavin, 1986; (48) Polak and Bloom, 1980; (49) Grunditz et al., 1988; (50) Hedge et al., 1984; (51) Hokfelt et al., 1981; (52) Uddman et al., 1989; (53) Bloom et al., 1979; (54) Lundberg et al 1980b; (55) Uddman et al., 1979; (56) Uddman et al., 1982; (57) Terenghi et al., 1982; (58) Uddman et al., 1980e.

TABLE 1.5 Localization of NPY-immunoreactive perivascular nerves

Tissue	Species	References
Brain	Man, pig, cat, guinea-pig rat, gerbil	1 - 6
Heart and Systemic vessels	Man, monkey, dog, cat guinea-pig, rat	7 - 12
Respiratory tract	Man, cat, guinea-pig, rat	10, 13 - 15
Gastrointestinal tract	Man, pig, cat, guinea-pig, rat	10, 13, 16, 17
Hepatobiliary system	Man, cat, guinea-pig, rat	10, 13, 18
Spleen	Man, cat, guinea-pig, rat	10, 19, 20
Kidney and Urogenital tract	Man, pig, cat , rabbit guinea-pig, rat	8, 10, 13, 21-24
Skeletal Muscle	Man, dog, guinea-pig, rat	10, 13, 14, 20,25
Endocrine glands	Guinea-pig, rat	5, 16, 26
Exocrine glands	Man, cat, rat	8, 13
Ear	Cat, guinea-pig, rat	14
Eye	Guinea-pig, rat	27

(1) Edvinsson et al., 1983b; (2) Edvinsson et al., 1984a; (3) Edvinsson et al., 1985a; (4) Edvinsson et al., 1988b; (5) Schon et al., 1985; (6) Alafaci et al., 1986b; (7) Sternini and Brecha, 1985; (8) Lundberg et al., 1982; (9) Gu et al., 1984; (10) Ekblad et al., 1984; (11) Wharton and Gulbenkian, 1987; (12) Hassal and Burnstock, 1987; (13) Lundberg et al., 1983; (14) Uddman et al., 1984; (15) Springall et al., 1988; (16) Sundler et al., 1983; (17) Dahlstrom et al., 1988; (18) Allen et al., 1984b; (19) Lundberg et al., 1985a; (20) Lundberg et al., 1985b; (21) Adrian et al., 1984; (22) Mattiason et al., 1985; (23) Morris et al., 1985; (24) Papka et al., 1985; (25) Pernow et al., 1988., (26) Hedge et al., 1984; (27) Bjorklund et al., 1985.

TABLE 1.6 Localization of CGRP-immunoreactive perivascular nerves

Tissue	Species	References
Brain	Man, cat, guinea-pig, rat rabbit	1 - 6
Heart and Systemic vessels	Dog, cat, guinea-pig, rabbit, rat	7 - 13
Respiratory tract	Guinea-pig, rat	8,10,14
Gastrointestinal tract	Guinea-pig, rat	7, 11, 12, 15
Hepatobiliary system	Guinea-pig	10 - 12
Kidney and Urogenital tract	Guinea-pig, rat	7, 8, 11, 12
Skeletal Muscle	Guinea-pig	7, 11, 12
Exocrine glands	Rat	16
Eye	Monkey, cat, guinea-pig, rat	17

(1) Alafaci et al., 1986b; (2) Hanko et al., 1985; (3) Lee et al., 1985a; (4) Matsuyama et al., 1986; (5) Uddman et al., 1985a; (6) Wanaka et al., 1986; (7) Dhall et al., 1986; (8) Gibbins et al., 1985; (9) Gubelkian et al., 1986; (10) Lundberg et al., 1985c; (11) Mulderry et al., 1985; (12) Uddman et al., 1986; (13) Weihe, 1991; (14) Cadieux et al., 1986; (15) Clague et al., 1985; (16) Ekstrom et al., 1988; (17) Terenghi et al., 1985.

TABLE 1.7 Localization of peptides other than SP, VIP, NPY and CGRP in nerves associated with blood vessels

Peptide	Tissue	Species	References
Neurotensin	Brain	Rat	1
	Kidney	Man, monkey, pig, dog opossum, guinea-pig cat, rat	2
	Heart and associated vessels	Man, monkey, dog, guinea-pig, chicken, lizard, frog, fish	3, 4
Somatostatin	Kidney	Man, monkey, dog, pig, cat, guinea-pig, opossum	2
	Gastrointestinal tract	Cat, guinea-pig	5
Gastrin/cholecystokinin	Brain	Cat, guinea-pig, rat	8, 9
Gastrin-releasing peptide	Brain and spinal cord	Cat, guinea-pig, rat, mouse	10
	Urogenital tract	Rabbit, guinea-pig, mouse	11
	Ear	Guinea-pig	12
Enkephalin	Brain	Cat	7,13
	Gastrointestinal tract	Cat, guinea-pig, rat	9
Vasopressin-like peptide	Brain	Rat	1
	Liver	Rat	15
	Kidney	Mouse	15
Peptide histidine isoleucine	Brain	Cat	16
Galanin	Gastrointestinal tract	Man, guinea-pig, rat	19
	Urogenital tract	Guinea-pig	20

(1)Chan-Palay, 1977; (2) Forssman et al., 1982; (3) Reinecke et al., 1982a; (4) Reinecke et al., 1982b; (5) Dahstrom et al., 1988; (6) Hendry et al., 1983; (7) Moskowitz et al., 1984; (8) Lundberg et al., 1980a; (9) Schultzberg et al., 1980; (10) Uddman et al., 1983; (11) Stjernquist et al 1983; (12) Uddman et al ., 1982; (13) Kapadia and deLanerolle, 1984; (14) Jojart et al., 1984; (15) Hanley et al., 1984; (16) Edvinsson and McCulloch, 1985; (17) Ekblad et al., 1985; (18) Melander et al., 1985; (19) Moskowitz et al., 1985; (20) Morris et al., 1985.

1.6 NERVE TRUNK CIRCULATION

1.6.1 Historical Background

The presence and functional significance of vasa nervorum was noted as early as 1627 by van der Spiegel and subsequently by Ruysch (1701) and von Haller (1756). It was not until Isenflamm and Droeffler (1768) that these nerve trunk vessels received experimental scrutiny. By the turn of the century, details about their origin and pattern of distribution within the nerve trunk were assessed (Quenu and Lejars, 1890, 1892; Bartholdy, 1897) and attention was directed towards their physiological role as well as their response to injury. The effects of vascular abnormalities on peripheral, autonomic and cranial nerve function were not as yet appreciated. Even with the large number of nerve injuries during World War I, the possibility that vasa nervorum and nervi nervorum could be important in nerve regeneration was not considered until the works by Ramage (1927) who examined the blood supply of large peripheral nerves of the upper limbs in man. Nerve injuries during the second World War renewed interest in the study of vasa nervorum. Physiologists started to address for the first time their functional significance, and pathological changes in these vessels were thought to be responsible for some peripheral nerve disorders. While the importance of vasa nervorum in maintaining the integrity of nerve conduction is well established, their neurogenic regulation, pivotal in the control of the vascular tone and in responding to functional demands, has until now been neglected.

1.6.2 Anatomy Of Nerve Trunk Circulation

Origin and distribution of vasa nervorum:

The application of injection techniques using plastic dyes, improved histochemical methods, and microangiography have been instrumental in displaying the finer details of the anatomical distribution of vasa nervorum (Olsson, 1972; Nobel and Black, 1974; Bell and Weddell, 1984). The anatomy of the circulation of cranial, peripheral and autonomic nerves has been extensively reviewed by Sunderland (1978) and Pallie (1982) and will only be discussed in general terms in this chapter. An anatomical study of the sciatic nerve vasa nervorun in various animals has also been described (Bell and Weddell, 1984).

The term 'vasa nervorum' is used here collectively for all the blood vessels which enter and terminate within the nerve trunk. Additionally, a distinction can be made between the extraneural (nutrient) and intraneural vessels. The latter can be further subdivided into epineural, perineural and endoneural vasa nervorum depending on their distribution within one of the three connective tissue layers present in a nerve trunk. These intraneural vessels all arise from nutrient arteries. There are two types of nutrient arteries, the more common being those which originate as branches of the main limb arteries, and which provide a direct and exclusive blood supply to the nerve. Nutrient arteries that arise as branches of subsidiary arteries which primarily supply extraneural tissues (muscle and skin) are less common.

The origin of nutrient arteries is consistent for any particular nerve, but the exact take off from the main artery, their number, size and length vary considerably between species and are usually asymmetric on the two sides of the body (Sunderland,

1945a,b.c). The length of these extraneural arteries in man varied from 5 to 15 mm, although a length of 25mm has been reported for branches of the radial artery supplying the median nerve of the forearm (Sunderland, 1978). The variability in the length of the nutrient arteries implies that long segments of the main nerve trunk may be without external blood supply, but the circulation to these segments is maintained by descending intraneural vessels. It is thought that the size of the first nutrient artery or group of nutrient vessels determines the size and number of subsequent arteries along the nerve trunk (Sunderland, 1978).

Nutrient arteries reach the nerve trunk and distribute within it to create four distinct intraneural plexuses of vasa nervorum arranged concentrically, paralleling the long axis of the nerve. The first plexus is found on the surface of the nerve trunk, followed by the epineural, perineural and endoneural plexuses.

The epineural blood vessels consist of nutrient arteries which, after reaching the nerve, divide into ascending and descending branches communicating by an epineural plexus of longitudinally oriented vessels. Glomerular-like loops and arteriovenous shunts have been described within nerves (Lundberg and Branemark, 1968; Lundborg, 1970). Epineurial vasa nervorum are predominantly arterioles, but larger vessels are found especially in the median and sciatic nerves.

The perineural blood vessels have been examined ultrastructurally (Olsson and Reese, 1969), but these and other studies have merely confirmed the anatomical features described a century earlier by Key and Retzius (1876) and Ranvier (1871). These vessels, which mostly consist of arterioles and precapillaries, run an oblique course within the perineurium. This results in a valve mechanism which becomes shut by swelling of the fascicles (Lundborg, 1970).

The vascular anastomotic network between the different layers of the nerve trunk is very well developed and no particular direction of blood flow predominates in the endoneural vessels within any segment of the nerve (Lundborg, 1970) The direction of endoneural blood flow can change in response to nerve compression or damage to a regional nutrient artery.

Compared to skeletal muscle, the endoneural microvasculature has a surprisingly large calibre and wide spacing, the smallest endoneural capillaries having diameters of between 6 - 10um, while those in muscle have diameters between 3 - 6 um (Bell and Weddell, 1984). This allows for a considerable reserve capacity observed in the extensive intraneural microcirculation whereby only a fraction of the circulatory network is functioning during normal conditions. A large number of other vessels are recruited following minor insults to the nerve (Lundberg, 1979).

Except for the decrease in the density of endoneural capillaries with age, due to advancing myelination and consequent separation of capillaries (Lang, 1962), there are no postnatal developmental changes in the vasculature of peripheral nerves (Sunderland, 1978).

The perineurium consists of layers of flattened cells with their basement membranes on both sides which are in close apposition, particularly toward the pointed ends of the cells. A fibrous outer layer encircles perineural cells and gradually merges into the connective tissue of the epineurium. An interfascicular plexus of arterioles, capillaries and venules is found within the perineurium.

The anatomy of veins of peripheral nerves is not well understood as most studies have concentrated on arterial and capillary networks. It is suggested that venous drainage accompanies the intraneural arterial pattern and that veins emerge from the nerve trunks at the sites of arterial entries.

The structure of vasa nervorum:

Vasa nervorum are composed of the same elements as other blood vessels, but their ultrastructural features are less well defined. Endothelial tight junctions have been found at points of cellular contacts (Olsson and Reese, 1969). Vasa nervorum are encircled by connective tissue containing substantial extracellular space.

Nutrients from the circulation must pass through the vessel wall, enter the extracellular space and penetrate basement membranes of Schwann cells before reaching their destination in nerve fibres. This arrangement is quite different from that in the central nervous system where the extracellular space is small and glia are closely apposed to the vessel wall.

Diffusion barriers:

Tight junctions between endothelial cells of the endoneural capillaries confer an important barrier to the passage of many molecules, particularly proteins. Although epi- and perineural vessels do not have such tight junctions and allow leakage of many substances into the surrounding tissues, the perineurium itself acts as an effective diffusion barrier. This has been demonstrated using tracer techniques whereby many substances are unable to penetrate the perineural layer when applied externally (Olsson and Reese, 1969; 1971; Lundborg, 1970). This function is attributed to the innermost layers of the perineural lamellae where tight junctions are present between individual perineural cells. These cells exhibit a rich activity of a number of dephosphorylating and oxidative enzymes, and ATP has been demonstrated in micropinocytic vesicles as well as in the basement membranes of perineural cells (Shantaveerappa and Bourne, 1966). These studies suggest that the perineural membrane constitutes a metabolically active diffusion barrier.

As blood vessels enter the endoneurium, they are invested by a short sleeve of perineural tissue which is thought to play an important role in maintaining the blood-nerve-barrier (Olsson, 1972; Rosenberg et al., 1984). Distally, the close apposition of endothelial cells within the endoneural capillaries further aid in protecting the endoneurium and nerve fibres contained within from external influences. Experimental dyes, proteins and even diphtheria toxin have shown the effectiveness of this barrier (Waksman, 1961). Intravenously injected peroxidase does not pass from the blood stream into the endoneural compartment. Similarly, the mast cell degranulator compound 48/80 (Olsson, 1968) is able to degranulate the mast cells within the nerve trunk except for those within the endoneurium. This can only be achieved by direct endoneural injection of the compound.

Nerve blood flow:

The above sections have described the microvascular anatomy and structure of the vasa nervorum. This section aims to outline our knowledge of blood flow within nerve trunks. Intravital microscopic studies in rabbit tibial nerves suggest that there is no specific direction of blood flow which predominates any particular segment of the nerve trunk (Lundborg and Branemark, 1968). Manipulation of the nerve appears to alter the direction of blood flow. Interestingly, at normal conditions, only part of the nerve trunk vasculature was seen to be working, while the remainder could be recruited with application of warm saline or local trauma.

An adequate supply of nutrients and oxygen is esential for the normal functioning of nerves. Ischaemia leads to a rapid deterioration in nerve function, and there are numerous studies which show a consequent reduction in nerve conduction velocity as well as impairment of the blood-nerve barrier with oedema formation

(Lundborg, 1970; Roberts, 1948; Sjostrand et al., 1978; Low et al., 1989; Parry and Linn, 1986). Axonal transport is also partially or completely blocked by local ischaemia or nerve compression (Minckler et al., 1976; Quigley and Anderson, 1976). Unless prolonged, the deterioration in nerve function resulting from a period of ischaemia is normally recoverable upon restitution of the blood supply. Controversy however, surrounds the issue of how much devascularization a nerve can withstand before its function is significantly affected. Ligation of the gluteal artery or segmental sciatic nerve nutrient arteries in rabbits has been shown to cause marked sciatic nerve degeneration and diminished nerve function (Okada, E, 1905; Causey and Stratman, 1953). In contrast, similar studies by Adams (1943) in the rabbit sciatic nerve revealed only minimal changes in ths sciatic nerve. Other studies in dogs and cats, also show a resistance to nerve dysfuction upon ligation of nutrient arteries (Bentley and Schlapp, 1943; Deenny-Brown and Brenner, 1944). Even in the clinical setting, where Bateman (1962) states that the intraneural blood supply is sufficient on its own to fully maintain the nutrition and function of a nerve trunk, a contradictory opinion is put forward by Bunnell (1948). Further studies using vital microscopic techniques have helped in resolving this controversy. There appears to be a critical ratio of 1:45 between width and length, such that within this ratio all external blood supply can be interrupted and the intrinsic anastomosing system is capable of maintaining adequate intraneural microcirculation (Roberts, 1948; Lundborg and Branemark, 1968; Lundborg and Sweden, 1975).

The perivascular innervation of the vasa nervorum is briefly mentioned in the next section, and fully discussed in chapters 3 and 4, both of which address the issue of neurogenic control of the nerve trunk vasculature.

Innervation of vasa nervorum:

The distribution of nerves in the connective tissue layers of nerve trunks have received much interest in the past (see Hromada, 1963) using histochemical stains. These studies identified the presence of both nervi nervorum and nerves running along vasa nervorum. Evidence for a sympathetic innervation of the vasa nervorum comes from the marked vascular responses seen in the tibial nerve of the rabbit upon stimulation of the lumbar sympathetic chain (Lundborg, G. (1972). However since then, this has received little attention. Given the ever increasing number of neurotransmitter substances which have been recognized and the evidence for important roles for them in the context of neurovascular transmission, it is surprising that the nerve trunk circulation has not yet received enthusiastic scrutiny in an attempt to identify the nerves innervating vasa nervorum.

1.7 HYPERTENSION

1.7.1 Introduction

A satisfactory definition of hypertension remains difficult with ongoing controversy regarding the dividing line between what is normotensive and hypertensive. It is a feature of some underlying pathology with probable multiple aetiological factors and represents a quantitative rather that a qualitative diagnosis, such that elevated blood pressures are related to increased morbidity and mortality. The major morbidities include heart disease (coronary artery disease, congestive heart failure and left ventricular failure), cerebral infarction and haemorrhage, and renal failure. These are in turn strongly associated with several arterial pathologies in the hypertensive state, namely; atheromatous or nodular arteriosclerosis; intracerebral microaneurysms; fibrinoid necrosis of the small arteries and arterioles; and more generalised arteriosclerosis.

Atheromatous or nodular arteriosclerosis is characterized by the presence of fibrofatty plaques which increase the risk of thrombus formation and thereby lead to ischaemic or embolic events predominantly in the cardiac circulation and less frequently in the cerebral, renal and limb supplying arteries. Intracerebral microaneurysms (Charcot, 1868, 1879) which result from an abnormality of the media in the hypertensive state are a significant cause of cerebral haemorrhage particularly in the elderly (Cole and Yates, 1967). Fibrinoid necrosis of the small arteries and arterioles which primarily affects the young, was first recognized by Fahr (Fahr, 1919), and is characterized by the exudation of plasma into the arterial wall which subsequently causes luminal stenosis or blockage. This reduction in luminal diameter has been shown to be reversible upon control of

arterial pressure in experimental animals (Allison et al, 1967). It is a major factor in the development of malignant hypertension, the predominant feature of which is that of renal failure.

Hypertension is classified either with respect to absolute values of arterial blood pressure (National Institute of Health, 1993) or by virtue of its underlying pathology. The latter can be subdivided into conditions which cause the elevation of systolic blood pressure only, and those causing elevation of both diastolic and systolic pressures (Ellis, 1942). The latter category also includes the classification of essential hypertension where there is no identifiable cause.

1.7.2 The sympathetic nervous system in hypertension

There is a vast wealth of literature implicating a role for the sympathetic nervous system in both clinical (De Quattro & Miura, 1973; DeChamplain, 1978; Victor & Mark, 1995) and experimental (Bevan et al., 1975; Lee and Saito, 1984a; Head, 1991) hypertension. It is unclear whether alterations in the sympathetic nervous system are primary or secondary events in the pathophysiology of hypertension. This is made more complex by the multifactorial aetiology in hypertension alluded to earlier, and therefore a precise role for the sympathetic nervous system remains elusive.

Several studies demonstrate an increased sympathetic vasoconstrictor tone in skeletal muscle of hypertensive subjects which result from increased sympathetic nerve discharge and subsequent raised plasma noradrenaline levels (Goldstein, 1983; Floras and Hara, 1993; Egan et al., 1987). Furthermore, this increase in the sympathetic nerve activity appears to be more marked in patients with accelerated essential hypertension as compared with those with less severe hypertensive states (Matsukawa et al., 1993).

Despite these findings, the importance of the sympathetic nervous system in the development of sustained hypertension has been challenged (Abboud, 1982), given the lack of hypertension in numerous states with enhanced sympathetic nerve activity such as heart failure and cirrhosis. However, these challenges have been made with, what is now in retrospect, a simplistic analysis of the sympathetic nervous system confined to alterations in catecholamines and α-adrenoceptor mediated vascular neuroeffector mechanisms. The increased prevalence of hypertension in the elderly population and a decreased incidence in the female gender (Ng et al., 1993), as well as racial differences (Whelton and Klag, 1989) would suggest, in addition to any genetic predisposition, a trophic role for the sympathetic nervous system. The considerable complexity of the autonomic nervous system as described earlier in this chapter, with ever increasing substances which appear to have a role in modulating sympathetic nerve activity, means that our efforts must be directed towards other neuroeffector mechanisms than simply on classical adrenergic neurotransmission.

1.7.3 Cerebrovascular circulation in hypertension

The cerebrovascular endothelium which confers the bood-brain barrier, and the cerebral capillary density which is matched to meet the local blood flow and metabolic needs (Kuschinsky and Paulson, 1992), both serve to maintain a homeostatic microenvironment with regulatory mechanisms for controlling cerebral blood flow. Most important of these is the autoregulatory one, which is very well developed in the cerebral circulation such that over a considerable range of arterial pressure, or more correctly over a range of perfusion pressure, cerebral blood flow remains constant. With falls in perfusion pressure, there is vasodilatation of the resistance vessels maintaining

constant flow concomitant with increased oxygen extraction from the blood (Haggendal and Johansson, 1965; MacKenzie et al, 1979a), but only upto a minimal critical pressure. Below this, autoregulation fails. Similarly, with increase in the perfusion pressure there is a tendency for the resistance vessels to constrict as a protective mechanism. However, above the upper threshold of autoregulation, these vessels are unable to adjust flow thereby allowing for forceful structural changes which then predispose for cerebral insults (Skinhoj and Strandgaard, 1973; Strangaard et al., 1974; MacKenzie et al., 1976). Other mechanisms for maintaining constant cerebral blood flow include a chemical regulatory process coupled with the arterial CO_2 tension and thus the local pH , and a metabolic process (Tominaga et al., 1976; Wahl et al., 1970).

Although there is evidence that the sympathetic nervous system does not affect the autoregulatory process over its physiological range (Harper et al., 1972; Busija et al., 1980), it does shift the autoregulatory range to the right with elevated systemic blood pressures (Bill and Linder, 1976; Heistad and Marcus, 1979) thus protecting the brain from injury consequent to disruption of the blood-brain barrier or from hypertensive structural changes.

The sustained or chronic hypertensive state is associated with structural alterations which are both adaptive and degenerative, which include arteriosclerosis, medial hypertrophy and luminal narrowing, as well as areas of medial weakness which predispose the formation of microaneurysms. These morphological features and the rightward shift of the autoregulatory mechanism in hypertension, mean that despite similar absolute values of cerebral blood flow as in normotensive subjects (Kety et al., 1948; Strangaard and Paulson, 1994), there is a diminished capacity for adaptive vasomotor response by the resistance vessels at the extremes of cerebral perfusion pressures.

1.7.4 Experimental models of hypertension

Given the likelihood of a multifactorial aetiology in the pathogenesis of clinical hypertension, there is great difficulty in defining what are the important genetic, biochemical and physiological mechanisms to be assessed in animal models. Even with improved classifications of hypertension and therefore the ability to subdivide the hypertensive population, the lack of our present knowledge regarding the pathogenesis of this disease warrants caution in extrapolating animal data to the bedside. With this caveat, the experimental study of hypertension in animal models has proved to be extremely valuable, particularly in trying to overcome the problem of aetiological and genetic heterogeneity in the clinical setting.

There are at least eight animal strains which are used as models for studying various forms of genetic hypertension (see Lovenberg 1987 for an extensive review). This excludes numerous other non-genetic models where hypertension is established by interfering with renal or adrenal mechanisms. In view of the rapidly advancing techniques in probing for genetic abnormalities, the hope is to be able to marry the observed biochemical and physiological changes in these hypertensive models with precise abnormalities at the molecular level. In the study described in this thesis, the spontaneously hypertensive rat (SHR) model was used to assess the cerebrovascular innervation in hypertension. There is evidence in this model, for an increased sympathetic activity (Judy et al., 1976; Head, 1991). Findings of increased noradrenaline in nerve terminals and a greater density of noradrenergic nerve fibres in the sympathetic nervous system (Mangiarua and Lee, 1990), as well as an enhanced vasoconstrictor response to sympathetic nerve stimulation (Lais and Brody, 1978; Lokhandwala and Eikenburg, 1983) further support this association. Additionally, the

prevention of hypertension developing in young SHR animals following neonatal sympathectomy, with less structural vascular changes in later adulthood also strongly implicate the sympathetic nervous system in the pathogenesis of hypertension (Lee et al., 1987). Other relevant aspects of this relationship between hypertension and the sympathetic nervous system, particularly with reference to the cerebral circulation is discussed in Chapter 6.

1.8 DIABETES

1.8.1 Introduction

Historical descriptions of diabetes can be found in texts as early as the 15th century B.C. (Ebers papyrus), and the sweet taste of diabetic urine is mentioned around the 4th century B.C. in Indian medical history. The first complete description of diabetes was made in the first century A.D. by Aretaeos of Kappadokia of Asia Minor. He gave the disease its name diabetes (Gk: "I pass through") in describing the copious volume of urine which is produced. It was not until the 17th century that Willis differentiated between two forms of the disease by the presence or lack of sweet urine. The term 'mellitus' (Lt: "honeyed") was added a century later by Dobson who identified sugar as being responsible for the sweet taste. An excellent historical account of diabetes can be found in Medvei's text on the history of endocrinology (Medvei, 1993).

Diabetes is divided into two major forms, juvenile onset insulin-dependent (IDDM) and maturity or adult onset non-insulin-dependent (NIDDM) disease. Although there are differences between the two forms with respect to the prevalence of various diabetic complications, this thesis will refer to diabetes in general without distinction. Despite the availability of animal models which appear to reflect abnormalities to specific diabetic groups in the clinical situation, our limited knowledge of the pathogenesis of diabetic neuropathy makes it difficult to extrapolate the majority of experimental laboratory data to any one form of the disease. The complex and heterogenious clinical conditions arising from variable abnormalities of insulin resistance and pancreatic beta-cell function raises the possibility that we may need a more detailed subclassification of the disease than solely on the presence or not of

insulin-dependence. Furthermore, diabetes itself should be recognized as being more complex than merely in relation to an abnormality of glucose metabolism. It represents a clinical syndrome characterized by large and microvascular disease, as well as neuropathy in addition to the background hyperglycaemic state.

1.8.2 Diabetic neuropathy

Isolated descriptions if diabetic neuropathy with respect to vasomotor and trophic skin abnormalities were noted towards the end of the last century. It was not until 1936 that diabetic neuropathy became a recognized pathology (Jordan, 1936), and gained wider acceptance after detailed descriptions by Rundles (1945).

Diabetes commonly affects the peripheral nervous system, and the ensuing abnormalities which are responsible for significant morbidity, have been the focus of extensive efforts to elucidate the underlying mechanisms involved in this form of neuropathy. While a greater prevalence of the clinically apparent disease is seen with an expanding elderly population, the prevalence of subclinical diabetic neuropathy has also increased with more sophisticated diagnostic tools.

There is growing evidence for a role for the autonomic nervous system in diabetes. In the cardiovascular system, a major feature of diabetic autonomic neuropathy is postural hypotension which is defined as a 30 mmHg fall in the systolic blood pressure from supine to the standing position. It is thought to be secondary to efferent sympathetic denervation of the splanchnic bed (Stevens et al., 1991). Another feature is the heart rate which may remain fixed despite changes in respiration, posture

and on gentle exercise (see Ewing, 1988). The neuropathy has also been implicated in the increased incidence of painless myocardial infarction in the diabetic population (Niakan et al., 1986), in whom electrocardiographic abnormalities are also more prevalent (Khan et al, 1987). Diabetics also exhibit deranged cardiovascular circadian rhythms with respect to heart rate and blood pressure (Hornung et al., 1987; Felici et al., 1991). Diabetic neuropathy is also thought to play a role in peripheral vasomotor control, sudomotor abnormalities, bladder and gastrointestinal dysfunction (see Spallone et al., 1995). A more detailed account of the probable pathogenesis of diabetic neuropathy particularly with respect to microvascular changes in peripheral nerves appears later in this section.

A satisfactory classification of diabetic neuropathy has been hampered by the multiple peripheral nerve disorders with varying symptomatology and the probable multifactorial mechanisms which may be involved in its pathogenesis. This issue has been aided by the recent recommendations for a suitable classification (Thomas, 1997; Sima et al, 1997). This basically divides diabetic neuropathy into three broad categories: (i)- Rapidly reversible (hypergycaemic) neuropathy, (ii)- Persistent symmetrical polyneuropathies, and (iii)- Focal/multifocal neuropathies. Also of interest in the clinical setting, have been the developments of various scoring systems (see Thomas, 1997) which have helped to diagnose and stage the presence and extent of diabetic neuropathy.

Although the pathogenesis of diabetic neuropathy is most likely to be multifactorial encompassing genetic predisposition, endoneurial hypoxia/ischaemia, increased oxidative stress and metabolic derangements, there are classicaly two main theories for the pathogenesis of diabetic neuropathy, namely the vascular and

metabolic hypotheses (see Thomas, 1992). The current feeling is however, that these two pathogenic mechanisms are not mutually exclusive, and in addition to abnormalities of neurotrophic (Vinik et al., 1995) and autoimmune (Rabinowe, 1990) function, they are part of a possibly multifactorial aetiology in diabetic neuropathy. Without a clear understanding of the factors which trigger the development of diabetic neuropathy, and those factors which may be involved in maintaining the disease, our knowledge of its natural history remains obscure. We also need to consider the possibility that the pathogenic mechanisms may be diverse for the different subclasses of diabetes.

Vascular Theory

The vascular theory has been alive ever since Pryce (1893) described the findings of nerve degeneration associated with microvascular occlusion in nerves supplied by markedly atheromatous arteries. The original studies which suggested that the neuropathy was secondary to ischaemia resulting from large vessel occlusive disease (Woltman and Wilder, 1929) are now generally out of favour, given that the studies were based on post-mortem and amputation specimens from long-standing diabetics without exclusion of concomitant atherosclerotic pathology. The descriptions of epi- and endoneurial lesions in the form of luminal occlusion and vessel wall thickening in diabetic patients with neuropathy (Fagerberg, 1959) started the initial interest in the microvascular circulation. This was followed by much controversy over which of the neuronal (Greenbaum et al., 1964), axonal (Dolman, 1963), Schwann cell (Thomas and Lascelles, 1965) or vascular abnormalities came first.

There is now a substantial volume of evidence for abnormalities in peripheral vasa nervorum. Both small vessel occlusive disease and endothelial cell hyperplasia have been shown in diabetic neuropathy (Dyck et al., 1985; Timperly et al., 1976;

Williams et al., 1980). Increases in sural nerve capillary basement membrane and endothelial cell profile number have been reported in a recent morphometric study (Malik et al., 1993). Experimental work has revealed reduced oxygen tension and nerve blood flow in diabetic animals (Tuck et al., 1984; Cameron et al.,1991). The reduction in nerve blood flow has been further substantiated by doppler and nuclear imaging techniques (Pugliese et al., 1991; Monafo et al., 1988). These findings are consistent with observations in the clinical setting demonstrating a reduction in nerve oxygen tension (Newrick et al., 1986) and reduced nerve blood flow (Tesfaye et al., 1993).

These vascular abnormalities, together with haemorheological abnormalities characterized by increased plasma viscosity (McMillan, 1982), sedimentation rate (Wautier et al., 1981) platelet aggregability (O'Mally et al 1975), fibrin deposition (Timperly et al., 1985) and more rigidity of red blood cells all contribute to an impairment of nerve blood flow. This then causes endoreurial hypoxia leading on to abnormalities in nerve function and finally presenting as a neuropathy in the diabetic state.

Metabolic Theory

The metabolic mechanisms implicated in diabetic neuropathy are centered around four areas,- abnormalities in the polyol pathway, non-enzymatic glycation of structural proteins, changes in axonal transport, and alterations in neurotrophic function.

Accumulation of both sorbitol and fructose in peripheral nerves is found in both human and experimental diabetes (Gabbay et al., 1966). Sorbitol is produced in one of the two steps of the polyol pathway, in which glucose is enzymatically converted to sorbitol by aldose reductase, and sorbitol converted to fructose by sorbitol

dehydrogenase. This pathway appears to be activated by the hyperglycaemic state. The accumulation of sorbitol and fructose is thought to cause hyperosmotic stress and thus lead to the depletion of other intracellular compounds such as myo-inositol or taurine (Nishimura et al., 1987) which then is followed by axonal shrinkage and nerve dysfunction. The ensuing deficit of nerve conduction has been shown to be prevented by treatment with aldose reductase inhibitors (Yue et al., 1984; Judzewitch et al., 1983).

Another mechanism for the reduction of intracellular myo-inositol, which is structurally similar to the glucose molecule, is through competitive inhibition of its uptake by glucose (Greene et al., 1990). Reduced levels of myo-inositol are then responsible for impairing the phosphoinositide metabolism causing a diminished diacylglycerol and protein kinase C activity. The latter then markedly impairs the Na^+K^+-ATPase activity (Greene et al., 1992). Although there is experimental evidence for a benefit with myo-inositol supplementation (Greene and Lattimer, 1983; Tomlinson and Mayer, 1985), no benefit has been observed in human trials (Gregersen et al., 1983).

There is experimental evidence that hyperglycaemia is associated with non-enzymatic glycation of structural proteins such as tubulin and neurofilaments (Kennedy and Baynes, 1984; Tarsio et al., 1985). This enhanced glycation is suggested to cause neuropathy by a number of modes. Either resulting from the oxidative stress of the glycation process itself, by the accumulation within nerves of end-products of such glycation and finally via the release of free radicals and other inflammatory products such as cytokines (Brownlee et al., 1988; Brownlee, 1994).

Derangement of protein synthesis and axonal transport have also been implicated in diabetic neuropathy. Experimental studies have shown defective axonal transport in diabetes involving both the fast and slow transport mechanisms and in both

directions (Schmidt et al., 1975, 1983; Brimijoin and Dyck, 1979; Jacobsen et al., 1981). Some of there defects seem to be preventable with insulin or myoinositol treatment (Mayer and Tomlinson, 1983a,b).

1.8.3 Experimental models in diabetes

Animal models have so far been of immense value in the study of diabetic neuropathy where there appears to be substantial similarity with the clinical findings in many of the structural and functional derangements (see Sharma and Thomas, 1987). These models allow us to investigate the genetic factors, which in man would be difficult given the difficulty of controlling environmental influences, and also to tease out specific mechanisms in a way that would not be ethically possible in the clinical setting. Ultimately it gives us a controlled way to study the natural history of the disease in a longitudinal way.

Animal models exist for both type 1 and type 2 forms of diabetes (see Sharma and Thomas, 1987). Majority of the experimental data pertaining to diabetic neuropathy has come from studies on chemically induced diabetic models. The two most common agents are alloxan and streptozotocin (STZ). Alloxan is a pyrimidine compound which has a structural resemblance to glucose and uric acid. Its diabetic actions were originally noted by Jacobs (1937). STZ, is a N-nitroso derivative of D-glucosamine which was isolated from streptomyces achromogenes and shown to have diabetogenic properties on intra-venous injection in dogs and rats (Rakieten et al., 1963). The mechanism by which these drugs cause the diabetic state is not fully appreciated, although the evidence is that they act on the pancreatic beta cells causing a reduction or loss of cellular

granules (Rerup, 1970), shrinkage of the beta cells and cause nuclear pyknosis (Arison et al., 1967; Rakieten et al., 1963).

STZ has been of particular value in studying type 1 diabetic neuropathy because of the pathological similarities in the rat model. The STZ-rat model exhibits polyol pathway activation with accumulation of sorbitol and reduction of myo-inositol levels and reduced $Na^{+}K^{+}$-ATPase activity similar to the clinical setting (see Yagihashi, 1995 for a review). It also shows impaired nerve conduction velocities which is reversed by insulin treatment (Greene et al., 1975). In contrast to human diabetes, the peripheral neuropathy characterized by distal myelinated nerve fibre atrophy, does not appear to be associated with any fibre loss. Whether the myelinated fibre atrophy is secondary to a defect in maturation (Sharma et al., 1981; Thomas et al., 1990) or a direct result of the metabolic abnormalities remains controversial (Sima et al., 1988; Yagihashi et al., 1990). In view of the ease of reproducibility of diabetic abnormalities and their resemblance to human type 1 diabetic neuropathy, the STZ-rat diabetic model was used in the work presented in this thesis.

1.9 EVALUATION OF MAIN FINDINGS

Although I have outlined the aims and main findings of the work presented in this thesis both in the Abstract (p2-3) and Preface (p 10-11) sections, it seems necessary here at the end of the General Introductory chapter to re-emphasise those points before embarking on the experimental sections.

The purpose of the studies described in this thesis, was to study the innervation of the nerve trunk and cerebral circulations, by classical and putative neurotransmitter substances, using histochemical techniques. Broadly, this was divided into firstly, assessing the pattern and density of innervation in development, and secondly to study this innervation in disease states of diabetes and hypertension.

Apart from establishing the presence of variour perivascular nerve types along the vasa nervorum, the main findings of these studies were those of differential expression of pervascular nerves innervating the nerve trunk and cerebral circulations in both development and disease.

I am mindful, at the outset, of the obvious criticisms of having confined the diabetic work to the vasa nervorum, and the hypertensive work to the cerebral circulation. Also indeed, against the use of different experimental animal species, making it difficult to extrapolate results from one to another. In the discussions that follow after each chapter, in the final General Discussion, and particularly in the section entitled 'Limitation of the Study' , I have alluded to this weakness.

For the sake of improving on the shortcomings of these studies, which became apparent as they progressed, and to take them further, I have included a section entitled 'Future Directions' at the very end.

CHAPTER 2

GENERAL METHODOLOGY

2.1 HISTOCHEMICAL DEMONSTRATION OF PERIVASCULAR NERVES

2.1.1 Fluorescence Histochemical Localization of Noradrenaline

The demonstration of neuronal transmitter substances first became possible with the development of the Falck-Hillarp fluorescent histochemical method using formaldehyde vapour (Falck et al., 1962). This was based on the findings that biogenic catecholamines reacted with formaldehyde to produce a fluorescent product which was observable upon exposure to ultraviolet light. The formation of this fluorescent product involves a two-step process; an initial formaldehyde-induced condensation of catecholamines to 1,2,3,4-tetrahydroisoquinolines, which in the final step became highly fluorescent and insoluble (Falck et al., 1962). This method however, had its limitations. It did not always produce consistent results; it was time consuming, and while it was good for the visualization of cell bodies and axon terminals, it was not sensitive enough for staining non-terminal regions of catecholaminergic axons in central nervous tissue.

The introduction of the glyoxylic acid (GA) method (Lindvall and Bjorklund, 1974; Lindvall et al., 1974) provided an alternative way that was of greater sensitivity particularly for the primary amines noradrenaline and dopamine. It was also very rapid and highly reproducible in demonstrating neuronal catecholamines. Modification of this method using aqueous GA was later found to be particularly suitable for the localization of peripheral adrenergic nerves in whole-mount stretch preparations (Furness and Costa, 1975; Waris and Partanen, 1975).

Formation of the GA-induced fluorophore from primary catecholamines follows a two-stage process; the first step involves a cyclization reaction leading to the formation of a weakly fluorescent 1,2,3,4-tetrahydroisoquinoline-1-carboxylic acid derivative; in the second step, the tetrahydroderivative reacts with another GA molecule to form the highly fluorescent compound 2-carboxymethyl-6,7-dihydroxy-3,4-dihydroisoquinolinium (Lindvall et al., 1974). The more recent addition of pontamine sky blue (PSB) which counterstains for background autofluorescence has further improved this technique (Cowen et al., 1985). Using the GA method, the limitation for the detection of NA in nerve varicosities is of the order of $5x\ 10^{-4}$ femtomoles (Falck et al., 1982). The GA-induced catecholamine fluorophores show maximal excitation and emission spectra at wavelengths of 415 and 475 - 480 nm respectively (Lindvall and Bjorklund, 1974).

Method

Immediately following sacrifice of the experimental animal by ether overdose, relevant tissues were carefully dissected and immersed in 2% glyoxylic acid in 0.1 M phosphate buffer saline (PBS) at a pH of 7.2 and room temperature for 70 minutes. In the case of the cerebral arteries, the circle of Willis together with the basilar artery was dissected off the brain and pinned out on small squares of silicone elastomere (Sylgard) during this incubation time. Similarly, in the case of nerves, one centimeter length of each nerve was immersed in the GA solution for incubation. During this time, a longitudinal cut was made along the nerve trunk thus opening out the epineurium with nerve fascicles running along it. Each nerve fascicle was then opened longitudinally to allow for the removal of all intrafascicular nerve fibres. Some fascicles were too small to open, and these were left intact but nerves contained within them were pulled out

from one of the ends. This dissection was facilitated by pinning the tissues to Sylgard. At the end of the dissection, the resulting flat sheet of connective tissue comprising the epi- and perineurium and the vasculature contained within was stretched out by pins to a length of one centimeter which was the original length of excised nerve.

Following this initial incubation period in GA solution, the tissues were then further incubated in a fresh GA solution containing 0.05% PSB and 0.1% dimethylsulphoxide (DMSO) for ten minutes. The samples were then rinsed in more fresh GA solution to remove excess PSB counterstain before being transferred to glass slides on which they were stretched and allowed to air dry in a dust free environment. Once dried onto the slides, they were incubated in a 100°C oven for 4 minutes and then mounted in liquid paraffin and kept in the dark refrigerated until the time of viewing. Careful measurements showed that there was only minimal but uniform shrinkage of the tissues because of the procedure.

At all times the tissues were handled with extreme care to reduce any possibility of damage during dissection and processing. The tissues were always handled only at the edges by fine forceps. Any

For assessment the slides were viewed with a Zeiss photomicroscope fitted with a 3RS epi-illumination system,. A high pressure mercury light source was used (Osram HB50) with excitation filters (BP 436/8), barrier filter (LP470 and a dichroic mirror (FT460).

2.1.2 Immunohistochemical Localization of Peptides and 5-hydroxytryptamine

The development of immunohistochemistry as a powerful tool for the localization of tissue antigens is credited to Albert Coons, who in 1941 introduced a technique of labeling specific antibodies with a fluorescent dye named fluorescein isocyanate (see Coons, 1958). This dye has now been replaced by the isothiocyanate derivative due to its ease of conjugation to antibodies (Riggs et al., 1958). Previous attempts at antibody labeling with ordinary dyes had proved largely unsuccessful (Marrack, 1934). Coons, further developed the original 'direct' method, which involved the labeling of the primary antibody to a far more sensitive 'indirect' method (Coons et al., 1955). The subsequent use of enzymes as antibody markers (Nakane and Pierce, 1967), and the introduction of monoclonal antibodies by Cesar Milstein (Milstein et al., 1979) has turned immunohistochemistry into a highly selective and sensitive microscopical technique which now has a widespread use both as a research tool as well as an important procedure in diagnostic histopathology.

The principle of immunohistochemistry is based on the principle that an antibody will only bind with the antigen which stimulated its production. Labeling of the antibody will thus allow for the visualization of its specific antigen. Antibodies, largely of the IgG class, are raised by immunizing an animal with a pure or preferably synthetic antigen. In the case of small molecules (haptens) such as numerous peptides, or those which are non-antigenic, an immune response can be mounted by conjugating it to a larger carrier molecule such as thyroglobulin or bovine serum albumin. Antibodies produced in this manner are polyclonal in nature, as they are not solely directed at the immunizing antigen. The donor serum will contain antibodies directed against various

regions of the antigen and the carrier molecule. In addition, there will be numerous natural antibodies which can attach to other tissue antigens. The use of these antibodies necessitates rigorous control studies to assess their specificity to the required antigen. Hybridoma technology, which consists of fusing spleen cells from immunized to plasmacytomas from the same strain of animal in culture, allows for the continual production of a pure antibody.

In the 'indirect' method, antibody is raised against IgG of the species that produced the primary antibody. This second antibody is then tagged with a marker. The most commonly used fluorescent marker, fluorescein isothiocyanate (FITC), exhibits excitation and emission maximal at wavelengths of 490 and 520 nm respectively (Larsson, 1981).

Immunohistochemical methods are used extensively in both basic and clinical science, and this indirect method, like other immunocytochemical techniques, has its weaknesses. There is a potential for non-specific reactions resulting from the degree of antibody specificity and cross-reactivity. Standard controls are required to ascertain that the observed immunofluorescence under the microscope does indeed represent the site of antigen location against which the primary antiserum was directed.

Non-specificity of antisera normally results from two possible causes: firstly, the heterogeneity of antibodies in the donor serum which may react with tissue compounds and give non-specific immunoreactivity, and secondly, due to possibly shared aminoacid sequences between related substances. This latter problem is particularly true for peptides. Standard controls to ensure that there are no non-specific

reactions are: firstly, a negative stain after preabsorption of the antibody at a maximal dilution with its specific antigen, and secondly, a positive stain when the antibody is preabsorbed with non-specific antigens.

Method

All the tissues described in this thesis were examined as whole mount slide preparations, except those which underwent biochemical assay. The tissues were dissected as described in the previous section. Once pinned to silicone elastomere squares, the tissues were fixed in 4% wt/vol paraformaldehyde in PBS at a pH of 7.4 for 90 minutes. The tissues were then subjected to graded dehydration and then rehydration in alcohol (80%, 90%, 100%, 90%, 80% in that order) by immersion in each concentration for 10 minutes. This process is carried out to render the tissues more permeable to the antibodies. Following 3 x 10 min washes in PBS containing a non-ionic detergent (1% Triton X-100), the tissues were incubated overnight (12 to 18 hrs) with the appropriate primary antisera in a humidified chamber at room temperature. This 'first layer' antibody was prepared by dilution with antibody diluting medium (which contains bovine serum albumin, lysine and Triton X-100 to minimize background fluorescence) to an optimal concentration determined previously in preliminary experiments. Following overnight incubation , the tissues were then washed again in PBS (3 x 10 min) containing 1% Triton X-100 before incubation with the 'second layer' antibody (conjugated to fluorescein isothiocyanate)at a dilution of 1:50 for one hour. After this period, the tissues were rinsed further in PBS and counter stained with 0.005 - 0.01% pontamine sky blue/0.1% DMSO in PBS for 5-10 min to reduce background fluorescence (Cowen et al., 1985). The tissues were then unpinnned from the Sylgard pieces and stretched unto glass slides where they were allowed to dry

before being mounted under coverslips with PBS/glycerol (Citifluor) for eventual examination.

All immunohistochemical specimens were examined under a Zeiss fluorescence microscope equipped with an epi-fluorescence condenser (3RS), using a high pressure mercury light source (Osram HB 50) with excitation filter (BP 450 - 490), barrier filter (KP 560) and a dichromatic beam splitter (FT 510).

2.2 MEASUREMENTS OF NERVE DENSITY

The density of nerve fibres projecting to the vasa nervorum, those fibres without vascular contacts terminating in the epi- and perineurium (nervi nervorum) and of the intrafascicular fibres was measured semi-quantitatively. The inconsistency of vascular pattern at the microscopic level and the usually sparse nature of fluorescent nervi nervorum makes it difficult to apply any other method in this tissue preparation, except that of subjecting the tissue to biochemical assay, in which case it is impossible to comment on the proportion of peri- and non-perivascular innervation of the nerve sheath.

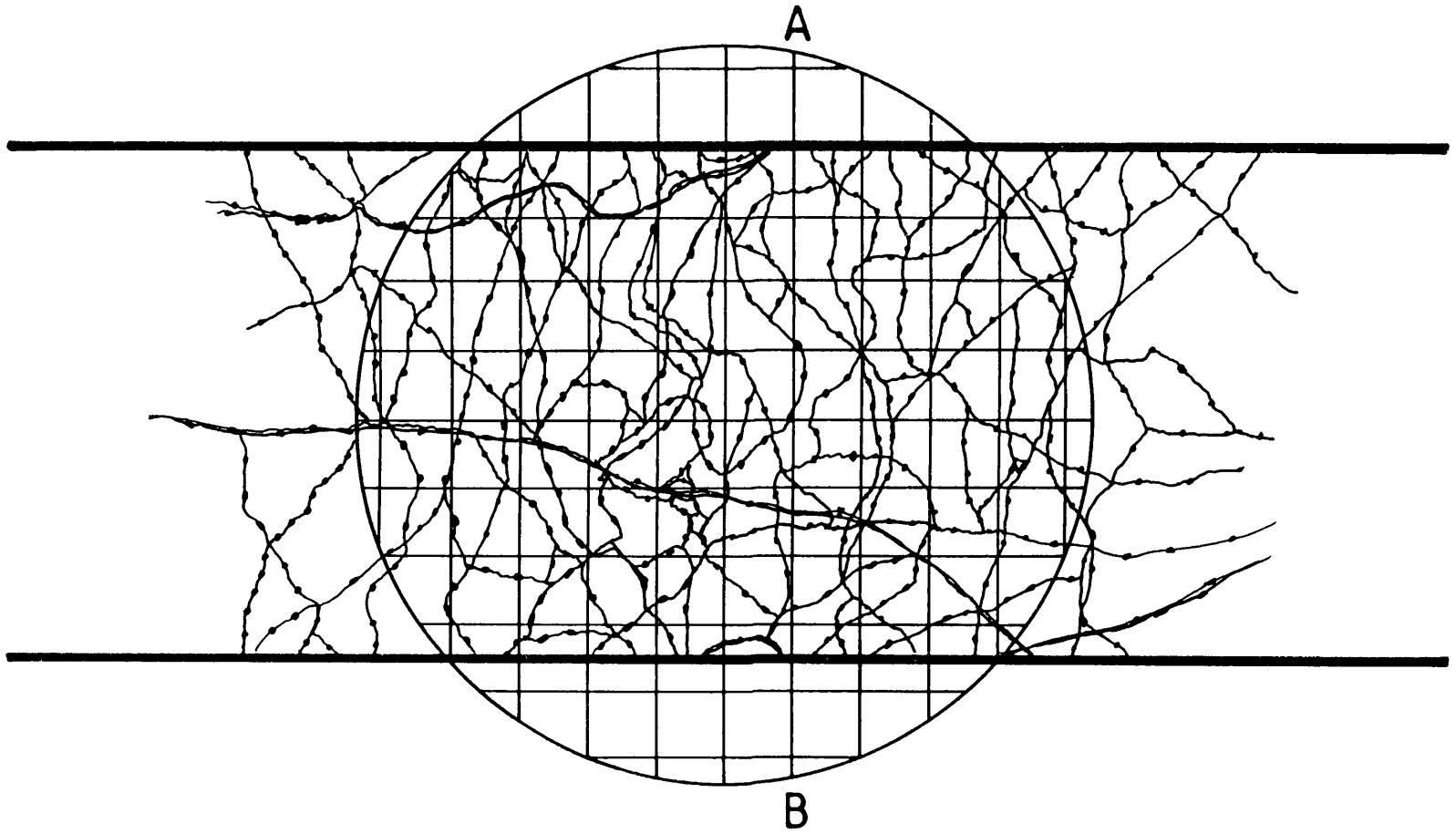
The size of the cerebral arteries allowed for a better method of determining nerve density. Using an ocular grid and a 16 x Neofluor objective lens, a count was made of the number of nerve fibres intersecting an ocular grid line (eg line AB in fig. 2.1) along the circumference of each vessel at three different places, at constant distances from its origin. The density of innervation was expressed as mean number of

fibres showing positive reactivity for a particular substance per mm of vessel circumference. The differential effect on vessel circumference between preparations processed for immunohistochemistry and those for GA-induced fluorescence was negligible (about $\pm$ 2 - 6% of unit circumferences). Control preparations consisted of vessels bathed only in physiological saline before they were mounted on slides. Bearing in mind the difficulty in obtaining exact 'in vivo' cerebral parameters and the semi-quantitative method used , it seemed inappropriate to apply any correction factor to the results. These small changes did not significantly alter the relative values of our measurements(i.e. the error is well within the standard error range) or affect the interpretation of the results.

The following cerebral arteries were studied: basilar artery (BA), superior cerebellar artery (SCA), posterior cerebral artery (PCA), internal carotid artery (ICA), middle cerebral artery (MCA) and the anterior cerebral artery (ACA). Two measurements were made along the BA, one at the proximal end (at the initiation of the vertebral arteries) and the second at the distal end (at its termination at the circle of Willis). Adjacent age stages were tested using the unpaired Student's t-test in order to establish any significant differences in the density of innervation. A level of probability of <0.05 was considered to be of significance.

FIGURE 2.1

Schematic illustration of semi-quantitative measurement of perivascular nerve density. In this example, there are 10 nerve intercepts along the line AB which measures a vessel diameter of 7.5 units.


A
B

2.3 BIOCHEMICAL ASSAYS

Following careful dissection of the relevant tissues as described above, those to be processed for biochemical assays were briefly rinsed in Krebs solution, blotted dry on filter paper and rapidly frozen in liquid nitrogen for storage until the time of assay.

2.3.1 HPLC with Electrochemical Detection of Noradrenaline

Assays for noradrenaline content using high performance liquid chromatography with electrochemical detection were kindly carried out by Dr. Jill Lincoln. Once removed from the liquid nitrogen store, the tissue samples were transferred to ice then weighed prior to homogenization in 500µl 0.1 M perchloric acid containing 0.4 mM sodium bisulphite and 25 ng/ml dihydroxybenzylamine (DBHA). This latter compound was added as an internal standard. Although DBHA does not exist biologically, it behaves in the same manner as noradrenaline or dopamine in terms of its extraction by alumina and its electrochemical properties. The addition of a known amount of DBHA at this stage thus allows correction for any losses of noradrenaline during the extraction procedure.

The samples were then centrifuged at low-speed and the supernatant was pipetted off and then subjected to alumina extraction method of Keller et al (Keller et al., 1976) with a modification of the addition of 0.1 mM EDTA in the solution used for washing the alumina. This extraction separates the catecholamines from the indoleamines (5-HT and 5-HIAA), which are very slow to come off the chromatography system. NA, DBHA and dopamine bind to alumina at neutral pH; when the alumina is washed the 5-HT and 5-HIAA are removed and the catecholamines can then be eluted

with 0.1 M perchloric acid containing 0,4 mM sodium bisulphite, which protects the amines from oxidation during the process.

The compounds were separated by reverse phase paired-ion high performance liquid chromatography. In reverse phase chromatography, the most polar of the compounds binds to the column least and thus comes off quickest. The mobile phase consisted of 0.1 M sodium dihydrogen phosphate, 0.1 mM EDTA, 5 mM heptone sulphate (pH 5.0) containing 13% methanol. This liquid phase produced overall polarity of the compounds allowing for good separation; the methanol reduces the polarity of all the compounds and thus speeded up the run. EDTA prevents electrochemically active metal ions released from the steel tubing from binding to the column and producing large contaminating peaks on the chromatogram. Heptone sulphate was used as the ion-pair agent, whereby it alters the polarity of NA, DBHA and dopamine to differing extents. Dopamine being the most affected thus binds more strongly to the column. The concentration of heptone sulphate used in the mobile phase was sufficient to allow good separation of the compounds without causing the dopamine to bind excessively and hence reduce the speed and sensitivity of the method. Chromatography was carried out at a flow rate of 2 ml/min on a radial pak μ-bondpak C-18 reverse phase column. NA and DBHA were detected electrochemically using a glassy carbon electrode set at a potential of +0.72 V. The eluent from the column flowed through a cell containing this electrode, and on passing through the potential difference, electrochemically active compounds such as catecholamines are oxidised resulting in the formation of electrons and hence producing a current. The magnitude of the generated current, which is proportional to the concentration of the compound, and the eluent time was compared with standards of known concentration to give a sample concentration for NA.

2.3.2 Inhibition Enzyme-linked Immunosorbant Assay (ELISA) of NPY

This assay for the determination of NPY content was kindly performed by Dr. Pam Milner. After removing the samples from liquid nitrogen and carefully and rapidly weighing them, they were plunged into 0.5 M acetic acid in polypropylene tubes in a boiling water bath for 15 min (~ 20 mg tissue/ml acetic acid) to extract the neuropeptide. The samples were then homogenized and centrifuged for 30 min at 3500 g, following which the supernatant was pipetted off and lyophilized.

Dynatech polystyrene microelisa plates were coated with the peptide diluted in a 0.1 M bicarbonate-carbonate buffer at pH 9.6, containing 0.2% sodium azide. This assay peptide was then stored at a 1:10 concentration and 10µl of it was diluted upto 12.5 ml for NPY. 100 µl of the peptide solution was added to each well and then incubated for 18 h at 4°C to allow the peptide to adhere to the wells. When the plates were prepared, the lyophilised tissue samples were reconstituted in polypropylene tubes in PBS containing units/ml aprotinin (sample buffer) at 0°C, then centrifuged for 10 min at 3500 g to remove sediments. NPY peptide standard was also prepared using the sample buffer at a range of dilutions (0, 5, 10, 25, 50, 100, 250, 500, 1000, and 10 000 pg/well).

The coated plates were then washed three times in PBS containing 0.05% Tween 20 to remove excess peptide and incubated for one hour at room temperature in PBS-Tween containing 0.1% gelatin to coat all the remaining areas and prevent non-specific binding. The plates were emptied by vigorous inversion onto absorbent paper. 50 µl of extracted sample and standard was then added to each well (each sample being

assayed in triplicate at two dilutions) followed by 50 µl of antiserum against the peptide, diluted 1:12 500 in sample buffer. The plates were then covered and incubated at 4°C for three days. The principle of this assay is that the antiserum will bind to the peptide in the tissue sample and any surplus unbound antiserum will bind to the peptide coating the wells. Thus, the higher the concentration of the peptide in the tissue sample, the more the antiserum is bound to the sample and less is available to bind to the coated plates. After incubation, the plates were washed three times with PBS-Tween containing 0.02% sodium azide to remove the tissue sample and any antiserum bound to it. Goat anti-rabbit IgG conjugated to alkaline phosphatase, diluted 1:500 in sample buffer, was added to each well and the plates were then incubated in a humidified chamber at 37°C for two hours. This second layer antibody will bind to any primary antiserum which was conjugated to the peptide coating the wells and thus was not removed when the well were washed. After three washes in PBS-Tween to remove excess goat anti-rabbit IgG, the plates were then washed once in a glycine buffer containing 0.001 M $MgCl_2$ and 0.001 M $ZnCl_2$, at pH 10.4. 100 µl of p-nitrophenol phosphate (1 mg/ml glycine buffer) was finally added to each well and left at room temperature; this compound interacts with the conjugated alkaline phosphatase to produce an intense colour.

The absorbance at 405 nm was read on a TiterteK Multiscan automatic spectrophotometer. The readings obtained with the standards were plotted against peptide concentrations on logarithmic graph paper to give a straight line from which the sample NPY value was obtained. The minimal detectable concentration was less than 0.5 fmol and the intra-assay variability was about 4%.

2.4 INDUCTION OF DIABETES

The diabetogenic action of STZ can be induced in rats at any age, but in trying to keep the study relevant to the neuropathy seen in the juvenile-onset form in man, young rats were used in this study. STZ will cause diabetes at doses of 25 - 100 mg/kg (Junod et al., 1969). The animals become rapidly hyperglycaemic, and show signs of weight loss and intermittent diarrhoea.

In the work presented in Chapter 4, diabetes was induced in adult male Wistar rats (400-450 g) by intra-peritoneal injection of buffered STZ at a dose of 65 mg/kg body weight. STZ was kindly donated by the Division of Cancer Treatment, National Institute of Health, Bethesda, USA. Onset of diabetes was known by the presence of polyuria, glycosuria and weight loss. These animals did not receive any insulin treatment. Similar weight animals of the same breed were used as controls and they were kept in identical conditions to the treated animals. All animals had access to food and water ad libitum.

2.5 PREPARATION OF SOLUTIONS

1. Modified Krebs Solution

Substances to weigh out for making 8 L of modified Krebs solution:

NaCl	62.40g
KCl	2.80g
NaH_2PO_4	1.68g
$NaHCO_3$	10.96g
$MgSO_4.8H_2O$	1.20g
Glucose	11.20g

Add and make up to 5 L with double distilled water in a large container with a bottom tap. Shake and stir to dissolve all substances.

Add 15.2 ml of 1 M $CaCl_2$.

Oxygenate with 95% oxygen/5% Carbon dioxide to achieve a final pH of 7.2-7.4.

The solution should be made freshly as needed, but will keep for upto 24 hours at 4°C.

2. Phosphate Buffered Saline (PBS)

To make 0.1 M Phosphate Buffer:

Solution A: 42.60g of NaH_2PO_4 in 3L distilled water

Solution B: 31.20g of NaH_2PO_4 in 2L distilled water

Add soln. B to soln. A until pH is 7.4-7.6

Usually takes under 1L soln.B for 3L soln.A

To make up final PBS solution (1L), add 50 ml 0.2 M phosphate buffer, 0.2g KCl and 8.76g NACl to 950 ml distilled water (pH 7.4-7.5).

3. Sylgard

10 parts Sylgard resin

1 part curing agent

Mix well with care as this tends to foam. Pour into plastic container (not glass as the polymer sticks too strongly) to a depth of 3-4mm and allow to dry. On drying the bubbles should all have disappeared. This curing process can be accelerated at 37-40°C.

4. Glyoxylic Acid Solution

Initially make 0.1 M Phosphate Buffer:

Solution A:	NaH_2PO_4	2.75g in 100 ml distilled water
	$NaH_2PO_4.2H_2O$	3.12g in 100 ml distilled water
Solution B:	Na_2HPO_4	2.83g in 100 ml distilled water
	$Na_2HPO_4.2H_2O$	3.56g in 100 ml distilled water

Make up 25 ml of buffer solution by adding 3.5 ml of Soln.A and 9 ml of soln.B to 12.5 ml of distilled water. To this 25 ml of buffer solution, add 0.5g glyoxylic acid to achieve a 2% w/v glyoxylic acid solution. This solution has a low pH (about 3.5) so needs titrating drops of 5 M Sodium Hydroxide to achieve a final pH of 7.5. The final solution can be kept for 4 hrs at room temperature of for upto 24 hrs at 4°C.

For every 10 ml of this solution, 5-10 mg of pontamine sky blue and 100 µl of DMSO is added for adequate counterstain.

4. Antibody Diluting Medium

To make 500 ml of diluting medium:

Sodium azide	0.50 g (0.1%)
Bovine serum albumin	0.05 g (0.01%)
Lysine	0.50 g (0.1%)
Triton X-100	0.50 ml (0.1%)

Add and make upto 500 ml with Phosphate Buffered Saline. This solution should be made up every six weeks at the latest as it is prone to bacterial contamination.

5. 4% Paraformaldehyde

Initially make a stock solution of 20% paraformaldehyde

200 mg paraformaldehyde (weigh in fume cupboard) in a flask to which add 700 ml distilled water. Using a refluxing equipment, dissolve the mixture in a fume cupboard. This takes about 5 hours. Allow to cool and add drops of NaOH until the solution clears.

Top up the solution with distilled water to make a final volume of 1 litre. Store in a glass bottle and dilute to the required 4% solution with PBS.

6. 0.1 M Carbonate-Bicarbonate Buffer

Solution A: Na_2CO_3 1.06 g

Sodium azide 0.02 g in 100 ml distilled water

Solution B: $NaHCO_3$ 0.84 g

Sodium azide 0.02 g in 100 ml distilled water

Mix each solution. Add A to B until pH is 9.6. Store at 4°C.

7. Aprotonin Gelatin Tween PBS Azide (Sample Buffer)

PBS-Tween: 10 tablets into 1 Litre distilled water to which add 500 ml Tween 20.

Gelatin PBS-Tween azide: Add 0.1 g gelatin to 100 ml of the above PBS-Tween solution. Stir on a hot plate and allow to cool. Then add 0.02 g Sodium azide.

This solution can be stored for one week at 4°C.

To make the sample buffer: Add 1 mg aprotonin to 100 ml gelatin PBS-Tween azide solution. Make up fresh of each day of use.

8. Glycine Buffer

To make 200 ml glycine buffer :

Glycine	1.5 g
$MgCl_2$ (1M)	200 µl
$ZnCl_2$	0.0272 g

Add the above to 150 ml distilled water and adjust pH to 10.4 with 1M NaOh, stirring constantly. Make up to 200 ml and store at room temperature.

CHAPTER 3

INNERVATION OF VASA NERVORUM

3.1 SUMMARY

Using a novel whole mount preparation of the epi- and peri-neurium of various nerve trunks and histochemical techniques, this study firstly describes the presence of perivascular innervation of the vasa nervorum in different species. These nerve fibres which innervate the vascular and non-vascular elements of the epi- and perineurium, are shown to contain NA, 5-HT, VIP, NPY, SP and CGRP.

In the rabbit ,the density of innervation was seen to increase with advancing age. In rats, the NA-containing nerves are sensitive to 6-OHDA -induced sympathectomy unlike theVIP-LI fibres which were resistant to this and anti-NGF, and whose density increased following peripheral nerve injury. These substances have yet to be attributed roles in neurovascular transmission in nerve trunks, although the findings of this study do suggest a possible trophic role for VIP.

3.2 INTRODUCTION

The repertoire of normal vascular function and that of the tissues they supply nutrients with, includes the role of neurotransmitters and hormones in their ability to alter vascular tone. Improvements in histochemistry and particularly in the techniques of immunohistochemistry have demonstrated the presence of definitive and putative neurotransmitters within perivascular nerve varicosities of many vascular beds as outlined in Chapter 1. Neurohumoral control of blood vessels supplying blood to nerves (vasa nervorum) is important in normal nerve function and it may play a role in the pathogenesis of both human and experimental neuropathies. The innervation of the vasa nervorum has so far not been examined. This chapter examines the innervation of the vasa nervorum in the sciatic and vagus nerves and the sympathetic chain of rabbits at various ages; the sciatic nerves of the guinea-pig; and the rat sciatic nerves following nerve injury and chemical sympathectomy.

3.3 MATERIALS AND METHODS

These experiments were carried out on locally bred animals. The rabbits were studied at the following age stages: 3 weeks, 8 weeks, 6-8 months and 3 years. The guinea-pigs were studied at 8-12 weeks of age. The rat sciatic nerves following nerve injury, and exposure to 6-hydroxydopamine (6-OHDA) and anti-nerve growth factor anti-NGF) were kindly provided by Dr. P. Anand from the Departments of Medicine and Histochemistry at the Hammersmith Hospital,London.

Tissues for the visualization of NA- containing fibres and those exhibiting immunofluorescence for 5-HT and peptides were processed as described in Chapter 2. For the immunohistochemical technique, the following primary and secondary antibodies were used:

Primary antisera:

Rabbit anti-5-HT (R.I.A., UK) diluted 1:500

Rabbit anti-VIP (R.I.A., UK) diluted 1:500

Rabbit anti-SP (Sera-Lab, UK) diluted 1:100

Second layer antibody:

Goat anti-Rabbit IgG-FITC (Nordic) diluted 1:50 for VIP and 5-HT

Rabbit anti-Rat IgG-FITC (Nordic) diluted 1:50 for SP

Nerve injury, chemical sympathectomy and anti-NGF treatment

Three groups of rats were used (n=6 in each experimental group). Littermates served as controls. All three experimental groups underwent sciatic neurectomy at various stages. All animals undergoing surgical procedures were under ether anaesthesia. One group underwent sham surgery where the sciatic nerve was mobilized but not injured. Where neurectomy was performed, this was carried out on the sciatic

nerve (right side for consistency) in the mid-thigh region. Nerve injury consisted of nerve transection at this level. Group I animals underwent nerve injury in adulthood and did not receive any drugs.

Group II animals underwent chemical sympathectomy with 6-OHDA (150μg/g body weight subcutaneously) for the first two weeks after birth and half underwent sciatic neurectomy at 2 weeks of age.

Group III animals received anti-NGF (100μl of antiserum subcutaneously, corresponding to 25 mg/kg body weight) at age 2 days. Half the animals underwent sciatic neurectomy at the age of 2 months. All control animals received equal volumes of normal saline.

Samples for the assessment of NA- and VIP-containing nerves supplying the sciatic vasa nervorum were obtained from animals sacrificed 2 weeks after nerve injury.

3.4 RESULTS

Perivascular innervation of Rat Vasa Nervorum

Noradrenergic innervation demonstrated by catecholamine fluorescence in the rat sciatic nerve is shown in Figure 3.1. The extraneural nutrient artery receives a dense supply of nerves exhibiting catecholamine fluorescence (Fig. 3.1a) and this density diminishes as the vessel gets smaller as shown by a sparser innervation of the epineural vasa nervorum (Fig. 3.1b). Nervi nervorum and the intrafascicular fibres within the main sciatic nerve trunk also show positive catecholamine fluorescence (Fig. 3.1c,d).

Perivascular Innervation of Rabbit Vasa Nervorum

The distribution of perivascular varicose nerves supplying vasa nervorum for the various nerves is detailed in Table 3.1, and the appearance of NA-, 5-HT-, VIP- and SP-containing nerves is shown in Fig. 3.2. These results show that there is an increased density of these nerve fibres innervating the vasa nervorum of the rabbit sympathetic chain, and sciatic and vagus nerves with advancing age. The epineurium of the intrathoracic portion of the vagus and paravertebral sympathetic chain contained, in addition to perivascular nerves, an independent plexus of nervi nervorum containing NA and SP. (fig 3.2d,f). A relatively rich plexus of perivascular NA-containing nerves was seen in arterioles greater than 25μm in diameter and around muscular arteries (Fig. 3.2e). Single peptide- and 5-HT-containing nerves only were present around some blood vessels (Fig. 3.2a,b,c). We were unable to demonstrate any innervation of veins or capillaries.

Innervation of Sciatic Nerve Vasa Nervorum in Guinea-pigs

Unlike the rabbit tissues where there was very sparse innervation, the guinea-pig demonstrated a far richer innervation of nerve trunk vasculature, by nerve fibres showing immunofluorescence for 5-HT, NPY, SP, VIP and CGRP, and showed a greater number of nervi nervorum. Additionally in these animals I also wanted to assess the intrafascicular nerve bundles. The innervation of the peri- and non-perivascular components of the nerve sheath and the presence of these substances within the main intrafascicular nerves are shown in Fig 3.3 to 3.7.

Effects of Nerve Injury, 6-OHDA and anti-NGF in Rat Sciatic Nerves

While there was a rich plexus of NA-containing fibres supplying the rat sciatic vasa nervorum, the VIP-innervation was extremely sparse. This meant that one had to look at the whole tissue sample to get an idea of the overall density of VIP-LI fibres, and looking merely at one field would fail to reveal small changes.

The density of NA- and VIP-containing fibres supplying the sciatic vasa nervorum following nerve injury alone (Group I) and additionally after chemical sympathectomy with 6-OHDA (Group II) and treatment with anti-NGF (Group III) is shown in Table3.2.

Both chemical sympathectomy and anti-NGF treatment resulted in the complete loss of NA-containing nerve fibres. Sciatic neurectomy caused an increase in the number of VIP-LI fibres, and this increase was maintained but to a lesser extent after treatment with 6-OHDA and anti-NGF. These changes are shown in Fig.3.8.

3.5 DISCUSSION

The distribution of blood vessels has been repeatedly examined and the number of nutrient arteries to nerve trunks and their origin has been tabulated (Sunderland, 1978). However, the importance of perivascular nerves of vasa nervorum in healthy nerves and in peripheral nerve disease has been largely overlooked, and their anatomical distribution and neurotransmitter content has so far not been addressed.

Previous studies have looked at the question of nerve-smooth muscle interaction of neurovascular plexuses but have not examined these relations in vasa nervorum (Bevan et al., 1980; Burnstock et al., 1980). The demonstration here of the presence of peptides, serotonin and NA in autonomic perivascular nerve terminals of the vagus and sciatic nerves and of the sympathetic chain, suggest an important role for these substances.

The distribution of all the fibres was similar in that they all innervated vasa nervorum and were all present in nervi nervorum and within intrafascicular nerve fibres. What was noticeable is the differential expression of these substances in terms of the density of innervation. The precise role/s of these nerves has yet to be elucidated. The demonstration of immunoreactivity within intrafascicular nerves is not a new finding, and this has been described before with reference to the axonal transport of many of these peptides in peripheral nerves (Brimijoin et al 1980; Gilbert et al., 1980). However, given the greater knowledge of these substances in perivascular nerves supplying other systemic and cerebral vessels, it would not be out of place to suggest that their presence in both the perivascular nerves of vasa nervorum and in nervi nervorum somehow may have a role in controlling nerve blood flow.

The increased density of innervation seen in the rabbit tissues with advancing age is in keeping with the immature vasomotor functions in man and animals at birth

and with pharmacological findings of increased neurotransmitter levels with development (Dalmaz et al., 1979; Lanpus et al., 1962).

Like NA-containing autonomic nerves elsewhere, those innervating the vasa nervorum as well as the non-perivascular elements of the nerve sheath connective tissue, are also sensitive to chemical sympathectomy with 6-OHDA which is known to cause a selective destruction of NA-containing nerve terminals (Theonen and Tranzer, 1968). The increase in the density of VIP-LI nerves was only marginally diminished by 6-OHDA and anti-NGF, implying that at least a population of VIP-LI fibres in the epi- and perineurium is sensitive to these agents. The increase in density of VIP-LI observed here is in keeping with capsaicin-insensitive increased levels of VIP content in the sciatic nerve portion proximal to the site of transection in these same animals (personal communication Dr. Anand). These findings would suggest a reparative role for VIP, either acting to alter local blood flow and/or having some trophic effect following peripheral nerve injury.

3.6 CHAPTER UPDATE

The findings of this chapter were the first to demonstrate the presence of NA- , 5-HT-, and peptide-containing nerves along vasa nervorum and to describe their change with increasing age. Since then there has been a resurgence in studies directed at the vasa nervorum, both with respect to descriptive anatomy and physiology.

The finding of increased density of NA-containing perivascular nerves supplying the vasa nervorum has been supported by similar finding in the rat (Mione et al., 1987). The innervation of vasa nervorum has since been described in different species and nerves: NA-containing nerves innervate vasa nervorum of both cranial and spinal nerve roots in the subarachnoid space in monkeys (Hara and Kobayashi, 1987). This study also showed the presence of NA-containing free nerve endings in the endoneurium, similar to the finding in this chapter of the presence of fluorescent nervi nervorum. Other examples include; the presence of CGRP- and peripherin-immunoreactivity in nerves which are distinct from those innervating vasa nervorum in rats where they are thought to have a nociception role (Bove and Light, 1995); and double-labeling studies in rat facial, vagus and sciatic nerves showing NA/NPY-immunoreactive fibres projecting to the epi- and endoneurial arterial blood vessels, and nerves showing only NA-immunoreactivity to project to the endoneurium between the axon of the main nerve bundle (Kummer et al., 1994). They also showed that in the rat, VIP-immunoreactivity was present along epineurial vessels of the facial and vagus nerves but were absent from the sciatic nerve. This fits in with the sparse innervation in general that was observed in the rat nerve sheath in this study unlike the guinea-pig which showed a rich innervation by most of the substances studied.

The presence of 5-HT-LI nerve fibres was very scanty throughout the developmental period studied. Although 5-HT-LI nerves have been seen along a number of blood vessels (Burnstock and Griffith, 1988; Griffith and Burnstock, 1983) it would

appear that 5-HT is not synthesized and stored in distinct nerve fibres, but is taken up, stored and released as a 'false transmitter' (Jackowski et al., 1989). The very few 5-HT nerve fibres seen in this study may therefore be secondary to 5-HT uptake by sympathetic nerves from either platelets and/or degranulated mast cells resulting from tissue damage during handling.

Similar to the increase in NA-containing fibres, those containing SP and VIP also showed an increase with development, and reached a peak at 6-8 months of age in the rabbits. Both SP and VIP are potent vasodilators. A reappraisal of the autonomic nervous system has led Burnstock to classify nerve fibre types as sympathetic, parasympathetic and sensory-motor (Burnstock, 1985a; also see page 36 of the General Introduction). In this context, the VIP-containing nerves would be parasympathetic, while those containing SP would be sensory motor. The perivascular and non-perivascular (nervi nervorum) SP-containing nerve fibres may play a role in nociception resulting from peripheral nerve damage. Asbury and Fields (1984) have put forward an hypothesis to explain such pain. They propose that two major types of pain can be distinguished in peripheral nerve disorders, namely dysaesthetic pain and nerve trunk pain. They argue that nerve trunk pain results from activation of normal nociceptive endings in the nerve trunk sheaths (nervi nervorum). Until now nervi nervorum had been demonstrated in experimental animals as well as in human nerves using simple histochemical stains (Hromada, 1963). The present findings of SP in the vasa and nervi nervorum would support this hypothesis.

Numerous physiological and pharmacological studies have shed more light on the mechanisms controlling nerve blood flow (NBF). It has been suggested that NBF responds to adrenergic manipulation (Zochodne and Low, 1990; Zochodne et al., 1990), but the question of autoregulation is still controversial. There is some evidence that transperineurial arterioles are able to regulate regional NBF (Kihara and Low, 1990)

which is thought to be under adrenergic control (Zochodne et al., 1990). There is also a possible role for CGRP in an axon-reflex like mechanism for the regulation of NBF. Its vasodilatory action which increases NBF (Zochodne and Ho, 1991a) is blocked by a CGRP-receptor antagonist (Hotta et al., 1996).

Following sciatic nerve section in rats, increased expression of VIP has been seen only in the cells which have had their axons cut in the periphery (McGregor et al., 1984; Shehab et al., 1986). VIP and galanin expression is also increased at the messenger RNA level after nerve injury, and this increase in expression is of the same magnitude whether the injury is mechanical or chemical (Hyatt-Sachs et al., 1996). The role of VIP in the nerves innervating the vasa nervorum is uncertain. Certainly, its potent vasodilatory action would suggest that an increase in its expression may lead to augmentation of nerve blood flow in the area of injury. An additional trophic role, in the peripheral and central regenerative changes following peripheral nerve injury, can not be discounted.

TABLE 3.1

Innervation of rabbit epi- and perineurial vasa nervorum

++++ = very dense, +++ = dense, ++ = moderate, + = sparse, 0 = none

* = In bracket is the non-perivascular innervation

Age	Nerves	NA	VIP	SP	5-HT
3 Weeks	Sciatic	+	0	+	0
	Vagus (T)	+ (+)*	+	+	0
	Vagus (N)	+	0	+	0
	Sympathetic	++	+	+	0
8 Weeks	Sciatic	++	+	++	+
	Vagus (T)	+ (+)*	+	++	0
	Vagus (N)	++	+	++	0
	Sympathetic	++	+	+ (+)*	0
6-8 Months	Sciatic	+++	++	+++	+
	Vagus (T)	++ (+)*	++	++ (+)*	+
	Vagus (N)	+++	++	+++	0
	Sympathetic	++ (+)*	++	++ (+)*	+
3 Years	Sciatic	++++	++	+++	++
	Vagus (T)	+++ (+)*	++	++ (++)*	+
	Vagus (N)	+++	++	+++	0
	Sympathetic	+++ (+)*	++	++ (+)*	+

Sympathetic = Sympathetic chain; Vagus (T) = Thoracic portion of vagus nerve; Vagus (N) = Neck portion of Vagus nerve.

TABLE 3.2 Innervation of vasa nervorum following nerve injury and 6-OHDA and anti-NGF treatment for NA- and VIP-containing fibres in rat sciatic nerve

	n		NA	VIP
Group I (vehicle treated)	5	Intact	++++	+
(Adult age)		Neurectomy	++++	+++
Group II (6-OHDA)	5	Controls	+++	+
(4 wk age)		Intact	0	+
		Neurectomy	0	++
Group III (anti-NGF)	4	Controls	++++	+
(10 wk age)		Intact	0	+
		Neurectomy	0	++

		No. of fibres /5 mm^2 tissue
++++	Dense	(> 10)
+++	Moderate	(5 - 10)
++	Sparse	(1 - 5)
+	Present	(Single fibre)
0	Absent	(None)

FIGURE 3.1

Catecholamine fluorescence showing four regions of peri- and non-perivascular innervation typically associated with a nerve trunk (here the sciatic nerve of the rat).

a) Extraneural (nutrient) artery of the sciatic nerve

b) Epineurial vasa nervorum

c) Non-perivascular perineurial nerve fibres (nervi nervorum)

d) Intrafascicular nerve fibres within the main nerve trunk

Calibration bars = 25μm.

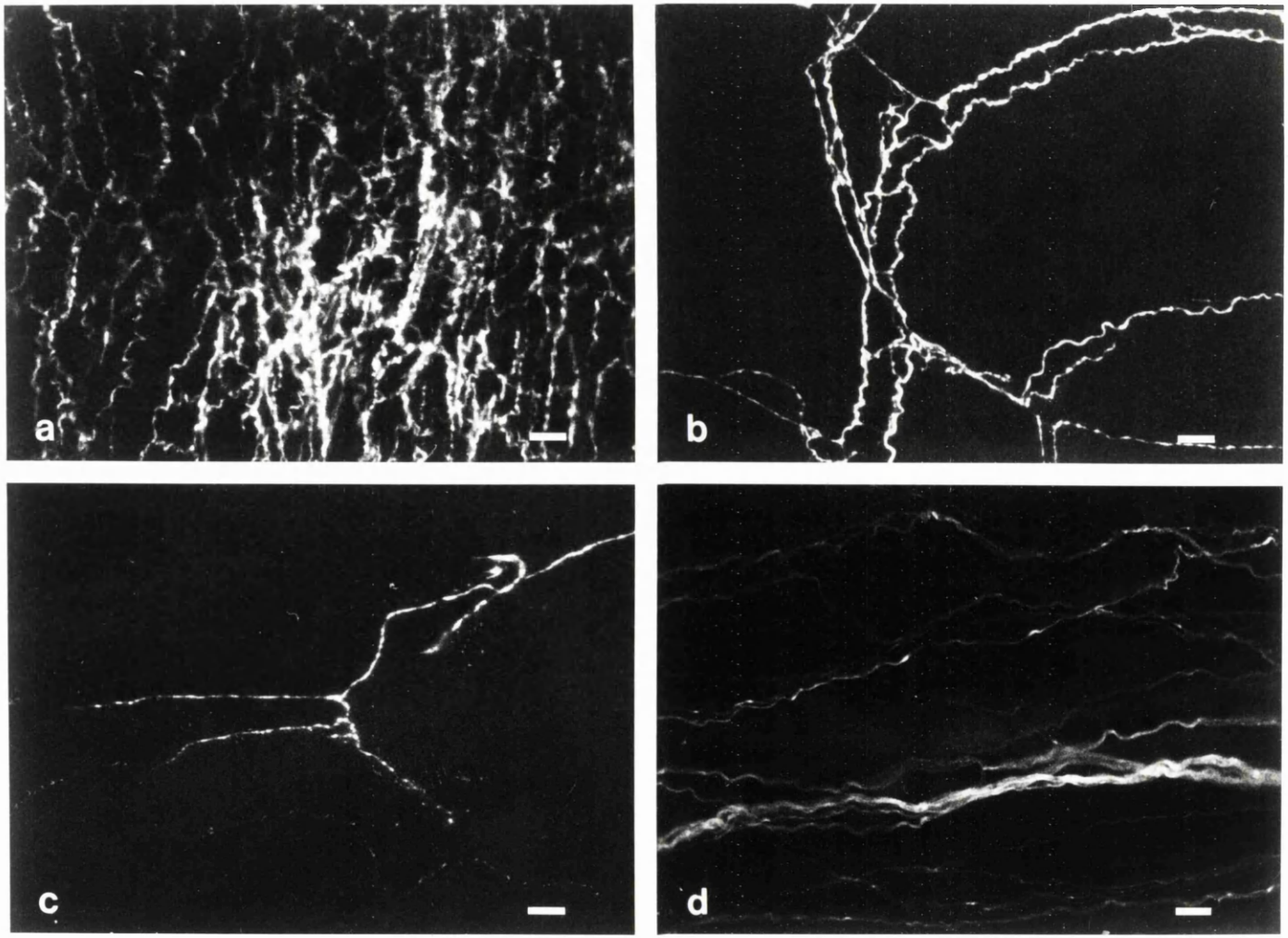

a
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FIGURE 3.2

a) SP in the sciatic nerve

b) 5-HT in the thoracic vagus nerve

c) VIP in the sciatic nerve

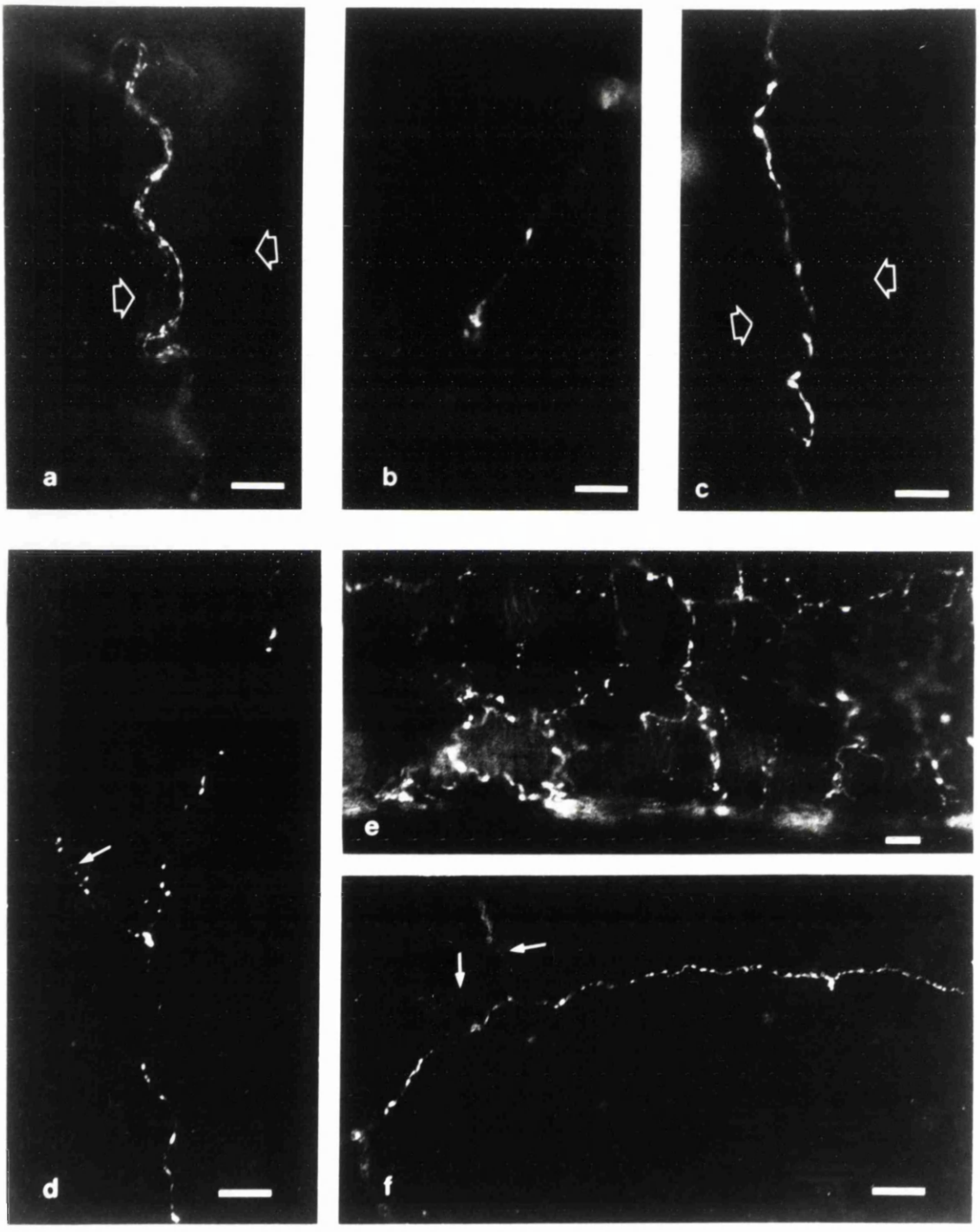
d) NA in the thoracic vagus nerve (non-perivascular plexus)

e) NA in the vagus nerve from the neck

f) SP in the sympathetic chain (non-perivascular plexus)

Varicose fibres of non-perivascular plexuses are shown by small arrows.Large arrows outline the outer margins of the vasa nervorum.

Calibration bars = 10 μm.


a
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e
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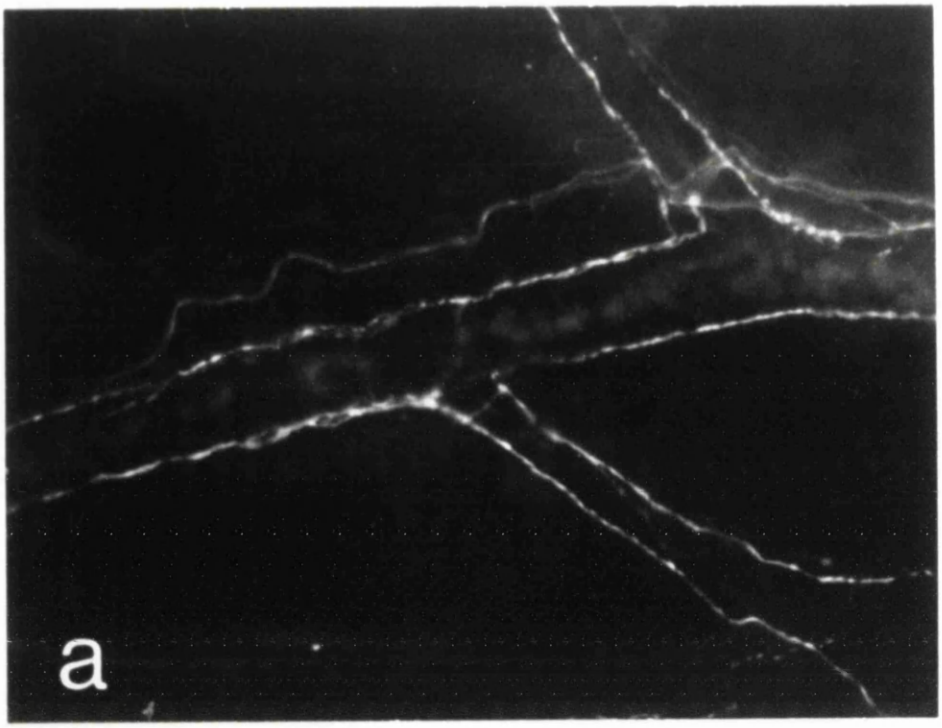
FIGURE 3.3

5-HT -like immunofluorescence of peri- and non-perivascular components of the guinea-pig sciatic nerve sheath.

a) 5-HT-LI around the sciatic vasa nervorum

b) 5-HT-LI nervi nervorum

Calibration bars = 50 μm


a


b

FIGURE 3.4

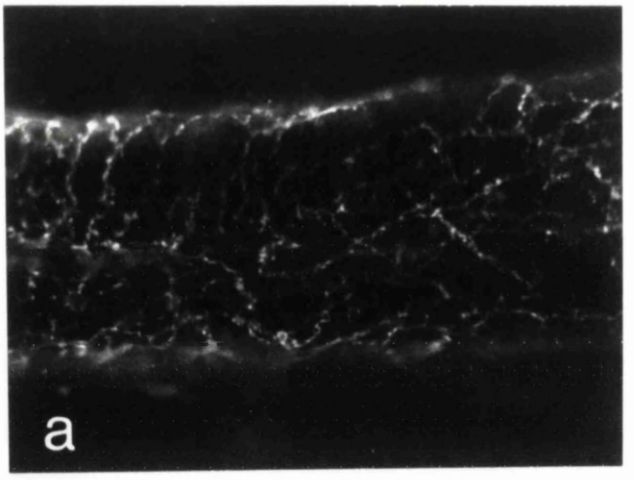
NPY-Like immunofluorescence of per- and non-perivascular components of the guinea-pig sciatic nerve sheath, and of the main intrafascicular nerve bundle.

a) NPY-LI of perivascular nerves of the extraneural nutrient vasa nervorum.

b) NPY-LI of perineurial vasa nervorum.

c) NPY-LI of the main intrafascicular nerve bundle.

Calibration bars = 50 μm 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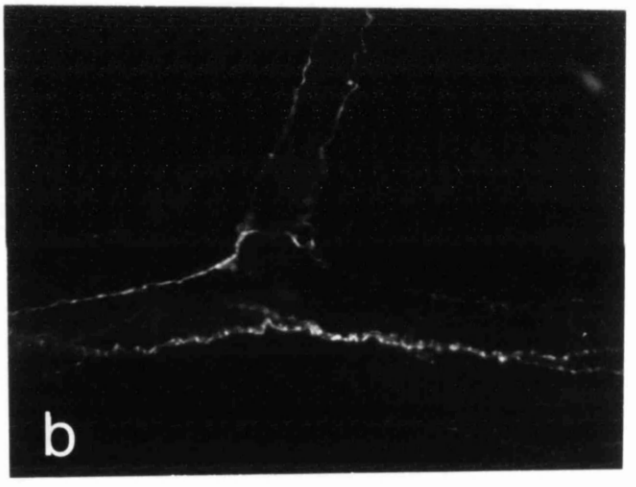
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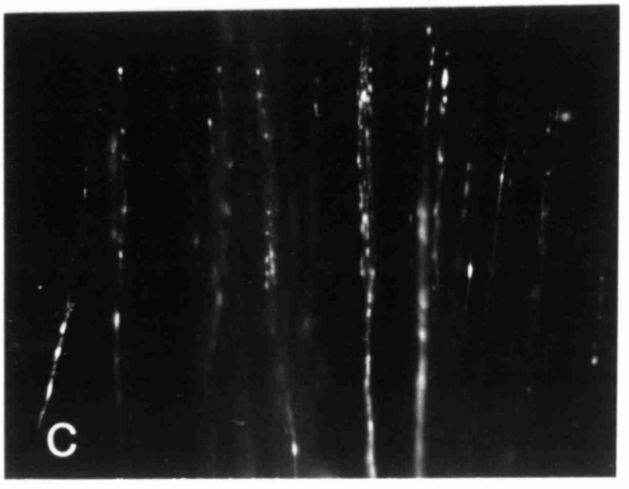

c

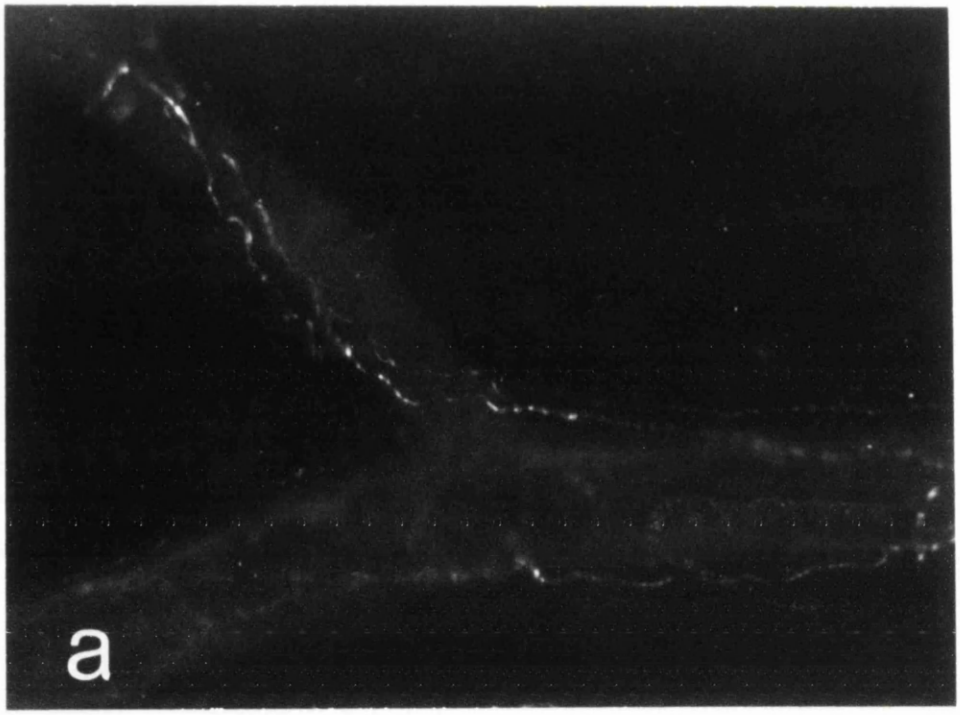
FIGURE 3.5

Substance P-like immunofluorescence of vasa nervorum and of the main intrafascicular nerve bundle of the guinea-pig sciatic nerve.

a) SP-LI of vasa nervorum.

b) SP-LI of the main nerve bundle intrafascicular fibres.

Calibration bars = 50 μm 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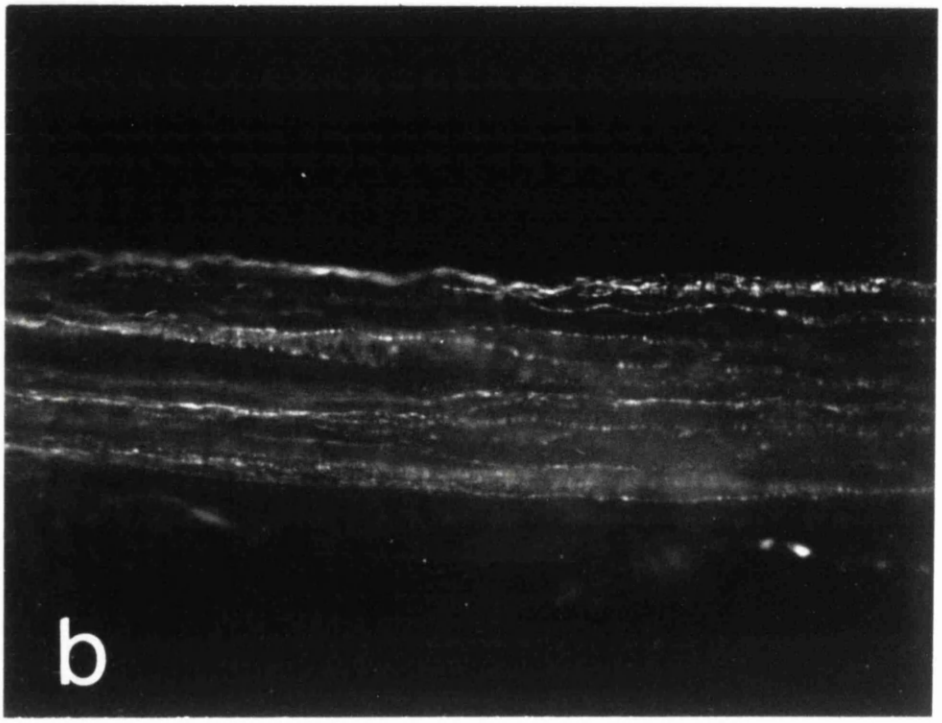

b

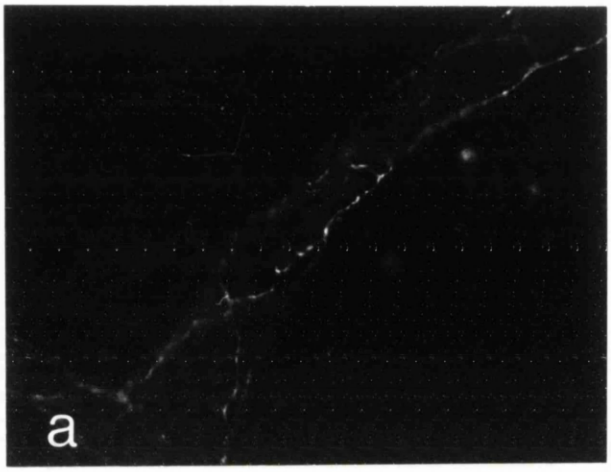
FIGURE 3.6

Vasoactive intestinal polypeptide -like immunoreactive nerve fibres along the guinea-pig sciatic nerve vasa nervorum, and in the main intrafascicular nerve bundle.

a) VIP-LI of sciatic vasa nervorum.

b) VIP-LI of the intrafascicular fibres.

Calibration bars = 50 μm.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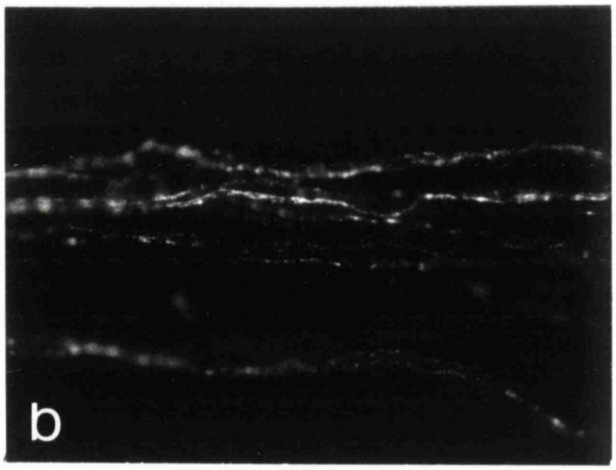
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FIGURE 3.7

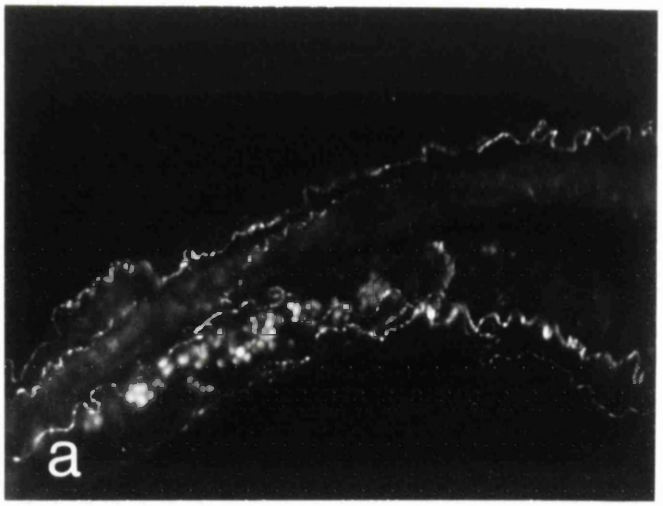
Calcitonin gene-related peptide -like immunofluorescence of peri- and non-perivascular components of the guinea-pig sciatic nerve sheath, and of the main intrafascicular nerve fibres.

a) CGRP-LI along the perineurial vasa nervorum.

b) CGRP-LI nervi nervorum.

c) CGRP-LI of the main intrafascicular nerve fibres.

Calibration bars = 50μm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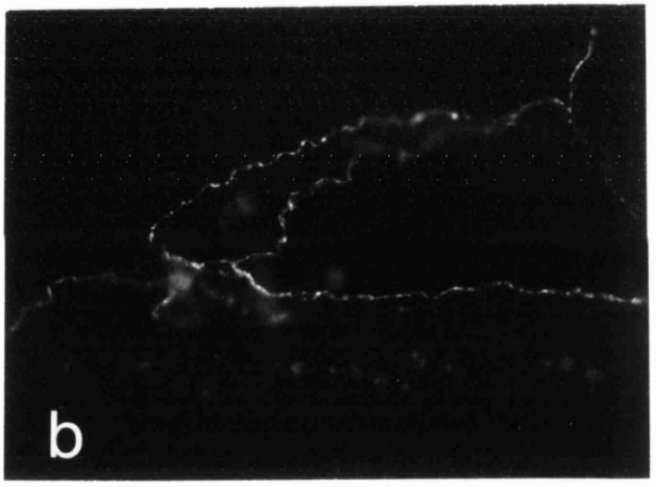
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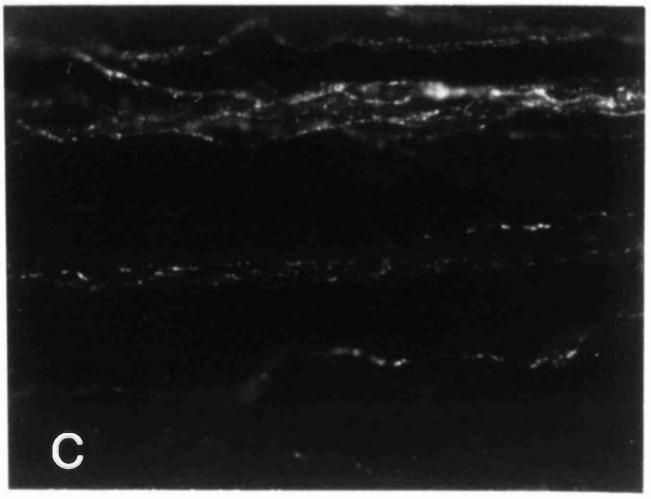

c

FIGURE 3.8

Effects of chemical sympathectomy and neurectomy on rat sciatic nerves.

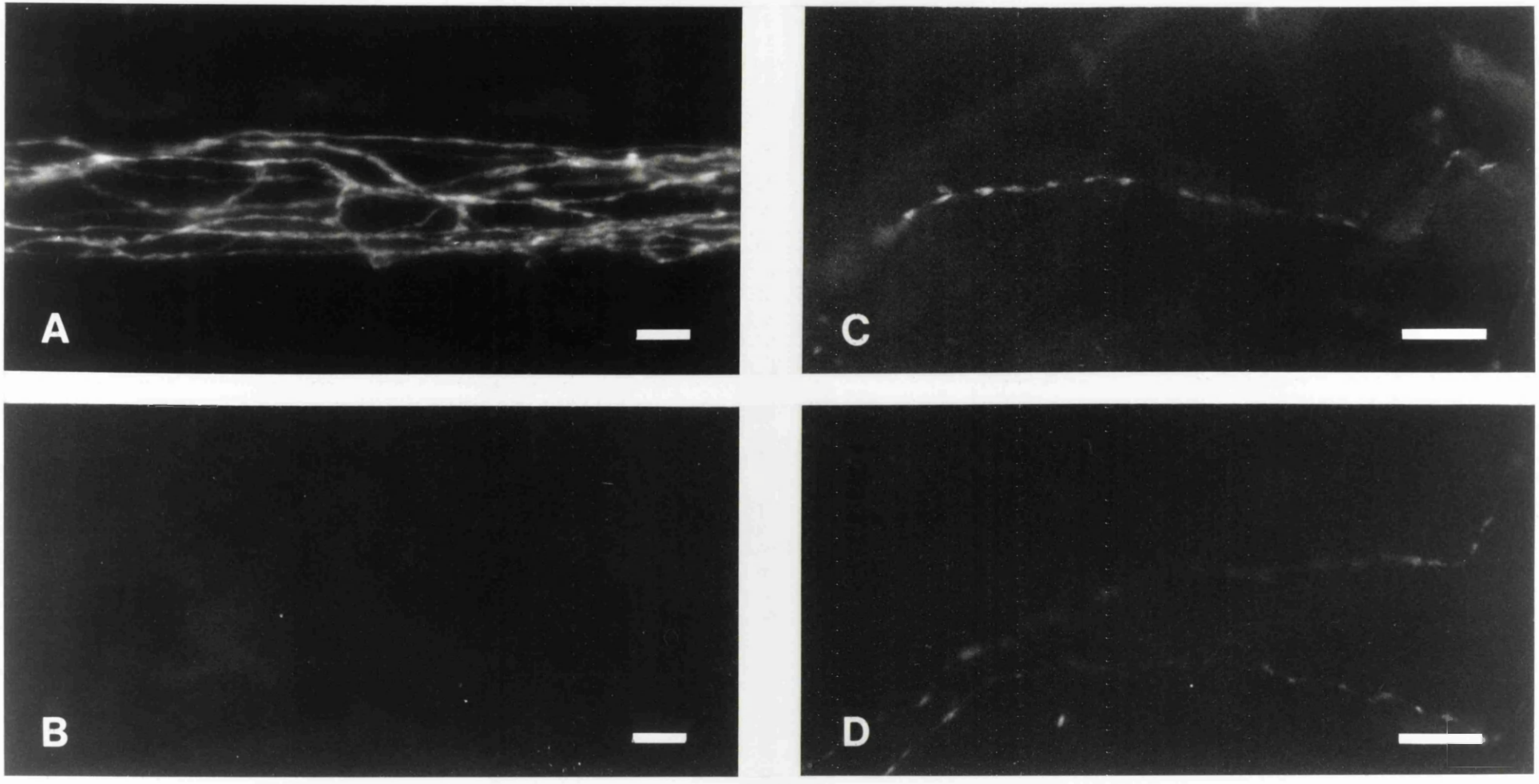
a) Catecholamine fluorescence of perivascular nerve plexus of vehicle treated control.

b) Complete loss of catecholamine fluorescence after chemical sympathectomy.

c) VIP-LI nervi nervorum of intact nerve. Only a single fibre was observed in a given preparation.

d) VIP-LI in proximal stump of neurectomized sciatic nerve; 5-10 fibres were observed
per preparation. In sympathectomized rats, VIP-LI fibres were also increased after neurectomy but were relatively sparse (1-5 fibres per preparation).

Calibration bars = 10 μm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CHAPTER 4

SYMPATHETIC INNERVATION OF VASA AND NERVI NERVORUM IN EXPERIMENTAL AND HUMAN DIABETES

4.1 SUMMARY

NA-containing nerves were studied in the perivascular nerve plexus of vasa nervorum and nervi nervorum, in whole-mount nerve sheath preparations of optic, sciatic and vagus nerves and the paravertebral sympathetic chain in normal and streptozotocin-treated diabetic rats using fluorescence histochemistry. At 8 weeks following induction of diabetes, there was a substantial or complete lack of fluorescent NA-containing fibres around blood vessels in the optic nerves. This was in marked contrast to the increase in perivascular nerve fibres in the sciatic, vagus and sympathetic chain nerve trunks of the same animals at the same time. An increase in the number of mast cells in the vicinity of vasa nervorum was also noted in the diabetic nerves.

Assays of noradrenaline levels in whole nerve segments also showed that they were not biochemically detectable in the optic nerves, but were significantly higher in the vagus nerves of diabetic animals ($P<0.05$).

A similar examination of sural nerves obtained as biopsies from diabetic patients revealed a loss of NA-containing fibres supplying the vasa nervorum.

4.2 INTRODUCTION

The demonstration of substantial innervation of the vasa nervorum as detailed in Chapter 1, and also shown independently by Amenta et al., (1983) would suggest an important role in normal nerve function for these perivascular nerves. Abnormalities in the neural control of vasa nervorum may contribute to mechanisms responsible for the pathogenesis of some human and experimental neuropathies.

Somatic and autonomic neuropathies account for many functional disorders in human diabetes. In a quantitative ultrastructural examination of sural nerves epineural arterioles, diabetic patients showed a greater separation of perivascular nerve profiles from the nearest arteriolar smooth muscle cells and fewer axons (Grover-Johnson et al., 1981). Reversible neurophysiological abnormalities in the retina (Frost-Larsen et al., 1983) and in pupillary function (Hreidarsson, 1981) have been related to the diabetic metabolic state. The debilitating effects of diabetic neuropathies and the limited knowledge of their pathogenesis, led to this investigation of the adrenergic innervation of the vasa nervorum and of the nervi nervorum in a variety of nerves of streptozotocin-induced diabetic and control rats, as well as in a limited number of human sural nerve biopsies.

4.3 MATERIALS AND METHODS

Twelve adult male Wistar rats were injected with buffered streptozotocin (i.p. 65 mg/kg) as described in Chapter 2. Induction of diabetes was assessed by the presence of rapid weight loss, polyuria and glycosuria. Twelve untreated controls were of the same initial weight range (400 - 450 g) as the diabetic animals. Both groups were maintained under identical conditions and sacrificed after 8 weeks.

Human sural nerve biopsies were kindly provided by Professor P K Thomas (Royal Free Hospital, London) and Dr. P Watkins (Kings College Hospital, London). 8 biopsies from diabetic patients and 2 control samples from nerve graft donors. Due to the limited size of the biopsy samples, these specimens were not processed for biochemical assay of NA.

Histochemistry

Tissues were processed for the visualization of NA-containing nerve fibres using the glyoxylic acid method as described in Chapter 2.

Noradrenaline assay

Whole nerve segments of the sciatic, vagus and optic nerves from the contralateral side to that used for histochemistry, were rapidly dissected after death and processed for noradrenaline assay using high-performance liquid chromatography as detailed in Chapter 2. Tissue content of noradrenaline was calculated both per gram of wet weight tissue and per centimetre length of nerve. Results have been expressed as mean +/- S.E.M. and the data were compared using Student's T-test. A level of probability of < 0.05 was considered to be significant.

4.4 RESULTS

Following 8 weeks of streptozotocin, the weights in grams (g)of the animals were as follows: Controls = 484 $\pm$ 8.5g vs Diabetics = 305 $\pm$ 12.7g ($p<0.001$).

The blood glucose levels were as follows: Controls = 125 $\pm$ 8.9 mg/dl vs Diabetics = 483 $\pm$ 18.4 mg/dl ($p< 0.001$).

The histochemical descriptions in this study are confined to changes in the epivascular NA-containing nerves of the intraneural circulation and of nervi nervorum. The density of NA-containing nervi nervorum and nerves innervating vasa nervorum was markedly increased in the sciatic and vagus nerves and in the paravertebral sympathetic chain of diabetic animals (Fig. 4.1a-d). In contrast, the optic nerves of diabetic animals showed a substantial loss of both peri- and non-perivascular nerves (Fig. 4.1e-f), particularly around small vessels. These observations are summarized in Table 4.1.

Quantitative data showing noradrenaline levels in whole segments of the sciatic, vagus and optic nerves are shown in Fig. 5.2. Noradrenaline content is expressed both in microgram per gram tissue, and in nanograms per centimeter length of nerve sample. There were no significant differences in the weights of control and diabetic nerves (mg/cm, Sciatic: C 10.52 +/- 0.70, D 9.37 +/- 1.13; Vagus:C 2.06 _/- 0.15, D 2.37 +/- 0.31; Optic: C 3.19 +/- 0.16, D 3.26 +/- 0.12, n=5).

Vagus nerves from diabetic rats had higher NA levels than controls, and this difference was significant ($P<0.05$) when expressed in ng/cm length. NA was present in control optic nerves but was not detected in any of the five diabetic optic nerves studied. The levels of NA in the diabetic sciatic nerves were significantly lower ($P<0.05$) than in controls when expressed both in μg/g tissue and in ng/cm length.

Human sural nerves showed a significant reduction or a complete loss of NA-containing perivascular nerve fibres (Fig. 4.3 and 4.4). The two control samples both exhibited a rich plexus of NA-containing nerves supplying vasa nervorum.

4.5 DISCUSSION

Fluorescence histochemical analysis in this study has demonstrated a lack of NA-containing nerve fibres around the vasa nervorum in diabetic optic nerve sheaths 8 weeks following treatment with streptozotocin. This was observed simultaneously with a proliferation of NA-containing fibres in the other nerve sheaths studied in the same animals. Quantitative HPLC assay results support the histochemical observations for the optic and vagus nerves. This was not true of the sciatic nerve where histochemistry showed a proliferation of epi- and perineural nerve fibres in diabetic animals while the assay results showed a significant decrease in the NA content of the whole nerve trunk. The sciatic nerve which is very much larger in diameter than the other nerves studied, contains a greater number of nerve bundles. These nerve fibres, distinct from those innervating the vasa nervorum, therefore account for a greater percentage of the total nerve weight when compared with the surrounding nerve sheath. The opposite is true of the vagus nerve. Small changes in the intrafascicular nerve fibre content in the sciatic nerve may then mask the histochemical changes in the epi- and perineurium. These findings indicate a differential vulnerability of the sympathetic nerve supply to the vascular and non-vascular components of the nerve sheath in different nerve trunks. It is possible that at an earlier stage the epi- and perivascular sympathetic innervation of the optic nerve sheath undergoes proliferation before disappearing and that, conversely, at a later stage there is loss of these perivascular nerves around the vasa nervorum of sciatic, vagus and sympathetic nerve trunks. Increase in the number of nerve profiles at the adventitial-medial border of rat jejunal arteries after 8 weeks of streptozotocin treatment has been shown to fall back to control levels after 12 weeks (Scott et al., 1984). Similarly, the increase in rat cardiac noradrenaline levels after 4 weeks duration of streptozotocin diabetes has been reported to have fallen back to control or lower levels after 4 months (Felten et al., 1982). Studies on the rat

myenteric plexus 8 weeks after streptozotocin treatment demonstrated degeneration of NA-containing nerves in the ileum and increased levels of noradrenaline in the colon (Lincoln et al., (1984). Whether such differential responses are characteristic of tissue function, varied susceptibility to noxious insults or depend on the origin of adrenergic fibres has not been determined.

The differences shown in this study are unlikely to be due to a toxic effect of streptozotocin, as there were no changes between the streptozotocin-treated non-diabetic and control rats. Further experiments with insulin treatment may aid in substantiating the claim that the findings presented here are due to the diabetic illness per se. This is certainly supported by the findings of a significant decrease or complete loss of NA-containing fibres innervating the vasa nervorum of sural nerves from diabetic patients. A loss of vasoconstrictor sympathetic tone may be responsible for the increased blood flow observed in the feet of patients with diabetic neuropathy (Watkins, 1983). The precise role of these sympathetic perivascular nerves of the vasa nervorum in the aetiology of clinical and experimental diabetic neuropathy needs further scrutiny.

An increase in the number of mast cells in the vicinity of the vasa nervorum was also observed in the diabetic condition. This was most noticeable in the vagus nerve. The presence of mast cells in the endoneurium, and peri- and epineurium where they are often in close proximity to vasa nervorum is well established, particularly in the rat (Olsson, 1966,1968, 1971; Hansson et al., 1971). By virtue of their ability to release histamine and 5H-T (Parratt and West., 1957), it has been suggested that they play a role in the regulation of the microcirculation (Selye, 1965). Certainly, both histamine and 5-HT have been shown to render the rat sciatic nerve more permeable (Olsson, 1966). The increased number of mast cells in the present study may reflect a pathological phenomenon associated with microcirculatory problems in diabetes.

4.6 CHAPTER UPDATE

The differential response of NA-containing nerves in to STZ-diabetes as shown in this study has been documented in the tibial and vagus nerves of the diabetic rat (Koistinaho et al., 1990). There was an increase in the density of NA-containing nerve fibres innervating the epi-and perineurial arterioles 5 to 12 weeks after inducing the diabetic state with STZ. This increase then subsided to normal levels after 16 weeks. In the endoneurium however, they observed a lack of NA-containing fibres following 5 weeks of treatment and at 12 weeks these fibres were absent from the endonerium in both nerves. Similar differential changes in the pattern of sympathetic innervation has been demonstrated in the gut also (Di Giulio et al., 1989), where there appears to be a complex denervation and hyperinnervation of the gut wall.

A differential vulnerability of neuropeptides in nerves supplying the vasa nervorum in STZ-diabetic rats has also been demonstrated (Milner et al., 1992a). This study showed that at 8 weeks after induction of diabetes, there was an increase in the density of NPY-immunoreactive nerve fibres in the optic nerve sheath. This was contrasted with a decrease in the NPY-immunoreactive fibres in the sciatic, vagus and sympathetic chain. VIP-immunoreactivity was increased in the nerve sheaths of all the nerves studied. Vasa and nervi nervorum, as well as the main intrafascicular nerve bundles of control human optic and sural nerves (obtained from post mortems) have shown the presence of NA, NPY, VIP and SP. In patients with diabetic neuropathy, there was a decrease in SP and NA fascicular/epineurial ratios (Lincoln et al 1993). The abnormal innervation of the vasa nervorum in sural nerves from diabetic patients has since been supported by ultrastructural and immunocytochemical studies (Beggs et al., 1993).

The exact mechanism and functional role of these changes are not as yet understood, but the increasing evidence for cotransmission, neuromodulation and the principle of chemical coding may help us to partly unravel the interplay

between transmitter substances present in perivascular plexuses supplying vasa nervorum.

With respect to the observes increase in mast cells in the diabetic samples, a recent study has shown that the intense hyperaemic response of rat sciatic vasa nervorum to the epineurial application of capsaicin, is in fact locally mediated and involves mast cells (Zochodne and Ho, 1991b). This response does not depend on central connections, but on an intact epineurial nerve plexus (Zochodne, 1993). Its inhibition by morphine suggests that there may be local opiate receptors perhaps on epineurial perivascular peptide-containing fibres. More recently, mast cells are thought to be involved in neovascularization both in microvessels in human coronary and carotid artery atheromas by possibly releasing angiogenic factors (Kaartinen et al., 1996; Jeziorska and Wooley, 1999)

A reduced nerve blood flow resulting in poor nerve perfusion, and consequent endoneurial hypoxia, has been suggested as a cause for the lower nerve conduction velocities seen in experimental diabetic animals (Low et al., 1989; Cameron and Cotter, 1994; Cameron et al., 1991). In the clinical setting, patients with diabetic neuropathy also show impaired nerve blood flow, epineurial arterio-venous shunting and reduced sural nerve oxygen tension (Tesfaye and Ward, 1994). Interestingly, the 50% reduction in nerve blood flow in diabetic animals observed after only one week of induction of diabetes, has been shown to be preventable or even corrected by vasodilator treatment (Cameron et al., 1991, 1992). Some improvement in clinical diabetic neuropathy following vasodilator treatment with an ACE inhibitor has also been reported (Reja et al., 1995).

Studies in rats using laser doppler flowmetry and the injection of vasoactive substances has shown that nerve blood flow is sensitive to adrenergic manipulation, thought to be via the α-adrenoceptors present on the vasa nervorum (Zochodne and Low, 1990). Although there is lack of evidence for any

autoregulation in peripheral nerve blood flow (Smith et al., 1977; Sugimoto and Monafo, 1987), the above studies suggest that at least in disease conditions the vasa nervorum may play an important role in the pathogenesis. The findings in this chapter of altered expression of NA-containing nerve fibres within the nerve trunk sheath, and the demonstration in the previous chapter of other transmitter substances also being present in nervi nervorum and perivascular nerves, further support a function for these nerves in regulating nerve blood flow. More recent studies also implicate an abnormality in the endothelial function of vasa nervorum in the diabetic state (Maxfield et al., 1997). These studies have demonstrated an impairment of the nitric oxide mediated relaxation of the vasa nervorum leading to increased vasoconstrictor sensitivity. This situation, in the background of increased adrenergic innervation as seen in this chapter, would lend itself to further embarrass nerve trunk perfusion thereby compounding the development of diabetic neuropathy.

TABLE 4.1

The distribution of NA-containing nervi vasorum and nervi nervorum in diabetic and control rat nerves

Nerve		Nervi vasorum	Nervi nervorum
Sciatic	Control	++	+
	Diabetic	+++	++
Vagus	Control	++	+
	Diabetic	++++	+++
Sympathetic	Control	++	+
	Diabetic	++++	+++
Optic	Control	+	+
	Diabetic	+/0	0

++++ = Very dense, +++ = dense, ++ = moderate, + = sparse, 0 = none

(n=12 for each group)

Sympathetic = paravertebral sympathetic chain.

FIGURE 4.1

Peri- and non-perivascular catecholemine fluorescence of nerve fibres along vasa nervorum and nervi nervorum of sciatic, vagus and optic nerves off control and diabetic rats.

a) Control sciatic vasa nervorum

b) Diabetic sciatic vasa nervorum showing increased innervation.

c) Control vagal vasa nervorum. (Arrows point to mast cells)

d) Diabetic vagal vasa nevorum showing increased innervation and mast cell numbers. (Small arrows point to mast cells; Larger arrows point to non-perivascular nerves)

e) Control optic nerve vasa nervorum.

f) Diabetic optic vasa nervorum showing loss of innervation.

Large open arrows indicate vessel margins.

Calibration bars = 25 μm

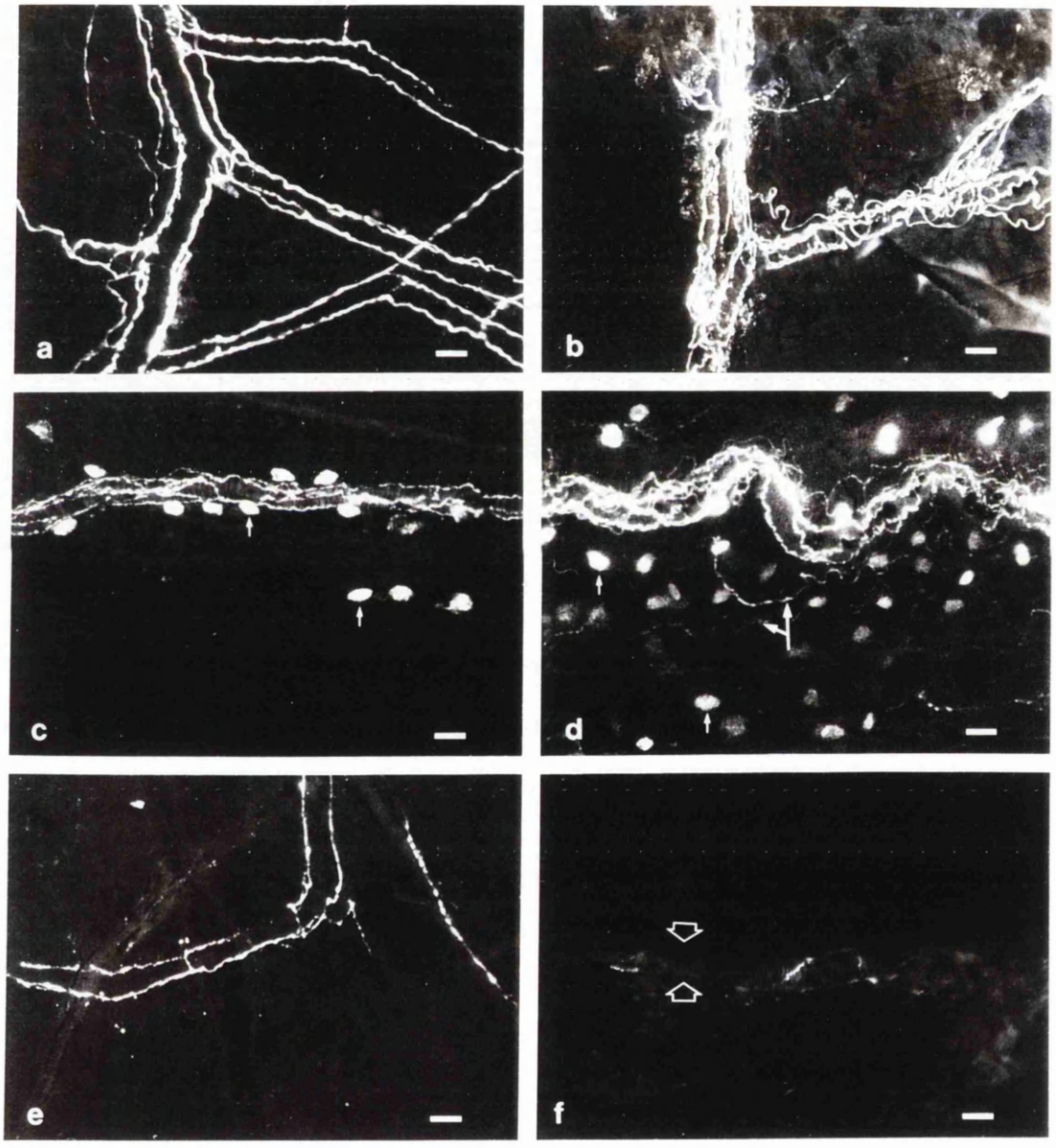
a
b
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FIGURE 4.2

Noradrenaline content in whole segments of sciatic, vagus and optic nerves of control and diabetic animals expressed in μg/g tissue (left side of figure) and in ng/cm nerve length (right side of figure) shown as a mean ± S.E.M. The number of animals in each experiment is indicated below each bar.

Open bars = Controls. Shaded bars = diabetic animals.

(nd = not detected; * = $p<0.05$)

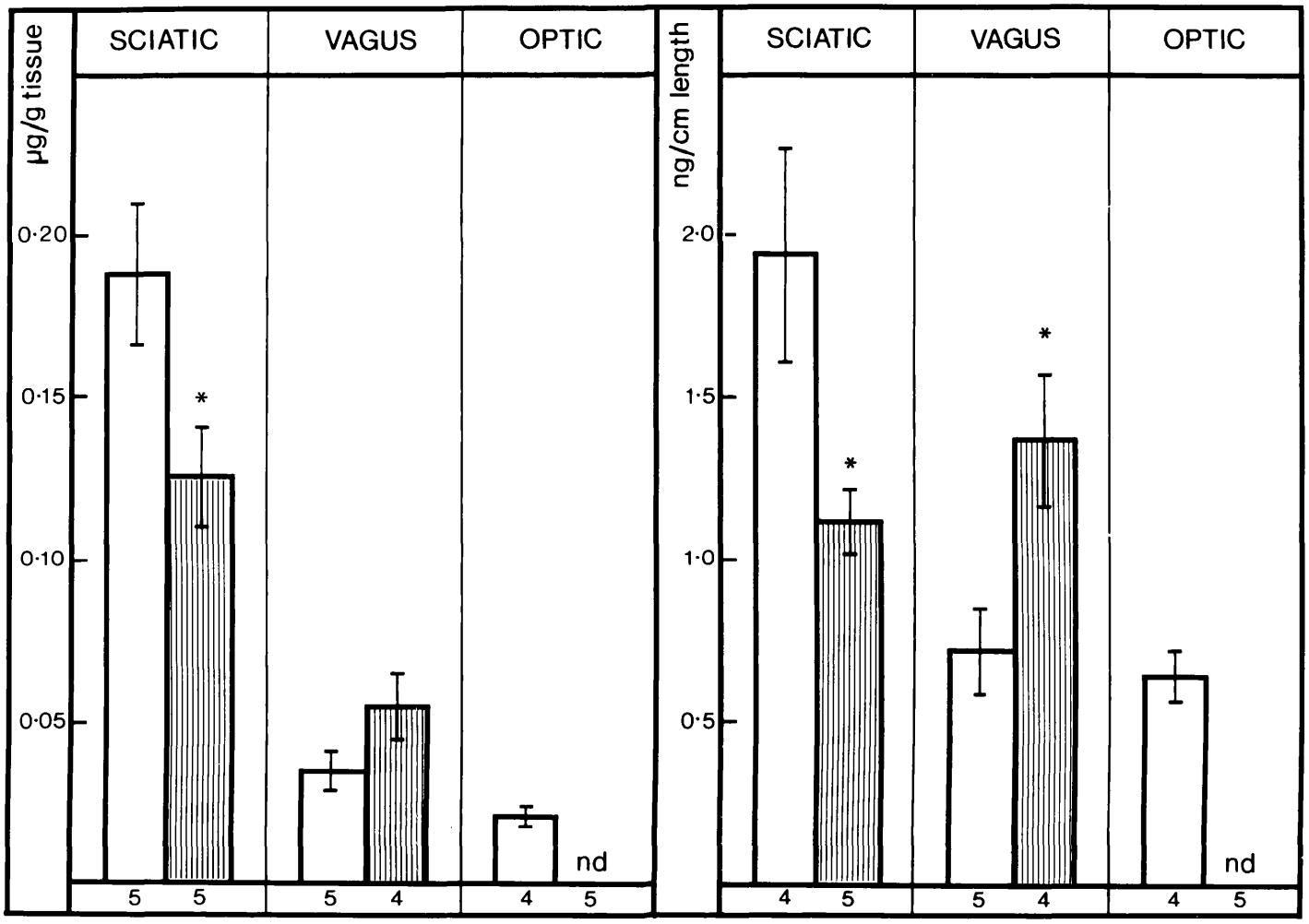

µg/g tissue
SCIATIC
VAGUS
OPTIC
0·20
0·15
0·10
0·05
*
nd
5 5
5 4
4 5
ng/cm length
SCIATIC
VAGUS
OPTIC
2·0
1·5
1·0
0·5
*
*
nd
4 5
5 4
4 5

FIGURE 4.3a

Catecholamine fluorescence in control human sural nerve biopsies.

Figure shows perivascular fibres along vasa nervorum.

Calibration bars = 50 μm.

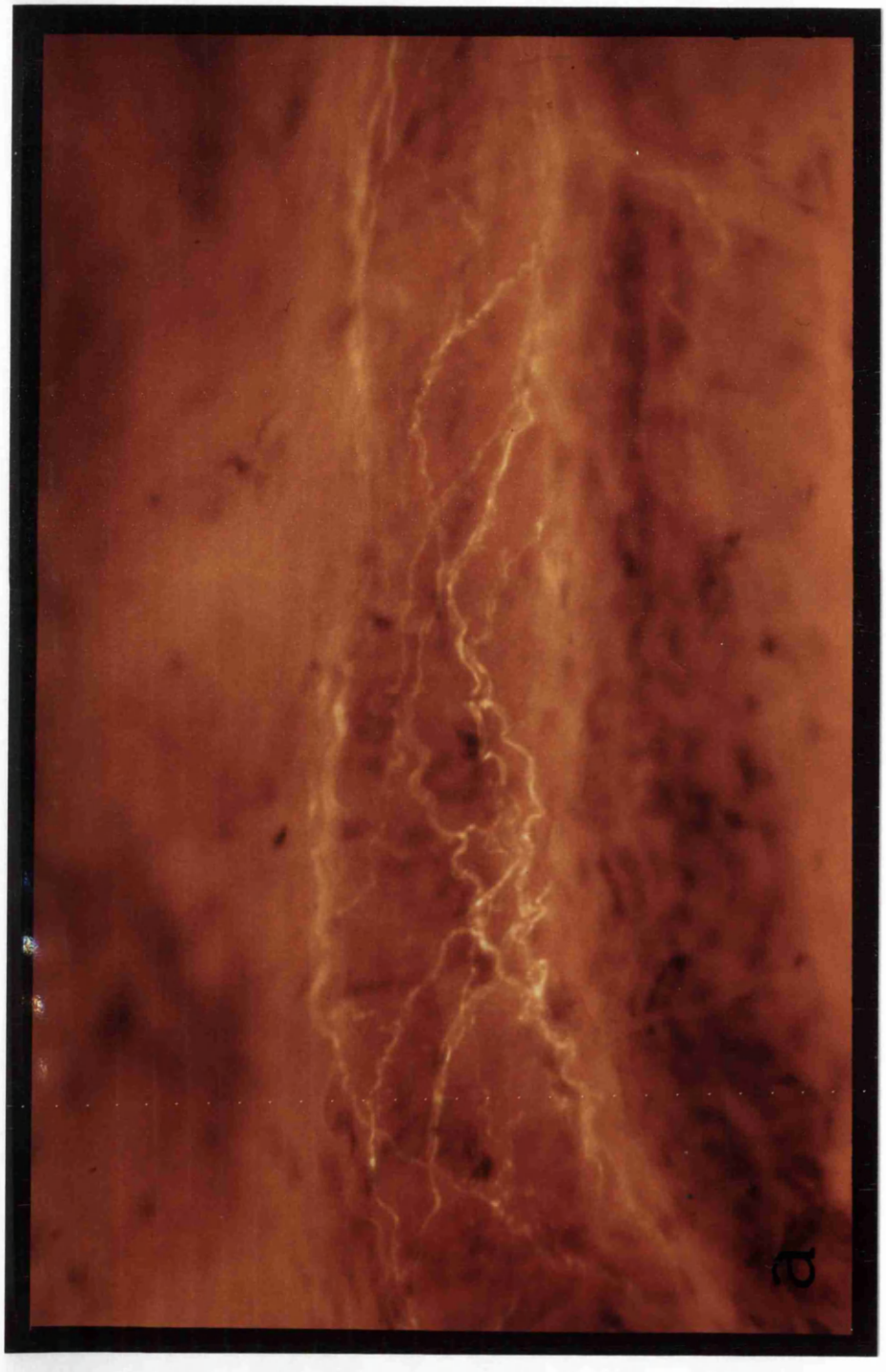


FIGURE 4.3b

Catecholamine fluorescence in control human sural nerve biopsies.

Figure shows perivascular fibres along vasa nervorum.

Calibration bars = 50 μm. ⟵——⟶

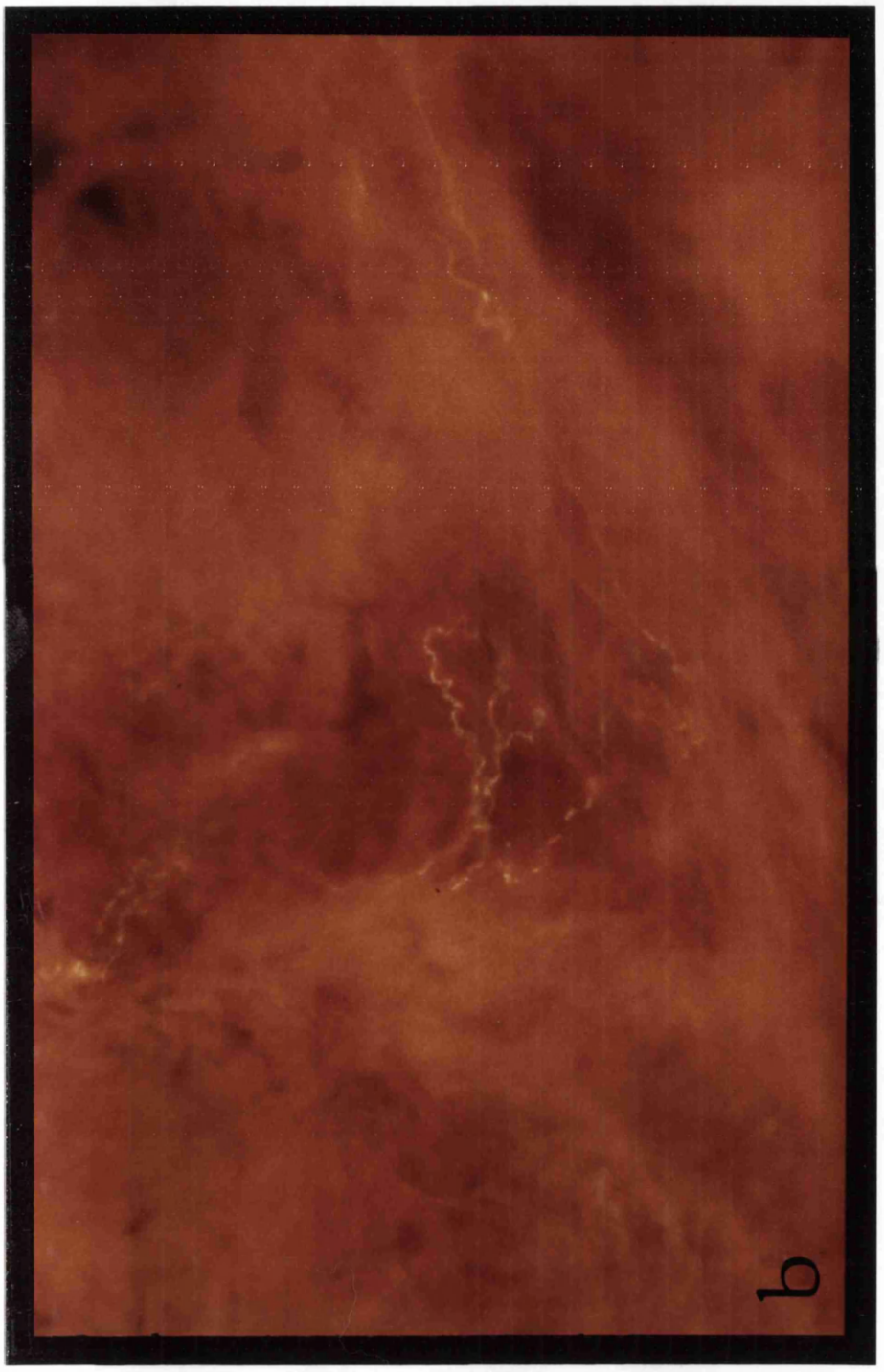


FIGURE 4.4a

Catecholamine fluorescence in diabetic human sural nerve biopsies.

Figure shows a marked loss of catecholamine fluorescent fibres.

Calibration bars = 50μm. ⟷

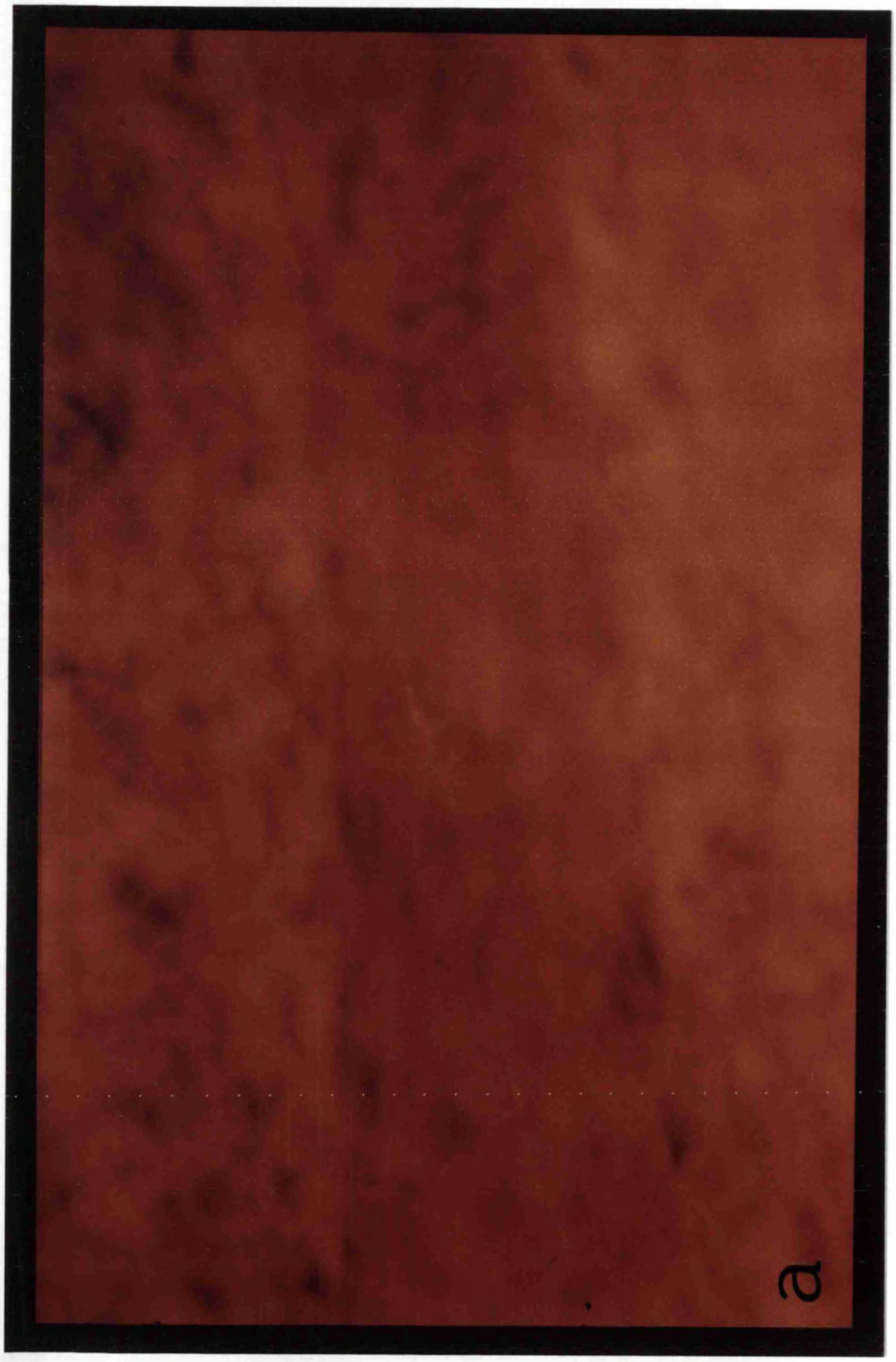


FIGURE 4.4b

Catecholamine fluorescence in diabetic human sural nerve biopsies.

Figure shows a marked loss of catecholamine fluorescent fibres.

Calibration bars = 50μm. ⟷

CHAPTER 5

AN INCREASE IN THE EXPRESSION OF NEUROPEPTIDE - CONTAINING VASODILATOR, BUT NOT VASOCONSTRICTOR, CEREBROVASCULAR NERVES IN AGING RATS

5.1 SUMMARY

Perivascular nerve fibres containing noradrenaline (NA), serotonin (5-HT), substance P (SP), vasoactive intestinal polypeptide (VIP), neuropeptide Y (NPY) and calcitonin gene-related peptide (CGRP) were localized in whole-mount stretch preparations of the major cerebral arteries of the rat using fluorescence and immunohistochemical techniques Changes in the pattern and density of these perivascular nerves were studied from birth through to 27 months of age. All perivascular nerve types reached a peak density of innervation at one month of age. This was followed by a general fall in the density of fluorescent nerve fibres. However with old age, there was a decrease in the expression of vasoconstrictor neurotransmitters (NA and 5-HT) in cerebrovascular nerves, whereas the expression of vasodilator neurotransmitters (VIP and CGRP) in perivascular nerves supplying the rat cerebral arteries was strikingly increased in old age. The density of NPY- and SP-containing nerve fibres were not significantly altered in old age.

5.2 INTRODUCTION

The higher occurrence of cerebrovascular disorders in the elderly still remains to be adequately explained in spite of the large amount of research carried out in this field. While many factors may play a role in the development of cerebrovascular diseases in old age, the localization of several putative neurotransmitters within perivascular nerve fibres has recently focused attention on the neurogenic mechanisms which regulate cerebrovascular tone (Burnstock, 1985a,b). Although the precise physiological role of these substances in the regulation of cerebral blood flow is not yet established, relationships between neurotransmitters and some cerebrovascular pathological conditions have been hypothesised. For example, serotoninergic nerve fibres have been associated with vasospasm (Vanhoutte et al., 1984), purinergic mechanisms with hyperaemia (Burnstock, 1981a) and SP with "axon reflex" vasodilatation (Lembeck and Holzer, 1979).

The innervation pattern of cerebral arteries by both classical and putative neurotransmitters in many species is well documented both in normal and some pathological conditions (Low et al., 1975; Edvinsson et al., 1978b; Lobato et al., 1980; Bannister et al., 1981; Lee and Saito, 1984a,b; Delgado et al., 1985). However, little is known about their developmental changes.

Studies concerning developmental changes of peptidergic perivascular innervation have been carried out on relatively few vessels (Llewellyn-Smith, 1984; Dhall et al., 1986), and much more information is available on the adrenergic innervation of several vasculatures in old age (Dolezel et al., 1974; Lundberg et al., 1976; Todd, 1980; Cowen et al., 1982a; Gallen et al., 1982; Santer, 1982; Duckles, 1983; Amenta et al., 1985b).

This chapter describes the developmental changes in noradrenergic, serotoninergic and peptidergic perivascular nerve fibres supplying rat cerebral vessels from birth through to 27 months of age.

5.3 MATERIALS AND METHODS

These experiments were carried out on locally bred male Wistar rats (n=150) at age stages : 1 day after birth and at 1, 4, 8 and 27 months. Animals were killed by an overdose of ether. The brains were rapidly removed and placed in Hanks solution (Gibco, Paisley, UK).

For the visualisation of noradrenergic fibres, the cerebral vessels were dissected out and processed according to the glyoxylic acid method described in Chapter 2.

For the demonstration of serotoninergic and peptidergic nerve fibres, cerebral arteries were fixed in situ and processed by the indirect immunofluorescent technique as described in Chapter 2. In situ fixation allowed for ease of dissection of cerebral arteries. It was possible to consistently remove the circle of Willis and basilar artery intact.

The following primary antisera were used:

- Rabbit anti-5-HT (R.I.A., UK) diluted 1:300
- Rabbit anti-VIP (R.I.A., UK) diluted 1:1000
- Rabbit anti-NPY (CRB) diluted 1:1000
- Rabbit anti-CGRP (CRB) diluted 1:400
- Rabbit anti-SP (Sera Lab., UK) diluted 1:200

The second layer antibodies used were:

- Goat anti-Rabbit IgG-FITC (Nordic) for 5-HT, VIP, NPY and CRGP
- Rabbit anti-Rat IgG-FITC (Nordic) for SP

Both were used at a dilution of 1:50.

Ten animals were used as controls for the specificity of immunostaining. The vessels were incubated with the antisera absorbed with an excess of the appropriate substance at 4°C prior to use. This resulted in the absence of any immunoreaction.

The density of perivascular innervation was quantitated using a 16X Neofluore objective lens according to the procedure described in Chapter 2.

5.4 RESULTS

In all the vessels of the rat Circle of Willis, noradrenergic nerve fibres showed the densest innervation at all age stages, followed by 5-HT- and NPY-containing nerve fibres, and then by VIP-, CGRP- and SP-containing nerve fibres in descending order.

With the exception of SP-containing nerve fibres, which were sparse and rarely formed plexuses, the pattern of innervation at birth was often different from that at other ages. At birth perivascular nerve fibres were organized in large, longitudinally orientated plexuses. From 1 month of age, the plexuses were orientated mainly in a circular fashion. Nerve varicosities were less dense but more prominent at birth.

NA-containing nerve fibres: NA-containing nerve fibres were well developed at birth. They showed a constant pattern of innervation from 1 to 8 months, and then significantly decreased at 27 months of age (Table 5.1, Figs. 5.1a and 5.2 a,b,c).

5-HT-containing nerve fibres: 5-HT-containing nerve fibres showed a well developed nerve plexus at birth, which reached a peak density at 1 month. The density was significantly diminished at 4 months, and more markedly at 27 months of age. There was a slight increase in the density of innervation in almost all the vessels between 4 and 8 months (Table 5.2, Figs. 5.lb and 5.2a, b, c). The density of 5-HT-containing nerve fibres was significantly greater in the proximal part of the basilar artery compared with the distal part from 1 to 8 months of age. No such regional variation in the density of the innervation along any one vessel was observed with regard to the other nerve types studied.

VIP-containing nerve fibres: At birth, VIP-containing nerve fibres were found only along the middle and the anterior cerebral arteries. The density of

innervation which reached a peak at 1 month, was maintained until the fourth month. Thereafter, in all vessels the number of VIP-containing nerve fibres declined at 8 months, followed by a significant increase at 27 months of age (Table 5.3, Figs. 5.3a and 5.4a,b,c).

CGRP-containing nerve fibres: The density of CGRP-containing nerve fibres at birth was very sparse in the cerebral arteries of the internal carotid system. Few CGRP-containing nerve fibres were visualized in the arteries of the vertebro-basilar system at this early age. Peak density of innervation was reached at 1 month, after which a significant decrease was observed. From the fourth month until old age there was a marked increase in the number of CGRP-containing nerve fibres innervating the cerebral arteries (Table 5.4, Figs. 5.3a and 5.4d,e,f).

NPY-containing nerve fibres: At birth, NPY-containing nerve fibres were present in almost all the examined vessels, with a maximum density along the basilar and the internal carotid arteries. Although the density of NPY-containing nerve fibres decreased significantly during development between 1 and 8 months, at 27 months the density was similar to that at 1 month (Table 5.5, Figs. 5.5a and 5.5a,b,c).

SP-containing nerve fibres: At birth, only one or two nerve fibres containing SP were found along the anterior and middle cerebral arteries. The density of SP-containing nerve fibres along these two vessels was significantly increased at 1 month. At this age, SP-containing nerve fibres were also present in the remaining vessels studied. The nerve density observed at 1 month was maintained throughout development until 27 months except in the middle cerebral artery where the density of innervation decreased significantly (Fig. 5.6d,e,f).

5.5 DISCUSSION

Based on fluorescence and immunohistochemical studies, results of this study show different patterns of innervation and differential expression for various perivascular neurotransmitters supplying the major cerebral arteries of the rat during development.

At all age stages, from birth through to 27 months of age, the densest innervation of cerebral vessels was shown by noradrenergic nerves, followed by those containing 5-HT, NPY, VIP, CGRP and SP, in that order. The innervation for all perivascular nerve types was denser in the vessels of the internal carotid system than in those of the vertebrobasilar system. In contrast to SP-, VIP- and CGRP-containing nerve fibres, those containing NA, 5-HT and NPY were well developed at birth and present in all of the vessels examined. Peak density of innervation by almost all these perivascular nerve types was reached at 1 month of age. Between 1 and 8 months, there was a decrease in the density of nerve fibres containing 5-HT, VIP, CGRP and SP in most of the vessels examined. A decrease in NPY-containing nerve fibres was only observed in the vessels of the internal carotid system. In terms of the relative life spans of humans and rats, this reduction in innervation density is consistent with decreased contents of NPY, VIP and SP in human middle cerebral arteries from the age of (1 year to an age group of 16 - 41 years (Edvinsson et al., 1985a, 1986). In the present study, this decreased cerebrovascular innervation in the rat, was followed by three kinds of developmental changes through to old age (27 months) namely an increase, a decrease and no change in the density of different perivascular nerve types.

Nerve fibres containing 5-HT and NA showed a significant reduction in the density of innervation from 8 months. The decrease in noradrenergic fibres with age is consistent with previous studies which have shown a reduction of noradrenergic nerve density in the arteries of old rats (Santer, 1982) and rabbits

(Cowen et al., 1982a; Duckles, 1983; Saba et al., 1984) Similarly, a reduction of catecholamine fluorescence, indicative of decreased NA levels, has been reported in sympathetic ganglia of both man (Hervonen et al., 1985) and rat (Santer, 1982) during old age. It is interesting to note that a physiological study has shown little change in vascular adrenergic function in old rats (Duckles et al., 1985). Both NA and 5-HT are potent vasoconstrictor substances in the cerebral circulation (Toda and Fujita, 1973; Edvinsson et al., 1978a, 1982). With the exception of a small population of nerve fibres which are thought to have a central origin the overwhelming majority of noradrenergic and serotoninergic innervation of the cerebrovasculature has a peripheral origin in the superior cervical ganglia (Alafaci et al., 1986a; Cowen et al., 1986; 1987). Superior cervical ganglionectomy in the rat leads to the disappearance of 5-HT- containing nerve fibres from all major cerebral arteries except the vertebral and basilar arteries (Cowen et al., 1987). The combination of 5-HT- containing nerve fibres from both peripheral and central origins supplying the posterior circulation is consistent with our observation of a significantly greater density of 5-HT- containing nerve fibres in the proximal compared with the distal part of the basilar artery between 1 and 8 months. There was no similar regional variation in the density of innervation along any one vessel by the other nerve types studied.

The reduction in the density of vasoconstriction nerve fibres containing NA and 5-HT was in marked contrast to the significant increase of innervation density shown by nerve fibres containing CGRP and VIP at 4 and 8 months respectively. The increase in CGRP- containing nerve fibres at a time when there is decreased noradrenergic innervation is consistent with a study showing a 70% rise in the CRGP content of rat pial arteries after bilateral excision of the superior cervical ganglia (Schon et al., 1985a). VIP and CGRP both exert potent vasodilatation in cerebral arteries (Said and Mutt, 1970; Edvinsson et al., 1980; McCulloch and

Edvinsson, 1980; Hanko et al., 1985. Immunohistochemical studies have shown the coexistence of VIP with AChE (Kobayashi et al ., 1983; Hara et al., 1985), and SP with CGRP (Uddman et al., 1985a) within perivascular nerves supplying the cerebral vasculature. VIP- and AChE-containing cerebrovascular nerves originate from the sphenopalatine ganglion (Hara et al., 1985, 1987), whereas nerve fibres containing SP and CGRP principally originate from the trigeminal ganglion (Lee et al., 1985a; Uddman et al., 1985). The present findings, which show a greater density of CGRP-containing nerve fibres as compared with those containing SP, are consistent with a greater number of CGRP-positive neurons in the trigeminal ganglion (Lee et al., 1985a; Skofitsch and Jacobowitz, 1985).

The density of SP-containing nerve fibres stayed constant with age, while the density of those containing NPY fluctuated during development. Nevertheless, the density of both these two nerve types at 27 months of age was similar to the peak density seen at 1 month of age. Along with VIP and CGRP, SP exerts potent vasodilatation of cerebral arteries (Burcher et al., 1977; Edvinsson and Uddman, 1982). SP-positive nerve fibres have also been attributed with a sensory role in the vasculature (Hokfelt et al., 1975, 1977; Moskowitz, 1984).

NPY, which has been shown to coexist with NA in perivascular nerve fibres (Ekblad et al., 1984; Lundberg et al., 1983; Sternini and Brecha, 1985) is a potent vasoconstrictor of cerebral vessels (Edvinsson et al., 1983b, 1984c; Dahlof et al., 1985; Edvinsson, 1985). The persistence of abundant NPY-containing nerve fibres in 27 month old rats indicates that this substance may be an important physiological vasoconstrictor in rat cerebral vessels in old age. NPY has also been shown to be a neuromodulator of the release and action of NA (Stjarne et al ., 1986).

The results from this study show that different perivascular nerve types follow markedly different developmental changes. Further work is needed to elucidate if certain developmental changes can be ascribed to perivascular nerve types according to their particular physiological role. The increasing evidence for the coexistence of a combination of classical and putative neurotransmitters in perivascular nerves strongly warrants studies to determine whether or not coexisting substances can show different developmental changes. Certainly, the present results would indicate that this is the case with regard to NA and NPY in sympathetic nerves and SP and CGRP in sensory nerves in the cerebral circulation. The extent to which these substances may serve a neuromodulatory or trophic role needs clarification (Burnstock, 1982a).

In general, the present study shows a decrease in perivascular vasocontrictory nerve fibres with age. These changes could be regarded consistent with a loss of sympathetic trophic regulation of vascular smooth muscle and an increased susceptibility to sclerotic and degenerative lesions in old age (Wexler and True, 1963). The concomitant increase in VIP- and CGRP-containing vasodilatory nerve fibres in 27 month old rat cerebral arteries may therefore represent a compensatory mechanism towards providing a greater blood flow to the brain in old age.

5.6 CHAPTER UPDATE

Although I have included relevant updates with respect to the cerebrovascular innervation in the General Introduction, the following section will briefly summarise the more interesting scientific findings with specific reference to development and ageing.

Some of the age related changes described in this chapter have since been confirmed by others. A reduction in 5-HT-like immunoreactive nerve fibres supplying cerebral vessels in old age has been shown in rabbits (Gale et al., 1989) and rats (Cowen and Thrasivoulou, 1990). Identical development of cerebrovascular nerves positive for noradrenaline and NPY, and a close correlation between neuropeptide Y and VIP-like immunoreactive cerebrovascular nerves in old age has been demonstrated (Tsai et al., 1992)

A better appreciation of the origin of various cerebrovascular nerve types has become apparent. There is considerable consistency amongst numerous studies demonstrating that NPY-LI fibres originate from the superior cervical ganglion, that VIP-LI nerve fibres originate from the sphenopalatine and otic ganglia, and that CGRP- and SP-LI nerve fibres originate from the trigeminal and upper cervical dorsal root ganglia (Saito and Moskowitz, 1989; Suzuki et al., 1989a; Uddman et al., 1989). A population of CGRP-LI nerve fibres innervating the cerebral vessels also seem to originate from the adventitial ganglion cells of the internal carotid artery (Okuno et al, 1994).

The controversy over 5-HT-LI nerve fibres remains controversial. Studies continue to localise 5-HT-LI cerebrovascular nerves with possibly dual origin from the superior cervical ganglion and the raphe nuclei differentially supplying the major cerebral arteries and the small pial vessels in the rat (Bonvento et al., 1991). At the same time, there is further evidence that cerebral vessels do not receive a true

serotonergic innervation, and that their presence is secondary to 5-HT uptake by sympathetic nerve endings (Yu et al., 1989).

Additional substances have also been localized to cerebrovascular nerves. GABAergic innervation has been found to be co-localised with those immunoreactive for CGRP, and appear to originate from the trigeminal ganglia as well as from the adventitial ganglion cells of the internal carotid artery (Okuno et al, 1994). Pituitary adenylate cyclase activating peptide (PACAP), which is a VIP-like peptide, has been localised along cerebral vessels (Uddman et al.,1993). Of greater interest has been the finding of nitric oxide synthase-immunoreactivity in perivascular nerves as well as endothelial cells of rat basilar artery (Loesch et al., 1994; Loesch and Burnstock, 1996). The presence of NADPH-diaphorase positive neurons within close proximity of arterioles descending through the cerebral cortex adds further evidence for a role for nitric oxide in cerebrovascular neuroeffector mechanisms (Regidor et al., 1993).

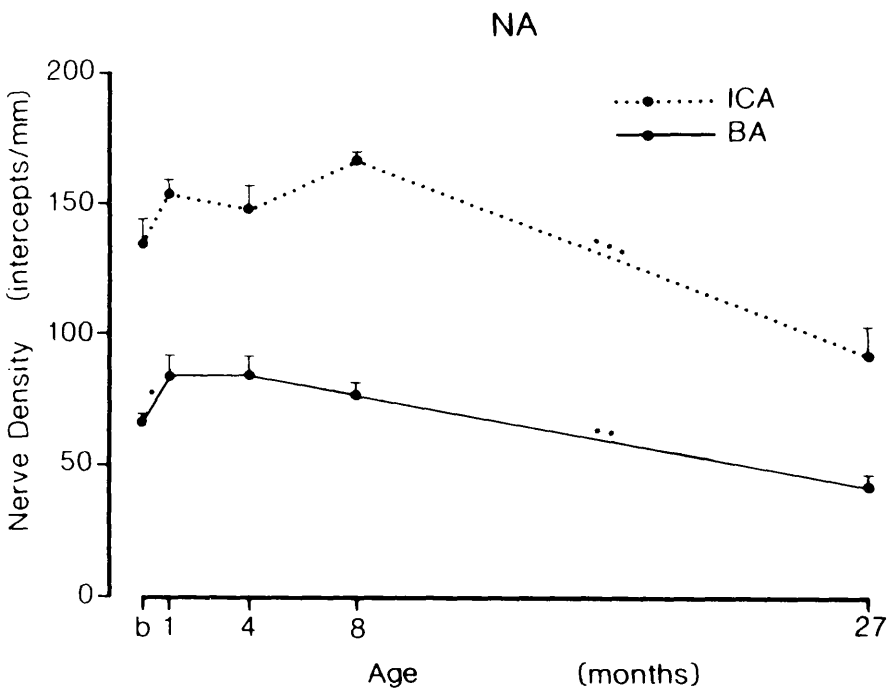
Perhaps the most important work concerning the plasticity of cerebrovascular nerves in development and ageing, has come from studies looking into trophic mechanisms involving the interaction between neurons and their target tissue. When rat superior cervical ganglia are explanted from young and old rats, and implanted *in-oculo*, the subsequent reinnervation of the host iris by the transplanted ganglia show no age differences (Gavazzi and Cowen, 1993a,b). Further evidence for such neurotrophic mechanism comes from the ability of old age cerebral vessels to receive 'young' type innervation in the presence of nerve growth factor treatment. Nerve growth factor also seems to increase the dendritic arborization of cerebrovascular sympathetic neurons in old age (Andrews and Cowen, 1994). The neurotrophic theory therefore suggests that aged neurons do not lose their ability to regenerate, but that the innervation pattern is determined by the quality of the target tissue (Cowen, 1993).

FIGURE 5.1

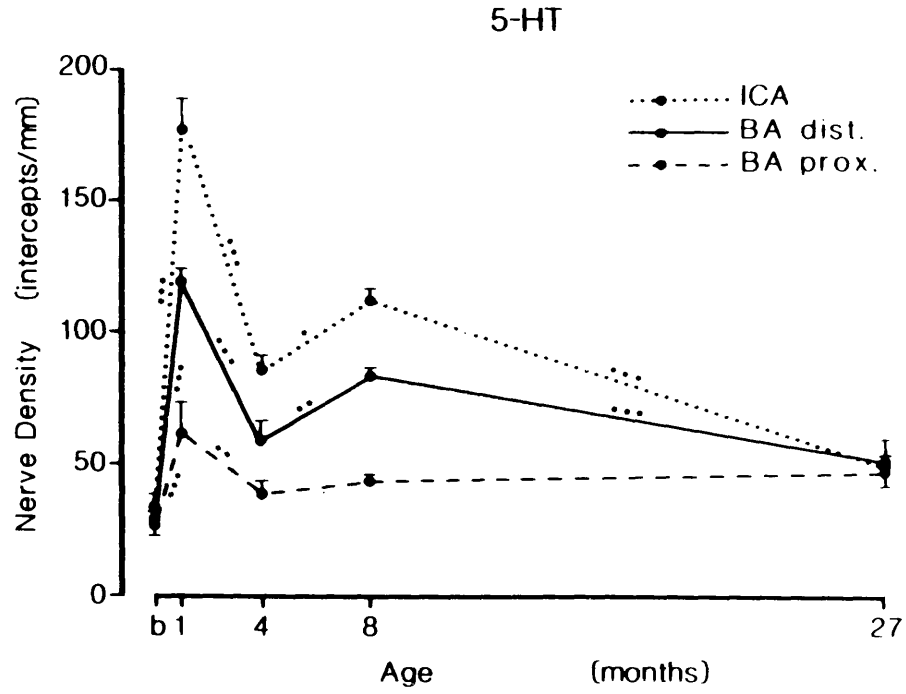
Graphs of age-related changes of (a) NA- and (b) 5-HT-containing nerve fibres in the internal carotid (ICA) and basilar (BA) arteries.

b = 1 day after birth. (Note different values for the Y-axis when compared with Figs. 7.3 and 7.5.)

*P <0.05, ** P <0.01, *** P <0.001.


NA
ICA
BA
Nerve Density (intercepts/mm)
200
150
100
50
0
b 1 4 8 27
Age (months)

a


5-HT
ICA
BA dist.
BA prox.
Nerve Density (intercepts/mm)
200
150
100
50
0
b 1 4 8 27
Age (months)

b

FIGURE 5.2

Photomicrographs of whole-mount preparations of cerebral arteries showing perivascular nerve fibres at various age stages.

NA-containing nerve fibres in the middle cerebral artery (MCA) at:

(a) 1 day

(b) 4 months

(c) 27 months

5-HT-containing nerves in the distal portion of the basilar artery (BA) at:

(d) 1 day

(e) 8 months

(f) 27 months

Nerve density shows a decrease in old age for both NA and 5-HT. Arrows indicate longitudinal axis of blood vessel . Scale bars = 25 um.

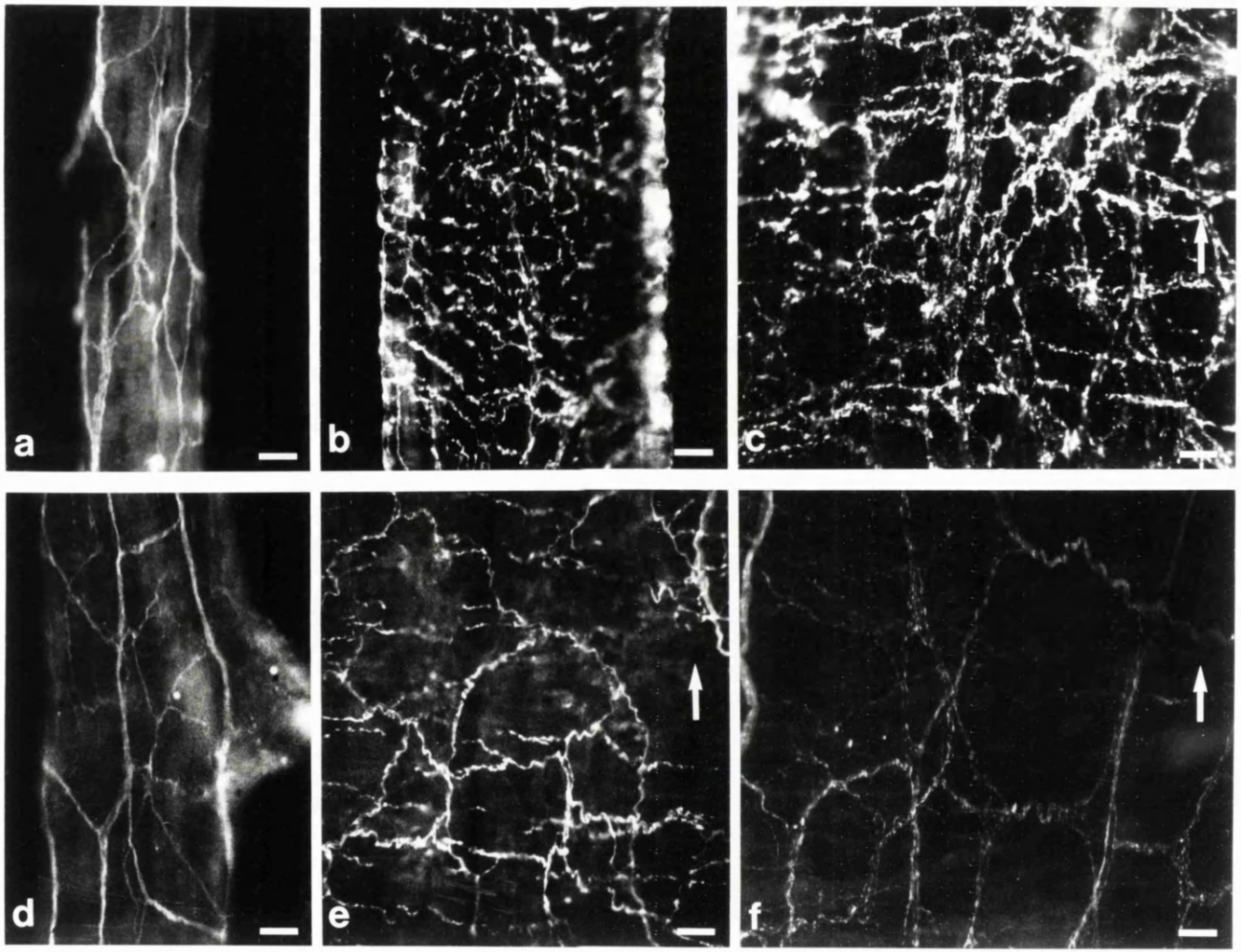
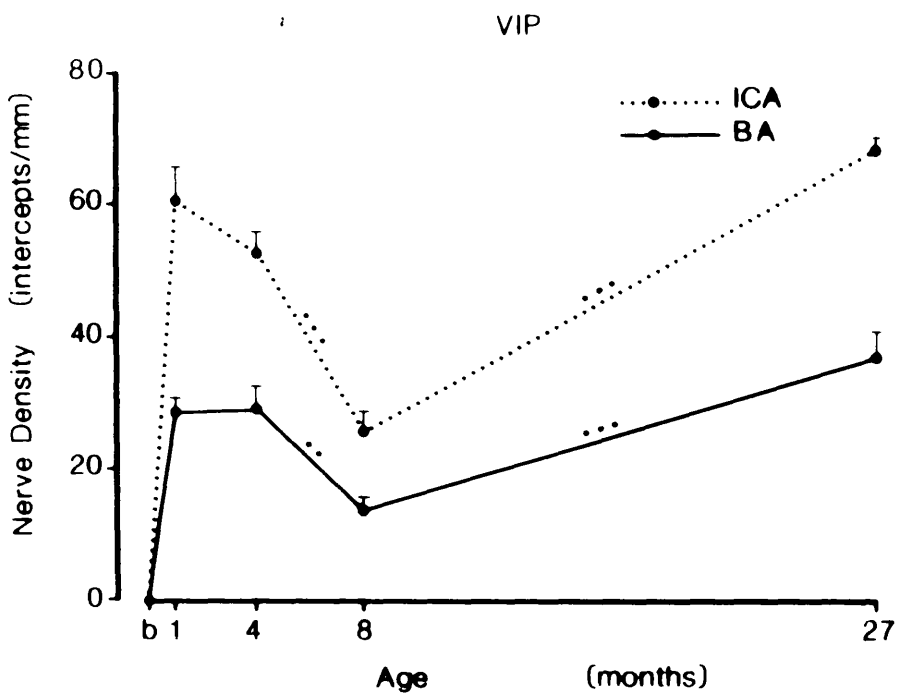

a
b
c
d
e
f

FIGURE 5.3

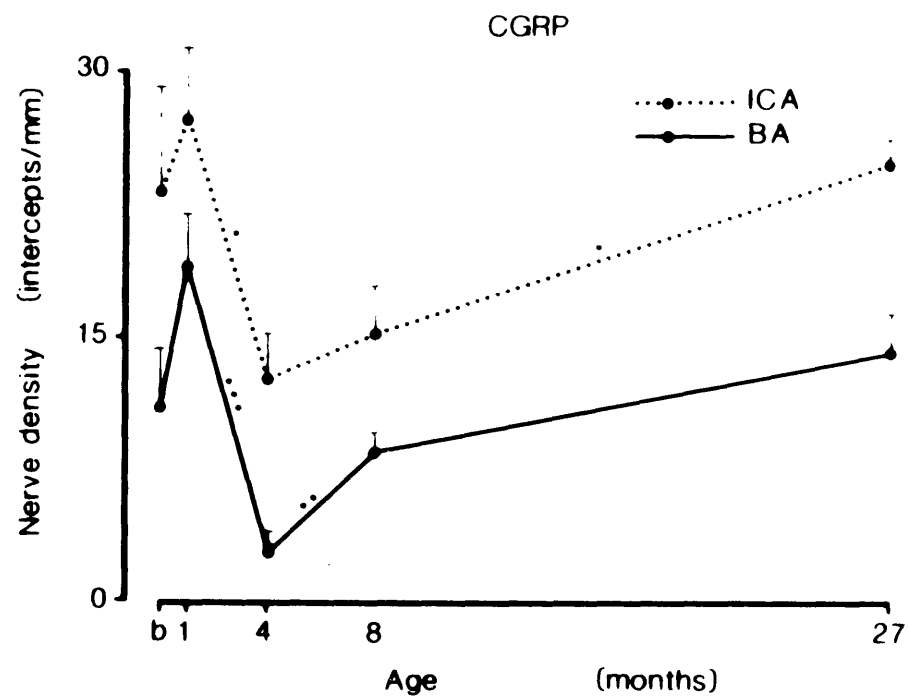
Graphs of age-related changes in the density of (a) VIP- and (b) CGRP-containing nerve fibres in the internal carotid (ICA) and basilar (BA) arteries.

b = 1 day after birth

(*P <0.05, ** P <0.005, ***P <0.001.)


VIP
ICA
BA
Nerve Density (intercepts/mm)
80
60
40
20
0
b 1 4 8 27
Age (months)

a


CGRP
ICA
BA
Nerve density (intercepts/mm)
30
15
0
b 1 4 8 27
Age (months)

b

FIGURE 5.4

Photomicrographs of whole-mount preparations of cerebral arteries showing perivascular nerve fibres at various age stages.

VIP-containing nerves in the middle cerebral artery (MCA) at:

(a) 1 day

(b) 8 months

(c) 27 months

CGRP-containing nerves in the posterior cerebral artery (PCA) at:

(d) 1 day

(e) 4 months

(f) 27 months

Nerve density is increased in old age for both VIP and CGRP.

Scale bar = 25 um.

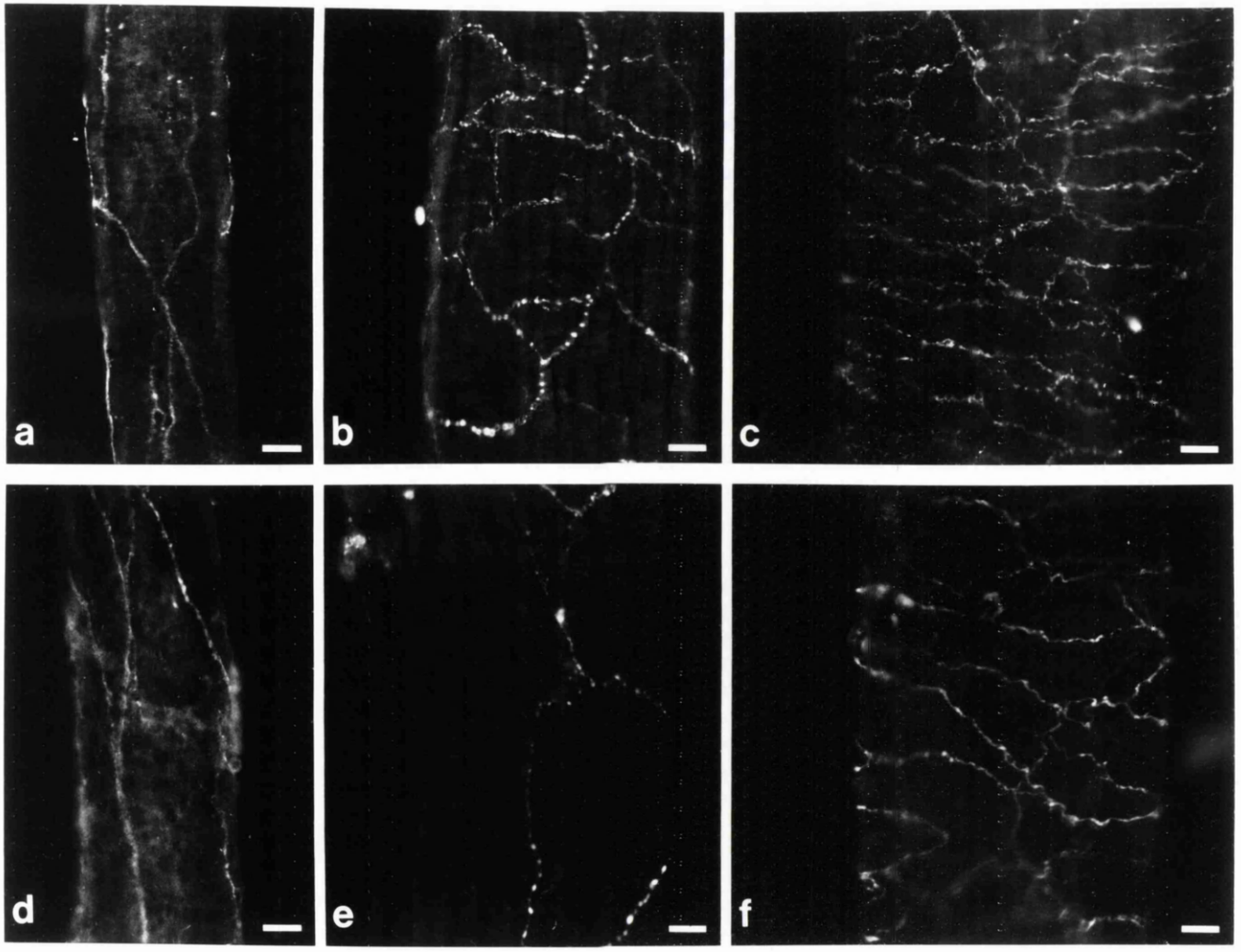

a
b
c
d
e
f

FIGURE 5.5

Graph of age-related changes in the density of NPY-containing nerve fibres in the internal carotid (ICA) and basilar (BA) arteries.

b = 1 day after birth

*P <0.05, ** P <0.0l, *** P <0.00l.

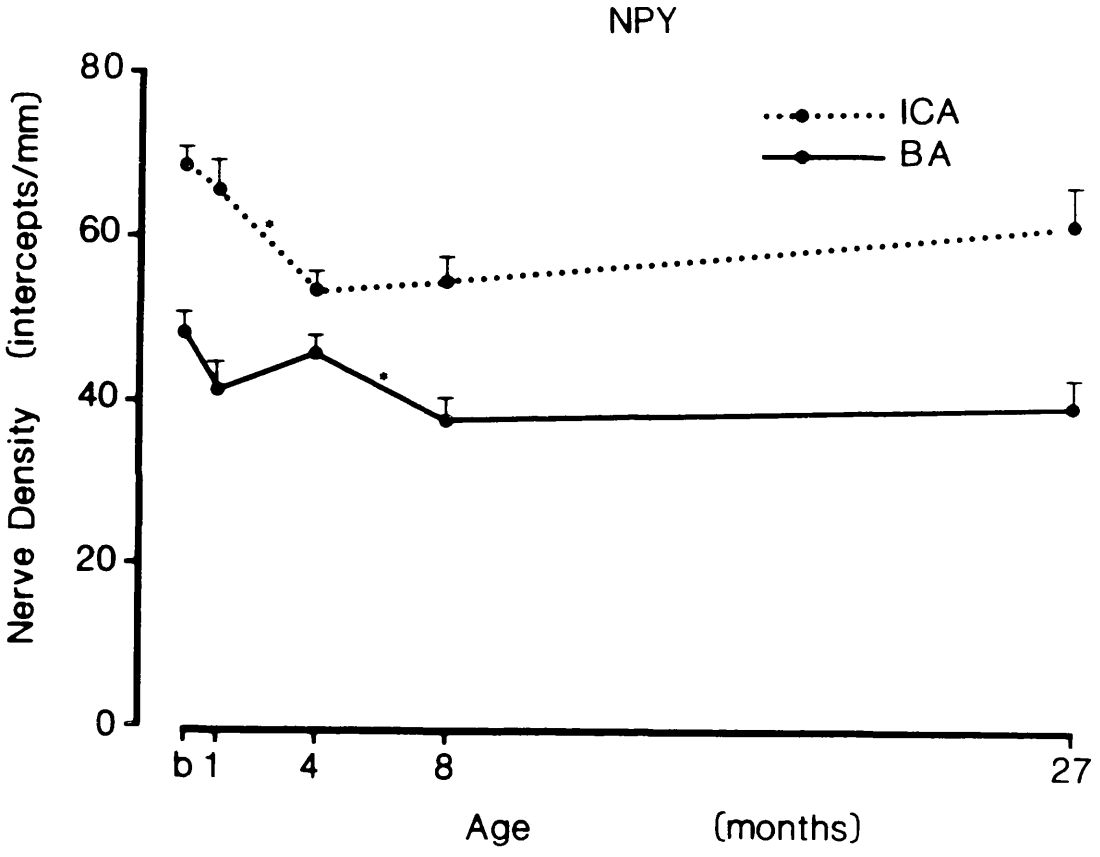

NPY
ICA
BA
Nerve Density (intercepts/mm)
80
60
40
20
0
b
1
4
8
27
Age (months)

FIGURE 5.6

Photomicrographs of whole-mount preparations of cerebral arteries showing perivascular nerve fibres at various age stages.

NPY-containing nerve fibres in the internal carotid artery (ICA) at:

(a) 1 day

(b) 1 month

(c) 27 months

SP-containing nerve fibres in the anterior cerebral artery (ACA) at:

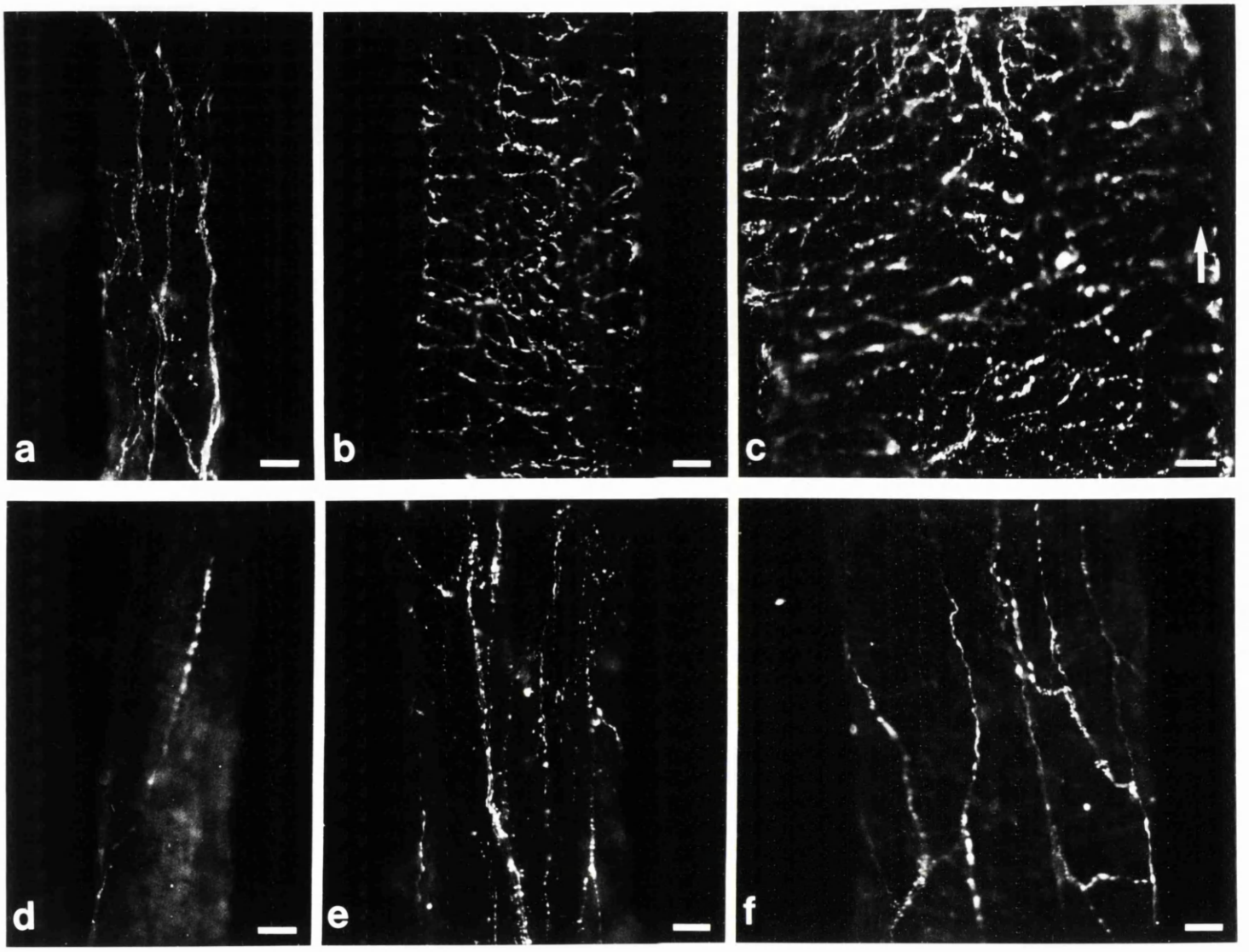
(d) 1 day

(e) 1 month

(f) 27 months

Nerve density is maintained with development for both NPY and SP. Arrow indicates longitudinal axis of blood vessel.

Scale bars = 25 um.


a
b
c
d
e
f

TABLES 5.1 - 5.5

In the following tables, the **p** values of significance refer to a comparison with the density of innervation of the preceeding developmental stage.

No comparison is made, in these tables, between the density of innervation in one vessel against another.

TABLE 5.1

Density of NA-containing nerve fibres supplying cerebral arteries of rats at different ages.
Nerve density is expressed as mean number of nerve intercepts per mm of vessel circumference ± S.E.M.

Vessel	1 day	1 month	4 months	8 months	27 months
BA	67.00 ± 2.34	84.89 ± 7.59	84.49 ± 7.06	76.39 ± 5.77	42.44 ± 4.88**
SCA	44.44 ± 6.08	73.84 ± 8.24*	58.89 ± 4.88	42.28 ± 4.67*	23.93 ± 0.39**
PCA	133.89 ± 4.66	124.72 ± 11.5	116.11 ± 6.27	118.0 ± 3.67	53.56 ± 2.11**
ICA	132.71 ± 9.82	152.28 ± 6.92	147.39 ± 9.87	165.89 ± 2.82	91.19 ± 11.63***
MCA	68.58 ± 5.55	104.61 ± 2.88***	113.67 ± 6.35	95.33 ± 8.6	46.52 ± 1.70***
ACA	161.06 ± 8.28	203.78 ± 11.9**	241.39 ± 12.76*	227.11 ± 10.84	83.33 ± 6.48***

* $P<0.05$, ** $P<0.01$, *** $P<0.001$ n = 5 at each age stage

BA = Basilar artery, SCA = Superior cerebrellar artery, PCA = Posterior cerebral artery, ICA = Internal carotid artery
MCA = Middle cerebral artery and ACA = Anterior cerebral artery.

TABLE 5.2

Density of 5-HT-containinmg nerve fibres supplying cerebral arteries of rats at different ages. Nerve density is expressed as mean number of nerve intercepts per mm of vessel circumference ± S.E.M.

Vessel		1 day	1 month	4 months	8 months	27 months
BA	P	26.84 ± 4.06	119.38 ± 5.41***	62.8 ± 3.64***	82.84 ± 3.86**	50.28 ± 2.06***
	D	27.94 ± 3.96	67.16 ± 5.83***	39.47 ± 4.18**	43.49 ± 2.12	45.87 ± 5.46
SCA		32.06 ± 2.5	51.02 ± 7.26*	23.78 ± 1.14**	44.00 ± 1.12***	33.72 ± 6.10
PCA		+	109.15 ± 4.10***	48.39 ± 2.77***	68.61 ± 3.28***	50.45 ± 5.62
ICA		32.39 ± 6.12	176.89 ± 11.09***	86.77 ± 4.84***	110.22 ± 6.92*	49.83 ± 8.90***
MCA		15.61 ± 5.16	120.89 ± 5.58***	69.50 ± 13.39**	69.51 ± 1.07	52.50 ± 10.64
ACA		18.22 ± 2.08	169.42 ± 8.79***	109.89 ± 6.48***	150.85 ± 4.21***	60.89 ± 5.84***

* $P<0.05$, ** $P<0.01$, *** $P<0.001$ n=5 at each age stage

P = Proximal, D = Distal, BA = Basilar artery, SCA = Superior cerebellar artery, PCA = Posterior artery, ICA = Internal carotid artery, MCA = Middle cerebral artery and ACA = Anterior cerebral artery.
+ = only single fibres were observed in some of the preparations.

TABLE 5.3

Density of VIP-containing nerve fibres supplying cerebral arteries of rats at different ages. Nerve density is expressed as mean number of nerve intercepts per mm of vessel circumference ± S.E.M.

Vessel	1 day	1 month	4 months	8 months	27 months
BA	-	28.05 ± 2.62	28.93 ± 3.37	13.51 ± 2.32**	36.76 ± 3.51***
SCA	-	24.82 ± 3.67	18.03 ± 1.30	10.76 ± 1.09**	27.37 ± 4.82**
PCA	-	62.65 ± 3.77	61.54 ± 1.04	14.09 ± 1.54***	37.77 ± 1.98***
ICA	-	60.58 ± 4.45	52.76 ± 3.21	25.07 ± 3.63***	67.62 ± 1.98***
MCA	23.74 ± 0.79	48.32 ± 2.80***	48.76 ± 1.46	20.74 ± 1.77***	44.35 ± 1.92***
ACA	27.14 ± 3.12	55.27 ± 2.48***	48.21 ± 2.17	34.13 ± 2.70**	46.86 ± 3.89*

* $P<0.05$, ** $P<0.01$, *** $P<0.001$, $n = 5$ at each age stage

D' Distal. P = Proximal, BA = Basilar artery, SCA = Superior cerebellar artery, PCA = Posterior cerebral artery, ICA = Internal carotid artery, MCA = Middle cerbral artery and ACA = Anterior cerebral artery. - = No nerve fibres were visualized.

TABLE 5.4

Density of CGRP-containing nerve fibres supplying cerebral arteries of rats at different ages. Nerve density is expressed as mean number of nerve intercepts per mm of vessel circumference ± S.E.M.

Vessel	1 day	1 month	4 months	8 months	27 months
BA	11.01 ± 3.31	19.06 ± 2.91	+	8.53 ± 1.16**	13.93 ± 2.27
SCA	+	7.00 ± 2.62	+	6.98 ± 1.04***	15.66 ± 1.51**
PCA	+	17.72 ± 2.51	+	7.89 ± 2.46	21.02 ± 2.00**
ICA	23.05 ± 6.02	27.34 ± 3.81	12.66 ± 2.65	15.16 ± 2.8	24.70 ± 1.32*
MCA	+	15.11 ± 3.36	5.28 ± 2.07*	7.28 ± 2.50	28.26 ± 3.31***
ACA	20.99 ± 7.16	31.37 ± 5.12	18.22 ± 0.92*	26.33 ± 2.37*	26.21 ± 1.82

* $P<0.05$, ** $P<0.01$, *** $P<0.001$, n=5 at each age stage

BA = Basilar artery, SCA = Superior cerebellar artery, PCA = Posterior cerebral artery, ICA = Internal carotid artery, MCA = Middle cerebral artery and ACA = Anterior cerebral artery.
+ = Only single fibres were observed in some of the preparations.

TABLE 5.5

Density of NPY-containing nerve fibres supplying cerebral arteries of rats at different ages.
Nerve density is expressed as mean number of nerve intercepts per mm of vessel circumference ± S.E.M.

Vessel	1 day	1 month	4 months	8 months	21 months
BA	48.03 ± 2.79	41.10 ± 3.39	45.97 ± 2.02	37.79 ± 2.31*	39.82 ± 3.07
SCA	-	28.03 ± 1.59	25.31 ± 2.72	29.25 ± 3.86	22.39 ± 1.64
PCA	62.2 ± 7.38	52.76 ± 3.26	50.57 ± 1.78	36.21 ± 4.69**	46.76 ± 0.9
ICA	68.99 ± 1.71	65.55 ± 3.58	53.26 ± 3.53*	54.55 ± 2.86	61.41 ± 4.68
MCA	46.16 ± 6.81	58.67 ± 5.36	39.74 ± 0.89**	48.07 ± 5.12	56.09 ± 4.84
ACA	48.02 ± 9.33	72.96 ± 4.35*	65.85 ± 2.62*	60.78 ± 2.69	73.98±3.34*

* $P<0.05$, ** $P<0.01$, *** $P<0.001$ n=5 at each age stage

BA = Basilar artery, SCA = Superior cerebellar artery, PCA = Posterior cerebral artery, ICA = Internal carotid artery, MCA = Middle cerebral artery and CA = Anterior cerebral artery.
- = No nerve fibres were visualized.

CHAPTER 6

INCREASED DENSITY OF PERIVASCULAR NERVES TO THE MAJOR CEREBRAL VESSELS OF THE SPONTANEOUSLY HYPERTENSIVE RAT: DIFFERENTIAL CHANGES IN NORADRENALINE AND NEUROPEPTIDE Y DURING DEVELOPMENT

6.1 SUMMARY

Fluorescence and immunohistochemical techniques were used to study the pattern and density of perivascular nerves containing noradrenaline (NA) and neuropeptide Y (NPY) supplying the major cerebral arteries of 4, 6, 8 and 12 week old spontaneously hypertensive rats (SHR) and normotensive Wistar (WIS) controls. Levels of NA and NPY in the superior cervical ganglia were measured. the density of nerves containing NA and NPY was greater in the hypertensive animals at all ages studied. However, the developmental changes in the density of innervation showed similar trends in both SHR and WIS groups. With few exceptions, there was a significant increase in the density of nerves containing NA from 4 to 6 weeks and from 8-12 weeks of age. This was in contrast to a low expression, and in some vessels a significant decrease in the number of NPY-containing nerves from 4-6 weeks. The density of nerve fibres containing NPY increased significantly in almost all vessels between 6-8 weeks of age and then stabilized. Thus there is a differential time-course for the appearance of NA and NPY during development. Furthermore, the hyperinnervation of cerebral vessels in SHR by nerves containing NA and NPY precedes the onset of hypertension and associated medial hypertrophy. High performance liquid chromatography and enzyme-linked immunosorbant assays show that the NA and NPY contents of the superior cervical ganglion do not reflect the changes in innervation pattern seen in the terminal fibres in the cerebral arteries. This tends to support the view that a local neurovascular mechanism is involved in the maintenance of hypertension. The possibility that increase in NPY as well as NA in cerebral perivascular nerves of hypertensive animals is involved in the protection of the blood-brain barrier against oedema and cerebral haemorrhage is raised.

6.2 INTRODUCTION

Past studies of the relationship between sympathetic nerves and blood vessels in hypertension have dealt almost exclusively with adrenergic nerves (Graham et al., 1970; Scott et al., 1982; Scott and Pang, 1983; Lee and Saito, 1984a; Scott and Galway, 1984; McLean et al., 1985; Haebara et al., 1986). Recent advances in histochemical techniques, particularly immunohistochemistry have revealed, in addition to the classical adrenergic and cholinergic neurotransmitters, a number of other substances (including serotonin, ATP and various peptides) within nerve fibres innervating the cerebral vasculature. These non-adrenergic, noncholinergic perivascular autonomic nerve types may also be of importance in the development and/or maintenance of essential hypertension in man and experimental animal models. Of these, NPY, a 36-amino acid peptide first isolated from porcine brain (Tatemoto et al., 1982), is present abundantly in a dense plexus of nerves supplying cerebral vessels, where it is a potent vasoconstrictor in both in vivo and in vitro preparations (Edvinsson et al., 1983b, 1984b, 1985a; Allen et al., 1984a; Edvinsson, 1985; Tuor et al ., 1985). In vessels such as the rat and rabbit basilar arteries, where NA is not implicated in the pressor effects, NPY induces a pronounced vasoconstriction at low doses, suggesting that in these vessels NPY may represent a major transmitter substance mediating this response (Edvinsson, 1985). The similarity in the distribution of NA and NPY in the peripheral nervous system has led to a number of pharmacological and, especially, immunohistochemical studies which have substantiated the coexistence of these two substances within many perivascular nerves (Lundberg et al ., 1982; Ekblad et al ., 1984; Sternini and Brecha, 1985; Uddman et al., 1985b). The coexistence of NA and NPY in some nerves

supplying the cerebral vasculature has been demonstrated in a recent ultrastructural study (Matsuyama et al., 1985). It is possible that some NPY-containing perivascular nerve fibres have their origin in neurons in the brain (Alafaci et al., 1985; Matsuyama et al., 1985). While the physiological importance of sympathetic nerves in the regulation of resting cerebral blood flow (CBF) is reported to be minimal (Mueller et al., 1977; Heistad and Marcus, 1978), there is now considerable evidence that they play an important role in keeping the CBF to normal levels during pronounced arterial hypertension (Edvinsson et al., 1976). This ability of sympathetic nerves to shift the upper limit of autoregulation towards higher blood pressure levels is thought to represent a protective mechanism against cerebral oedema and intracerebral haemorrhage (Bill and Linder, 1976; Sadoshima et al., 1981; Heistad, 1982; Mueller et al. 1982; Sadoshima and Heistad, 1982; Mueller and Ertel, 1983).

In the present study the distribution of NA and NPY in nerves is compared during the early development of cerebral vessels in both spontaneously hypertensive rats (SHR) and in normotensive Wistar (WIS) controls before and after the time when hypertension becomes clearly apparent after 5 weeks of age, when no intimal lesions are yet present at the cerebral level (Weber et al., 1986).

6.3 MATERIALS AND METHODS

Brains and superior cervical ganglia (SCG) were rapidly removed from 4-, 6-, 8- and 12-week old SHR and WIS rats after killing with ether overdose. The major cerebral arteries were dissected out and stretched on pieces of Sylgard (Dow Corning, Belgium) using micropins. The vessels were then processed for the demonstration of either noradrenergic nerve fibres using the glyoxylic acid fluorescence method, or of NPY-like immunoreactive nerve fibres using the indirect labelling technique. The SCG were quickly frozen and stored in liquid nitrogen until the time of assay. The left and right SCG were used for the detection of NA and NPY, respectively. These techniques and the method for the quantitation of perivascular innervation, are described in Chapter 2.

All these results are expressed as mean + S.E.M., and the data were compared using Student's unpaired t-test. A level of probability of $p < 0.05$ was considered to be of significance.

6.4 RESULTS

The density of adrenergic and NPY-LI innervation of the major cerebral arteries of the SH rats and normotensive WIS controls at 4, 6, 8 and 12 week old stages are shown in Tables 6.1 and 6.2. Although the density of perivascular nerves containing NA was consistently greater than those containing NPY, the pattern of innervation was similar for both nerve types. The innervation was denser in the vessels of the internal carotid system than in those of the vertebro-basilar system. The density of innervation was greatest in the anterior cerebral artery followed by the internal and posterior cerebral arteries, and then the middle cerebral artery, the basilar artery and the superior cerebellar artery in decreasing order. A schematic diagram of the pattern of adrenergic and NPY-LI innervation is shown in Fig. 6.1.

Although the developmental changes in both strains followed similar patterns, the density of perivascular nerve fibres containing NA or NPY was significantly greater in almost all vessels in the SHR as compared with the WIS controls at all age stages studied. Examples of these changes in a vessel of the internal carotid system (anterior cerebral artery) and a vessel of the vertebro-basilar system (basilar artery) for both NA- and NPY-containing nerve fibres are illustrated in Figs. 6.2a,b and 6.3a,b. Further examples of these changes are shown in Fig. 6.4. No immunostaining was observed in vessels preabsorbed with excess NPY.

4-6 weeks

There was a significant increase in the density of NA-containing nerve fibres in the BA, ICA and ACA of WIS rats and in the BA of SH rats. During this period, there was a significant decrease in the density of nerve fibres containing NPY in the PCA, ICA and ACA of WIS rats and in the SCA of SH rats. An increase in the density of NPY-containing nerve fibres during this stage was only observed in the ACA of hypertensive rats.

6-8 weeks

There were no significant changes in the density of NA-containing nerve fibres supplying the major cerebral vessels of both WIS and SHR groups except for an increase in the SCA of control rats. In contrast, the density of nerve fibres containing NPY increased significantly in all vessels of both groups except for the ICA of hypertensive rats.

8-12 weeks

During this period there was a further significant increase in the of NA-containing nerve fibres supplying the SCA, ICA and MCA of and ICA of SH rats. The density of NPY-containing nerve fibres did not undergo any significant change during this stage in any of the examined in either control or hypertensive animals.

Figures 6.5a and 6.5b show the levels of NA and NPY, respectively, in the SCG of SHR and WIS normotensive controls at various age stages. NA levels were significantly lower in the SHR animals at 4 weeks (40.00 $\pm$5.38 compared with 69.52 $\pm$ 10.24 pmol ganglion, $p < O.05$). There were no other significant

differences between the hypertensive and normotensive groups for either NA or NPY levels. There was, however, a significant increase in NA levels between 8 and 12 weeks (71.72 $\pm$ 10.83 to 117.22 + 15.62 pmol ganglion, $p < 0.05$) and a significant decrease in NPY levels between the same time period (2.01 $\pm$ 0.30 to 1.10 $\pm$ 0.13 pmol/ganglion) in the WIS group.

6.5 DISCUSSION

It is generally assumed that genetic hypertension in the SHR model, developed by Okamoto and Aoki (1963), closely resembles essential hypertension in man (Tripodo and Frohlich, 1981), although this has been debated (McGiff and Quilley, 1981). As such, it is the most commonly used animal model in the study of hypertension. There is much evidence for the involvement of the sympathetic nervous system in the development and maintenance of hypertension (Judy et al., 1976; Yamori, 1976; Abboud, 1982). The vascular abnormalities present in the SHR (Abboud, 1982), which account for the increased total peripheral resistance, are thought to be due to two, probably interacting, factors. Firstly, morphological alterations of the vessel wall and secondly, changes in the regulation of hypertensive vessels by neurohumoral agents. The aim of the present study was to see whether there are any changes in the pattern and density of nerves containing NA and NPY in the major cerebral arteries of the SH rat when compared with a population of normotensive WIS rats from which the hypertensive animals are derived.

The patterns of adrenergic and NPY-LI innervation of normotensive rat cerebral vessels were similar to that previously reported (Kobayashi et al., 1981; Lundberg et al., 1982; Edvinsson et al., 1984c, 1985b; Schon et al., 1985b; Uddman et al., 1985b). The present study shows that this pattern is similar in the SHR strain, but that the density of nerves containing NA and NPY is consistently greater in almost all vessels studied in the hypertensive animals at 4, 6, 8 and 12 weeks of age. The density of nerves containing NA was greater than that of nerves containing NPY. The increased density of NA- and NPY-containing nerve fibres

as early as 4 weeks suggests that the hyperinnervation of these cerebrovascular sympathetic nerves precedes the onset of hypertension and medial hypertrophy which occur after 5 weeks of age in the SHR (Pang and Scott, 1982). These results are consistent with the adrenergic hyperinnervation and increased NA content of cerebral (Lee and Saito, 1984) and other vascular beds (Grobecker et al., 1975; Scott and Pang, 1983; Scott and Galway, 1984: Donohue et al., 1986; Haebara et al., 1986) reported in hypertensive rats. Similarly, increased activity of catecholamine synthesizing enzymes (Nagatsu et al ., 1975 , 1976) are present in SH as compared with normotensive rats. The present results add further support to perivascular adrenergic hyperinnervation being a general feature of the SHR model, which may reflect the increased plasma NA levels in this strain (Grobecker et al., 1975) and in human essential hypertension (Louis et al ., 1973; Louis and Howes, 1990). Interestingly, there is ultrastructural evidence for the decrease in sympathetic and non-sympathetic nerve terminals in the adventitia of cerebral vessels in renal hypertensive rats (Saito and Lee, 1985). Two separate forms of the same disease appear to influence a given population of perivascular nerves in a different manner.

Cerebrovascular sympathetic nerves do not appear to play an important role in the regulation of resting CBF (Mueller et al., 1977; Heistad and Marcus, 1978). However, their ability to change the upper limit of autoregulation towards higher blood pressure and therefore keep CBF to normal levels during episodes of pronounced hypertension, indicates a protective role for sympathetic nerves against cerebrovascular insults (Mueller et al., 1982, 1983; Mueller and Ertel, 1983; Sadoshima et al., 1981; Sadoshima and Heistad, 1982). Numerous studies have suggested that adrenergic nerves exert a trophic influence in controlling vascular smooth muscle thickness, and as such may be causally related to the

development of medial hypertrophy (Bevan et al., 1975; Scott and Pang, 1983; Chamley and Campbell, 1986). It has been shown that sympathetic denervation markedly attenuates cerebrovascular hypertrophy (Hart et al ., 1980) and leads to a higher incidence of stroke (Sadoshima et al., 1981) in stroke-prone SH rats, and increases the permeability of the blood-brain barrier (BBB) in SH rats (Mueller et al., 1982). These findings further support a protective role for sympathetic nerves against stroke and disruption of the BBB during hypertension.

In contrast to young SH rats (4 weeks) (Mueller et al., 1982), chronic sympathetic denervation in adult (9-19 weeks) failed to alter the BBB protein transfer during acute cerebral vasodilatation (Mueller et al 1983), suggesting a different relationship between sympathetic nerves and the integrity of the BBB during development. These results are consistent with such an age-related physiological role for sympathetic nerves. The differences in the densities of cerebrovascular innervation by NA- and NPY-containing nerves, between SH and WIS rats, was most marked at 4 weeks and less so with increasing age. The persistence of adrenergic hyperinnervation in cerebral blood vessels of 24 week old SH rats (Lee and Saito, 1984) indicates that although the sympathetic fibres may no longer be involved in a protective role, they may still play an important part in maintaining the hypertensive state.

The demonstration of coexistence of NA and NPY in many perivascular sympathetic nerves (Lundberg et al ., 1982; Ekblad et al ., 1984; Matsuyama et al., 1985; Sternini and Brecha, 1985; Uddman et al., 1985b). and our present study showing hyperinnervation by both NA- and NPY-containing nerves, raise the possibility that NPY, in addition to NA, may be involved in the trophic control of vascular smooth muscle (Burnstock, 1982a; Chamley and Campbell, 1986) and play a role in the development of hypertension and medial hypertrophy

in the SHR (Henricks et al., 1980; Pang and Scott, 1982; Scott and Pang, 1983). Furthermore, these findings suggest that the protective role assigned to NA-containing cerebrovascular nerves against stroke and haemorrhage may also involve NPY contained in perivascular nerves.

There was a significant increase in the density of NA-containing nerves, coincidental with a low expression of NPY, from 4-6 weeks in both SHR and WIS strains. In contrast, NPY-containing nerves did not show an increase until 6-8 weeks of age, when the density of NA-containing nerves was stabilizing in both control and hypertensive animals. A further increase in the density of NA-containing nerve fibres from 8-12 weeks was observed simultaneously with a lack of any significant changes in the number of nerve fibres containing NPY. With very few exceptions, these results suggest that there is a differential time-course for the appearance and expression of developmental changes of NA and NPY in perivascular nerves during development.

The precise physiological function of NPY in circulatory homeostasis is still unclear, but studies so far suggest both neurotransmitter and neuromodulatory roles for this substance. In the peripheral circulation (Lundberg et al., 1985d), NPY has been shown to act as a prejunctional modulator inhibiting the release of NA from perivascular sympathetic nerve terminals, and as a postjunctional modulator potentiating the responses to vasoconstrictor substances and transmural nerve stimulation (Edvinsson et al., 1984b; Ekblad et al., 1984; Dahlof et al., 1985b; Wahlestedt et al., 1986). The differential expression of NA and NPY, seen in both hypertensive and normotensive animals during early development, may be a result of an interaction between these two substances,

particularly as they appear to co-exist in a population of perivascular sympathetic nerves(Lundberg et al., 1982,1983). Any significant alteration in the prejunctional neuromodulation by NPY, to explain this differential transmitter expression in the hypertensive state, seems unlikely in the light of the following studies. In the cerebral circulation, NPY does not induce an inhibition of field stimulation-evoked fractional release of [^{3}H]NA (Edvinsson and Skarby, 1984) and that no differences have been observed in the number of α_2-adrenoceptors in cerebral microvessels of SH rats (Kobayashi et al., 1985). Furthermore, in the rat portal vein, where NPY does have a prejunctional action, its inhibition of transmural nerve stimulation-induced [^{3}H]NA release is of a similar degree in both adult SH and WKY rats (Dahlof et al., 1985b).

With regard to its postjunctional transmitter action in some vessels, NPY-induced vasoconstriction has been shown to be independent of α -adrenoceptors, 5-HT_2 serotonin receptors and muscarinic cholinoceptors (Edvinsson et al., 1983b), and a lower concentration of NPY is required for its enhancing effect on the vascular response to other vasoconstrictor agents than that required for its direct vasoconstriction (Edvinsson, 1985). Although in contrast to peripheral vessels, NPY does not appear to significantly modulate the response to NA in cerebral vessels (Hanko et al., 1986), the recent findings of increased α_1-adrenergic receptors in neuronal rat brain cultures (Feldsten et al., 1986) and in cerebral microvessels (Kobayashi et al ., 1985) of SH rats, suggest that NPY-induced postjunctional effects may be altered in the hypertensive state. An enhanced postjunctional α -adrenoceptor-mediated vasoconstrictor component has been shown in human established essential hypertension (Amann et al ., 1981). This is consistent with a local mechanism for the maintenance of hyper-

tension in the SHR. Increased plasma adrenaline levels in hypertension (Franco-Morselli et al., 1978; McCarty and Kopin, 1978) also support such a local mechanism. The ability of sympathetic nerves to take up and release adrenaline (Majewski et al., 1981), and the finding that chronic adrenaline administration can lead to a self-sustaining increase in blood pressure (Majewski et al ., 1980) further support local mechanisms in maintaining hypertension. Furthermore, the stimulation of α - adrenoceptors by increased levels of adrenaline in the SHR may lead to a greater release of NA (Franco-Morselli et al., 1978).

Previous studies have shown that the sympathetic innervation of the major cerebral arteries of the rat originate from the SCG (Kobayashi et al., 1981; Schon et al., 1985b). In the present study the changes in densities of nerves containing NA and NPY supplying the cerebral vessels were not reflected by changes in the contents of NA and NPY in the SCG. Studies have shown that preganglionic electrical stimulation of sympathetic nerves leads to an increase in the NA content in the SCG and a reduction in the NA content at the adrenergic terminals in rat salivary glands (Anden et al., 1986). It appears that the biosynthesis of NA in the SCG is facilitated by preganglionic nerve impulses rather than utilisation at the terminals. Although enhanced preganglionic sympathetic nerve responses have been recorded in 16-20 week old SHR as compared with Wistar-Kyoto (WKY) animals (Morrison and Whitehorn, 1984), it is possible that at an early stage of development preganglionic activity is actually less in the SHR strain. This would be in agreement with the present observations of lower NA content in the SCG and denser adrenergic innervation of cerebral vessels in 4 week old SHR animals. An important observation was the significant increase of NA content of the SCG with the simultaneous decrease in NPY content at 8 weeks in the WIS control

group. No significant changes in the SCG content of NA and NPY were observed in the SHR group. However, a previous histochemical study has shown an increased catecholamine content of the SCG in 20 week old SH compared with WKY rats (Alho et al., 1984). These findings, and the importance of preganglionic nerve impulses for the biosynthesis of NA in the SCG are consistent with an abnormality of central cardio-vascular centres in hypertension (Howes, 1984; Morrison and Whitehorn, 1984). Unfortunately, many of these studies report conflicting neurochemical changes. A recent study has shown that NA levels in the 4 week old SHR were greater in some central autonomic nuclei than in the WKY, but that levels in the WIS normotensives were also greater than in the WKY and equalled levels in the SHR (Felton et al., 1984). This finding, and results of the present study using WIS normotensives as controls, suggest that the sole use of WKY as a control for SH rats is inadequate for a reliable interpretation of changes in neurochemical and possibly other parameters. Increased NPY levels (Maccarrone and Jarrot, 1985) and unchanged numbers of NPY binding sites (Chang et al ., 1986) in the striatum in adult SH rats compared with WKY animals, need to be interpreted with caution until it is clear that there are no significant genetic differences, unrelated to hypertension, between these two inbred strains. Nevertheless, the coexistence of NA and NPY in central as well as peripheral neurons (Lundberg et al., 1982, 1983; Everitt et al., 1984), and the importance of central autonomic nuclei in the control of blood pressure strongly warrants more detailed studies on the extent of central influence at the level of vascular neuroeffector mechanisms. This is further supported by the possibility of a central origin for a population of NA- and NPY-containing nerve fibres supplying some cerebral vessels (Alafaci et al., 1985; Matsuyama et al.,

1985), and by the hypotensive action of NPY when administered centrally (Fuxe et al ., 1983).

The coexistence of other peptides with NA in perivascular sympathetic nerves would suggest that they too may be of importance in the vascular abnormalities in hypertension. There is now substantial evidence for the coexistence of ATP and NA in perivascular sympathetic nerves (Katsuragi and Su, 1982; Kennedy et al., 1986; Burnstock and Warland, 1987) and abnormalities in purinergic mechanisms have been reported in hypertension (Kamikawa et al ., 1980; Hicks et al., 1985) VIP has recently been shown to coexist with NA in the guinea-pig uterine artery (Morris et al., l985). The finding of decreased VIP levels in cervical and thoracic spinal cord of SH rats (Lewis et al., 1985) raises the possibility that this vasoactive substance may also play a role in the aetiology of hypertension.

No differences in the density of perivascular SP-containing nerve fibres were observed in the mesenteric vasculature of adult SH rats compared with controls (Foote et al., 1986). Increased density of SP binding sites in brainstem nuclei (Shigematsu et al. 1986), as well as increased blood pressure and heart rate responses to centrally administered SP (Unger et al., 1980) have been reported in SH rats. Nevertheless, the possibility of changes in these nerves during different stages of development, and more importantly any changes with regard to vascular neuroeffector mechanisms in hypertension, need further elucidation.

6.6 CHAPTER UPDATE

This section aims to update the evidence for a role for Neuropeptide Y in hypertension. Much of the remaining advances in cerebrovascular innervation have already been mentioned in the General Introduction and in the 'Chapter Update' to Chapter 5.

Firstly, there have been several studies that have confirmed some of the findings described in this chapter. Similar to the findings described above, a denser cerebrovascular innervation of cerebral vessels by both noradrenergic and NPY-LI nerve fibres has been confirmed in the SHR model (Mangiarua and Lee, 1990;Kamura and Takebayashi, 1991). They additionally show that there was no difference in the cerebral innervation with respect to nerve fibres immunoreactive for SP and VIP. The reduction in NPY content on the superior cervical ganglia with development has also been confirmed but in a different hypetensive rat model (Gurusinghe et al., 1990). Interestingly, there appears to be no difference in the NPY-LI innervation of cerebral vessels in the stroke-prone-SHR model (Lee et al., 1988). This raises the possibility that the lack of cerebrovascular hyperinnervation by NPY-LI nerve fibres, therefore a loss of protective vasoconstrictor tone, may render the stroke-prone model more susceptible to cerebral insult. The increased sympathetic innervation seen in the cerebral vasculature in the SHR model, is in keeping with similar sympathetic hyperinnervation seen in other peripheral vascular beds (Scott and Pang, 1983., Scott and Galway, 1984., Grobecker et al., 1975., Haebara et al., 1986) and thus reflects a generalised vascular pathology/adaptation to the hypertensive state.

There is increasing evidence that blood levels of NPY are raised in clinical hypertension (Erlinge et al., 1992; Januszewicz et al., 1994; Lettgen et al., 1994) and after subarachnoid haemorrhage in man (Juul et al., 1990). Studies also show increased platelet concentration of NPY in hypertensive animals, as well as a greater release of NPY on stimulated platelet aggregation (Ogawa et al., 1992). A possibility is that following a haemorrhage, NPY released from aggregating platelets may serve an important role in controlling the bleed by inducing further vasoconstriction in addition to that achieved by NPY released from perivascular nerves.

FIGURE 6.1

A schematic diagram showing the pattern of perivascular

Innervation by nerves containing NA and NPY in the rat circle of Willis.

VA = vertebral artery; BA = basilar artery; SCA = superior cerebellar artery; PCA= posterior cerebral artery; ICA = -internal carotid -artery; MCA = middle cerebral artery; ACA = anterior communicating artery.

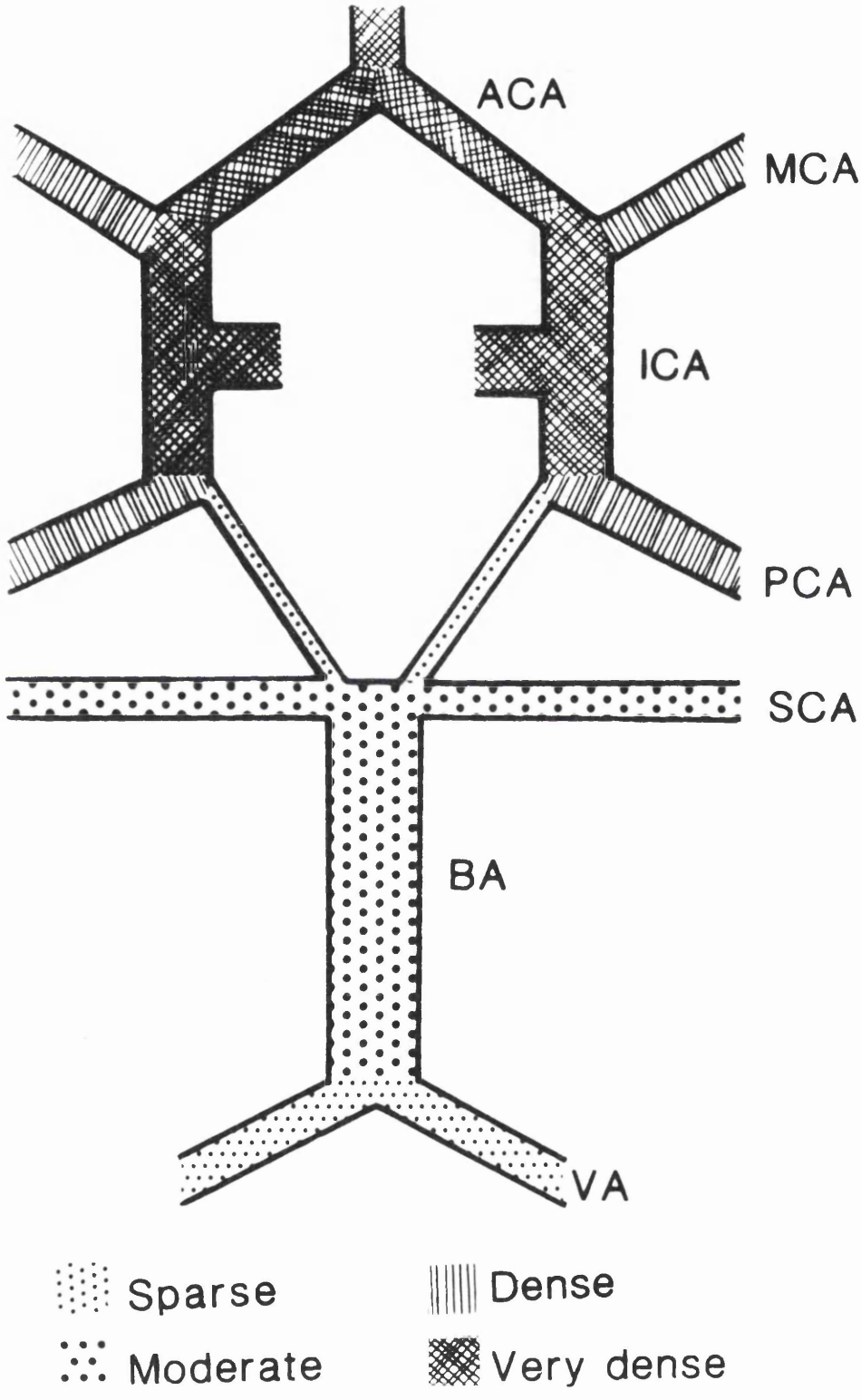
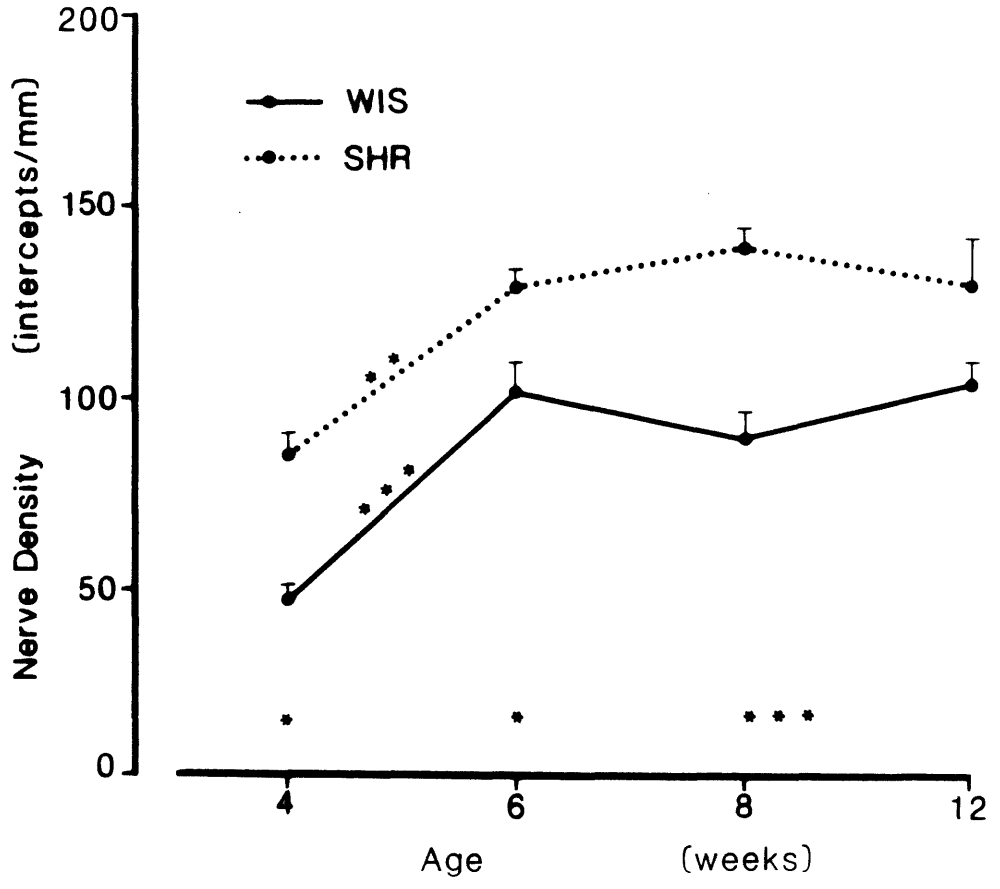

ACA
MCA
ICA
PCA
SCA
BA
VA
Sparse
Moderate
Dense
Very dense

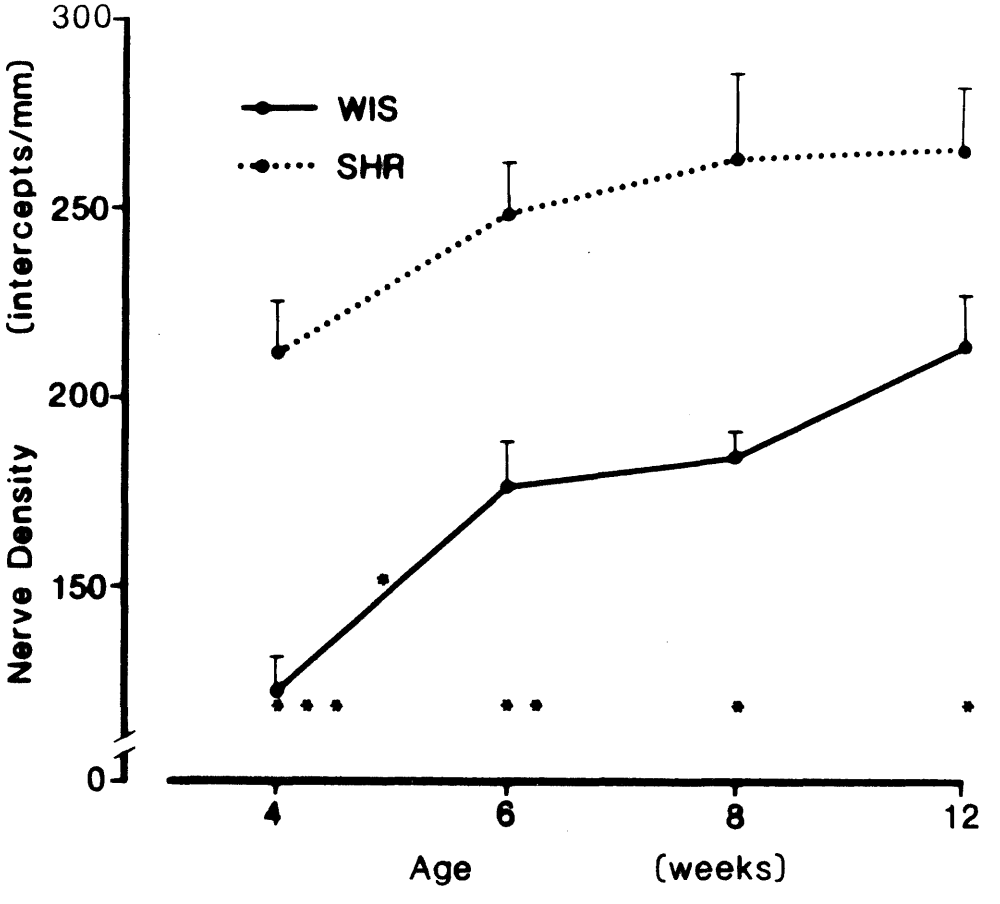
FIGURE 6.2

Graphs showing developmental changes in the density of NA-containing nerves supplying the basilar artery (a) and anterior cerebral artery (b) in WIS and SH rats.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.


200
150
100
50
0
Nerve Density (intercepts/mm)
WIS
SHR
4
6
8
12
Age (weeks)

a

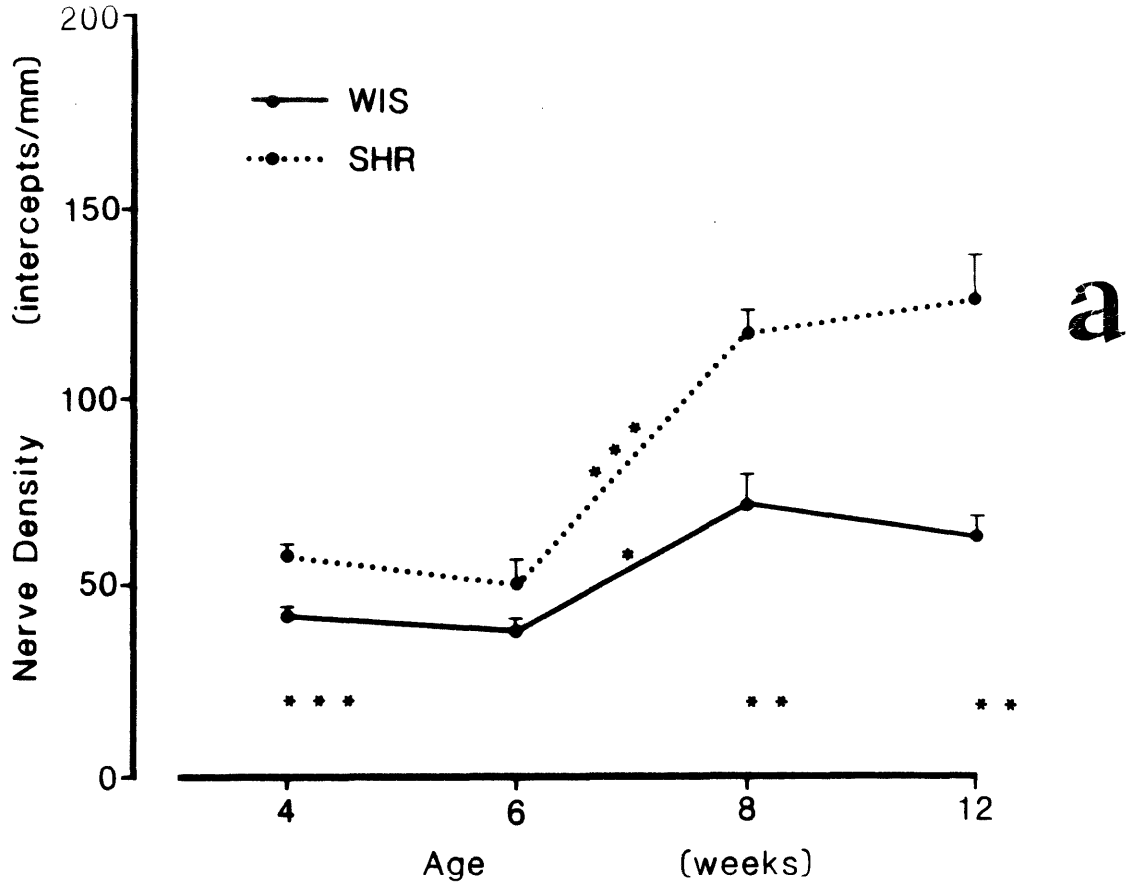

300
250
200
150
0
Nerve Density (intercepts/mm)
WIS
SHR
4
6
8
12
Age (weeks)

b

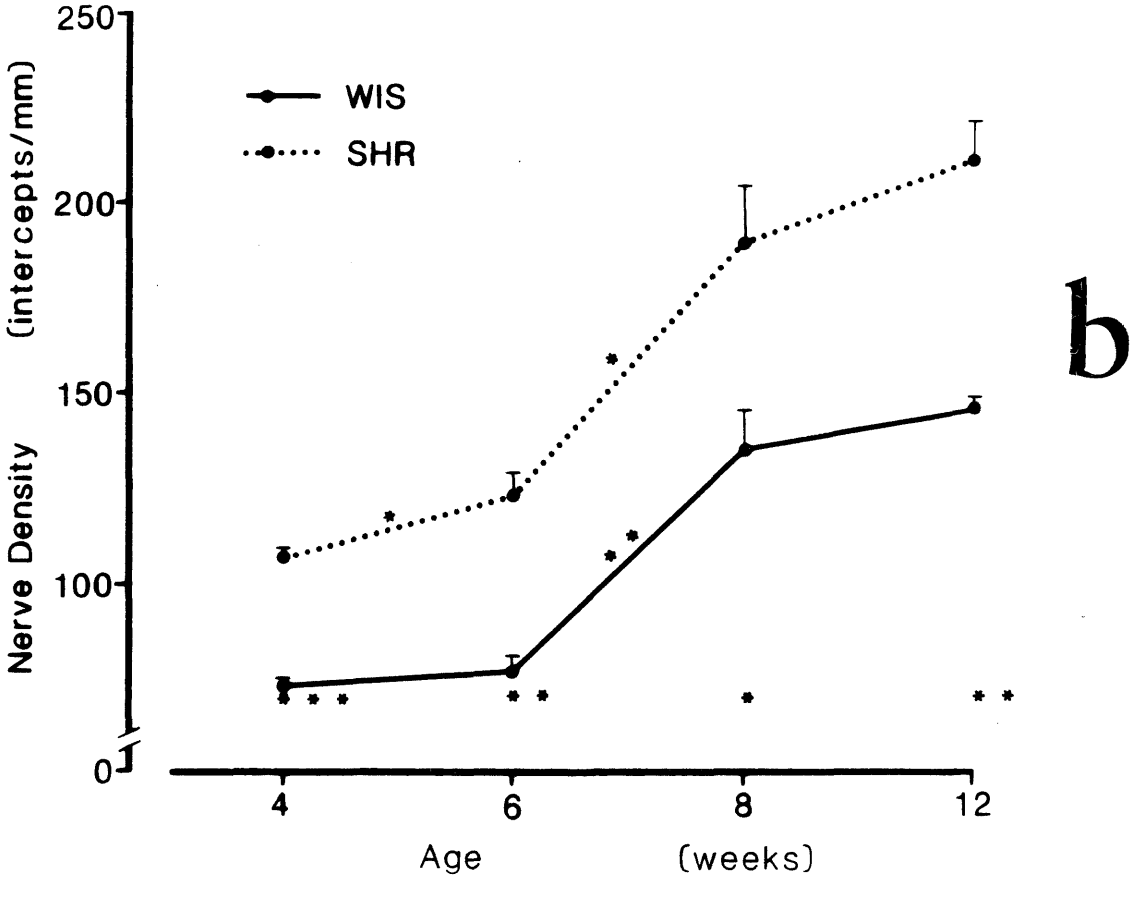
FIGURE 6.3

Graph showing developmental changes in the density of NPY-containing nerve fibres supplying the basilar artery (a) and anterior cerebral artery (b) in WIS and SH rats.

* $p < O.05$, ** $p < O.0l$, *** $p < O.00l$.


200
150
100
50
0
Nerve Density
(intercepts/mm)
WIS
SHR
4
6
8
12
Age
(weeks)

a


250
200
150
100
0
Nerve Density
(intercepts/mm)
WIS
SHR
4
6
8
12
Age
(weeks)

b

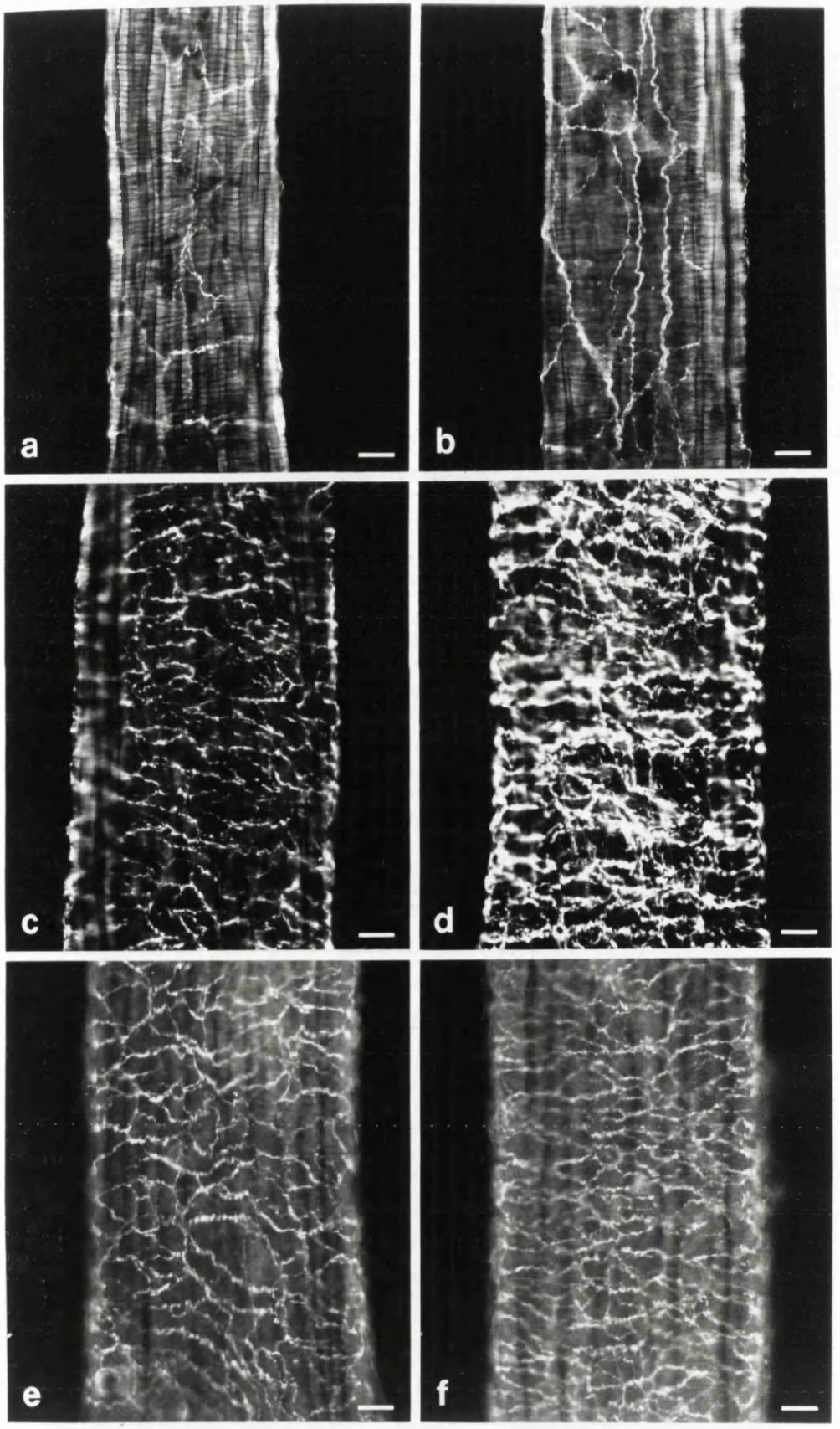

a
b
c
d
e
f

FIGURE 6.4

Photomicrographs showing cerebrovascular nerves containing NA and NPY in WIS and SH rats.

(a) NA-containing nerves supplying the superior cerebellar artery of 6-week old WIS rat.

(b) NA-containing nerves supplying the superior cerebellar artery of 6-week-old SH rat.

(c) NA-containing nerves supplying the anterior cerebral artery of 4-week-old SH rat.

(d) NA-containing nerves supplying the anterior cerebral artery of 12-week old SH rat. With the increased density of innervation there are more nerve fibres running close together in perivascular bundles giving the impression of increased nerve thickness.

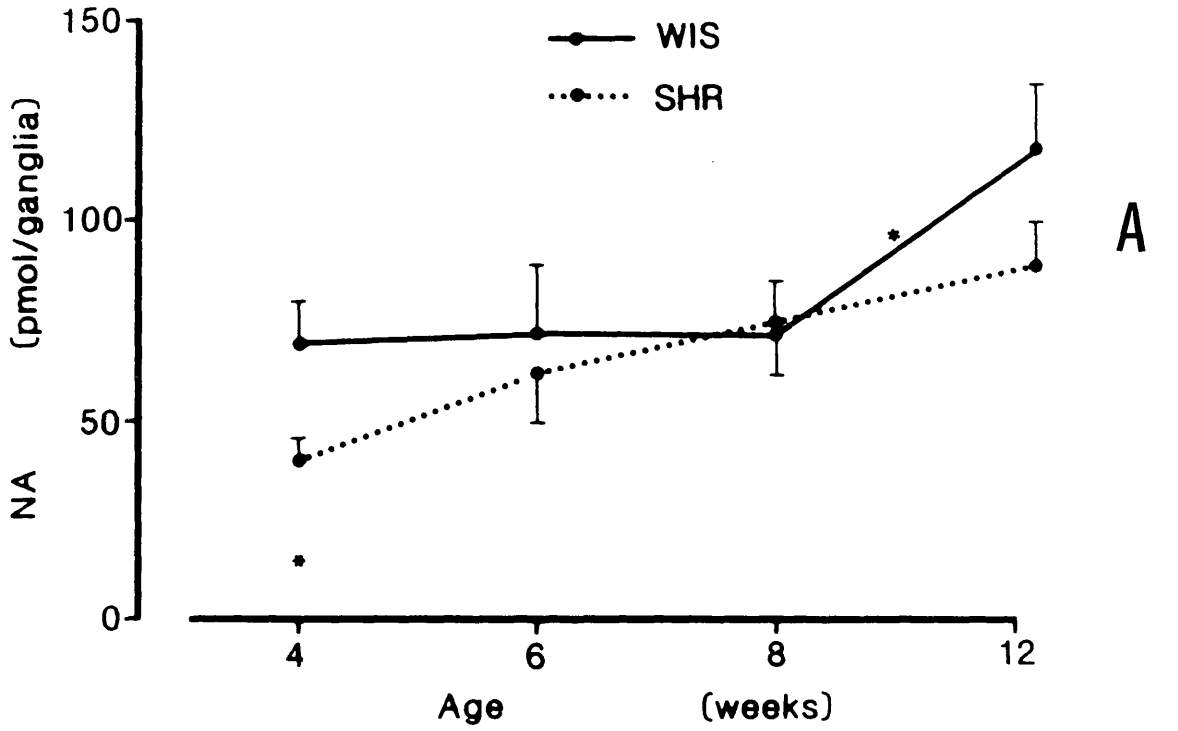
(e) NPY-containing nerves supplying the anterior cerebral artery of 4-week-old WIS rat.

(f) NPY-containing nerves supplying the anterior cerebral artery of 4- week-old SH rat.

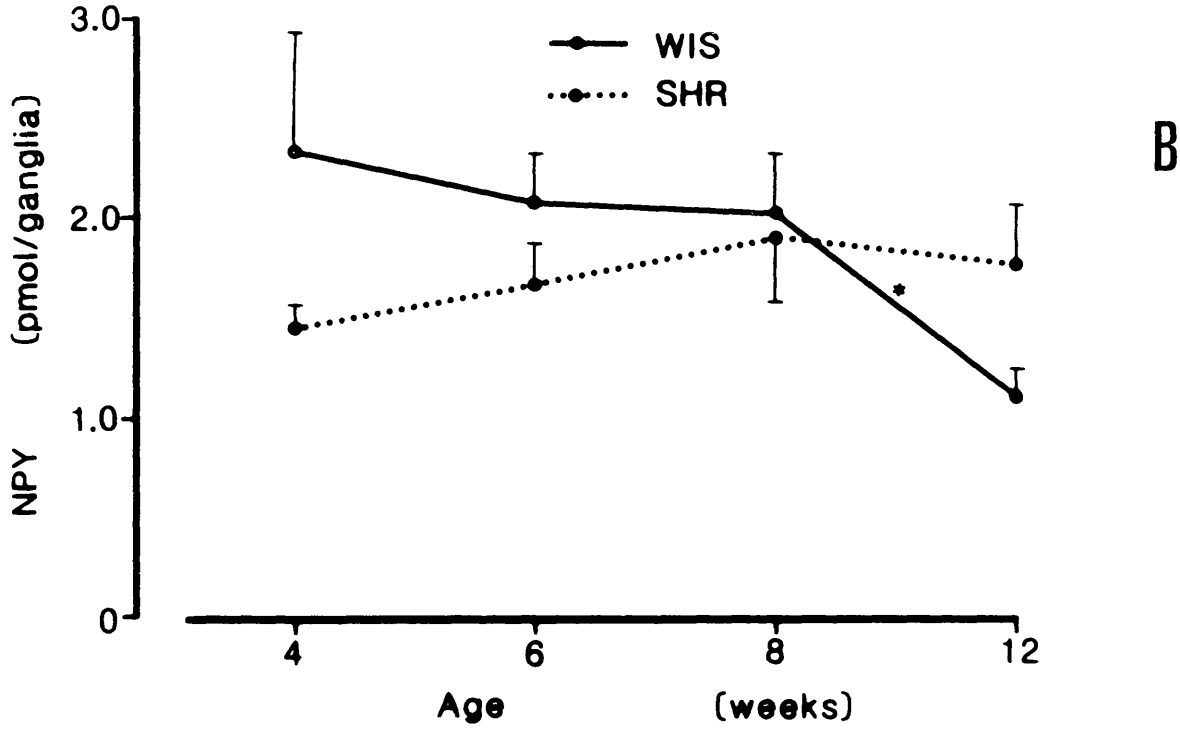
Calibration bars = 25μm

FIGURE 6.5

Graph showing developmental changes in the NA (a) and NPY (b) content of the SCG in WIS and SH rats. * = $p<0.05$.


150
100
50
0
NA (pmol/ganglia)
WIS
SHR
4
6
8
12
Age (weeks)

A


3.0
2.0
1.0
0
NPY (pmol/ganglia)
WIS
SHR
4
6
8
12
Age (weeks)

B

TABLE 6.1

Density of NA-containing nerve fibres supplying cerebral arteries of WIS and SH rats at 4, 6, 8 and 12 weeks of age. Nerve density is expressed as mean number of nerve intercepts per millimetre of vessel circumference ± S.E.M.

	4 weeks			6 weeks			8 weeks			12 weeks		
Artery	WIS	SHR	p	WIS	SHR	p	WIS	SHR	p	WIS	SHR	p
BA	47.12±3.81	80.58±11.60	*	100.33±8.81	128.67±4.79	*	88.09±7.59	139.67±3.63	***	102.42±5.66	128.00±12.66	NS
SCA	47.84±3.72	79.08±6.61	**	44.80±3.75	96.59±6.95	***	65.33±1.03	112.83±7.59	***	76.34±0.94	120.00±7.36	**
PCA	98.13±5.14	140.84±7.03	**	103.80±7.72	151.07±7.03	**	109.58±6.68	143.04±21.28	NS	137.25±10.26	177.42±12.55	*
ICA	107.84±9.79	159.75±16.18	*	139.00±7.35	166.73±12.85	NS	121.83±12.78	188.92±15.11	*	159.92±6.45	248.17±15.11	**
MCA	68.50±6.96	121.00±15.69	*	93.67±14.17	163.59±12.81	*	78.42±0.89	155.50±14.30	**	106.17±4.19	178.17±13.77	**
ACA	122.92±10.00	213.93±12.72	***	177.20±11.42	249.00±13.06	**	184.42±6.95	263.50±21.66	*	213.17±13.96	265.17±15.49	*

*$p<0.05$, **$p<0.01$, ***$p<0.001$, NS = not significant.

BA = basilar artery; SCA = superior cerebellar artery; PCA = posterior cerebral artery; TCA = internal carotid artery; MCA = middle cerebral artery; ACA = anterior cerebral artery.

TABLE 6.2

Density of NPY-containing nerves supplying cerebral arteries of WIS and SH rats at 4, 6, 8 and 12 weeks of age. Nerve density is expressed as mean number of nerve intercepts per millimetre of vessel circumference ± S.E.M.

	4 weeks			6 weeks			8 weeks			12 weeks		
ARTERY	WIS	SHR	p	WIS	SHR	p	WIS	SHR	p	WIS	SHR	p
BA	42.33±0.78	58.25±2.16	***	39.17±3.58	50.92±7.61	NS	71.50±9.14	116.67±6.47	**	62.50±5.52	125.04±12.19	**
SCA	30.33±3.29	50.25±2.12	**	23.17±1.40	34.09±2.20	**	74.25±8.38	99.00±5.11	*	55.59±3.83	78.83±3.93	**
PCA	51.75±2.43	68.42±1.93	**	37.67±4.52	71.75±5.39	**	94.08±6.49	124.83±8.95	*	90.83±6.44	137.83±8.67	**
ICA	56.75±2.39	93.83±2.40	***	36.93±4.06	100.50±4.61	***	100.17±9.22	141.50±17.65	NS	116.34±7.54	151.59±6.58	*
MCA	39.58±2.76	70.84±2.23	***	26.84±2.25	81.92±6.26	***	79.58±5.02	117.75±12.70	*	98.08±7.75	143.58±8.98	**
ICA	73.59±1.70	106.17±1.92	***	76.17±5.62	123.00±6.13	**	135.67±10.31	189.59±18.15	*	145.67±3.81	210.09±11.26	**

*$p<0.05$, **$p<0.01$, ***$p<0.001$, NS = not significant.

BA = basilar artery; SCA = superior cerebellar artery; PCA = posterior cerebral artery; TCA = internal carotid artery; MCA = middle cerebral artery; ACA = anterior cerebral artery.

CHAPTER 7

GENERAL DISCUSSION

7.1 INTRODUCTION

The goals of the work presented in this thesis which was confined to the circulation of nerve trunks and that of the major cerebral arteries were as follows:

1. To assess the presence of perivascular nerves (and their neurotransmitter contents) supplying the vasa nervorum.
2. To examine the changes in the pattern and density of perivascular innervation with development and aging in both vascular beds.
3. To study any changes in the innervation of the blood vessels in diabetes (vasa nervorum) and hypertension (cerebral arteries).

With the proviso that the experiments were limited to some animal models, with the exception of human sural nerves, and looked only at a fraction of an ever-expanding list of neurotransmitter substances, then I believe the work described in the preceding chapters has achieved the above mentioned aims. The development of the whole-mount preparation for nerve sheaths has demonstrated the presence of both peri- and non-perivascular nerve profiles which contain a number of neurotransmitter substances in addition to noradrenaline (Chapter 3). Within the confines of the histochemical techniques used here, there are changes in the innervation pattern, not only with normal development (Chapter 3 and Chapter 5), but also in disease (diabetes in Chapter 4 and hypertension in Chapter 6).

What then is the unifying theme of this work? This has to be the altered or differential expression of neurotransmitter substances. Such neuronal plasticity, which as a principle has recently come to the fore as a major inherent

characteristic of the autonomic nervous system, refers to the interactions between perivascular nerves and endothelial cells (see Burnstock, 1991a,b; Burnstock, 1993; Ralevic and Burnstock., 1996). Because these interactions are influenced by the size of the vasculature and therefore by the distance between the perivascular nerves and the endothelial layer, they can be divided into two types. Firstly into the short-term, dynamic, temporary and reversible interactions. These relate to the overall homeostatic balance between the multiple effects of different perivascular nerves (largely sympathetic vasoconstrictor) and endothelial cells (largely vasodilator). Short-term interactions also refer to pre- and postjunctional modulation of transmission release such as the effects of endothelium derived factors and the effects of neurotransmitters on endothelial cells. These interactions are distinct from the long-term effects which are sustained, and which result in morphological and/or functional changes in the endothelium and perivascular nerve plexus. The discussion which follows is largely limited to reviewing the evidence for this latter, long-term, type of plasticity with reference to the findings presented in this thesis. For clarity, I have divided the main body of this discussion into two parts.

1. A presentation of the background evidence for plasticity of perivascular neurotransmission in normal development including aging, and in experimental and pathological situations with reference to the findings of this thesis.

2. A review of the possible genetic and cellular mechanisms which may be involved in making possible the processes of altered neurotransmitters and neuromodulators.

Additional comments are also made with regard to firstly, the limitation of the methods and data described in this work, and secondly, to the future directions which may aid us in further clarifying this concept. The final aim being the possibility of selective analysis and modification of parts of this system for pathological diagnosis and treatment of many vascular disorders and neuropathies.

7.2 PLASTICITY OF PERIVASCULAR NEUROTRANSMISSION

The ability of the autonomic nervous system to alter the expression of its neurotransmitters, in response to influencing genetic and environmental factors is now a well established principle applicable to both developing and fully mature neurons. This ranges from a simple change in the substance being expressed, such as the change from noradrenaline to acetylcholine in rat sweat glands (Landis and Keefe, 1983), to differential changes in the expression of a combination of neurotransmitters/neuromodulators, and finally to the ability of some denervated targets to accept reinnervation by another type or "foreign" nerve (Burnstock, 1981b).

Changes in development and aging

The pattern of development of autonomic neuroeffector junctions follows a two-stage process, where an initial phase of neuronal migration and outgrowth towards target tissues is then followed by a phase termed 'recognition'. During this latter phase, interaction between neuronal elements and the target effector

cells leads to the structural and chemical differentiation of the neuromuscular junction.

The degree of maturation of autonomic innervation at birth varies markedly between species. For example, perivascular innervation in the sheep and guinea-pig is relatively mature at birth (Su et al., 1977a), while in the rat the development of perivascular innervation takes place at a later stage (Ljung and Stage, 1975).

There have been numerous studies which have addressed the issue of altered perivascular neurotransmission in development and aging (see Burnstock, 1990c; 1995 for reviews). Majority of this evidence is confined to NA-containing perivascular nerves. The pattern of NA-containing perivascular nerves in the rabbit varies markedly between different vessels during development (Cowen et al., 1982a), whereby the time taken to reach the peak density of innervation varies according to the vascular bed. In the same animal model it has been shown that the decline in the density of NA-containing nerves with advancing age is more pronounced in the left middle cerebral artery compared with the right side (Saba et al., 1984). The density of NA-containing nerves fibres supplying the guinea-pig renal artery , which are found to be non-functional, starts to decline postnatally, while in the rabbit the renal artery innervation continues to increase with development (Gallen et al., 1982). While these studies, in general, show a decline in the density of NA-containing perivascular nerves with age, pharmacological assessment reveals that both in man and the rat, there is no decline in the vascular adrenergic-transmission (Duckles et al., 1985).

Similarly, the perivascular innervation of rabbit cerebral and peripheral arteries by 5-HT containing nerves has shown an initial delay in the time to reach

peak density, and a greater reduction of innervation when compared with NA-containing nerves (Gale et al., 1989; Cowen and Thrasivoulou., 1990).

Perivascular nerves containing peptides have also been assessed in development in the rat cerebral (chapter 7 and 8), guinea-pig mesenteric, femoral, carotid and renal arteries (Dhall et al., 1986), and rat mesenteric vessels (Scott and Woolgar., 1987).

In the mesenteric vessels of the rat (Scott and Woolgar., 1987), and a variety of guinea-pig arteries, neuropeptide-containing nerves appear to develop earlier when compared to the NA- containing and TH-LI nerves. There is also evidence of early appearance of CGRP-containing fibres in human foetal skin (Terenghi et al., 1993). In the rat cerebral circulation however, NA-containing nerves innervated corresponding parts of the cerebral circulation earlier than CGRP-containing fibres. (Tsai et al., 1989). CGRP-LI nerve fibres develop earlier than the SP-LI nerves even though they may co-exist in the same nerve fibre (Scott and Woolgar., 1987).

A number of functional studies have supported the histological changes observed for some neuropeptide-containing nerves with advancing age. The decrease in CGRP-containing nerves supplying the mesenteric arteries of older rats is in keeping with age-related decrease in the sensory-motor vasodilatation of the same vascular bed (Kawasaki et al., 1990b; Li and Duckles., 1993). There is also evidence for a decrease in axon reflexes and repair processes with age suggesting a reduced sensory innervation with development (Helme and McKernan., 1985; Parkhouse and LeQuesne., 1988).

Hypertension

The sympathetic nervous system had been attributed a major role in the development and maintenance of hypertension both in experimental animal models and in man (Abboud, 1982; Judy et al., 1976; Yamori, 1976). Increases in perivascular sympathetic innervation and higher content of NA and NPY have been demonstrated in a number of vascular beds (chapter 8; Westfall and Meldrum, 1985; Mangiara and Lee, 1989; Lee and Saito, 1984b). This is in contrast to diminished NPY levels of the renal arteries in experimental hypertension (Ballesta et al., 1987). Increases in NPY- and VIP-nerve density with no changes in the SP-containing nerves of peripheral vessels, but decreased SP-containing nerve fibres innervating the cerebral circulation has been reported in the stroke-prone SHR (Lee et al., 1988).

In the mesenteric vasculature of the SHR model, there is a decrease in the density of nerves containing CGRP with a concomitant decrease in CGRP-induced sensory-motor vasodilatation (Kawasaki et al., 1990a and 1990b). In the same animal model, an enhanced sympathetic activity of the mesenteric arteries is thought to be secondary to diminished prejunctional inhibition by NPY along with an enhanced postjunctional potentiation by NPY of sympathetic neurotransmission (Westfall et al., 1990).

The ATP component of sympathetic cotransmission is enhanced in peripheral arteries of the SHR model to the extent that it becomes the dominant component of the sympathetic response (Vidal et al., 1986; Bulloch and McGrath, 1992). Increased sympathetic neurotransmission in hypertension is also likely to occur with defective prejunctional P_1-purinoceptor-mediated modulation (Kamikawa et al., 1980; Kubo and Su, 1983; Illes et al., 1989).

Sympathetic activity is associated with a hypertrophic effect on the developing blood vessels in hypertension (Bevan and Tsuru, 1981). Such morphological changes also occur in the endothelium whereby alterations in the size, shape and overall geometry of the endothelial cell layer (Luscher, 1988) can lead to an increased risk of developing vascular pathology like the intimal plaque (Todd,1992). There is certainly evidence for impaired endothelial cell function in hypertension (Van de Voorde and Leusen, 1986; Carvalho et al., 1987; Dohi et al., 1990).

Diabetes

It is clear from a number of studies that ,at least in the animal model, the diabetic state results in a differential expression of neurotransmitters within perivascular nerves, and that the extent and nature of this change varies in different vascular beds. The hyperinnervation of sciatic and vagus vasa nervorum by NA-containing nerves in the STZ-diabetic rats is contrasted with a loss of similar fibres supplying the vasculature of the optic nerve sheath (Chapter 4). A loss of perivascular NA-containing nerve fibres in vasa nervorum has also been demonstrated in human sural nerves (chapter 4; Lincoln et al., 1993). In the STZ rat model, physiological studies have demonstrated a marked reduction in the contractile responses of the mesenteric (Taguchi et al., 1986b) and tail (Hart et al., 1988) vasculature.

Similar changes are also seen with regard to neuropeptide-containing perivascular nerves. There is a loss of VIP- and ACh-containing nerves supplying the penile vessels in both STZ-diabetic rats and in diabetic impotent men (Crowe et al., 1983; Lincoln et al., 1987; Blanco et al., 1990). In the cerebral vessels of STZ-treated rats, there is a decrease in the expression of VIP and 5-HT

in the perivascular nerve plexus (Lagnado et al., 1987). In the same animal model, there is impaired sensory-motor vasodilatation but and intact response to exogenous CGRP (Ralevic et al., 1993). Further examples of this alteration in the expression of neuropeptide-containing nerves include the vasa nervorum (Milner et al., 1992), mesenteric vessels (Belai et al., 1995, 1996), regional variations in intestinal tissue (Di Giulio et al., 1989; Belai and Burnstock, 1987), prostate gland (Crowe et al., 1987) skin (Karanth et al., 1990) and the iris (Crowe and Burnstock, 1988). Susceptibility of the endothelium-dependent relaxing properties has been highlighted in both experimental clinical diabetes (De Tejata et al., 1989; Oyama et al., 1986; Meraji et al1987; Durante et al., 1988; Mayhan, 1989, 1992; Miyata and Kasuya, 1989; Kiff et al., 1991; Taylor et al., 1992).

Surgery, Trauma and Chronic exposure to drugs

The effect of some surgical procedures on arteries (mobilizing, clamping and division with re-anastomosis) has been assessed in the guinea-pig with respect to NA-containing perivascular nerve fibres (Cowen et al., 1982b). The application of a crush lesion resulted in a different degree of denervation in the carotid and mesenteric arteries. The rate of nerve fibre regrowth and the time taken to reach the preoperative density of innervation also varied between the two vessels studied. Mechanical injury to the endothelium of the dog coronary artery leads to an increase in the number of neuron-specific enolase-positive nerve fibres after one to three months (Taguchi et al., 1986). This correlates with an increased density of SP-containing nerve fibres following the same experiment (Taguchi et al., 1986). Following the induction of subarachnoid haemorrhage in experimental animals, there is an initial increase in the expression of 5-HT, and a reduction in the expression of NPY and CGRP in cerebrovascular nerves

(Jackowski et al., 1989; Nozaki et al., 1989). The time course for the recovery of these changes differed for the different transmitter substances.

Much more has been learnt from surgical ganglionectomies and exposure to neurotoxins. Unilateral superior cervical ganglionectomy, leads to the denervation of the ipsilateral cerebral circulation, and this is followed by reinnervation by fibres originating from the contralateral SCG (Edvinsson et al., 1972). The same surgical procedure also leads to increased number of nerve fibres containing SP in the ipsilateral iris and ciliary body (Cole et al., 1983), increased CGRP levels of pial vessels (Schon et al., 1985a), and increased numbers of NPY- and VIP-containing sympathetic nerves in the cerebral circulation (Gibbins and Morris, 1988). Increased density of CGRP-containing perivascular nerve fibres has also been reported following long term guanethidine-induced sympathectomy in developing rats (Aberdeen et al., 1988, 1990).

There is some evidence that the level of activity on the perivascular nerve plexus can have a trophic influence on the expression of some peptides in a subpopulation of endothelial cells. This is based on the appearance of NPY and CGRP in a group of endothelial cells following ten days of chronic in-vivo stimulation of perivascular nerves of the rabbit ear artery (Loesch et al., 1992).

The sympathetic innervation of the guinea-pig uterine blood vessels in late pregnancy, shows a switch from adrenergic vasoconstrictor to cholinergic vasodilator function (Bell, 1968). This is supported by the reduction of NA-containing perivascular nerve fibres, an increase in NPY-containing nerves, but no changes in nerves expressing SP, VIP and CGRP in guinea-pig uterine artery in pregnancy (Mione et al., 1990). Ultrastructural analysis of these vessels did not reveal any degeneration of serotonergic or peptidergic (NPY, VIP, SP and

CGRP)-containing nerves at similar gestational age (Mione et al., 1988). A four week exposure to oestrogen, but not progesterone, leads to a significant decrease in the density and varicosity diameters of 5-HT-containing nerves innervating the rabbit basilar artery (Dhall et al., 1988).

7.3 MOLECULAR MECHANISMS FOR NEURONAL PLASTICITY

It is evident from the above mentioned examples of altered neurovascular transmission, that there is a rich variety of phenotype expression in the autonomic nervous system, not only in early life, but maintained into maturity and influenced by both intracellular and environmental factors. In this thesis, some aspects of this diversity of neurovascular mechanisms have been assessed by morphological and biochemical means. These tools, in addition to physiological studies, give us more examples of plasticity in the nervous system only to add further complexity to any possible unifying interpretation of the possible mechanisms and significance of these changes. In order to address this problem, we must turn to investigations and explanations at the molecular level. The following discussion reviews our understanding of the cellular processes that may be involved in neuronal plasticity, and attempts where pertinent to give a mechanistic explanation and possible physiological significance of the findings observed in this thesis.

Intracellular mechanisms for neurotransmitter expression

An important concept to appreciate at the outset, is that all neurons possess the necessary genes for the expression of all the relevant neurotransmitter and neuromodulator substances present in any one organism.

Great variability of the ultimate phenotypic expression, as a consequence of this multipotentiality of cells, appears to be influenced by other controlling genes, which in turn are themselves controlled by a multitude of intracellular and environmental stimuli.. Thus in terms of the development of a multicellular organism, this process of maturation reflects a series of molecular events which are characterised by switching on a set of genes and/or removing the action of inhibitory or silencing genes. The presence of enhancer and suppressor genes and their ability to function into maturity, suggest a dynamic interplay, such that cells are not pre-committed to a single fate purely by virtue of their lineage. The extent of cellular commitment or restriction of possible fates vary in different parts of the nervous system depending on firstly the cellular lineage and secondly on external factors including the influence of target tissues.

The intracellular mechanisms, namely those of signal transduction, controlling the expression of neurotransmitter genes in neurons, are similar to those present in other eukaryotic cells. Studies subsequent to the important development of the patch-clamp technique (Hamill et al., 1981) have identified two major aspects of signal transduction (see Benham, 1992). Firstly, the identification of G-proteins as being molecular transducers by coupling receptor molecules to cell membrane channels or to second- messenger generating enzymes, and secondly the discovery that inositol phosphates, which along with cyclic nucleotides, are part of the intracellular messenger system. The generation of second-messengers leads to activation of protein kinases which in turn phosphorylate regulative proteins. These latter proteins, many of which are products of oncogenes (Adamson, 1987), then bind to appropriate regions of the DNA and control the process of gene transcription. Furthermore, the ability of some regulative proteins to autoregulate their own transcription (Angel et al.,

1988; Thayer et al., 1989); to activate each other's expression (Olson,1990); and act together in variable combinations (Landschulz et al., 1989) , allows for a vast range of phenotypic expression.

Neurotransmitter genes have been identified containing sequences which allow for the regulation of transcription. These sequences consist of enhancers, regulatory elements and promoters. (Dyman and Tjian, 1985; Maniatis et al., 1987), and have been identified for a number of neurotransmitter genes: somatostatin (Montminy et al., 1986; Hyman et al., 1988); proenkephalin (Seaholtz et al., 1986; Hyman et al., 1988); tyrosine hydroxylase (Hyman et al., 1988); VIP (Hyman et al., 1988; Dobson et al., 1994; Mulderry and Dobson, 1996); SP (Hershey et al., 1991; Kageyama et al., 1991); CGRP (Tverberg and Russo, 1992); and NPY (Minth and Dixon, 1990).

Extracellular factors influencing neurotransmitter expression

This section attempts to review the many extracellular factors which influence neuronal genes thereby influencing the processes which regulate neurotransmitter expression, neuronal growth, synaptic density and the activity of membrane receptors and ion channels.

Neurotransmitters

Neurotransmitters are able to modulate transcription in postsynaptic cells by interacting firstly with specific receptors located on the postsynaptic

membrane and thus initiating a second-messenger cascade usually involving the cyclic AMP or protein kinase signalling pathways. Observations of increased gene expression are not necessarily a result of increased transcription. Certainly there is evidence that stimulation of gene expression can take place without the activation of second-messenger signalling pathways, and need not be mediated by transcription.These changes are thought to be due to posttranscriptional mechanisms and have been shown to exist for somatostatin (Patel et al., 1995) and NPY (Lerchen et al., 1995). It seems then, that the expression of a neurotransmitter substance is regulated not only by the rates of synthesis and decay of its specific mRNA, but importantly, by other factors which control the steady state of the mRNA and the time it stays in the cytoplasm.

Among the multitude of neurotransmitters, NPY has received much interest with regard to elucidating these molecular mechanisms. This neuropeptide which is one of the more ubiquitous substances in both the central and peripheral nervous systems, is expressed by a great variety of cells where it acts as a hormone or a neurotransmitter (McDonald, 1988). Its role in the regulation of cardiovascular function is well documented (see chapter 1; Walker et al., 1991). Numerous agents including forskolin and 12-0-tetradecanoylphorbol-13-acetate (TPA) which activate adenylate cyclase and protein kinase C (PKC) respectively (Nishizua, 1986; Seamon et al., 1981), as well as nerve growth factor (NGF) are known to influence the steady state levels of NPY mRNA (Higuchi et al., 1988; Sabol and Higuchi, 1990; Minth-Worby, 1994). Promoter sequences which respond to some of the regulative substances have been identified for NPY (Higuchi et al., 1993; Minth and Dixon, 1990; Minth-worby, 1994).

Neurotransmitters can also influence each other's expression. The ability of VIP to stimulate NPY gene expression and cause neurite extension via c-AMP dependent protein kinase in pheochromocytoma cell lines (Colbert et al., 1994), and the inhibitory influence of 5-HT on VIP mRNA expression in foetal suprachiasmatic nucleus of rats (Ugrumov et al., 1994) are both examples interaction between neurotransmitters at the molecular level. Neurotransmitter agonists are also able to influence gene expression by regulating specific receptors. Receptor down regulation secondary to agonist interaction has been demonstrated to alter gene expression at both the transcriptional and posttranscriptional level (Hadcock and Malbon, 1991).

Trophic factors

Target tissues and factors released by non-neuronal cells have been found to play a major role in influencing neuronal phenotype. Co-culture of non-neuronal cells with sympathetic neurons has demonstrated a change from sympathetic to parasympathetic phenotype (Patterson and Chun, 1974; Potter et al., 1986). Furthermore, this ability to change to a parasmpathetic phenotype was maintained even in medium conditioned by non-neuronal cells (Patterson and Chun, 1977). *In vivo* studies on rat sweat glands have demonstrated the ability of sympathetic neurons to alter their transmitter property and express a cholinergic phenotype when innervating a cholinergic target (Schotzinger and Landis, (1988). This experiment further supports the concept that postnatal neurons are not irreversibly committed to expressing a particular neurotransmitter phenotype, but that they are able to modulate this expression in response to alterations in their environment. The following section briefly discusses our understanding of some of the major differentiation factors.

Elegant studies by Bueker and Levi-Montalcini in the late 1940s and early 1950s demonstrated that some diffusible factor was implicated in the development of both sympathetic and sensory ganglia and in the increased outgrowth of fibres arising from them (Bueker, 1948; Levi-Montalcini and Hamburger, 1951; Levi-Montalcini, 1987). Additional observations that many neurons are lost during development, and that the extent of this cell death can be modulated by altering the volume of the target tissue led to the competition hypothesis (Hamburger, 1958; Prestige, 1967).

The changes in perivascular innervation observed with development and aging (Chapter 7) could be due in part to this concept of competition. The volume of target tissue and hence the presence of a fixed number of recognition sites would lead to competition for neuromuscular contacts. The formation of such a contact would then confer survival advantage (Oppenheim, 1981), which as will be explained in a later section, is influenced not only by formation of a synaptic contact, but also by a variety of intracellular and extracellular differentiation and regulative factors. There is no reason to afford an exception of this competition hypothesis with regard to perivascular innervation (Burnstock,1981b). Developmental changes in the morphology of the target tissue may also cause a differential expression of neurotransmitters. We know that there are morphological changes in cerebral vessels of old rats (Hajdu et al., 1990). Interestingly, the sympathetic neurons from both young and old rats exhibit similar ability to survive, synthesize catecholamines and innervate host tissues when allotransplanted to a new and young target (Suhonen et al., 1991;Cowen, 1993).

In hypertension, and specifically with regard to the SHR model, there is increased wall-to-lumen ration in the hypertensive animals (Nordborg and Johansson, 1980; Sadoshima et al., 1986. In addition, there are increased number of vascular muscle cells in hypertensive cerebral vessels (Henrichs et al., 1980; Lee et al., 1987), thus allowing for a greater number of target sites for neural contact.

The decrease in the density of vasoconstrictor sympathetic nerves supplying cerebral arteries of old rats, may have led to the availability of new contact sites and thereby allow for the increase in vasodilatory parasympathetic nerves. The role of factors responsible for neurite differentiation and gene expression of neurotransmitter substances is discussed next.

Nerve Growth Factor

The most extensively studied differentiation factor is NGF. NGF along with brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) are considered to be members of a gene family by virtue of a greater than 50% homology shared between their primary structures (Leibrock et al., 1989; Maisonpierre et al., 1991). There is increasing evidence for NGF as a neurotrophic factor in the differentiation and development of neurons, and in promoting their survival (Levi-Montalcini, 1987; Rohrer, 1991). In the rat pheochromocytoma PC12 cell line, NGF not only induces neuronal transformation (see Greene and Tischler, 1982), but also activates some oncogenes such as c-fos and c-myc (Greenberg et al., 1985; Bartel et al., 1989). Additionally it has been shown more recently to increase the expression of the NPY gene (Sabol and Higuchi, 1990; Minth-Worby, 1994; Balbi and Allen,

1994). It has also been shown to stimulate TH activity in embryonic enteric neuroblasts (Kessler et al., 1979); lead to an increase in SP-, CCK- and VIP-immunoreactivity in primary neurons of neonatal rats (Otten and Lorez, 1982; Mulderry, 1994); counteract the depletion of SP-IR in dorsal root ganglia following treatment with capsaicin in guinea-pigs (Miller et al., 1982), as well as in axotomized rat sensory neurons (Wong and Oblinger, 1991); to induce target hyperinnervation (Kessler et al., 1985; Isaacson et al., 1990); enhance the mRNA transcriptions for SP- and CGRP-precursors in rat dorsal root ganglia (Lindsay and Harmar, 1989); upregulate the expression of its own receptor in both sensory (Wyatt et al., 1990) and sympathetic neurons (Miller et al., 1991).

NGF is involved in the development of perivascular nerve fibres, and in this context, it has been implicated in the sympathetic hyperinnervation seen in the perivascular nerves of SH rats (Lee et ., 1987). Anti-NGF induced sympathectomy prevented the development of vascular smooth muscle cell hyperplasia (Lee et al., 1987). Levels of endogenous NGF and NGF gene expression have also been shown to correlate with the degree of sympathetic innervation in the peripheral tissues (Korching and Thoenen, 1983; Shelton and Reichardt, 1984). In the mesenteric arteries of young SHrats, there is a 5-fold increase in NGF-mRNA concentrations (Curto et al., 1991) which is accompanied by an increase in the level of NGF (Donohue et al., 1987). It has even been suggested that the elevated level of NGF in the hypertensive model is the primary cause of the hyperinnervation with secondary influence in affecting muscular hyperplasia (Rush et al., 1992). While raised NGF levels may well be responsible for the hyperinnervation, it does not seem to cause hypertension. Chronic administration of NGF to WKY control rats resulted in increased volumes of sympathetic ganglia, increase in neuronal population, and resulted in raised

catecholamine levels . However, the animals remained normotensive (Zettler et al., 1991). Knowledge of any developmental changes in the concentration of endogenous NGF may help to explain the changes seen in the experiments outlined in this thesis.

Neurotransmitter expression in development, disease and nerve injury.

The experimental work for this thesis falls mainly into this category. As such, the following discussion aims, when possible, to explain the findings described in the experimental chapters in terms of molecular mechanisms. This is supported by referring to examples of altered neurotransmitter gene expression during development, in diabetes and hypertension, following nerve injury and in a number of miscellaneous physiological states.

Although much work has been done to identify neurotransmitter genes, their controlling factors and the relevant signalling pathways, there is little information about their developmental expression. It was mentioned earlier that serotonin had an inhibitory influence on the expression of VIP mRNA in foetal suprachiasmatic nuclei (Ugrumov et al., 1994). In Chapter 5, there was a decrease in the density of 5-HT-containing cerebrovascular nerve density concomitant with an increase in the density of VIP-containing nerves with aging. The possibility that the reduction in 5-HT-mediated inhibition allows for a greater expression of VIP within cerebrovascular nerve varicosities remains speculative. As discussed previously, 5-HT appears to be a 'false transmitter' and is taken up from the surrounding tissue. Whether this uptake is confined to sympathetic nerves or not is uncertain. 5-HT can be taken up from that released by degranulated mast cells and from platelets. The latter may occur when there is

disruption of the endothelium as in atheromatous plaques, or during bleeds suchas a subarachnoid haemorrhage.

Reduction in the density of immunohistochemically detectable nerve fibres as seen in various nerve types in the work presented in this thesis can be secondary to a number of mechanisms. While it most probably reflects an impairment of the expression of the neurotransmitter gene, its detectability may also be influenced by abnormalities of transmitter production, packaging, its transport from soma to axon, and as a result of enhanced transmitter release.

Studies on primary sensory neurons of the STZ-treated rat model shows a decrease in the levels of SP and CGRP to correlate with a reduced expression of NGF mRNA in sensory neuron target tissues (Fernyhough et al., 1994). A decreased expression of CGRP and SOM has also been reported in primary sensory neurons of the same diabetic animal model (Rittenhouse et al., 1996).

In renal hypertensive rats, there is a reduced expression of TH mRNA in paravertebral ganglia and in the adrenal gland (Krukoff and Zheng, 1991). No changes were detected in the expression of NPY mRNA. In the SHR-rat model, there is a decreased expression of NPY mRNA in adrenal glands, but the marginal decrease in NPY mRNA expression in the cerebral cortex did not occur until the animals were at least 17 weeks old (Higuchi et al., 1993). Increased levels of NPY mRNA have been observed in the arcuate nucleus of SH rats (Mclean et al., 1996). We do not know what the expression of NPY mRNA is in the cerebrovascular nerves of SH rats. Nevertheless, the increased density of NPY-containing cerebrovascular nerves observed in Chapter 6 may well be determined by alterations in the expression of the NPY gene. Such an increase in the density of innervation could reflect a proliferation of axons or a greater

concentration of transmitter substance thus improving the detectability of previously non-immunoreactive nerve fibres.

Numerous studies have shown altered expression of neurotransmitter substances following nerve injury. Enhanced expression of galanin, corticotropin releasing factor, angiotensin II and NPY have been shown following axotomy (Palkovits, 1995), and this was associated with decreased expression of co-existing transmitters (acetylcholine, aspartate, glutamate) and neurohormones (Vasopressin and oxytocin). The increase in the expression of VIP, galanin and NPY following axotomy and partial nerve injury have also been demonstrated by other studies (Anand et al., 1990; Mohney et al., 1994; Nahin et al., 1994). Axotomy also induces the transcription factor c-jun which appears to regulate the transcription of VIP in primary sensory neurons (Mulderry and Dobson, 1996). This may be a mechanism for the increased numbers of VIP-LI nervi nervorum in the proximal sciatic nerve stump following axotomy as described in Chapter 3.

The functional consequences of such diverse chemical coding in the autonomic nervous system appear bewildering. However, the rational division into sympathetic, parasympathetic and sensory-motor nerves has allowed some sense to be made from the puzzling combinations of transmitter substances which have been found to co-exist. It appears that the same transmitter substance can be found in different populations of neurons innervating the same target tissue, but having different post-junctional effect. A population of neurons may also contain different transmitter substances which have opposing actions on the same target. Additionally, neurons having similar functions may contain a different

mixture of transmitters. The multitude of such transmitter combinations is further compounded by the findings that some transmitters have diverse actions depending on their concentration and target tissue. NA and 5HT cause vasoconstriction of large cerebral vessels, but they both mediate dilatory effects on small pial vessels and arterioles (Lincoln, 1995). In the SHR hypertensive model, substance P appears to have a range of vascular effects depending its concentration. At low concentrations, it causes a reduction in blood pressure; at medium doses, it causes a biphasic response (initial depressor followed by a pressor component); and at high doses, it produces hypertensive effects (Oehme et al., (1981).

Finally, it has become increasingly clear that the neurotrophic theory is gaining a wider audience with respect to the complexity of neuronal plasticity, aspects of which have been demonstrated in the work described in the preceding chapters. This appears particularly true of those changes observed in development and ageing, but may apply equally well in various disease situations. Indeed, if the innervation pattern is determined by the target tissue, then in both hypertension and diabetes there is ample evidence for alterations of the morphology of the blood vessel wall and of nerve sheaths. Such morphological change may then alter the balance of local factors thereby preferentially attracting a more protective combination of innervation. An example of this is the finding of a reduction in the density of catecholamine- and 5-HT-containing cerebrovascular nerve fibres in old age concomitantly with an increase in vasodilatory nerve fibres containing VIP and CGRP (Chapter 5). The reduction in vasoconstrictor nerve fibres in old age, could be a result of the impaired elasticity and reduced vascular smooth muscle cell mass, thus creating an

environment that is less sensitive to sympathetic vasoconstriction. This loss of requirement for a vasoconstrictor tone in a less distensible aged vessel could then reflect in a lesser supply of vasoconstrictor substances. The target tissue thus needs to attract vasodilatory nerve fibres to allow for increase in blood flow in a region served with rigid and possibly atheromatous vessels. These possible mechanisms remain speculative at present, but are increasingly supported by allotransplant studies of sympathetic ganglia and cerebral vessels to different environments thus altering the target tissue, or by the use of local trophic factors to prevent abnormal innervation pattern from developing in development or disease (see Cowen, 1993).

7.4 LIMITATIONS OF THE STUDY

Although the findings described in this thesis have now largely been substantiated by others, I am conscious of the many limitations of the techniques and animal models used in them, which consequently cloud the final interpretations. While some of the limitations are intrinsic to the methods used, others have only become obvious in retrospect, and which I hope, as perhaps necessary evils of such experimentation, will spurn increasingly more focused investigations in the future.

Studies on nerve trunk circulation

The densest innervation of vasa nervorum and nervi nervorum for all substances examined was in the guinea-pig. Unfortunately neither the established models for diabetes or old age involve this specie. The rabbit is also not a commonly used model for aging, and in hindsight, the rat model may have been the best compromise.

Because of the inherent difficulty in adequately quantifying the density of innervation due to the large variation in intraneural microvascular anatomy, the findings are necessarily descriptive with only semi-quantitative data. Following the demonstration of all the regions where fluorescent nerve fibres could be detected within the nerve trunk, sampling of nerve sections for biochemical assays, such as in the study on diabetic nerves, would have added quantitative data.

The sural nerve biopsies were necessarily minute samples and I was unable to divide it into several sections to study other substances with any

consistency. These samples were also very difficult to prepare as whole-mount specimens thus penetration of the immunofluorescent antibodies was a problem such that I was unable to detect any substances other than catecholamines innervating the vasa nervorum. A baseline of what is the natural innervation pattern could be obtained from post-mortem tissues and in the case of biopsies, the best utilization would probably be to make cryostat sections for fluorescent histochemistry.

The use of animal models

Such models of disease allow an in depth assessment of the natural history of the disease, with particular value in determining genetic abnormalities, in allowing a study at the onset of the relevant disease and make it possible to assess any biochemical or physiological process in a longitudinal fashion that would be ethically prohibitive in the clinical setting. Given the frequent lack of consistency between species and the possible anatomical differences, the findings from animal studies can not be extrapolated directly to the human condition. As was mentioned with the hypertensive rat. There appears to be a difference in the innervation pattern of cerebral blood vessels by NPY-LI nerve fibres not only between two strains of rats (SHR and its stroke-prone variant.), but also regionally as seen in the denser innervation of anterior cerebral artery as compared with that of the basilar artery by NPY-LI nerve fibres. Given the complexity of co-transmission, and the multiple transmitter combinations contained within cerebrovascular nerves, it is difficult to confer any functional consequence to this regional variance in the density of innervation.

Fluorescence histochemistry

Fluorescence histochemistry has served a valuable tool in mapping the perivascular nerve types and their pattern of distribution. Immunohistochemistry has clearly increased the specificity of detecting transmitter substances within nerve fibres. However, it too has its limitation with respect to its powers of resolution with respect to the specificity of each different antibody. The lack of immunohistochemical demonstration of a substance within nerve fibres, does not necessarily mean that those fibres are not present. As discussed before, the concentration of the substance may be too low for detection. Similarly the presence of a substance within nerve fibres does not imply that it is intrinsic to that neuron. The substance may have been made elsewhere and taken up by the nerve endings. An example of this is the suggestion that 5-HT localised within cerebrovascular nerve fibres is that which has been taken up by the sympathetic nerve terminals. Despite this, majority of the studies find good correlation between immunohistochemistry and more powerful molecular techniques such as 'in situ' hybridization in localizing substances within cell bodies. Supported by biochemical and ultrastructural studies, and with the use of modern quantitative computer-driven image analysers, immunohistochemistry will remain a valuable tool in the continuing study of perivascular innervation.

7.5 FUTURE DIRECTIONS

There are two parts to consider with respect to the future direction of the work presented here. The first is in extending the present descriptive data with respect to gaining a fuller appreciation of the various nerve types that may influence the circulation of the brain and that of nerve trunks. While the cerebral circulation has and continues to receive extensive attention, less effort is spared towards the nerve trunk circulation. Unlike in the cerebral circulation, we do not have a full picture of all the substances which are present within nevi nervorum and those fibres supplying the vasa nervorum. Additionally, we still need to assess the various coexistence of neural substances that may be present within nerve trunks supplying the vasa nervorum. Denervation studies to elucidate more clearly the origin of these fibres, and pharmacological and physiological studies to assess the effect of these transmitter substances on vasa nervorum and the blood-nerve barrier still need to be carried out.

Secondly, the future direction needs to concentrate on more broader concepts. It is interesting that over a decade since some of the work presented here describing the differential expression or plasticity of transmitter substances within perivascular nerve fibres, we are still far from understanding the precise mechanisms which underpin these changes. There is little doubt that in order to get broad conceptual arguments for explaining the mechanisms involved in development, regeneration and plasticity in the autonomic nervous system, we need to look for explanations at the molecular level. Although we can subdivide perivascular nerves into sympathetic, parasympathetic and sensory-motor types, the complexity of the number of transmitter substances found within them and

their differential pattern depending on the target tissue, which varies with age and disease, compels us to decipher the chemical coding which governs them. Advances in molecular biology now allow us to not only assess the expression of neurotransmitter substances, but more importantly to find out specifically when these genes responsible for expressing various substances are actually switched on and off, and how they are influenced by development and disease. These studies need to go hand- in- hand with those assessing the expression of receptors for the various substances so as to better understand the effector processes in blood vessel control. The enormous possibilities of interplay between different perivascular nerve types, and influences on them and on target tissues by local and neuronal trophic factors adds further complexity to this field. And complex as it is, current and future investigations aimed at unraveling these mysteries primarily at the molecular level should hopefully bear fruit and allow us the luxury of therapeutic strategies in numerous human diseases.

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