PLASTICITY OF PERIVASCULAR NERVES

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Maria Caterina Mione

Department of Anatomy and Developmental Biology

University College London

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ABSTRACT

This thesis presents a study on the plasticity of perivascular nerves, and is divided in two sections. The first concerns changes in expression of perivascular nerves during development and ageing and in the nerves that remain after selective sympathetic and/or sensory denervation of the rat cerebral, meningeal and irideal vessels. In the second section changes in the innervation of the guinea pig uterine artery during pregnancy and after chronic treatment with progesterone are described.

Changes in the expression of neurotransmitters in cerebrovascular nerves were studied in rats from birth to 27 months of age, using histochemical and immunohistochemical techniques, followed by quantitative analysis of the density of innervation. The study revealed an early development of sympathetic cerebrovascular nerves, while sensory nerves and parasympathetic nerves developed later. Ageing resulted in a decrease of the expression of vasoconstrictor neurotransmitters in cerebrovascular nerves, whereas the expression of vasodilator neurotransmitters (vasoactive intestinal polypeptide, VIP, and calcitonin gene-related peptide, CGRP) was strikingly increased. Long-term sympathectomy produced by chronic treatment with guanethidine caused degeneration of the sympathetic neurons in the superior and inferior cervical ganglia and disappearance of glyoxylic acid-induced fluorescent nerves and 5-hydroxydopamine (5-OHDA) labelled vesicles in rat cerebral, meningeal and irideal vessels. The results also suggest that there is a compensatory increase of the expression of neuropeptide Y (NPY)-immunoreactivity (IR) in non-

sympathetic perivascular axons in the brain and iris of developing rats subjected long-term guanethidine treatment, but not in those of the dura mater. Using a dual immunostaining technique at the ultrastructural level, the NPY-IR present in cerebrovascular nerves of developing rats sympathectomized with long-term guanethidine treatment was localized in VIP-IR nerve fibres. Their origin, from pterygopalatine ganglion neurons, was traced using a fluorescent neuronal tracer (fast blue) and immunohistochemistry revealed an increase in the expression of NPY-IR in pterygopalatine ganglion neurons after long-term sympathectomy. An increased number of CGRP- and substance P (SP)-IR nerves was observed in the anterior cerebral artery, iris and dura mater of guanethidine sympathectomized rats. These nerve fibres could arise from sensory and/or parasympathetic cranial neurons storing CGRP- and/or SP-LI. Rats were also subjected to long-term guanethidine treatment afer capsaicin treatment. Projection of sensory CGRP- , but not SP-IR neurons which did not degenerate after capsaicin treatment again increased dramatically after guanethidine treatment.

In the second section of this thesis, the number of noradrenaline (NA)-containing nerves in the uterine artery, which formed the densest plexus in virgin animals, was much reduced in late pregnancy, a finding supported by a significant reduction in noradrenaline levels. In contrast, the innervation of the uterine artery by NPY-IR nerve fibres was increased in pregnancy, while the other peptidergic nerves and peptide levels were unchanged. Systemic progesterone treatment did not mimic these changes. An immunocytochemical analysis of uterine arteries from virgin and pregnant guinea-pigs injected

with 5-OHDA to label noradrenergic nerves showed that, unlike the pregnant uterus, no degeneration of nerves containing large and small dense-cored vesicles occurred in pregnant uterine arteries. The results suggested that, in pregnant animals, 5-OHDA labelled vesicles were also present in non-noradrenergic perivascular nerves. In the uterine arteries from pregnant guinea-pigs, the neuronal uptake of ^{3}H-NA was found to be increased compared with that of control arteries, despite the reduction of noradrenergic nerves. The autoradiographic localization of ^{3}H-NA taken up by guinea pig uterine arteries, further processed for immunocytochemistry of dopamine β hydroxylase (DBH)-, NPY-, VIP-, SP- and CGRP-IR, revealed that the uptake of ^{3}H-NA in pregnant animals occurred also in non-sympathetic nerves.

This study showed that : a) compensatory changes in the expression of neurotransmitters in perivascular nerves occur both in physiological (development, aging, pregnancy) and experimental (selective denervation) conditions; b) the changes that have been observed are complex : one clear example is a marked increase in CGRP/SP in sensory nerves following destruction of sympathetic nerves; another is the increase of NPY in perivascular nerves supplying the uterine artery in late pregnancy; c) plasticity is a property of perivascular nerves that is retained throughout life.

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PUBLICATIONS ARISING FROM THE WORK PRESENTED IN THIS THESIS

Mione, M.C., Dhital, K.K., Amenta, F., Burnstock, G. (1988) An increase in the expression of neuropeptidergic vasodilator, but not vasoconstrictor cerebrovascular nerves in aging rats. **Brain Res.**, 460:103-113

Mione, M.C., Ralevic, V., Burnstock, G. (1990) Peptides and vasomotor mechanisms. **Pharmacol. Ther.**, 46:429-468

Mione, M.C., Cavanagh, J.F.R., Lincoln, J., Milner, P., Burnstock, G. (1990) Long-term sympathectomy leads to an increase of neuropeptide Y-immunoreactivity in cerebrovascular nerves and iris of the developing rat. **Neuroscience**, 34:369-378

Cavanagh, J.F.R., Mione, M.C., Burnstock,G (1990) Use of enhanced silver stainingcombined with electron microscopical immunolabelling to demonstrate the colocalization of neuropeptide Y and vasoactive intestinal polypeptide in cerebrovascular nerves. **Neuroscience**, 39:775-785

Mione, M.C., Cavanagh, J.F.R., Lincoln, J., Milner, P., Burnstock, G. (1990) Pregnancy reduces noradrenaline, but not neuropeptide levels in the uterine artery of the guinea-pig. **Cell Tissue Res.**, 259:503-509

Mione, M.C., Cavanagh, J.F.R., Kirkpatric, K. Burnstock, G. (1991) Plasticity in expression of calcitonin gene-related peptide- and

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Mione, M.C., Lincoln, J., Cavanagh, J.F.R., Burnstock, G. - ^{3}H-Noradrenaline uptake in sensory and parasympathetic nerves of uterine arteries of late pregnant guinea-pigs. In preparation

GENERAL INTRODUCTION

The subject of this thesis is the perivascular innervation of two arterial districts, chosen as model for the study of changes occurring in physiological condition or induced by experimental manipulation.

Rat cerebral vessels have been chosen because their perivascular nerve supply : a) has been classified as sympathetic, parasympathetic and sensory on the basis of the origin and neurotransmitter(s) phenotype, being the three components segregated in specific ganglia and characterized by a well defined pool of neurotransmitter(s) ; b) can be easily and specifically denervated by surgical or chemical manipulation. These vessels were also used for evaluating changes in the density and pattern of innervation taking place during development and ageing.

The uterine artery of the guinea-pig was chosen since dramatic changes were shown to occur to both nerve pattern and vessel size during pregnancy, so that it represents a good model for the study of the adaptability of perivascular nerves in physiological condition.

The changes under study were: a) quantitative and qualitative variations in the density and pattern of nerve fibres, characterized on the basis of neurotransmitter expression and revealed using histochemical and immunohistochemical techniques at the light and electron microscopical level in both cerebral and uterine arteries; b) changes in the expression of neurotransmitter(s) type in neuronal cell bodies which give raise to the cerebrovascular, meningeal and irideal nerves under study; c) 5-OHDA and ^{3}H-NA uptake ability in the uterine artery of control and pregnant guinea pig and its autoradiographic localization in immunostained perivascular nerves.

Section III A describes experiments carried out on cerebral vessels. In Chapter one, cerebrovascular nerves containing noradrenaline (NA), serotonin (5-HT), substance P (SP)-, neuropeptide Y (NPY)-, calcitonin gene-related peptide (CGRP)- and vasoactive intestinal polypeptide (VIP)-immunoreactivity (IR) were quantified at various age stages from birth to 27 months of age. In Chapter two, the expression of NPY, a cotransmitter in NA-containing nerves was studied after long-term chemical sympathectomy with guanethidine in cerebral arteries, dura mater and iris, all targets of sympathetic nerve fibres originating from cervical ganglia. In Chapter three, the parasympathetic nature and origin of the cerebrovascular nerves expressing NPY-immunoreactivity after sympathectomy was demonstrated using a dual immunostaining technique at the ultrastructural level and retrograde tracing experiments. In Chapter four, the increase of CGRP- and SP-IR in cerebral vessels, dura mater and iris after long-term sympathectomy was quantified and compared with the changes of the expression of those neurotransmitters in neurons of sensory ganglia. This study was carried out also in sensory denervated (capsaicin treated) rats in order to quantified the involvement of sensory neurons.

In section III B, the changes in the expression of neurotransmitters in perivascular nerves of guinea-pig uterine arteries occurring during pregnancy were studied (Chapter five). Quantitative analysis of the density of nerve fibres and neurotransmitter levels took into account the dramatic morphological changes of the smooth muscle coat of the artery. The ultrastructural study described in Chapter six revealed some features of perivascular

uterine nerves, that suggested that, during pregnancy, perivascular non-noradrenergic nerves developed the ability to take up the false noradrenergic neurotransmitter 5-hydroxydopamine (5-OHDA), used as a tool for the demonstration of dense cored vesicles at the electron microscopical level. In Chapter seven, this ability was challenged with ^{3}H-NA, which is taken up by noradrenergic nerves, its uptake was measured and localized with an autoradiographic technique for electron microscopy, in arteries that had been immunostained for various neurotransmitters.

SECTION I :

HISTORICAL BACKGROUND

1. INTRODUCTION

For many years, studies on neurohumoral control of the vasculature have been dominated by consideration of the role of catecholamines released from sympathetic perivascular nerves and from the adrenal medulla into the bloodstream. The availability over the past two decades of new and improved techniques in immunohistochemistry, electron microscopy, electrophysiology and pharmacology has led to a wealth of discoveries that have profoundly reshaped our understanding of the autonomic nervous system (ANS) (see Burnstock, 1986a,b). While the classical view of the ANS was formulated largely from consideration of antagonistic cholinergic and adrenergic nerves, about 16 new putative neurotransmitters have been proposed in the ANS in the last few years, including various monoamines, polypeptides, purines and amino acids. Several modulatory transmitter mechanisms have been recognized, including prejunctional inhibition or enhancement of transmitter release, postjunctional modulation of transmitter action, and the secondary involvement of locally synthesized hormones and prostaglandins. The existence of more than one transmitter substance in some nerves, cotransmission, is now also widely recognized. Modulatory mechanisms appear to represent the dominant role of neuropeptides in the vasculature, such that they may produce short- or long-term influences on the release or action of other neurotransmitters, either directly or indirectly, through their action on degradative pathways.

Not only has an increasing complexity in neuroregulation of

blood vessels (and other tissues) been revealed in recent years, but the understanding of local humoral regulation has also rapidly progressed. This has largely stemmed from the discovery that endothelial cells, forming the innermost layer of blood vessels, play a crucial role in the vasodilatory response of the vessel to acetylcholine (ACh) (Furchgott and Zawadzki, 1980) and to a growing number of other substances (see Vanhoutte and Rimele, 1983; Furchgott, 1984). The considerable interest in vascular control mechanisms arising from these studies has led to the concept of a dual regulation of blood vessel tone, whereby both nerves and the endothelium are involved (Burnstock and Kennedy, 1986; Burnstock, 1988).

In this section some of the above-mentioned recent discoveries in neurohumoral control of the vasculature are reviewed, with special emphasis being placed on the localization and vasomotor roles of peptides. Attention will be given largely to those peptides most prominent in perivascular nerves, including neuropeptide Y (NPY), vasoactive intestinal polypeptide (VIP), substance P (SP) and calcitonin gene-related peptide (CGRP), although mention will be made of other biologically active peptides claimed to be in some perivascular nerves, such as neurotensin, somatostatin, bradykinin, vasopressin, galanin, atrial natriuretic peptide, gastrin-releasing peptide, cholecystokinin, dynorphin and the opioid peptides.

2. VASCULAR NEUROEFFECTOR MECHANISMS

2.1 Vascular neuroeffector junction

The model of the autonomic neuroeffector junction, as proposed by Burnstock and Iwayama (1971), differs from the classical neuromuscular junction in skeletal muscle and from the synapses in the central and peripheral nervous systems. Both of these latter sites are characterized by the presence of specializations of the pre- and postsynaptic membranes, separated by a cleft of 20-50 nm.

Studies on the relationship between autonomic nerve fibres and vascular smooth muscle cells (Burnstock, 1970; Gabella, 1981) have shown that terminal axons branch extensively at the level of the adventitial-medial border to form "plexus-like" structures. These terminal axons are devoid of Schwann cell covering and are rich in varicosities (0.5-2 μm in diameter), separated by intervaricose regions (0.1-0.3 μm in diameter). Neurotransmitters are mainly stored in vesicles within these varicosities and are released "en passage" during the conduction of nerve impulses. Specialized thickened areas are sometimes seen on prejunctional, but not on postjunctional membranes, and the junctional cleft can vary from 50 to 2000 nm, depending on the size of the vessel (Burnstock, 1975; Burnstock et al., 1980). Active substances released from the varicosities reach specific receptors situated on smooth muscle cells which are in electrical continuity with neighbouring muscle cells via intercellular "gap junctions" or "nexuses" (Burnstock, 1986b).

Attempts have been made to correlate the features of the nerve terminals, in particular with respect to the types of vesicles within

these terminals, with the neurotransmitters they contain. So far, only the preferential occurrence of noradrenaline (NA) in small dense-cored vesicles, of ACh in small agranular vesicles and peptides in large dense-cored vesicles has been established (Lee et al., 1984a; Fried et al., 1985a,b; Gulbenkian et al., 1986). The newly discovered membrane proteins synaptosin and synaptophysin appear to be specifically associated with small granular vesicles (Navone et al., 1984, 1986; Metz et al., 1986).

2.2 Cotransmission and neuromodulation

The earliest hint that nerve cells might release more than one transmitter came in 1957, when Abrahams et al. suggested that impulses conducted along the hypothalamo-neurohypophysial neurosecretory fibres might liberate ACh at their terminals to provide the stimulus for the release of oxytocin and vasopressin from the same terminals. This was followed by reports of other inconsistencies in the principle that nerve cells make and release only one transmitter (Burn and Rand, 1959; Gerschenfeld et al., 1960). A major challenge to the one neurone one neurotransmitter principle, known (albeit fallaciously) as Dale's principle, came in a commentary in Neuroscience by Burnstock in 1976. Subsequent experimental evidence largely supported this hypothesis and it is now known that the majority of nerve fibres, if not all, can store and release more than one neurotransmitter (see Cuello, 1982; Osborne, 1983; Chan-Palay and Palay, 1984; Hökfelt et al., 1986).

Most commonly, coexistence involves the classical neurotransmitters NA and ACh, together with various peptides and

adenosine 5'-triphosphate (ATP) (Burnstock and Griffith, 1988). There is usually a preferential association between neurotransmitters, and the roles exerted by each of them are beginning to be understood. Several examples will be given either in this section, or in the sections dealing with description of the individual transmitters.

For any two substances identified as coexisting within nerve cells to fulfil the criteria for cotransmitters, it must be established that each of these has a direct action on specific postjunctional receptors. A neuromodulator, on the other hand, is defined as a substance which modifies the process of transmission. It can do this in two ways: either by acting on prejunctional receptors to reduce or enhance transmitter release, or by acting postjunctionally to alter the magnitude or time course of the action of the transmitter on the postjunctional cell. Since many cotransmitters have been shown to exert such effects, these are, by definition, also neuromodulators. Several characteristics of cotransmission and neuromodulation have been described and are represented diagramatically in Fig. 1.

When a particular transmitter, in addition to its postjunctional effects, modifies its own release, invariably attenuating it, this is termed autoinhibition (Fig. 1a). As a consequence of its effects on the nerve terminal, the release of any cotransmitters will also be affected, such as occurs with the inhibition of NA and ATP from sympathetic nerves by coreleased NPY (Fig. 2). In addition to the effects the neuromodulator may have on its own terminal, it may also act on a closely juxtaposed terminal

such that an interaction, or "cross-talk" occurs (Fig. 1b). This is likely to occur, for example, between perivascular adrenergic and cholinergic nerves, where the transmitters released from the nerves not only produce antagonistic actions on the vascular smooth muscle, but also act prejunctionally to reduce each others release. Neurotransmitters, circulating hormones or local agents such as prostanoids, bradykinin and histamine may also act as neuromodulators.

When each of two transmitters, released from different nerve terminals (Fig. 1c) or as cotransmitters from the same nerve terminal (Fig. 1d), have the same postjunctional effect and in addition there is a reinforcement of their effects, this is known as synergism. Opposite actions have also been described, where the effect depends on the target organ (Fig. 1e), such as occurs with VIP in the cat salivary glands, where it produces inhibitory effects on blood vessels, but stimulant effects (via the enhancement of ACh action) on acinar cells (Fig. 3) or where the preferred effect depends on the tone of the vessel (Fig. 1f).

Another form of neuromodulation is that recently described in sensory nerves where CGRP has been shown to prolong the behavioural effects of SP injected intrathecally in the rat, through the inhibition of an SP-endopeptidase present in the cerebrospinal fluid (CSF) (Wiesenfeld-Hallin et al., 1984; Le Greves et al., 1985) (Fig. 4). This situation results in a different rate of survival of different substances in the junctional cleft, which is also the case in the coexistence of a classical neurotransmitter (with a briefer life) and a peptide (having a longer life).

Specific combinations of substances appear to be associated with different populations of neurones projecting to different sites (Lundberg et al., 1982a; Costa and Furness, 1984; Macrae et al., 1986; Furness and Costa, 1987; Gibbins et al., 1987a). An example of this "chemical coding" is in the rat salivary gland, where sympathetic nerve fibres containing predominantly NA project to the secretory ducts and to the acini, while those that also contain NPY-immunoreactivity (NPY-IR) project to the arterioles (Lundberg et al., 1982a). In the guinea-pig dorsal root ganglia, the presence of additional neurotransmitters in the SP/CGRP-containing sensory neurones which supply the systemic arteries is target related, such that SP/CGRP/dynorphin (DYN) neurones supply the pelvic viscera, neurones containing SP/CGRP/cholecystokinin (CCK) supply the arterioles of skeletal muscles and those containing SP/CGRP/DYN/CCK supply the skin (Gibbins et al., 1987a).

A neuromodulator may affect the expression of a certain neurotransmitter within a population of neurones by effects exerted at the level of transcription of its gene. This may be the mechanism underlying the compensatory changes that occur during development and disease. Alterations in gene expression may also be reasponsible for the increased synthesis of receptors such as has been shown in cultured chicken myotubes, where CGRP stimulates the synthesis of receptors for ACh (Fontaine et al., 1986; New and Mudge, 1986) (Fig. 5). This mechanism can also operate <u>in vivo</u>, since CGRP has been shown to coexist and cooperate with ACh in spinal motorneurones (Gibson et al., 1984; Takami et al., 1985a,b).

3. PERIVASCULAR NERVES AND PEPTIDES

3.1 Neuropeptide Y

General Distribution of Neuropeptide Y

NPY is the most widely distributed neuropeptide in perivascular nerves of mammals (see Table I). Initially isolated for porcine brain by Tatemoto et al. (1982), it is a 26 amino acid peptide with tyrosine at both N and C terminals (Tatemoto, 1982). NPY is a member of the pancreatic polypeptide family; it shares considerable sequence homology with pancreatic polypeptide and peptide YY. These two peptides had already been described in mammalian perivascular nerve fibres prior to the identification of NPY (Hökfelt et al., 1980; Lundberg et al., 1980b; Hunt et al., 1982; Jacobowitz and Olschowka, 1982). Their presence in perivascular nerves has now been attributed to a cross-reactivity of their antibodies with those to NPY (Lundberg and Tatemoto, 1982; Lundberg et al., 1984b). In man, the gene encoding for pancreatic polypeptide has been localized on chromosome 17, while NPY is part of a 97 amino acid precursor, pre-pro-NPY, encoded for by a gene localized on human chromosome 7 (Takeuchi et al., 1985). The precursor pre-pro-NPY comprises three peptide segments: a putative leader sequence of 28 amino acids, the biologically active NPY and a 30 amino acid carboxy flanking peptide (CPON) (Minth et al., 1984). Immunocytochemistry has shown that NPY and CPON coexist in the same vesicles as well as in the same neurones (Allen et al., 1985a; Gulbenkian et al., 1985). No physiological role has yet been attributed to CPON, but its localization can be a

useful tool for the demonstration of metabolic pathways of NPY in nerve cells.

Neuropeptide Y in Sympathetic Neurones

Of major interest has been the observation that NPY coexists with catecholamines in both central (Hökfelt et al., 1986) and peripheral neurones (Lundberg et al., 1982b, 1983; Lundberg and Hökfelt, 1983; Ekblad et al., 1984). Several studies have confirmed the sympathetic origin of NPY-containing perivascular nerve fibres. Thus, surgical removal of the stellate ganglia (Dalsgaard et al., 1986) or of the superior cervical ganglia (Edvinsson et al., 1983; Ekblad et al., 1984; Schon et al., 1985a; Uddman et al., 1985) as well as reserpine or 6-hydroxydopamine treatment, which depletes NA store (Allen et al., 1985b; Lundberg et al., 1985c, 1986a), affect NPY levels in the cardiovascular system. However, these treatments never cause a complete depletion of NPY immunoreactivity, as they do with NA (Lundberg et al., 1985c, 1986a; Schon et al., 1985a, Edvinsson et al., 1987b; Nagata et al., 1987; Papka and Trauring, 1988). This was initially attributed to a different sensitivity, and content, of terminal and preterminal axons, the latter being less sensitive to reserpine treatment (Morris et al., 1986), or to the different metabolism (synthesis, transport, uptake) of the two transmitters within the same nerve fibres (Nagata et al., 1987).

The storage of NA and NPY in different vesicles within sympathetic nerve varicosities has also been claimed to be responsible for their different sensitivities to chemical treatment. Electron microscopical and fractionation studies carried out in the

rat vas deferens and cat spleen (Fried et al., 1985a,b) and in human atrial appendage (Wharton and Gulbenkian, 1987) have shown that NPY is preferentially localized, along with NE and ATP, in large dense-cored vesicles, which are the major storage sites for NE and ATP (Stjärne et al., 1986). Recently, this arrangement has also been demonstrated in the bovine stellate ganglia, where large dense-cored vesicles have been shown to contain NPY, NE and [Met]enkephalin (Met-ENK) (Miserez et al., 1988).

That NPY, ATP and NA are all released, by nerve stimulation, from sympathetic nerve terminals has been confirmed by use of the sympatholytic agent, guanethidine, which prevents the release of each of these transmitters (Lundberg et al., 1986b). NPY release is optimal at high-frequency intermittent bursts of stimulation (Lundberg et al., 1986b). While in the vas deferens and in many vessels it has little direct postjunctional action (Pernow et al., 1986; Stjärne et al., 1986), it does have potent prejunctional actions reducing the release of NA, and postjunctionally enhancing the actions of NE (Glover, 1985; Pernow et al., 1986; Wahlestedt et al., 1986) (Fig. 2). Thus, the electrical activity of the neurone is able to modulate the release of coexisting neurotransmitters, and so affects the relative amounts of these substances at the level of the neurovascular junction. This will be discussed in more detail in the section on NPY as a neuromodulator.

NPY does not always occur in coexistence with NA, as has been shown by the presence of NA-containing nerves that completely lack NPY in the cardiovascular system of certain marsupials (Morris et al., 1986; Gibbins et al., 1988b) and in rat brown adipose tissue (Cannon

et al., 1986). Conversely, evidence for the existence of neurones containing NPY-IR but not NA, particularly in intrinsic and parasympathetic neurones, is continually emerging.

Neuropeptide Y in Intrinsic Neurones

The importance of the intrinsic neurones disseminated in the walls of many peripheral organs, including the bladder, the heart and the trachea, is starting to be recognized largely due to extensive studies carried out on the enteric nervous system (Furness and Costa, 1982). The enteric nervous system may be considered as a large plexus of intrinsic neurones which are responsible for control of the motility, secretory activity, mucosal transport and local blood flow in the gut (Gershon, 1981).

Intrinsic neurones contain several peptidergic substances, often coexisting with the classical neurotransmitters NA and ACh (Furness and Costa, 1982). NPY is present, along with VIP and peptide histidine isoleucine (PHI), in neurones of the myenteric ganglia, and in a population of amine-handling (5-hydroxytryptamine; 5-HT) intracardiac neurones that do not contain dopamine beta-hydroxylase (DBH) (Hassall and Burnstock, 1987). Neurones of the paratracheal ganglia also display immunoreactivity for NPY (Hassall, unpublished observations) as do those in the guinea-pig bladder (James and Burnstock, 1988).

Although their effects are largely unknown, intrinsic neurones may participate in the neural control of local blood flow, together with the extrinsic supply arising from sympathetic ganglia or from the spinal cord. From the use of animal models of specific extrinsic

denervation (Galligan et al., 1988; Mulderry et al., 1988), together with studies on transplanted organs (Springall et al., 1988), an involvement of intrinsic neurones in the perivascular innervation of blood vessels is now starting to be understood.

Neuropeptide Y in Parasympathetic Neurones

Some of the ganglia connected with the Vth, VIIth and IXth cranial nerves are classically defined as parasympathetic. Typically, their neurones are acetylcholinesterase positive, they contain choline acetyltransferase (ChAT)- and VIP-IR and they exert antagonistic roles to noradrenergic sympathetic neurones (see Landis et al., 1987; for review). Quite surprisingly, the presence of NPY-IR has been revealed in some of the ChAT/VIP-containing neurones of the parasympathetic ciliary, sphenopalatine, otic (Leblanc et al., 1987) and pterygopalatine ganglia (Kuwayama et al., 1988), which do not display any sympathetic markers [NE, DBH, tyrosine hydroxylase (TH)]. The targets of these neurones, including the iris and cerebral vessels, are normally also innervated by sympathetic noradrenergic/NPY-IR nerve fibres. The minor amount of NPY present in these neurones has been shown to increase enormously after surgical (Gibbins and Morris, 1988) and chemical sympathectomy (Björklund et al., 1985), thus indicating a close link between the two NPY-IR neuronal systems.

NPY-IR has also been found in VIP/DYN-IR neurones in the guinea-pig uterine artery (Morris et al., 1985) and in a population of non-noradrenergic nerves which supply the rat uterine cervix, but not in its vasculature (Papka and Trauring, 1988). These axons are

likely to originate from the paracervical ganglia, where noradrenergic and non-noradrenergic neurones are present (Costa and Furness, 1983; Bell, 1974). In these ganglia, NPY-IR is present in half of the catecholaminergic neurones (5% in total), that are surrounded by NPY-IR extrinsic nerve fibres, and in the majority of the VIP/DYN-, somatostatin (SOM)-, and DBH-IR neurones, which receive SP-IR afferents (Morris and Gibbins, 1987).

Actions of Neuropeptide Y

The major role of NPY in the vasculature appears to be that of a neuromodulator of noradrenergic transmission (Ekblad et al., 1984; Dahlöf et al., 1985a,b; Lundberg et al., 1985a; Wahlestedt et al., 1985; Hanko et al., 1986; Pernow et al., 1986) (Table II). At the prejunctional level, NPY exerts an inhibitory effect on the release of NA (Dahlöf et al., 1985a), which is Ca^{++}-independent (Pernow et al., 1986) and requires very low concentrations of the peptide. Not very much is known about this mechanism, but it has been shown that in the rat dorsal root ganglia, inhibitory prejunctional receptors exert their effects through the blockage of intracellular Ca^{++} mobilization following the activation of the guanine triphosphate complex (Ewald et al., 1988). In turn, NA acting on α_2-adrenoceptors located on the prejunctional membrane of sympathetic nerves, inhibits both its own release and that of NPY (Dahlöf et al., 1985b). The lack of prejunctional inhibitory control in reserpine-pretreated animals, as demonstrated in perivascular nerves of dog skeletal muscle (Pernow et al., 1988a), has been attributed to a depletion of NPY in this preparation.

The mechanism of postjunctional enhancement of NA-induced vasoconstriction, demonstrated in many vascular beds (Edvinsson et al., 1984; Dahlöf et al., 1985a; Pernow et al., 1986), is also not fully understood. Calcium also appears to play a role in this mechanism, since the calcium entry blocker nifedipine antagonizes the NPY-enhanced noradrenergic vasoconstriction (Lundberg et al., 1985a; Pernow et al., 1986). In the central nervous system, NPY increases the number of α_2-adrenoceptors (Agnati et al., 1983), although a similar mechanism is unlikely to occur in the vasculature (Pernow et al., 1986). In the rabbit ear artery, NPY potentiates the noradrenergic constriction only in those arteries with an intact endothelium (Lüscher and Vanhoutte, 1986; Daly and Hieble, 1987), while in the rat aorta the potentiation has been attributed to the release of a third substance from the prejunctional terminals (Mabe et al., 1987). Postjunctional inhibitory effects of NPY have been demonstrated in canine cerebral arteries, where NPY was shown to suppress the contractile effect of exogenously applied NA (Suzuki et al., 1988).

While NPY does not seem to have direct effects on the smooth muscle cells of many vessels (Hanko et al., 1986; Pernow et al., 1986; Wahlestedt et al., 1986), it does exert a direct, non-adrenergic, calcium-dependent vasoconstriction <u>in vitro</u> and <u>in vivo</u>, in vessels of the spleen, kidney and in the coronary and cerebral arteries of several animals species including humans (Allen et al., 1983; Edvinsson et al., 1983, 1987b; Franco-Cereceda et al., 1985; Lundberg et al., 1986b; Leys et al., 1987). It exerts similar effects, though independent of the presence of calcium, on arteries

supplying skeletal muscles (Pernow, 1988) and in the guinea-pig uterine artery (Morris et al., 1985; Morris and Murphy, 1988). These effects are slow in onset, long lasting, independent of the presence of an intact endothelium, and can be evoked by both exogenous application of NPY and by transmural stimulation at high frequency. The response to transmural stimulation is blocked by guanethidine, while α_2-adrenoceptor and β-adrenoceptor antagonists do not affect the vasoconstriction induced by NPY application (Edvinsson et al., 1983; Pernow et al., 1988).

An involvement of NPY-IR perivascular nerves has been demonstrated in animal models of hypertension, where NPY content was shown to decrease markedly in the kidney and renal artery (Ballesta et al., 1984), while NPY-IR cerebrovascular nerves increased in density in parallel with NA-containing nerves (Dhital et al., 1988). An increase in NPY levels also occurs in the cerebral spinal fluid (CSF) of rabbits that have received intracisternal blood injection to simulate subarachnoid haemorrhage, and a potentiation of NA-induced vasoconstriction was shown to occur in rabbit cerebral vessels as a consequence of incubation of the arteries with CSF from the treated animals (Abel et al., 1988).

3.2 Vasoactive intestinal polypeptide

General Distribution of Vasoactive Intestinal Polypeptide

Several lines of evidence indicate that VIP is the most widely diffused vasodilator neuropeptide in perivascular nerves of mammals (Larsson et al., 1976a,b; Uddman et al., 1981; Bevan et al., 1984). VIP is a 28 amino acid peptide which was originally isolated from the porcine small intestine (Said and Mutt, 1970) and exhibits potent vasodilator activity, in vivo and in vitro, in many vascular beds. It belongs to a family of peptides including secretin, glucagon, corticotropin-releasing factor, gastrin-inhibitory peptide, growth hormone-releasing factor and the structurally related peptide histidine isoleucine (PHI) (or methionine in man, PHM), all of which share several classes of homologies with VIP. PHM and VIP are derived from the same 170 amino acid precursor (pre-pro-VIP, Itoh et al., 1983), for which the human gene has recently been isolated, purified and cloned (Bodner et al., 1985).

In the peripheral nervous system, VIP-IR has been localized in the gastrointestinal tract, lungs, genitourinary structure and heart, in addition to its localization in blood vessels (Costa and Furness, 1973; Dey et al., 1981; Barja and Mathison, 1982; Ottesen et al., 1984). The distribution of VIP-IR in the vasculature of several species is shown in Table III. Perivascular VIP-IR nerve fibres are usually sparse in large conducting fibres, but form very dense plexuses in the vessels supplying regional vascular trees (Uddman et al., 1981; Della et al., 1983), and in man the density of these fibres lies between that of NPY- and CGRP-IR nerve fibres. The

systemic veins usually lack VIP-IR, with the exception of mesenteric veins in the rat (Edvinsson et al., 1985c), the guinea-pig caval vein (Della et al., 1983) and the rat portal vein (Barja and Mathison, 1982). Within perivascular nerves, VIP-IR is localized in varicosities containing mainly large vesicles (Lee et al., 1984a). The peptide precursor is synthesized at the level of the cell body and packed in vesicles which are carried to the nerve terminals by fast axonal transport, during which time cleavage mechanisms are carried out (Lundberg et al., 1980a).

Cerebrovascular VIP-IR nerve fibres have been shown to persist after surgical or chemical sympathectomy (Edvinsson et al., 1980; Kobayashi et al., 1983; Matsuyama et al., 1983) and after neonatal capsaicin treatment (Matsuyama et a., 1983), indicating that the nerve fibres are neither sympathetic nor sensory in nature. The origin of VIP-IR nerve fibres supplying the cardiovascular system is frequently attributed to local ganglia; VIP-IR nerve fibres supplying the respiratory tract (and its vessels) arise from clusters of VIP-IR cells within the lungs (Dey et al., 1981). The heart and coronary arteries also receive VIP-IR from local ganglia (Weihe et al., 1984). VIP-IR nerve fibres innervating the cerebral arteries originate from the cranial parasympathetic ganglia, in particular from the sphenopalatine ganglion (Gibbins et al., 1984a,b; Hara et al., 1985), while those in the genitourinary tract originate from pelvic and paracervical ganglia (Gu et al., 1984). Sparse VIP-IR neurones have been described in the wall of the rat portal vein (Ishi et al., 1982) and in the stroma of the guinea pig iris (Björklund et al., 1985), as well as in intrinsic ganglia of the bladder (Crowe et al., 1986) and

of the heart (Weihe et al., 1984), where they contribute to the nerve supply to local vessels (Björklund et al., 1985; Terenghi et al., 1982a; Galligan et al., 1988).

Vasoactive Intestinal Polypeptide in Parasympathetic Neurones

In the same way that NPY has been considered to be the main peptidergic cotransmitter in sympathetic neurones, so has VIP been attributed with a cotransmitter role in cholinergic parasympathetic neurones. In addition to the colocalization of VIP-IR with acetylcholinesterase (AChE) (Hara et al., 1985) and, more recently, with ChAT-IR (Leblanc et al., 1987), the most convincing evidence for this comes from pharmacological experiments performed on cat salivary glands (Lundberg, 1981). In this preparation, VIP and ACh are released from the same nerve terminal, though probably from separate vesicles, in response to transmural nerve stimulation (TNS). During low-frequency stimulation, ACh is released to cause an increase in salivary secretion from acinar cells and also to elicit some minor dilatation of blood vessels in the gland (Lundberg et al., 1980a, 1984a; Lundberg, 1981). VIP is preferentially released at high frequencies to cause marked vasodilatation of blood vessels and, while it has no direct effect on acinar cells, it acts as a neuromodulator to enhance substantially both the postjunctional effect of ACh on acinar cell secretion and the release of ACh from the nerve varicosities via prejunctional receptors (see also Fig. 3). The differential release of VIP and ACh is probably a reflection of the differential storage of classical and peptidergic coexisting neurotransmitters, as already described for NPY and NA. VIP and ACh

have also been shown to cooperate in the rat colon and rectum, with the rectal vasculature being more sensitive to VIP than to ACh (Andersson et al., 1983a,b).

VIP has also been shown to coexist with the vasoconstrictor NPY in some cranial parasympathetic ganglia (Leblanc et al., 1987; Cavanagh et al., 1989) and cerebrovascular nerves (Gibbins and Morris, 1988), as well as in nerve fibres originating from the thyroid ganglia that supply the rat thyroid gland and its vasculature (Grunditz et al., 1988). VIP/NPY coexistence has also been shown in the paracervical ganglia of the guinea-pig, where DBH-, DYN- and SOM-IR have also been found (Morris and Gibbins, 1987).

Vasoactive Intestinal Polypeptide in Sensory Neurones

There is increasing evidence for the localization of VIP-IR in sensory neurones of the dorsal root and trigeminal ganglia in the rat, cat and guinea-pig (Hökfelt et al., 1980; Kawatami et al., 1982; Fuji et al., 1983; Leah et al., 1985; Kummer and Heim, 1986). While the peripheral distribution of their axons has not yet been investigated, the existence of central branches of sensory neurones containing VIP-IR terminating on the neurones of the dorsal horn is known (Gibson et al., 1981; Kawatami et al., 1982). Both these axons and their cell bodies show an increase in VIP-IR, and in VIP levels, following peripheral axotomy or crush (McGregor et al., 1984; Atkinson and Shehab, 1986; Shehab and Atkinson, 1986), in contrast to the level of another sensory neurotransmitter (SP), or to the fluoride-resistant acid phosphatase activity, both of which undergo a marked reduction (McGregor et al., 1984; Shehab and Atkinson, 1986).

The mechanism by which peripheral axotomy or crush induces changes in the expression of neurotransmitters with sensory origins is unknown, but a recent report showed that the induction of VIP-gene expression in rat sensory neurones following nerve injury is due to the loss of a retrogradely transported factor, which is not nerve growth factor (NGF) (Keen et al., 1988). Whether the VIP-IR sensory neurones correspond to any of the other sensory peptide-containing neurones, or represent a different population of cells, is still unknown.

Actions of Vasoactive Intestinal Polypeptide

VIP exerts a direct vasodilator action on several vessels (see Edvinsson and Uddman, 1988, for review) (see also Table IV). Its effects have been extensively studied in cerebral vessels: in feline cerebral arteries, VIP and the structurally related PHI induce a dose-dependent vasodilatation when applied to isolated vessels or infused in vivo, with small arterioles being more sensitive than the larger ones (Larsson et al., 1976a; McCulloch and Edvinsson, 1980; Duckles and Said, 1982; Edvinsson and McCulloch, 1985; Edvinsson and Ekmann, 1984; Lee et al., 1984a; Suzuki et al., 1984).

VIP has been claimed to be the non-adrenergic, non-cholinergic vasodilator neurotransmitter responsible for the endothelium-dependent relaxation of cerebral vessels induced by TNS (Lee et al., 1984a). Several lines of evidence support the role of VIP as vasodilator neurotransmitter in cerebral vessels, including: the close relationship between levels of endogenous VIP and the ability of a vessel to relax to TNS (Duckles and Said, 1982), the release of

VIP following TNS (Bevan et al., 1984), the presence of characterized VIP receptors coupled with adenylate cyclase (Suzuki et al., 1985) and the successful use of anti-VIP antiserum to antagonize the vasodilatation induced by TNS (Bevan and Brayden, 1986). However, the fact that TNS is still able to relax cerebral vessels following tachyphilaxis with exogenous VIP, together with the demonstration that TNS-induced relaxation may be completely blocked with specific guanosine 3,'5'-phosphate (cyclic GMP) blocking agents (Lee, 1986), indicates that other neurotransmitters are also involved in the endothelium-independent relaxation of cerebral vessels.

An endothelium-dependent vasodilatation induced by VIP has been demonstrated in the bovine pulmonary artery, and this has been associated with an increase in both adenosine 3',5'-phosphate (cyclic AMP) and cyclic GMP. The increase in cyclic AMP is abolished by indomethacin, suggesting a prostacyclin-mediated mechanism for the vasodilatation (Ignarro et al., 1987). Other authors, however, have been unable to demonstrate a role for the endothelium in VIP-mediated relaxation either in bovine or in human pulmonary arteries (Barnes et al., 1986; Greenberg et al., 1987).

An involvement of perivascular VIP-IR nerves has been demonstrated in experimental diabetes in the rat intestine (Belai et al., 1985), cerebral vessels (Lagnado et al., 1987) and iris (Crowe and Burnstock, 1988). Specifically, a loss of VIP has been reported in nerves supplying penile vessels in diabetic impotence (Crowe et al., 1983). Reduced levels of VIP in cerebrospinal fluid have also been reported in humans with recent cerebral infarction (Wikkelsö et al., 1985) and a disappearance of VIP-IR from cerebrovascular nerves

has been observed in dog cerebral arteries following subarachnoid haemorrhage (Uemura et al., 1986). During aging a decrease (rat pulmonary artery, Geppetti et al., 1988) in the density of perivascular VIP-IR nerves has been reported.

3.3 Substance P

General Distribution of Substance P

Of the neuropeptides, SP has the longest history, having been discovered in 1931 by Von Euler and Gaddum. They reported that a powder, subsequently termed SP (P for powder), extracted from the brain and from the gut, exerted potent hypotensive and spasmogenic effects which could not be blocked by antagonism of established neurotransmitters. In 1970 the peptide was isolated from bovine hypothalamus (Chang and Leeman, 1970), characterized (Chang et al., 1971) and later synthesized (Tregear et al., 1971).

SP consists of an undecapeptide belonging to the family of tachykinins, which have in common many homologies in their C-terminal amino acid sequences (see Table V). t was considered to be the only tachykinin present in mammals, until neurokinin A (NKA), neurokinin B (NKB) (Kimura et al., 1983; Minamino et al., 1984) and neuropeptide K (NPK, a 36 amino acid polypeptide that contains the NKA sequence in the C-terminal portion) (Tatemoto et al., 1985) were isolated from the porcine central nervous system.

At least three peptide precursors of tachykinins have been identified from work on the rat - the alpha, beta and gamma pre-pro-tachykinins (Nawa et al., 1983, 1984), the alpha containing only the sequence of SP, and the beta and gamma containing the sequences of

SP and NPK in the ratio 1:1. Differences in the levels of individual tachykinins occurs as a result of alternative RNA splicing in the expression of the single pre-pro-tachykinin A gene. As shown in neurones of the guinea-pig myenteric plexus, NKA is formed from NPK, and this occurs during axonal transport (Deacon et al., 1986). Earlier studies in the dorsal root ganglia (DRG) (Gamse et al., 1979; Gilbert et al., 1980; Harmar et al., 1981) have shown that SP is synthesized in the ribosomal fraction of the perikaryon and is carried to the spinal cord and to the periphery mainly by fast axonal transport (Gilbert et al., 1980).

Substance P in Sensory Neurones

The widespread distribution of SP in both central and peripheral nervous systems, together with its pronounced pharmacological effects, initiated the interest in its putative physiological role. It was largely due to the immunohistochemical localization of SP in defined groups of small-diameter cell bodies in sensory ganglia, as well as in nerve fibres in the periphery and in the dorsal horn of the spinal cord, that led Lembeck in 1953 to suggest that SP might function as a transmitter in primary sensory neurones. Since then, many other studies have substantiated this hypothesis, and in addition, many other substances, including CGRP, have been proposed to have roles as sensory transmitters (see Maggi and Meli, 1988).

Capsaicin sensitivity has been used as a marker for sensory neurones and the loss of SP-IR following capsaicin treatment correlates well with a role for SP as a sensory transmitter. Both

chronic treatment with capsaicin, inducing selective degradation of C-fibre afferents, and its acute effects, causing stimulation of chemosensitivie C-fibres (and concomittantly releasing SP), have been used to investigate the nature and distribution of sensory neurotransmitters.

SP-containing perivascular nerves have their roots in numerous SP-IR cell bodies in the spinal ganglia and in the jugular, nodose and trigeminal ganglia. The cell bodies represent a population of small neurones (about 20% of the total cell bodies) that give rise to unmyelinated C-fibres. In a recent study (Lawson and McCarthy, 1988), SP-LI has been localized in electrophysiologically characterized neurones of the DRG, having both C-type and A-delta fibres. Immunohistochemical and radioimmunoassays have demonstrated that CGRP coexists with SP in a large number of perivascular nerve fibres (Gibbins et al., 1985; Lee et al., 1985a). SP has been shown to be released in a Ca^{++}-dependent manner from the spinal cord of the rat and cat during stimulation of sensory nerves (Otsuka and Konishi, 1976; Yaksh et al., 1980) and to act on receptors localized on the postsynaptic membrane of dorsal horn neurones and motoneurones (Helke et al., 1986). On these cells, SP causes excitatory postsynaptic potentials that are very slow in onset and last for seconds (Akagi et al., 1985) and are inhibited by tachykinin antagonists and anti-SP antibodies (Randic et al., 1986). This kind of slow excitatory postsynaptic potential agrees well with the transmission of delayed pain that is typically attributed to non-myelinated C-type fibres.

As shown by immunocytochemistry, the peripheral nerve endings of primary sensory neurones are extensively associated with the

cardiovascular system (Table VI). These have been identified in the heart, around large arteries and veins, and in smaller vessels supplying vascular beds. SP-IR nerve fibres are present in the adventitia and in the adventitial-medial border of almost all the arteries and veins of guinea-pigs (Furness et al., 1982) and rats (Barja et al., 1983), with the larger vessels receiving more innervation than the smaller, and arteries receiving more innervation than veins. Treatment with capsaicin cuases the loss of SP-LI perivascular nerves in guinea-pigs (Gamse et al., 1980; Furness et al., 1982; Holzer et al., 1982), whereas rats are reported to be less sensitive (Helps and Reilly, 1985). Tachykinins other than SP have also been shown to be present in capsaicin-sensitive nerves of the guinea-pig (Hua et al., 1985).

Coexistence of SP with CGRP occurs extensively in the guinea-pig cardiovascular system (Gibbins et al., 1985), but in rat sensory neurones this is less common, with half of the CGRP-immunoreactive neurones also containing SP (Wharton et al., 1986a,b). It has also been suggested that ATP may coexist with SP (Burnstock, 1977) and this has been substantiated by later proposals of a sensory role for ATP (Jahr and Jessel, 1983; Salt and Hill, 1983; Fyffe and Pearl, 1984). SP-CGRP interactions have been described biochemically with the demonstration that CGRP inhibits the degradation of SP (Le Greves et al., 1985; Nyberg et al., 1988) (Fig. 4), and <u>in vivo</u>, where it has been shown that SP may modulate the effect of CGRP through the release of protease from mast cells (Brain and Williams, 1988).

Substance P in Intrinsic Neurones

SP is present in intrinsic neurones of the gut (Furness et al., 1980), where it mediates non-adrenergic, non-cholinergic excitatory transmission to both circular and longitudinal muscle (Costa et al., 1985). Capsaicin-insensitive perivascular SP-LI fibres, which are likely to arise from these enteric neurones, have been found supplying arterioles of the distal colon and rectum of rats (Holzer et al., 1980; Cuello et al., 1981). The number of SP-IR perivascular nerves originating from enteric neurones has been shown to increase in response to extrinsic denervation of the gut (Galligan et al., 1988), although the majority of SP-IR nerve fibres in rat salivary glands have been shown to disappear after sectioning of the auriculotemporal nerve (Ekstrom et al., 1988). A small number of SP-IR neurones have been found in intrinsic neurones of the guinea-pig heart (Baluk and Gabella, 1989) and in solitary neuronal cell bodies along the ciliary nerves in the guinea-pig (Gibbins and Morris, 1987).

Actions of Substance P

Specific receptor sites for SP on the smooth muscle cell membrane have been identified by autoradiography (Stephenson et al., 1986; Stephenson and Summers, 1987) and characterized pharmacologically (Regoli et al., 1987). SP receptors have also been localized on the endothelial cell surface (Stephenson and Summers, 1987). On the basis of their affinity for SP, NKA and NKB, the tachykinin receptors have now been identified and classified as NK-1, NK-2 and NK-3 respectively (Regoli et al., 1987). NK-1 receptors

have been shown to occur on endothelial cells of the guinea-pig basilar artery (Edvinsson and Jansen, 1987), dog carotic artery (Regoli et al., 1987) and rabbit pulmonary artery (D'Orleans-Juste et al., 1985). Relaxations mediated by these receptors may be coupled to the formation of cyclic GMP, as suggested in the guinea-pig mesenteric artery, where the SP-induced relaxation was abolished by haemoglobin (Bolton and Clapp, 1986). In contrast, NK-3 receptors appear to be located on smooth muscle cells and cause vasoconstriction, at least in the portal vein of the rat (Mastrangelo et al., 1986).

Since its first description, SP has been considered to be a potent vasodilator both in vivo and in vitro (Pernow and Rosell, 1975; Edvinsson et al., 1981) (Table VII) and more recently, this effect has been shown, in arteries, to usually require the presence of an intact endothelium (Furchgott, 1984a,b; D'Orleans-Juste et al., 1985; Edvinsson et al., 1985b). However, vasoconstriction (Barja et al., 1983; Lee and Saito, 1984; Regoli et al., 1984a,b) or no effects (Barja et al., 1983) have also been described. Moreover, there is a lack of correlation between the density of innervation and vascular reactivity - the rat carotid artery, where SP innervation is very sparse, exhibits a dose-dependent relaxation to SP, whereas the well-innervated superior mesenteric artery does not (Barja et al., 1983). Veins are also relaxed by SP application, but this effect is endothelium independent (Regoli et al., 1984a,b). SP has also been described to have an indirect action on vascular tone, as a modulator of the effects of NE in rat mesenteric arteries (Gulati et al., 1983).

As a putative sensory neurotransmitter, SP is believed to be responsible for mediating nociceptive and neurogenic vasodilatory responses and plasma extravasation. The route by which this occurs, that of antidromic vasodilatation, has been known since 1876, when Stricker showed that stimulation of the peripheral parts of the dorsal roots, or of the distal end of cut sensory nerves, cause arteriolar vasodilatation. Intradermal injection of SP in man induces flare, wheal and itching. These effects on the vasculature are thought to be partially mediated by the release of histamine from mast cells (Johnson and Erdos, 1973), however, evidence for specific receptor sites for SP on the arterial wall suggest that direct action may also be involved. Furthermore, a greater potency of NKB over SP and NKA in inducing plasma extravasation in human skin (Fuller et al., 1987), lends support to the hypothesis of an involvement of NK-3 receptors in the neurogenic plasma extravasation caused by tachykinins.

The peripheral effects of SP (vasodilatation, plasma extravasation and release of histamine from mast cells) are in accordance with a role for the peptide in the control of vascular tone in response to sensory stimulation. In addition, SP-IR nerves in the trigeminovascular system have been claimed to have a role in headache (Uddman et al., 1985). Trophic roles have also been attributed to SP, in relation to a mitogenic effect on epithelial cells in culture (Hanley, 1985), and a decrease of SP has been shown to occur during the closure of a cutaneous wound (Senapati et al., 1986).

3.4 Calcitonin gene-related peptide

General Distribution of Calcitonin Gene-Related Peptide

The existence of this peptide was predicted on the basis of the alternative processing of the calcitonin gene (Amara et al., 1982; Rosenfeld et al., 1983). In the nervous system, the main product of the calcitonin gene is the 37 amino acid peptide CGRP, which has been identified, in the rat, in molecular forms, alpha and beta, differing by only a single amino acid (Amara et al., 1985; Steenberg et al., 1985). The two molecular forms of CGRP have been shown to preferentially occur in particular populations of neurones, alpha-CGRP in sensory, and beta-CGRP in enteric neurones, exhibiting differential sensitivites to capsaicin treatment (Mulderry et al., 1988).

Biochemical and immunohistochemical studies have demonstrated the wide distribution of CGRP in perivascular nerves (Hanko et al., 1985; Mulderry et al., 1985; Sasaki et al., 1986; Uddman et al., 1986; Wanaka et al., 1986) (Table VIII). Species differences in the distribution of CGRP-LI perivascular nerves in the vasculature have been described, with guinea pigs having the richest innervation (Gibson et al., 1984; Mulderry et al., 1985; Uddman et al., 1986; Franco-Cereceda et al., 1987).

Calcitonin Gene-Related Peptide in Sensory Neurones

The distribution of CGRP-IR neurones closely resembles that of SP-IR neurones, as does the sensitivity of these neurones to capsaicin treatment (Gibson et al., 1984; Gibbins et al., 1985; Lee

et al., 1985b; Lundberg et al., 1985b; Terenghi et al., 1985; Uddman et al., 1986; Wanaka et al., 1986). Recently, the colocalization of SP- and CGRP-IR has also been demonstrated at the ultrastructural level, the two peptides being shown to coexist in the same large vesicles in various tissues (Gulbenkian et al., 1986; Wharton and Gulbenkian, 1987). While in the guinea pig capsaicin treatment leads to the disappearance of 90% or more of the CGRP-IR in perivascular nerves (Gibbins et al., 1985; Lundberg et al., 1985b; Terenghi et al., 1986; Wharton et al., 1986a, b), CGRP-IR nerve fibers in the rat show varying degrees of sensitivity to capsaicin treatment (Mulderry et al., 1985, 1988; Wharton et al., 1986a). In the rat trigeminal ganglia, two different populations of CGRP-IR neurones have been identified, one consisting of small to medium-sized neurones that also contain SP and are sensitive to neonatal capsaicin treatment, and the other consisting of larger neurones, which do not exhibit SP-IR and are insensitive to capsaicin (Matsuyama et al., 1986).

Calcitonin Gene-Related Peptide in Intrinsic Neurones

CGRP-IR has also been found in intrinsic neurones of the gut (Clague et al., 1985; Gibbins et al., 1985), the heart (Gerstheimer and Metz, 1986) and the bladder (Moss, 1988). At least in the gut, CGRP-IR cell bodies have been shown to be insensitive to capsaicin treatment, to lack SP-IR and to express mainly the beta form of the peptide (Mulderry et al., 1988). Their involvement in the innervation of local blood vessels is debated, but seems unlikely, since after extrinsic denervation, intestinal blood vessels receive only SP- and VIP-IR nerve fibers from the submucosal and myenteric

neurones (Galligan et al., 1988). A selective damage of intrinsic CGRP-IR enteric nerve fibers has been demonstrated in streptozotocin-induced diabetic rats (Belai and Burnstock, 1987).

Calcitonin Gene-Related Peptide in Parasympathetic and Motor Neurones

Parasympathetic cholinergic neurones located in the pontine and sacral parasympathetic nuclei and the ventral horn (as well as in the trigeminal motor nucleus) contain CGRP-IR (Takami et al., 1985a; Batten et al., 1988; Semba and Toyama, 1988). CGRP is released with ACh at the level of the motor endplates (Takami et al., 1985b), where its interaction with specific receptors has been shown to be responsible for an increase in the number of postsynaptic ACh receptors in cultured chicken myotubes (Fontaine et al., 1986; New and Mudge, 1986) (Fig. 5).

Actions of Calcitonin Gene-Related Peptide

CGRP is a potent vasodilator both in vivo and in vitro (Brain et al., 1985; Hanko et al., 1985; McCulloch et al., 1986; McEwan et al., 1986; Uddman et al., 1986) (see Table IX). In all the vessels that have been studied, including the coronary, cerebral, gastroepiploic and mesenteric arteries, CGRP causes a dose-dependent relaxation of precontracted arteries, which is not influenced by propanolol, cimetidine or atropine (Hanko et al., 1985; Uddman et al, 1986), and which is likely to mediated by its interaction with specific receptors. CGRP is a potent dilator of the uterine artery (Brizzolara, personal communication) and recently, vascular receptor sites have been identified with an autoradiographic technique in the

uterine vasculature of the guinea pig (Power et al., 1988). In a study of forearm blood flow, CGRP was unable to change the venous tone, in contrast to SP, but was more effective than SP in inducing arterial vasodilatation (Benjamin et al., 1987). In rat tail arterial strips, CGRP was found to inhibit NE-induced contraction by a mechanism involving the mobilization of intracellular calcium (Kline and Pang, 1987).

Although in many vessels the vasodilatation caused by CGRP is not dependent on an intact endothelium (Hanko et al., 1985), this is not the case in rat aortic rings where CGRP has been shown to cause relaxations that are entirely endothelium dependent (Brain et al., 1985; Kubota et al., 1985). Unlike the endothelium-mediated relaxations to ACh, which induce the production of cyclic GMP, CGRP-endothelium-mediated responses cause an increase in cyclic AMP and prostacyclin (Kubota et al., 1985; Crossman et al., 1987), suggesting different underlying mechanisms for these two endothelium-mediated responses.

On human skin, intradermal injection of CGRP causes a long-lasting erithema that has been attributed to a direct vasodilator effect (Fuller et al., 1987; Wallengren and Hakanson, 1987), and wheals, the formation of which can be prevented by H-1 antagonists, suggesting the involvement of mast cells (Piotrowski and Foreman, 1986). When SP is injected with CGRP into human skin, it is able to convert the CGRP-mediated long-lasting vasodilatation into a transient response by a mechanism which is dependent on the action of proteases released from mast cells by SP (Brain and Williams, 1988). In contrast, CGRP has been shown to potentiate SP transmission in the

spinal cord by two unique mechanisms: by inhibiting a specific SP-endopeptidase (Le Greves et al., 1985), and by increasing the release of SP from capsaicin-superfused slices of dorsal spinal cord in vitro (Oku et al., 1987).

The increase in CGRP content and CGRP-IR in the nerves of the trigeminovascular system 6 weeks after bilateral removal of the superior cervical ganglion in the rat, lends support to the hypothesis that a differential expression of CGRP in perivascular nerves after sympathetic denervation is implicated in pain transmission in human sympathalgia (Schon et al., 1985b). However, the recent demonstration of an increase in CGRP-IR in perivascular nerves in response to chronic chemical sympathectomy (Lamano-Carvalho et al., 1986; Aberdeen et al., 1988; 1990) indicates that the increase in the expression of CGRP occurring under these conditions may be a general property of the ANS.

3.5 Other peptides

Many other peptides, in addition to those described above, have been attributed with a vasomotor role on the basis of pharmacological and immunohistochemical evidence (Table X).

Somatostatin

SOM was originally isolated from the hypothalamus in 1968 (Krulich et al., 1968) as a potent growth hormone-inhibiting factor, and it is now known that it also inhibits the release of thyroid-

stimulating hormone (Guillemin, 1976). Outside the brain, endocrine glands and gut, SOM-IR has also been detected in the heart (Day et al., 1985) and kidney of many species (Forssman et al., 1982), in neurones of sensory and sympathetic ganglia (Hökfelt et al., 1975b; Luft et al., 1978) and in perivascular nerves in the brain (Duckles and Buck, 1982).

SOM causees vasoconstriction in almost all of the vascular beds that have been studied, as well as in pial and extracerebral arteries (Hanko et al., 1981, 1982), the mesenteric artery and vein (Törnebrandt et al., 1987), and the portal vein (Jaspan et al., 1979). Another known action of SOM is the inhibition of SP release from sensory nerve endings (Gazelius et al., 1981).

Gastrin-Releasing Peptide

Gastrin-releasing peptide, which shares many homologies with bombesin, a peptide present in invertebrates, exhibits vasomotor actions in many species (Erspamer et al., 1972). Gastrin-releasing peptide and another gut hormone, CCK, are present in cerebrovascular nerves (Uddman et al., 1983; McCulloch and Kelly, 1984), although there is a lack of evidence for vasomotor effect on the cerebral vasculature (Uddman et al., 1983, McCulloch and Kelly, 1984). In the cerebral cortex of the rat and monkey, CCK-IR neurones have been shown to be in intimate contact with intraparenchymal blood vessels (Hendry et al., 1983). Capsaicin treatment of the guinea pig and trigeminal ganglionectomy of the cat both caused a complete disappearance of CCK-IR in cerebrovascular nerves (Liu-Chen et al., 1985). However, evidence for CCK-IR neurones in the trigeminal

ganglion is controversial (Lee et al., 1985a), particularly since some CCK antisera have been shown to cross-react with CGRP (Ju et al., 1987).

Neurotensin

Neurotensin, a tridecapeptide isolated from bovine hypothalamus (Carraway and Leeman, 1973), is present in perivascular nerve fibers in the heart (Weihe et al., 1984; Reinecke et al., 1982b), brain (Chan-Palay, 1977) and kidney (Forssman et al., 1982). The actions of neurotensin are varied; it has been shown to cause vasoconstriction in a number of vessels (Quirion et al., 1979),but has no effect in rat cerebral vessels (Hanko et al., 1981).

Opioid Peptides

Opioid peptides have a wide distribution outside the brain. Of these, enkephalins (ENK) are present in sympathetic neurones, where they coexist with NE (Wilson et al., 1981). Consequently, treatment with 6-hydroxydopamine in the guinea pig has been shown to reduce, by about 70%, the cardiac ENK content (Lang et al., 1983). Nerve fibers displaying immunoreactivity for both Met- and Leu-ENK are present in perivascular nerves of the brain (Kapadia and De Lanerolle, 1984), and of the gut (Schultzberg et al., 1980). In feline cerebral arteries, ENK induces a naloxone-blocked vasodilatation (Hanko et al., 1982), whereas no effect of either Met- or Leu-ENK could be demonstrated on human mesenteric blood vessels (Törnebrandt et al., 1987). A decrease in perfusion pressure following Leu- or Met-ENK application has been observed in the cat hindlimb preparation (Moore

and Dowling, 1982).

The presence of prejunctional opioid receptors on noradrenergic nerves has been demonstrated in the ear artery (Illes et al., 1983) and in the splenic artery (Gaddis and Dixon, 1982). As already described for SOM, opiates may also inhibit the release of SP from peripheral sensory nerve endings (Lembeck et al., 1982) and in the rat trigeminal nucleus (Jessel and Iversen, 1977).

Dynorphin

DYN, a peptide contained in the proenkephalin A sequence, has been demonstrated in perivascular nerves of the guinea pig uterine artery and iris (Morris et al., 1985; Gibbins and Morris, 1987) as well as in pial vessels (Moskowitz et al., 1987). A DYN analogue (dynorphin 1-13) has been shown to reduce SP release from trigeminal nerve endings in the eye (Ueda et al., 1985).

Vasopressin

Vasopressin-IR nerves have been shown to be present in the vasculature of mammals (Hanley et al., 1984; Jojart et al., 1984). Vasopressin exerts a potent vasoconstriction in all of the vessels in which it has been tested (Nakano, 1973; Altura and Altura, 1977), probably through a V-1 receptor site situated on smooth muscle cells (Penit et al., 1983).

Galanin

Galanin-IR has been detected in CGRP-containing neurones of the dorsal root ganglia (DRG) (Ju et al., 1987) and in perivascular

nerves of the gut (Ekblad et al., 1985; Melander et al., 1985; Fehér and Burnstock, 1988) and airways (Cheung et al., 1985), but its action (if any) on the vasculature is unknown.

4. PEPTIDES AND ENDOTHELIAL CELLS

4.1 Endothelium-mediated vasodilation

In recent years it has become apparent that many of the agents which relax blood vessels do so by endothelium-dependent mechanisms. This was first demonstrated in 1980, when Furchgott and Zawadzki reported that the endothelium was requisite for relaxation induced by ACh in the rabbit aorta. Since then, endothelium-dependent vasodilatation has also been shown to occur in response to: ATP, adenosine 5'-diphosphate (ADP), arachidonic acid, SP, NKA, bradykinin, histamine, neurotensin, vasopressin, renin-angiotensin, thrombin (Zawadzki et al., 1981; Cherry et al., 1982; Furchgott, 1984; Vanhoutte and Rimele, 1983; Vanhoutte and Miller, 1985; Edvinsson and Jansen, 1987; Förstermann and Dudel, 1988), VIP in the bovine pulmonary artery (Ignarro et al., 1987), VIP and PHI in isolated human splenic and transverse cervical arteries (Hughes et al., 1986), CGRP in rat aortic strips (Kubota et al., 1985; Grace et al., 1987) and NA in the canine coronary, pulmonary and femoral arteries (Cocks and Angus, 1983; Matsuda et al., 1985; Miller and Vanhoutte, 1985).

It was suggested that the activation of specific receptors on the endothelial cell surface causes the release of an EDRF(s), which

acts on the smooth muscle to cause relaxation. Autoradiographical analysis has demonstrated cell surface receptors for SP (Stephenson and Summers, 1987), but somewhat surprisingly, not for ACh. While most endothelium-dependent agents act via EDRF, some, for example bradykinin in the cat and rabbit arteries (Förstermann et al., 1986) and in human mesenteric arteries (Cherry et al., 1982), induce relaxations that are blocked by indomethacin, suggesting that in these cases prostacyclin, rather than EDRF, mediates the response.

Investigations into the nature and mechanism of action of EDRF have not been facilitated by either its short half life (6-45 sec, see Lüscher, 1988) or by the marked heterogeneity displayed in endothelium-dependent responses between vessels, amongst species, and between arteries and veins (Vanhoutte and Miller, 1985; Rubanyi and Vanhoutte, 1988). In general, the venous system tends to be less sensitive to endothelium-dependent relaxing agents (Furchgott and Zawadzki, 1980; De Mey and Vanhoutte, 1981) and this has been proposed to be due to inherent differences in the venous endothelium, rather than in the ability of smooth muscle to respond to EDRF (Rubanyi and Vanhoutte, 1988).

Most workers regard nitric oxide (NO) as the most likely contender for EDRF (Ignarro et al., 1986; Moncada et al., 1987; Palmer et al., 1987) and it has been proposed that L-arginine is its physiological precusor (Rees et al., 1989). Consistent with NO as an EDRF is the demonstration of bradykinin- and ACh-evoked release of NO from the guinea pig and rabbit heart in quantities sufficient to account for at least part of the resulting vasodilatation (Amezcua et al., 1988; Kelm and Schrader, 1988). However, other substances which

might also be involved as EDRFs include: (a) metabolities of arachidonic acid (Furchgott, 1984); (b) an oxygen-derived free radical (Furchgott, 1984); (c) some other yet unknown substance. The production and/or the release of EDRF(s) appears to be dependent on an increase in intracellular Ca^{++} in endothelial cells (Singer and Peach, 1982; Long and Stone, 1985; Winquist et al., 1985; Collins et al., 1986). Accordingly, an increase in extracellular Mg^{++} has been shown to inhibit the release of EDRF in canine coronary arteries (Ann and Ku, 1986). Oxygen also appears to be important in the production and/or the release of EDRF, since anoxia prevents the relaxations induced by ACh, but not those evoked by endothelium-independent vasodilators (De Mey and Vanhoutte, 1983; Furchgott, 1984).

The relaxation of smooth muscle cells caused by EDRF is usually coupled with the production of cyclic GMP in a time- and concentration- dependent manner (Holzmann, 1982; Rapoport and Murad, 1983; Furchgott et al., 1984) and is antagonized by the inhibitors of guanylate cyclase, hemoglobin and methylene blue (Griffith et al., 1985; Martin et al., 1985; Ignarro et al., 1988). Cyclic GMP in turn is suggested to elicit relaxation through phosphorylation and dephosphorylation of myosin light chains. On the other hand, $Na^{+}K^{+}$ATPase has also been proposed as a mediator of the smooth muscle relaxation. In contrast, VIP and CGRP endothelium-mediated relaxation occurs concomitantly with an increase in cyclic AMP (Kubota et al., 1985; Ignarro et al., 1987).

In addition to the endothelium-dependent relaxations of peptides discussed in the earlier sections, a number of other peptides also exert their effects through the vascular endothelium.

Bradykinin, which belongs to the kallikrein-kinin family, exerts its effects on vascular tone through two specific types of receptors, B-1 and B-2, which were suggested to be located on the smooth muscle cells and endothelial cells respectively (Couture et al., 1980; Regoli and Barabè, 1980; Gandreau et al., 1981). B-2 receptors are the most widely distributed of the two and can mediate relaxation, and sometimes contraction, as well as an increase in vascular permeability (Marceau et al., 1981; Regoli, 1984). The endothelium-dependent relaxation evoked through B-2 kinin receptors caused an accumulation of cyclic GMP in smooth muscle cells (Toda, 1977; Wahl et al., 1983; Furchgott et al., 1984; Whalley et al., 1984, 1987; Hardebo et al., 1985; Katusic et al., 1986b; Ignarro et al., 1987). Recently, the endothelial location for B-2 receptors mediating vascular responses has been cast in doubt, since intraluminally infused bradykinin was unable to exert any vasomotor action, although it did lead to an increase in vascular permeability (Untaberg et al., 1984).

In the rabbit basilar artery, bradykinin causes vasoconstriction, acting on muscular B-1 receptor sites which are synthesized "de novo" under continued exposure (10 hr) to bradykinin (Whalley et al., 1983). Such a mechanism has also been described in other systems (Regoli et al., 1978, 1981; Regoli and Barabe, 1980).

4.2 Endothelium-mediated vasoconstriction

The endothelium has been proposed to mediate vasoconstriction via production of an endothelium-derived vasoconstrictor factor(s) [EDCF(s)] in response to various chemical and physical stimuli such

as NE, thrombin, high extracellular potassium, hypoxia and stretch (De Mey and Vanhoutte, 1983; Rubanyi and Vanhoutte, 1985; Katusic et al., 1986a, 1987a, b). In response to stretch, endothelial Ca^{++} channels have been described and suggested to operate as mechanotransducers (Lansman et al., 1987). Thus, the role of endothelial cells is two-fold, sensory and effector, such that vasoconstriction may occur independently of the action of extraneous vasoactive substances.

Although the nature of EDCF(s) is still uncertain, and appears to be different in blood vessels of different anatomical origin, at least three different classes of endothelial vasoconstrictor substances have been recognized: (a) metabolities of arachidonic acid; (b) a polypeptide-like factor (or factors) produced by cultured endothelial cells; (c) a still unidentified diffusable factor(s) released from anoxic/hypoxic endothelial cells (see Lüscher, 1988 for review). That a polypeptide, produced by the endothelium in response to the above stimuli could represent the EDCF, was demonstrated using cultured bovine aortic endothelial cells. These were shown to release a potent vasoconstrictor substance which was unaffected by inhibitors of receptors for known vasoactive substance and by inhibitors of prostaglandin synthesis, but the effect of which was abolished by several treatments (sodium dodecyl sulfate, trypsin, alkali or acid hydrolysis) known to affect proteins (O'Brien and McMurthy, 1984; Hickey et al., 1985; Gillespie et al., 1986).

Recently, an endothelium-derived 21-residue vasoconstrictor peptide, endothelin, has been isolated from porcine aortic endothelial cells, and the complementary DNA of its precursor, pre-

pro-endothelin, has been cloned and sequenced (Yanagisawa et al., 1988). It has been shown to be a potent constrictor in, for example, the rabbit skin microvasculature (Brain et al., 1988), in isolated human resistance vessels (Hughes et al., 1988) and in rat mesenteric resistance vessels (Warner, 1988). Recent studies have shown that endothelin is expressed in neurones of the human spinal cord and dorsal root ganglia (Giaid et al., 1989). Receptors for endothelin have been localized by autoradiography on cultured rat aortic smooth muscle cells (Hirata et al., 1988), rat kidney (Kohzuki et al., 1989), and human and porcine coronary arteries (Power et al., 1989). One of the initial events in its action appears to be to induce phospholipase C-stimulated phosphatidylinositol 4, 5-bisphosphate hydrolysis at the plasma membrane (Marsden et al., 1989; Sugiura et al., 1989), and both endothelin and the polypeptide demonstrated in cultured bovine aortic endothelial cells appear to exert vasoconstriction by a mechanism dependent on the presence of extracellular calcium (Hickey et al., 1985; Yanagisawa et al., 1988). Interestingly, a high degree of sequence homology has been demonstrated between endothelin and the snake venom vasoconstrictor peptides, the safratoxins, and binding studies have suggesting that these share common sites and mechanisms of action (Ambar et al., 1989).

4.3 Source of peptides acting on endothelial cells

Some of the substances acting to cause release of EDRF are present in the circulation (hormones, catecholamines, renin-

angiotensin, vasopressin), while others may be locally produced, mainly from aggregating platelets (thrombin, arachidonic acid, ATP, ADP, 5-HT). The source of other vasoactive substances (ACh, SP, NKA, VIP, CGRP), however, for which endothelium-dependent relaxations have been demonstrated, remains unclear. Some of these may also be present in the circulation, but the fact that they are rapidly broken down by their degrading enzymes makes it physiologically unlikely that a sufficiently high concentration can be achieved to cause endothelial activation. Since periarterial nerves are confined to the adventitial-medial border of blood vessels, it is also unlikely that their transmitters traverse the vascular wall (including media and elastic lamina) to reach specific receptors on the endothelial cell surface. Adventitial application of SP has been shown to be some 50-100 times less potent in relaxing the canine femoral artery than when applied luminally (Angus et al., 1983). ACh and ATP in particular would be subject to degradation by AChE and 5'-nucleotidase, respectively, located at the adventitial-medial border.

A local source for vasoactive agents from within endothelial cells themselves was first suggested by the electron microscopic immunocytochemical localization of ChAT (the ACh synthesizing enzyme) in vascular endothelial cells in the rat brain (Parnavelas et al., 1985). A neuronal origin of vasoactive Ach in pial vessels has, however, been alternatively suggested (Hamel et al., 1987). Other electron microscopic studies have shown that endothelial cells may additionally be a source of SP and 5-HT in the rat femoral and mesenteric arteries (Loesch and Burnstock, 1988) and of 5-HT in the rat coronary artery (Burnstock et al., 1988), and SP and ChAT in the

rat coronary artery (Milner et al, 1989).

Release studies also suggest that the endothelium is a source of vasoactive substances: ATP has been shown to be released during the period of hypoxic vasodilatation in the guinea pig heart (Paddle and Burnstock, 1974), and in the rat heart, hypoxic insult causes the release of 5-HT (Burnstock et al., 1988), as well as of SP and ACh (Milner et al., 1989). It has been suggested that this may represent a pathophysiological mechanism whereby ACh, ATP, 5-HT and SP may be released from endothelial cells during hypoxia, to act on specific endothelial cell receptors with a resulting protective vasodilatation via EDRF (Burnstock, 1987). Studies with cultured endothelial cells have shown these to be a source of ATP (Pearson and Gordon, 1984; Gordon, 1986) and current studies strongly suggest that ATP, SP and ACh may be released from columns of endothelial cells in culture (Milner, personal communication).

Endothelial cells appear to be very sensitive to various mechanical and chemical stimuli such as changes in flow, stretch and hypoxia, as well as to various substances. In the canine femoral artery, flow causes dilatation and induces the release of EDRF (Hull et al., 1986; Rubanyi et al., 1986), which is blocked by the EDRF inhibitors methylene blue and 5,8,11,14-eicosatetraynoic acid (Kaiser et al., 1986). Flow-dependent, endothelium-mediated dilatation, that is unaffected by cyclooxygenase inhibition, has been demonstrated in canine epicardial coronary arteries (Holtz et al., 1984). Flow-induced dilatation has also been shown to occur at the microvascular level in rat mesenteric arterioles (Smiésko et al., 1987) and in resistance vessels from the rabbit cerebral and ear, and the cat

lingual circulations (Bevan and Joyce 1988). Endothelium-derived vasoactive substances may also be implicated in flow-induced regulation of vascular tone, in a manner similar to that already proposed to occur in response to hypoxia (Burnstock et al., 1988; Milner et al., 1989). Figure 6 illustrates some of the mechanisms discussed in this section.

5. PLASTICITY OF PERIVASCULAR NEUROPEPTIDES

The ability to change the expression of a neurotransmitter (e.g. from NA to ACh in rat sweat glands (Landis and Keefe, 1983) is a well established feature of the ANS. This has been shown to occur during development and in culture (Landis, 1980), and also after transplantation of undifferentiated neural crest cells to a different region of the neurotaxis (Le Douarin and Teillet, 1974; Le Douarin et al., 1975; Le Douarin, 1981). This property has also been shown to belong to the mature neurones of the autonomic and sensory ganglia, which can not only change the expression of their neurotransmitters qualitatively and quantitatively, but can also spread to reinnervate denervated targets, which, in turn, can accept "foreign" nerves (Burnstock, 1981). The regulation of neuronal plasticity is under the control of several factors, some genetic and some environmental. NGF(s) is one of the most studied "signals" and it is known to initiate the intracellular transduction of messages activating gene expression (Raynaud et al., 1988).

For some of the neuropeptides, including VIP (Tsukada et al., 1985), SOM (Montminy et al., 1984) and proENK (Kley et al., 1987), and for the enzyme TH, which represents the rate-regulating step in the synthesis of NE (Harrington et al., 1987), synthesis has been shown to be controlled at the level of transcription by receptor-mediated processes that involve the intracellular production of cyclic AMP. Related regulatory 5' flanking regions, all cyclic AMP responsive and all containing the same sequence of 5'-nucleotides, have been found in the genes encoding for human proENK (Kley et al., 1987) and rat TH (Harrington et al., 1987). The differences in the surrounding nucleotides are probably responsible for the bidning of different cyclic AMP-regulated trans-acting factors (Comb et al., 1987).

5.1 During development and ageing

There are relatively few studies on the development of perivascular nerves and the majority of these deal with noradrenergic nerves (see Cowen and Burnstock, 1986, for review). The development of peptidergic perivascular nerves has been studied in rat cerebral vessels (Dhital et al., 1988) in guinea pig mesenteric, femoral, carotid and renal arteries (Dhall et al., 1986), and the rat mesenteric vascular bed (Scott and Woolgar, 1987). Few generalizations are possible: in guinea pig arteries (Dhall et al., 1986) and in the rat mesenteric vascular bed (Scott and Woolgar, 1987), neuropeptide-containing nerve fibers developed earlier than the NA-containing and the TH-IR perivascular nerves respectively. Moreover, the latter study showed that CGRP-IR nerve fibers developed

first, while the expression of SP-IR, presumable in the same nerve fibers, started much later (Scott and Woolgar, 1987).

In rat cerebral vessels, there is no overlap in the expression of coexisting neurotransmitters in perivascular nerves (Dhital et al., 1988). The levels of NPY-, VIP- and SP-IR in human cerebral vessels have been found to decrease from the age of 1 through to 46 years (Edvinsson et al., 1985a). Unfortunately, morphological changes in the arterial wall due to development and aging, which are manifested as an increase in the surface of the adventitial-medial border, a thickening of the muscular layer and a spreading out of the existing nerves (Cowen and Burnstock, 1986), often cause difficulties in the comparison of results obtained with different methods.

A pharmacological approach, as well as a study of the affinity of the binding sites for the neuropeptides is strongly warranted, to clarify the relevance of age-related changes in vascular innervation. In fact for NE, despite a general reduction in noradrenergic perivascular nerve density and NE levels in the periphery (see Cowen and Burnstock, 1986, for review), vascular adrenergic neuroeffector function does not decline with age (Duckles et al., 1985).

5.2 After trauma, surgery and selective denervation

There are few reports on the involvement of perivascular nerve fibers in local trauma on mixed peripheral nerves. In a study carried out on patients with spinal cord injury (Milner et al., 1987), an increase of NA-containing, NPY-, and VIP-IR nerve fibers

was found in the striated muscle of the intrinsic urethral sphincter. Much information on the plasticity of the ANS has come from the use of animal models, in which the ANS is confined to a particular system (i.e. sympathetic, parasympathetic or sensory), and from studies on the local nerve supply under well-defined experimental conditions.

Surgical removal of autonomic ganglia has been used as a tool for the demonstration of the origin of a certain population of nerve fibers (Edvinsson, 1982). Recently, the effects of ganglionectomy on the "surviving" nerve fibers has been evaluated at various time intervals after the operation. Thus, in the rat and rabbit iris, removal of the superior cervical ganglion (SCG), which is the source of noradrenergic nerves in the iris, led to an increase in sensory nerve fibers containing SP-IR, which originate from the trigeminal ganglia (Cole et al., 1983; Kessler et al., 1983). Conversely, sectioning of the ophthalmic branch of the trigeminal nerve caused an increase in TH and ChAT activity in the iris (Kessler, 1985a). These events are likely to be due to an increase in the production of NGF in the denervated iris, since the injectin of NGF into the anterior chamber mimics the effect of both sympathetic and sensory denervation. Moreover, anti-NGF antiserum, injected in the denervated iris (either by means of sympathectomy or sensory denervation), was able to prevent the increase of any nerve fibers (Kessler, 1985a).

The amount of NGF in the rat iris increases several fold after sympathetic and/or sensory denervation and elevated levels of NGF mRNA have been found in the denervated iris (Shelton and Reichardt, 1984). Studies utilizing colchicine to block fast axonal transport

and 6-hydroxydopamine to destroy the sympathetic nerve terminals (Korsching and Thoenen, 1985) demonstrated that NGF is produced in target organs, bound to specific receptors on nerve terminals, internalized and transported retrogradely to the cell body (Korsching and Thoenen, 1985), where it induces several changes, including the expression of neurotransmitters and their synthesizing enzymes (Thoenen and Barde, 1980). Thus, two different neuronal populations (sympathetic and sensory) utilize the same trophic factor produced by the same target organs. During fetal life, both sympathetic and sensory neurones need NGF for their survival, but while sensory nerves require NGF during early fetal life, sympathetic neurones need the trophic factor during the perinatal period (see Black, 1986, for review).

A similar effect, that of increased levels of CGRP, has been shown to occur in the trigeminovascular system 6 weeks after removal of the SCG (Schon et al., 1985b). Recently, Aberdeen et al., (1988; 1990) showed that an increased in levels and density of perivascular CGRP-IR occurred in several vascular beds and in the SCG of rats treated as neonated with guanethidine.

The rat iris has also been a useful model for studies in changes of expression. This has been used to demonstrate the appearance of NPY-and TH-IR in cholinergic nerves originated from the ciliary ganglia after long-term sympathectomy (Björklund et al., 1985). Changes in expression have also been described as: an increase in DBH-IR in non-noradrenergic axons supplying the guinea pig uterine artery after 6-hydroxydopamine treatment (Morris et al., 1987), a loss of expression of DBH-IR in guinea pig intracardiac

neurones when transferred in culture (Hassall and Burnstock, 1987), and the appearance of SP-IR in unmyelinated sensory afferents when cross-anastomized with the skin (McMahon and Gibson, 1987). The expression of NPY-IR in 70% of VIP-containing axons innervating guinea pig cerebral vessels is also altered after superior cervical ganglionectomy (Gibbins and Morris, 1988).

After sectioning or, to a lesser extent, after crushing the rat sciatic nerve, an increase of VIP-IR and VIP levels in the L-4 segment of the homolateral spinal cord was demonstrated (McGregor et al., 1984). These VIP-IR nerve fibers arose from sensory neurones in the corresponding dorsal root ganglia of the operated side, while VIP-IR neurones were completely absent in the ganglia of the unoperated side or in control animals (Shehab and Atkinson, 1986). A similar effect has been demonstrated in the rat trigeminal ganglia, where VIP-IR cell bodies appeared after section of the sensory root of the mandibular nerve (Atkinson and Shehab, 1986).

5.3 Plasticity in diseases

Although diseases such as diabetes, hypertension and atherosclerosis involve the cardiovascular system, the pattern of perivascular innervation during these diseases in largely unknown. Animal models, more or less similar to the human pathologies, have been created. Thus, in diabetic impotent males, a reduction of VIP-IR to the penile vessel has been demonstrated (Crowe et al., 1983). In streptozocin diabetic rats there is also a reduction of nerves containing VIP-IR (and 5-HT) but not NPY-IR (or NA) in cerebral blood vessels (Lagnado et al., 1987). The remarkable changes in the expression of

neuropeptides occurring in the nervous system of streptozocin diabetic rats (Belai et al., 1985, 1988), possibly involves intestinal vessels.

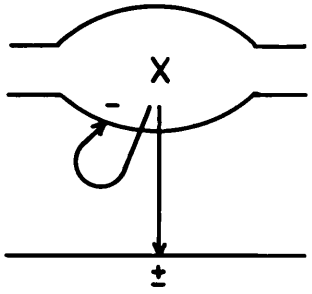
An involvement of NA-containing perivascular nerves in hypertension has been proposed, largely due to the demonstration of an increase in NA-containing nerves in several blood vessels (Abboud, 1982). In contrast, the few studies that have been carried out on neuropeptide-containing perivascular nerves in spontaneous hypertensive rats have shown a reduction of NPY levels in the kidney and in the renal artery (Ballesta et al., 1984), an increase of NPY-IR (and NA in cerebrovascular nerves (Dhital et al., 1988) and no changes in SP-IR nerves in the mesenteric vascular bed (Foote et al., 1986).

In the study of atherosclerotic vessels, interest has been centered on the development of morphological lesions and on the effects of drugs affecting the metabolism of lipids (Lüscher, 1988). The development of animal models for atherosclerosis (hypercholesterolemic fat-fed rabbits and Watanabe heritable hyperlipedemic rabbits), together with the demonstration that the trophic role exerted by sympathetic nerves can reduce lipid accumulation in the rabbit aorta (Fronek and Alexander, 1986), and the fact that endothelium-mediated processes are markedly attenuated in this disease (Jayakody et al., 1985; Harrison, 1988), has raised an interest in the roles exerted by vascular neuropeptides. In particular, this interest is focussed on those neuropeptides whose

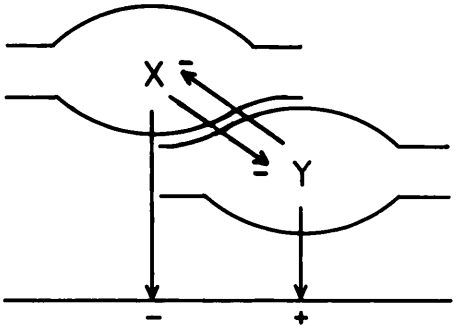
effects on vascular tone may be exerted through the endothelium.

Fig. 1 - Schematic representation of the different types of interactions that are features of cotransmission and neuromodulation. X and P represent two nominal vascular neurotransmitter substances. The different situations are described in detail in the text.

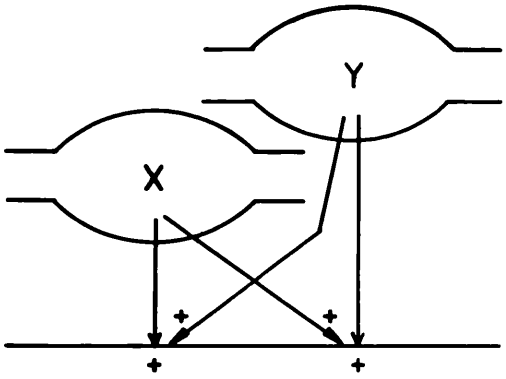
AUTOINHIBITION



CROSS-TALK

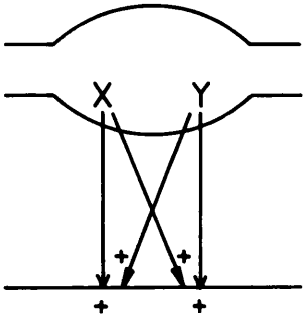


SYNERGISM



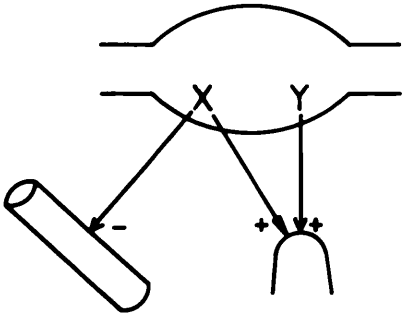
POSTJUNCTIONAL NEUROMODULATION

e.g. vas deferens



OPPOSITE ACTIONS

e.g. salivary gland



e.g. uterine artery

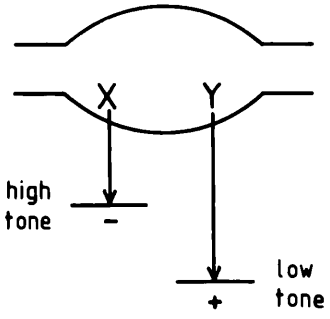


Fig. 2 - Top - Schematic representation of different interactions that occur between NPY and ATP and norepinephrine (NA) released from a single sympathetic nerve varicosity, in many blood vessels including the mesenteric artery. NA and ATP, probably released from small granular vesicles, act synergistically to contract the smooth muscle via $alpha_1$-adrenoceptors and P_2-purinoceptors, respectively. NPY, which is also released from the nerve, has little, if any, direct action on the muscle cell, but exerts potent neuromodulatory actions, both prejunctional inhibition (-) of the release of NA and postjunctional enhancement (+) of the action of NA.

Fig. 3 - Bottom - Schematic representation of transmission where VIP is a cotransmitter with ACh in parasympathetic nerves supplying the cat salivary gland. Note that ACh and VIP are stored in separate vesicles; they can be released differentially at different stimulation frequencies to act on acinar cells and glandular blood vessels. Cooperation is achieved by selective release of ACh at low impulse frequencies and of VIP at high frequencies. Pre- and postjunctional modulation is indicated.

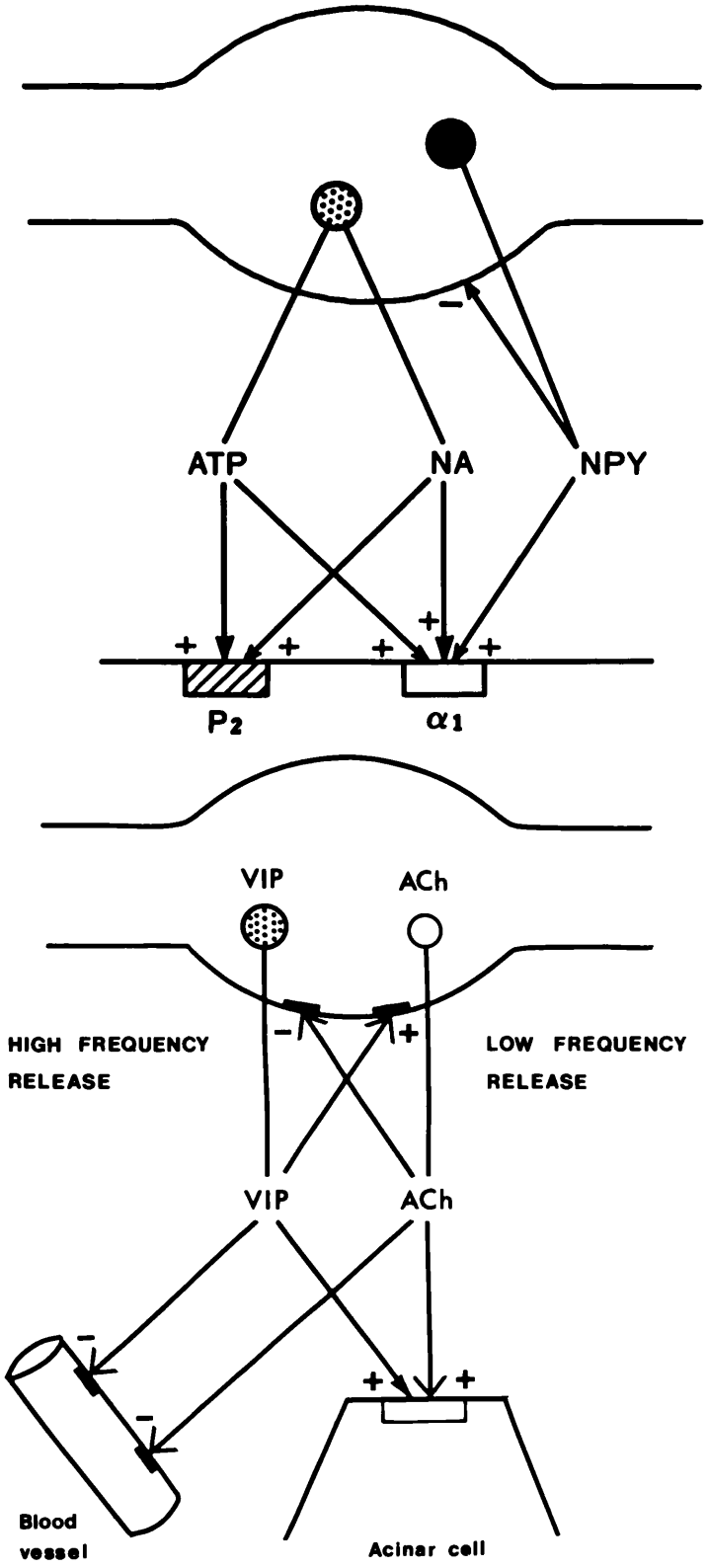
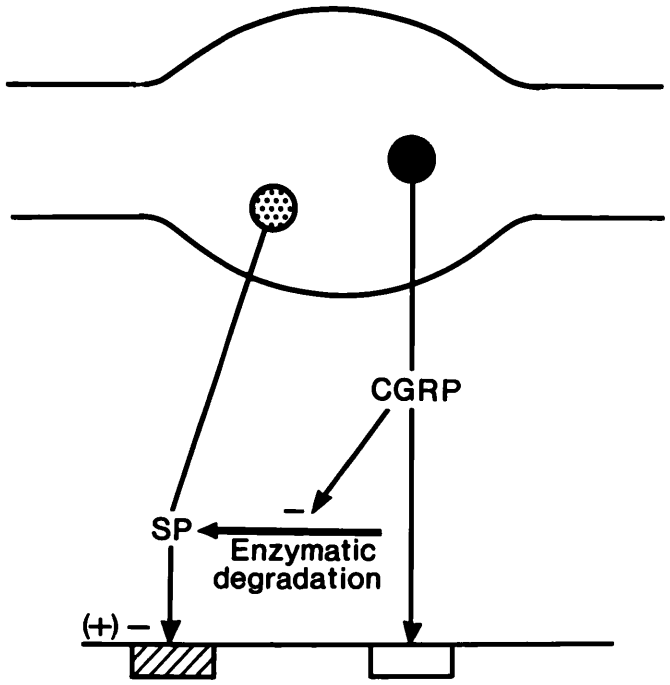

ATP
NA
NPY
P2
α1
VIP
ACh
HIGH FREQUENCY
RELEASE
LOW FREQUENCY
RELEASE
VIP
ACh
Blood
vessel
Acinar cell

Fig. 4 - Top - Schematic representation of neuromodulation in sensory nerves, whereby CGRP modulates the amount of SP in the neurovascular junction by reducing its breakdown by inhibition of SP endopeptidases.

Fig. 5 - Bottom - Schematic representation of trophic effects of CGRP as occurs in chicken myotubes, where the action of ACh is enhanced through an increased synthesis of its postjunctional receptors.


CGRP
–
SP
Enzymatic
degradation
(+) –

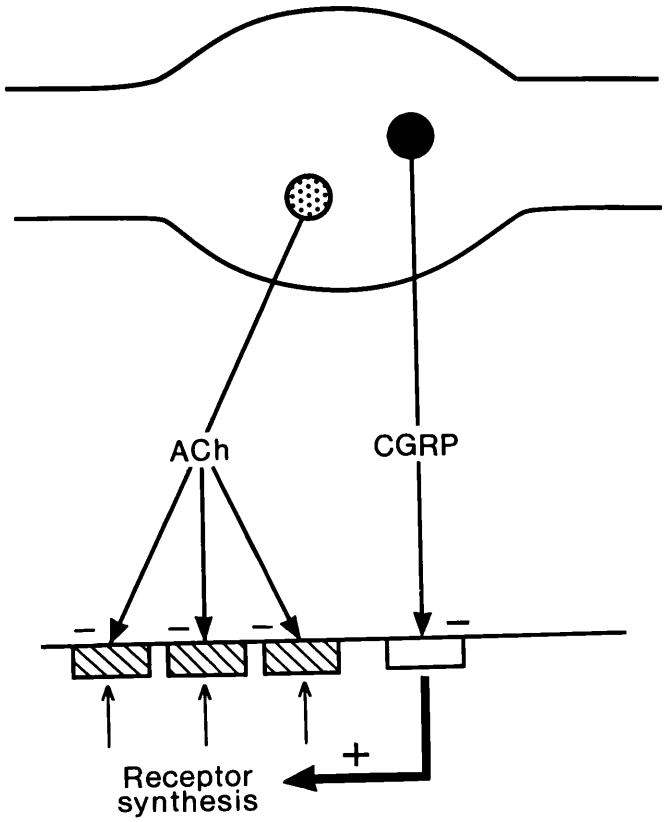

ACh
CGRP
– – – –
+
Receptor
synthesis

Fig. 6 - Schematic representation of potential modes of regulation of vascular tone by endothelial-related mechanisms. Noradrenaline (NA), ATP, CGRP, SP and VIP can be released from nerves in the adventitia (ADV) to act on their respective receptors in the media (MED) to cause vasoconstriction or vasodilation. ATP, ACh, 5-HT and SP released from endothelial cells (END) by shear stress or hypoxia act on their receptors on endothelial cells to cause release of EDRF or prostaglandins (PG), which in turn act on the smooth muscle to cause relaxation. In areas denuded of endothelial cells, opposite effects may be produced, by receptors on the smooth muscle. α = norepinephrine receptor, P_{2x} = P_{2x}-purinoceptor, P_{2y} = P_{2y}-purinoceptor, M = muscarinic receptor. Modified from Burnstock (1989).

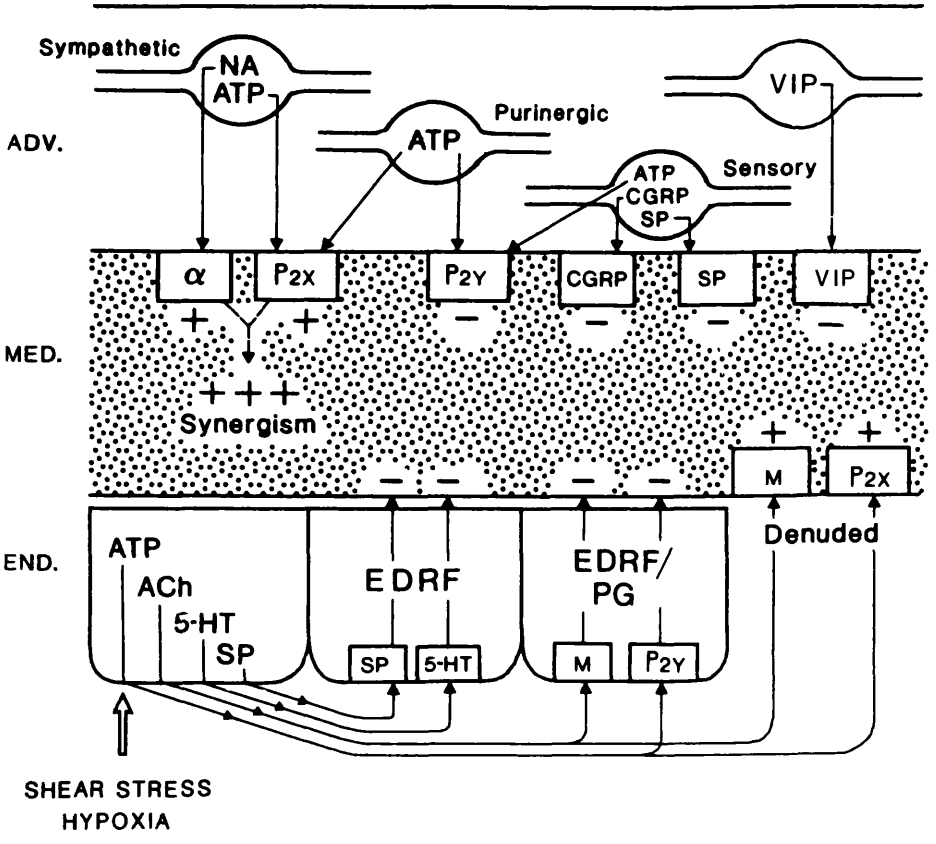

Sympathetic
NA
ATP
Purinergic
ATP
VIP
ADV.
ATP
CGRP
SP
Sensory
α
P2X
P2Y
CGRP
SP
VIP
MED.
+ + +
Synergism
M
P2X
Denuded
END.
ATP
ACh
5-HT
SP
EDRF
SP
5-HT
EDRF/
PG
M
P2Y
SHEAR STRESS
HYPOXIA

TABLE I. Localization of NPY immunoreactivity in perivascular nerves

Tissue	Species	References
Brain	Rat, man, pig, cat, gerbil, guinea pig	1,2,3
Heart and systemic arteries	Man, monkey, dog, cat, rat, guinea pig	4,5,6,7,8
Respiratory tract	Man, cat, guinea pig, rat	9,10
Gastrointestinal tract	Rat, cat, guinea pig, chicken	11,12.13
Liver and gallbladder	Rat, guinea pig	14
Spleen	Man, cat, rat, guinea pig	15,16
Kidney and urogenital tract	Rat,guinea pig,man,mouse	17,18,19,20
Skeletal muscle	Dog	9,21
Endocrine glands	Rat, guinea pig	22,23
Exocrine glands	Rat	24
Eye	Guinea pig, rat	26,27
Ear	Cat, guinea pig, rat	25

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

Modified from Dhital and Burnstock (1987).

TABLE II. Main effects of NPY on blood vessels

Effect	Vessel	Reference
Neuromodulation		
Inhibition	Cerebral arteries	1
	Femoral artery	2
	Basilar artery	3
	Mesenteric artery	4
	Portal vein	5
Potentiation	Ear artery	6
	Aorta	7
	Cerebral vessels	8
	Mesenteric artery	9
Constriction	Cerebral artery	10
	Uterine artery	11
	Coronary arteries	12
	Skeletal muscle arteries	13
	Intrarenal arteries	14

(1) Suzuki et al., 1988; (2) Lundberg et al., 1987; (3) Pernow et al., 1986; (4) Westfal et al., 1987; (5) Dahlof et al., 1985; (6) Lüscher and Vanhoutte, 1986; (7) Mabe et al., 1987; (8) Edvinsson et al., 1984; (9) Andriantsitohaina and Stoclet, 1988; (10) Edvinsson et al., 1983; (11) Morris et al., 1985; (12) Franco-Cereceda et al., 1985; (13)) Pernow, 1988; (14) Leys et al., 1987.

TABLE III. Localization of VIP immunoreactivity in perivascular nerve

Tissue	Species	References
Brain	Monkey, cow, pig, dog, cat,rabbit	1,2,3,4,5,6,7
Heart and systemic vessels	Cat, guinea pig, rat	8,9,10,11
Respiratory tract	Man, dog, cat, rat	12,13,14,15
Gastrointestinal tract	Man, monkey, pig, dog, cat, guinea pig, rat, mouse, chicken	8,16,17,18
Liver and gallbladder	Man, guinea pig, pig, rat, cat	19,20
Kidney and urogenital tract	Man, monkey, pig, dog, cat, rabbit, guinea pig, rat, mouse	21,22,23,24,24, 26,27,28,29,30
Skeletal muscle	Guinea pig, rat	8,31
Endocrine glands	Rat	32,33
Exocrine glands	Pig, cat, rat	34,35,36
Ear	Cat, guinea pig	37
Eye	Cat, guinea pig, rat	38

(1) Edvinsson et al., 1989; (2) Edvinsson et al., 1985a; (3) Kobayashi et al., 1983; (4) Gibbins et al., 1984; (5) Hara et al., 1985; (6) Lee et al., 1984; (7) Alafaci et al., 1986; (8) Dhall et al., 1986; (9) Weihe et al., 1987; (10) Weihe, 1987; (11) Gibbins et al., 1988; (12) Uddman and Sundler, 1979; (13) Uddman et al., 1980b; (14) Springall et al., 1988; (15) Geppetti et al., 1988; (16) Jessen et al., 1980; (17) Saffrey et al., 1982; (18) Schultzberg et al., 1980; (19) Sundler et al., 1977b; (20) Barja and Mathison, 1982; (21) Alm et al., 1980; (22) Crowe et al., 1983; (23) Diani et al., 1985; (24) Forssman et al., 1982; (25) Gu et al., 1983; (26) Larsson et al., 1977a; (27) Larsson et al., 1977b; (28) Papka et al., 1985; (29) Stjerquist et al., 1983; (30)Vaalasti et al., 1980; (31) Azanza and Garin, 1986; (32) Uddman et al., 1989a; (33) Grunditz et al., 1988; (34) Bloom et al., 1979; (35) Lundberg et al., 1980a; (36) Lundberg et al., 1980c; (37) Uddman et al., 1982; (38) Terenghi et al., 1982.

Modified from Dhital and Burnstock, 1987.

TABLE IV. Main effects of VIP on blood vessels

Effect	Vessel	Reference
Vasodilation		
Endothelium-independent	Cerebral arteries	1
	Pulmonary artery	2
	Vessels of salivary glands	3
	Mesenteric artery	4
	Skeletal muscle arteries	5
Endothelium-dependent	Pulmonary artery	6

(1) Lee et al., 1984; (2) Greenberg et al., 1987; (3) Lundberg, 1981; (4) Ganz et al., 1986; (5) Järult et al., 1984; (6) Ignarro et al., 1987.

TABLE V. Mammalian and nonmammalian tachykinins

Mammalian tachykinins	
Substance P	Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2
Neurokinin A*	His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH_2
Neurokinin B+	Asp-Met-His-Asp-Phe-Phe-Val-Gly-Leu-Met-NH_2
Nonmammalian tachykinins	
Eledoisin	<Glu-Pro-Ser-Lys-Asp-Ala-Phe-Ile-Gly-Leu-Met-NH_2
Physalaemin	<Glu-Ala-Asp-Pro-Asn-Lys-Phe-Tyr-Gly-Leu-Met-NH_2
Kassinin	Asp-Val-Pro-Lys-Ser-Asp-Gln-Phe-Val-Gly-Leu-Met-NH_2

* This peptide is identical to peptides that have been previously designated as substance K, neurokinin alpha or neuromedin L.

+ This peptide is identical to neuromedin K.

From Leeman (1987).

TABLE VI. Localization of SP immunoreactivity in perivascular nerves

Tissue	Species	References
Brain	Man, pig, cat, guinea pig, rat gerbil, monkey	1,2,3,4,5,6,7 8,9,10
Heart and systemic vessels	Man, monkey, dog, cat, rat guinea pig	11,12,13,14,15 16,17,18,19
Respiratory tract	Man, cat, guinea pig, rat	12,20,21,22,23,24
Gastrointestinal tract	Man, cat, rat, guinea pig chicken	12,25,26,27,28
Liver and gallbladder	Rat, guinea pig	29,30,31
Spleen	Rat, guinea pig	29,30
Kidney and urogenital tract	Man, pig, dog, cat, rat, rabbit, guinea pig, mouse	32,33,34,35
Skeletal muscle	Guinea pig, rat	12, 29, 30
Endocrine glands	Rat	36
Exocrine glands	Rat	37
Ear	Guinea pig	38
Eye	Monkey, cat, rabbit, guinea pig, rat	39, 40, 41

(1)) Edvinsson et al., 1981; (2) Edvinsson and Uddman, 1982; (3) Edvinsson et al., 1985a; (4) Furness et al., 1982; (5) Itakura et al., 1984; (6) Alafaci et al., 1986; (7) Liu-Chen et al., 1983; (8) Matsuyama et al., 1984; (9) Yamamoto et al., 1983; (10) Simons and Ruskell, 1988; (11) Helke et al., 1980; (12) Hökfelt et al., 1977; (13) Lundberg et al., 1985b; (14) Reinecke et al., 1980; (15) Reinecke et al., 1982; (16) Wharton et al., 1981; (17) Gibbins et al., 1988; (18) Dalsgard et al., 1986; (19) Weihe, 1987; (20) Baluk and Gabella, 1989; (21) Lundberg and Saria, 1981; (22) Lundberg et al., 1980c; (23) Sundler et al., 1977a; (24) Springall et al., 1988; (25) Jessen et al., 1980; (26) Polak and Bloom, 1980; (27) Saffrey et al., 1982; (28) Schultzberg et al., 1980; (29) Barja et al., 1983; (30) Galligan et al., 1988; (31) Goheler et al., 1988; (32) Forssman et al., 1982; (33) Stjernquist et al., 1983; (34) Sikri et al., 1981; (35) Papka et al., 1985; (36) Hedge et al., 1984; (37) Ekström et al., 1988; (39) Uddman et al., 1982; (40) Cole et al., 1983; (41) Stone et al., 1982.
Modified from Dhital and Burnstock, 1987.

TABLE VII. Main effects of SP on blood vessels

Effect	Vessel	Reference
Relaxation (endothelium-dependent)	Cerebral artery	1
	Pulmonary artery	2
	Carotid artery	3
	Mesenteric artery	4
	Coronary artery	5
	Femoral artery	6
Constriction	Cerebral artery	7
Neuromodulation	Mesenteric artery	8

(1) Edvinsson et al., 1981; (2) D'Orleans-Juste et al., 1985; (3) Barja et al., 1983; (4) Furchgott, 1983; (5) Beny et al., 1986; (6) Angus et al., 1983; (7) Lee et al., 1984; (8) Gulati et al., 1983.

TABLE VIII. Localization of CGRP immunoreactivity in perivascular nerves

Tissue	Species	References
Brain	Man, cat, rat, rabbit, guinea pig	1,2,3,4
Heart and systemic vessels	Guinea pig, rat, cat, dog rabbit	5,6,7,8,9, 10,11
Respiratory tract	Guinea pig, rat	6,8,12
Gastrointestinal tract	Guinea pig, rat	5,10,11
Liver and gallbladder	Guinea pig	5,10,11
Kidney and urogenital tract	Guinea pig	5,6,11
Skeletal muscle	Guinea pig	5,11
Exocrine glands	Rat	14
Eye	Guinea pig	15,16

(1) Hanko et al., 1985; (2) Lee et al., 1985a; (3) Matsuyama et al., 1986; (4) Wanaka et al., 1986; (5) Uddman et al., 1986; (6) Gibbins et al., 1985; (7) Gubelkian et al., 1986; (8) Lundberg et al., 1985b; (9) Weihe, 1987; (10) Mulderry et al., 1985; (11) Dhall et al., 1986; (12) Cadieux et al., 1986; (13) Goehler et al., 1988; (14) Ekström et al., 1988; (15) Terenghi et al., 1985; (16) Terenghi et al., 1986.

Modified from Dhital and Burnstock, 1987.

TABLE IX. Main effects of CGRP on blood vessels

Effect	Vessel	Reference
Relaxation		
Endothelium-dependent	Aorta	1
	Pulmonary artery	2
Endothelium-independent	Cerebral vessels	3
	Coronary artery	1
	Mesenteric artery	3

(1) Brain et al., 1985; (2) Ignarro et al., 1987; (3) Uddman et al., 1986.

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

Peptide	Vessel	Species	References
Neurotensin	Brain	Rat	1
	Kidney	Man, monkey, pig, dog, guinea pig, rat, cat	2
	Heart and associated vessels	Man, monkey, dog, frog, guinea pig, fish, lizard, chicken	3,4
Somatostatin	Kidney	Man, monkey, pig, dog cat, guinea pig	2
	Gastrointestinal tract	Cat, guinea pig	5
Gastrin/ cholecysto- kinin	Brain	Monkey, rat, cat	6,7
	Gastrointestinal tract	Cat, guinea pig	8,9
Enkephalin	Brain	Cat	7
	Gastrointestinal tract	Cat, guinea pig, rat	8,9
Gastrin-releasing peptide	Brain and spinal cord	Cat,guinea pig,rat,mouse	10
	Urogenital tract	Rabbit, guinea pig	11
	Ear	Guinea pig	12
Vasopressin	Brain	Rat	13
	Liver	Rat	14
	Kidney	Rat	14
PHI	Brain	Cat	15
Galanin	Gastrointestinal tract	Pig, guinea pig, rat	16,17
Dynorphin	Urogenital tract	Guinea pig	18
	Brain	Rat, guinea pig, man	19

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

SECTION II :

GENERAL METHODS

This section describes the methods that I used in this study. The neurochemical methods used by my colleagues are described in the relevant chapters.

1. FLUORESCENCE HISTOCHEMISTRY

This method has been used for the visualization at the light microscope level of catecholamine-containing nerve fibres in rat cerebral vessels, dura mater, and iris; in guinea-pig uterine arteries and in neurons of rat sympathetic cervical ganglia.

Two different methods for catecholamine fluorescence were used : a) whole mount stretch preparations (cerebral arteries, dura mater, iris, uterine arteries) were stained with the glyoxylic acid fluorescence technique, as described by Lindvall and Björklund, 1974, and modified in accordance with Cowen et al., 1985; b) cryostat sections of cervical ganglia were treated with the SPG method, described by de La Torre and Surgeon, 1976.

The use of glyoxylic acid represented a step forward in the development of histochemical methods for catecholamine fluorescence because of its reproducibility, for the higher sensitivity compared with the previous methods and for decrease in background fluorescence. The addition of pontamine sky blue (PSB) to counterstain background autofluorescence (Cowen et al., 1985) further improved the quality of this histochemical method.

In the present study, because fluorescence histochemistry was often followed by quantitative evaluation, care was taken in order to

standardize both the histochemical procedure and the preparation of the tissues.

Methods

Animals were killed by anaesthetic (pentobarbital,i.p.) overdose. For cerebral vessels, the brain was removed and immersed in 2% glyoxylic acid in 0.1 M phosphate buffer, pH 7.2, at room temperature for a total of 1.5 hrs; during this time the whole circle of Willis, including the basilar artery, was carefully dissected from the surrounding meninges and incubated for 10 min in 0.05% PSB and 0.1% dimethylsulphoxide (DMSO) in the glyoxylic acid solution. The arteries were then washed in fresh solution and transferred to a clean glass slide, stretched and dried at room temp. in a dust free atmosphere. The same procedure was applied to the rat dura mater, which was removed from the skull just before going into the PSB solution and to the rat iris, dissected from the eye and from the attached cornea at the end of the incubation in the glyoxylic acid solution. The slides with the dried whole mount stretch preparations were then heated at 100 °C for 4 min, and mounted in liquid paraffin. Only small changes occurred in cerebral vessel calibre, due to shrinkage during the procedure, as shown by comparing the circumference of these vessels at different steps of the procedure (i.e. before drying, before heating, at the end) or with the circumference of similar arteries from perfusion-fixed animals. Similarly, the area of the rat dura mater and iris, as measured before drying and after mounting, did not change significantly.

Guinea-pig uterine arteries had their length and diameter measured

in vivo. The arteries were then cut in segments of 1 cm, as marked "in situ" by micropins dipped in the PSB solution. The segments of arteries were opened longitudinally and pinned, adventitial side uppermost on a square of silicone elastomer (Sylgard, BDH Ltd., Poole, U.K.). A piece of graph paper was put underneath and the vessel segments were stretched to the original size. Graph paper was also used when vessels were transferred to the glass slides for drying. Final shrinkage after heating was of about 5%, but no correction factor was applied.

Superior cervical and stellate ganglia were removed from non perfused animals, dipped in O.C.T. compound (Gurr, BDH Ltd., Poole, U.K.), frozen inside the cryostat chamber at - 25 °C, as recommended by de La Torre and Surgeon, 1976. 10 µm thick sections were cut and processed as suggested by these authors.

2. IMMUNOHISTOCHEMISTRY

Indirect immunohistochemical methods (Coons, 1955) were used for both light and electron microscopic visualization of the immunoreactivity of neurotransmitters and related enzymes in nerve fibres and cell bodies.

All the antibodies used were from commercial sources and details about them, their specificity and control experiments that were carried out, are given in the relevant chapters.

Two methods have been used : a) immunofluorescence for whole mount stretch preparations and cryostat sections for the light microscope;

b) immunoperoxidase, using a biotinylated anti IgG antiserum, followed by a biotin-streptavidin-horseradish peroxidase (HRP) conjugated solution, for light and electron microscopy for whole mounts and paraffin sections.

The advantages of the latter method over other immunoperoxidase techniques derive from the high affinity of the biotinilated IgG for the Fc fragment of the primary antibodies and especially from the "avidity" of the streptavidin for the vitamin (binding energy 2.3 kcal/mol, Green, 1986). Thus, both primary and secondary antisera can be used at higher dilution; moreover, because of the amplification of the final signal (the biotin-streptavidin-HRP complex bears a greater number of peroxidase molecules than the conventional peroxidase anti-peroxidase (PAP) complex, Hsu et al., 1981) the occurrence of non specific background, with the low concentration of solution that is required to produce a visible reaction, is negligible. As the size of the complex can become very large, with consequent reduction of penetrability, the damage of the immunostained axons usually seen at the electron microscope with the use of diaminobenzidine (DAB) to reveal peroxidase localization, is much lower than with the conventional PAP technique.

2.1 Immunofluorescence

Animals, injected with anaesthetic overdose as before, were perfusion-fixed with 4% paraformaldehyde in phosphate buffered saline (PBS). The brain, skull, eyeballs, cranial and cervical ganglia or uterine arteries were dissected out from surrounding tissue and immersed in the same fixative, while further dissection was carried out, for a

total of 2-12 hrs. Tissues to be sectioned were then washed with PBS for at least 3 hrs and stored in 15% sucrose, 0.1% sodium azide in PBS for a maximum of 24 hrs. Tissues to be processed as whole mounts, after washes with PBS, were dehydrated to 100% alcohol and rehydrated to PBS to increase penetration of antibodies through the damage produced by this procedure. All the steps were carried out before removing the cerebral vessels from the brain, the dura mater from the skull and the iris from the eye, but after opening and pinning the segments of uterine arteries on pieces of Silgard, procedure that was carried out immediately after perfusion and before further fixation.

The dissected cerebral vessels, dura mater, iris and uterine artery segments were then exposed to 0.1% triton x100 in PBS for 15 min, and incubated in vials of convenient size in the antibody solution, overnight. All the antibodies were diluted in PBS containing 0.1% lysine, 0.1% sodium azide, 0.1% triton X100 and 0.01% bovine serum albumin.

Blocks for cryostat sections were prepared by embedding the tissue in O.C.T. compound and freezing in isopentane cooled in liquid nitrogen. The temperature of the cryostat chamber was not lower than -15 °C and sections were cut at 7-10 μm thickness. They were thaw-mounted on gelatine-coated slides (prepared by dipping acid-alcohol cleaned slides in a solution containing 0.5% gelatine and 0.05% chrome alum), encircled by a strip of DPX mounting medium and, when dried, covered by a drop of the diluted antiserum. Sections were incubated with the antisera overnight in a moist chamber. The following morning, sections and whole mounts were washed in three changes of PBS and incubated with the second layer, usually a goat

anti-rabbit IgG conjugated with fluoresceine isothiocyanate (FITC) (Nordic- Immunoreagents, Denmark) diluted 1:100 with the same antibody diluting medium as above, for 1 hr at room temperature. After washing, whole mounts were transferred to gelatine-coated slides and air-dried. Both sections and whole mounts were mounted with Citifluor (Citifluor Ltd., London, U.K.) and stored, in the dark, at 4 °C until viewed and photographed.

2.2 Immunoperoxidase

The fixative used for perfusion was a modified Zamboni solution, composed as follows : 4% paraformaldehyde, 15% picric acid, 0.05% glutaraldehyde in 0.1 M phosphate buffer (PB). After removal, tissue was stored in this fixative for a maximum of 1.5 hrs, while dissection was carried out.

Cerebral vessels, dura mater, iris, cranial and cervical ganglia or uterine arteries were cleared of picric acid with several washes in 50% alcohol. Ganglia, for paraffin embedding were further dehydrated (70%, 80% x 3, 90% x 3, 100% x 3, chloroform x 3, each step of 1 hr) and wax embedded. Serial sections, 10 µm thick were cut with a microtome and collected, in sequences of ten, on poly-L-lysine-coated slides (Huang et al., 1983). After dewaxing in histoclear and rehydration, sections were incubated with 0.1% trypsin and 0.1% CaCl2 in PBS for 20 min and washed with PBS.

The whole mounts, when cleared of picric acid, were treated with 0.1% sodium cyanoborohidride (NaCnBH3) in PB for 30 min at room temperature, to increase penetration of antibodies (Llewellyn-Smith,

1984).

The following steps are common to both sections and whole mounts that were processed as floating tissue throughout all steps. Endogenous peroxidases were blocked by incubating sections and whole mounts in 0.3% H_2O_2 in 50% methanol for 10 min, followed by three washes in PBS, 10 min each. A preincubation with 10% normal donkey serum (NDS), since the second layer was produced in donkey, was always carried out for 30 min, in order to block non-specific antigenic sites. Incubation with the first layer, diluted in PBS containing 5% NDS was carried out overnight at room temperature, in vials under gentle agitation for whole mounts and in a moist chamber for sections. At the end of the incubation, sections and whole mounts were washed with PBS and incubated with donkey anti-rabbit, biotin-conjugated IgG (Amersham, U.K.), diluted 1:200 in PBS containing 5% NDS, for 2 hrs at room temperature. After washes with PBS, the biotin-streptavidin-HRP complex, diluted 1:300 in PBS, was applied for 2 hrs. Sections and whole mounts were washed with PBS, preincubated with DAB, 0.04% in Tris buffer (pH 7.2; 0.1 M) for 10 min and then incubated with the latter solution with the addition of 0.01% H_2O_2 for 10 min in the dark. DAB-end products were sometimes enhanced by the addition of 0.3% ammonium nickel sulphate to the final solution or by silver intensification (see paragraph 2.4 in this Chapter). Sections and whole mounts, transferred to gelatine-coated slides, were dehydrated and mounted with DPX.

3. ELECTRON MICROSCOPY AND IMMUNOCYTOCHEMISTRY

Conventional electron microscopy and immunocytochemistry have been applied to the ultrastructural study of perivascular nerves, in order to visualize neurotransmitter-related features [i.e. 5-hydroxydopamine, 5-OHDA- labelled vesicles for noradrenaline (NA) or peptide-immunoreactivity (IR)].

The use of the "false" neurotransmitter 5-OHDA to mark NA-containing varicosities was introduced by Tranzer and Thoenen, 1967. This substance has some advantages on the analogue 6-hydroxydopamine, that is also taken up by noradrenergic axons and visualized at the electron microscope, but causes a more severe degeneration of sympathetic nerves.

Method

5-OHDA, diluted in sterile saline containing 0.2% ascorbic acid with a concentration of 10 mg/ml, was injected in the jugular or femoral vein of the anaesthetized animal at a dosage of 10 mg/Kg. After 15-20 min, 100 ml of warm Krebs solution (Bülbring, 1955) containing 1,000 units of heparin and 0.1 g of sodium nitrite was flushed into the ascending aorta through an incision on the left ventricle with a large cannula. The right atrium was cut. Two different fixatives were used : 5% glutaraldehyde in 0.1 M cacodylate buffer for conventional electron microscopy and the modified Zamboni's fixative described in paragraph 2 in this section. Arteries were rapidly removed and immersed in the same fixative for 1.5 hrs. Segments of arteries for conventional electron microscopy were rinsed in cacodylate buffer, osmicated (2% OSO_4 in the same buffer),

dehydrated, including "en bloc" staining with 1% uranyl acetate in 70% alcohol for 20 min, and embedded in Spurr resin. Transverse semithin (0.5 µm thick) sections were stained with 0.5% toluidine blue in 0.5% borax in 70% alcohol, or with a methylene blue-Azur II-basic fuchsin recipe (Humphrey and Pittman, 1974). Ultrathin transverse sections were cut with glass knives, stained with methanolic (12%) uranyl acetate (5-10 min), followed by 0.4% lead citrate (5 min), viewed and photographed with a Philips 300 electron microscope.

For immunocytochemistry, the pre-embedding method described in paragraph 2.2 was followed. After the DAB reaction, some arteries were subjected to silver intensification as described below. A procedure for double labelling using silver intensification of DAB-end products of the first immunoreaction is described in Chapter three of the Results' section.

3.1 Silver intensification

This procedure is derived from the methods described by Gallyas et al., 1982 and by Maegawa et al., 1987. Briefly, after completing the DAB-reaction, arteries were washed with 2% sodium acetate and immersed, overnight in 10% thioglycolic acid. After several rinses with 2% sodium acetate, arteries were exposed to the physical developer, prepared just before use, by adding to a 5% solution of sodium carbonate (anhydrous) an equal volume of a solution containing 0.2% ammonium nitrate, 0.2% silver nitrate, 1% phosphotungstosilicic acid, 1.7% paraformaldehyde, for 10 min at room temperature in the light. The reaction is stopped by a brief rinse in 0.1% acetic acid,

followed by 2% sodium acetate. A gold toning is obtained by exposing the vessels to cold (0-5 °C) 0.05% gold chloride for 5 min, again followed by rinses with sodium acetate. To fix the silver grains, 2 changes of 3% sodium thiosulphate are used. Vessels were rinsed with 2% sodium acetate before further processing.

Both silver intensified and non intensified, immunostained vessels were fixed in 5% glutaraldehyde in cacodylate buffer, before osmication, dehydration and embedding, as described for conventional electron microscopy.

4. QUANTITATIVE METHODS

Quantitative methods have been used in different histological preparations, including whole mounts and sections for light and electron microscopy, in order to compare the density of perivascular nerves in different biological situations (ageing, pregnancy, denervation).

Quantitative methods in histochemistry and immunohistochemistry are affected by some of the limits of these methods : i.e. reproducibility and non-specific background. When applied to whole mount stretch preparations (and in spite of the increased yield of histochemical and immunohistochemical methods in these preparations compared to sections) quantitative analysis suffers other variables, which are concerned mainly with the dimensional changes of the sample during the preparation.

In order to minimize these problems, care was taken during the

preparation of the samples. The results of the quantitative studies reported here represent the mean of the results obtained by two different researchers on the same preparations. Morphometric methods, including computer-assisted image analysis, have been used throughout the study.

4.1 In rat cerebral vessels

This method was applied to whole mount stretch preparations of rat cerebral arteries stained for NA-fluorescence or peptide-IR. Three regions of each artery (see Chapter one in the Results for details) are chosen to carry out the quantitative analysis (i.e. the areas of the middle cerebral artery :a) proximal to its origin from the internal carotid artery; b) just before its bifurcation; c) in between these two areas). Using a x16 objective lens and 1.25 optovar, a square grid was applied to the ocular of the microscope and the number of stained nerve fibres intersecting 3 grid lines perpendicular to the major axis of the vessels were counted. This operation was repeated at the three levels of the vessel chosen for quantitative analysis. The mean of the results obtained by two observers for each vessel was transformed from number of nerve intersections x vessel circumference (the minor axis of the vessel is considered as 1/2 circumference and measured using the previously calibrated grid) to density of nerve fibers (nerve fibre intersections x mm^{-1}). This value was used to calculate the mean ± S.E.M. of nerve density in the vessels of the same age group and used

for all the comparisons.

4.2 In the guinea-pig uterine artery

The same method was applied to guinea pig uterine arteries. Whole mount stretch preparations of the vessels, opened longitudinally and stretched to the "in vivo" size (± 6%), stained for NA-fluorescence or peptide-IR were used. All the nerves along three lines perpendicular to the major axis of the vessels were counted, in three different segments (each of them of ≈1 cm length). These values were used to evaluate the mean ± S.E.M. of nerve fiber intersections x vessel circumference. Nerve density (nerve intersections x mm^{-1} of vessel circumference) was not considered to be representative of the ratio between nerves and target area (smooth muscle cells in the media). In fact, a parallel morphological study (Chapter five, Results section) has shown that a dramatic increase occurred to the area of the medial layer of the uterine artery during pregnancy, involving hypertrophy and, perhaps, hyperplasia of smooth muscle cells. Also levels of peptides and NA in this study were expressed per cm of artery rather than per g tissue, to obviate to inconsistencies due to the increase in weight of components that did not represent the innervation (i.e. luminal content, adventitial elements). It is not possible to speculate on the changes occurring to the functional unit nerve-smooth muscle cell in vascular hypertrophy, that would need a knowledge of the cellular distribution of the receptors and their activity, on the responsiveness and contraction ability of the hypertrophic cells and, furthermore, on the intercellular connections that could facilitate (gap junctions)

or inhibit (intercellular matrix elements) nerve transmission and muscle contraction. In view of the lack of knowledge, any overinterpretation of the results was avoided. For example, in the case of the guinea pig uterine artery during pregnancy, the marked decrease of nerve density stressed by expressing the results as nerve intersection x mm-1 of vessel circumference could be equally reported as a dramatic sprouting of nerve fibres along the surface of the vessel, because of an increase of adventitial surface of about 400%.

4.3 Computer assisted image analysis

A computer-assisted image analysis system was used to quantify the density of SP- and CGRP-IR nerves in whole mount stretch preparations of dura mater, iris and middle cerebral artery of rats subjected to different denervation procedures. It is exaustively described in Chapter four in the Results section and so will not be given here.

4.4 Number of immunoreactive neurons

Quantitative analysis was carried out on one 10 µm thick wax section of several cranial ganglia. The ganglia were serially sectioned throughout and adjacent sections were immunostained for the investigated peptide and conventionally stained with toluidine blue or cresyl violet for counting the neurons. Immunostained neurons were expressed both as absolute number and as percentage of the total number of neurons in the same or in the adjacent section. Counting of neurons was usually restricted to few representative sections, taken at regular distances to cover all levels of the ganglia, untill

a sufficient number of neurons (over 500) was evaluated. For smaller ganglia (i.e. the pterygopalatine ganglia) a minimum of 200 neurons, representing 1/5 of the whole population of those ganglia, was evaluated.

4.5 Number of nerve varicosities

This method was used to quantify the number of nerve varicosities, their subtypes and their density in uterine arteries from virgin and pregnant guinea pig (Chapter six, Results section) and it is based on the method used by Cowen, 1984. Briefly, ultrathin transverse sections of conventionally fixed uterine arteries, some of them from 5-OHDA injected animals were viewed with a 300 Philips electron microscope. All nerves, as nerve bundle or single axons, lying within the adventitial layer (so excluding the large paravascular nerves present in the loose, fatty connective tissue surrounding the artery) were photographed and printed at a final magnification of 13,000. From tracings of these micrographs or from the negatives, the number of varicosities (axons containing more than 10 vesicles) and the subtypes of vesicles present were counted. Three principal subtypes of varicosities were recognized : those containing exclusively small (<60 nm) empty vesicles, called small agranular vesicle-containing varicosities; those containing predominantly small dense-cored vesicles, sometimes associated with a few small empty vesicles or with large (>60 nm) dense-cored vesicles, and called dense-cored vesicle-containing varicosities; those containing predominantly large dense-cored vesicles usually associated with small empty vesicles and mitochondria and defined as large dense-cored vesicle-containing

varicosities.

4.6 Morphometry

When the evaluation of the area of a whole mount stretch preparation was needed, this was carried out from drawings or photographs using a Summagraphic, Summasketch digitizing tablet connected to an Archimedes PC, running software written by Mr. Peter Trigg.

Statistical analysis was carried out using either the unpaired Student's t test or the Dunnet's analysis for multiple comparison (1964) run on an Apple II PC.

5. HIGH-RESOLUTION AUTORADIOGRAPHY

This technique was used, alone or in combination with pre-embedding immunocytochemistry to demonstrate the localization of ^{3}H-NA uptake sites in nerves of guinea pig uterine arteries.

For a description of the combined methods, see Chapter seven in the Results section. For reference see Rogers, 1979.

Method

After the incubation with ^{3}H-NA (see Chapter seven, Results section), the segments of uterine arteries were fixed either in 5% glutaraldehyde in cacodylate buffer or in modified Zamboni's

fixative, and processed either for conventional electron microscopy or for immunocytochemistry, usually followed by silver intensification, as described in the previous section.

Semithin sections, 0.5 and 1 μm, were collected on alcohol-acid washed glass slides and heated on a hot plate at ≈60 °C. Rows of ultrathin sections were transferred, with the aid of a loop, to formvar-coated slides and dried at room temperature, covered from dust. The sections were sited in the distal 2/3 of the slides, in two groups from two different blocks, usually from a virgin and a pregnant guinea-pig, in order to reduce the significance of the variability of the thickness of the emulsion layer in different slides. The following steps were carried out in a darkroom, equipped with a safelight filter (Ilford, S safelight) and a 15 watt bulb. Ilford L4 nuclear emulsion (Ilford, U.K.) with a mean crystal diameter of 0.14 μm, was used. It was diluted in distilled water at 44 °C in a water bath in the dark, so as to obtain a final emulsion layer of 3-5 μM, for semithin sections, and a deep purple-coloured layer (<140 nm) for ultrathin sections. All the slides were withdrawn manually, after achieving a reproducible speed, suitable for the desired layer thickness. Slides were drained for 30 sec in vertical position; then the emulsion from the back of the slide was wiped away with tissue paper. The emulsion on coated slides was allowed to gel by placing them horizontally, face up, on metal plates, cooled by contact with ice-filled containers. After gelification (for a variable time, depending on the thickness of the emulsion layer i.e. 2-12 hrs), the slides were dried over silica gel for 1 hr and the placed in light-tight plastic boxes with dry silica gel, wrapped in

velin tissue. As necessary controls, sections obtained from immunostained, silver intensified, uterine arteries, not exposed to ^{3}H-NA and emulsion coated sections exposed to the light, were stored together with the others. The boxes were sealed with adhesive tape, wrapped in black plastic photographic bags and stored at 4 °C for variable times (from 2 weeks to 2 months). From test slides developed at variable intervals, the exposure time of 4 weeks appeared to be satisfactory in all the experiments.

Development of autoradiographs was carried out at 20 °C with undiluted D-19 (Kodak, U.S.A.). Time of development varied : 4 min for semithin sections and 2 min for ultrathin sections. This was followed by 2 rinses in distilled water with very gentle agitation, and fixation (4 min) with 25% sodium thiosulphate. Semithin sections were then mounted with DPX for phase contast observation or stained with toluidine blue before mounting.

Ultrathin sections were recovered, on uncoated 100 mesh grids, by floating the formvar-emulsion layer on clean distilled water and placing the grids on the rows of sections, visualized with the aid of reflecting light against a black background. The layer of formvar-emulsion, bearing the grids, was recovered from the water using a square of "nescofilm" gently pressed over the formvar and pushed down into the water, inverted and withdrawn slowly. After drying, the grids were removed by scoring the formvar layer around them. They were stained with methanolic uranyl acetate and viewed with the electron microscope.

6. SURGICAL PROCEDURES

These procedures are restricted to the removal of the superior cervical ganglion (SCG) bilaterally in adult rats, as described in Chapter two of the Results section.

Method

Rats (7 weeks old) were anaesthetized with fentanyl citrate and fluanisone (Hypnorm, Janssen, Oxford, U.K.; 50 mg/Kg i.m.). Through a median incision on the neck, the common carotid arteries were exposed and gently pulled aside. The SCGs were localized, dissected from surrounding tissues and their post-ganglionic branches were cut with microscissors. Holding one of these branches with microforceps, the ganglia were carefully separated from the adventitia of the carotid arteries, and so were their preganglionic nerves, down to the proximal ends (postganglionic) of the stellate ganglia, that were left in situ. Animals were sutured and dusted with antibiotic powder. Recovery was quick, accompanied by the development of a Bernard Horner's syndrome bilaterally. They were killed two days later.

7. CHEMICAL SYMPATHECTOMY

The use of guanethidine to cause specific sympathetic denervation was firstly described by Burnstock et al., 1971. In the studies on developing rats described in Chapters two, three and four (Results

section) guanethidine treatment was preferred to 6-hydroxydopamine treatment, that also causes sympathetic denervation, for the lower mortality, for the persistence sympathetic denervation in adults and for the effectiveness of the treatment at the level of the cell bodies in cervical sympathetic ganglia.

Method

Seven day old rats were treated subcutaneously with guanethidine (Ismelin - CIBA) 60 mg/Kg dissolved in sterile saline subcutaneously, daily for 6 weeks. Controls were injected with saline over the same period. The most relevant difference with their matched controls was a weight loss of about 10 % and a light intolerance. All animals were killed on the same day of the last injection.

8. SENSORY DENERVATION

This procedure is used in the study reported in Chapter four of the Results section, alone or in combination with guanethidine treatment, as described in the relevant Chapter. It was introduced after the finding of an increase of CGRP/SP-IR nerves in cerebral vessels, dura mater and iris after guanethidine treatment, as a tool to demonstrate their sensory nature.

Method

Newborn rats were treated with capsaicin (50 mg/Kg in 10 % ethanol, 10% tween 80 in sterile saline) subcutaneously (Jancso et al., 1977) under ice or light ether anaesthesia on day

1,2,4,6,8,10,12 and 14. Animals were killed at three months of age.

9. USE OF NEURONAL TRACERS

Neuronal tracers have consistently improved the knowledge of neuroanatomical connections at the level of both central and autonomic nervous systems.

For the purpose of the study described in Chapter 3 (Results section), a fluorescent neuronal tracer, taken up by nerve terminals and retrogradely transported to cell bodies, was chosen.

Method

The purpose of the study was to trace the origin of NPY/VIP-IR nerve fibres supplying the rat middle cerebral artery after long-term sympathetic denervation with guanethidine. In brief, adult, guanethidine-treated rats were anaesthetized as described in paragraph 6 (this section). Using a dental drill, a window in the parietal bone was opened and the dura mater covering the parietal branch of the middle cerebral artery was carefully cut on three sides and lifted to expose the artery. 1 mm strip of parafilm was pulled under the artery and pieces of gelatine sponge, soaked in 2% aqueous solution of fast blue (SIGMA, U.K.) were applied to the exposed artery and left in situ. The parafilm strip was brought over in order to minimize the contact between the fast blue solution and the dura mater, that was reapposed. The skull window was sealed with dental wax and the skin was sutured. Animals were killed after 3 days,

perfusion fixed with 4% paraformaldehyde, the ipsilateral pterygopalatine ganglion was removed, frozen and serially sectioned (7 μm). Every second or third section was mounted with PBS, coverslipped and observed under a fluorescent microscope (Zeiss), equipped with the appropriate filter setting for fast blue observation (excitation 380 nm, emission 420 nm). When retrogradely labelled neurons were found in a section, that section was further processed for immunofluorescence of NPY and the following one for VIP-IR.

SECTION III :

RESULTS

A: RAT CEREBRAL VESSELS

CHAPTER ONE

AN INCREASE IN THE EXPRESSION OF NEUROPEPTIDERGIC VASODILATOR, BUT NOT VASOCONSTRICTOR, CEREBROVASCULAR NERVES IN AGING RATS

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 arteries of the rat Circle of Willis 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 of one month of age. This was followed by a general fall in the density of fluorescent nerve fibres. However, with aging, 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 nerve fibres 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. These changes are discussed in relation to the increased incidence of cerebrovascular disorders in the elderly.

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 focussed attention on the neurogenic mechanisms which regulate cerebrovascular tone (Burnstock, 1985). 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 hypothesized. For example, serotoninergic nerve fibres have been associated with vasospasm (Vanhoutte et al., 1984), purinergic mechanisms with hyperemia (Burnstock, 1981) and substance P (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., 1978; Lobato et al., 1980; Bannister et al., 1981; Edvinsson, 1982; Lee and Saito, 1984; 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 (Appenzeller et al., 1984; Llewellyn-Smith, 1984; Dhall et al., 1986), and much more information is available on the adrenergic

innervation of several vasculatures in old age (Lundberg et al., 1976; Todd, 1980; Duckles, 1983; Gallen et al., 1983; Dolezel et al., 1984; Amenta et al., 1985).

This study describes the developmental changes in rat cerebrovascular nerve fibres containing noradrenaline (NA), serotonin (5-HT), SP, vasoactive intestinal polypeptide (VIP), neuropeptide Y (NPY), and calcitonin gene-related peptide (CGRP), from birth through to 27 months of age.

MATERIALS AND METHODS

Male Wistar rats (n = 150) were used in this study. The animals were studied at 5 age stages : 1 day after birth and at 1, 4, 8 and 27 months. Animals were killed by an overdose of ether. The skull was carefully opened and the brain rapidly removed. For the demonstration of adrenergic nerves, brains were immersed in a 2% w/v glyoxylic acid (GA) solution in 0.1 M phosphate buffer at pH 7.2 for 1.5 h at room temperature (Lindvall and Björklund, 1974). During the incubation time, brain vessels of the circle of Willis were dissected from surrounding meninges and incubated in 0.05% Pontamine Sky Blue (PSB) and 0.1% dimethylsulphoxide (DMSO) in the glyoxylic acid solution for 10 min, in order to reduce background autofluorescemce (Cowen et al., 1985). After drying, the vessels were stretched on glass slides and heated at 100 °C for 4 min before mounting in liquid paraffin.

Serotoninergic and peptidergic nerves were visualized by the indirect immunofluorescent technique (Coons et al., 1955). For the visualization of 5-HT -containing nerve fibres, brains were incubated for 3 h in Krebs' solution, aerated with 95% O_2 and 5% CO_2 and containing 5×10^{-5} pargylin, prior to fixation in 4% paraformaldehyde in phosphate buffered saline (PBS). In order to avoid any difference in stretching, the cerebral arteries were fixed in situ. After fixation, brains were washed with PBS, dehydrated in 80% ethanol for 1.5 h and stored in PBS containing 0.1% sodium azide. At this stage the whole circle of Willis was carefully dissected out from surrounding tissue with the aid of a stereomicroscope. The preparations, washed in PBS containing 0.1% Triton X-100, were

incubated with the specific antisera overnight at room temperature (rabbit anti-NPY, 1:400, CRB, U.K.; rabbit anti-CGRP, 1:400, CRB; rat anti-SP, 1:200, Sera Lab, U.K.;rabbit anti-5-HT, 1:300, RIA, U.K.; rabbit anti-VIP, 1:1000, RIA, U.K.). Next, they were incubated with anti rabbit IgG, conjugated with fluoresceine isothiocyanate (FITC; 1:50) for 1 hr at room temperature, washed in PBS and stained with PSB for 10 min. The preparations were stretched on glass slides and mounted in buffered glycerol. GA-induced fluorescence for NA-containing nerve fibres and FITC-induced fluorescence for 5-HT and peptide-immunoreactive (IR) fibres were observed under a Zeiss photomicroscope equipped with epi-illumination.

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

Measurements

Using an ocular grid and a x16 Neofluor objective lens, a count was made of the number of nerve fibres intersecting an ocular grid line along the circumference of each vessel at three different places, at constant distance 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 preparation processed for immunohistochemistry and those for GA-induces fluorescence was negligibile (about ± 2-6% of unit circumference). 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 vessels parameters and the semi-quantitative analysis used in this study, it seemed inappropriate to apply any correction factor to the results. These small changes did not significantly alter the relative values of the measurements (i.e. the error is well within the standard error range) or affect the interpretation of the results.

The following cerebral vessels 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 its initiation from 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 <0.05 was considered to be of significance.

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.

It should be made clear that changes in nerve density observed in this study do not necessarily reflect morphological alterations in the overall population of perivascular nerves, but are limited to describing the density of nerves which express a particular substance at a given developmental stage.

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 XI, Figs. 7 and 10a,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 XII, Figs. 7 and 10d,e,f). 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 reached a peak at 1 month and was maintained until the fourth month. Thereafter, there was a decline in the number of VIP-containing nerve fibres in all vessels at 8 months, followed by a significant increase at 27 months of age (Table XIII, Figs. 8 and 11a-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 vertebrobasilar 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 XIV, Figs. 8 and 11d-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, the density at 27 months was similar to that at 1 month (table XV, Figs. 7 and 12a-c).

SP-containing nerve fibres

At birth, only one or two nerve fibres were found along the anterior and middle cerebral arteris. 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 (Figs. 7 and 12d-f).

DISCUSSION

Based on fluorescence and immunohistochemical studies, the present results 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 formed 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 formed a distinct perivascular plexus in all of the vessels examined at birth. 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 immunoreactive 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 about 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 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., 1982; 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., 1978) and rat (Santer, 1979) 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., 1978; 1982; Edvinsson, 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., 1986; 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 vasoconstrictor nerve fibres containing NA and 5-HT was in marked contrast to the significant increase of innervation density shown by nerve fibres containing the vasodilators 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 recent study showing a 70% rise in the CGRP content of rat pial arteries after bilateral excision of the superior cervical ganglia (Schon et al., 1985a). VIP and CGRP both exert potent vasodilation 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 acetylcholinesterase (AChE; Kobayashi et al., 1983; Hara et al., 1985), and SP with CGRP (Uddman et al., 1985) 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., 1985; Uddman et al., 1985). The present results, 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., 1985; 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 (Hökfelt et al., 1977; Moskowitz, 1984).

NPY, which has been shown to coexist with NA in perivascular nerve fibres (Lundberg et al., 1983; Ekblad et al., 1984; Sternini and Brecha, 1985), is a potent vasoconstrictor of cerebral vessels (Edvinsson et al., 1983; 1984; Edvinsson, 1985; Dahlöf et al., 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 (Stjärne et al., 1986).

The present results show that different perivascular nerve types follow markedly different developmental changes. Further work is needed to elucidate whether 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 results of the present study 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, 1982).

In general, the present study shows a decrease in the expression of perivascular vasoconstrictor nerve fibres with age. These changes could be regarded as being 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.

TABLE XI. 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 nm 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**	241.11±12.76	227.11±10.84	83.33±6.48***

* P<0.05, ** P<0.01, *** P<0.01 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, ACA = Anterior cerebral artery.

TABLE XII. Density of ^{5}HT-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 P	26.84±4.06	119.38±5.41***	62.8±3.64***	82.84±3.86**	50.28±2.06
BA 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 cereballar artery, PCA = Posterior artery, ICA = Internal carotid artery, MCA = Middle cerebral artery, ACA = Anterior cerebral artery. + = only single fibres were observed in some of the preparations.

TABLE XIII. 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±2.77***	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

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

TABLE XIV. 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	27.343.81	12.66±23.65	15.16±2.8	24.70±1.32*
MCA	+	15.11±3.36	5.28±2.07*	7.27±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, ACA = Anterior cerebral artery. + = Only single fibres were observed in some of the preparations.

TABLE XV. 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	27 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	62.02±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, ACA = Anterior cerebral artery, ACA = Anterior cerebral artery. - = No nerve fibres were visualized.

Fig. 7 - Top - Graph of age-related changes of (left) NA- and (right) 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. 8 and 9).
* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

Fig. 8 - Middle - Graph of age-related changes in density of (left) VIP- and (right) CGRP-containing nerve fibres in the internal carotid (ICA) and basilar (BA) arteries. b = 1 day after birth. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

Fig. 9 - Bottom - Graph of age-related changes in density of NPY-containing nerve fibres in the internal carotid (ICA) and basilar (BA) arteries. b = 1 day after birth.
* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

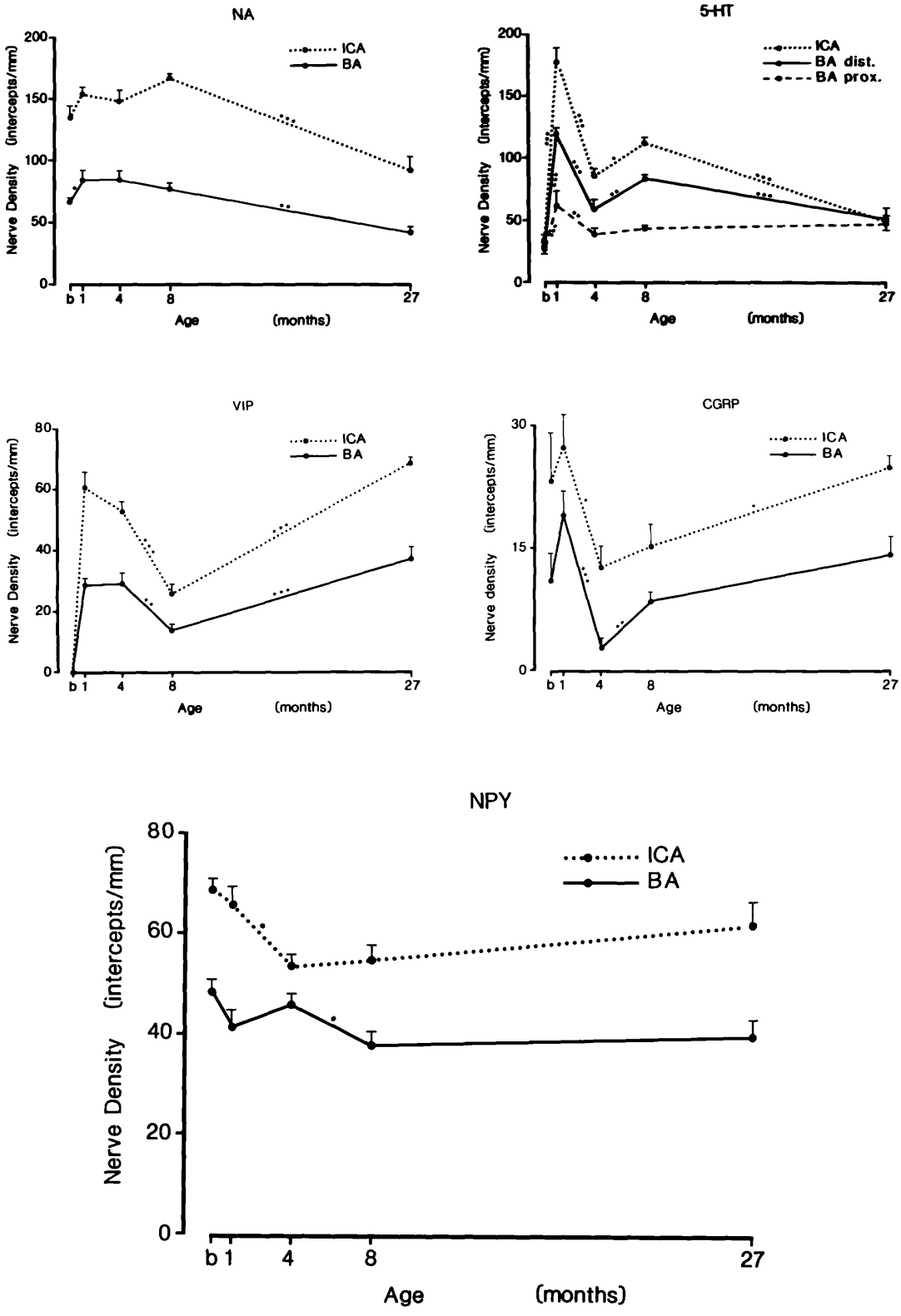

NA
ICA
BA
Nerve Density (intercepts/mm)
200
150
100
50
0
b 1 4 8 27
Age (months)
5-HT
ICA
BA dist.
BA prox.
Nerve Density (intercepts/mm)
200
150
100
50
0
b 1 4 8 27
Age (months)
VIP
ICA
BA
Nerve Density (intercepts/mm)
80
60
40
20
0
b 1 4 8 27
Age (months)
CGRP
ICA
BA
Nerve density (intercepts/mm)
30
15
0
b 1 4 8 27
Age (months)
NPY
ICA
BA
Nerve Density (intercepts/mm)
80
60
40
20
0
b 1 4 8 27
Age (months)

Fig. 10 - Photomicrographs of stretch preparations of cerebral arteries showing perivascular nerve fibres at various age stages. NA-containing nerves in the middle cerebral artery (MCA) at 1 day (**a**), 4 months (**b**) and 27 months (**c**). 5-HT-containing nerves in the distal portion of the basilar artery at 1 day (**d**), 8 months (**e**) and 27 months (**f**). Nerve density shows a decrease in old age for both NA and 5-HT. Arrows indicate longitudinal axis of blood vessel. Scale bars = 25 µm.

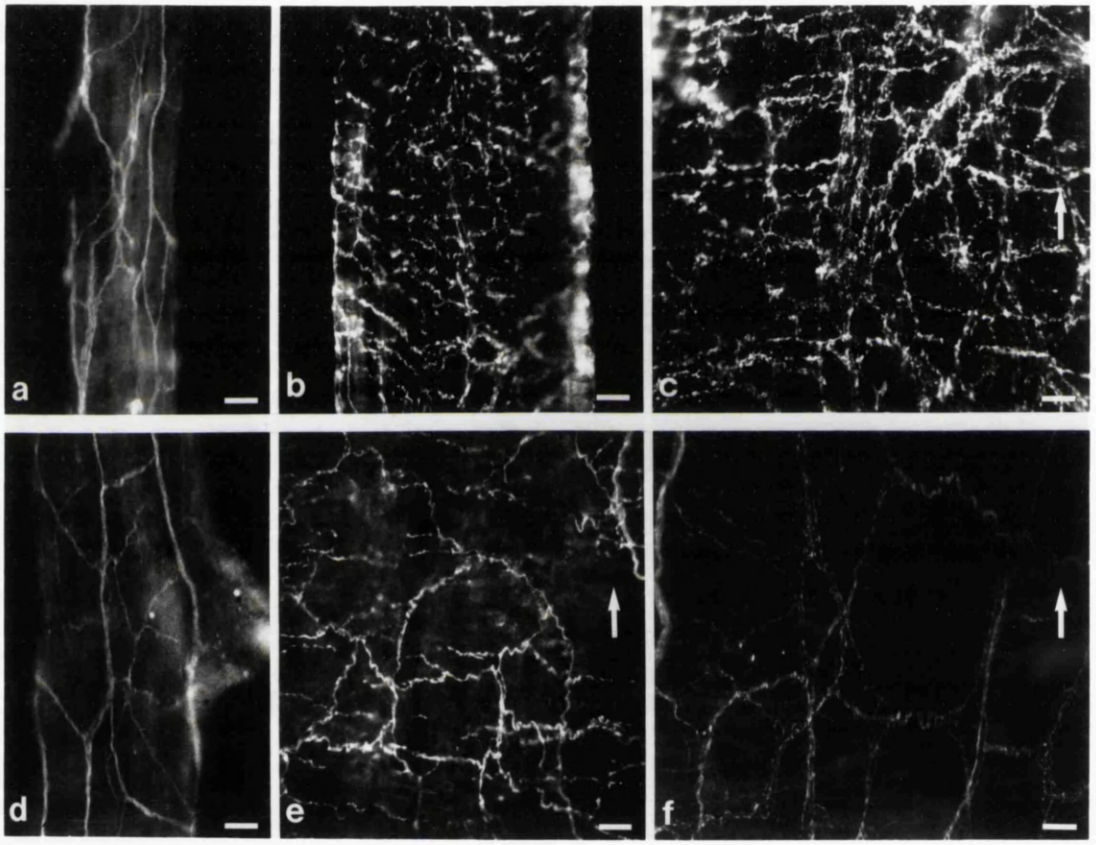
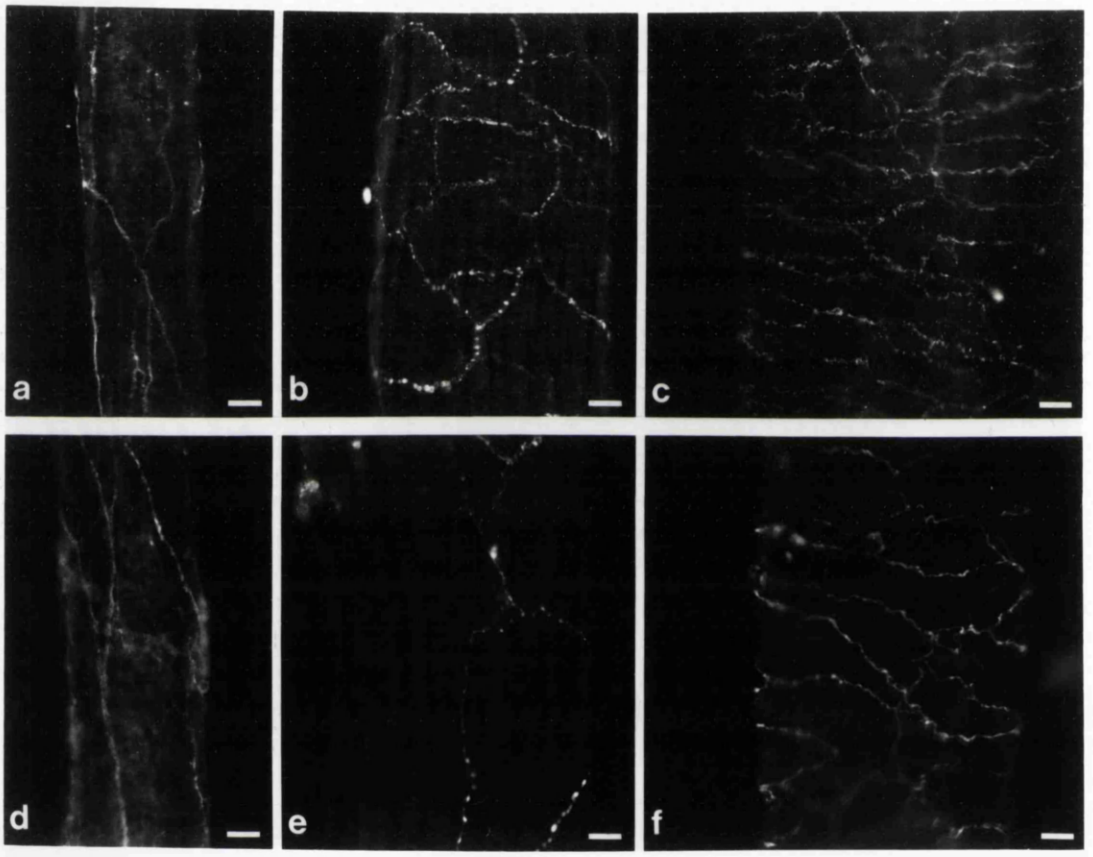

a
b
c
d
e
f

Fig. 11 - Photomicrographs of stretch preparations of cerebral arteries showing perivascular nerve fibres at various age stages. VIP-containing nerves in the middle cerebral artery at 1 day (**a**), 8 months (**b**) and 27 months (**c**). CGRP-containing nerve fibres in the posterior cerebral artery at 1 day (**d**), 4 months (**e**) and 27 months (**f**). Nerve density is increased in old-age for both VIP and CGRP. Scale bars = 25 μm.


a
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e
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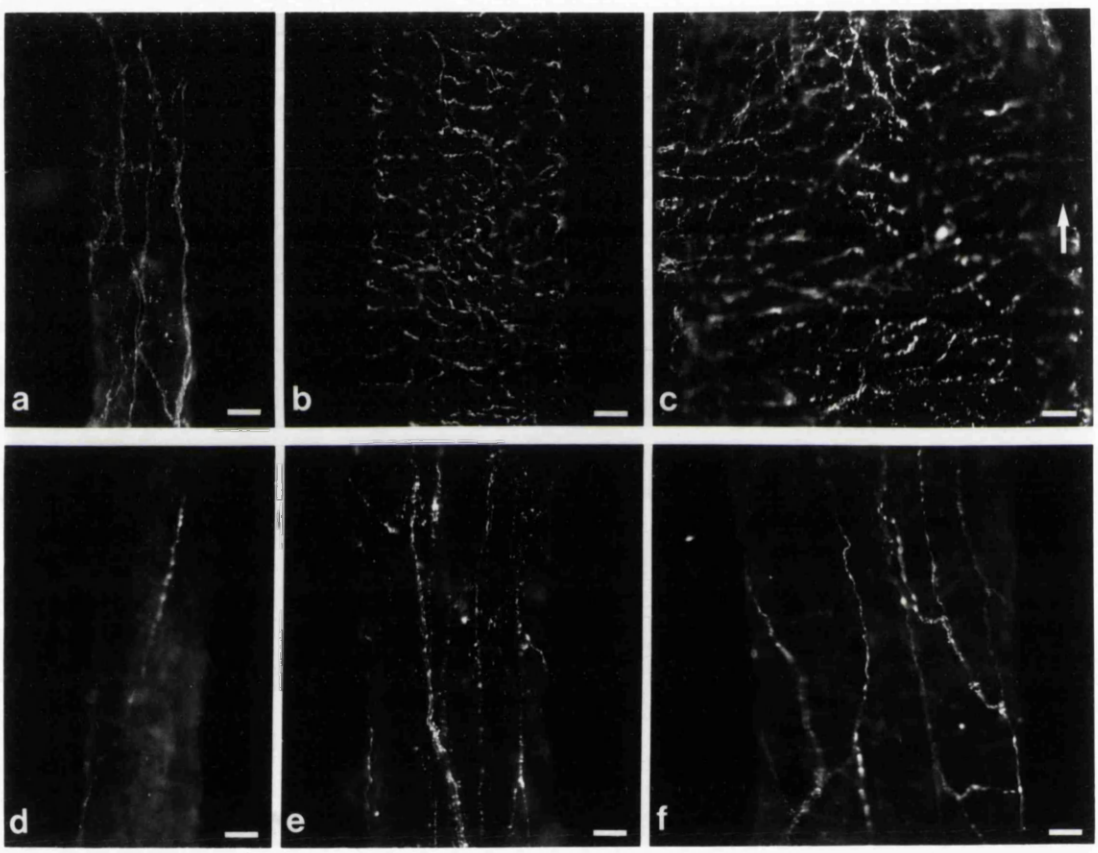
Fig. 12 - Photomicrographs of stretch preparations of cerebral arteries showing perivascular nerve fibres at various age stages. NPY-containing nerve fibres in the internal carotid artery at 1 day (**a**), 1 month (**b**) and 27 months (**c**). SP-containing nerves in the anterior cerebral artery at 1 day (**d**), 1 month (**e**) and 27 months (**f**). Nerve density is maintained with development for both NPY and SP. Arrow indicates longitudinal axis of blood vessel. Scale bars = 25 μm.


a
b
c
d
e
f

CHAPTER TWO

LONG-TERM CHEMICAL SYMPATHECTOMY LEADS TO AN INCREASE OF NEUROPEPTIDE Y IMMUNOREACTIVITY IN CEREBROVASCULAR NERVE AND IRIS OF THE DEVELOPING RAT

SUMMARY

Short-term (surgical) and long-term (chemical) sympathectomy have revealed the presence of a population of neuropeptide Y-immunoreactive nerve fibres which do not degenerate in parallel with noradrenaline-containing nerves supplying cerebral vessels and the iris of the rat. Two days after bilateral removal of the superior and middle cervical ganglia of 7 week old rats, noradrenaline-containing nerves could not be detected along any of the arteries of the rat circle of Willis or of the iris, but 18-31% of neuropeptide Y-immunoreactive nerves remained.

Long-term treatment (6 weeks) with guanethidine commencing in developing 1 week old rats caused degeneration of the sympathetic neurones in cervical ganglia and disappearance of 5-hydroxydopamine-labelled nerves (that showed dense-cored vesicles at the electron microscope level) from rat cerebral vessels, but did not significantly change the density of neuropeptide Y-immunoreactive axons on the vessels. Furthermore, whilst in control rats neuropeptide Y-immunoreactivity was localized largely within 5-hydroxydopamine-labelled cerebrovascular nerves, after long-term sympathectomy with guanethidine, neuropeptide Y-immunoreactivity was seen only in nerves lacking small dense-cored vesicles. A small number of catecholamine-containing nerves appeared along the internal carotid and anterior cerebral arteries after long-term sympathectomy; these may arise from neurones of central origin.

These results suggest that as a consequence of long-term sympathectomy with guanethidine, compensatory changes occur,

involving an increase in the expression of neuropeptide Y-immunoreactivity in non-sympathetic axons in cerebrovascular nerves and iris of the rat. In contrast, the neuropeptide Y-immunoreactive nerves in the dura mater appear to be entirely sympathetic, since none were present after short-term sympathectomy and none appeared after long-term sympathectomy.

INTRODUCTION

Neuropeptide Y-immunoreactivity (NPY-IR) has been shown to be present in the cerebrovascular tree of the rat, forming a dense plexus of perivascular axons which also contain the catecholamine-synthesizing enzyme, dopamine β-hydroxylase (Edvinsson et al., 1987b). The localization of NPY-IR in sympathetic nerves has been confirmed at the ultrastructural level (Matsuyama et al., 1985) and NPY-IR nerve varicosities have been identified with those containing catecholamines, using the false neurotransmitter 5-hydroxydopamine (5-OHDA). The origin of NPY-IR cerebrovascular nerves has been attributed to the sympathetic neurones of the superior cervical ganglia (Edvinsson et al., 1987b), although the loss of NPY-IR from cerebral arteries following superior cervical ganglionectomy is not complete (Schon et al., 1985b; Edvinsson et al., 1987b).

There is now increasing evidence for the presence of NPY-IR in non-sympathetic axons (Gibbins and Morris, 1987; 1988; Mione et al., 1988), including neurones in cranial parasympathetic ganglia, which also contain vasoactive intestinal polypeptide (VIP)- and choline acetyltransferase-IR (Leblanc et al., 1987). In guinea-pig cerebral vessels, the proportion of fibres showing VIP-IR which also contain NPY-IR increased from 18 to 70% 10-12 days after surgical sympathectomy (Gibbins and Morris, 1988).

I have used bilateral cervical ganglionectomy to evaluate the percentage of NPY-IR nerves remaining in the cerebral arteries following loss of noradrenergic nerves, and long-term treatment with guanethidine to study of the compensatory expression, if any, of NPY

in non-sympathetic cerebrovascular nerves in the rat. Two different approaches to sympathectomy have been used in order to achieve clear-cut results. Short-term sympathectomy, was used to define the amount of NPY immunoreactivity "normally" expressed by non-sympathetic nerves in cerebral vessels of young-adult rats. This was only possible by surgical bilateral cervical ganglionectomy. On the other hand, chemical sympathectomy following chronic treatment with guanethidine was preferred to the surgical approach when compensatory aspects of reinnervation of by NPY immunoreactive nerves were investigated, since all sympathetic nerves were destroyed by this treatment so there is no possibility of reinnervation by any other sympathetic nerves.

MATERIALS AND METHODS

Three groups of 20 male Wistar rats were used in this study. All animals were killed at seven weeks of age, their average weight being 150-200 g. Guanethidine (Ismelin, CIBA) treatment was by daily injection, 60 mg/Kg s.c. for 6 weeks, starting when the animals were 8 days old (Burnstock et al., 1971; Johnson and O'Brien, 1975). Controls were injected with saline over the same period. Animals were killed on the same day as the last injection. Ganglionectomy was carried out under anaesthesia using fentanyl citrate and fluanisone (Hypnorm; Janssen; Oxford; 50 mg/Kg i.m.), and involved removing the superior cervical ganglia, together with the sympathetic trunk up to the stellate ganglia, which were left in situ, because it was difficult to excise them cleanly. These animals were killed two days later. The left iris, half of the dura mater, half of the circle of Willis and the left superior cervical ganglion (SCG) were processed for the histochemical detection of catecholamines as whole mounts (Lindvall and Björklund, 1974) or, for the SCG, as cryostat sections (De la Torre and Surgeon, 1976). The other side of the dura mater, circle of Willis, the right iris and SCG were fixed with 4% paraformaldehyde and processed for the immunohistochemistry of NPY, as described in the previous chapter. Nerve fibres density in the cerebral vessels was expressed as the mean number of nerve intercepts per mm of vessel circumference ± S.E.M. The density of fluorescent nerves in the iris was expressed in arbitrary units from 0 (absent) to 4 (very dense).

The effectiveness of the guanethidine treatment was assessed by

assay for noradrenaline (NA) and NPY in the SCG using high performance liquid chromatography with electrochemical detection (Keller et al., 1976; Moyer and Jiang, 1978) and an inhibition enzyme-linked immunosorbant assay (Belai et al., 1988), respectively. There was an approximately 80% reduction in NA levels and 90% reduction in NPY content of the SCG following guanethidine treatment.

For electron microscopy, one group of five guanethidine- and five saline-treated rats were injected with 5-OHDA (150 mg/Kg i.p. in Krebs' solution containing 0.2% ascorbic acid) for visualization of dense cored vesicles 1 hr before perfusion with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4. The cerebral vessels, and the superior and inferior cervical ganglia of these animals were fixed for a total of 2-3 hrs, postfixed in 2% OsO_4, stained en bloc with uranyl acetate, dehydrated and embedded in Spurr resin. Some of the 5-OHDA-injected animals were perfused with a fixative containing 4% paraformaldehyde, 0.1% glutaraldehyde and 15% saturated picric acid in 0.1 M phosphate buffer. After 2 hrs fixation, the vessels were cleared through several washes in 50% alcohol, treated with 0.1% $NaCNBH_3$ in phosphate buffer and then with 0.3% H_2O_2 in 50% methanol for 10 min. A preincubation with 10% normal donkey serum (NDS; Sera Lab, U.K.) in phosphate buffered saline (PBS) was followed by incubation with the anti-NPY antiserum (Immunodiagnostic System Limited, U.K.) diluted 1:600 in 3% NDS in PBS for 18 hrs at room temperature. The biotin streptavidin horseradish peroxidase system (A-B-C; Amersham, U.K.) followed by diaminobenzidine reaction was used to detect the sites of the immunoreaction. No immunoreactivity was detected in cerebral vessels using anti-NPY antiserum preabsorbed

with an excess of the neuropeptide, or by replacing it with normal rabbit serum. The stained cerebral arteries were then osmicated, dehydrated and embedded as above. Semi-thin and ultra-thin sections were cut with an ultramicrotome (LKB) and stained with toluidine blue or lead citrate, respectively. Ultrathin sections were examined with a Philips 300 electron microscope.

RESULTS

Effect of the long-term treatment (6 weeks) with guanethidine on the density of noradrenergic and NPY-LI nerves.

Nerve fibres densities of cerebral vessels have been quantified and are shown in Figs. 13 and 14. The reduction of NA-containing nerves in all the arteries of the rat circle of Willis was almost complete, except the internal carotid and anterior cerebral arteries where a few (18%) remained (Fig. 15c). However, there was no significant reduction of NPY-IR cerebrovascular nerves (with the exception of the internal carotid artery; see Figs. 13 and 15f). The density of NPY immunoreactivity in nerve fibres supplying the cerebral vessels and the iris is increased from 18-32% of the control 2 days after superior cervical ganglionectomy to 80-90% of the control value 6 weeks after guanethidine treatment began. In the dura mater both NA-containing and NPY-IR nerves were absent in guanethidine-treated rats (see Fig. 17), while the iris, which was devoid of NA-containing nerve fibres (Fig. 16b), displayed a number of NPY-IR nerve fibres (Fig. 16e and Table XVI).

Effect of superior and middle cervical ganglionectomy on the NA- and NPY-containing nerves

Two days after surgery, NA-containing nerves had completely disappeared from the cerebral arteries, the iris and the dura mater (Fig. 15a, b, and Table XVI). In contrast, some (18-32%) NPY-LI nerves were still present on the cerebral vessels and a comparable percentage in the iris (Figs 13, 15d, e, 16c, d, and Table XVI), but

not in the dura mater.

Histochemistry, immunohistochemistry and morphology of SCG and stellate ganglia. No fluorescent neurones or nerve fibres were detected in the SCG and stellate ganglia after guanethidine treatment; on the other hand there was an increase in the number of small intensely fluorescent cells. In an examination of several 1 um semithin sections of the SCG and of the stellate ganglion, only one or two neurones were seen and these were damaged (Fig. 18). The total neuronal population had been replaced by small glial cells and by many non-myelinated and myelinated nerve bundles. In ultrathin sections, the abundance of these non-terminal axons was confirmed (Fig. 19).

Electron microscopy of nerve varicosities in cerebral vessels using 5-OHDA as a marker of sympathetic nerves. 5-OHDA-labelled varicosities were abundant in control rats and absent in treated rats (Fig. 20). Almost all NPY-IR varicosities showed 5-OHDA-labelled vesicles in control animals (Fig. 20c), while in treated rats NPY-IR varicosities were commonly seen in the absence of 5-OHDA-labelled nerves (Fig. 20d).

DISCUSSION

In this study the effects of long-term sympathectomy caused by chronic treatment with guanethidine on the NPY-IR nerve fibres supplying the cerebral vessels, the iris and the dura mater in the rat were evaluated. The results obtained with the long-term sympathectomy were compared with those found in animals that had undergone short-term sympathectomy achieved by surgical cervical ganglionectomy.

After long-term sympathectomy, a few catecholamine-containing nerves were found in the internal carotid and anterior cerebral artery but not in other cerebral vessels, in the dura mater, iris (present work) or systemic arteries (Aberdeen et al., 1988; 1990). Following guanethidine treatment, virtually no neurones were left in the superior and inferior cervical ganglia (which represent the origin of the sympathetic nerve supply to the rat cerebral vessels, dura mater and iris) and the glyoxylic acid induced fluorescence was confined to small intensely fluorescent cells. These cells may represent the source of the NA still detectable with high performance liquid chromatography in the SCG. The catecholamine-containing nerves present in the cerebral vessels after long-term treatment with guanethidine may be of central origin (Edvinsson, 1982).

The rise in NPY immunoreactivity in nerve fibres supplying the cerebral vessels and the iris after long-term sympathectomy was striking. The increase of NPY immunoreactivity in VIP-IR nerves found in guinea-pig cerebral arteries 10-12 days after bilateral superior cervical ganglionectomy (Gibbins and Morris, 1988) is of the

same magnitude as the increase in NPY immunoreactivity we describe after long-term sympathectomy compared to short-term sympathectomy.

During surgical sympathectomy, great care was taken in order to remove as much as possible of the cervical sympathetic trunk, as it is known that part of the sympathetic innervation of the vertebro-basilar system originates from the stellate ganglia (Arbab et al., 1988). Indeed, no NA-containing nerves could be demonstrated in the basilar artery, or in any of the other tissues examined, 2 days after this surgical procedure.

The origin of the NPY-IR nerves surviving superior cervical ganglionectomy, and which represent 18-32% of the whole NPY-IR nerve population in rat cerebral vessels, is unknown. However, in the rat iris, removal of the ipsilateral ciliary ganglion (a parasympathetic ganglion providing cholinergic innervation to the intrinsic muscles of the eye, see Landis et al., 1987) lead to the disappearance of NPY-IR nerve fibres in the iris after superior cervical ganglionectomy (Björklund et al., 1985). Since NPY-IR has been localized in other cranial parasympathetic ganglia (the rat otic, Leblanc et al., 1987; the rat and pterygopalatine, Leblanc et al., 1987; Kuwayama et al., 1988; the guinea-pig ciliary ganglion, Gibbins and Morris, 1987b) that commonly supply VIP-IR nerves to the cerebral vessels (Gibbins et al., 1984; Hara et al., 1985; Suzuki et al., 1989), in the following chapter, evidence will be presented of these ganglia being a source of the cerebrovascular NPY immunoreactivity surviving sympathectomy.

NPY-IR was absent from the dura mater of all sympathectomized rats, in parallel with NA-containing nerves, thus indicating a

sympathetic nature for all the meningeal NPY-IR nerves. It is noteworthy that compared to the dense VIP-immunoreactive innervation of cerebral vessels and iris, which could be the alternative source of NPY-immunoreactivity in these tissues, the rat dura mater is very poorly supplied by VIP-IR nerves (unpublished observation).

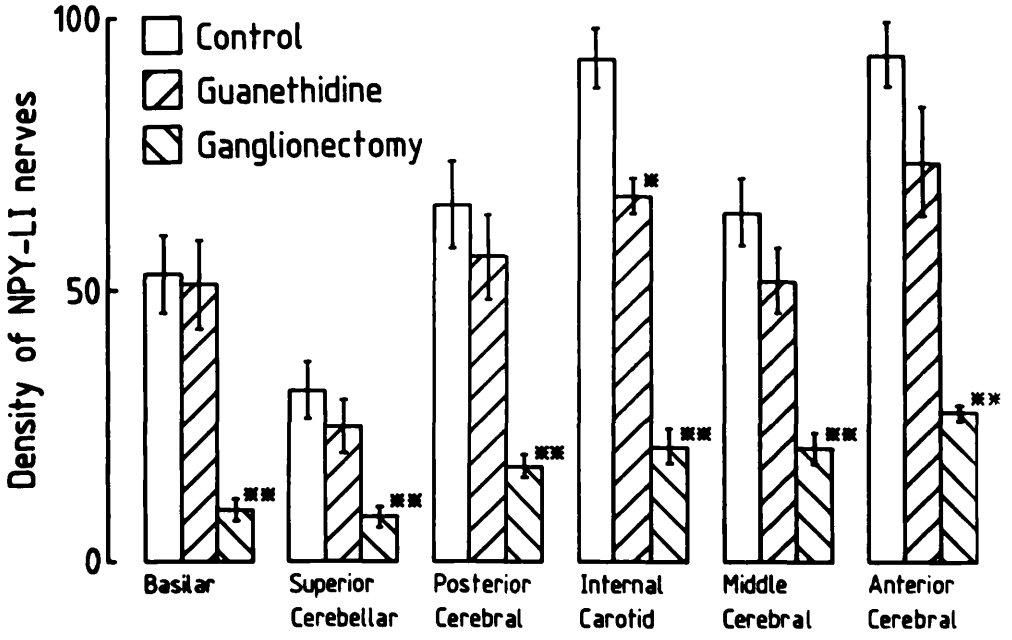
TABLE XVI. Effect of superior cervical ganglionectomy and guanethidine treatment on the density of NA-containing and NPY-LI nerves in the rat iris

	Control	Ganglionectomy	Guanethidine
NA	++++	-	-
NPY	++++	+	+++

- = absent; + = rare; +++ = dense; ++++ = very dense

Fig. 13 - Top - Histogram of NPY-immunoreactive nerve densities in the arteries of the rat circle of Willis in control, guanethidine-treated and ganglionectomized animals. (**clear bars**) controls; (**left cross-hatched bars**) guanethidine; (**right cross-hatched bars**) ganglionectomy. Nerve density is expressed as mean number of nerve intercepts per mm of vessel circumference ± S.E.M. (n=6) in all groups. **P<0.001 *P<0.01.

Fig. 14. - Bottom - Histogram of NA-containing nerve densities in the arteries of the rat circle of Willis in control and guanethidine-treated rats. (**clear bars**) control; (**cross-hatched bars**) guanethidine.Nerve density in the internal carotid and anterior cerebral vessels is expressed as the mean number of nerve intercepts per mm of vessel circumference ± S.E.M. (n=6) in both groups of animals. **P<0.001. Statistical comparisons were only made on these two vessels, since in the remaining guanethidine-treated vessels only the occasional nerve fibre was seen. Bilateral ganglionectomy led to a complete absence of NA-containing nerves in all the cerebral vessels (not shown).


100
50
0
Density of NPY-LI nerves
Control
Guanethidine
Ganglionectomy
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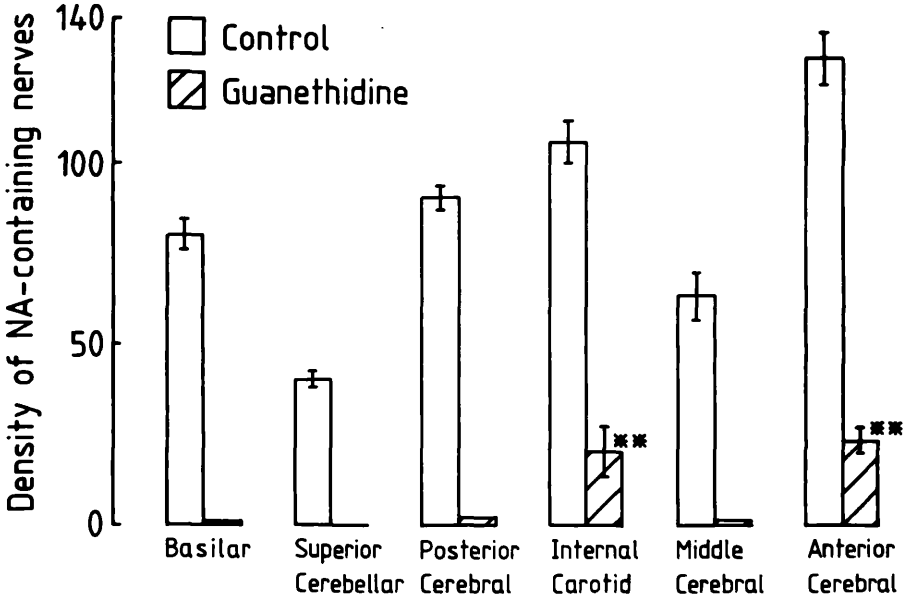
Basilar
Superior Cerebellar
Posterior Cerebral
Internal Carotid
Middle Cerebral
Anterior Cerebral


140
100
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Density of NA-containing nerves
Control
Guanethidine
**
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Basilar
Superior Cerebellar
Posterior Cerebral
Internal Carotid
Middle Cerebral
Anterior Cerebral

Fig. 15 - Fluorescence micrographs of NA- (**a-c**) and NPY- (**d-f**) containing nerves in the rat anterior cerebral artery from control (**a**, **d**), ganglionectomized (**b**, **e**) and guanethidine-treated (**c**, **f**) rats. Note the absence (**b**) and the dramatic reduction (**c**) of NA-containing nerves in the artery of ganglionectomized and guanethidine-treated animals, while NPY-immunoreactive nerves, very few in number after ganglionectomy (**e**), are only slightly reduced by guanethidine treatment (**f**). Calibration bar = 100 μm.

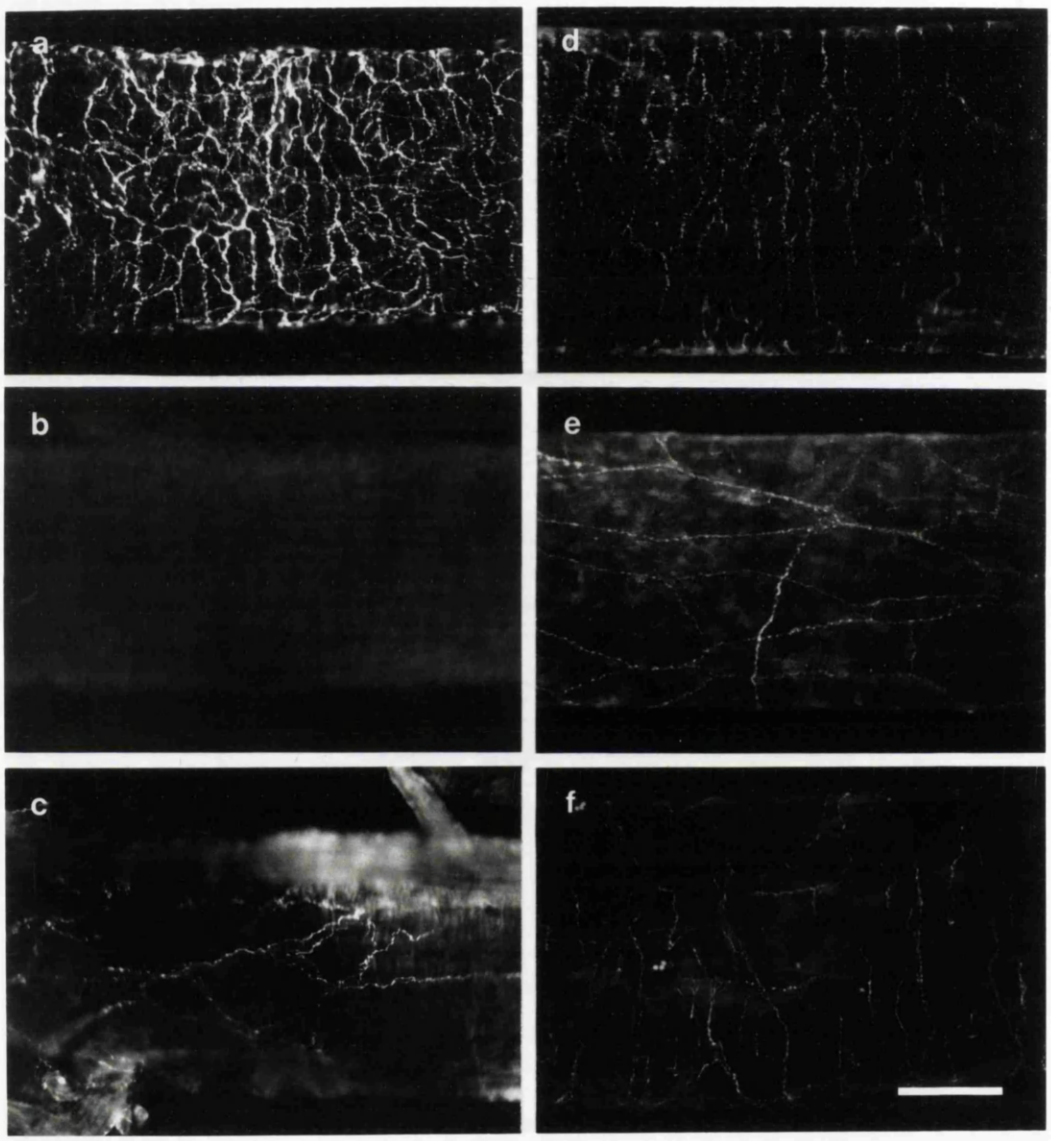
a
d
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Fig. 16 - Fluorescence micrographs of NA- (**a**, **b**) and NPY- (**c-e**) containing nerves in the rat iris from control (**a**, **c**), ganglionectomized (**d**) and guanethidine-treated (**b**, **e**) rats. Note the absence of NA-containing nerves and the increase of background autofluorescence in ciliary processes and blood vessels, after guanethidine treatment (**b**), while NPY-immunoreactive nerves formed a dense plexus (**e**). No NA-containing nerves were present in the rat iris after ganglionectomy, but a few NPY-immunoreactive nerve fibres could be visualized (**d**). Calibration bar = 100 µm.

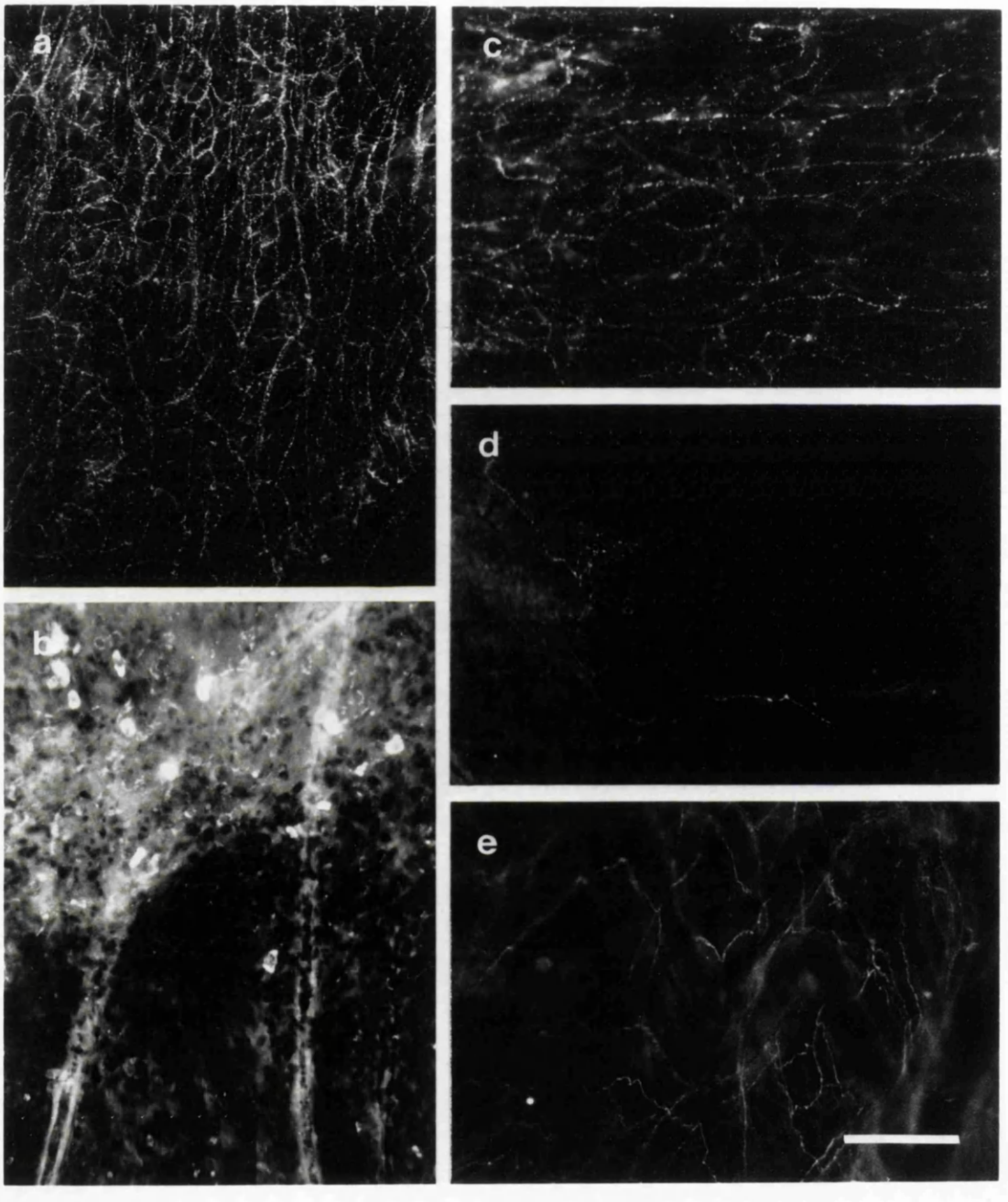
a
c
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Fig. 17 - Fluorescence micrographs of NA- (**a**, **b**) and NPY- (**c**, **d**) containing nerves in the rat dura mater from control (**a**, **c**) and guanethidine-treated (**b**, **d**) rats. Middle meningeal artery. Both NA- and NPY-containing nerves were eliminated by guanethidine treatment (**b**, **d**). Note the increase in fluorescent cells (probably mast cells) in the dura mater of the guanethidine-treated rat (**b**, glyoxylic acid technique). Calibration bar = 100 µm.

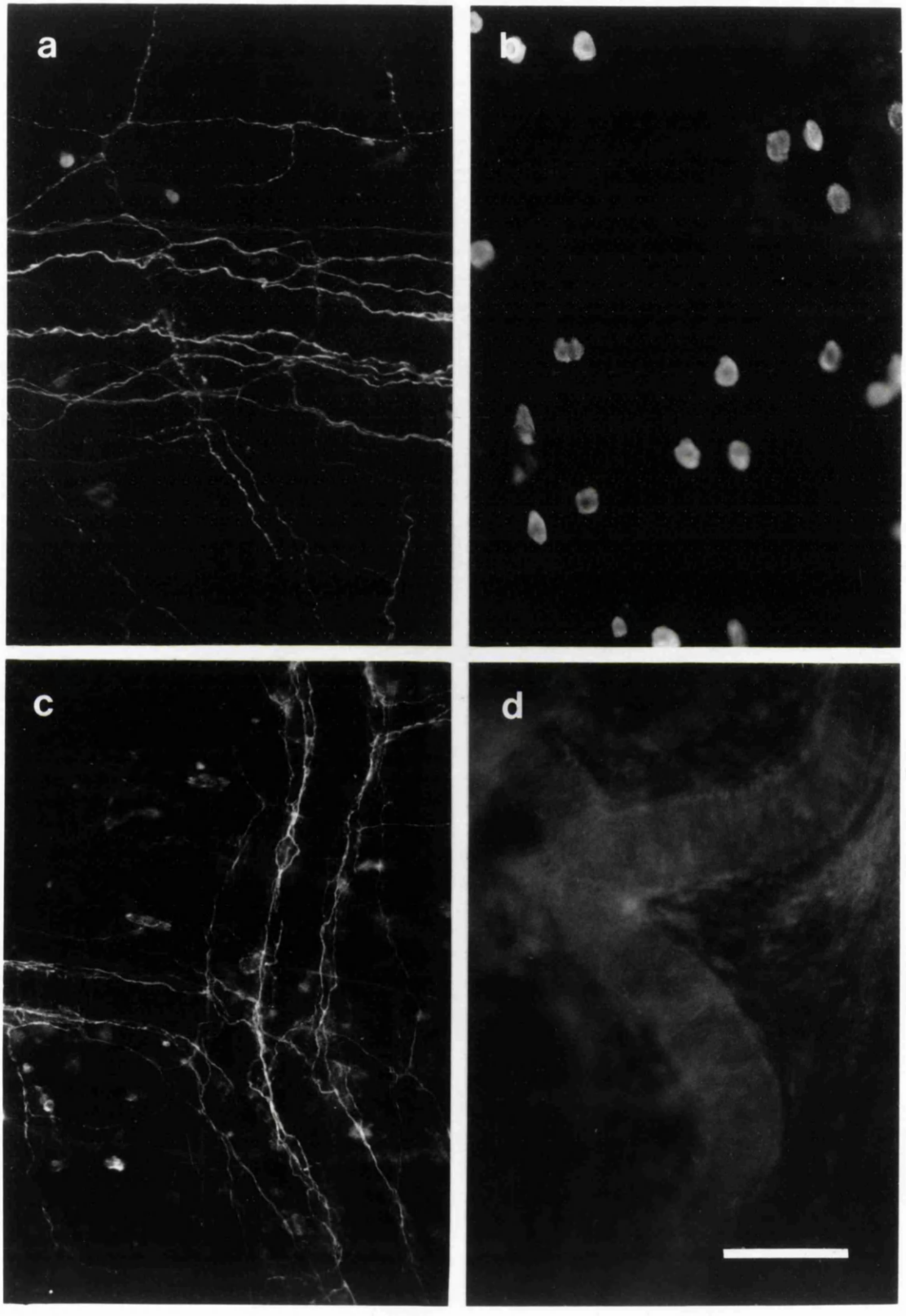
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Fig. 18 - Section (1 µm thick) of superior (**a**, **c**) and inferior (stellate) (**b**, **d**) cervical ganglia of control (**a**, **b**) and guanethidine-treated (**c**, **d**) rats.
Note the marked reduction in the number of neurones in both ganglia after treatment and an apparent increase in glial cells in both the SCG and the stellate ganglion (**c**, **d**). Calibration bar = 50 µm.

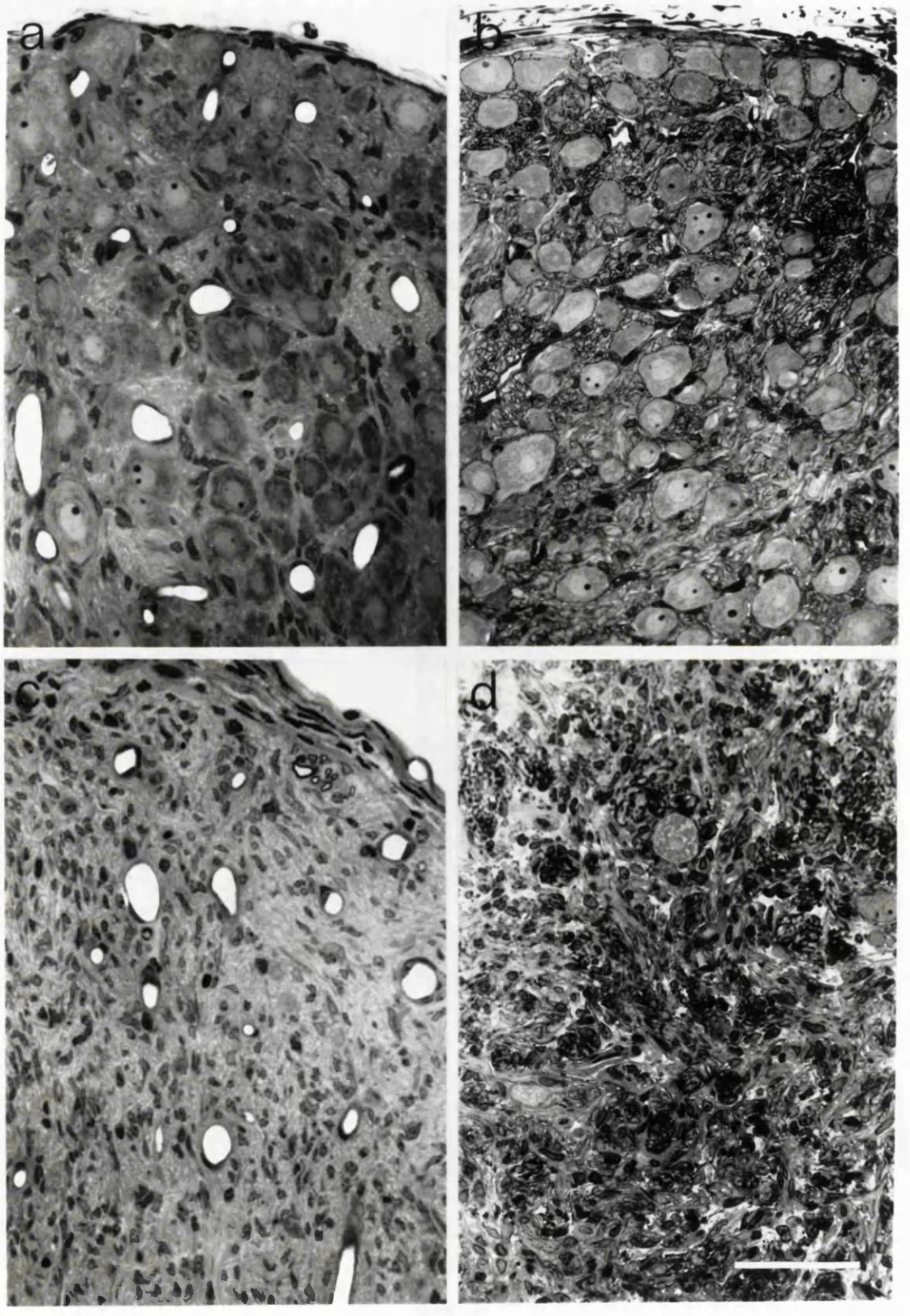
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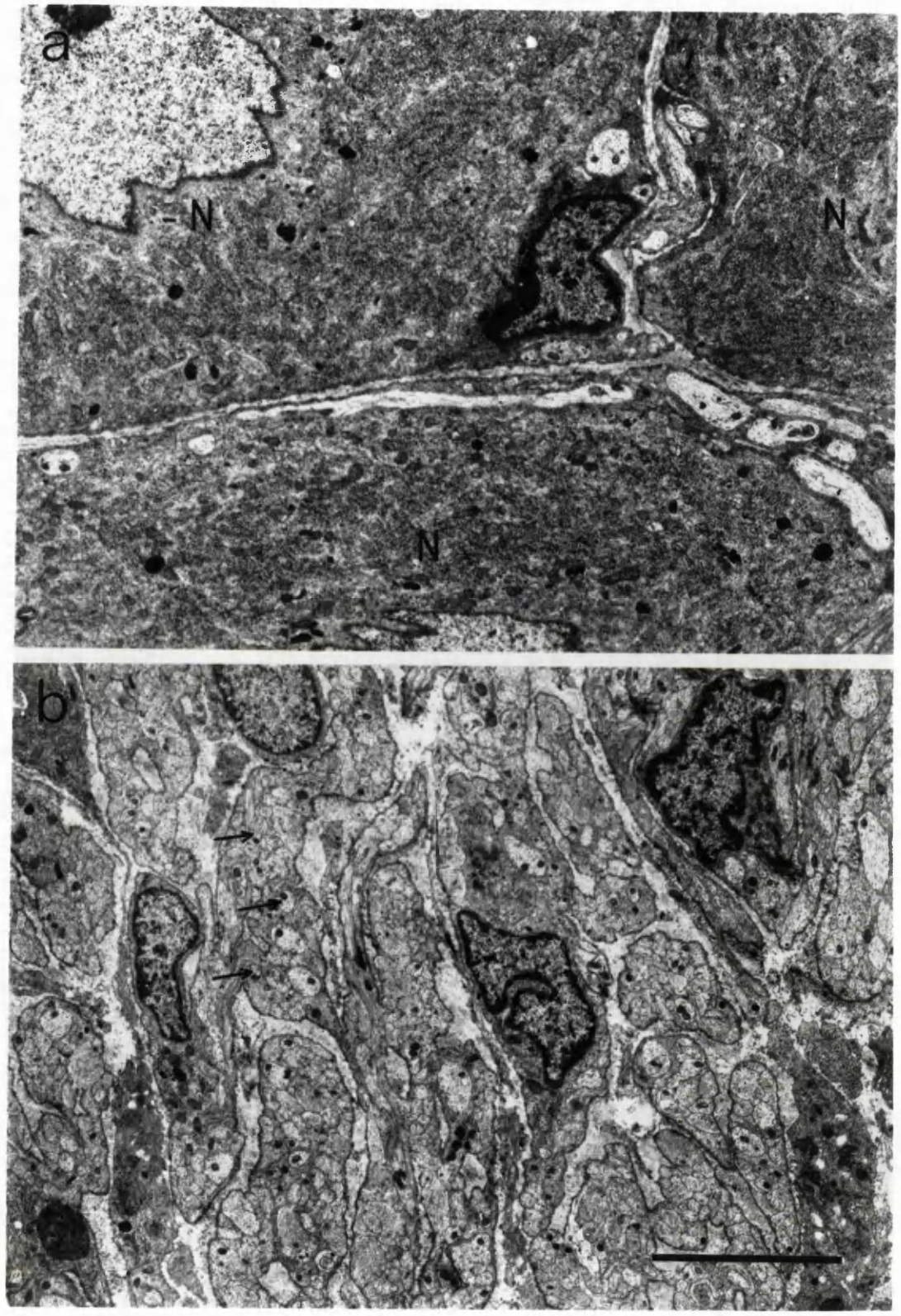
Fig. 19 - Electron micrographs of the SCG of control (**a**) and guanethidine-treated (**b**) rats. Note the abundance of non-myelinated axons (**arrows**) in the guanethidine-treated SCG. Calibration bar = 5 μm. N, neurones.


a
N
N
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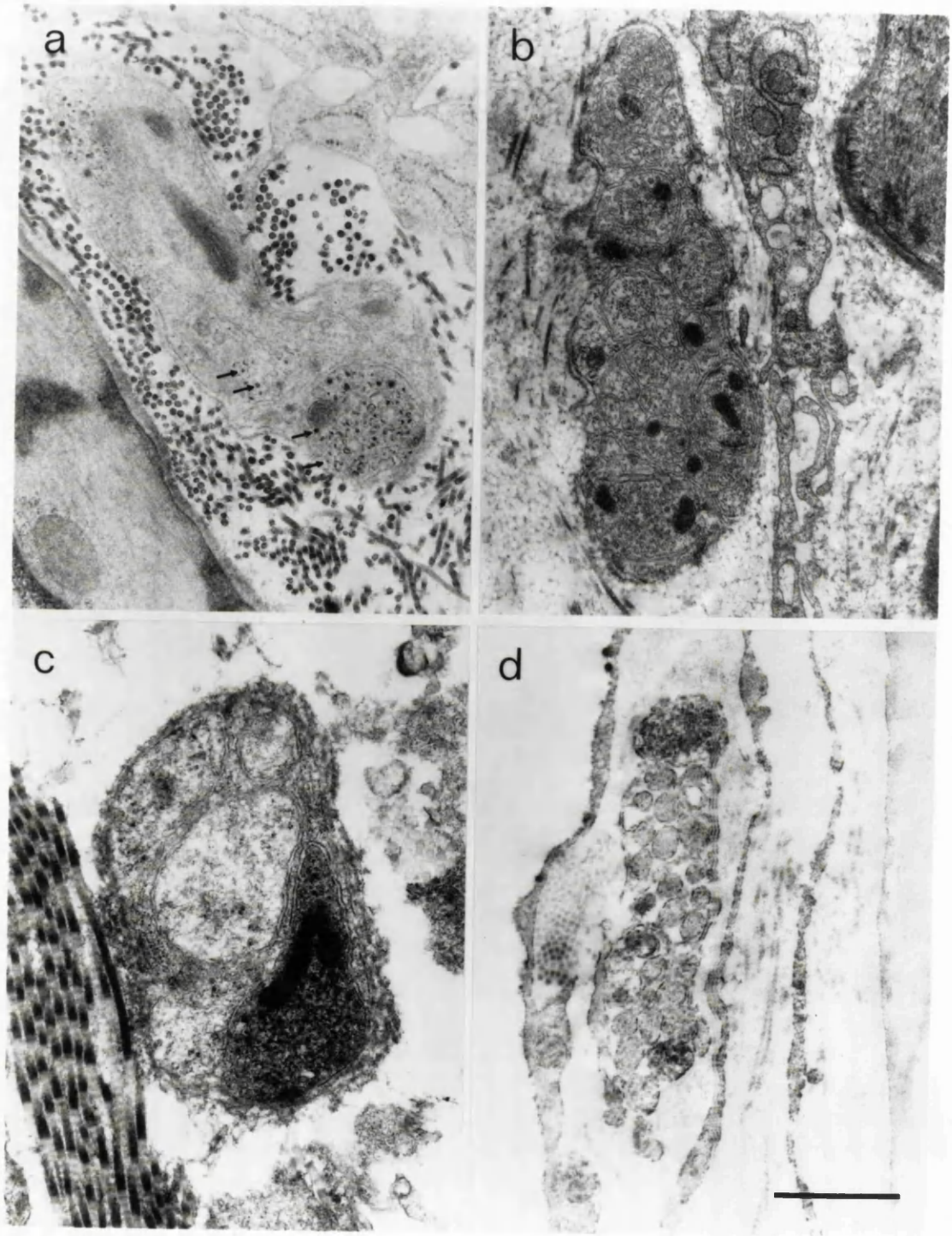
b

Fig. 20 - Electron micrographs of representative nerve bundles in the adventitia of the anterior cerebral artery of control (**a**, **c**) and guanethidine-treated (**b**, **d**) rats, after 5-OHDA-loading and (**c**, **d**) after immunostaining for NPY. Varicosities with 5-OHDA labelled vesicles (**arrows**) were frequent in the control (**a**, **c**) but very rare (if any) in the treated animals. Note the extreme electron density of mitochondria in **b**. NPY-immunoreactive nerves (open arrows) are usually labelled with 5-OHDA in control arteries (**c**), but not after guanethidine treatment (**d**).
Calibration bar = 1 µm.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CHAPTER THREE

USE OF ENHANCED SILVER STAINING COMBINED WITH ELECTRON MICROSCOPICAL IMMUNOLABELLING TO DEMONSTRATE THE COLOCALIZATION OF NEUROPEPTIDE Y AND VASOACTIVE INTESTINAL POLYPEPTIDE IN CEREBROVASCULAR NERVES

SUMMARY

The combination of immunolabelling at the electron microscope level and enhanced silver staining has been used to demonstrate the colocalization of neuropeptide Y and vasoactive intestinal polypeptide in perivascular nerves supplying cerebral arteries of the rat. This has been shown in control tissue, but it is easier to demonstrate after long-term sympathectomy since that leads to an enhancement of neuropeptide Y in vasoactive intestinal polypeptide-containing parasympathetic nerves supplying these vessels. Immunolabelling of the antigens for these peptides was performed sequentially with the biotin-streptavidin-diaminobenzidine method, and the end product to the first antiserum was gold-silver intensified before the visualization of the second antigen. Using this technique, it was shown that all the neuropeptide Y-immunoreactivity present in the rat cerebral vessels after long-term sympathectomy with guanethidine was localized in vasoactive intestinal polypeptide-containing nerves. Furthermore, an immunohistochemical analysis of the parasympathetic pterygopalatine ganglia in guanethidine-treated rats showed an increase in the percentage of neurons displaying neuropeptide Y-immunoreactivity. In order to clarify if the pterygopalatine ganglion was the origin of those neuropeptide Y/vasoactive intestinal polypeptide-immunoreactive cerebrovascular nerves, which had increased in number after sympathectomy, a fluorescent neuronal tracer (fast blue) was applied to the right middle cerebral artery of rats which had undergone guanethidine treatment for 6 weeks. Immunohistochemical analysis of

the ipsilateral ganglion 72 hrs after application of the tracer revealed the presence of immunoreactivity against both these peptides in retrogradely labelled neurons.

It is concluded that neuropeptide Y and vasoactive intestinal polypeptide are colocalized in perivascular parasympathetic nerves supplying the middle cerebral artery of the rat, which have their origin in the pterygopalatine ganglion. Furthermore, long-term sympathectomy with guanethidine leads to an increase in the expression of neuropeptide Y in these vasoactive intestinal polypeptide-immunoreactive neurons.

INTRODUCTION

Recent immunohistochemical studies have revealed the existence of a population of neuropeptide Y (NPY)-immunoreactive cerebrovascular nerves that survive sympathectomy achieved either by bilateral removal of the superior cervical ganglia (Gibbins and Morris, 1988) or guanethidine treatment (Mione et al., 1988; Chapter two). Moreover, an increase of NPY-immunoreactivity (IR) in vasoactive intestinal polypeptide (VIP)-containing cerebrovascular nerves following superior cervical ganglionectomy has been demonstrated in the guinea pig (Gibbins and Morris, 1988), even though surgical sympathectomy leads to a decrease of NPY-immunoreactive cerebrovascular nerves. Using long-term guanethidine treatment in developing rats, the compensatory expression of NPY in non-sympathetic nerves was shown to maintain the pattern and density of NPY-immunoreactivity in cerebrovascular nerves (Chapter two).

The present study was undertaken to localize at the ultrastructural level the immunoreactivity for NPY present in rat cerebral vessels after long-term sympathectomy with guanethidine. The origin of these non-sympathetic NPY-immunoreactive cerebrovascular nerves was also studied. The pterygopalatine ganglion, a parasympathetic ganglion, known to be the origin of VIP-IR supply to rat cerebral vessels (Suzuki et al., 1988) and also to contain NPY-immunoreactive neurons (Leblanc et al., 1987; Kuwayama et al., 1988), was studied immunohistochemically in control and guanethidine-treated animals. Finally, the pathway of NPY/VIP-immunoreactive axons from the middle cerebral artery to this ganglion

was studied in sympathectomized rats using a fluorescent neuronal tracer.

MATERIALS AND METHODS

Seven-day-old Wistar rats of both sexes were injected daily with guanethidine (Ismelin, CIBA), 60 mg/Kg, s.c. for six weeks. Control rats were injected with saline over the same period. Animals were perfusion-fixed on the same day of the last injection with 300 ml of 4% paraformaldehyde, 0.1% glutaraldehyde and 15% saturated picric acid in 0.1 M phosphate buffer. Dissection of the cerebral vessels was carried out and fixation continued from a further two hrs. The cerebral vessels were cleared in 50% ethanol, treated with 0.1% $NaCNBH_3$, in phosphate buffer and incubated with 0.3% H_2O_2 in 50% methanol for 10 min.

The double immunostaining of NPY and VIP was carried out as described by Maegawa et al., 1987. A preincubation with 10% normal donkey serum (NDS) in phosphate buffered saline (PBS) was followed by incubation with either anti-NPY (Eugene Tech., U.S.A.; diluted 1:400) or anti-VIP (Peninsula Lab, U.K.; diluted 1:1000) for 18 h at room temperature. Cerebral vessels were then treated with biotin-streptavidin-horseradish peroxidase complex (Amersham, U.K.) and reacted with diaminobenzidine (DAB) and H_2O_2. After several washes in PBS, vessels were incubated with the other first layer (i.e. anti-VIP antiserum, if anti-NPY was used in the previous immunoreaction) and then reacted with the biotinilated donkey anti-rabbit antiserum for 12 hrs. At this stage, the silver-gold intensification of the DAB end- products to the first antiserum was performed as described by Gallyas et al., 1982. Briefly, after 3 hrs in 15% thioglycolic acid, the vessels were reacted with a freshly prepared physical developer

(by mixing an equal volume of 5% sodium carbonate with a solution containing 0.1% ammonium nitrate, 0.1% silver nitrate, 1% phosphosilicotungstic acid and 1.8% paraformaldehyde) for 10 min. After a brief wash with 1% acetic acid, the deposited silver particles were replaced with gold, by immersion in 0.05% gold chloride for 8 min at 4 °C and finally fixed in 3% sodium thiosulphate (3 x 10 min). Vessels were then reacted with streptavidin horseradish peroxidase complex for 12 hrs followed by DAB plus H_2O_2 to visualize the second applied antiserum. Some photographs were taken at the light microscope either before the silver intensification or after the whole procedure. Samples of cerebral vessels were osmicated, dehydrated, stained en bloc with 2% uranyl acetate in 70% ethanol and embedded in Spurr resin. Ultrathin sections were cut with an ultramicrotome (LKB), stained with lead citrate and examined with a Philips 300 electron microscope.

Some experiments were carried out in order to establish the following : (i) the effectiveness of guanethidine-treatment in inducing sympathectomy. The glyoxylic acid technique was applied to fresh cerebral arteries from animals of the same litters and subjected to the same treatment, and semithin sections of the superior an inferior cervical ganglia of the same animals used for immunocytochemistry were cut and the absence of neurons (see Chapter two); (ii) whether one of the two antigens (NPY or VIP) could be damaged by the chemicals used during the silver intensification. To test this possibility, vessels were incubated with the first antiserum, reacted with the biotinilated donkey anti-rabbit antiserum and processed as for the silver intensification. Both immunoreactivi-

ties for NPY and VIP were unaffected by the treatment; (iii) whether all the DAB-end products formed during the first immunoreaction had undergone silver-gold intensification, so that they were clearly distinguishable from those formed in the second immunoreaction, if this occurred in different nerves. In vessels subjected to only one immunostaining sequence followed by the silver-gold intensification, all the DAB-end products were found covered by variable amounts of silver granules. No silver particles and/or DAB end-products were seen if the procedures were carried out using antisera preabsorbed with an excess of the appropriate neurotransmitter or by replacing any of the antisera with normal rabbit serum.

For the pterygopalatine ganglion, dissection from the surrounding tissue was carried out during the fixation time (2 hrs in 4% paraformaldehyde) occasionally using a gross anatomical preparation of cranial nerves stained with a modified thiocholine method as an aid to the identification of the ganglion (Kuwayama et al., 1987). The ganglia were rinsed in PBS several times and equilibrated in 20% sucrose in PBS overnight. Cryostat sections, 7 µm thick, were collected on gelatine-coated microscope slides and alternatively processed for VIP or NPY immunoreactivity. A goat anti-rabbit fluoresceine isothiocyanate (FITC)-conjugated antiserum (Nordic, Denmark) was used at a dilution 1:100. Pair of sections stained with anti-VIP or anti-NPY antisera were compared and the immunoreactive neurons counted. The coverslips were then removed, the sections stained with toluidine blue and the total number of neurons in the sections counted. At least 100 neurons for each of the five ganglia were examined.

For the neuronal tracing study, five guanethidine-treated rats, seven weeks old, were anaesthetized with fentanyl citrate and fluanisone (Hypnorm, Janssen, Oxford, U.K.; 50 mg/Kg). Using a dental drill, a window was opened in the skull of the rats at the level of the parieto-temporal region. The dura mater covering the parietal branch of the middle cerebral artery was carefully opened on three sides and 1 mm-strip of parafilm was pulled under the artery. A 1 mm^2 gelatine sponge sheet soaked in a 2% acqueous solution of fast blue (Sigma) was applied to the exposed artery and left in situ. The parafilm strip was brought over, as well as the dura, and the skull window sealed with dental wax. After three days, animals were anaesthetized and perfused as above; the ipsilateral pterygopalatine ganglion was removed and serially cut (7 µm) with cryostat. Every second or third section was coverslipped and examined under a fluorescent microscope equipped with the appropriate filter setting for alternate observation of fast blue (excitation 380 nm; emission 420 nm) and FITC (excitation 490 nm; emission 525 nm) fluorescence. When retrogradely labelled neurons were found in a section, that section was stained for NPY-IR and the following one for VIP-IR.

RESULTS

Cerebral vessels - light microscopy

The distribution of NPY- and VIP-immunoreactive perivascular nerves in rat cerebral vessels, illustrated in Fig. 21a,b, resembled that described in earlier studies. In vessels labelled for both antigens (Fig. 21c) the density of immunoreactive nerves appeared as the sum of NPY- and VIP-immunoreactive nerve fibres.

In animals sympathectomized with long-term guanethidine treatment, noradrenaline-containing cerebrovascular nerves have been shown to disappear, while the pattern and distribution of NPY-immunoreactive nerves was little changed (Fig. 21d). No changes in density and pattern occurred to VIP-immunoreactive cerebrovascular nerves after guanethidine treatment (Fig. 21e). In this situation, the pattern of NPY-immunoreactive cerebrovascular nerves clearly resembled that of the VIP-immunoreactive nerves (cf. Figs. 21d and 21e) and when these arteries were double immunostained for both antigens (Fig. 21f), no further increase in the density of immunopositive nerves was seen, compared with vessels stained for one antigen only.

Cerebral vessels - electron microscopy

In ultrathin sections of the middle cerebral artery of control animals subjected to the double immunostaining, regardless of which antigen was applied first, two kinds of immunoreactive nerves could be visualized: those containing DAB end-products plus a variable amount of silver granules and those that lacked silver granules (Fig.

22a,b). Some NPY-immunoreactive nerves clearly lacking VIP-immunoreactivity containined dense-cored vesicles, indicating a possible coexistence with noradrenaline (Fig. 22b).

In animals sympathectomized by long-term guanethidine treatment, the results obtained were different according to whether NPY- or VIP-immunoreactivity was silver-gold intensified. When NPY-immunoreactivity was intensified, a number of immunoreactive nerves lacking silver granules (and corresponding to nerves containing only VIP-IR) were always present (Figs. 23a, 24b). Nerve profiles with conclusively only NPY-IR were shown by omitting the second immunoreaction (Fig. 24a). However, when the VIP-antiserum was applied first and the DAB end-products were silver intensified followed by anti-NPY immunostaining, all the immunostained nerves also showed silver deposition (Fig. 23b), thus indicating that all the NPY-immunoreactivity, visualized at both the light microscope (Fig. 21d) and at the electron microscope (Figs. 23a, 24a) is contained in VIP-immunoreactive nerves.

Pterygopalatine ganglia

In pterygopalatine ganglia from control animals, 91 neurons out of 638 exhibited immunoreactivity for NPY (14.23%; Figs. 25b and 26a) and 276 neurons out of 639 were immunoreactive for VIP (43.26%; Fig. 25a).

In guanethidine-treated animals, no changes in the total number of pterygopalatine ganglion neurons were apparent, and no degenerating neurons were found in conventionally stained cryostat sections of the ganglia. 540 neurons from five different animals

were evaluated: 236 were NPY-immunoreactive (43.70%; Fig. 26b) and 250 were VIP-positive (46.29%; Fig. 26c). By comparing serial sections it became clear that almost all the NPY-immunoreactivity was confined to VIP-immunopositive cells (Figs. 26b,c).

Neuronal tracing

Following application of the neuronal tracer fast blue to the middle cerebral artery of guanethidine-treated rats, an average of 5 neurons per ganglion were found retrogradely labelled after 72 hrs (Fig. 27a). These neurons were invariably immunoreactive for both NPY and VIP as shown in that section and in the following one (Figs. 27b,c).

DISCUSSION

In Chapter two it was shown that the NPY-immunoreactivity in cerebral vessels remained, or was even enhanced, after chemical sympathectomy with guanethidine, and this was due to an increase of the expression of NPY-immunoreactivity in non-sympathetic nerves. In the present study, the origin of the non-sympathetic cerebrovascular nerves that express NPY-immunoreactivity after sympathetic denervation was studied using a double immunolabelling technique at the electron microscope level to localize precisely the NPY-immunoreactivity in cerebrovascular nerves of sympathectomized animals. The finding that, in guanethidine-treated rats, all the NPY-immunoreactivity was localized in VIP-containing axons is consistent with the report of Gibbins and Morris, 1988, who found an increase in the expression of NPY-immunoreactivity in VIP-containing cerebrovascular nerves of surgically denervated guinea-pigs using double immunolabelled at the light microscope. Recently, the origin of VIP-immunoreactive nerves was traced in rats and shown to be the sphenopalatine ganglion (Suzuki et al., 1988) which is now known as the pterygopalatine ganglion.

In this study NPY-immunoreactivity was found to be present in about 15% of the rat pterygopalatine neurons and this is consistent with the results of two other groups (Leblanc et al., 1987; Kuwayama et al., 1988).

After long-term sympathectomy with guanethidine in the rat, we found an increase in the percentage of NPY-immunoreactive neurons in the rat pterygopalatine ganglion when stained sequentially for NPY-

and VIP-immunoreactivity. It was evident that the increase concerned the expression of NPY-immunoreactivity in VIP-containing neurons. In our preparations, VIP-containing neurons representing up to 50% of the total number of neurons, while Leblanc et al., 1987, claimed that VIP-immunoreactive neurons represent the majority of the neuronal population of the rat phenopalatine ganglion. Following guanethidine-treatment, no increase in the number of VIP-immunoreactive neurons was found in the pterygopalatine ganglion, but almost all of them now expressed NPY-immunoreactivity. It is not known how the sympathectomy produced by long-term guanethidine treatment is able to turn on the expression of NPY, in parasympathetic cerebrovascular neuroness. The role of NPY coexisting with VIP in parasympathetic nerves needs investigation.

Fig. 21 - Light micrographs of NPY- (**a**,**d**), VIP- (**b**,**e**) and NPY- plus VIP- (**c**,**f**) containing nerves on the middle cerebral artery of control (**a**,**b**,**c**) and guanethidine-treated (**d**,**e**,**f**) rats. Biotin-streptavidin-horseradish peroxidase technique. The DAB end-products of NPY- (**a**,**c**,**d**,**f**) or VIP- (**b**,**e**) immunoreactivity were subjected to silver intensification in order to enhance the immunoreaction. Note that: (i) some NPY-containing nerves clearly remained after long-term sympathectomy, although at a reduced density (cf. **a** and **d**); (ii) with double-labelling for NYP and VIP, the density of nerves is greater than that of the individual labels in controls (cf. **a**,**b** and **c**), but has a similar density to the individual labels after long-term sympathectomy with guanethidine (cf. **d**,**e** and **f**). Calibration bar: 50 μm.

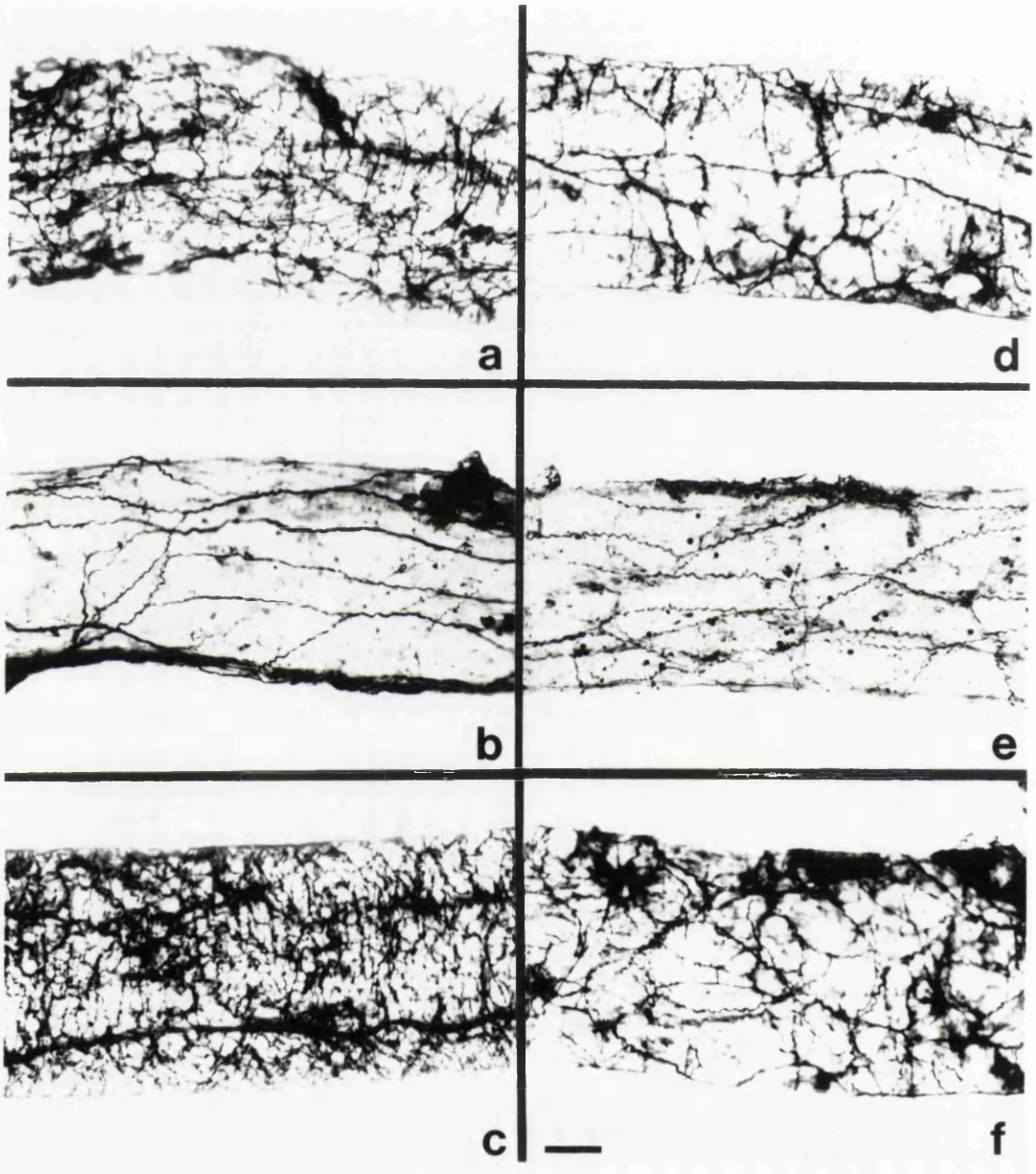
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Fig. 22 - Electron micrographs of immunopositive nerve bundles within the wall of the middle cerebral artery of control rats. Double immunostaining for NPY- and VIP-immunoreactivity. (a) NPY-immunoreactivity was further subjected to silver intensification so that the NPY-immunopositive nerve (**long arrow**) is clearly distinguishable from the other immunopositive nerves, which contain only VIP-immunoreactivity (**short arrows**). (b) In this case it was the VIP-immunoreactivity that was further subjected to silver intensification, silver granules being deposited on two VIP-immunopositive nerve profiles (**long arrow**) and not onto nerves that were only positive for NPY (**short arrows**). Some dense-cored vesicles (**small arrows**) were seen in sympathetic NPY-immunoreactive nerves that did not contain VIP-LI. **Asterisks** indicate unstained nerves. Calibration bars: 1 μm.

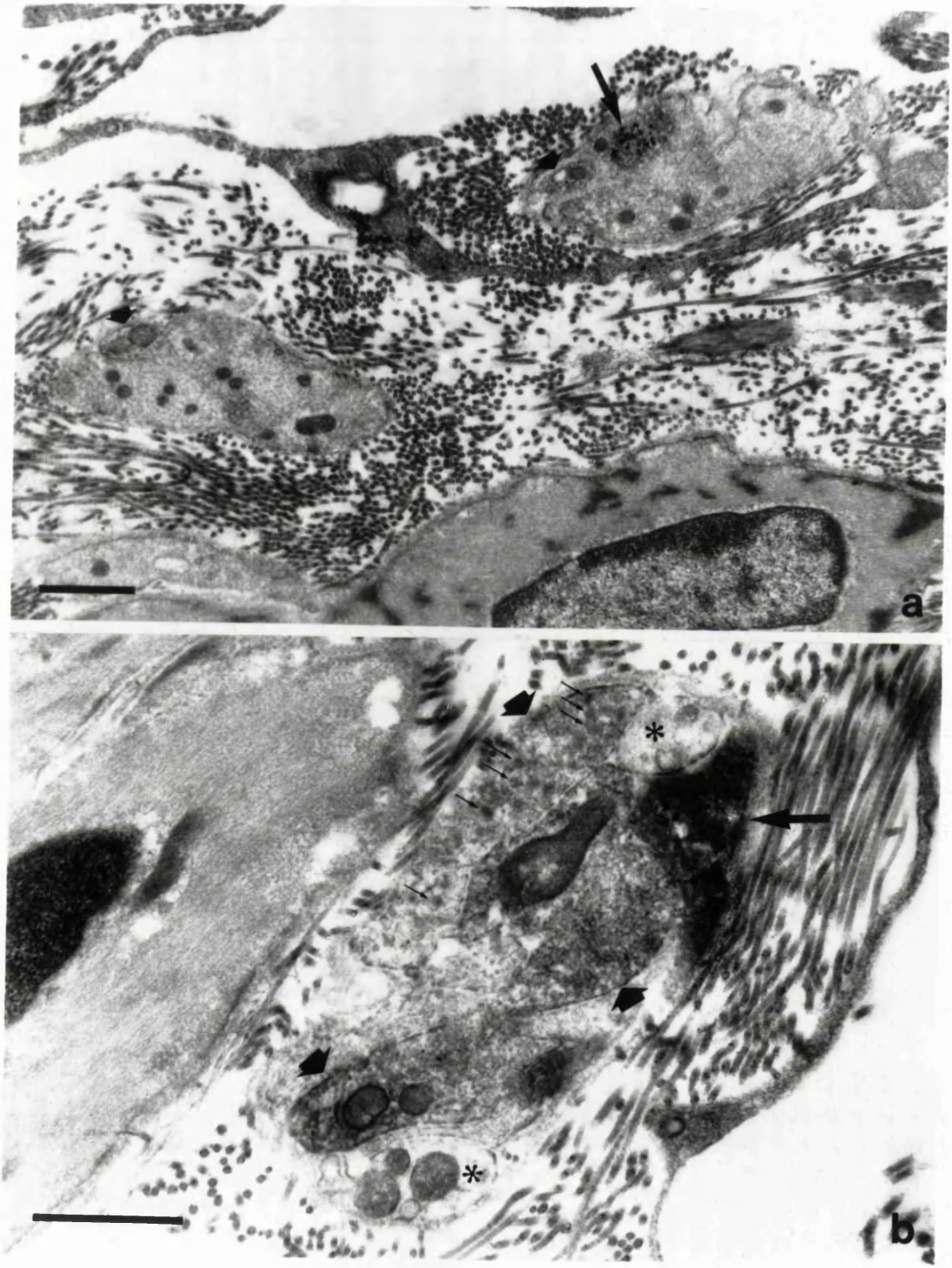
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Fig. 23 - Electron micrographs of immunopositive nerves, double-stained for NPY and VIP, within the wall of the middle cerebral artery of guanethidine-treated rats. (**a**) NPY-immunoreactive nerves were subjected to silver granule deposition (**long arrow**), followed by the VIP-immunoreaction; two nerves that were immunoreactive only for VIP and did not contain silver granules are indicated by short arrows. (**b**) The VIP-immunoreaction with silver deposition preceded the NPY-immunoreaction (**long arrows**). No nerves containing only NPY, which would have lacked silver granules, could be seen in double immunostained vessels.
Calibration bars: 1 μm.

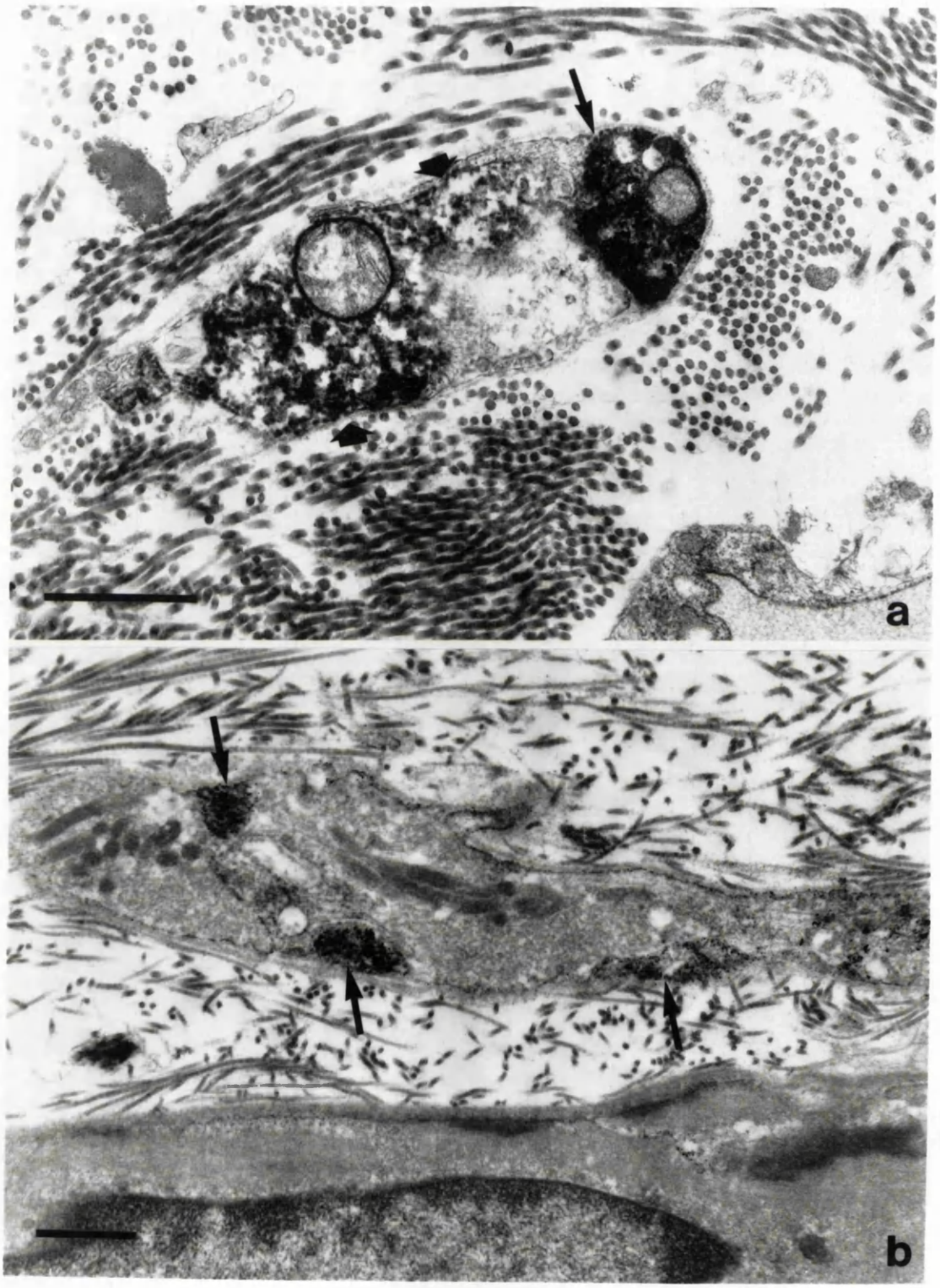
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Fig. 24 - Electron micrographs of immunostained nerves within the wall of the middle cerebral artery of guanethidine-treated rats, for comparison with Fig. 23. (**a**) The NPY-immunoreaction was followed by silver intensification, with no immunostaining for VIP; all the immunostained nerves had silver granules. (**b**) A VIP-immunopositive nerve from an artery that had first been immunostained for NPY and silver intensified. Calibration bars: 1 μm.

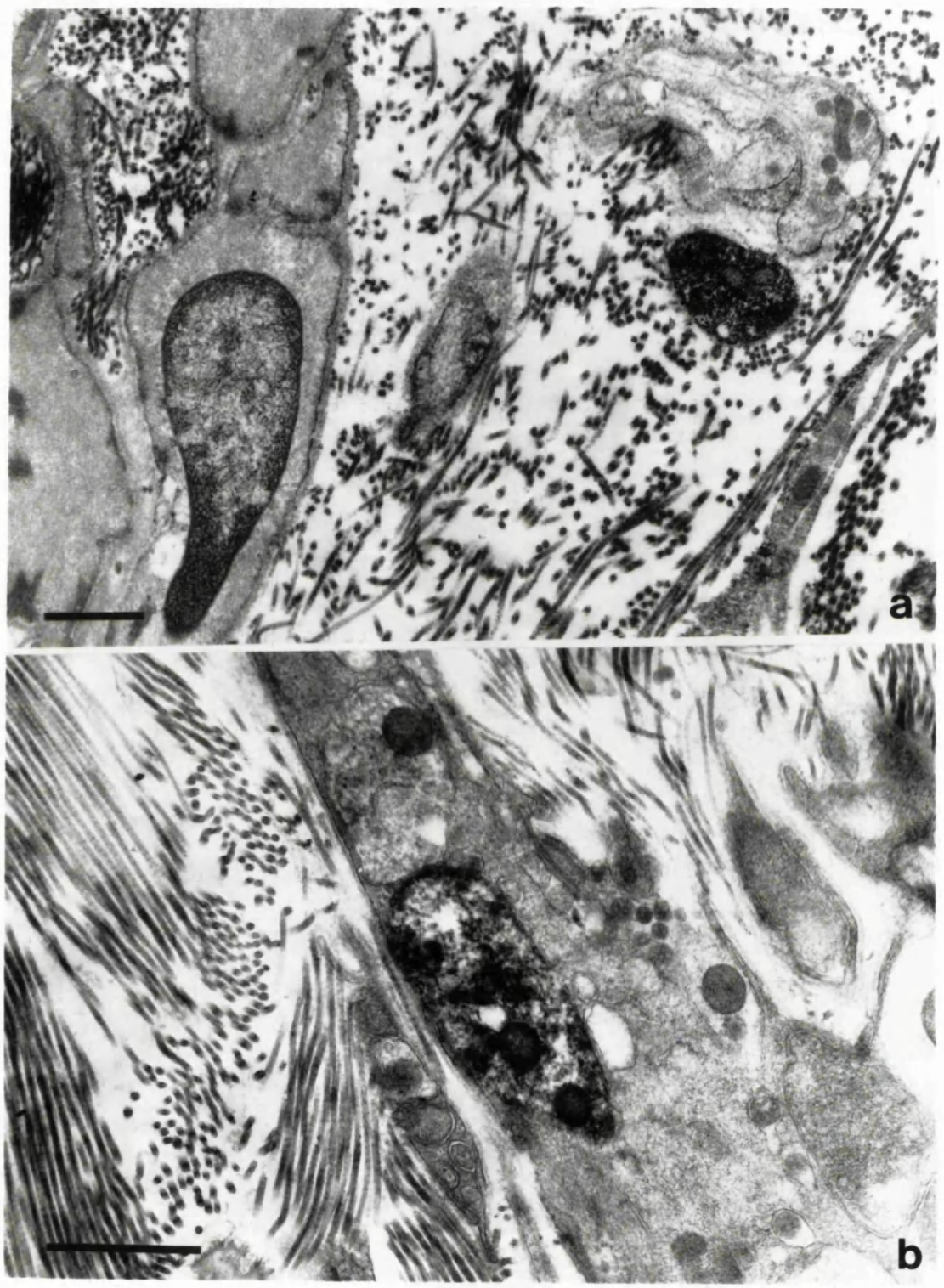
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Fig. 25 - Fluorescence micrographs of pterygopalatine ganglia from control rats. (**a**,**b**) are consecutive sections immunostained for VIP-IR (**a**) and NPY-IR (**b**) and show the degree of coexistence of the two neurotransmitters. In control rats only some neurons contained both neurotransmitters (**short arrows**) and many were immunoreactive for only one peptide (**long arrows**). Calibration bar: 50 µm.

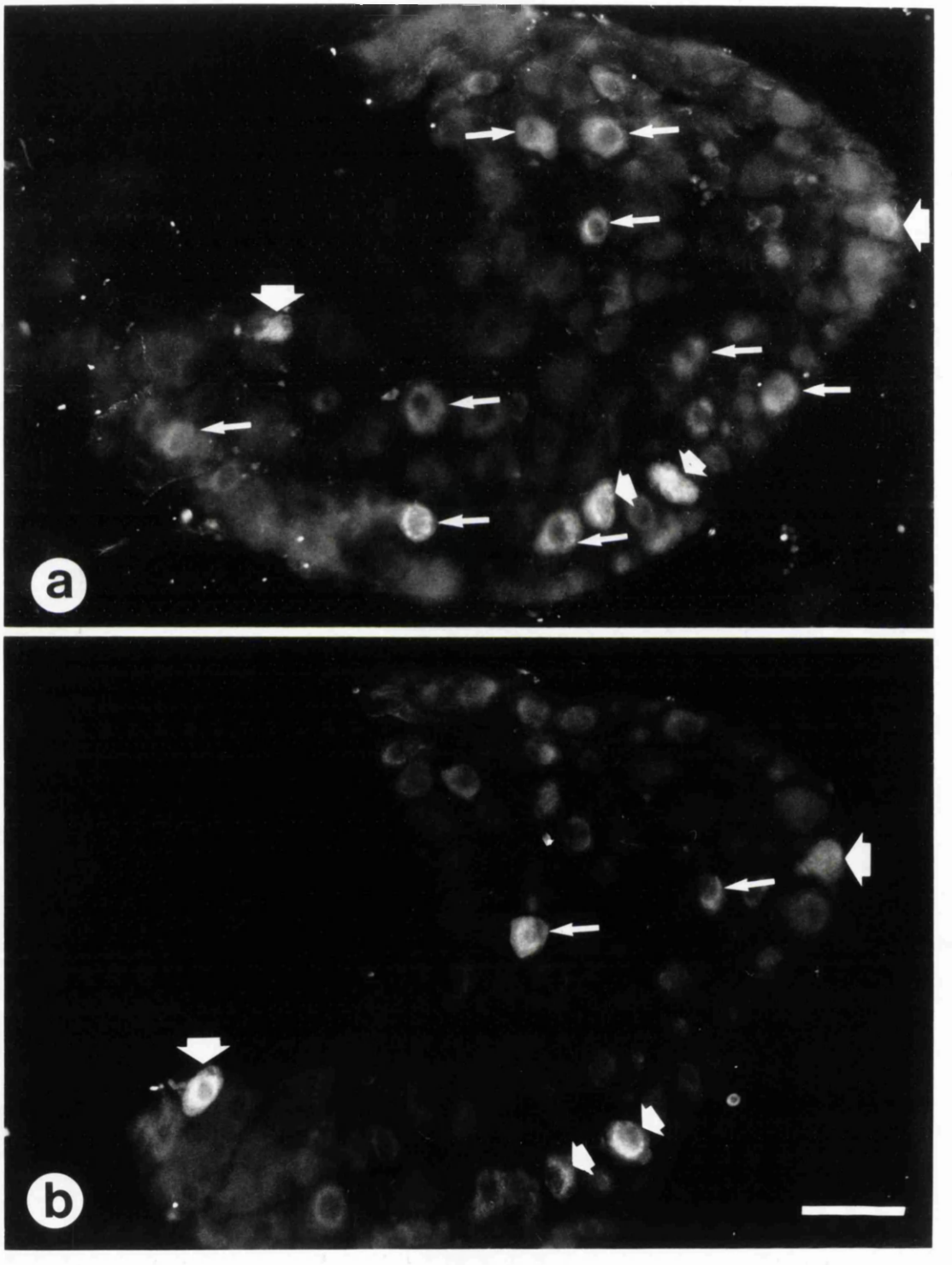
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Fig. 26 - Fluorescent micrographs of pterygopalatine ganglia from (**a**) a control rat and (**b,c**) a guanethidine-treated rat, showing NPY-IR (**a,b**) or VIP-IR (**c**). Note the increase of NPY-immunoreactive neurons in the pterygopalatine ganglion from a long-term guanethidine-treated rat (**b**) compared with a control ganglion (**a**) and the almost complete overlapping of NPY- and VIP-immunopositive neurons in consecutive sections of the ganglion from a guanethidine-treated rat (**b,c**).
Calibration bar: 50 µm.

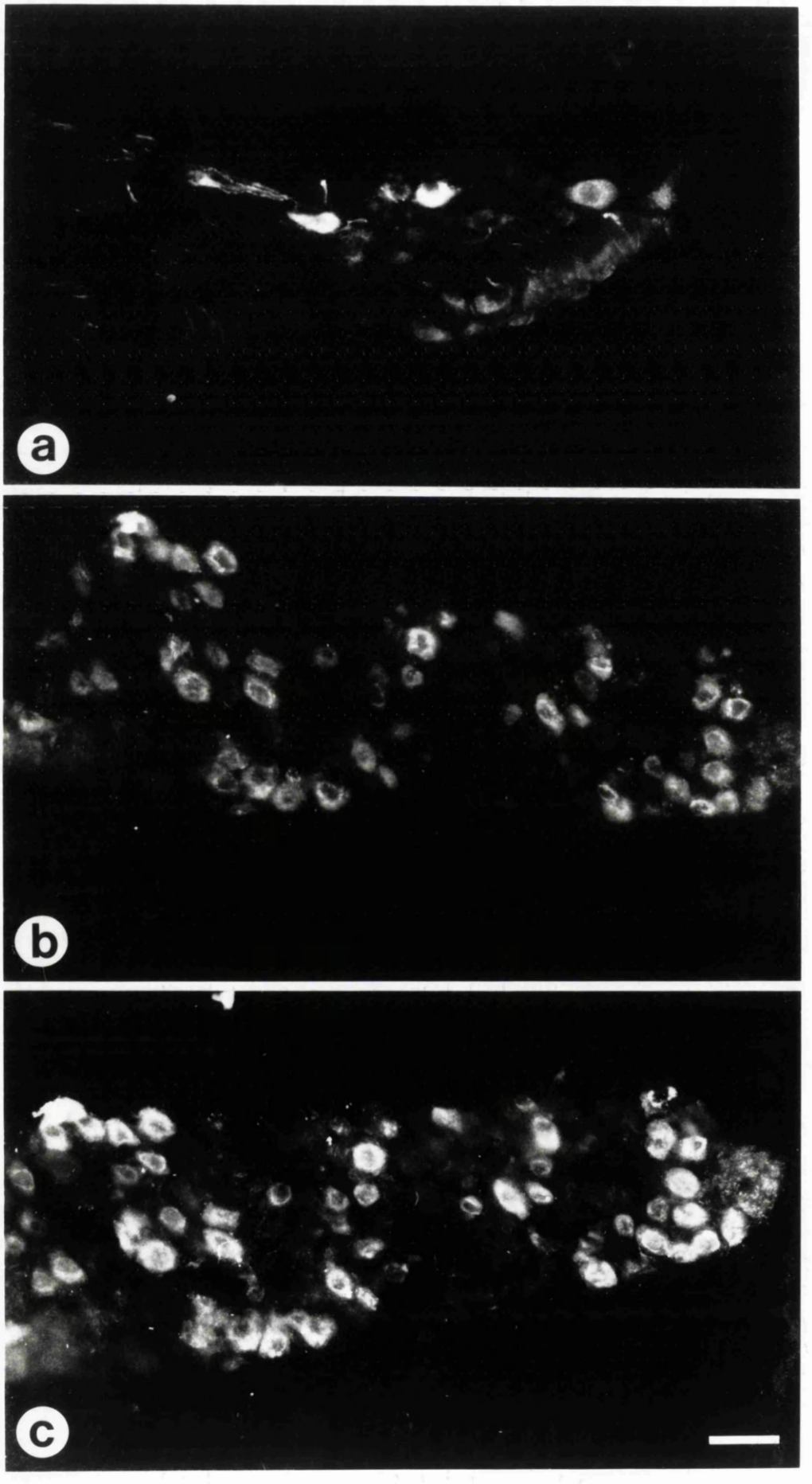
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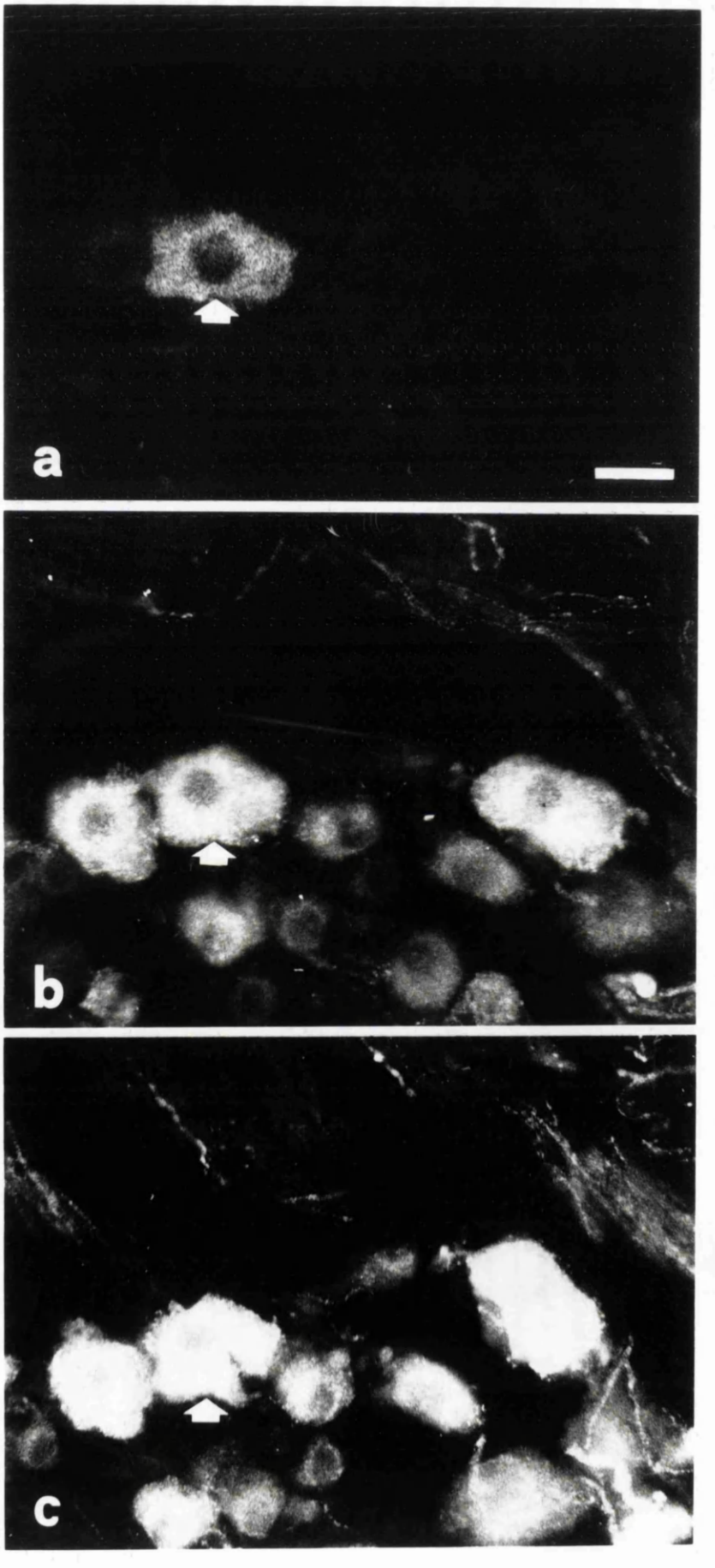
Fig. 27 - A neuron (**arrow**) showing (**a**) fast blue positivity and (**b**) NPY-IR in the same section of the pterygopalatine ganglion from a guanethidine-treated rat 72 hrs after the application of the tracer to the ipsilateral middle cerebral artery. In the consecutive section (**c**) the same NPY-IR neuron is also shown to contain VIP-immunoreactivity. Calibration bar: 20 µm.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CHAPTER FOUR

PLASTICITY IN EXPRESSION OF CALCITONIN GENE-RELATED PEPTIDE- AND SUBSTANCE P-IMMUNOREACTIVITY IN GANGLIA AND FIBRES FOLLOWING SYMPATHETIC AND/OR SENSORY DENERVATION

SUMMARY

The distribution of calcitonin gene-related peptide (CGRP) and substance P (SP) immunoreactivity in sympathetic (superior and inferior cervical) ganglia and sensory (trigeminal and first three dorsal root) ganglia was studied in rats subjected to selective sympathetic denervation (by long-term guanethidine treatment), sensory denervation (by capsaicin injections) or combined (sensory + sympathetic) denervation procedures. Changes in the density of immunoreactive nerve fibres in selected cranial targets (dura mater, anterior cerebral artery and iris) were quantified with the aid of an image analyzer.

The results of the study in rats indicate that:

(1) long-term guanethidine sympathectomy induced a pronounced increase of CGRP- and SP-immunoreactive (IR) sensory nerve fibres in all the tissues investigated in contrast to a significant reduction of CGRP- and SP-immunoreactivity in sensory nerve cell bodies in both trigeminal and dorsal root ganglia;

(2) neonatal capsaicin treatment abolished SP-immunoreactivity in many nerve fibres in the dura mater, anterior cerebral artery and sympathetic ganglia and caused a dramatic reduction of SP-IR nerve cell bodies in both trigeminal and dorsal root ganglia; however, while CGRP-IR nerve density was decreased, the number of CGRP-IR sensory nerve cell bodies was unchanged;

(3) guanethidine treatment of capsaicin injected rats reversed the loss of CGRP-IR nerve fibres in the dura mater, anterior cerebral artery and sympathetic ganglia, but not that of SP-IR nerve fibres.

The number of sensory neurons expressing CGRP-immunoreactivity was increased;

(4) in the iris, the increase of substance P- and calcitonin gene-related peptide-immunoreactive nerve fibres after sympathectomy was not affected by previous capsaicin treatment suggesting that in rats the majority of substance P- and CGRP-IR fibres originate from capsaicin insensitive pterygopalatine and ciliary ganglion neurons.

INTRODUCTION

The neuropeptide, calcitonin gene-related peptide (CGRP) (Rosenfeld et al., 1983) in the periphery has been implicated mainly with sensory functions and in particular with pain transmission (Gibson et al., 1984; Skofitsch and Jacobowitz, 1985). CGRP immunoreactivity was shown to be present in small neurons of the trigeminal and dorsal root ganglia (Gibbins et al., 1985; Rosenfeld, 1983; Gibson et al., 1985; Franco-Cereceda et al., 1987) and in areas known to be rich in sensory nerve fibres (Terenghi et al., 1985; Gibson et al., 1984) and to be markedly depleted by neonatal capsaicin treatment (Skofitsch and Jacobowitz, 1985), which causes selective degeneration of C- and Aδ-fibres and type B neurons (Jancso et al., 1977; Scadding, 1980). With respect to cranial structures, CGRP immunoreactivity has been shown to be localized also in neurons of cranial parasympathetic (pterygopalatine, otic, ciliary and submandibular) ganglia (Lee et al., 1985a; Silverman and Kruger, 1989).

In a recent study from our laboratory (Aberdeen et al., 1990) a dramatic increase of CGRP-immunoreactive (IR) nerve fibres in various peripheral vessels and organs was shown to occur after long-term chemical sympathectomy. This finding is consistent with previous reports of the increase in CGRP immunoreactivity levels and distribution after surgical (Schon et al., 1985a) or chemical (both 6-hydroxydopamine and guanethidine treatments) sympathectomy in rat cerebral vessels (Schon et al., 1985a), iris (Terenghi et al., 1986) and genital organs (Lamano-Carvalho et al., 1986). So far, an

increase of substance P (SP) immunoreactivity after sympathectomy was found in rat salivary glands (Ekström et al., 1988) or the rat iris after surgical or 6-hydroxydopamine treatment (Cole et al., 1983; Kessler, 1985a).

In the present study, the relative changes in CGRP and SP immunoreactivity following sympathectomy, neonatal capsaicin treatment, or both, were analysed in sympathetic and sensory ganglia as well as in the dura mater, anterior cerebral artery and iris.

MATERIALS AND METHODS

Eight litters of Wistar rats (8-12 pups each) were used in this study. Two litters were treated with capsaicin (Sigma, Poole, UK) 50 mg/kg dissolved in saline containing 10% ethanol and 10% Tween 80, subcutaenously, under ice or light ether anaesthesia on days 1, 2, 4, 6, 8, 10, 12 and 14. Five out of 22 rats died in the first week. 2 litters were treated with guanethidine (Sigma), 60 mg/kg dissolved in saline s.c. from day 7 for 6 weeks daily. Only two out of 24 animals died at the beginning of the treatment. Two litters were treated with capsaicin as above, and from day 7 they were also treated with guanethidine, 60 mg/kg s.c. as above. 7 out of 22 of these rats died in the first two weeks of life. The remaining two litters were used as controls, and treated with both the capsaicin vehicle and guanethidine vehicle over the same period. None of these rats died. All the animals were sacrificed at 3 months of age. Two rats from each group were used for catecholamine histofluorescence (Lindvall and Björklund, 1974; De la Torre and Surgeon, 1976) of whole-mounts of dura mater, anterior cerebral artery and iris: no fluorescent nerves were visualized in guanethidine or capsaicin + guanethidine-treated animals, while vehicle- and capsaicin-treated rats displayed noradrenaline-containing fluorescent nerve plexuses in all the whole-mounts studied. However, the degree of sympathetic denervation was also assessed by a histological study of the superior cervical and stellate ganglia in all the animals; this showed an almost complete disappearance of neurons in these ganglia from guanethidine and capsaicin and guanethidine-treated rats. All the other animals were

perfusion-fixed under pentobarbital anaesthesia. The fixative was 4% paraformaldehyde in 0.1 M phosphate buffer. The superior cervical and stellate ganglia, dorsal root ganglia C1-3, skull, eyes and brains were dissected out and immersed in the same fixative for 2-9 h, while dissection of the trigeminal ganglia was carried out. Finally, the dura mater covering the parietal and frontal bones, the arteries of the Circle of Willis, and the iris were carefully dissected from the surrounding tissue and dehydrated in 50% ethanol for 30 min, rinsed in phosphate buffer, incubated in $NaCnBH_3$ 0.1 M in phosphate buffer for 30 min, rinsed in phosphate-buffered saline (PBS), treated with 50% methanol containing 0.3% H_2O_2 for 10 min, incubated with 10% normal donkey serum (NDS) for 30 min and finally left overnight in the anti-CGRP (Cambridge Research Biochemicals, Cambridge UK) or in the anti-SP (Eugene Tech, Allendale, NJ, USA) antiserum diluted 1:600 in PBS containing 5% NDS. Biotinylated anti-rabbit IgG and streptavidin- horseradish peroxidase (HRP) conjugated complex (Amersham International, Amersham, UK) followed by diaminobenzidine + H_2O_2 were used to reveal the sites of the immunoreaction. The whole-mounts were flat mounted on polylysine-coated glass slides, dehydrated and mounted with DPX (BDH, Poole, U.K.). The above ganglia superior cervical and stellate ganglia, trigeminal and C_{1-3} dorsal root ganglia were dehydrated and embedded in paraffin. 7 µm longitudinal sections of the ganglia were serially cut with a microtome and collected on polylysine-coated slides. One section every 10 was saved for histological examination after staining with cresyl violet. The other sections were dewaxed, rehydrated and incubated with PBS containing 0.1% crude trypsin (Sigma) and 0.1 M

$CaCl_2$ for 30 min. Blockage of endogenous peroxidases and preincubation with 10% NDS preceded the incubation of alternate sections with the first layers.

Coexistence experiments

Two whole-mount stretch preparations of the dura mater, iris and anterior cerebral artery of control animals were used in this study. They were firstly incubated with anti-SP antiserum, following the protocol already described. 0.1% nickel ammonium sulphate was added to the Tris buffer used to dilute the diaminobenzidine (DAB) (Sigma, UK) in both the preincubation and incubation media (Hsu and Soban, 1982). SP-IR nerve fibres appeared blue-black rather than brown. The whole-mounts were then washed with PBS and incubated with anti-CGRP antiserum for 2 days at room temperature. The second layer and the streptavidin-horseradish peroxidase complex was also used for a longer time. DAB was dissolved in the standard buffer and the CGRP-IR axons that lacked SP-IR were distinguishable by their brown-red colour.

Coexistence of CGRP- and SP-IR was also studied in ganglion neurons, as described in the main section of Materials and Methods of this chapter.

Specificity tests

Both SP- and CGRP-antisera were preincubated with an excess of the appropriate neurotransmitter (10^{-4}M) prior to being used in these experiments, on whole-mounts of dura mater of control rats. In some

experiments, antisera were incubated with the other neurotransmitter involved before use, in order to confirm that there was no cross-reactivity. In this case, neither reduction in the expected number of immunopositive nerves, nor a fainter staining seemed to occur, while immunoreactivity was totally abolished in the first group of tests.

Quantitative studies

Quantitative analysis of the density of CGRP- and SP-IR nerves in whole-mounts of dura mater, iris and anterior cerebral artery was performed by an ASBA-Leitz Image Analyzer connected to a Leitz Orthoplan microscope via a video camera. A x10 objective lens was used. The area analysed by the computer each time was 186,624 μm^2, which corresponded to 256 x 256 pixels. The levels of sensitivity and light intensity were adjusted until optimum and kept constant during the measurements. Two parameters were analysed: (a) the area occupied by immunopositive nerves (and their percentage over the total area); (b) the number of immunopositive intercepts x mm along an axis (times 3) perpendicular to the longitudinal sinus for dura mater, to the pupillary border for irides, and to the longitudinal axis of the vessels for anterior cerebral arteries.

Six areas have been evaluated in the dura mater stretch preparations, as shown in Fig. 28. Three areas (MMA-1, MMA-2, MMA-3) were selected along the middle meningeal artery (MMA) from its origin in the medial cranial fossa. The other three areas were devoid of large vessels and were: T-1, between the terminal bifurcation of the MMA and the longitudinal sinus; T-2, 5 mm anterior to MMA-2; T-3, 5 mm posterior

to MMA-2.

In the iris, three zones underwent quantitative analysis of immunopositive structures (see Fig. 29). They corresponded to the pupillary border (zone 1), the constrictory muscle (zone 2) and to the region usually covered by ciliary processes (zones 3), which had been removed.

In the anterior cerebral artery, the portion of the vessel between the internal carotid and the internal etmoidal artery was studied.

The total areas of irides from control-, guanethidine-, capsaicin- and capsaicin + guanethidine-treated rats was measured with the aid of camera lucida drawings and a graphics tablet connected to a MacIntosh PC.

Counting of immunoreactive cell bodies in sensory ganglia was performed on four pairs of sections of each ganglion, alternatively stained for SP- and CGRP-IR, every 100 µm (every 200 µm for trigeminal ganglia, only the V-1 region was evaluated), in order to avoid counting the same neurons twice. Sections were photographed with a x16 objective lens in phase contrast, on a Zeiss photomicroscope using Technical Pan film (Kodak), exposed at 50 ASA. Montages of consecutive sections of the ganglia were prepared and used for counting.

The number of CGRP- and SP-IR neurons were related to the total number of cell bodies in that section and expressed as a percentage of immunoreactive neurons; the total number of neurons that we evaluated was over 500 for each group of ganglia. The number of

neurons co-storing both immunoreactivities was also evaluated; the large variability of the number of such neurons per section obviated any statistical analysis. Hence, the evaluation of the degree of coexistence is on a semiquantitative scale.

Statistics

All the data were compared using Dunnet's Test for multiple comparisons (1964). Differences between values of control and treated rats were tested for significance at $P<0.05$.

RESULTS

Control rats

A rich plexus of CGRP-IR nerve fibres was seen in dura mater, anterior cerebral artery and iris of control rats. In the dura mater, varicose CGRP-IR nerve fibres both in bundles and single axons ran along the main vessels (middle and anterior meningeal arteries, dural veins and dural sinuses) and in vessel-free areas, where some of them terminated as "free nerve endings" (Fig. 30a).

Quantitative analysis of the density of CGRP-IR dural nerves showed no spatial heterogeneity (Table XVII) but nerve fibre orientation changed from a loose network in parietal, avascular areas to a pattern of parallel-orientated bundles and single axons along arteries and in the proximity of dural sinuses.

SP-IR nerves in the dura mater of control rats showed a similar pattern but were less dense (Table XVIII). Very few SP-IR "free nerve endings" were seen in control of guanethidine-treated animals (Figs. 31a, c). Examination of the coexistence between CGRP- and SP-IR showed that almost all SP immunoreactivity was localized in CGRP-IR nerves, while many nerves contained only CGRP-IR (not shown).

In sympathetic ganglia, no CGRP- or SP-IR cell bodies were seen, but a moderate plexus of immunoreactive axons surrounded unstained sympathetic neurons (Fig. 32).

CGRP-IR nerve fibres in the anterior cerebral artery formed a loose plexus longitudinally orientated with a few axons branching from the bundles and running trasversely (Fig. 33a). SP-IR nerves were sparser (see Table XXI). Coexistence experiments showed that all SP-

IR axons were also immunoreactive for CGRP. However, a small number of CGRP-IR axons lacked SP immunoreactivity.

The iris was supplied with a very dense CGRP- and SP-IR nerve plexus, distributed to both vascular and avascular areas, with a more dense pattern at the level of the pupillary border (Figs. 34a, 35a, 36a; Tables XIX, XX). Although whole-mount, processed for coexistence, although difficult to read because of a very dense nerve plexus, they did show some independent CGRP- and SP-IR nerve fibres.

In trigeminal ganglia (Tables XXII, XXIII, see also Fig. 38a and b), SP-IR neurons accounted for less than 20% of the neuronal population, while CGRP-IR cells were more numerous. Both immunoreactivities were mainly found in small, presumably type B, sensory neurons, but a few large neurons also exhibited CGRP-, and less frequently, SP-IR. Coarse CGRP- or SP-axons were seen along the nerves branching rostrally to the trigeminal ganglion.

The percentage of IR neurons, for both CGRP and SP, was higher in the first three dorsal root ganglia than in trigeminal ganglia (Tables XXII, XXIII; Fig. 37). The degree of coexistence was similar to that in trigeminal ganglia.

Guanethidine treatment

In all of the whole-mount preparations of dura mater, iris and anterior cerebral artery, as well as in sections of superior cervical and stellate ganglia, the increase of both CGRP- and SP immunoreactivity was striking. (Figs. 30c, 31c, 33c, 34c, 35c, 36c; Tables XVII-XXI). Quantitative analysis showed the change was by at least 2-3 fold. This increase occurred in the above structures,

without any obvious localization. A remarkable increase of CGRP-IR "free nerve endings" (Fig. 30c) was seen in the dura mater. In the anterior cerebral artery, a number of fine, curled and smooth single CGRP-IR axons appeared (Fig. 33c). By contrast, the percentage of both CGRP- and SP-IR neurons in sensory ganglia significantly decreased in guanethidine-treated rats (Figs. 37c; 38c,d; Tables XXII, XXIII). No more large- or medium-sized IR neurons were seen.

Capsaicin treatment

This treatment severely affected SP-IR nerve fibres in the dura mater, anterior cerebral artery and sympathetic ganglia. SP-IR nerve fibres could rarely be detected in the above structures (e.g. Fig. 31b), while SP-IR nerves in the iris were only slightly reduced (Fig. 35b).

In sensory ganglia, the number of SP-IR neurons in both trigeminal and dorsal root ganglia fell dramatically (Table XXIII).

CGRP immunoreactivity both in the target areas and sensory ganglia was not significantly affected by capsaicin treatment (Figs. 30b, 33b, 34b, 36b, 37b and Tables XVII, XIX, XXI).

Capsaicin and Guanethidine treatment

The sympathetic ganglia of the rats underwent degenerative changes similar to those caused by guanethidine treatment only (Fig. 39).

When guanethidine treatment was carried out in capsaicin-treated rats, the rise in CGRP-IR nerve density in the dura mater,

anterior cerebral artery, iris and sympathetic ganglia still occurred (Figs. 30d, 33d, 34d, 35d; Tables XVII, XIX, XXI). However, only one to two SP-IR nerve fibres could be detected in the dura mater (Fig. 31d), and no SP-IR nerves were seen in the anterior cerebral arteries or the sympathetic ganglia. The number of SP-IR sensory neurons still remained very low (Fig. 38f and table XXIII).
An increase in both CGRP- and SP-IR nerves was measured in the iris of capsaicin and guanethidine-treated rats (Figs. 34d, 35d, 36d; Tables XIX, XX).

The percentage of CGRP-IR neurons in sensory ganglia of capsaicin- and guanethidine-treated rats was significantly higher than in control or guanethidine-treated rats (Fig. 37d, 38d; table XXII).

The <u>total area of irides</u> of control, guanethidine, capsaicin, and capsaicin and guanethidine-treated rats showed interesting changes: a slight, but not significant, increase was present after capsaicin treatment, and irides of guanethidine- and especially of capsaicin + guanethidine-treated rats were significantly larger than control (Fig. 40; Table XXIV).

DISCUSSION

In this study, a detailed investigation of CGRP- and SP-immunoreactivities in both the nerve fibres of some selected cranial target structures and cell bodies of sensory ganglia, known to project to those targets, was carried out in control rats and after sympathetic, sensory or combined denervation.

Control rats

The distribution of CGRP- and SP-IR nerve fibres in the dura mater, anterior cerebral artery and iris described in this study is in agreement with previous reports (Edvinsson and Uddman, 1982: Edvinsson et al., 1987a; Terenghi et al., 1982; 1985; Tsai et al., 1988; Suzuki et al., 1989; Silverman and Kruger, 1989; Chapter one). The "free nerve endings" seen in dural tissue stained with silver impregnation techniques (Rossi and Scevola, 1935) or with anti-CGRP antibodies (Silverman and Kruger, 1989 and the present study) have been described in an electron microscopical study by Andres et al. (1987), who hypothesized a possible sensory role, at least for some of them.

CGRP- and SP-IR nerve fibres were also seen in sympathetic ganglia, thus confirming previous findings. These fibres are likely to be extrinsic, because no immunoreactive cell bodies were observed within the sympathetic ganglia. In a recent report, anterograde and retrograde tracing studies excluded projection of trigeminal ganglion neurons to the superior cervical ganglion (tenTuscher et al., 1989). However, there are alternative sources for CGRP/SP-IR nerve fibres in

both superior cervical and stellate ganglia, such as visceral sensory neurons in the nodose and jugular ganglia (Lee et al., 1985a; Silverman and Kruger, 1989; Helke and Niederer, 1990).

Guanethidine-treated rats

The increase of sensory nerve fibres after long-term sympathectomy seen in this study and previously reported by other authors (Cole et al., 1983; Schon et al., 1985a; Terenghi et al., 1986; Carvalho et al., 1986; Aberdeen et al., 1990) has been related with an increased availability of nerve growth factor(s) for sensory neurons as a consequence of the degeneration of sympathetic neurons (Kessler et al., 1983; Kessler, 1986; Aberdeen et al., 1990). A hyperinnervation of cerebral vessels has been demonstrated after intracerebral infusion of nerve growth factor (Isaacson et al., 1990). However, the number of trigeminal and dorsal root ganglion neurons expressing CGRP and SP immunoreactivity was reduced after guanethidine treatment. Aberdeen et al. (1990) showed that CGRP immunoreactivity in nerve cell bodies in the nodose ganglia disappeared after guanethidine treatment. These changes in sensory ganglion neurons seem to be the result of a reduction in immunoreactivity at the level of the cell bodies, since no sign of neuronal death was seen either in this study or in that of Aberdeen et al. (1990). This may reflect an increased transport of the neurotransmitters to the varicosities, coupled with an inadequate fast synthesis of the peptides (see changes of the number of CGRP-IR neurons in trigeminal ganglia after colchicine treatment, Lee et al., 1985a).

Capsaicin-treated rats

Sensory denervation by capsaicin treatment (Jancso et al., 1977; Scadding, 1980) preceding long-term sympathectomy, was introduced to find out whether it was the sensory neurons that gave rise to the increased CGRP- and SP-IR nerve fibres. As a control, capsaicin treatment alone was also carried out. This study showed that CGRP-IR nerve cell bodies in trigeminal and dorsal root ganglia showed a greater resistance to capsaicin treatment than SP-IR neurons.

It is well known that capsaicin treatment in the rat gives a variable degree of sensory denervation depending on the protocol used (see Fitzgerald, 1983, for a review). Some authors, using a different protocol, found a marked decrease of CGRP-IR nerves in cerebral arteries (Saito and Goto, 1986) and of CGRP-IR neurons in trigeminal ganglia (Skofitsch and Jacobowitz, 1985). However, other authors have previously reported the sparing of CGRP-IR neurons in the trigeminal ganglion and in the iris of rats subjected to neonatal capsaicin treatment (Matsuyama et al., 1986), in contrast with the disappearance of SP-IR neurons.

Capsaicin and guanethidne-treated rats

The major finding in rats treated with both capsaicin and guanethidine was the increase of CGRP-IR nerve fibres in the dura mater and anterior cerebral arteries, while SP-IR nerves were still absent. This discrepancy between CGRP- and SP-IR nerves could only be due to the effects of capsaicin pretreatment, which was likely to be the cause of the irreversible degeneration of SP-IR sensory

neurons. It also adds support for the view that while SP and CGRP coexist in many sensory nerves, others contain a predominance of one peptide or another (Matsuyama et al., 1986).

In the iris, both CGRP- and SP-IR nerve fibres increased, like after guanethidine treatment only; however, little change was seen after capsaicin treatment alone. The rat iris receives projections from trigeminal ganglion neurons (Terenghi et al., 1982; 1985; tenTuscher et al., 1989) and parasympathetic pterygopalatine and ciliary ganglion neurons (Kuwayama et al, 1987; tenTuscher et al., 1990). The changes observed in nerve density in the iris after different treatments suggest that SP-IR nerve fibres, which increase after guanethidine treatment, are not sensitive to capsaicin treatment. It seems likely that this subpopulation of SP-IR nerves originate from the pterygopalatine and/or ciliary ganglia, rather than from remaining SP-IR trigeminal neurons.

CGRP-IR neurons in sensory ganglia were the most numerous seen in this study; this finding indicates that capsaicin-treatment preceding guanethidine sympathectomy altered the levels of detectable immunoreactivity in these neurons, in comparison with guanethidine alone. Again, mechanisms related to the transport of the neurotransmitter are likely to be involved, since it is well known that capsaicin interacts with the axonal transport system in sensory neurons (Fitzgerald, 1983).

In conclusion, this study has shown that sensory hyperinnervation of target organs in guanethidine sympathectomized rats is associated with reduced detectability of SP and CGRP immunoreactivity in nerve

cell bodies. Nevertheless, the number of sensory neurons involved in the hyperinnervation of target organs is likely to be greater than in non-treated animals; this is suggested by the increase of CGRP-IR neurons after combined capsaicin and guanethidine treatment, but not after capsaicin treatment alone. Sensory CGRP-IR nerve cell bodies responding to guanethidine treatment are not destroyed by capsaicin treatment, unlike SP-IR neurons. It is likely that capsaicin causes both destruction of SP-IR neurons and increase of CGRP-IR neurons in guanethidine treated rats by impairing axonal transport mechanisms. A blockade of retrogradely transported nerve growth factor (NGF) by capsaicin (Miller et al., 1982) is responsible of the death of SP-IR neurons, which are NGF-dependent (Kessler and Black, 1980; Lindsay and Harmar 1989), while a slowing down of the anterograde transport of CGRP may allow the visualization of the greater number of IR nerve cell bodies seen after capsaicin + guanethidine treatment. Further studies are needed to confirm this hypothesis.

Table XXVII. CGRP-IR nerve density in dura mater after sympathetic, sensory or combined denervation

Area	N	C	G	C+G
MMA-1 (n=5)				
p (μm^2)	3796±642	2448±295	7410±578*	4366±681
n.int/mm	15.58±2.48	7.03±0.86*	26.29±1.80	17.20±0.64
MMA-2 (n=5)				
p (μm^2)	3162±239	2230±275*	8630±245*	4791±242*
n.int/mm	12.42±1/78	7.67±0.85	29.53±1.63*	17.27±1.63
MMA-3 (n=5)				
p (μm^2)	3178±319	2818±212	7426±511*	5262±564*
n.int/mm	13.03±1.76	5.89±0.99	26.98±2.98*	18.14±2.28
T-1 (n=5)				
p (μm^2)	3713±399	3709±304	10340±480*	6493±209*
n.int/mm	16.11±2.07	10.97±1.18	30.30±1.54	21.44±3.19
T-2 (n=5)				
p (μm^2)	2992±428	3093±438	10000±493*	7413±308*
n.int/mm	13.71±2.34	12.30±1.23	25.77±2.49	25.23±2.86
T-3 (n=5)				
p (μm^2)	3987±524	3900±217	10270±669*	6442±156*
n.int/mm	15.21±2.23	11.33±0.89	29.07±2.26*	25.22±4.42

See text and Fig. 28 for illustration of the above areas. p = mean ± SEM of CGRP-immunopositive areas over 186624 μm^2 of each region of dura mater. n.int/mm = mean ± SEM of number of intercepts (positive nerve fibres seen as single axons) per mm of tissue. N = control; C = capsaicin; G = guanethidine; C+G = capsaicin+guanethidine. See text for detail on treatments. * Significant versus control at $P<0.05$

Table XVIII. SP-IR nerve density in dura mater after sympathetic, sensory or combined denervation

AREA	N	C	G	C+G
MMA-1p (μm^2)	1918±195	-	9.64±365*	-
n.int/mm	5.43±1.09	-	9.63±0.51	-
MMA-2 (n=5)				
p (μm^2)	1639±141	-	3687±183*	-
n.int/mm	5.43±0.45	-	9.65±0.57	-
MMA-3 (n=5)				
p (μm^2)	1769±122	-	3407±130*	-
n.int/mm	5.41±0.62	-	9.76±0.53*	-
T-1 (n=5)				
p (μm^2)	2046±55	-	4140±120*	-
n.int/mm	6.81±0.46	-	9.91±0.38*	-
T-2 (n=5)				
p (μm^2)	1921±4	-	4159±153*	-
n.int/mm	5.73±0.53	-	9.92±0.39*	-
T-3 (n=5)				
p (μm^2)	1910±75	-	4079±311*	-
n.int/mm	6.60±0.73	-	9.83±0.62*	-

See text and Fig. 28 for illustration of the above areas. o = mean ± SEM of SP-immunopositive areas over 186624 μm^2 of each region of dura mater. n.int/mm; mean ± SEM of number of intercepts (positive nerve fibres seen as single axons) per mm of tissue. N = control; C = capsaicin; G = guanethidine; C+G = capsaicin+guanethidine.

* Significant compared with control at $P<0.05$.

Table XIX. CGRP-IR nerve fibres irides after sympathetic, sensory or combined denervation

Zone	N	C	G	C+G
Z-1 (n=5)				
p (μm^2)	18910±514	17040±576	29890±1901*	28890±1861
n.int/mm	55.05±1.32	54.97±3.75	100.82±3.63	86.15±3.91*
Z-2 (n=5)				
p (μm^2)	17890±871	15690±1272	27500±908*	24670±1050*
n.int/mm	53.00±3.10	54.26±4.19	98.31±6.21*	73.30±1.47*
Z-3 (n=5)				
p (μm^2)	15840±599	12940±789	22750±2194*	2220±774*
n.int/mm	43.51±1.20	36.90±3.87	76.88±5.50*	66.98±3.74*

See text and Fig. 29 for illustration of the above zones. p = mean ± SEM of CGRP-immunopositive areas over 186624 μm^2 of each region of the iris. n.int/mm - mean ± SEM of number of tissue. N = control; C = capasaicin; G = guanethidine; C+G = capsaicin + guanethidine. See text for detail on treatments. * Significant compared with control at $P<0.05$.

Table XX. SP-IR nerve fibres in irides after sympathetic, sensory or combined denervation

Zone	N	C	G	C+G
Z-1 (n=5)				
p (μm^2)	12410±515	11090±433	23690±750*	20050±550*
n.int/mm	39.36±0.63	40.17±0.38	74.28±2.40*	67.90±1.07*
Z-2 (n=5)				
p (μm^2)	10050±430	10140±112	21890±655*	18670±540*
n.int/mm	36.24±1.04	38.58±0.64	68.38±2.11*	66.50±1.30*
Z-3 (n=5)				
p (μm^2)	9459±373	9121±326	21250±441*	16470±388*
n.int/mm	35.19±0.4	36.10±1.05	66.01±1.12*	64.74±0.87*

See text and Fig. 29 for illustration for the above zones, p = mean ± SEM of CGRP-immunopositive areas over 186624 μm^2 of each region of the iris. n.int/mm = mean ± SEM of number of intercepts (positive nerve fibres seen as single axons) per mm of tissue. N = control; C = capsaicin; G = guanethidine; C+G = capsaicin + guanethidine. See text for detail on treatments. * Significant compared with control at $P<0.05$.

Table XXI. Immunoreactive nerve fibres in anterior cerebral arteries after sympathetic, sensory or combined denervation

-IR	N	C	G	C+G
CGRP-IR (n=5)				
p (%)	8.64±0.66	2.20±0.61*	21.09±0.92*	6.33±1.75
n.int/mm	18.70±1.27	6.56±0.54*	32.90±0.27*	6.36±2.22
SP-IR (n=5)				
p (%)	7.76±0.5	-	16.44±1.11*	-
n.int/mm	17.87±1.57	-	36.96±1.79*	-

See text for details on vessels and treatments. p (%) = mean ± SEM of percentages of immunopositive area for each vessel. n.int/mm = mean ± SEM of the number of intercepts (positive nerve fibres seen as single axons) per mm of vessel circumference. N = control; C = capsaicin; G = guanethidine; C+G = capsaicin + guanethidine. * Significant compared with control at $P<0.05$.

Table XXII. CGRP-IR neurons in trigeminal and dorsal root ganglia C_{1-3} after sympathetic, sensory or combined denervation

Ganglion	N	C	G	C+G
TG (n=5)				
%	31.69±2.15	30.51±2.57	23.10±2.23*	38.54±1.90
Total	198/575	186/567	152/624	203/526
DRG (n=5)				
%	39.96±3.11	35.19±4.84	27.77±0.85*	53.02±1.77*
Total	436/1082	307/932	201/718	271/526

TG, trigeminal; DRG, dorsal root C1-3 ganglia. N = normal; C = capsaicin; G = guanethidine; C+G = capsaicin + guanethidine. See text for details on pharmacological treatments. % = the mean ± SEM of the percentage of immunoreactive neurons counted in 4 sections from each ganglion from 5 different animals for each treatment. Total = the total number of immunopositive neurons/total number of neurons counted in 4 sections of 5 different ganglia for each group. Statistical analysis was performed with Dunnet's Test for multiple comparisons. * Significant compared with control at $P<0.05$.

Table XXIII. SP-IR neurons in trigeminal and dorsal root ganglia C_{1-3} after sympathetic, sensory or combined denervation

Ganglion	N	C	G	C+G
TG (n=5)				
%	19.71±1.87	2.10±0.58	7.79±0.27*	5.44±0.75*
Total	106/578	13/566	49/619	26/458
DRG (n=5)				
%	15.79±2.48	5.45±1.77*	8.47±1.21	4.87±3.39
Total	193/1080	73/936	60/720	107/522

TG, trigeminal; DRG, dorsal root C1-3 ganglia. N = normal; C = capsaicin; G = guanethidine; C+G = capsaicin + guanethidine. See text for details on pharmacological treatments. % = the mean±SEM of the percentage of immunoreactive neurons counted in 4 sections from each ganglion from 5 different animals for each treatment. Total = the total number of immunopositive neurons/total number of neurons counted in 4 sections of 5 different ganglia for each group. Statistical analysis was performed with Dunnetts Test for multiple comparisons. * Significant compared with control at $P<0.05$.

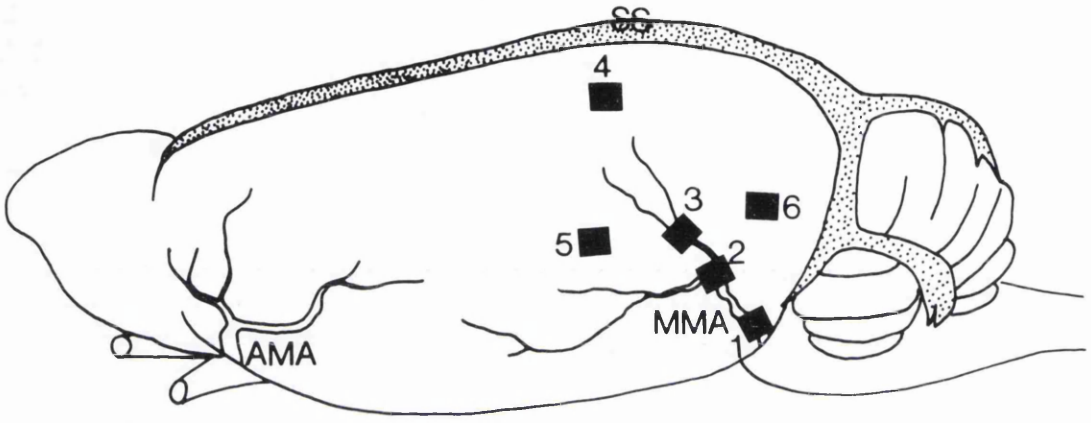
Table XXIV. Area of irides after sympathetic, sensory or combined denervation

(N=5)	N	C	G	C+G
Area (mm^2)	46.86±3.79	58.692±5.99	75.21±4.33*	84.03±7.11*

See text for detail on treatments. N = control; C = capsaicin; G = guanethidine; C+G = capsaicin + guanethidine. * Significant compared with control at $P<0.05$.

Fig. 28 - Top - Schematic topography of the areas of rat dura mater, in which the densities of CGRP- and SP-IR nerve fibres were evaluated. MMA = middle meningeal artery: AMA = anterior meningeal artery; SS = sagittal sinus. 1 = MMA-1; 2 = MMA-2; 3 = MMA-3; 4 = T-1; 5 = T-2; 6 = T-3. Redrawn from Andres et al., 1987.

Fig. 29 - Bottom - Schematic drawing illustrating the rat iris with the three areas in which the densities of CGRP- and SP-IR nerve fibress were evaluated. CP = ciliary processes. 1 = Z-1; 2 = Z-2; 3 = Z-3. Redrawn from Cole et al. (1983).


SG
4
3
6
5
2
MMA
1
AMA

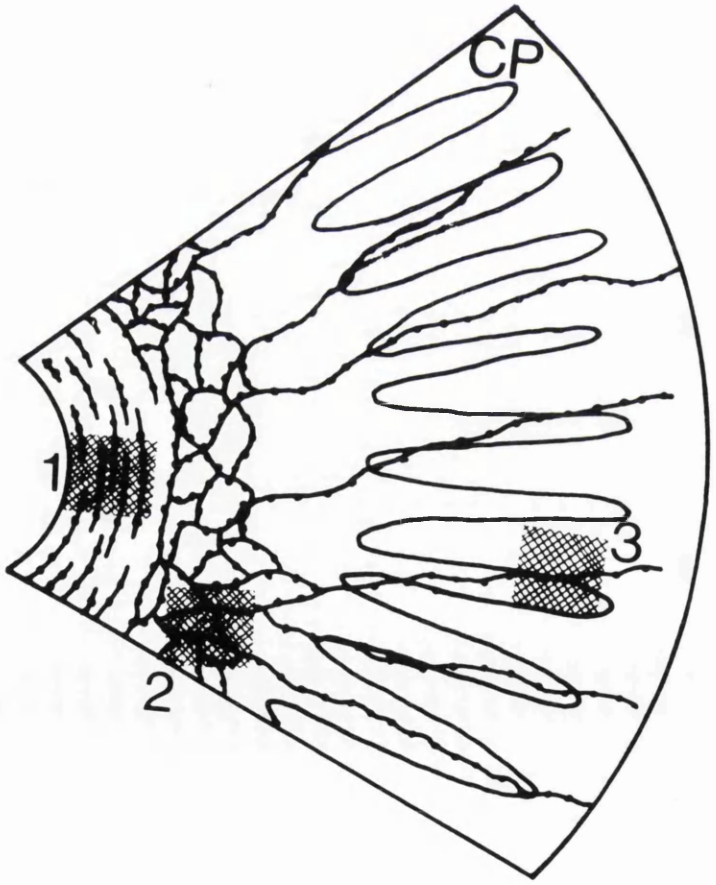

CP
1
3
2

Fig. 30 - CGRP-IR nerve fibres in whole-mount stretch preparations of rat dura mater, corresponding to the area T-1 (see text). (**a**) control rat; (**b**) capsaicin-treated rat; (**c**) guanethidine-treated rat; (**d**) capsaicin + guanethidine-treated rat. Note the increase of CGRP-IR nerves in (**c**) and (**d**). Calibration bar = 50 µm.

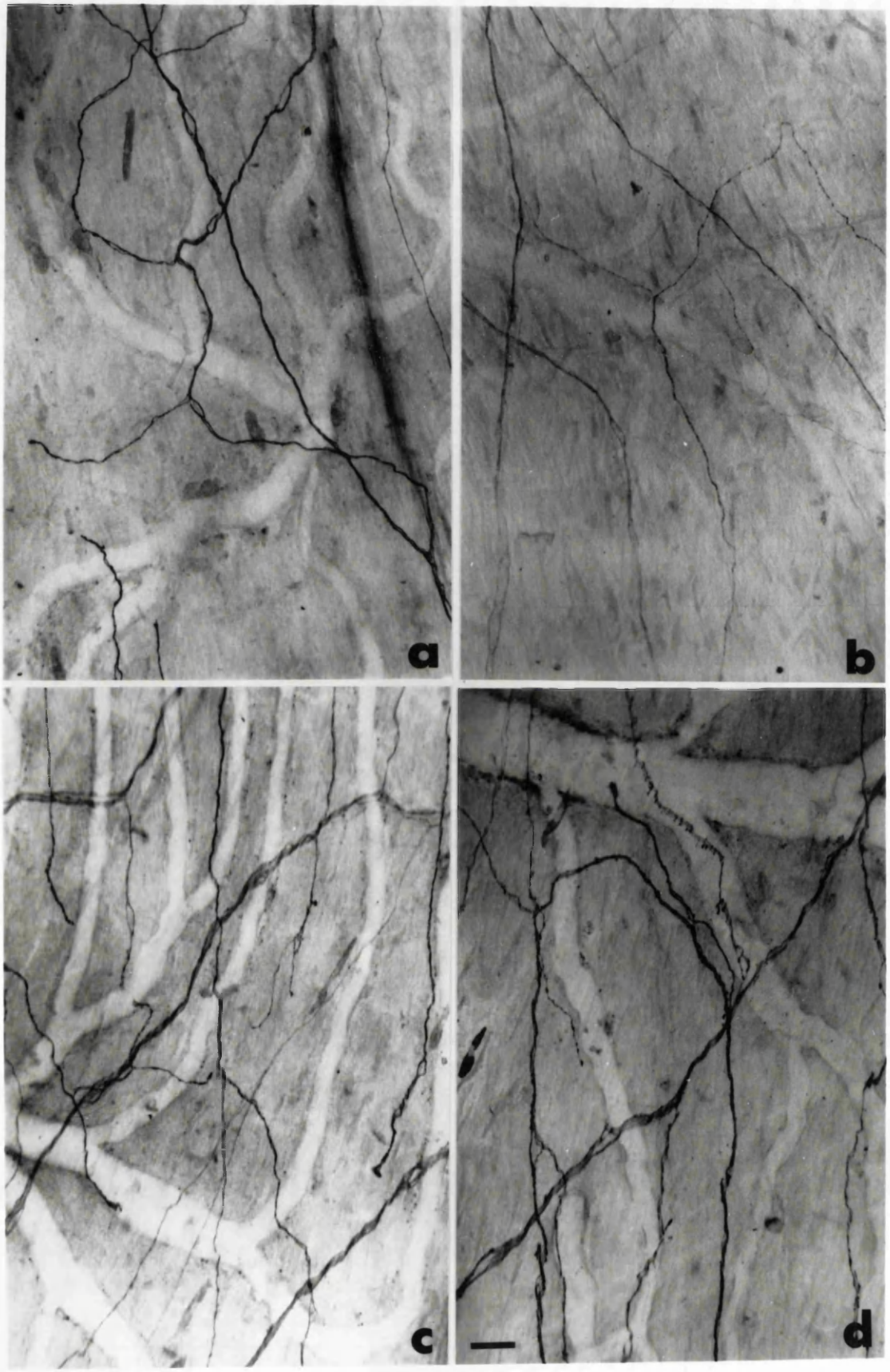

a
b
c
d

Fig. 31 - SP-IR nerve fibres in whole-mount stretch preparations of rat dura mater. The photomicrographs represent the area MMA-2. (**a**) control rat; (**b**) capsaicin-treated rat; (**c**) guanethidine-treated rat; (**d**) capsaicin + guanethidine-treated rat. Note the almost complete disappearance of SP-IR nerve fibres in capsaicin and capsaicin + guanethidine-treated rats. Calibration bar = 50 µm.

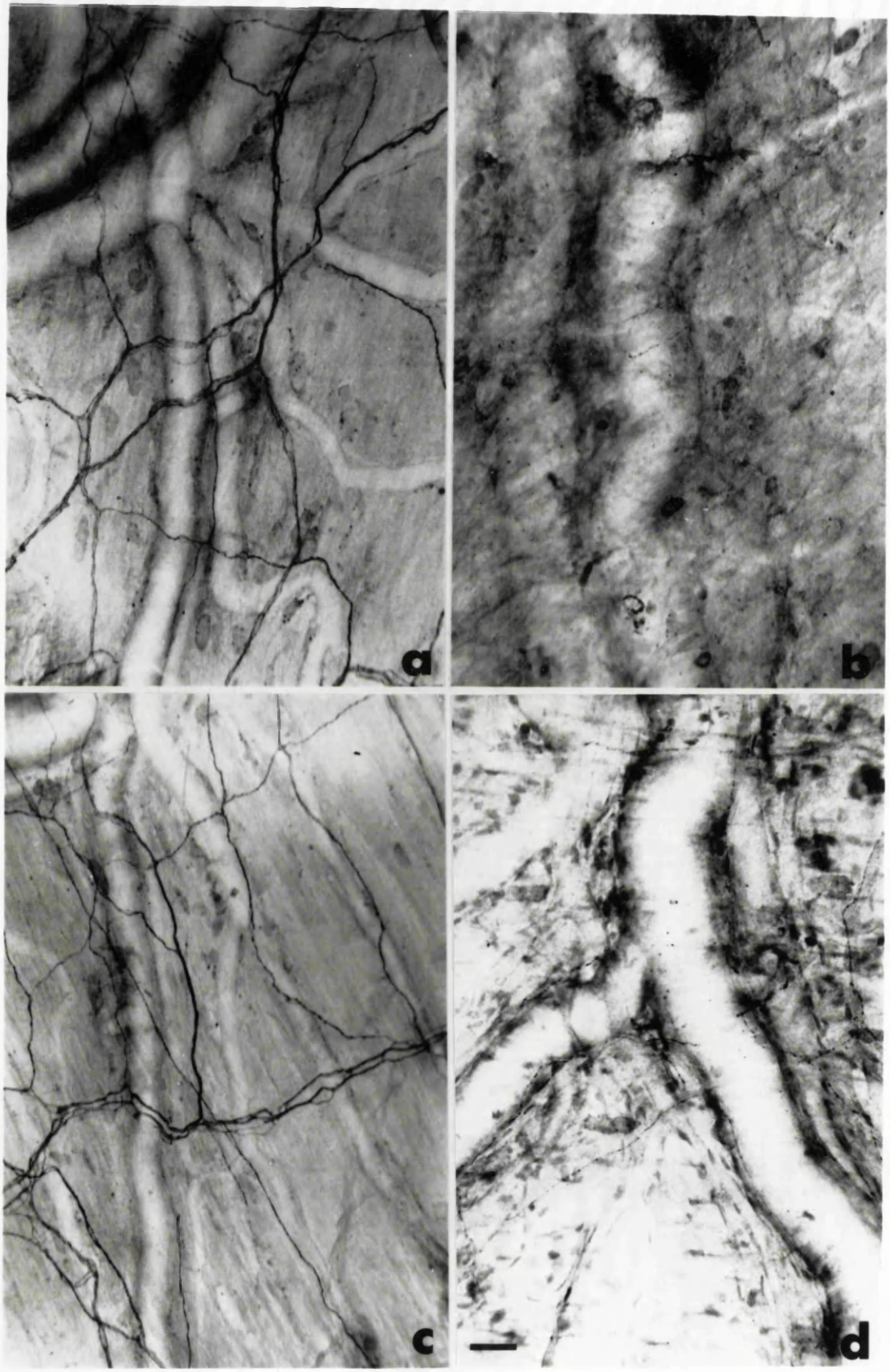

a
b
c
d

Fig. 32 - Paraffin sections of stellate ganglion of a control rat, immunostained for SP-IR (**a**) and CGRP-IR (**b**). Note the presence of a dense bundle of IR nerve fibres in the bottom half of both micrographs (**arrowheads**) and several CGRP-IR axons surrounding unstained neurons in (**b**). Calibration bar = 50 µm.

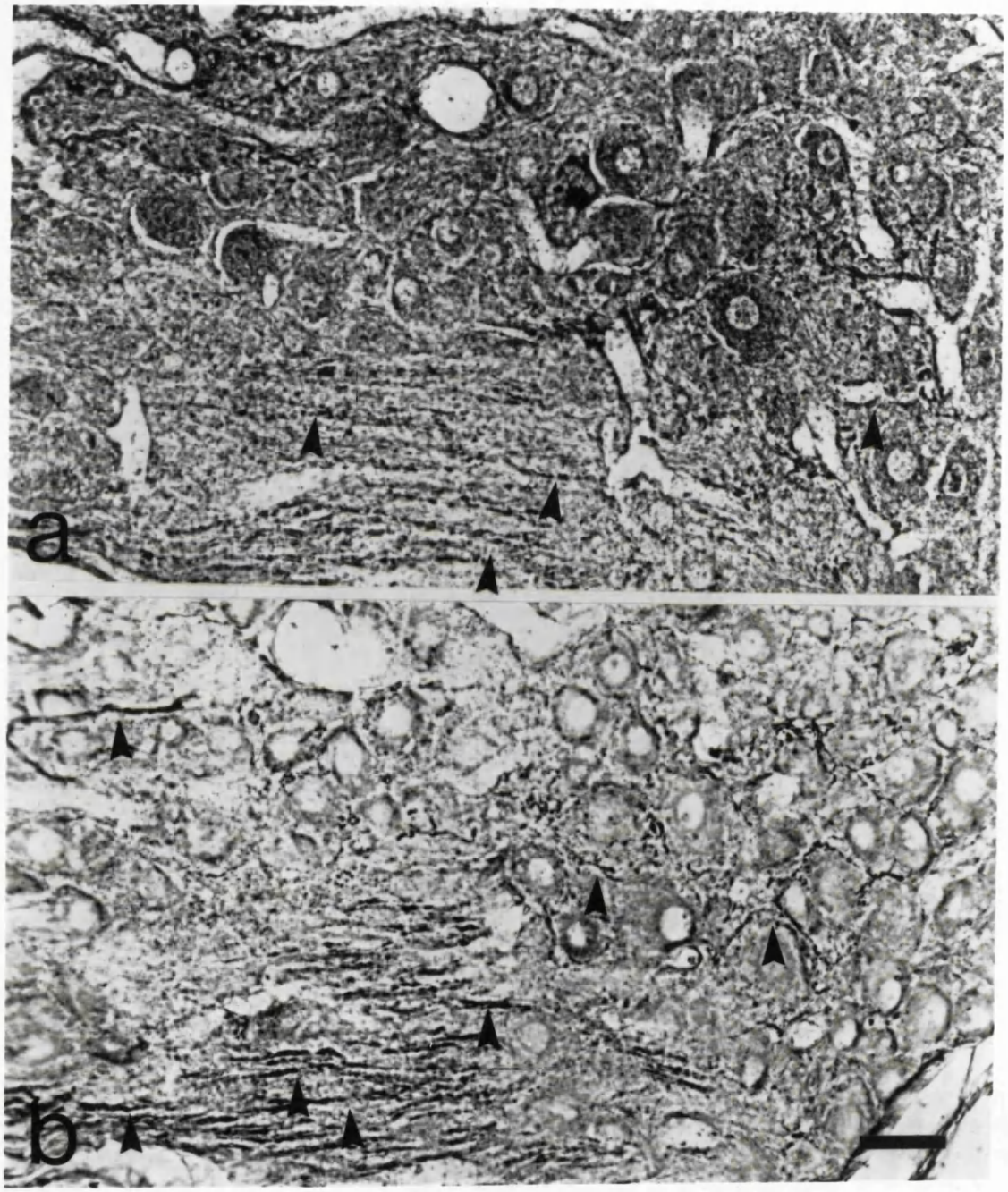

a
b

Fig. 33 - Whole-mount stretch preparations of rat anterior cerebral arteries immunostained for CGRP-IR. (a) control rat; (b) capsaicin-treated rat; (c) guanethidine-treated rat; (d) capsaicin + guanethidine-treated rat. Note the increase of CGRP-IR nerve fibres, which appear smooth and curled, after long-term guanethidine treatment. Calibration bar = 50 μm.

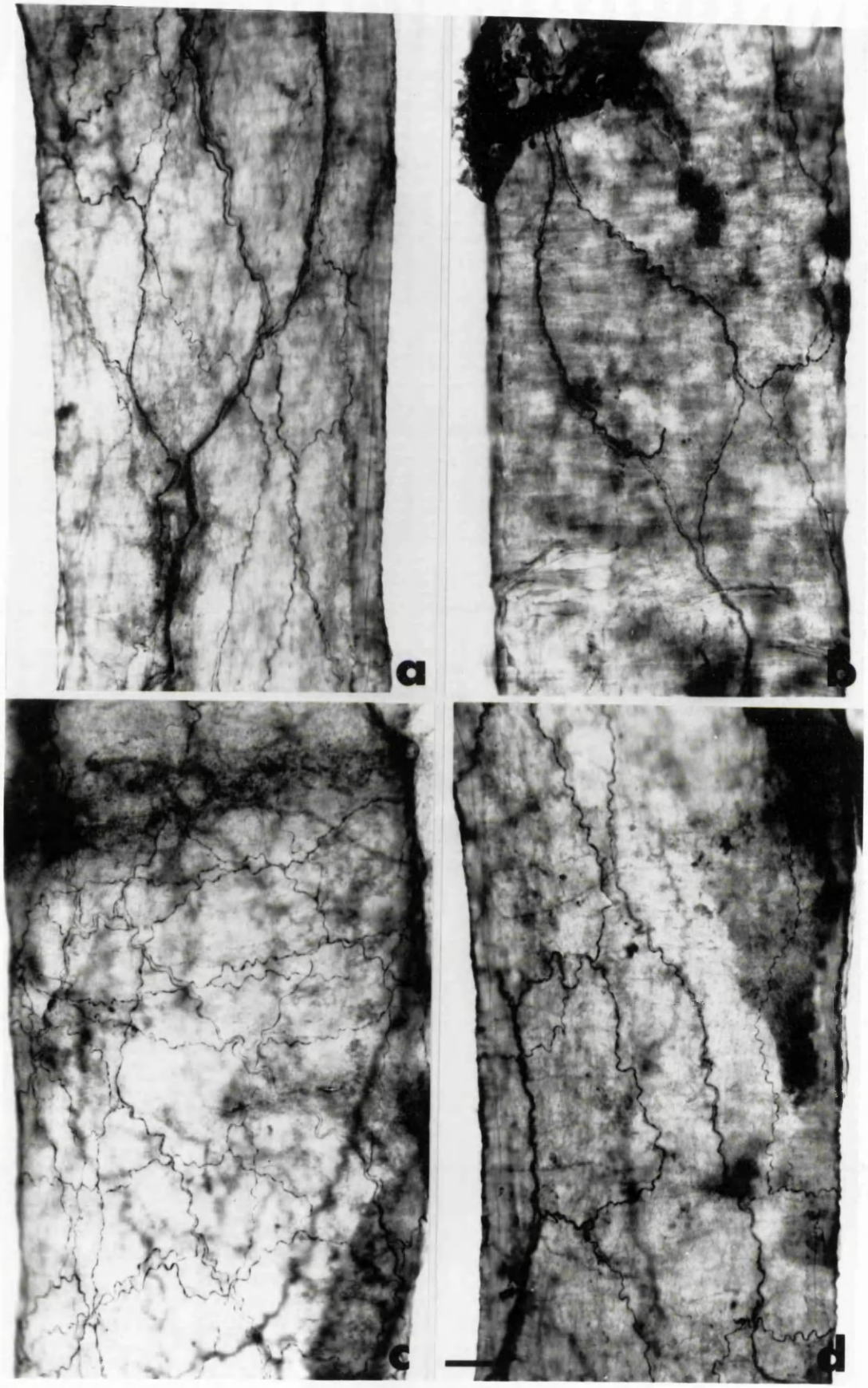

a
b
c
d

Fig. 34 - CGRP-IR nerve fibres in whole-mount stretch preparations of the rat iris. Representative photographs of the area Z-1 (see text). (**a**) control rat; (**b**) capsaicin treated rat; (**c**) guanethidine-treated rat; (**d**) capsaicin + guanethidine-treated rat. Note the increase of CGRP-IR nerve density especially evident in the guanethidine-treated rat. Calibration bar = 50 μm.

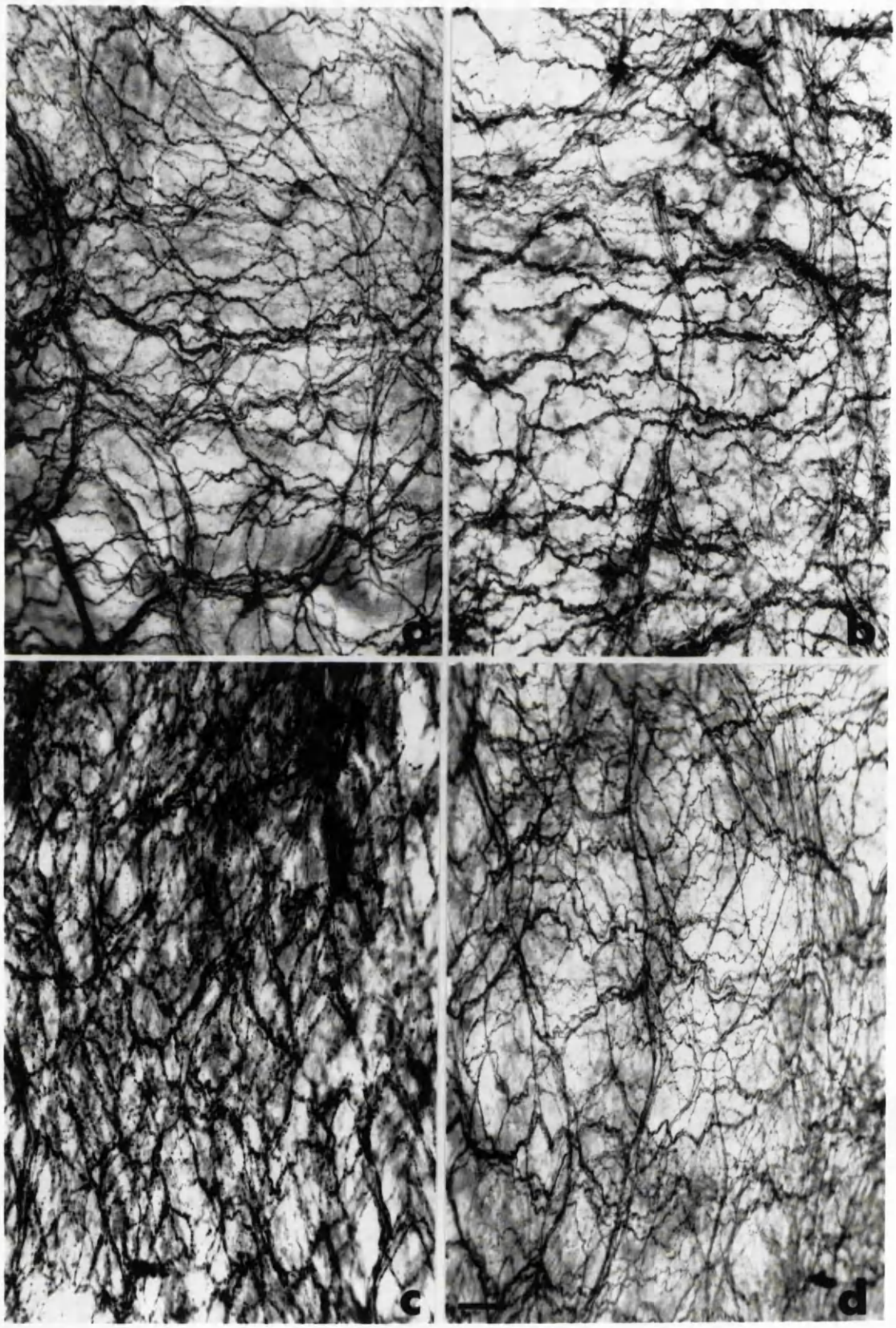

a
b
c
d

Fig. 35 - SP-IR nerve fibres in whole-mount stretch preparations of the rat iris. The area shown is from Z-2 (see text) (**a**) control rat; (**b**) capsaicin-treated rat; (**c**) guanethidine-treated rat; (**d**) capsaicin + guanethidine-treated rat. Note the increase of SP-IR nerve fibres in ((**c**) and (**d**). Calibration bar = 50 µm.

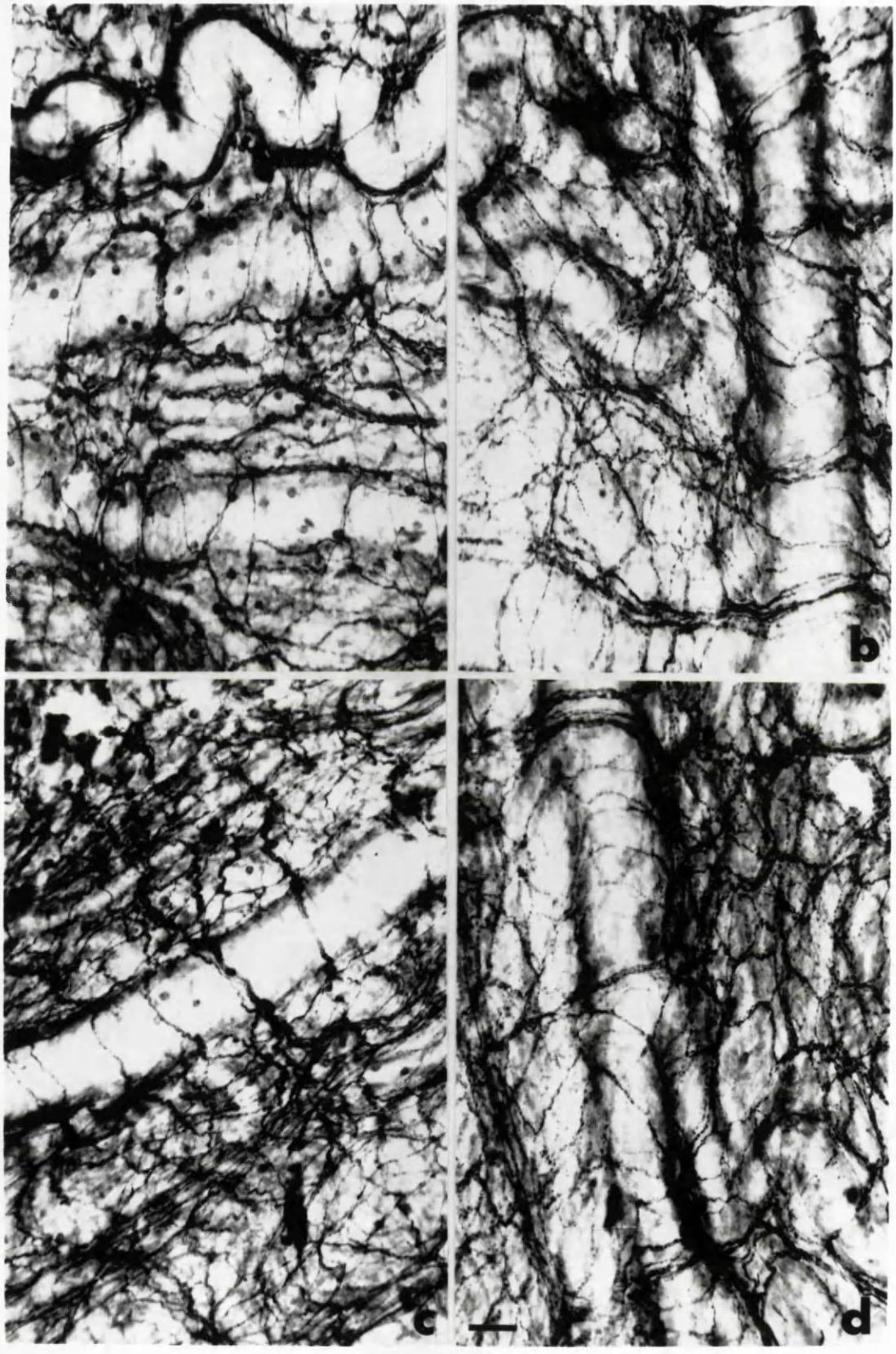

b
c
d

Fig. 36 - CGRP-IR nerve fibres in whole-mount stretch preparations of the rat iris. The area shown is from Z-3, i.e. the area under the ciliary processes, which were removed before staining. (**a**) control rat; (**b**) capsaicin-treated rat; (**c**) guanethidine-treated rat; (**d**) capsaicin + guanethidine-treated rat. Calibration bar = 50 μm.

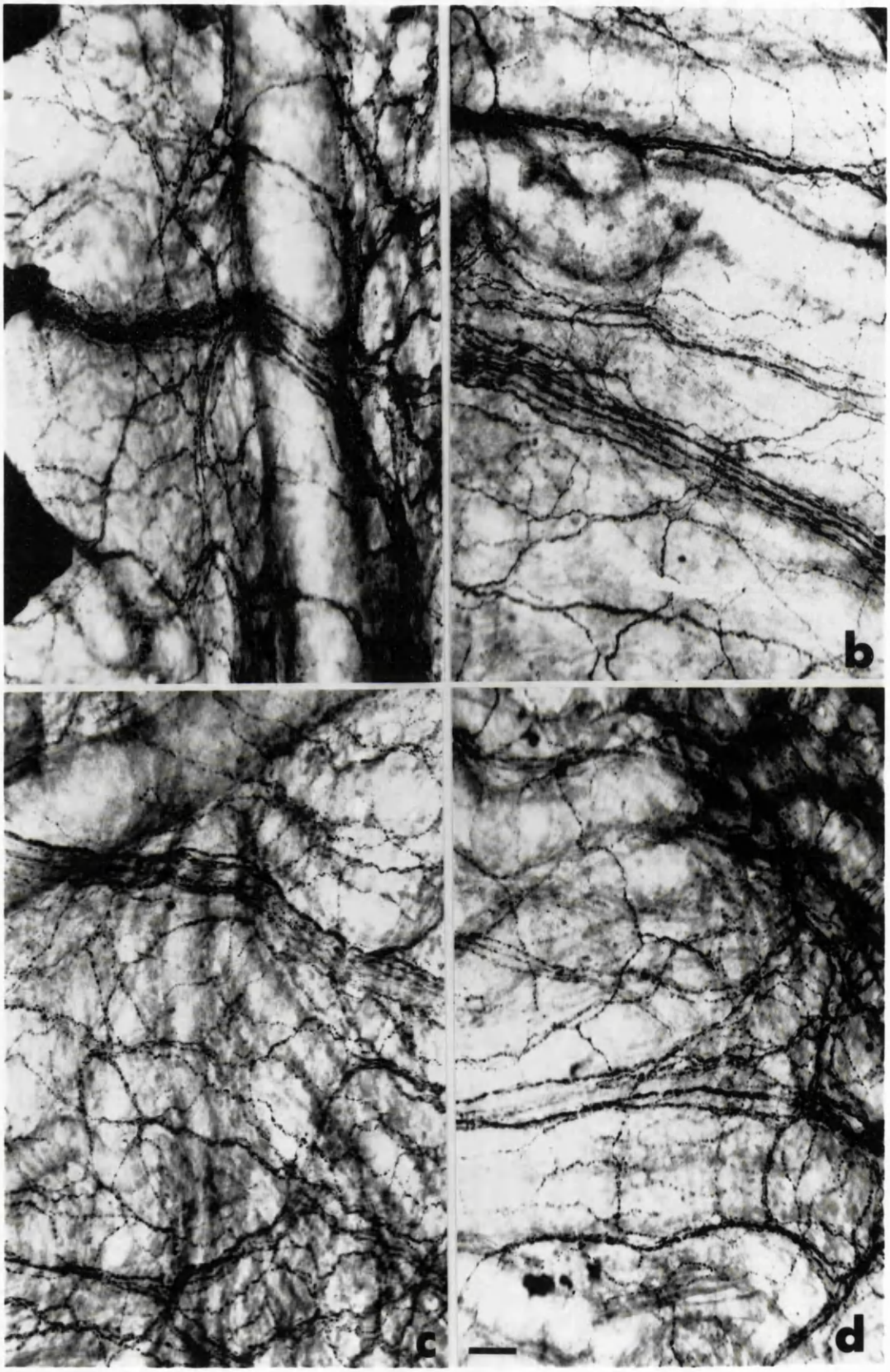

b
c
d

Fig. 37 - Montages of representatives sections of C-3 dorsal root ganglia, stained for CGRP-IR. (**a**) control rat; (**b**) capsaicin-treated rat; (**c**) guanethidine-treated rat; (**d**) capsaicin + guanethidine-treated rat. Note the reduction of IR neurons in (**b**) and (**c**), the absence of large-sized CGRP-IR neurons in (**c**) and the labelling of large bundles of nerve fibres in (**c**) and (**d**) (**arrowheads**). Calibration bar = 50 µm.

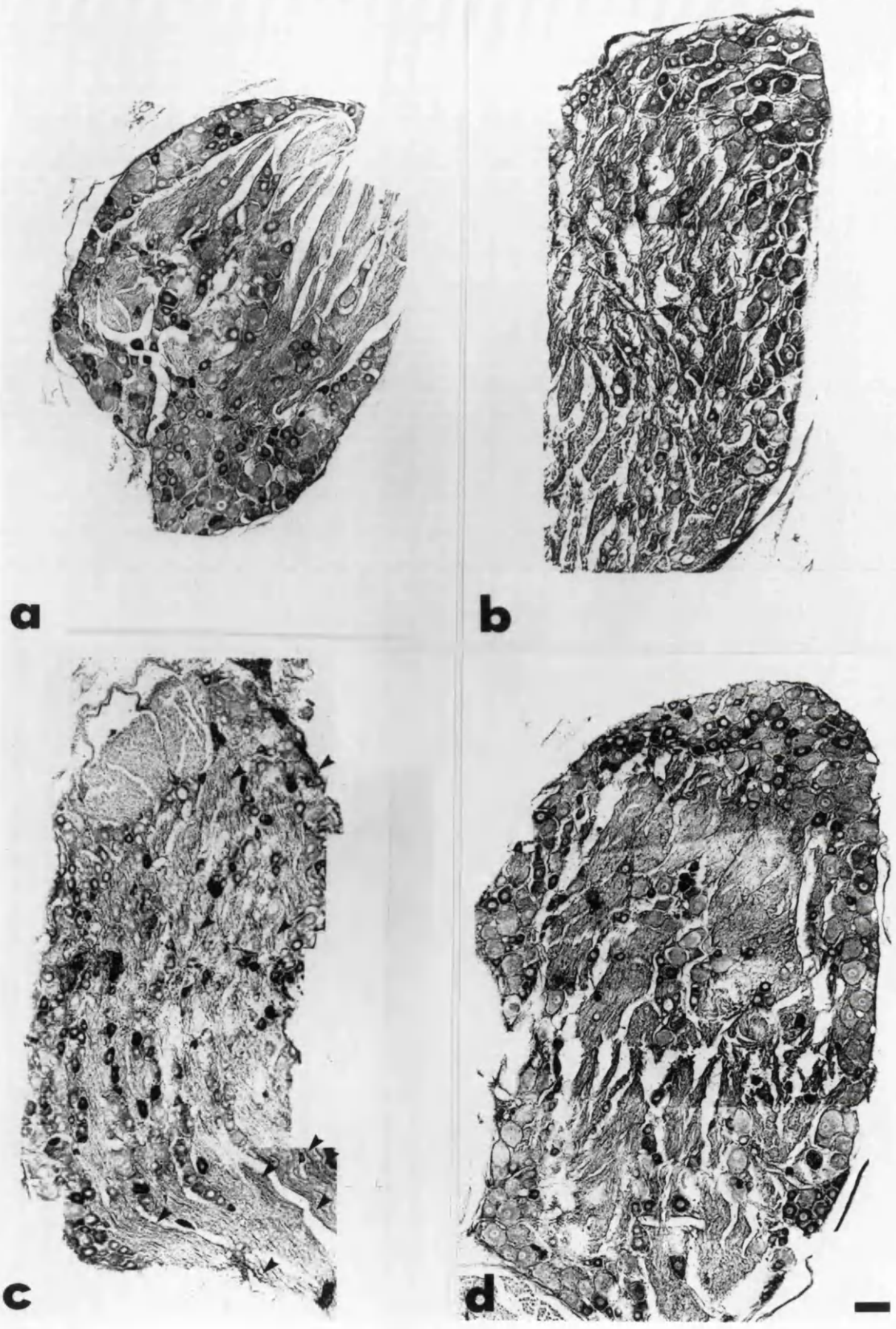

a
b
c
d

Fig. 38 - Montages of representative areas of rat trigeminal ganglion sections, V-1 region. (**a**) and (**b**) sections from a control rat, immunostained for CGRP-IR (**a**) and SP-IR (**b**). (**c**) and (**d**) section from a guanethidine-treated rat, immunostained for CGRP-IR (**c**) and SP-IR (**d**) . Note the absence of large CGRP-IR neurons in (**c**), while they are normally present in control animals. (**e**) and (**f**), sections from a guanethidine + capsaicin treated rat. Note the large number of CGRP-IR neurons (**e**) and the few SP-IR (**f**) neurons. Calibration bar = 50 μm.

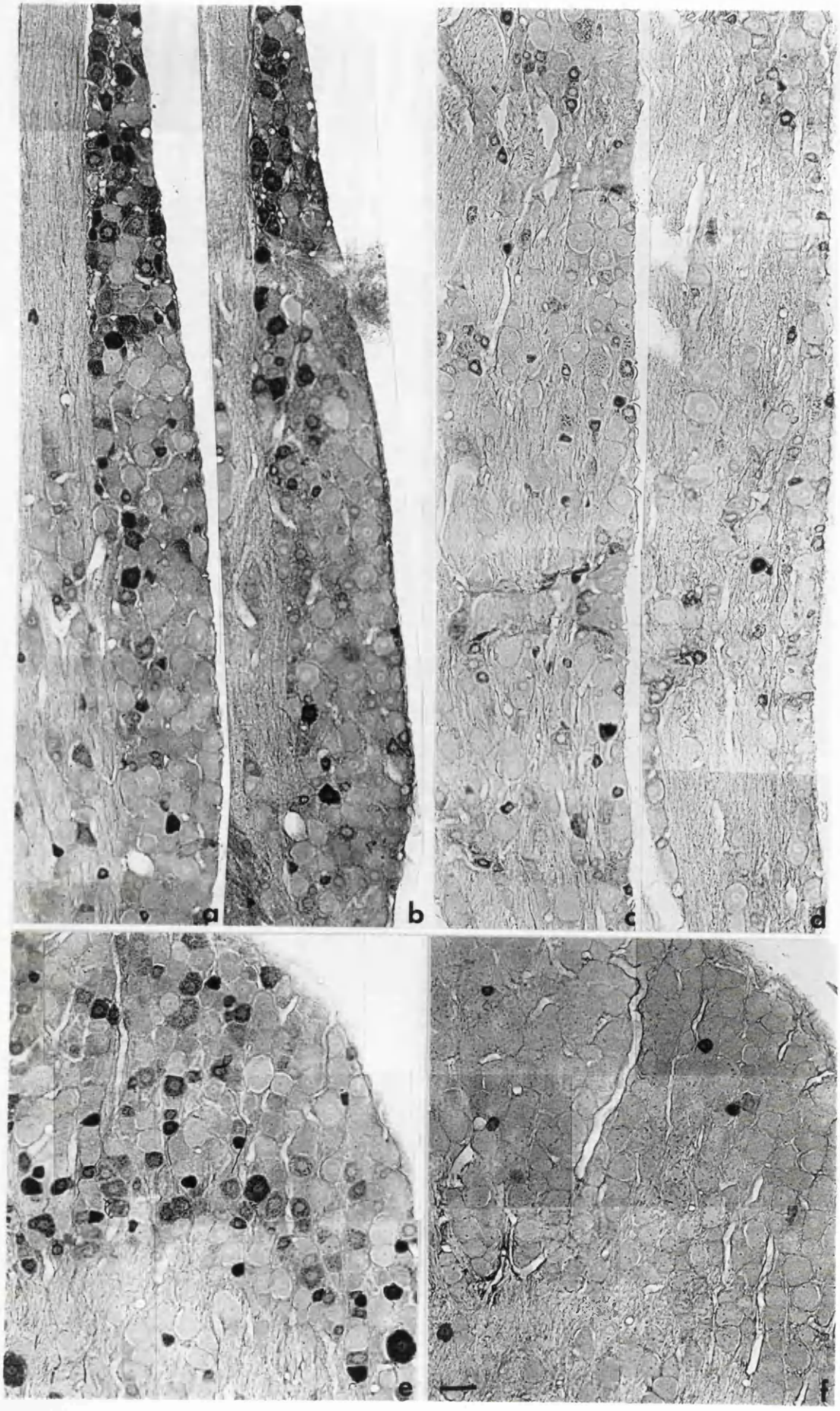

a
b
c
d
e
f

Fig. 39 - Resin-embedded sections of superior cervical ganglia from a control rat (**a**) and a capsaicin + guanethidine-treated rat (**b**). Toluidine blue staining shows the degree of neuronal depopulation in sympathetic ganglia after capsaicin + guanethidine treatment. Calibration bar = 50 µm.

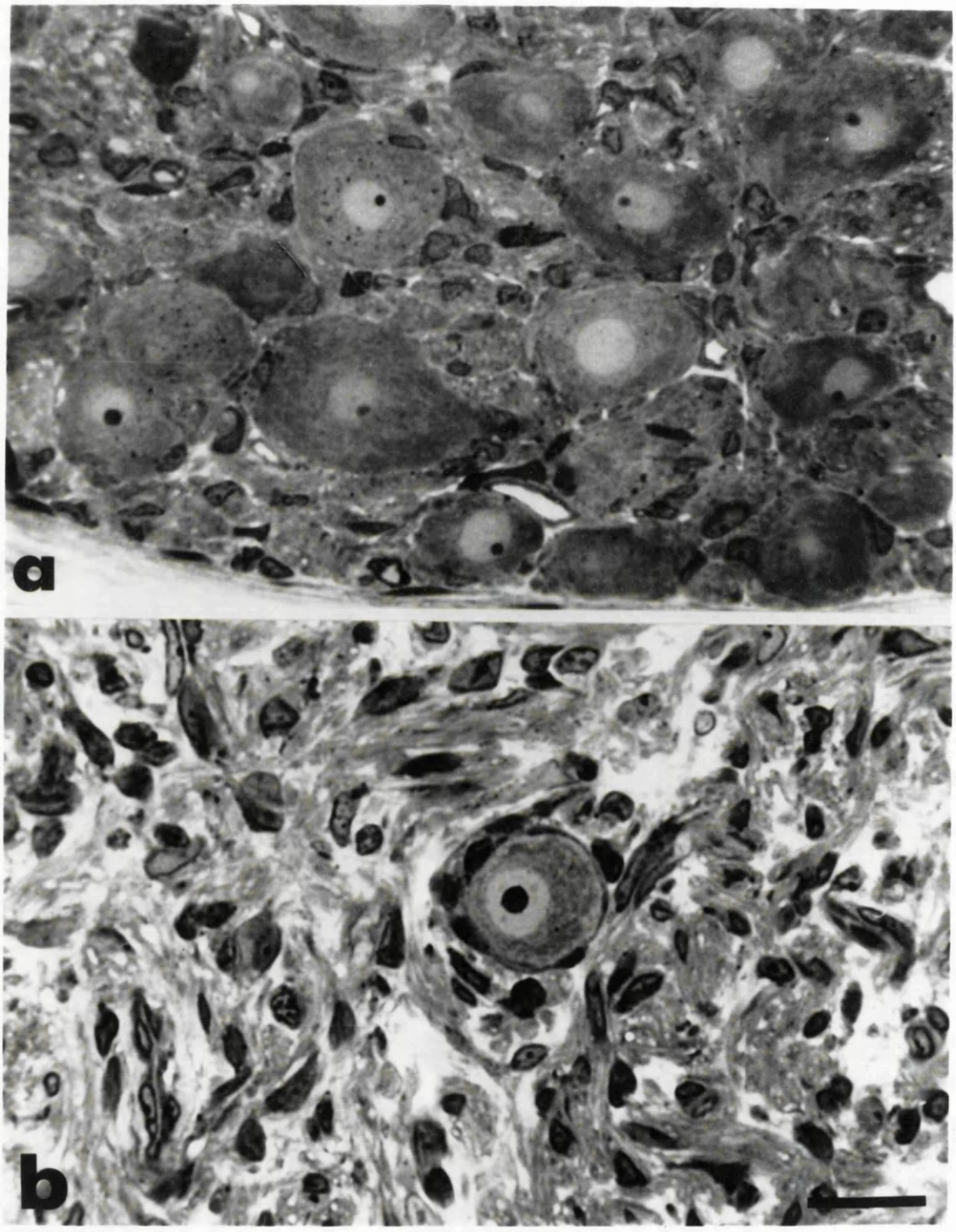
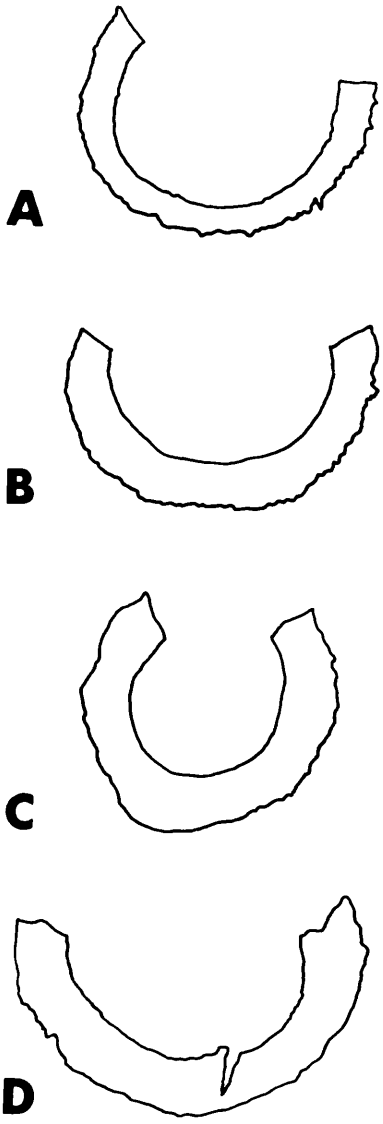

a
b

Fig. 40 - Tracing of typical whole-mount preparations of irides from control (**a**), capsaicin-trated (**b**), guanethidine-treated (**c**) and capsaicin + guanethidine-treated (**d**) rats. Irides have been cut to allow flattening, after in situ fixation. Calibration bar 50 µm.


A
B
C
D
10 mm

B : GUINEA-PIG UTERINE ARTERY

CHAPTER FIVE

PREGNANCY REDUCES NORADRENALINE BUT NOT NEUROPEPTIDE LEVELS IN THE UTERINE ARTERY OF THE GUINEA-PIG

SUMMARY

Using histochemical, immunohistochemical and biochemical techniques, noradrenaline-, neuropeptide Y-, vasoactive intestinal polypeptide-, substance P- and calcitonin gene-related peptide-containing nerve fibres were studied in the uterine artery of virgin, progesterone-treated and pregnant guinea-pigs. Morphological changes following hormone treatment or in pregnancy were also evaluated in a quantitative study of semithin sections of the uterine artery. In late pregnancy, the number of noradrenaline-containing nerve fibres, which formed the densest plexus in virgin animals, was significantly decreased, a finding supported by a significant reduction in noradrenaline levels. This reduction was not mimicked by systemic progesterone treatment. In contrast, the innervation of the uterine artery by neuropeptide Y-containing nerve fibres was increased in pregnancy, while the other peptidergic nerves and peptide levels were unchanged after progesterone treatment and in pregnancy. These changes led to a predominance of innervation by nuropeptide Y- rather than noradrenaline-containing nerve fibres in late pregnancy. No morphological changes were detected following progesterone treatment, but pregnancy led to a marked increase in the cross-sectional area of the vessel accompanied by an increase in the thickness of the media.

INTRODUCTION

It has been reported that noradrenergic nerves disappear from the uterus of several animal species during late pregnancy (Sjöberg 1968; Owman et al., 1975; Alm et al., 1979a,b; Thorbert et al., 1979). By use of electron microscopy (Sporrong et al., 1981), a pregnancy-induced degeneration of uterine noradrenergic nerves has been shown to occur in the guinea-pig. A concomitant decrease of neuropeptide Y (NPY)- and vasoactive intestinal polypeptide (VIP)-immunoreactive nerves and levels has been demonstrated in the uterus of guinea-pigs (Fried et al., 1985) and rats (Stjernquist et al., 1985), respectively. In addition, pharmacological responses of the uterine artery to noradrenaline (NA) are unchanged, while cholinergic responses are increased in late pregnancy (Bell, 1968; 1969). It has been reported that transmural nerve stimulation of the uterine artery fails to evoke vasomotor responses in pregnancy (Tare et al., 1988). Progesterone treatment has also been shown to cause a reduction of NA levels in the uterus and uterine artery (Bell and Malcolm, 1978).

Since perivascular nerves in the uterine artery contain various neuropeptides, including VIP, NPY, dynorphin and calcitonin gene-related peptide (CGRP), as well as the catecholamine-synthesising enzyme, dopamine β-hydroxylase (Morris et al., 1985, 1987; Uddman et al., 1986), the expression of neuropeptides as well as NA in the uterine artery of the guinea-pig in late pregnancy was investigated. In addition, the ability of progesterone treatment to mimic the effects of pregnancy was examined. A combination of quantitative histochemical, immunohistochemical and neurochemical techniques was

used to investigate nerve distribution and neurotransmitter levels in the uterine artery from control, pregnant and progesterone-treated guinea-pigs.

A quantitative morphological study was also carried out on semithin sections and all the results were evaluated in the light of the morphological changes that were shown to occur in the uterine artery during late pregnancy.

MATERIALSAND METHODS

The study was carried out in Dunkin-Hartley guinea-pigs. Three groups of animals were used :

(1) virgin guinea-pigs (3-5 months of age);

(2) late pregnant guinea-pigs (about 50 days gestation);

(3) guinea-pigs injected with 5 mg progesterone (Paines & Byrne Ltd., Greenford, U.K.) i.m. every day, for 45 days.

The treatment with progesterone was started at the end of the oestrus phase (as checked by the presence of leucocytes in vaginal smears); the gestation time was estimated from the crown-rump length of the fetuses (Draper, 1920). Only bilaterally pregnant animals were used in this study.

Animals were killed by cervical dislocation. The right uterine artery of each animal was taken for biochemical analysis (immediately frozen in liquid nitrogen). The left uterine artery was taken for morphological studies (see below).

Immunohistochemical study

Pieces of tissue, 1 cm in length, corresponding to the uterine horn, were removed, opened longitudinally and stretched to their original length and circumference onto sheets of silicone elastomer (Sylgard; BDH Ltd., Poole, U.K.) with micropins.

To localize noradrenergic nerves, vessels were incubated in 2% w/v glyoxylic acid as described by Lindvall and Björklund, 1974, followed by counterstain with pontamine sky blue (Cowen et al., 1985). After drying, vessels were stretched on glass slides to their

original size, heated at 100 °C for 4 min, and mounted in liquid paraffin.

For immunohistochemistry, whole mount stretch preparations of the uterine arteries were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for 1.5 h, dehydrated in 80% ethanol and incubated with 0.1% Triton X 100 in PBS, before incubation with the specific antisera overnight at room temperature.

All the antisera were polyclonal and raised in rabbits : anti-NPY (CRB; Cambridge, U.K.) diluted 1:400; anti-VIP (Peninsula Lab, St. Helens, U.K.) diluted 1:1000; anti substance P (CRB, U.K.) diluted 1:400; anti-CGRP (CRB, U.K.) diluted 1:400. The vessels were then washed with PBS and incubated with anti-rabbit IgG conjugated with fluoresceine isothiocyanate (FITC) diluted 1:50 for 1 h at room temperature. Before mounting, vessels were stained with PSB. The preparation were stretched to the original size on glass slides, mounted in PBS/glycerol (Citifluor; City University, London, U.K.) and observed under a Zeiss photomicroscope equipped with epi-illumination.

Measurements

Using an ocular grid and a x16 Neofluor objective lens, the nerve fibres that intersected an ocular grid line along the opened circumference of each vessel in three different regions were counted. The mean number of nerve fibre intercepts per vessel circumference in the uterine artery of the three groups of animals was compared. Nerve bundles were also examined with a x40 objective lens in order to distinguish single fibres within the bundles.

Other segments of the uterine arteries were also used : (i) as a control for the specificity of the immunostaining, by incubating them with the antisera preabsorbed with an excess (10^{-3} M) of the appropriate neurotransmitter (all from CRB, U.K.) for 24 h at 4 °C prior to use, and (ii) to study regional differences in the density of neurotransmitter-containing nerves along the uterine artery corresponding to the uterine horn, in all three groups of animals.

Biochemical studies

Noradrenaline assay Na levels were measured using high performance liquid chromatography with electrochemical detection. The length and weight of the arteries were measured before extraction. Samples were homogenized in 500 µl of 0.1 M perchloric acid containing 0.4 mM sodium bisulphite and 12.5 ng of an internal standard, dihydroxybenzylamine (DHBA). The extraction procedure, slightly modified by the addition of 0.1 mM ethyendiaminotetracetate (EDTA) in the solution used for washing, was that of Keller et al., 1976. Chromatography was carried out using a mobile phase consisting of 0.1M sodium dihydrogen phosphate, 0.1 mM EDTA, 5 mM heptane sulphonate (pH 5.0) containing 10% (v/v) methanol on a µ-Bondapack C-18 reverse phase column (Moyer and Jiang, 1978). Detection and quantification was accomplished with a glassy carbon electrode set at a potential of +0.72 V. NA levels were corrected for loss during the extraction procedure using the DHBA internal standard. Results were expressed as nmol/cm length.

Peptide assay After measuring the length and weight of the arteries,

samples were placed in 0.5 M acetic acid (1ml) in polypropylene tubes in a boiling water bath for 15 min. The samples were homogenized, centrifuged for 30 min at 3,500 x g and lyophylized. VIP, NPY, SP and CGRP levels were quantified using an inhibition-linked immunosorbent assay (Stjernschantz et al., 1982) as described by Belai et al., 1985. Results were expressed as fmol of VIP, NPY, SP or CGRP per cm of artery. Samples from virgin, progesterone-treated and pregnant animals were extracted and assayed together to counteract any interassay variabilities.

Morphological study

Lengths of right uterine arteries (2 cm) were pinned to Sylgard-lined dishes and fixed by immersion in 5% glutaraldehyde in 0.1 M sodium cacodylate buffer. Using a fine syringe, fixative was also passed through the lumen of each vessel. Excess surrounding fat deposits were removed and fixation continued for a total of 2 hrs. After washing in buffer, the samples were cut into convenient lengths, osmicated (1% osmium tetroxide in sodium cacodylate buffer, 1 hr), washed, stained en bloc (1% uranyl acetate, 1 h), dehydrated in ethanol and embedded in Spurr resin.

Transverse section (1 µm thick) of the arteries were cut on an LKB ultramicrotome, dried and mounted directly in DPX or after staining with a methylen-blue/azur II/basic fuchsin recipe (Humphrey and Pittman, 1974).

Counts of the total number of smooth muscle cell nuclei per cross-section were made directly from the stained material and the mean tickness of the muscle and connective tissue layer of the artery wall

was determined from several measurements on each section.

Fron tracings of photomicrographs at x 560 magnification, areas of the adventitial and medial layers were determined using a digitizing system (Houston Instrument tablet connected to an Apple IIe computer). As the cross-sectional area of the lumen could not be accurately measured, due to contraction of the artery wall to an unknown extent, the length of the convoluted internal elastic lamina was measured. Cross sectional area of the lumen at maximal dilation could then be considered as that of a circle with a perimeter of this length (Todd et al., 1983).

Although the cross-sectional areas of the adventitia and media will remain constant with dilation of the vessel (Lee et al., 1983), the thickness of these layers will change. To overcome the inconsistencies due to contraction of the samples prior fixation, thickness measurements were converted to the fully dilated condition.

Statistical analysis

Results were expressed as the mean ± S.E.M. and the three groups of data were compared using analysis of variance followed by Dunnet's test for multiple comparison with a control (Dunnet, 1964). A level of probability of 0.05 or less was considered to be significant.

RESULTS

Quantitative analysis of the distribution of NA- and peptide-containing nerves in the uterine artery of virgin guinea-pigs, expressed as a number of nerve fibre intercepts per vessel circumference, showed that NA is present in the highest number of nerve fibres, followed by NPY-, VIP-, CGRP- and SP-immunoreactive (IR) nerve fibres in descending order (Table XXV).

Treatment with progesterone had no effect on any of the parameters studied of the perivascular nerves; however, the pregnant guinea-pigs had a significant reduction in the number of catecholamine-fluorescent nerve fibre intercepts per vessel circumference ($p<0.01$, Table XXV) and a significant increase in the numbers of NPY-IR nerve fibre intercepts per vessel circumference ($p<0.05$, Table XXV; Fig. 41).

No differences were observed in the pattern of innervation by VIP- SP- and CGRP-containing nerve fibres in virgin and pregnant guinea-pigs (Table XXV, Fig. 42); this resulted in a relative predominance of NPY- and VIP-containing nerve fibres in the uterine artery in late pregnancy (see Table XXV). Data from the biochemical assay for NA showed a marked reduction of NA content in the pregnant uterine artery ($p<0.01$, Fig. 43). The changes in NPY content during progesterone treatment and pregnancy were not significant (Fig. 43).

Measurements of the weight per cm of the uterine artery of pregnant guinea-pigs showed a significant increase ($p<0.001$) when compared with control and progesterone-treated animals (Fig. 44). Results from the morphological studies (Table XXVI, Fig. 45) showed

that there were no statistically significant changes in the uterine arteries of progesterone-treated animals compared with controls, for any of the parameters measured. Pregnancy, however, led to marked changes. The cross-sectional area of the lumen of arteries during pregnancy was increased by nearly three-fold, with the media increasing in size to a greater extent than that required simply to maintain the same thickness of muscle around the lumen. Although there was also a significant increase in the number of smooth muscle cells in the media during pregnancy, cell density (expressed as number of cells/1000 um^2), in fact, decreased considerably (Table XXVI).

The surrounding adventitial layer increased substantially in cross sectional area during pregnancy, but always remained the same thickness (Fig. 45). Comparison of the luminal areas, recalculated to simulate the condition of an internal elastic lamina forming a perfect circle, showed large differences in late pregnancy. The luminal area of uterine arteries during pregnancy increased by 157%, i.e. 2.5 times greater than controls (Table XXVI, Fig. 46).

DISCUSSION

The quantitative analysis carried out in this study on guinea-pig uterine arteries demonstrated that NA-containing nerve fibres formed the densest perivascular plexus, compared with NPY-, VIP-, SP- and CGRP-IR nerve fibres. The action of NA in virgin as well as pregnant uterine arteries has been studied in detail previously (Bell, 1968; Tare et al., 1988).

NPY, which often coexists with dopamine β-hydroxylase and tyrosine hydroxylase in noradrenergic nerves in the uterine artery (Morris et al., 1985; 1987), causes a long-lasting vasoconstriction, similar to NA, when infused in the isolated uterine artery of virgin guinea-pigs (Morris et al., 1985; Morris and Murphy, 1988). However, in the uterine artery, NPY-immunoreactivity is also localized in non-noradrenergic axons containing the vasodilator neurotransmitter VIP, and/or with somatostatin- or dynorphin-immunoreactivity (Morris et al., 1985; 1987). VIP and SP are potent vasodilators of the uterine vasculature (Clark et al., 1981; Ottesen and Fahrenkrug, 1981; Gram and Ottesen, 1982) and the same function can be attributed to CGRP, which is one of the most potent vasodilator neurotransmitters (Girgis et al., 1985). SP and CGRP are contained in sensory nerve fibres in the uterine artery, since both are depleted by capsaicin treatment (Gibbins et al., 1985).

These data, showing unchanged noradrenergic innervation and NA levels in the uterine artery of progesterone-treated animals, indicate that systemic treatment with progesterone, unlike the direct applicaton of the hormone (Bell and Malcolm, 1978), was unable to

affect noradrenergic nerves supplying the uterine artery.

Of major interest is the finding of an increase in the number of NPY-IR nerve fibres in perivascular uterine axons in late pregnancy, despite the decrease of NA levels and noradrenergic nerve density. The presence of NPY-immunoreactivity in non-noradrenergic axons supplying some vascular beds (Morris et al., 1985; Gibbins and Morris, 1988) would explain the lack of a disappearance of NPY-immunoreactivity after chemical or surgical sympathectomy in the guinea-pig uterine artery and cerebral vessels, respectively. In the pregnant uterine artery, it is possible that the expression of the potent vasoconstrictor NPY increases in the perivascular nerve fibres as a consequence of the reduced availability of NA. Further studies are needed to establish whether the production of NPY increases in noradrenergic axons, which have decreased levels of NA, or whether it occurs in non-noradrenergic nerve fibres, where NPY coexists with the vasodilator neurotransmitter VIP (Morris et al. 1985).

Great care was taken in the analysis of nerve density and transmitter content to ensure that the changes detected reflect true changes in the nerve and do not represent a false impression as a consequence of muscle hypertrophy or distention. The influence of hypertrophy and distention on biochemical, histochemical and immunohistochemical results has been discussed by Lincoln et al., 1984. There was an increase in the number of smooth muscle cells observed in pregnant animals. In contrast, cell density, expressed as number of nucleate cells/1000 μm^2, showed a marked reduction during pregnancy.

These values probably provide a more correct evaluation of the increase in the size of smooth muscle cells and lend support to the hypothesis that both hyperplasia and hypertrophy may contribute to the increase in the cross-sectional area and thickness of the muscular layer in uterine arteries in pregnancy. It is for this reason that the data for the innervation were expressed per vessel circumference rather than per unit surface area, and transmitter content was expressed per cm vessel length rather per g wet weight tissue.

At this stage it is not possible to determine what effect the morphological changes in the muscle during pregnancy could have on neuromuscular transmission in the uterine artery. However, the findings of the present study provide evidence for a shift in the balance of the innervation of the uterine artery from a situation where nerves mediating vasoconstriction (NA-containing nerve fibres) predominate, to one where peptidergic nerves with multiple functions are dominant.

TABLE XXV. Innervation of the uterine artery in virgin, progesterone-treated and pregnant animals expressed as number of nerve fibre intercepts per vessel circumference ± S.E.M.

Neurotransmitter	Virgin		Progesterone-treated		Pregnant	
NA	78.27 ± 5.16	(8)	82.60 ± 5.51	(6)	54.58 ± 4.35**	(6)
NPY-IR	73.10 ± 2.42	(11)	74.35 ± 7.45	(4)	90.08 ± 6.68	(6)
VIP-IR	68.83 ± 2.60	(11)	70.17 ± 2.17	(5)	65.17 ± 6.47	(6)
CGRP-IR	58.52 ± 2.57	(6)	51.44 ± 3.18	(5)	50.39 ± 2.57	(6)
SP-IR	31.73 ± 1.50	(13)	32.82 ± 1.75	(7)	33.98 ± 2.39	(7)

The number of animals is given in brackets. *$p<0.05$ virgin versus pregnant; **$p<0.01$ virgin versus pregnant.

TABLE XXVI. The effect of pregnancy and progesterone treatment on the morphology of the uterine artery.

Parameter	Virgin	Progesterone-treated	Pregnant
No. nuclei/ cross-section	88.0 ± 4.2	94.6 ± 4.4	141.7 ± 6.0**
Cell density/ 1,000 μm^2	3.58 ± 0.39	3.27 ± 0.32	2.03 ± 0.16**
Media area $x10^{-3}$ μm^2	25.6 ± 2.7	30.1 ± 3.5	72.0 ± 7.2**
Media thickness (μm)	19.2 ± 1.59	22.4 ± 1.74	30.8 ± 3.48*
Adventitia area $x10^{-3}$ μm^2	25.1 ± 1.6	27.8 ± 2.0	46.2 ± 4.9**
Adventitia thickness (μm)	17.6 ± 1.07	19.6 ± 1.16	20.2 ± 1.93
Lumen area (dilated) $x10^{-3}$ μm^2	125.0 ± 8.1	125.9 ± 11.7	321.4 ± 27.8**

Results are the mean of five animals for each group ± S.E.M. **$p<0.01$ virgin versus pregnant; *$p<0.05$ virgin versus pregnant.

Fig. 41 - Fluorescent micrographs of NA- (A-C) and NPY- (D-F) containing nerves in the uterine artery from virgin (A,D), progesterone-treated (B,E) and pregnant (C,F) guinea-pigs. Note the marked decrease of catecholamine fluorescence in pregnancy. Calibration bar = 50 µm.

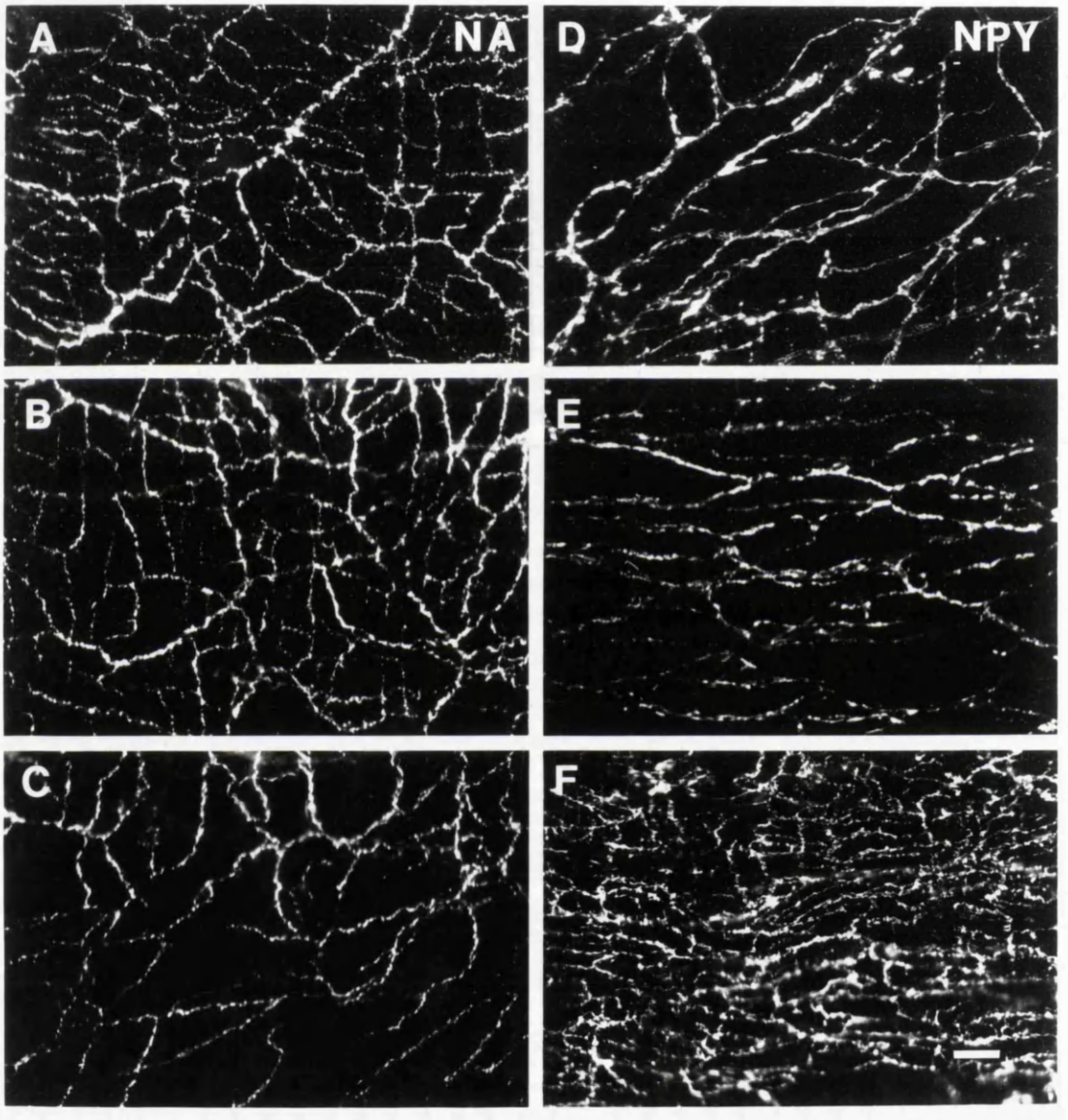

A
NA
D
NPY
B
E
C
F

Fig. 42 - Fluorescent micrographs of VIP- (A-C), CGRP- (D-F) and SP- (G-I) containing nerves in the uterine arteries from virgin (A,D,G), progesterone-treated (B,E,H) and pregnant (C,F,I) guinea-pigs. Calibration bar = 50 µm.

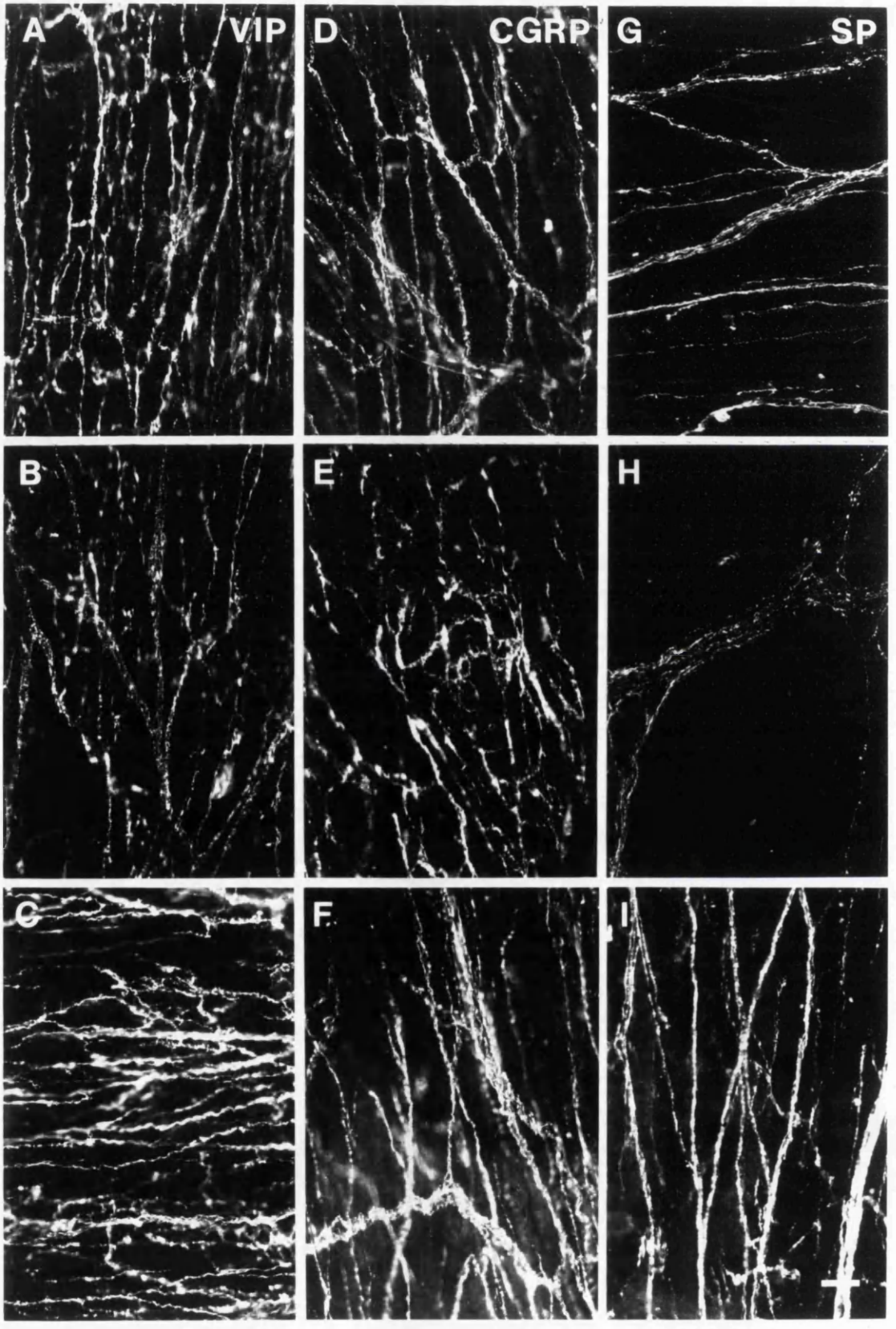
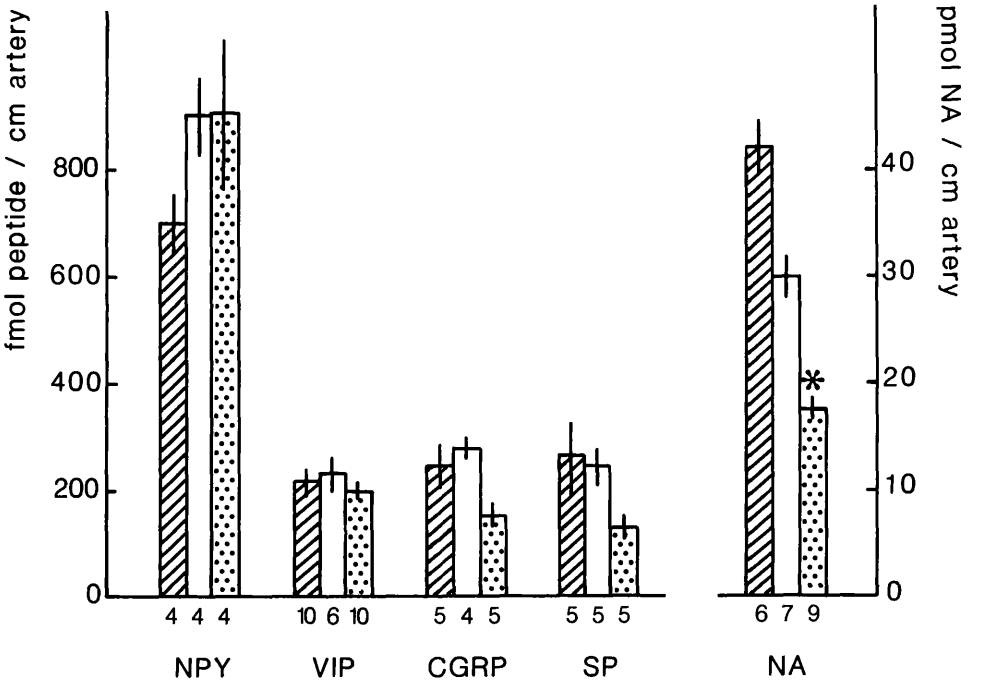

A
VIP
D
CGRP
G
SP
B
E
H
C
F
I

Fig. 43 - Histogram of neurotransmitter levels in the guinea-pig uterine artery, expressed as fmol (peptides) or pmol (NA) per cm of artery. (**cross-hatched bars**) controls; (**clear bars**) progesterone treated; (**spotted bars**) pregnant. The number of animals used in each group is given under each bar and the results given as the mean ± SEM.
*$p<0.01$ virgin versus pregnant.

Fig. 44 - Weight of the guinea-pig uterine artery per cm of vessel. (**cross-hatched bar**) controls; (**clear bar**) progesterone treated; (**spotted bar**) pregnant. The number of animals for each group is given under each bar and the results given as the mean ± SEM.
*$p<0.01$ virgin versus pregnant.


fmol peptide / cm artery
pmol NA / cm artery
800
600
400
200
0
40
30
20
10
0
4 4 4
10 6 10
5 4 5
5 5 5
6 7 9
NPY
VIP
CGRP
SP
NA
*

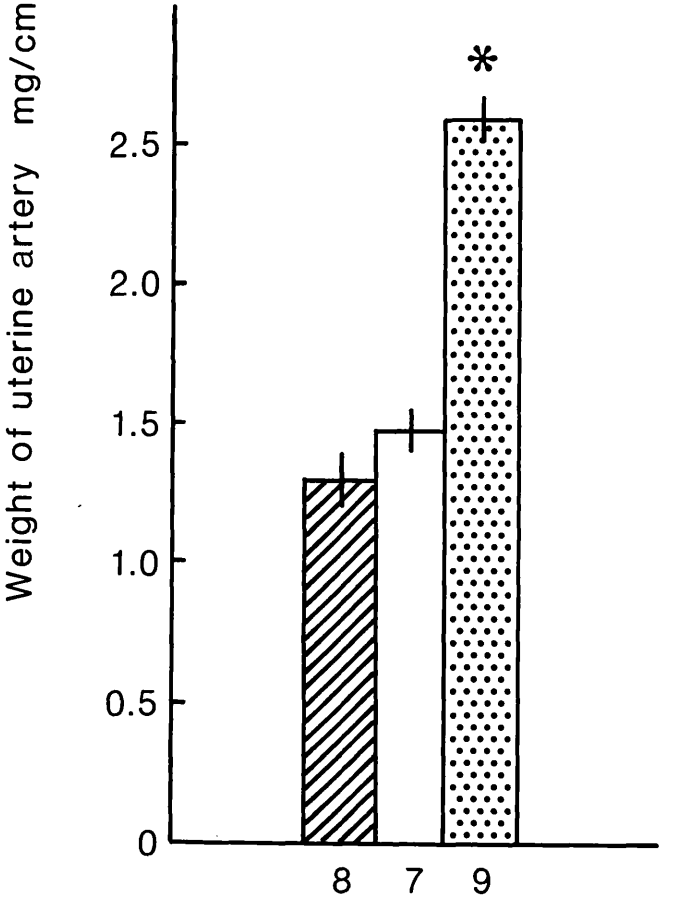

Weight of uterine artery mg/cm
2.5
2.0
1.5
1.0
0.5
0
*
8 7 9

Fig. 45 - Transverse sections (1 µm) of the uterine artery from virgin (A), progesterone-treated (B) and pregnant (C) guinea-pigs. a adventitia; m media; iel internal elastic lamina. Calibration bar = 50 µm.

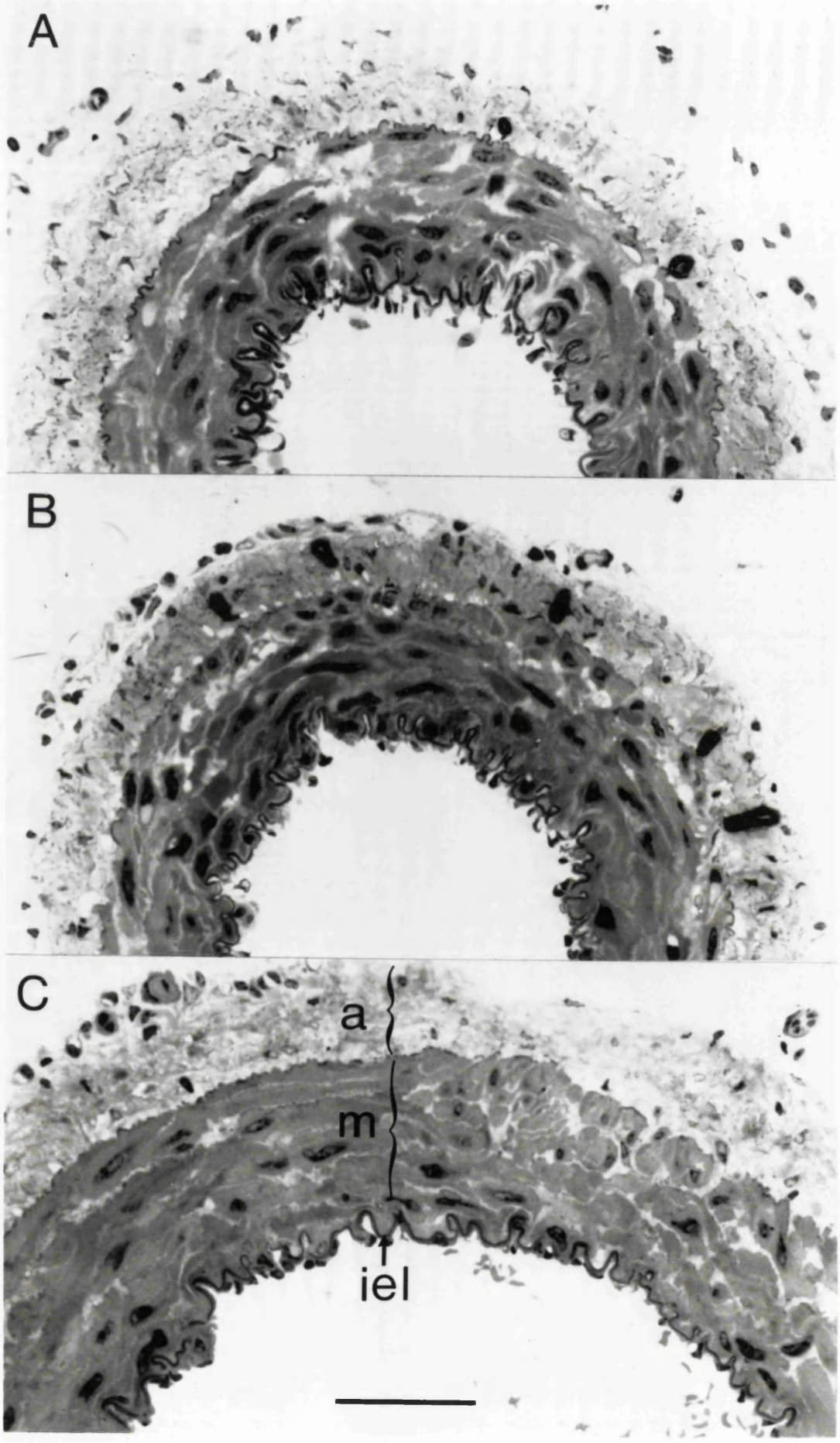
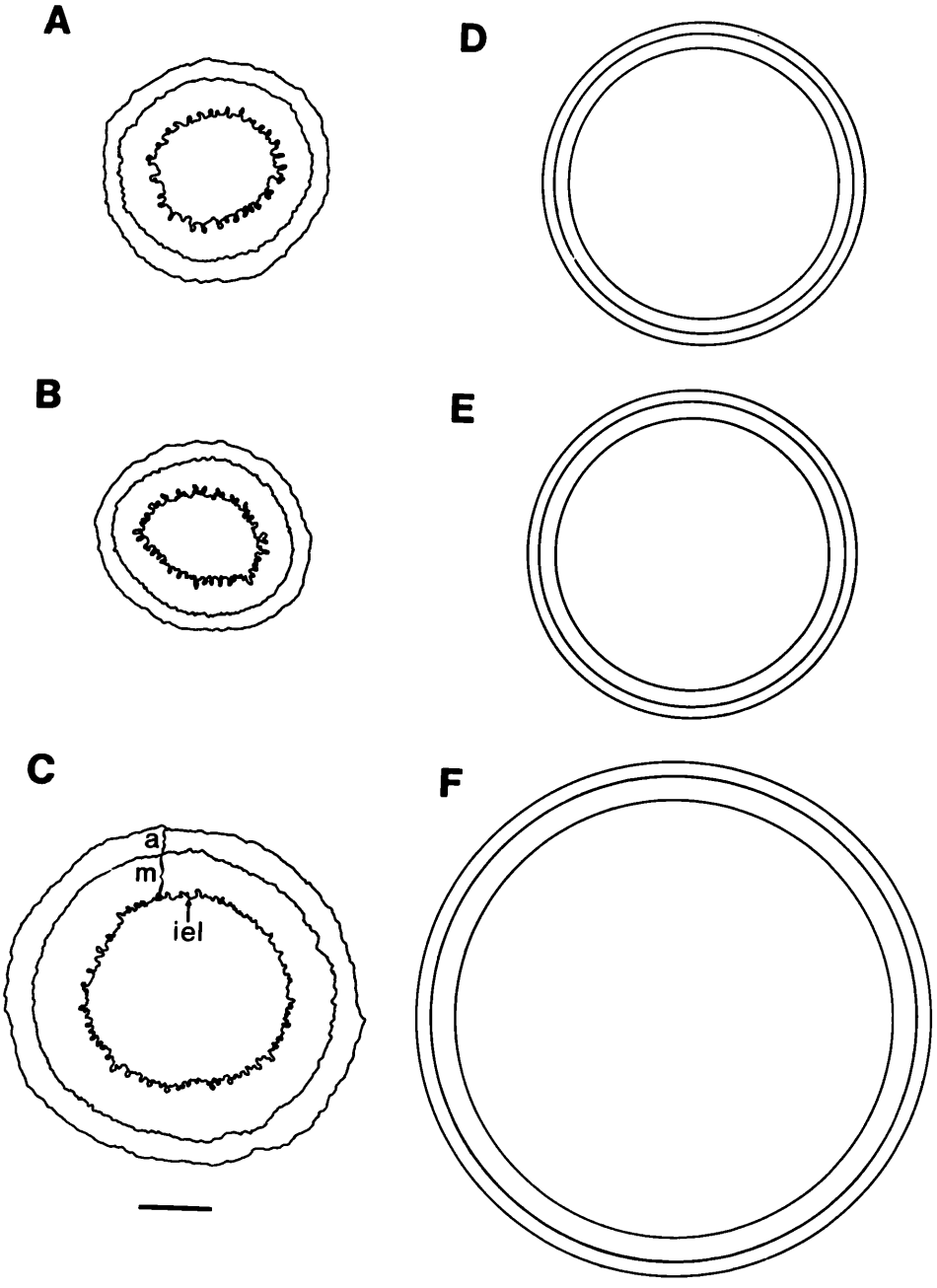

A
B
C
a
m
iel

Fig. 46 - Tracings of typical transverse sections (A,B,C) of the uterine artery from virgin (A,D), progesterone-treated (B,E) and pregnant (C,F) guinea-pigs, and after expanding the internal elastic lamina (iel) to form a perfect circle (D,E,F) without changing the areas of the media (m) or adventitia (a). Calibration bar = 100 μm.


A
B
C
a
m
iel
D
E
F

CHAPTER SIX

UPTAKE OF 5-HYDROXYDOPAMINE IN SENSORY NERVES OF GUINEA-PIG UTERINE ARTERIES IN LATE PREGNANCY

SUMMARY

Perivascular nerves of the uterine artery of virgin and late pregnant guinea-pigs were examined under the electron microscope following loading with 5-hydroxydopamine (5-OHDA), a marker for catecholamine uptake. Varicosities, loaded with 5-OHDA labelled vesicles, were counted in whole transverse sections of uterine arteries. Localization of immunoreactivity for dopamine-β-hydroxylase (DBH), neuropeptide Y (NPY), vasoactive intestinal polypeptide (VIP), substance P (SP) and calcitonin gene-related peptide (CGRP) in 5-OHDA labelled nerve varicosities was studied with an immunocytochemical method at the electron microscopical level. In addition, the colocalization of DBH- and SP-immunoreactivity was investigated with a double immunostaining technique at the light microscopical level.

The total number of varicosities with 5-OHDA labelled vesicles per section in uterine arteries increased in late pregnancy (53 ± 6.4 versus 24 ± 2.2 in virgin). In contrast, 5-OHDA labelled nerves were very sparse in the uterus of late pregnant guinea-pigs and, when present, showed severe signs of degeneration. In arteries immunostained for DBH, NPY, VIP, SP and CGRP, immunoreactivity was seen within varicose and non-varicose axons. After injection with 5-OHDA in virgins, small dense cored vesicles were prevalently found in DBH- and NPY-immunoreactive (IR) varicosities, while in late pregnancy, 5-OHDA labelled vesicles were clearly seen additionally in VIP-, SP- and CGRP-IR axons. Double immunolabelling for DBH- and SP-immunoreactivity showed that DBH-immunoreactivity was not present in SP-IR axons of the uterine artery, neither of virgin or late pregnant

guinea-pigs; rather SP-IR largely coexisted with CGRP-immunoreactivity.

It is concluded that the reduction of noradrenaline-containing nerves and levels previously shown in uterine arteries of late pregnant guinea-pigs was not accompanied by a loss of 5-OHDA labelled nerves; on the contrary, they were increased. This increase was attributed partly to growth of perivascular sympathetic nerves in conjunction with vascular hypertrophy and partly to the development of the ability of sensory and parasympathetic perivascular nerves containing SP/CGRP- and VIP-immunoreactivity, respectively, to take up 5-OHDA in late pregnancy. The absence of DBH-immunoreactivity in the sensory nerves of the uterine artery in late pregnancy indicates that, while they develop the ability to take up noradrenaline, they do not synthesize catecholamines. The relevance of these findings in pregnancy is discussed.

INTRODUCTION

Perivascular nerve fibres supplying the guinea-pig uterine artery have been divided into at least three groups : one population, which presumably correspond to those visualized by formaldehyde-induced fluorescence as noradrenaline (NA)-containing (Sjöberg, 1967), shows immunoreactivity to dopamine-β-hydroxylase (DBH) and also to neuropeptide Y (NPY) (Morris et al., 1985; 1987) and originate from short adrenergic neurons in the paracervical ganglia and from cell bodies in paravertebral sympathetic ganglia (aorto-renal and inferior mesenteric ganglia, Alm and Lundberg, 1988). Another population, classified as "non-noradrenergic" and originating from parasympathetic paracervical ganglion neurons (Gu et al., 1984; Alm and Lundberg, 1988), shows immunoreactivity to vasoactive intestinal polypeptide (VIP), dynorphin, NPY and, in a few fibers in some preparation, DBH (Morris et al., 1985; 1987). A third group, which are likely to be sensory, shows immunoreactivity to substance P (SP) and calcitonin gene-related peptide (CGRP), sensitivity to capsaicin treatment (Gibbins et al., 1985; Uddman et al., 1986) and have their cell bodies in lumbar dorsal root ganglia (Inyama et al., 1986; Alm and Lundberg, 1988).

It has been reported that NA-containing nerves in the guinea-pig uterine artery are greatly reduced in late pregnancy (Bell and Malcolm, 1978; Tare et al., 1988; Chapter five) in parallel with the disappearance of NA-containing nerves in the uterine wall (Sjöberg, 1968; Owman et al., 1975; Lundberg et al., 1987; 1988). At the ultrastructural level, the disappearance of NA-fluorescent nerve

fibres in the uterus has been associated with degeneration of 5-OHDA labelled nerves (Sporrong et al., 1981; Lundberg et al., 1987), since 5-OHDA- enhancement of vesicle-density has been widely used as a tool to demonstrate catecholamine-uptake ability at the electron microscopical level (Tranzer and Thoenen, 1967).

In this study, 5-OHDA labelled nerve varicosities in the uterine arteries of virgin and late pregnant guinea-pigs, were quantified; the colocalization of the immunoreactivities for the noradrenaline-synthesizing enzyme, DBH, and for SP was also investigated.

MATERIALS AND METHODS

Two groups of Dunkin-Hartley guinea-pigs were used : virgin sexually mature females (3-5 months of age) and those in late pregnancy (over 50 days of gestation, as estimated from the crown-rump lengths of the foetuses, Draper, 1920). The animals were injected with 5-OHDA (10 mg/Kg, dissolved in saline containing 0.2% ascorbic acid, i.v.) 20 min before perfusion (Tranzer and Thoenen, 1967), except for a few animals, that were not injected. Animals were perfusion-fixed with a modified Zamboni's fixative (see General Methods). Lengths of uterine arteries of about 1 cm, corresponding to that part of the artery facing the middle region of the uterine horn, and small pieces of uterine wall (in pregnant animals, the uterine wall overlaying a foetus) were postfixed in OsO_4 in sodium cacodylate buffer (0.1 M , pH 7.4), dehydrated in alcohol, stained "en block" with 2% uranyl acetate in 70% alcohol and embedded in Spurr resin.

Quantitative electron microscopy

Ten arteries from 5 virgin and 5 pregnant guinea-pigs were used in this study. Three animals of each group had been injected with 5-OHDA. The arteries were transversely sectioned and all the nerves (as nerve bundles or single axons) within the adventitial layer of the vessels were photographed and printed at a final magnification of x 13.000. Areas of sections covered by grid bars were reconstructed with the aid of adjacent sections. The number of varicosities (axon profiles with more than 10 vesicles) containing mainly small (< 50 nm) agranular vesicles, small and large (>60-80 nm) 5-OHDA labelled

vesicles and large dense cored vesicles, that did not take up 5-OHDA, were counted. Statistical analysis was performed with the unpaired Student's T test.

Immunocytochemistry

Some segments of uterine arteries (from 5 different animals for each group) were immunostained for DBH-, NPY-, VIP-, SP- and CGRP-IR, following the pre-embedding immunostaining protocol (described in section II of the General Methods). Polyclonal antisera, from commercial sources (Cambridge Research Biochemical, Cambridge, U.K. for anti-NPY, -SP and -CGRP antisera; Peninsula Laboratories, St. Helen, U.K. for anti-VIP antiserum; Eugene Tech., Allendale, N.J.,U.S.A., for anti-DBH antiserum) were used at a dilution 1 : 1,000. A biotin anti-IgG antiserum followed by streptavidin horseradish-peroxidase conjugated complex (Amersham, U.K.) was used to reveal the sites of the immunoreaction, at dilutions and incubation times recommended by the manufacturers. Immunostaining was sometimes followed by silver intensification of diaminobenzidine (DAB)-end products (section II, General Methods). Some specificity tests were performed, by omitting the first layers, by preincubating them with the appropriate antigen, when available, or by replacing it with normal rabbit serum. No immunoreactivity was found in these samples.

Immunostained segments of uterine arteries were washed in sodium cacodylate buffer, postfixed in 5% glutaraldehyde in the same buffer, osmicated, dehydrated and embedded as before. Ultrathin, transverse sections were cut with a OM 2 Reichert ultramicrotome, stained with

uranyl acetate and lead citrate, viewed and photographed with a Philips 300 electron microscope.

Double immunostaining

Segments of uterine arteries (of non-perfused virgin and late pregnant guinea-pigs) were opened longitudinally, stretched on sheets of Sylgard (BDH, Poole, England) and fixed with 4% paraformaldehyde in phoshate buffered saline (PBS). After dehydration in 80% ethanol, vessels were incubated with anti-DBH or anti-CGRP antisera, both raised in rabbit, diluted 1:400 in PBS for 12 h at room temperature. A goat anti-rabbit IgG, conjugated with tetramethylrhodamine (TRITC; Nordic Immunoreagents, Denmark) at a dilution 1:100 was used for 1 h at room temperature. After several washes with PBS, arteries were incubated with an anti-SP antiserum, raised in rat (Sera Lab, U.K.) at a dilution 1:200 for 12 h, followed by a rabbit anti-rat IgG conjugated with fluoresceine isothiocyanate (FITC; Nordic Immunoreagent, Denmark), as for the other second layer. The segments of arteries were then mounted flat on polylysine-coated slides with Citifluor (City University, London, U.K.). Observation and photography was carried out with a Zeiss photomicroscope equipped with epi-illumination and adequate filter setting for alternate observation of FITC- (excitation 490 nm, emission 525 nm) and TRITC- (excitation 520 nm, emission 590 nm) fluorescence.

RESULTS

Virgin guinea-pigs

Paravascular nerve bundles of uterine arteries were located in the outer adventitia and were ensheathed by connective tissue; up to 50 axons, almost all unmyelinated, were intermingled with Schwann cells and collagen fibrils. The axons in these nerves very rarely contained vesicles. Smaller nerves were present in the deeper adventitia, lining the external elastic lamina or facing smooth muscle cells. These nerves were made up of a few axons, usually bearing vesicles, partially ensheathed by the Schwann cell cytoplasm. No nerves were seen inside the media.

In nerves from animals not injected with 5-OHDA, the majority of vesicles (≈ 90%) were small (≤50 nm) and agranular (fig. 47a and table XXVIII). A few varicosities (< 10%) contained a predominance of large (>80 nm) dense-cored vesicles (fig. 47c). Nerves containing small dense-cored vesicles were very rare in these preparations. After injection with 5-OHDA, a number of axons (24 ± 2.2 per section, fig. 47b and table XXVII) within the adventitial bundles, contained small and few large dense-cored vesicles. In the uterus, small intramuscular bundles of 2-3 axons were common. Some of the axons contained a variable number of small dense-cored vesicles, although the majority contained small agranular vesicles (not shown).

In arteries immunostained for DBH- or NPY-IR, the peroxidase products in varicosities were usually associated with dense-cored vesicles, while immunoreactivity for VIP, SP or CGRP was confined to axons and varicosities containing predominantly small agranular

vesicles (fig. 48c). However, immunopositivity for any of the above peptides occurred whether vesicles were present or not within the axon profile (figs. 48a, b, d; see also fig. 51a).

Pregnant guinea-pigs

The general appearance of nerves was similar to that in virgin animals. After injection with 5-OHDA, a large number of axons (53 ± 6.4 per section; table XXVII) displayed small dense-cored vesicles. These axons had a normal ultrastructural appearance (fig. 49a) while, in the uterine wall, the very few nerves that were found, and in which 5-OHDA labelled vesicles were recognizable, showed signs of severe degeneration, in contrast with nerves containing small agranular vesicles, that were preserved (fig. 49b).

After immunostaining, positive axons were found in all the preparations. There was often more than one positive axon per nerve bundle, giving the impression of an increase of immunoreactive axons, but no quantitative evaluation was attempted. Nerves were similar to those seen in arteries of virgin animals; however, 5-OHDA labelled vesicles were not confined to DBH- or NPY-IR nerves only, but were consistently found in VIP- (fig. 50b), SP- (fig. 50d) or CGRP-IR nerve varicosities (fig. 51b). However, a number of axons, including some immunoreactive for NPY (fig. 50a), SP (fig. 50c), DBH, VIP or CGRP (not shown) were devoid of vesicles. The presence of some damage by DAB-end products and the occurrence of immunoreactivity in non varicose axons makes any evaluation on the preferential association of type of vesicles with a certain immunoreactivity difficult to resolve.

Colocalization

In double immunostained arteries, no coexistence was found between SP- and DBH-immunoreactivity in arteries from either virgin or late pregnant guinea-pigs (figs. 52a, b). However, all SP-IR axons also exhibited CGRP-immunoreactivity (figs. 52c, d).

DISCUSSION

By combining morphometry, histochemistry and uptake studies it was possible to quantify the number of axons with 5-OHDA labelling and to assess the extent to which peptide-immunoreactivity and NA or catecholamine-related enzymes (I.E. DBH) coexist in the same axons of the uterine artery of virgin and late pregnant guinea-pigs.

It is well established that in the myometrium, the number of axons detectable by catecholamine-fluorescence is dramatically reduced in late pregnancy (Alm et al., 1979b; Owman, 1983; Bell and Malcolm, 1988). Ultrastructural studies confirm that this is due to loss by degeneration of intramuscular sympathetic nerve fibres (Sporrong et al., 1981) and periaxonal Schwann cells (Alm and Lundberg, 1988). Proposed mechanisms include: hormonal (progesterone) inhibition of catecholamine synthesis (Malcolm, 1986) or a concurrence of mechanical (dilation) and hormonal factors (Owman, 1983).

Similarly, a marked reduction in the number of NA-fluorescent nerve fibres was shown in the uterine artery in late pregnancy (Bell and Malcolm, 1978; Chapter five). However, in the present experiments, no signs of degenerating nerve fibres were found in the uterine artery of the late pregnant guinea-pigs, and, in this respect, the changes affecting sympathetic nerves during pregnancy appear different in the myometrium than in the uterine artery. The fall in the number of nerve fibres displaying NA-fluorescence in the uterine artery in late pregnancy is not interpreted as an indication of nerve fibres loss (as in the case of the myometrium) but of a reduction in the amine content of the nerve fibres, so that many of

these sympathetic nerve fibres become undetectable by histochemistry. The conclusion that there is no loss of perivascular sympathetic nerves in the artery during pregnancy is consistent with two further observations made in the course of these experiments, namely that many nerve fibres in the artery of pregnant guinea-pigs remain positive for DBH-immunoreactivity and many fibres do take up the false transmitter 5-OHDA. Neither of these two latter observations, however, is conclusive proof that the sympathetic nerves are exclusively involved. In fact: a) DBH, a classical marker of sympathetic nerves, is also detected in many non-sympathetic neurons (see Mione et al., 1991, for review), including parasympathetic paracervical ganglion neurons and their projection to the uterine artery in guinea-pigs (Morris and Gibbins, 1987; Morris et al., 1987); b) 5-OHDA uptake, another classical marker of NA-containing nerves, has been found in cholinergic sympathetic neurons, in which NA stores and NA-related enzymes have been lost (Landis and Keefe, 1983).

An intriguing observation concerning the uptake of 5-OHDA is that the number of axons so labelled is significantly higher in the uterine artery of the pregnant guinea-pigs by comparison with the virgins (53 ± 6.4 versus 24 ± 2.2). Two hypothesis can be put forward to explain the increased number of axons taking up 5-OHDA. The first one is that there is an increase in the number of sympathetic axons, by the branching and/or lengthening of the existing fibres. This possibility is in agreement with the observation that the <u>total</u> number of perivascular nerve fibres increases significantly in the uterine artery in late pregnancy (Mione and Gabella, 1991). The

second hypothesis is that perivascular nerve fibres that are not sympathetic (i.e. sensory SP/CGRP-IR and parasympathetic VIP-IR nerves), which were unable to take up 5-OHDA in normal conditions (virgin animals) develop the ability to do so in late pregnancy. This ability is due to the presence of a specific, high affinity NA-transporter on the plasmamembrane. The NA- (and presumably 5-OHDA) transporter of human sympathetic nerves is a membrane-bound protein of 617 aminoacids, that has been recently cloned (Pacholczyk et al., 1991). Northern blot analysis of tissue and cell expression of the NA-transporter mRNA with cDNA probes, has shown the presence of two different mRNAs, one of which, a 5.8 kilobases (Kb) mRNA, is preferentially expressed in noradrenergic neurons of the locus coeruleus and in the adrenal gland; the other, a 3.6 Kb mRNA, was found in all the other brain areas, that lack, or contain very few noradrenergic cell bodies and axons, in addition to locus coeruleus neurons and adrenal gland (Pacholczyc et al., 1991). It is hypothesized that an inactive form of the NA-transporter may be present on the membrane of non-noradrenergic neurons and glial cells (Kimelberg et al., 1983). By analogy with some neurotransmitter receptors (i.e. the β-adrenergic receptor in the rat liver, Schleifer et al., 1989; and the muscarinic receptor in the chick heart, Galper et al., 1984) and with the nerve growth factor (NGF) receptor (Hempstead et al., 1991) that can be present on the cell membranes in an inactive form (defective of the intracellular transducer, i.e. of the GTP-proteins for the neurotransmitter receptors - Lumford and Talamo, 1983; Halvorson and Nathanson, 1984 - and of the tyrosin kinase for the NGF receptor - Hempstead et al., 1991) it can be

speculated that pregnancy may activate a NA-transporter already present in sensory and parasympathetic nerves of the guinea-pig uterine artery.

Lastly, the unlikely possibility needs to be considered that coexistence of VIP- and SP/CGRP-immunoreactivity with 5-OHDA labelled vesicles is expressed in newly grown sympathetic axons that have lost their ability to synthesize NA, but gained the ability to synthesize VIP and/or SP/CGRP. However, since no DBH-immunoreactivity was found in these SP/CGRP-IR axons, the present study suggests that at least SP/CGRP-immunoreactivity is confined to non-sympathetic perivascular nerves with a sensory origin in virgin (Alm and Lundberg, 1988) as well as in late pregnant guinea-pigs; this finding also suggests that these are the nerves that acquire the ability to take up 5-OHDA (and presumably NA) in late pregnancy, perhaps in addition to some parasympathetic axons.

It is likely that these additional stores of NA play a role in the vasoconstriction of the uterine artery during parturition. This hypothesis is discussed in details in the next Chapter.

TABLE XXVII. Number of nerve varicosities in the guinea-pig uterine artery

GROUP	5-OHDA-labelled (3)	Agranular (3) (non-labelled)	LDC (5) (non-labelled)
Virgin (5)	24 ± 2.2	58 ± 3.8	9 ± 2.1
Pregnant (5)	53 ± 6.4*	92 ± 10.3*	20 ± 5.6*

The number of animals is given in brackets. Three out of five animals per group were injected with 5-OHDA and the number of varicosities (those containing more than 10 vesicles) with 5-OHDA labelling (5-OHDA) and varicosities containing agranular vesicles (agranular) was counted in these arteries. In non-injected animals, up to 90% of varicosities contained agranular vesicles, while the number of varicosities containing large dense-cored vesicles (LDC), was similar in 5-OHDA injected and non-injected animals of the same group. * $p<0.1$

Fig. 47. - Micrographs of representative nerve varicosities in the wall of the uterine artery of virgin guinea-pigs. (**a**) two varicosities containing small agranular vesicles in a nerve bundle facing a smooth muscle cell with the interposed external elastic lamina; (**b**) a varicosity containing small and large dense cored vesicles, after 5-OHDA loading; (**c**) three axons predominantly filled with large dense cored vesicles. Calibration bar : 0.5 µm

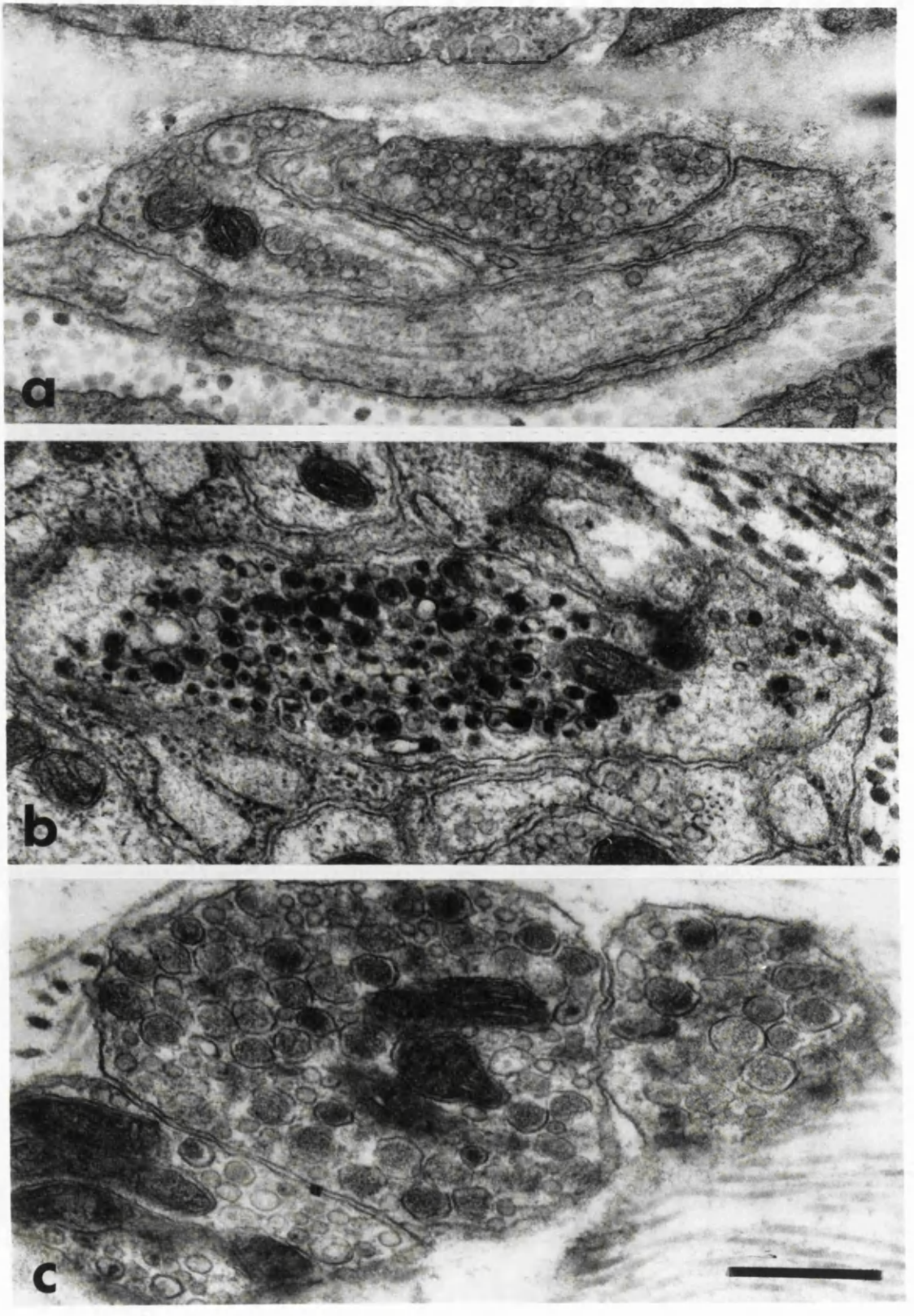

a
b
c

Fig. 48 - Immunoreactive nerves in the wall of the uterine artery of virgin guinea-pigs. (**a**) DBH-IR; (**b**) SP-IR; (**c**) VIP-IR; (**d**) NPY-IR. In (**b**) and (**d**), the DAB-end products were silver-gold intensified (**black particles**). Note the localization of VIP-IR in a varicosity packed with small clear vesicles in (**c**), a common feature for VIP-IR nerves in virgin animals. Calibration bars : 0.5 µm

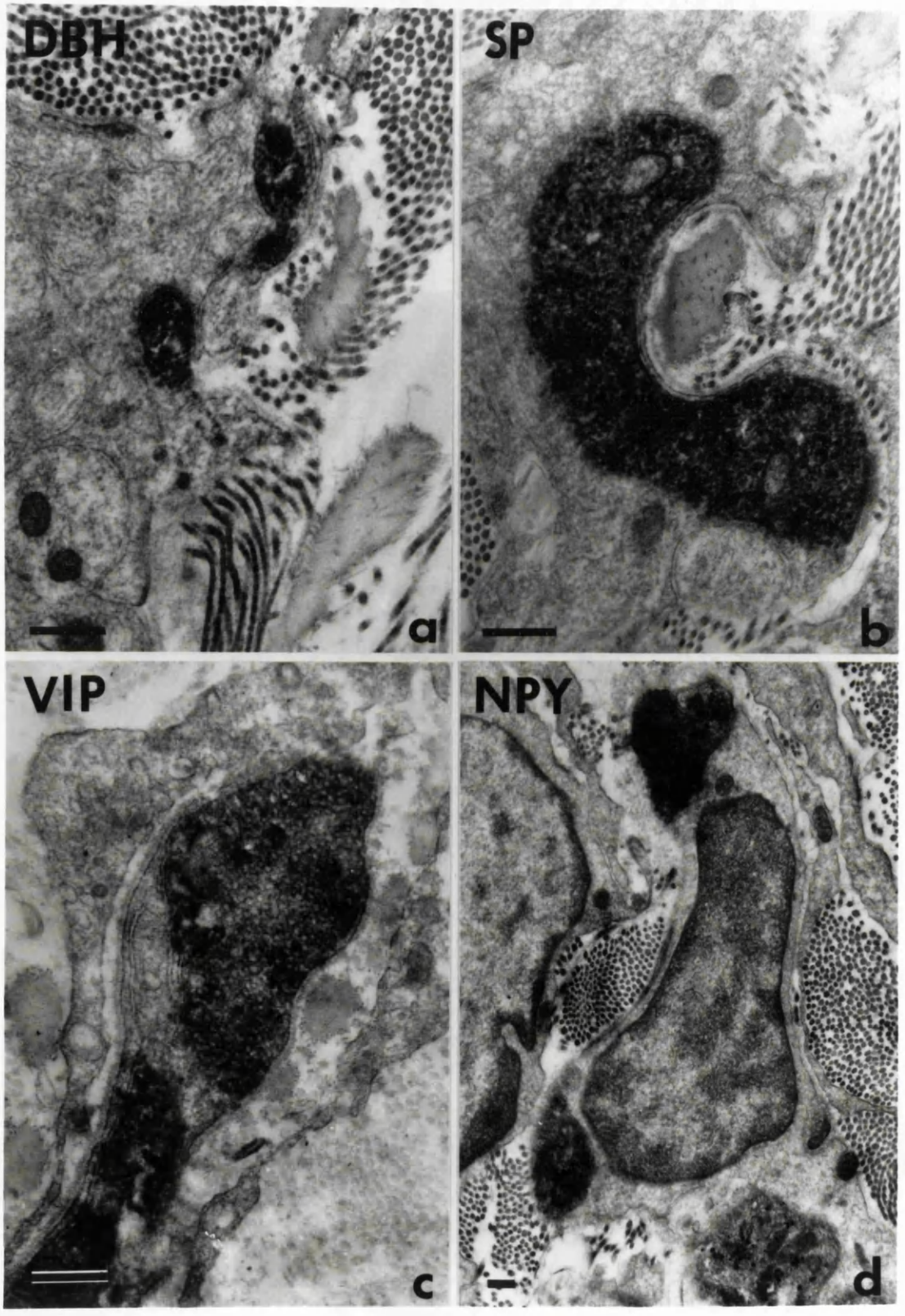

DBH
a
SP
b
VIP
c
NPY
d

Fig. 49 - Late pregnant guinea pig. (**a**) uterine artery; (**b**) uterus. Note the presence of varicosities filled with either small agranular vesicles or small dense cored vesicles in the uterine artery (**a**), while in the uterus (**b**) a large area of degeneration, likely including a varicosity with dense cored vesicles (**asterisk**) is present. The preservation of a varicosity containing clear vesicles (**arrows**) in the same nerve bundle is in striking contrast. Calibration bars : 1 µm

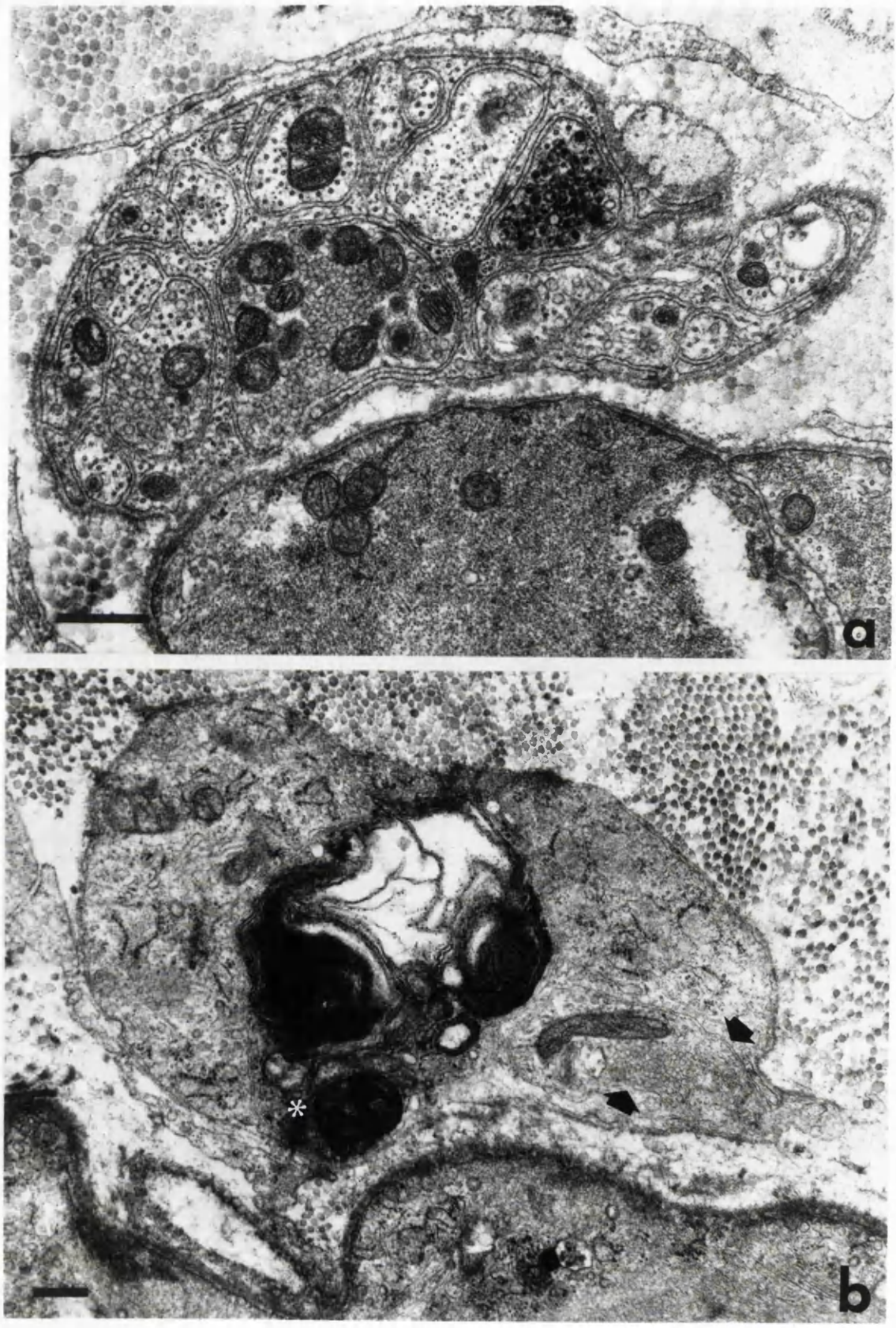

a
b
*

Fig. 50 - Micrographs of immunoreactive axons in the uterine artery of pregnant guinea pigs, injected with 5-OHDA. (**a**) NPY-IR; (**b**) VIP-IR; (**c**) and (**d**) SP-IR. Dense cored vesicles are present in VIP- (**b**) and SP- (**d**) immunoreactive varicosities (**asterisk**). Note that: i. NPY-immunoreactivity in (**a**), was associated with a non-varicose axon, as frequently seen for all neuropeptide immunoreactivities; ii. SP-immunoreactivity in (**c**) was also present in a non-varicose axon, while a neighbouring varicosity (**arrowheads**), containing large dense cored vesicles, was devoid of SP-immunoreactivity. Calibration bars : 0.5 µm

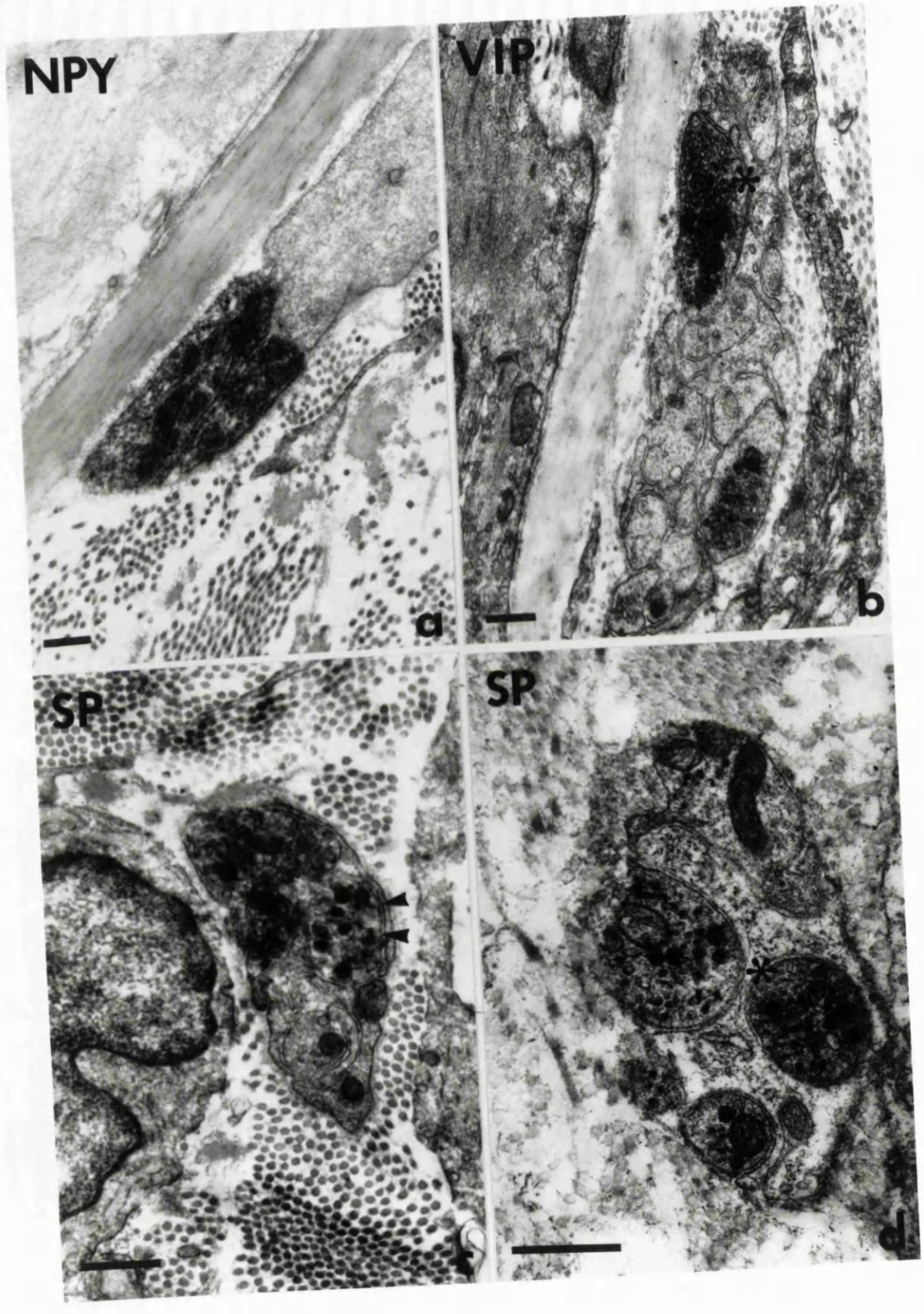
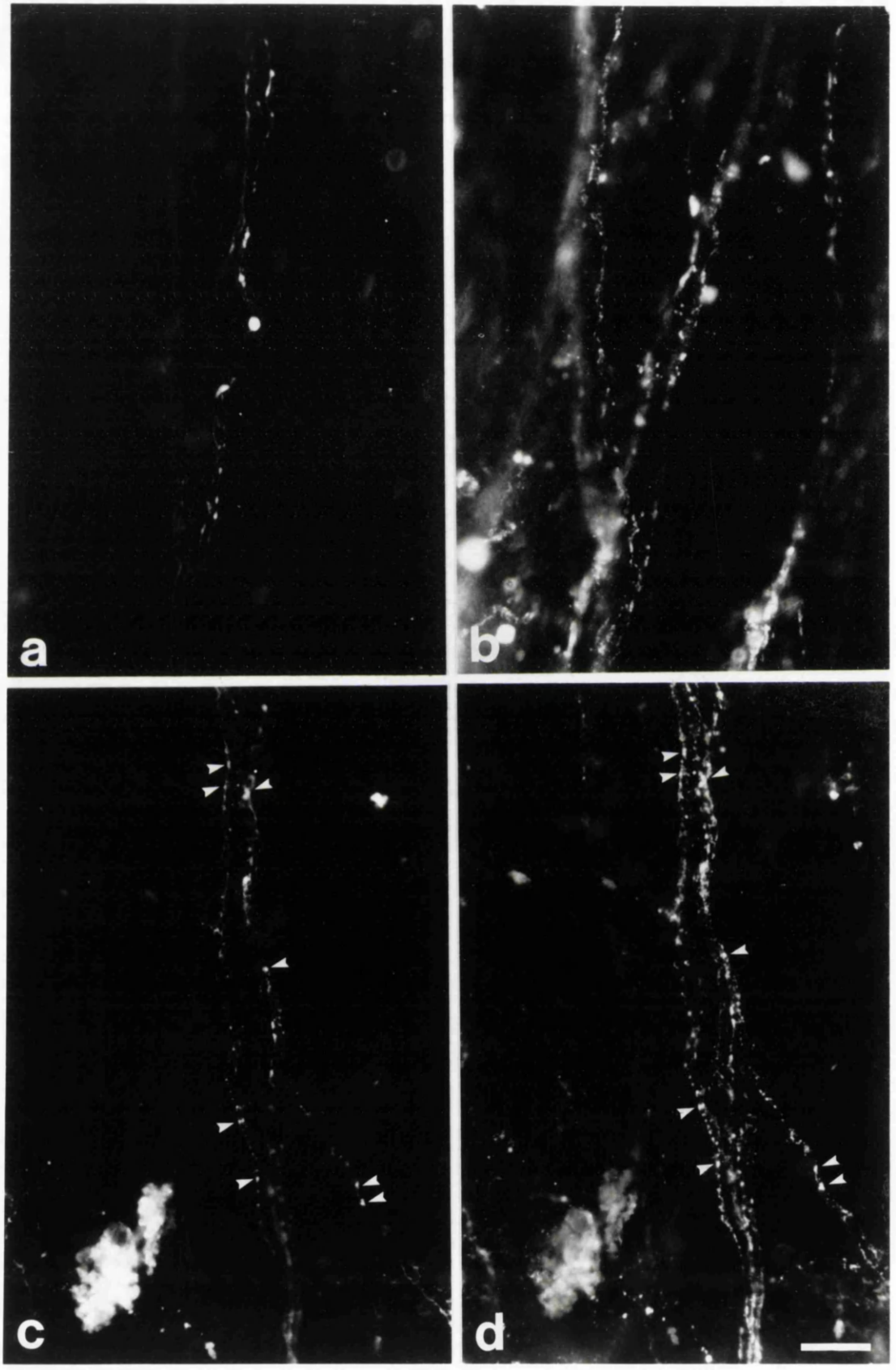

NPY
a
VIP
b
SP
c
SP
d

Fig. 51 - CGRP-immunoreactive nerves in the uterine arteries of virgin (**a**) and late pregnant (**b**) guinea-pigs injected with 5-OHDA. Note the colocalization of the immunoreactivity in a varicosity containing mainly small dense cored vesicles in late pregnancy (**b**), while this colocalization never occurred in virgin animals. Calibration bars : 0.5 µm

Fig. 52 - Fluorescent micrographs of double stained whole mount preparations of uterine arteries of a pregnant guinea-pigs. (**a-b**) and (**c-d**) represent the same microscopic fields. In (**a**), SP-IR nerve fibres visualized with a FITC-conjugated second layer are shown, while in (**b**), a TRITC-conjugated antiserum stained the DBH-IR nerve fibres. Note that immunoreactivity for SP (**a**) and DBH (**b**) are confined to different nerve fibres. In (**c**) immunoreactivity for SP (FITC-conjugated) shows a localization identical to CGRP-immunoreactivity (TRITC-conjugated) in (**d**). **Arrowheads** point to some varicosities containing both immunoreactivities. Calibration bar : 50 µm


a
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c
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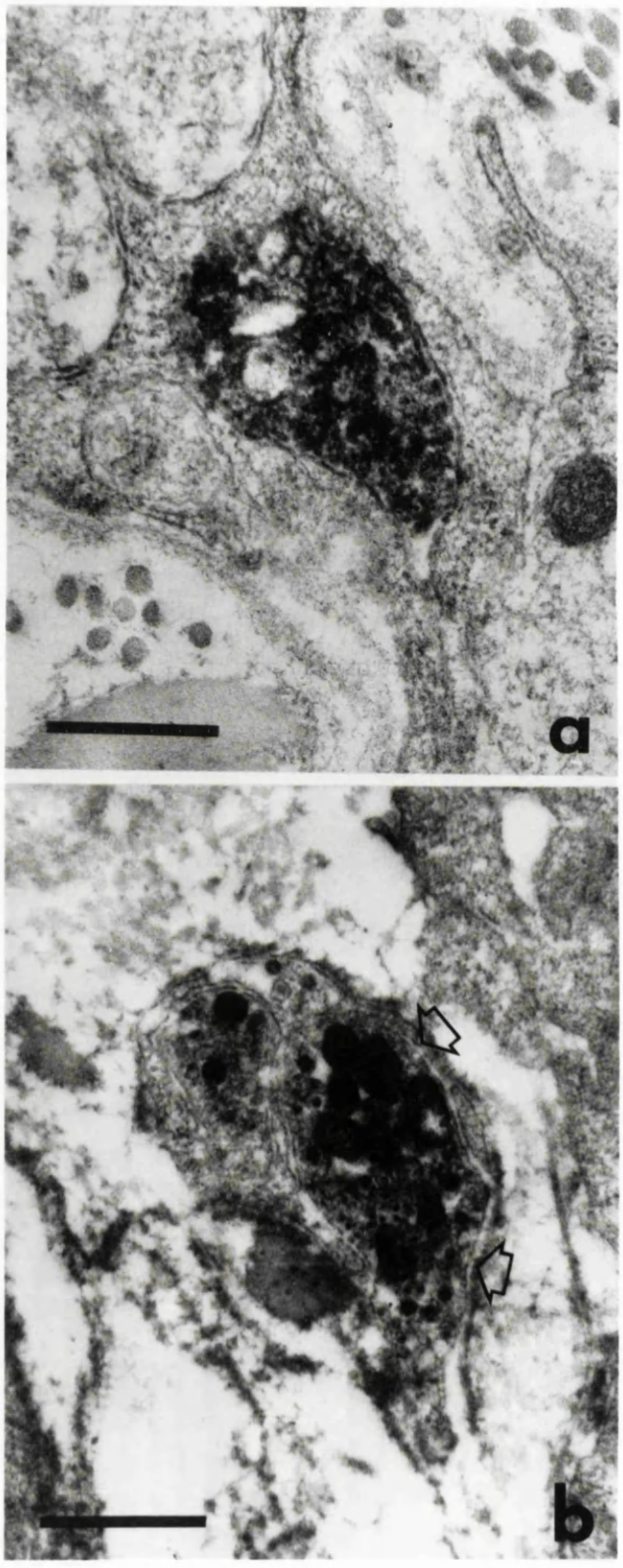

a
b

CHAPTER SEVEN

UPTAKE OF ^{3}H-NA IN SENSORY AND PARASYMPATHETIC NERVES OF GUINEA-PIG UTERINE ARTERIES IN LATE PREGNANCY.

SUMMARY

Fluorescent noradrenaline (NA)-containing nerves of uterine arteries of virgin and late pregnant guinea-pigs were visualized with the glyoxylic acid technique following incubation with 10^{-5} M NA. The uptake of ^{3}H-noradrenaline (^{3}H-NA, 10^{-8} M) was measured biochemically and localized at the light and electron microscope with an autoradiographic technique. This was combined with the immunolabelling of dopamine beta hydroxylase (DBH)-, neuropeptide Y (NPY)-, vasoactive intestinal polypeptide (VIP)-, substance P (SP)- and calcitonin gene-related peptide (CGRP)-immunoreactive nerves for visualization with the electron microscope.

Prior to NA loading, the number of NA-containing nerves appeared to be reduced in uterine arteries of late pregnant animals. However, NA loading increased the number of fluorescent perivascular nerves in late pregnant, but not in virgin guinea-pigs. The neuronal uptake (cocaine-sensitive) of ^{3}H-NA per cm of artery was significantly higher in late pregnant than in virgin animals, as was the high affinity ^{3}H-NA uptake inhibited by desipramine. Removal of the endothelium prior to incubation did not affect the significant increase in cocaine-sensitive ^{3}H-NA uptake. The biochemical data were confirmed with autoradiography, by the presence of a dense and thick coat of silver granules for ^{3}H-NA within the adventitia and adventitial-medial border of arteries of pregnant animals and not within the vascular smooth muscle or endothelial cells. The ultrastructural study showed silver grains on nerve bundles and varicosities. In arteries from virgins, further processed for

immunocytochemistry, ^{3}H-NA uptake sites were found almost exclusively on DBH- and NPY-IR nerves, while, in late pregnancy, a number of VIP-, SP- and CGRP-IR nerves also displayed deposition of silver granules.

These results suggest that neuronal NA-uptake is not impaired in uterine arteries of late pregnant guinea-pig, but rather is increased, and that NA can be taken up into parasympathetic and sensory nerves containing VIP-, SP- and CGRP-IR, which is not observed in virgins.

INTRODUCTION

Pregnancy-related changes of noradrenergic mechanisms in the guinea-pig uterine artery have been described extensively in pharmacological and morphological studies. It has been shown that there is a reduction of fluorescent, NA-containing perivascular nerves (Bell and Malcolm, 1978; Chapter five, this thesis) and that transmural nerve stimulation is less efficient in evoking excitatory responses (Tare et al., 1988). Reduced sensitivity of smooth muscle cells to NA has been ruled out by Fallgreen et al., 1988, who showed that pharmacological responses to exogenous applied NA are increased in late pregnancy.

It is still a matter for debate whether the reduction of NA-containing nerves and NA levels in the uterine artery in late pregnancy is due to degeneration of nerves, as in the uterus (Sporrong et al., 1981) or to reduced levels of detectable NA. Therefore, in the present study, the effect of pregnancy on the uptake of NA into perivascular nerves of the uterine artery was investigated. An incubation with 10^{-5} M NA was carried out before fluorescence histochemistry with the glyoxylic acid technique and uptake of ^{3}H-NA was measured in the presence and absence of cocaine and desipramine (blockers of neuronal uptake and the high affinity NA-uptake mechanism, respectively). In addition, autoradiography at the light and electron microscopical level was used to localize the sites of ^{3}H-NA uptake; this was combined with immunocytochemistry for DBH-, NPY-, VIP-, SP- and CGRP-IR to investigate the localization of ^{3}H-NA uptake sites within specific subpopulations of nerves.

MATERIALS AND METHODS

Animals

12 virgin (3-4 months of age) and 11 bilateraly pregnant (in the last 10 days of pregnancy, as estimated from the number and crown-rump length of the foetuses) Dunkin-Hartley guinea-pigs were used. Animals were killed by anaesthetic (pentobarbital) overdose, the left uterine artery was rapidly dissected out from surrounding fat and processed for the study of ^{3}H-NA uptake as described below.

Na-fluorescence

The right uterine arteries were cut in segments of approximately 1 cm and alternatively incubated in oxigenated Krebs solution, containing or not, 10^{-5} M NA hydrochloride (Sigma), 1 mg % pargilin (Sigma) and 0.1% ascorbic acid, for 20 min at 37 °C, before being processed with the glyoxylic acid fluorescence technique (Lindvall and Björklund, 1974), as whole mount stretch preparations (Chapter five).

^{3}H-NA uptake

Uterine arteries were kept in a modified Krebs' solution, bubbled with 95% O_2, 5% CO_2. The composition of Krebs' was as follows (millimolar): NaCl:122.0; KCl:5.2; $CaCl_2$:2.4; $MgSO_4$:1.2; $NaHCO_3$:25.6; K_2HPO_4:1.2; disodium EDTA (ethylendiaminotetracetato):0.03; glucose:11.0; ascorbic acid: 0.1; pargyline (monoaminoxidase inhibitor):0.1, according to Fukuda et al., 1986. The uptake

inhibitors cocaine and desipramine were made in Krebs' solution at a concentration 10 μM and 0.2 μM and used to distinguish whether uptake was neuronal or non-neuronal.

Segments of uterine arteries (approximately 1 cm long) were preincubated for 30 min in Krebs' solution, in the presence and absence of inhibitors (at room temperature). The segments of arteries were then transferred to 2.97 ml of fresh Krebs' solution, again with and without inhibitors at 37 °C and uptake was initiated by the addition of 30 μl of ^{3}H-NA (specific activity 36Ci/mmol, Amersham International, U.K.) to give a final concentration of 10 nM. This concentration was chosen as it was in the range for the high affinity neuronal uptake system. Incubation was carried out at 37 °C in Krebs' solution for 5 min, after which the arterial segments were washed three times in Krebs' solution, blotted dry and their weights and lengths measured. The arteries were solubilized in 0.5 ml Optisolv (LKB, Scintillation products) overnight, 5 ml Optiphase 'MP' (LKB, Scintillation Products) were added and the radioactivity was measured on a Beckman L 7500 Liquid Scintillation Counter equipped for automatic quench calibration. Uptake was calculated for the 5 min incubation period. Cocaine- and desipramine-sensitive uptake was calculated as the difference between the uptake in the presence and in the absence of the inhibitors measured in the same arteries. Since it has been reported that the endothelial cells may possess a high affinity Na uptake system which is sensitive to cocaine (Rorie and Tyce, 1985), in a second series of experiments (carried out in 6 virgin and 5 pregnant guinea pigs) the endothelium was removed prior to performing the ^{3}H-NA uptake measurements as described above.

Histological examination of portion of arteries confirmed the removal of the endothelium.

All results were expressed per cm length, statistical analysis was carried out with the unpaired student T test.

Autoradiography

For the autoradiographic analysis, segments of arteries incubated as above were immediately fixed in 5% glutaraldehyde in 0.1 M sodium cacodylate buffer or in the modified Zamboni fixative for immunocytochemistry. Anti-DBH (Eugene Tech, Allendale, N.Y., U.S.A.), anti-NPY, anti-VIP, anti-SP or anti-CGRP (Cambridge Res Biochemical, U.K.) antisera, diluted 1:600, were used, following a standardized protocol. In some arteries, the diaminobenzidine (DAB)-end products were silver intensified (Chapter three, this thesis) in order to allow a easier visualization of the immunoreactivity. This procedure did not cause any false positivity in the development of silver granules as seen by the absence of autoradiographic granules in segments of uterine arteries, that had been subjected to immunostaining, followed by silver intensification, but not incubated with ^{3}H-NA.

After staining, the arteries were osmicated, dehydrated and embedded in Spurr resin. Semithin section were collected on acid-washed glass slides, while rows of ultrathin sections were transferred on formvar coated slides and dried in a dust-free atmosphere, at room temperature. The slides were dipped in a diluted L4 Ilford nuclear Emulsion (Ilford,Ltd,U.K.) and withdrawn manually so to obtain a final emulsion layer of 3-5 μm for semithin sections and

a deep-purple coloured layer (<140 nm) for ultrathin sections. The slides were incubated at 4 °C for approximately 4 weeks, developed with Kodak D-19 and fixed with 25% sodium thiosulphate. After recovering of ultrathin sections onto uncoated grids, these were stained with 1% uranyl acetate in 70% alcohol and viewed with a Philips 300 electron microscope.

RESULTS

Catecholamine-fluorescence

Following incubation in Krebs solution, arteries from virgin animals had a denser NA-fluorescent nerve plexus than those from late pregnant guinea-pigs (fig. 53a, c). After NA-loading, the number of NA-fluorescent nerves in arteries of late pregnant animals increased remarkably, but not in those from virgin animals, so that the density of NA-fluorescent nerves in the two groups of arteries became very similar (fig. 53b, d).

^{3}H-NA uptake

Total ^{3}H-NA uptake in the absence of inhibitors was significantly greater ($p<0.01$) in uterine arteries of pregnant animals ($0.71 \pm 0.06 \times 10^{-13}$ moles/cm, n=6) compared with those of virgin controls ($0.49 \pm 0.02 \times 10^{-13}$ moles/cm, n=7). Under the conditions used, approximately 75% of the ^{3}H-NA taken up by uterine arteries of both virgin and late pregnant guinea pigs was inhibited by preincubation with cocaine or desipramine. Cocaine-sensitive uptake (i.e. the difference in uptake in the presence and absence of cocaine) was used to indicate neuronal uptake, whereas desipramine-sensitive uptake represents the uptake via the specific high affinity NA uptake mechanism of sympathetic nerves. The results have been given in Figure 54. Both cocaine- and desipramine-sensitive uptake, which were very similar, were significantly increased in uterine arteries of pregnant guinea-pigs compared with those of virgin animals.

In the second series of experiments, where the endothelium of the uterine arteries was removed prior to incubation with ^{3}H-NA, a similar result was obtained (Fig. 55). Cocaine-sensitive uptake was significantly increased in pregnant arteries. In contrast, neither cocaine-resistant nor desipramine-resistant ^{3}H-NA uptake was significantly altered in pregnancy, being approximately 25% of the total ^{3}H-NA uptake in all samples.

Autoradiography

Semithin sections of uterine arteries displayed ^{3}H-NA uptake sites almost exclusively on the adventitia and adventitial-medial border (Fig. 56). Levels of grains in endothelium and smooth muscle cells were similar to background, while autoradiography of uterine arteries incubated with ^{3}H-NA in presence of cocaine displayed very few grains (Fig. 56d), thus indicating that, under the incubation conditions used, ^{3}H-NA was almost exclusively taken up by the high affinity neuronal uptake system. Comparison between arteries from virgin (Figs 56a,b) and late pregnant animals (Figs. 56e,f) showed always a thicker and denser coat of autoradiographic granules on the adventitia of pregnant arteries.

At the electron microscopic level, silver granules were found in close proximity to, or just over nerves and varicosities (Fig. 57). Periadventitial nerve bundles had granules less frequently than intradventitial nerves. In late pregnancy, a common feature was the presence of a greater number of granules per positive axons (Figs 57b, d). In control arteries, silver granules were found almost exclusively on DBH- and NPY-IR varicosities (Figs 58a, c), while VIP-

, SP- and CGRP-IR nerves were usually devoid of autoradiographic granules (Fig. 58e). In contrast, in pregnant arteries, a number of VIP-, SP- and CGRP-IR nerves and varicosities, in addition to DBH- and NPY-IR nerves, had taken up ^{3}H-NA (Fig. 58b, d, f). A large variability and unpredictability of the immunostaining occurred, so that no quantitative analysis was attempted.

DISCUSSION

Pregnancy has often been considered to cause sympathetic denervation of the uterine arteries (Bell and Malcolm, 1978; Tare et al., 1988; Lundberg et al., 1987). In Chapter five a decrese of NA-levels and NA-fluorescent nerves in late pregnancy was shown. In the search of possible causes for the reduction of NA-levels, the integrity of NA uptake mechanisms was investigated by measuring the uptake of ^{3}H-NA and localizing it with high resolution autoradiography. I found that ^{3}H-NA uptake mechanisms are not impaired, like it has been shown to occur in the uterus (Alm et al., 1979a) and, instead, that perivascular nerves take up more ^{3}H-NA in late pregnancy. This suggests that a more likely explanation for the reduction of detectable NA-containing fibres in late pregnancy is reduced synthesis (or storage) of NA. Supersensitivity of uterine artery in late pregnancy to NA (Fallgren et al., 1988) is consistent with this explanation. An increased density of NA-fluorescent nerves in arteries of late pregnant guinea-pigs after "in vitro" loading with NA was also found. One possible interpretation for the increase of ^{3}H-NA and NA uptake in late pregnancy is an increase of the number of sympathetic perivascular nerves equipped with an uptake system for NA; another is the increase of the affinity for NA of sympathetic perivascular nerves; a third possibility is that non-sympathetic nerves can take up NA in late pregnancy. In support of the first hypothesis, an increase of perivascular nerves in the uterine artery of late pregnant guinea pigs has been found (Mione and Gabella, 1991); moreover, the visualization of ^{3}H-NA uptake sites in VIP-, SP-

and CGRP-IR nerves in late pregnancy suggests the development of a specific uptake system for NA in both parasympathetic and sensory nerves in keeping with the third hypothesis. The possibility of an increase of the affinity for NA of sympathetic perivascular nerves in late pregnancy has not been investigated; however, all three mechanisms may operate in these conditions.

This study is the first to show NA uptake sites on parasympathetic (VIP-IR) and sensory (SP/CGRP-IR) perivascular nerves. Neuronal noradrenaline uptake "in vivo" has been reported only in sympathetic nerves (Iversen, 1967), and the autoradiographic localization of ^{3}H-NA has been often used as a definitive tool for the demonstration of noradrenergic nerves at the ultrastructural level (Graham et al., 1968). However, there are examples "in culture" or in embryonic transplants, of parasympathetic (Coulombe and Bronner-Fraser, 1986) and sensory (Jonakait et al., 1984; 1985) neurons, that transiently exhibit amine-uptake ability and synthesize catecholamines, when exposed to appropriate stimuli (like hormones).

The suggestion that in late pregnancy, NA can be taken up in parasympathetic and sensory nerves must be confronted with evidence that : 1) SP- and VIP-IR has been demonstrated in some prevertebral sympathetic neurons (Kessler et al., 1981; Wright and Luebke, 1989); 2) prevertebral sympathetic ganglia (i.e the aorto-renal ganglion, the inferior mesenteric ganglion and the coeliac-superior mesenteric ganglion) have been found to project to the uterus in guinea pigs (Alm and Lundberg, 1988); however, no SP-or CGRP-IR neurons were seen in these ganglia by the same authors; 3) fibres containing NA and some peptides project to the uterus from the paracervical ganglia

(Marshall, 1970; Gu et al., 1984; Alm and Lundberg, 1988). Furthermore, there are atypical combinations of transmitter substances coexisting in some axons to the uterine artery (Morris et al., 1985; 1987) and uterine nerves are known to be sensitive to hormones (Owman and Sjoberg, 1977; Thorbert et al., 1978).

Changes in the expression of neurotransmitters and, above all, the reduction of NA levels, have been related with the maintenance of vasodilation in the uterine artery during pregnancy, in order to ensure adequate blood supply to the foetuses (Chapter five). However, vasoconstriction is likely to be needed suddenly, during and after partum. The increase of the uptake sites for NA, together with the supersensitivity of smooth muscle cells to NA in late pregnancy (Fallgreen et al., 1988), could represent the key mechanism to ensure NA-mediated vasoconstriction when needed, since NA could be released from parasympathetic and sensory nerves, as well as sympathetic. A simple mechanism has been shown for the rat mesenteric vascular bed, where serotonin taken up into noradrenergic nerves, acts as a reinforcing cotransmitter with NA (Kawasaki and Takasaki, 1984).

In summary, this study suggests that the increase of ^{3}H-NA uptake found in late pregnancy is due to development of an uptake system for NA in nerves that were previously devoid of this mechanism, in addition to the growth of new nerves. The possibility that part of the ingrowing nerves has a sympathetic origin and shows immunoreactivity for the above peptides in late pregnancy, while loosing the ability to produce NA, cannot be ruled out, but seems unlikely.

Fig. 53 - Whole mount stretch preparations of adjacent segments of uterine arteries of virgin (**a,b**) and late pregnant (**c,d**) guinea pigs. (**b**) and (**d**) were incubated in Krebs solution containing 10^{-5} M NA and 1 mg/100 ml pargilin, before being processed with the glyoxylic acid technique. Calibration bar : 50 µm

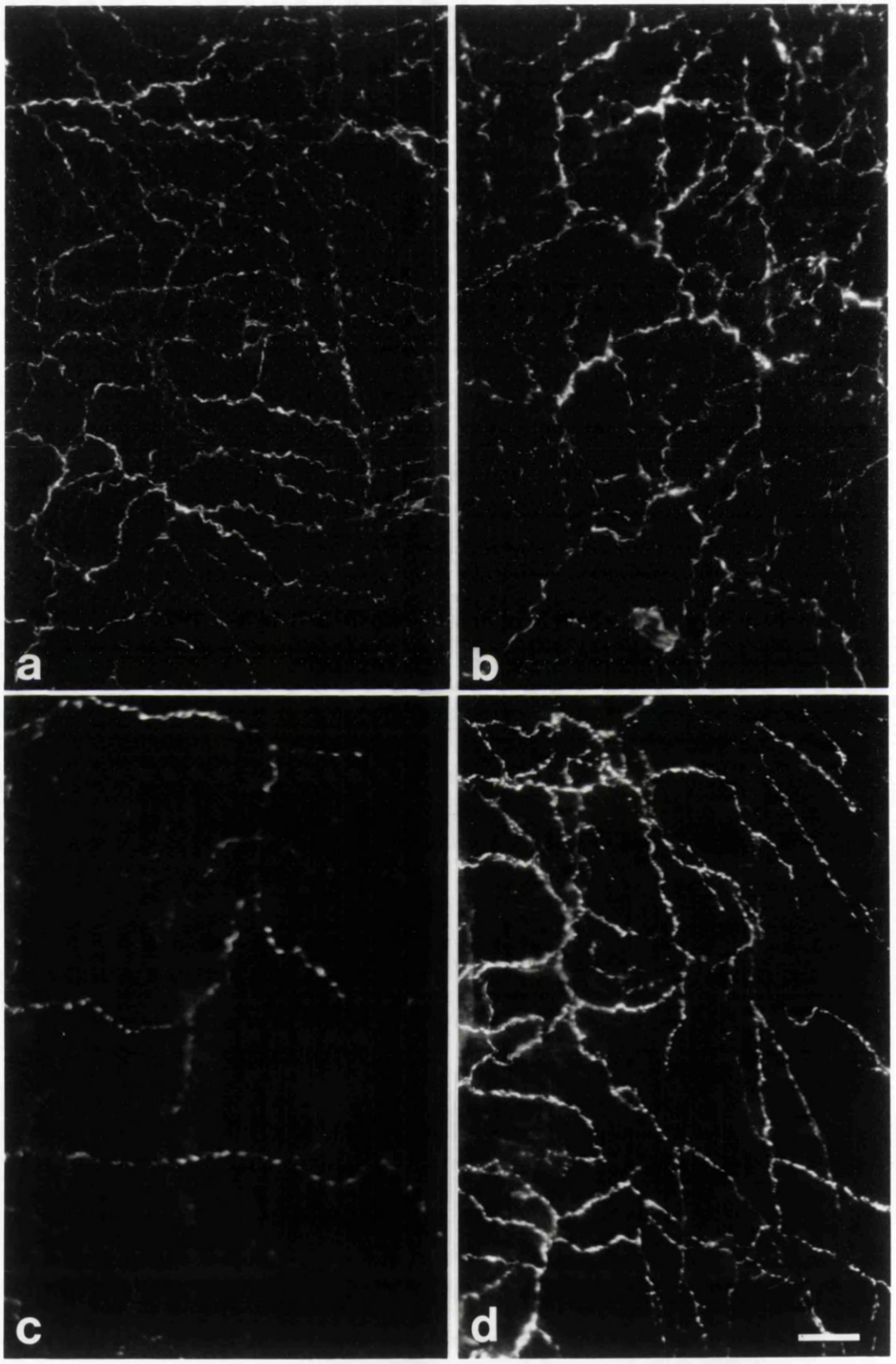
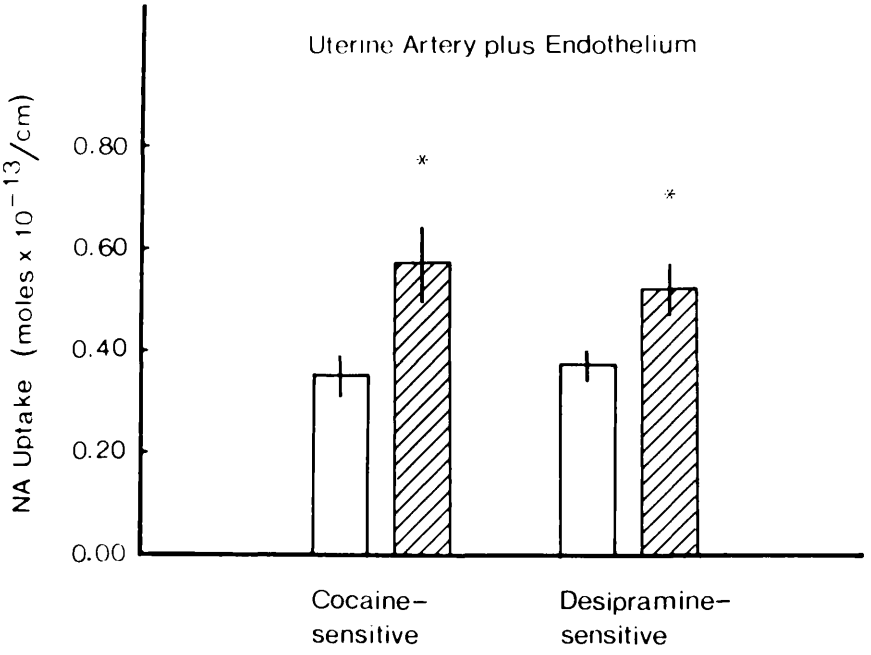

a
b
c
d

Fig. 54 - Top - Histogram showing the neuronal uptake of ^{3}H-NA in uterine arteries from 6 virgin (**clear bars**) and 5 pregnant (**cross-hatched bars**) guinea-pigs. The uptake was calculated using 10^{-8} M ^{3}H-NA. The neuronal uptake blockers, cocaine (10 μM) and desimipramine (0.2 μM) were added to the incubation medium, as described in the text. The results given are the mean ± S.E.M. * $p < 0.05$

Fig. 55 -Bottom - Histogram of cocaine-sensitive ^{3}H-NA uptake in uterine arteries of 6 virgin (**clear bars**) and 5 pregnant (**cross-hatched bars**) guinea-pigs, after removal of the endothelium, that has been described to possess a cocaine-sensitive uptake system for NA, similar to sympathetic nerves. The results are the mean ± S.E.M.
* $p<0.05$


Uterine Artery plus Endothelium
NA Uptake (moles x 10^{-13}/cm)
0.80
0.60
0.40
0.20
0.00
*
*
Cocaine-sensitive
Desipramine-sensitive

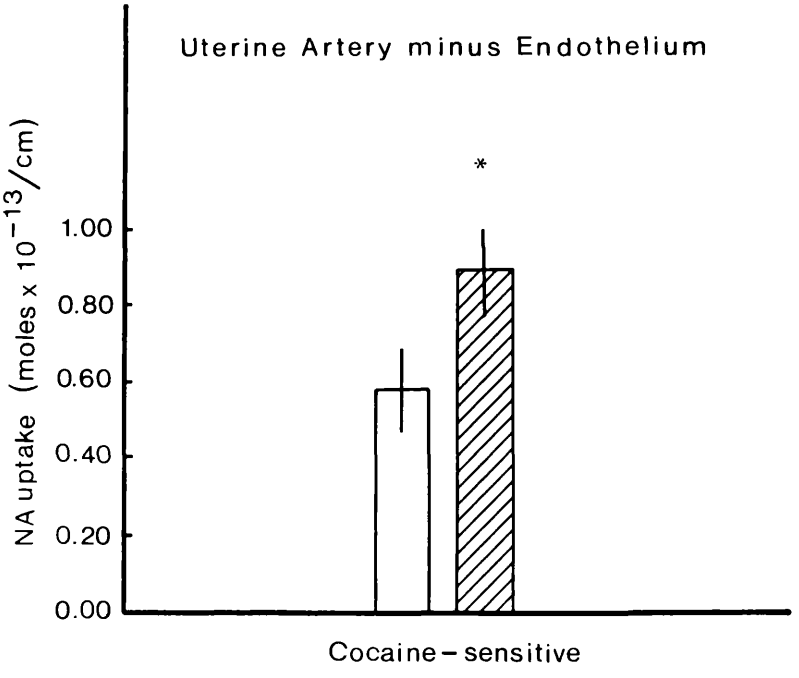

Uterine Artery minus Endothelium
NA uptake (moles x 10^{-13}/cm)
1.00
0.80
0.60
0.40
0.20
0.00
*
Cocaine-sensitive

Fig. 56 - Bright and dark fields microphotographs of the same sections of uterine arteries of virgin (**a**, **b**, and **c**, **d**) and pregnant (**e**, **f**) guinea-pigs. The vessels were incubated with 10^{-8} M ^{3}H-NA, in the absence (**a**,**b**,**e**,**f**) or in the presence (**c**,**d**) of 10 µM cocaine. (**arrowheads**) indicate the luminal border.
Calibration bar = 10 µm

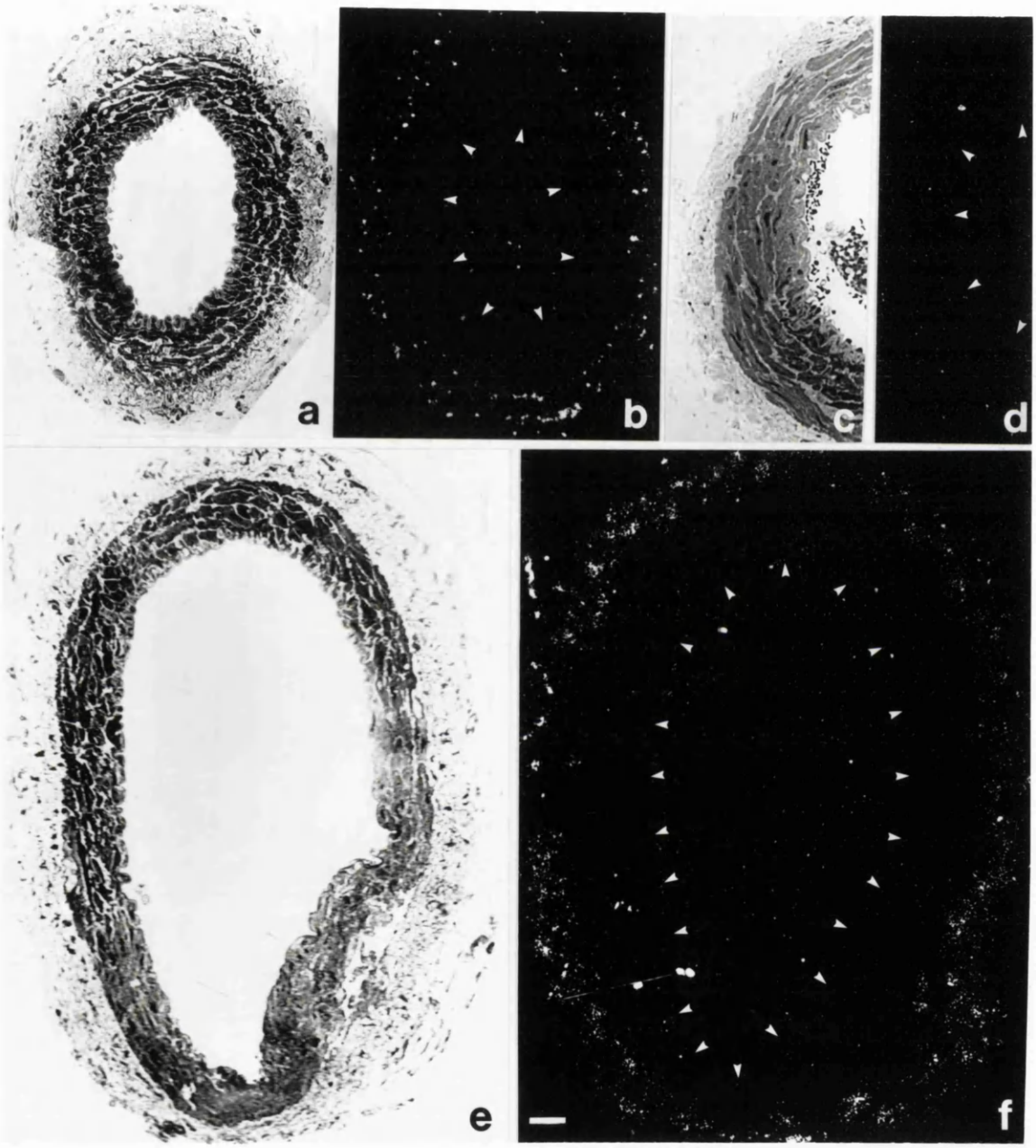

a
b
c
d
e
f

Fig. 57 - High resolution autoradiography of ^{3}H-NA uptake sites in perivascular uterine nerves from virgin (**a, c**) and late pregnant (**b, d**) guinea-pigs. A greater number of silver grains is present over some axons in arteries of pregnant animals (**b, d**). Silver grains are associated with clear vesicle-containing varicosities in arteries of both virgin and pregnant guinea-pigs (**b, c**) , as well as with nerve varicosities containing large dense cored vesicles (**d**).
Calibration bar = 0.5 μm

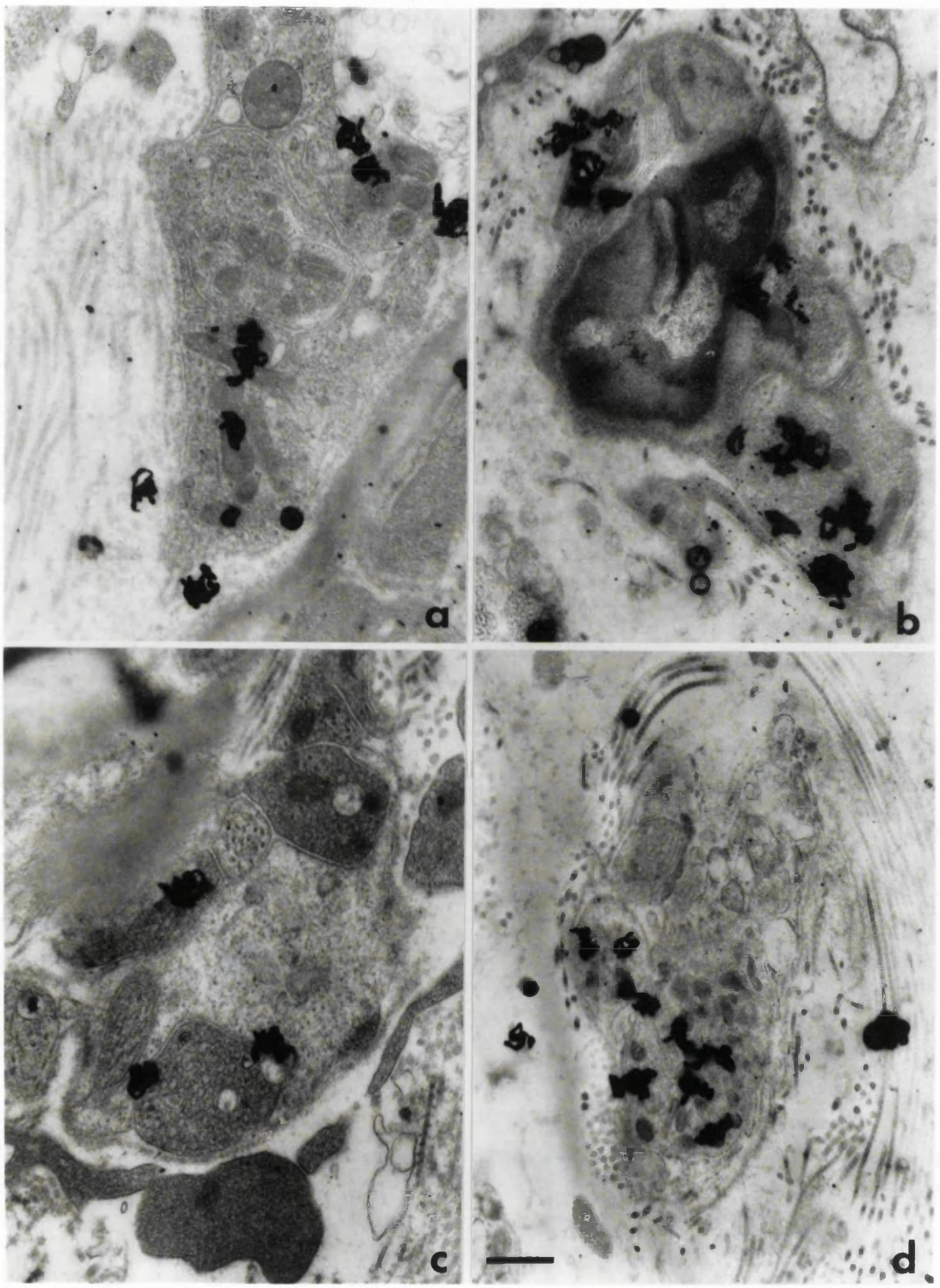
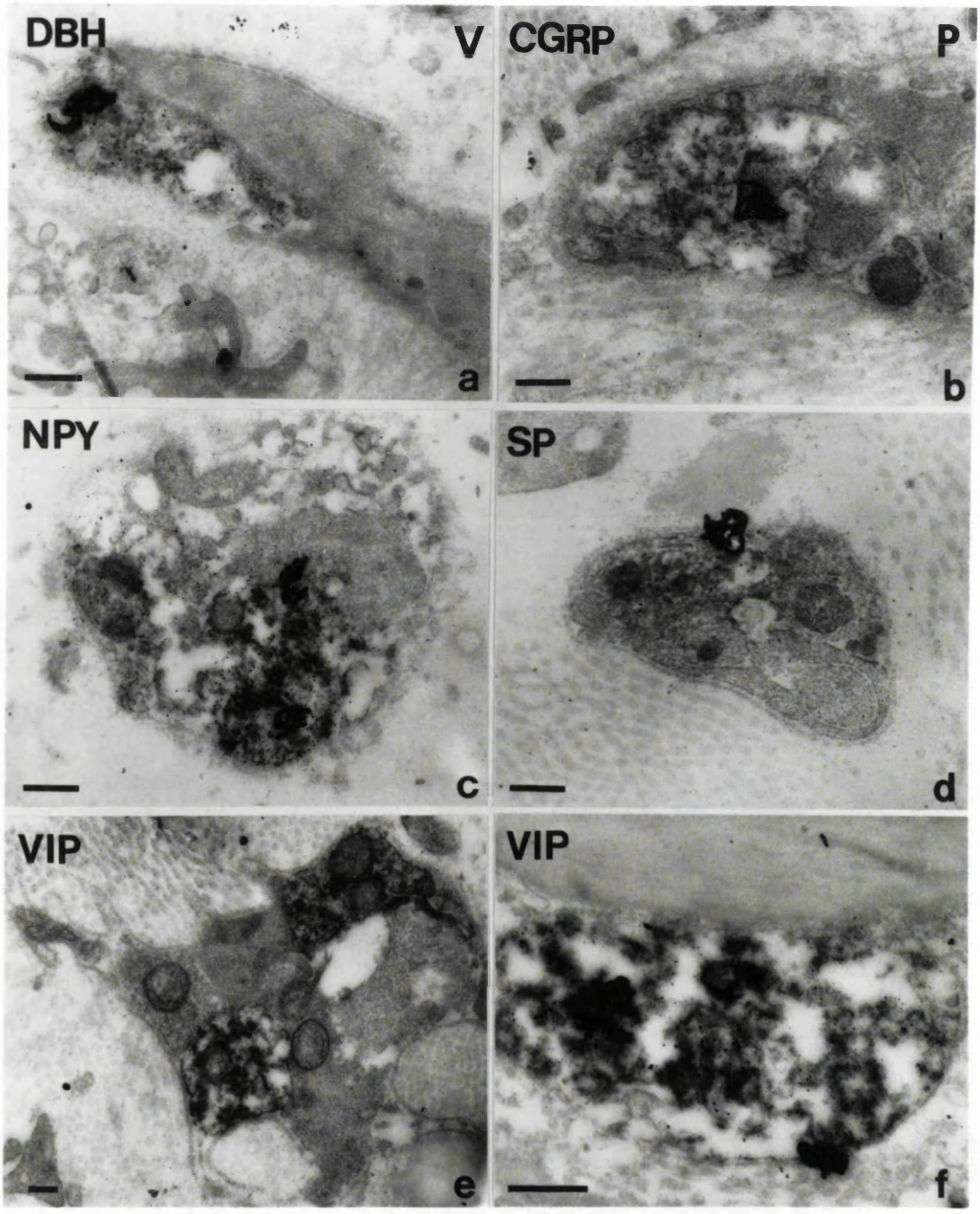

a
b
c
d

Fig. 58 - High resolution autoradiography of ^{3}H-NA uptake sites in nerve fibres of uterine arteries of virgin (**a**, **c**, **e**) and late pregnant (**b**, **d**, **f**) guinea-pigs. Segments of arteries were processed also for the immunocytochemical detection of DBH-IR (**a**), CGRP-IR (**b**), NPY-IR (**c**), SP-IR (**d**) and VIP-IR (**e**,**f**). Autoradiographic grains are seen over immunoreactive nerves. Note in (**e**), two VIP-IR axons of an artery of a virgin guinea-pig both lack ^{3}H-NA uptake sites, in contrast to (**f**), where silver grains are associated with a VIP-IR axon of a late pregnant animal. Calibration bars = 0.25 µm.


DBH
V
a
CGRP
P
b
NPY
c
SP
d
VIP
e
VIP
f

SECTION IV :

GENERAL DISCUSSION.

The aim of this thesis was to detect changes in the expression of neurotransmitters, their related enzymes and uptake systems in perivascular nerves and nerve cell bodies in response to altered levels of other neurotransmitters or the disappearance of subpopulations of nerve fibres.

In this section, some selected questions arising from these studies are discussed under the following headings :

1) Are there common features in the trophic response of perivascular nerves to the decrease or disappearance of sympathetic nerves?

2) What are the causes and mechanisms regulating neurotransmitter plasticity and how can they account for the changes detected in this study?

3) Is immunocytochemistry a suitable technique to detect plasticity of neurotransmitter expression in perivascular nerves?

4) Which future directions in the study of plasticity of perivascular nerves may be fruitful?

1 - COMMON FEATURES OF TROPHIC RESPONSES OF DIFFERENT VESSELS AND ANIMAL MODELS TO LOSS OF SYMPATHETIC NERVES

A common finding, stressed by these studies, is the invariable activation of perivascular parasympathetic and sensory fibres, in response to the disappearance or reduction of sympathetic perivascular nerves. This activation leads to qualitative changes in the expression of neuropeptides and sprouting of parasympathetic and/or sensory perivascular nerves. Examples are: a) the reduction of sympathetic cerebrovascular nerves in aged rats (probably sustained by a decrease of the number of sympathetic neurons, Baker and Santer, 1988), that was accompanied by a detectable increase of the number of VIP- and CGRP-IR, vasodilatory nerves (Chapter one); b) the disappearance of sympathetic nerves, following long-term sympathectomy with guanethidine, which caused an increase of the expression of NPY immunoreactivity in parasympathetic neurons arising from the pterygopalatine ganglia (Chapter two and three) and sprouting of sensory CGRP-/SP-IR nerves (Chapter four). In addition, the reduction of NA-synthesis in sympathetic perivascular uterine nerves in late pregnancy, which led to the expression of a specific uptake system for NA and related amines in perivascular sensory and parasympathetic nerve fibres (Chapters six and seven).

A simple speculation about these observations is that there is a fixed number of recognignition's sites for neuro-muscular contacts in a given tissue and that there is competition among nerve fibres with different phenotypes for occupation of these sites. Indeed, the

development of the autonomic nervous system, as studied by several researchers (Malmfors et al., 1971; Burnstock, 1974, and see Rohrer et al., 1991, for review) provides many examples of competition for the target : it is the establishment of contacts that guarantee the survival of a neuron (Oppenheim, 1981) through the retrograde flow of NGF (Hendry et al., 1974a; Hendry, 1977b; Claude et al., 1982; Richardson and Riopelle, 1984) and vascular nerve fibres are not an exception (Burnstock, 1981). From the same studies, it also became clear that sympathetic nerves are, for some intrinsic or extrinsic properties, favoured in innervating many target organs either because fewer contacts are "allowed" to parasympathetic and sensory nerves or because growth factor(s) produced by the targets favour "attraction" of sympathetic nerves.

An increase of nerve fibres immunoreactive for NPY, VIP, CGRP and SP in cranial targets in old rats or after long-term sympathectomy, took place in conjunction with the decrease or disappearance of NA-containing nerves. This proliferation of non-sympathetic perivascular nerves could be either due to new axons (through branching of preexisting nerves or projections of neurons previously committed to other targets) or reflects the detectability of the neurotransmitter along axons that were previously non-immunoreactive (i.e. because of a low level of the peptide).

In long-term sympathectomized rats (Chapters two and three), there is an increase in the number of cell bodies expressing NPY-IR in parasympathetic pterygopalatine ganglia in parallel with the increase of parasympathetic cerebrovascular nerve fibres immunoreactive for NPY. Both cell bodies and nerve fibres seem to correspond to

preexisting VIP-IR cell bodies and nerve fibres (this study and Gibbins and Morris, 1988). Concerning the expression of NPY-IR in parasympathetic nerves after long-term sympathectomy, it seemed unlikely that there was recruitment of cell bodies of the pterygopalatine ganglion projecting to other targets. It seems more likely that there was expression of the neuropeptide in previously non-immunoreactive cell bodies with consequent detectability in the axons. Increased levels of NPY in the target were achieved by simply turning on the production of the neuropeptide in a subpolpulation of neurons already projecting to the same target; the newly instaured coexistence of two neurotransmitters with different (contrasting) actions on vascular tone apparently not being an obstacle to this economic repair to the loss of sympathetic NPY-IR axons (see also below for NPY/VIP coexistence). Recently, an increase of DBH-IR in cranial parasympathetic neurons following long-term sympathectomy with 6-OHDA, similar to the increase of NPY after guanethidine treatment, has also been shown (Mione et al., 1991), thus indicating the activation of several genes in pterygopalatine ganglion neurons.

The expression of CGRP/SP immunoreactivity in perivascular nerves also increases in aged and sympathectomized rats. The mechanisms underlying this increase are not known. However, in contrast with NPY-IR parasympathetic neurons, nerve cell bodies expressing CGRP/SP-immunoreactivity in trigeminal and dorsal root ganglia of long-term sympathectomized rats are decreased in number, with the few immunoreactive neurons belonging to a homogenous population of small cells.

A possible interpretation is that branching and sprouting of axons together with an increase of number of varicosities require higher production of neurotransmitters and faster transport, with resultant lower detectability or shorter permanence of the IRs at the level of the cell bodies. Alternatively, the production of immunoreactive neurotransmitters (for example, following cleavage from a non-immunoreactive precursor) no longer takes place in the cell bodies. The use of blockers of the axonal transport could help solve this question.

Following such a hypothesis of a faster neurotransmitter transport, the increase of IR cell bodies seen after combined denervation with capsaicin and guanethidine, could be interpreted with a block by capsaicin of axonal transport of neurotransmitters. Indeed, capsaicin has been shown to interfere with the retrograde transport of NGF (Miller et al., 1982), but I am unable to establish if capsaicin + guanethidine treatment increases the number of CGRP-IR neurons by slowing down the axonal transport of the neurotransmitter.

In the study by Aberdeen and collegues (1990), the level and density of CGRP-IR nerves increased in several targets (including the SCG, similarly to the findings of this study). This confirms that sensory neurons of long-term sympathectomized rats undergo axonal growth, and synthesis of several structural and functional proteins and cellular reorganization, besides an increased amount of neurotransmitter to fill the varicosities.

In the uterine artery of late pregnant guinea pigs, there is a reduction of NA-levels and an enhancement of cholinergic mechanisms (Bell, 1968; 1969). It is not known whether the level of the

sympathetic cotransmitter ATP is also reduced in these circumstances. Levels of other neuropeptides per cm of artery do not change significantly, similarly to immunoreactive nerves detected with light microscopy immunocytochemistry. However, uterine arteries undergo dramatic increase in size, and a remarkable increase in the number of perivascular nerves (Mione and Gabella, 1991). Interestingly, the number of VIP- and NPY-IR axons per nerve bundle (that is not evaluable at the light microscope) is increased in late pregnancy (observation reported in Chapter six), while the number of IR-nerve bundles (visible at the light microscope) is not significantly changed. In late pregnancy, there was also a remarkable increase of the number of varicosities taking up 5-OHDA, some of them with immunoreactivity for SP, CGRP or VIP.

Thus, high resolution immunocytochemistry showed that in the uterine artery of late pregnant guinea pigs, the reduction of NA levels in combination with the increase in size of the artery induced axonal sprouting and branching, in a similar manner to that seen previously in cerebral vessels. Both VIP- and NPY-IR are expressed in paracervical ganglion neurons, projecting to the uterine arteries (Morris and Gibbins, 1987). In a recent study (Mitchell and Stauber, 1990), an apparent small decrease in number of NPY- and VIP-IR neurons in paracervical ganglia of late pregnant guinea pigs, has been mentioned.

Therefore, it appears that reduction of sympathetic perivascular nerves of both rat cerebral arteries and guinea pig uterine arteries, is accompanied by an increase of the expression of neuropeptides in parasympathetic and/or sensory nerves, regardless of the cause,

degree of NA reduction or age of the animals.

The second common feature in all models, was the maintenance of NPY-IR nerve density. These fibres, especially in the vasculature, are commonly considered as sympathetic, being NPY, together with ATP, the most frequent cotransmitter in sympathetic nerves (O'Donohue et al., 1985). Its role is mainly the neuromodulation (postjunctional enhancement and/or prejunctional inhibition) of noradrenergic vasoconstriction (see Introduction), but in some vessels, it has been shown to exert a potent direct vasoconstrictor action (Franco-Cereceda et al., 1985). It was expected to follow the fate of NA, and to decrease or disappear in conjunction with sympathetic nerves. Instead, when reduction of sympathetic neurons occurred (ageing) , the density of NPY-IR nerves in cerebral arteries was unchanged (Chapter one); when sympathectomy was achieved by long-term guanethidine treatment, its expression was taken over by parasympathetic neurons (Chapter two and three); when NA level decreased in intact nerves of late pregnant uterine arteries, NPY level and NPY-IR axons increased (Chapters five and six).
The ubiquity of this neurotransmitter (including component of the enteric nervous system and intrinsic ganglia of the heart, bladder, lung), the persistence of its expression in both lines of sympathetic and parasympathetic neurons (Leblanc et al., 1987, this study), the early expression (embryonic day 16, in the rat, Larhammar et al., 1987) and the maintenance of constant levels throughout development, aging (Chapter one) and pathological conditions, lead to the hypothesis that it has some unknown and necessary roles, awaiting

further study. In the cerebral cortex and in the striatum, NPY-IR neurons correspond to a well defined class of projecting neurons (Vincent et al., 1983), with the unique property to be resistant to various injuries, like hypoxia or excitotoxic damage (Beal et al., 1986) , and are selectively preserved in Huntington's disease (Ferrante et al., 1985). Those NPY-IR neurons also exhibit nitric oxide synthase activity, as recently shown by Hope and collegues (1991) through the characterization of NADPH-diaphorase activity typical of these cells. It would be interesting to know if NPY-IR neurons of the ANS share the same features with those in the CNS. In the ANS, coexistence between NPY and VIP is found with increasing frequency (see Introduction), although the two peptides seem always to exert contrasting effects to the targets (vasodilation/vasoconstriction in the vasculature; stimulation/inhibition of secretion in the salivary glands, see Introduction). Also in the rat brain, where the two neuropeptides coexist in a subpopulation of cortical neurons, they have opposite effects on the synthesis of cAMP (Westlind-Danielsson et al., 1987), with VIP activating adenylate cyclase and NPY inhibiting it. However, the inhibition exerted by NPY, through Y1 receptors, , became measurable only when the enzyme was activated (e.g. by VIP, Bartfai et al., 1988). Thus the two coexisting and coreleased neurotransmitters express both the "on" and "off" signals for cAMP synthesis, producing a sharp, time-defined increase of cAMP. It would be interesting to study if a similar interaction is present in vascular smooth muscle, where VIP is also known to induce cAMP synthesis (see Introduction). Alternatively, the answer for the

preservation of NPY in different situation may be found in some properties of its gene, rather than in its role. Studies carried out in cultures of SCG neurons have shown that NPY and NA synthesis are differently regulated, so that when changes of the culture medium induce the switching from a noradrenergic to a cholinergic production (Iacovitti et al., 1982; Kessler, 1985b), the synthesis of NPY is unchanged (Marek and Mains, 1989). Recently, a differential regulation of NPY and TH/DBH synthesis in noradrenergic neurons in vivo, has also been shown (Schalling et al., 1991). The NPY gene has been sequenced in three species (rat, man, pig) and it shows few substitutions (Larhammar et al., 1987); its sequence is compatibile with that of a precursor, containing also a COOH terminus which is known to be costored and coreleased with NPY, named CPON, that has signal sequence for cytoplasmic sorting and processing (Minth et al., 1984). However, in the structure or in the localization of the NPY gene, there are no indications that could suggest reasons for its ubiquity and preservation. The question is still open.

In conclusion, in different vessels and experimental conditions, two common points have been highlighted by this study : 1) the invariable activation of parasympathetic and sensory perivascular nerves, following reduction or disappearance of sympathetic nerves; 2) the maintainance of NPY-IR nerve density, in spite of the reduction or disappearance of sympathetic nerves.

2 - MECHANISMS, CAUSES AND SIGNIFICANCE OF NEUROTRANSMITTER PLASTICITY

This study has stressed the wide occurrence of neurotransmitter plasticity in perivascular neurons of adult animals in vivo. We are now beginning to understand the mechanisms that regulate the expression of neurotransmitter genes, while the signals triggering these mechanisms are still poorly understood.

In this section the molecular basis for plasticity of neurotransmitter expression will be reviewed and applied to the findings reported in the present study.

2.1. Intracellular mechanisms regulating the expression of neurotransmitter genes

It is clear that all the neurons carry the genes for all the neuropeptides and for the enzymes that synthetize the classical low molecular weight neurotransmitters. Some of these gene are silenced in certain subpopulations of neurons. Silencing of genes, that are "inappropriate" to a given differentiated neuron, is obtained with an "active" control mechanism; this results in a neurotransmitter(s) choice, that is neither pre-committed nor stable, but is continously influenced by environmental conditions.

How is the expression of neurotransmitter genes regulated intracellularly ?

Similarly to many types of eukaryotic cells, neurons use signal

transduction pathways which are common to membrane receptors and involve proteine phosphorylation. Signals received at the cell surface are transduced through G-protein coupled receptors to regulate the production of a second messenger, such as cAMP and diacylglycerol. These second messengers activate protein kinases, which in turn phosphorylate a regulative protein (regulator or trans-acting factor) that binds to a specific region of DNA and regulates transcription. The discovery that the products of many oncogenes are, in reality, regulative proteins (see Adamson, 1987 for review) and the continuous characterization of new regulators, allow a better understanding of their mechanism of action.
Some of these regulative proteins have been shown to be able to cause a complete phenotypic conversion, as for the regulator MyoD, which was shown to activate several muscular silenced genes in fibroblasts and other cell lines, converting them in myoblasts, stably and inheritably (Davis et al., 1987).

Some of the general properties of regulative proteins highlight their mechanism of action. For example, it is known that many of them, including the signal transducer c-jun (Angel et al., 1988) and the helix-loop-helix family of MyoD (Thayer et al., 1989) can regulate their own transcription. In this way, autoregulation provides stability and limits the number of regulative proteins required. Moreover, it is known that many regulators exist in the form of families, with overlapping functions, that can activate each others' expression (Olson, 1990). A second general property is that regulators act in combination (like in the case of heterodimers of jun(s) and c-fos to form leucine zippers, Landschulz et al., 1989)

which ensures a greater specificity of action and a finer control of their activation.

Neurotransmitter genes, similarly to many other genes transcribed by Pol II, contain sequences that regulate their own transcription. These elements include a promoter, located 25-30 base pairs upstream from the transcriptional starting point, other regulatory elements, and the enhancer, which may be located in any position or orientation within the transcriptional element (Dyman and Tjian, 1985; Maniatis et al., 1987). Promoters usually consist of AT-rich segments (the TATA box, Breathnach and Chambon, 1981). The regulative elements are usually part of the promoter and include fixed sequences, as the CAAT box and the GC box (McKnight and Tjian, 1986) for bindind a number of regulative factors. Enhancers too, may provide a binding site for regulatory proteins, through a sequence of 8-10 base pairs, located within 150 base upstream of the coding region. This region have been considered as confering cAMP responsiveness to the gene, until the discovery of trans-acting factors, the majority of which needs phosphorylation, often mediated by protein kinase A (cAMP-dependent), to bind to the enhancer sequence. Such sequences have been identified for a number of neurotransmitter genes : rat somatostatin (Montminy et al., 1986); human proenkephalin (Comb et al., 1986); rat tyrosine hydroxylase (Lewis et al., 1987); human VIP (Tsukada et al., 1987) inter alia. In many case, also the regulative protein (or the constellation of proteins) and their active and inactive forms have been determined : at least for somatostatin, proenkephalin and VIP genes, they include the heterodimers jun(s)/c-fos. The ability of c-jun to stimulate its own transcription (Angel et al., 1988) and the

participation of c-fos in the regulative function provide several potential mechanisms for amplifying and terminating signals from cell surface receptors. The initial step is the phosphorylation of pre-existing jun(s) (or changes in the association with c-fos) by protein kinase C (Lee et al., 1987; Angel et al., 1987) Activated jun(s) may then induce expression of the c-jun gene with self-sustained increase of transcription, while the association of c-jun and c-fos can also down-regulate c-fos gene expression (Schontal et al., 1988; Sassone-Corsi et al., 1988) thus terminating the signal transduced by the two associated regulators.

Studies to demonstrate the involvement of regulators (such c-fos and c-jun) during the activation of neurotransmitter genes are now continously emerging.

2.2 Extracellular mechanisms activating/silencing gene expression

Neuronal gene expression can be modulated by membrane electrical activity, neurotransmitters, neurotrophic factors, hormones and many other mechanisms, and it is likely to play an important role both in the development and adaptative plasticity of the nervous system. Gene regulation is responsible for neuronal sprouting, synaptic density, expression of neurotransmitters, receptors and ion channels.

i. Membrane electrical activity.

The ability of membrane depolarization of regulating the expression of neurotransmitters was suggested by Kessler et al.,

1981, who showed that membrane depolarization through presynaptic nerve activity in vivo, and through veratridine treatment in vitro, downregulated the expression of SP-IR in neurons of rat SCG. However, it was known since earlier studies (reviewed by Costa et al., 1974) that electrical stimulation caused an increase of TH activity in adrenal gland and SCG. The delayed, long-lasting portion of increased activity of the enzyme was shown to be due to an increase of mRNA levels, thus probably to increased expression (Faucon Biguet et al., 1989). Recently, short, peroperative electrical stimulation of human sympathetic ganglia has shown to induce the increase of the mRNAs for TH, DBH and NPY (Schalling et al., 1989), while it is known since 1986 that activation of somatic or visceral nociceptive sensory afferents by thermal, chemical, mechanical or electrical stimuli increase the expression of proenkephalin, prodynorphin and protachikinin A (reviewed by Narajo et al., 1991) in the external and mediodorsolateral layers of the dorsal horn. The mechanisms involved in the regulation of gene expression through membrane depolarization are attributed to the opening of voltage-sensitive Ca++ channels (Greenberg et al., 1986; Morgan and Curran, 1986; Sheng et al., 1988). Ca++, acting as a second messenger, induces a Ca/calmodulin regulating kinase to phosphorylate a trans-acting factor, that, for being also a substrate of cAMP-sensitive, protein kinase A, has been named CREB (cAMP responsive element binder, Gonzales and Montminy, 1989). Phosphorylated CREBs form homodimers via a leucin-zipper domain (Dwarki et al., 1990) and bind the consensus elements (CRE) in a number of neurotransmitter genes, including somatostatin (Montminy et al., 1986), VIP (Tsukada et al.,

1987), proenkephalin (Comb et al., 1986) and in immediate early genes (Christy et al., 1988; Changelion et al., 1989). It is of interest to note firstly that two different second messenger patways (the Ca/calmodulin kinase and the cAMP/phosphokinase A) converge on the same trans-acting factor, suggesting a dual control (by membrane depolarization and cAMP-related receptor activation) of the same elements in a number of genes, and secondly, that both systems also activate the expression of immediate early genes (including c-fos, nur/77, zip/268) with consequent amplification and specialization of the response.

In addition, K+ depolarization, as well as the occupation by the agonist of many neurotransmitter receptors, are known to stimulate phosphatidylinositol turnover (Audigier et al., 1988), another second messenger pathway utilizing phosphokinase C to activate a different set of trans-acting factors (see following section).

ii. Neurotransmitters.

Several neurotransmitters are known to activate gene expression or enzyme activities in neurons through specific receptors and/or membrane depolarization. Thus, Ach and VIP, released by preganglionic nerves following different patterns of stimulation, are shown to increase TH activity in SCG neurons (Ip and Zigmond, 1984; Ip et al., 1985) and target organs (Schwarzschild and Zigmond, 1989); VIP also regulates mitoses, differentiation and survival of sympathetic neuroblasts (Pincus et al., 1990). The blockage of opioid receptors in neuronal cultures regulates both TH and ChAT activities (Varnadakis and Kentroti, 1990), while stimulation of beta-adrenergic

receptors, induces NGF gene expression in glial cells (Schwarz and Mishler, 1990; Furukawa and Furukawa, 1990). Studies on the effects of neurotransmitters on gene expression are in their infancy, but it is easy to speculate that a neverending list of effects will be uncovered soon. This convincement is based on : a) the large number of studies on the effects of removing presynaptic inputs (e.g. presynaptic neurotransmitters) on structural (Purves and Hume, 1981, Hadley, 1990) and functional (Smith et al., 1987 and for changes in neurotransmitter expression, Landis et al., 1987; Landis, 1991) properties of neurons; 2) the coupling of neurotransmitter receptors to second messenger pathways, that have been shown to link extracellular signals to the regulation of gene expresssion; 3) the wide demonstration of the ability of neurotransmitters to regulate not only their own synthesis and uptake, but also the synthesis, uptake, receptors and degrading mechanisms for different neurotransmitters in the same or in other neurons. The molecular basis for mechanisms like "autoregulation" and "neuromodulation" will be soon uncovered and possibly attributed to specific intracellular signals, including protein phosphorylation, immediate early gene expression and long-term transcription of new genes.

An interesting aspect of the regulation of gene expression by neurotransmitters is that exerted by agonists on receptors and membrane tranporters. Recent studies have highlighted some peculiarities of the interaction between neurotransmitters and their receptors. One of these concerns the down regulation of receptor expression by the agonist (both at transcriptional and post-transcriptional levels, see Hadcock and Malbon, 1991), a feature

common to different classes of receptors. Another aspect is the expression of "immature" receptors in target organs, preceedind the arrival of nerve fibres. These receptors are irresponsive to the agonist, but rapidly acquire sensitivity, including the ability to mediate the intracellular effects, with the establishement of the innervation of the organ (Large et al., 1985; Schleifer et al., 1989; Feng et al., 1989). It is speculated that the development of sensitivity of these receptors (beta-adrenergic receptors in the liver, Schlefer et al., 1989, and parotid gland, Lunford and Talamo, 1983; muscarinic receptors in the heart, Galper et al., 1984; Feng et al., 1989) is correlated with the appearance of a G-protein, functionally related to the mature receptor (see Grant and Landis, 1991, for review). Its synthesis and/or incorporation in the receptor already present in the plasma membrane could probably be stimulated by the availability of the agonist, released by the nerve fibres, or by other unknown factors.

iii. Trophic factors.

Enviromental conditions are known to influence neurotransmitter choice : factors produced by target organs or by non-neuronal elements are able not only to support growth and differentiation of neuronal cells, but also to influence their phenotype. Earlier studies were carried out in cultures (see Patterson, 1978, for review), but elegant experiments have been conducted in vivo, for instance those by Landis and collegues (reviewed in Landis, 1991) on the innervation of sweat glands. Some of the best characterized trophic factors are listed below.

NGF - The recent discovery of the high affinity NGF receptor as a membrane complex including the proto-oncogene tyrosin kinase (a trans-acting factor, Hempstead et al., 1991) led to the initial understanding of the intracellular mechanisms of action of NGF. At least three different trophic factors, with over 50% homologies in their primary structures, are known : nerve growth factor (NGF); brain derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) (Leibrock et al., 1989; Maisonpierre et al., 1991). These protein are regarded as members of a gene family, and bind with different affinities to the NGF receptor (Rodriguez-Tebar et al., 1990; Ernfors et al., 1990), but the requirements of developing neurons, the developmental regulation and the sites of synthesis for these three factors are quite distinct (Maisonpierre et al., 1991).

The roles of NGF as a neurotrophic factor, promoting survival, differentiation and growth of sympathetic neurons, subpopulations of sensory neurons and cholinergic neurons of the basal forebrain (Buck et al., 1987) are well documented (Levi-Montalcini, 1987; Rohrer, 1991 for review). On the rat pheochromocytoma cell line, PC-12, NGF induces neuronal tranformation (reviewed in Greene and Tischler, 1982) and expression of several genes (induction of : pre-pro-NPY expression, Sabol and Higuchi, 1990; increase of dynamin levels, Schaife and Margolis, 1990; specific recognition sites for 5-HT3 agonists, Gordon et al., 1990). Both c-fos and c-myc are activated in PC-12 cells after NGF treatment (Greenberg et al., 1985; Bartel et al., 1989), thus indicating that the expression of these immediate early genes mediates some of the responses to NGF.

On neuronal cells, NGF has been shown to : stimulate TH activity in embryonic neuroblasts of the gut, Kessler et al., 1979; increase SP-, CCK- and VIP-IR in primary neurons of newborn rats, Otten and Lorez, 1982; prevent the loss of SP-IR in dorsal root ganglia of capsaicin-treated guinea pigs, Miller et al., 1982, and in axotomized rat sensory neurons, Wong and Oblinger, 1991; induce target hyperinnervation, Kessler, 1985a; Hill et al., 1988; Saffran et al., 1989; Isaacson et al., 1990; increase the transcription of mRNAs for SP- and CGRP-precursors in rat dorsal root ganglion neurons, Lindsay and Harmar, 1989; stabilize mRNA transcripts for TH, Raynaud et al., 1988; upregulate the expression of NGF receptors in both sensory neurons, Wyatt et al., 1990; and sympathetic neurons, Miller et al., 1991.

Cholinergic differentiating factors - Using cultured peripheral neurons, a number of instructive factors, have been identified and characterized. These include : a) ciliary neurotrophic factor (CNF, Adler et al., 1979; Lin et al., 1989), originally identified for its ability to support ciliary neurons in culture, has been found to induce ChAT-, and decrease TH-activity in cultured rat sympathetic neurons (Saadat et al., 1989) and to cause VIP-expression in chick sympathetic neurons (Ernsberg et al., 1989). It is found in extract of CNS and various target organs (Saadat et al., 1989); b) heart cell conditioned medium (HCCM), which induces the expression of several neuropeptides (SP, SOM, VIP, CCK, ENK and ChAT in cultured sympathetic neurons (Nawa and Sah, 1990). At least three factors with different specificity are present in it (Nawa and Patterson, 1990).

One of them appear to be identical to a hemopojetic differentiating factor, the leukemia inhibitory factor (Gering et al., 1987) and to increase c-fos and jun B transcript in sympathetic neurons (Yamamori et al., 1990); c) membrane-associate neurotransmitter stimulating factor (MANSF), that has been solubilized and partially purified from rat spinal cord as a 29 KD membrane protein (Wong and Kessler, 1987; Adler et al., 1989). MANSF causes reduction of TH activity, and increase of SP, ChAT and SOM expression in cultured sympathetic neurons.

Target-derived factors - The most throughly studied and partially characterized is the sweat gland-derived cholinergic factor from developing rats (Rao and Landis, 1990), inducing ChAT-activity and VIP-IR and reducing TH-activity and NPY-IR in cultured sympathetic neurons, mimicking the shift from catecholaminergic to cholinergic phenotype that occurs in vivo.

A choroid-layer derived 10 KD factor, that stimulate SOM expression in cultured ciliary ganglion neurons has been recently described (Coulombe and Nishi, 1991). Other target derived factors are considered to be responsible for : the change of SP-IR expression in sensory nerves resulting from cross-innervation of inappropriate tissue (MaMahon and Gibson, 1986); the increase of TH-IR in petrosal ganglion neurons after ligature of the carotid sinus nerve (Katz and Black, 1986); the differential expression of CGRP mRNA in axotomized motor and sensory neurons during regeneration (Dumoulin et al., 1991).

iv. Hormones

Glucocorticoids are known to stimulate the transcription of TH-gene in PC-12 cells (Lewis et al., 1987). However, the consensus sequence responsive to glucocorticoid activation has not been identified. In previous studies (McLennan et al., 1980), glucocorticoids were seen influencing neurotransmitter choice (NA) in cultured sympathetic neurons.

In the uterus of pregnant guinea pigs, progesterone is considered to inhibit TH-activity, by comparison with the effects of an implanted wax pellet slowly releasing progesterone in the uterus or other tissue of virgin animals, that causes a reduction of TH-activity similar to that seen in the pregnant uterus (Malcolm, 1986).

Recently, the mechanism of action of some hormones and vitamins has been elucidated by the discovery of the supergene family of steroid receptors, that works at the nuclear level (see Hadcock and Malbon, 1991, for review). This family includes receptors for sex steroids, glucocorticoids, thyroid hormones and retinoic acid. Steroid receptors, bound to their agonists, act as trans-acting factors, enhancing or repressing gene transcription by binding specific hormone-sensitive DNA-sequences. Interestingly, the complex steroid receptor/agonist, in the form of a homodimer, usually replace another trans-acting factor stably bound to the hormone-sensitive DNA sequence (probably with an opposite effect on gene expression), that belongs to the family of heat-shock proteins (see Hadcock and Malbon, 1991, for review).

v. Immunoregulators

In addition to the homology between one of the HCCM factors with LIF (see above), some immunomodulators (i.e. the cytokines, glycoproteins stimulating immune responses) showed an interesting relation with SP (Reusch et al., 1988; Kimball, 1990). Cytokines have been shown to modulate SP levels under inflammatory conditions (O'Birne et al., 1990) and to increase the level of SP expression in dissociated cultures of rat SCG (Freidin and Kessler, 1991).

2.3 How the mechanisms and causes described above apply to the findings of this study

Several lines of evidence seem to indicate that the changes of neurotransmitter levels detected in the course of this study are due to modification of the expression of neurotransmitter genes. However, post-transcriptional and post-translational mechanisms are also able to influence both quality and quantity of the immunoreactive neuropeptides. Dysfunctions occuring during production and packaging of neuropeptides (see Russell, 1987; Andrews et al., 1987; Sossin et al., 1989, for excellent reviews) can alter their immunoreactivity, hence their detectability with immunocytochemical methods. In the case of the disappearance of a neurotransmitter from perivascular nerves, once both death or degeneration of cell bodies and decrease of gene expression are ruled out, an explanation could be that of a rapid relocation of the immunoreactive neuropetide from soma to axons followed by fast and continous release (see the effects of reserpine on NPY-IR in sympathetic nerves, Lundberg et al.,1985; 1986; Schalling et al., 1991). A similar mechanism has been suggested to explain the reduced detectability of enkephalin-IR in SCG neurons and nerve fibres in the submandibulary gland of 8 week-old rats, despite no detectable reduction of the expression of mRNA for pre-pro-enkephalin and levels of ENK-IR similar to 3 week-old rats were obtained using colchicine to block axonal transport (Watanabe et al., 1991). In the present study, the reduction of SP- and CGRP-immunoreactive neurons in sensory ganglia, that follows sympathectomy induced by guanethidine treatment and is accompanied by

an increase of the density of sensory fibres in target organs (Chapter four), has been interpreted as due to the same mechanism.

In all the other experiments of the present work, either the appearance and/or the increase of neuropeptide-IR was described. To explain these findings in terms of post-traslational modifications, the presence of stores of non-immunoreactive precursors for the neuropeptides, should be hypothesized, with the sudden activation of post-traslational mechanisms leading to the production of IR-peptides, as a response to experimental conditions, which seems unlikely. The type of the neurotransmitter can be determined during the transcription, for alternate DNA splicing, providing that the peptide products originate from the same gene (see pre-protachikynin A and B gene, Nawa et al., 1983; 1984; VIP and PHI/PHM, Itoh et al 1983; Bodner et al., 1985), whereas this study deals with the expression of peptides encoded by different genes (see NPY/VIP in Chapter three; SP/CGRP in Chapter four). The stability of the mRNAs could, however be modified post-trascriptionally, leading to an increase of its product (see TH expression by NGF, Raynaud et al., 1988).

Either upregulation of gene expression or post-traslational modification of the cocaine-sensitive noradrenaline transporter may be responsible for its incorporation into the plasma membrane of sensory nerve fibres in uterine artery of late pregnant guinea pigs (Chapters six and seven). In fact, very little is known about this protein, the gene encoding for which has been recently sequenced and cloned, leading to the production and correct incorporation of an active transporter transfected in HeLa cells (Pacholczyk et al.,

1991). It appears to belong to a class of Na+/amino acid transporters, that includes the GABA transporter (Guastella et al., 1990; Nelson et al., 1990), and shows numerous amino acid homologies, including the domain related with the Na+/Cl- pump used to accumulate neurotrasmitter molecules against their gradient. Other possible member of this family include the transporter for glutamate and dopamine (Blakely et al., 1991). The cloned cocaine-sensitive NA/transporter also showed a strong ability to accumulate 6-OHDA, thus suggesting that 5-OHDA loaded vesicles, described in Chapter six, also represent sites of NA-uptake.

However, the transporters responsible for intravescicular accumulation of amino acid transmitters are different to those associated with the plasma membrane (De Camilli and Jahn, 1990; Sternbach et al., 1990). Carriers for NA, dopamine, glutamate, GABA etc. have been characterized as belonging to the class of vacuolar proton-pumps, the V-ATPase (see Stone et al., 1989, for review), active transporters generating proton gradients in separate cellular compartments by ATP hydrolysis (Maycox et al., 1990). Thus, the uptake of ^{3}H-NA and 5-OHDA occuring in sensory nerves in late pregnancy, implies the activation of at least two different mechanisms. Although there is no experimental evidence to exclude the new expression of the two (or more) genes encoding for these transporters in sensory neurons, this event should be integrated by the transport of the newly synthesized proteins along the axon and by their incorporation in the plasma and vescicular membranes. Alternatively, inactive transporters for NA could be present in the plasma and/or vescicular membranes of all nerves and acquire the

ability to take up NA and related compound through an unknown mechanism, perhaps by binding an energy-generating subunit to the complex (for analogy with the incorporation of G-proteins to inactive receptors, see Grant and Landis, 1991). Both the increase of sex hormones and decrease of NA concentration in the uterine artery of late pregnant guinea pigs could represent competent signals for gene expression and/or "integration" of an activating factor.

The age-related changes of subpopulations of nerve fibres in rat cerebral vessels reported in Chapter one, are also attributable to changes in expression of neurotransmitter and synthetic enzyme genes. Rules governing the developmental regulation of gene expression are likely to play a role also in age-related changes. Firstly, the modifications of the target requirements during aging : both the mechanics and structural composition of cerebral arterioles have been found greatly altered in old rats (Hajdu et al., 1990), while responses of cerebral blood flow to electrical sympathetic stimulation are well maintained in aged rats (Hervonen et al., 1990). Thus, a reduced elasticity and distensibility (due also to a reduction of smooth muscle cell mass) of cerebral vessels, induce an impairment of vasoconstrictor mechanisms (reduced availability of NA and 5-HT, Chapter one) in order to protect the brain and the arteries themselves, against the mechanical difficulty in evoking and sustaining vasodilation after contraction. In addition, the increase of vasodilatory neurotransmitters (Chapter one), may reflect the necessity to potentiate neurogenic vasodilation. An important finding is that of Suhonen et al., 1991, who showed that sympathetic neurons from both young and old rats exhibited a similar ability to survive,

synthetize cathecholamines and innervate the host tissue, when allotransplanted into the submandibular gland of young rats. Thus both morphological and functional age-related alterations of sympathetic neurons "in situ" (Hervonen et al., 1978; Santer et al., 1980) are overcome by the substitution of their "aged" target with a younger one!

Studies on gene expression for neurotransmitters and their related enzymes during aging have not yet been carried out in the ANS. However, it has been reported that long-term potentiation is reduced in the SCG of old rats (Wu et al., 1991), indicating an impairment of responses mediated by gene expression (Goelet et al., 1986).

In the CNS, several intraneuronal second messenger pathways have been found impaired in aged animals (see Magnoni et al., 1991); these changes have been held responsible for the reduction of receptor responsiveness, of neurotransmitter expression, of synaptic plasticity and memory.

3. IS PEPTIDE IMMUNOCYTOCHEMISTRY A SUITABLE TECHNIQUE TO DETECT PLASTICITY OF PERIVASCULAR NERVES?

The availaibility of techniques derived from molecular biology, has greatly enlarged the field of investigation for anatomists. In particular, "in situ" hybridization techniques have allowed the visualization of signals of nucletide sequences encoding for known proteins and peptides (see Penschow et al., 1987, for review) in neuronal cell bodies. Recent studies on neuropeptide expression frequently compare the results obtained with both "in situ" hybridization and immunocytochemistry. Almost invariably, when a long-lasting change of expression of a neurotransmitter was found, this was detectable by both techniques (Noguchi et al., 1989; Faucon-Biguet et al., 1989; Henken et al., 1990; Baetge et al., 1990; Watanabe et al., 1991; Schalling et al., 1991; Wong and Oblinger, 1991). Usually the change(s) detected by immunocytochemistry were preceeded by a similar alteration of the signals generated by the hybridized nucleotides, with an interval of about 24 hrs (Noguchi et al., 1989; Schalling et al., 1991; Watanabe et al., 1991), compatible with protein synthesis and posttraslational cleavage, apart for a report (Henken et al., 1990) where the results obained with immunocytochemistry (number of SP-IR neurons in DRG after sciatic nerve transection) matched those obtained with "in situ" hybridization for pre-protachikinin A mRNA after two weeks. All these studies are relevant to neuropeptide expression in cell bodies, while "in situ" hybridization is unsuitable to study neuronal processes (axon and dendrites) where only the synthetized and carried

neuropeptide can be detected. Similarly, the immunocytochemistry of trans-acting factors has to be confined to cell bodies, for the immediate nuclear migration of these factors (see Johnson and McKnight, 1989, for review).

Thus, immunocytochemistry will continue as a suitable technique to study qualitative changes of neurotransmitters in perivasculr nerves, while the increase or decrease of neurotransmitter expression within a fixed number of fibres, needs to be confirmed by biochemical studies and modifications of the number of nerve fibres expressing a certain neurotransmitter have to be assessed by quantitative analysis, possibly using high resolution light or electron microscopy.

4. FUTURE DIRECTIONS

Evidence of neurotransmitter plasticity in perivascular nerves brings about a major question about the components mediating these events. In some cases (cerebral vessels in aged rats and uterine arteries in pregnant guinea pigs) morphological changes of the target (either the whole vessel or the single smooth muscle cell) were reported (Hajdu et al., 1990; Moll et al., 1971; Mione and Gabella, 1991). However, in both sympathetic and sensory denervated cerebral vessels, no major alterations of the size and structure of the targets were noted (Chapters two, three and four), although an in depth examination of the different components of the vessel wall was not carried out. Other authors (Dimitriadou et al., 1988) have reported some changes in smooth muscle cells of sympathetically denervated cerebral arteries in rabbits.

Vascular smooth muscle cells are the common targets of different subpopulations of perivascular nerves. It is possible that they transduce messages from one nerve fibre to another, thus exerting a primary role in the regulation of neurotransmitter expression. Several features of smooth muscle cells need to be investigated to reveal their ability to mediate trans-neuronal communication. Are selective denervation procedures able to induce cellular responses leading to the production of releaseable or membrane-bound neurotrophic factors? The production of neuroactive substances (including NGF) that stimulate axonal sprouting and neurotransmitter expression in sensory or sympathetic denervated vessels should be investigated, as well as the intracellular signals usually preceeding

the expression of their genes (e.g. c-fos, Hengerer et al., 1990). It is known that stretching mediates axonal growth in culture (Bray, 1984). This mechanism may be relevant in vivo, during vascular hypertrophy (see uterine artery during pregnancy, Mione and Gabella, 1991 and present study). However, the signal(s) mediating axonal growth during stretching are not known and should be investigated.

Alternatively, or in addition to the target-mediated interneuronal communication, axons lying within the same nerve bundle, could influence each other directly. Morphological correlates, like interaxonal junctions or varicosity-related specializations, have been sought by several authors and not found, partly in an effort to explain well known mechanisms like prejunctional neuromodulation. However, interaxonal communication cannot be ruled out because of the absence of morphological specializations, this being a primary feature of the neuromuscular junction in the ANS (Burnstock, 1970; but see Luff et al., 1987). However, varicosities of different axons often concentrate in a limited length (personal observation) and the vesicles congregate so to face other axons, more frequently than smooth muscle cells. In addition, relation between varicosities may be temporary, being the varicosities of perivascular nerves interpreted as axonal swellings, where several organelles, mainly mitochondria and vesicles are concentrated because of a local slowing down of the intraaxonal flow (see Greenberg et al., 1990). This temporary relation may represent a morphological correlate for interaxonal exchanges of information, providing that a substance is released and a receptor is activated in adjacent axons.

However, the most important question arising from these and other studies on neurotransmitter plasticity remains that on how these changes are related to the effector mechanisms. All the studies on perivascular nerve plasticity need to be integrated by investigations on the expression and function of neurotransmitter receptors. The most interesting insight about the regulation of neurotransmitter receptors is the active role played by agonists (see Hadcock and Malbon, 1991, for review).

Neurotransmitters control the expression of their own receptors, hence the responsiveness of the target in several different ways, even if their initial presence is programmed by the target tissue itself. For example, during development of chick ciliary ganglion neurons, the synthesis and incorporation into the plasmamembrane of nicotinic cholinergic receptor, is triggered by the establishment of synapses with central cholinergic neurons (Jacob, 1991). In striated muscles, release of agrin (a protein that binds to membrane heparan sulphate, Wallace, 1990) from the developing motor axon terminals has been shown to be responsible for AChR phosphorylation (Qu et al., 1990; Wallace et al., 1991), with consequent lateral migration of the receptors along the plasmamembrane and formation of the typical AChR aggregations (reviewed by Schultze and Role, 1987). Beta-adrenergic receptors in the developing heart (Chen et al., 1979) and parotid glands (Lunford and Talamo, 1980) and cardiac muscarinic receptors (Galpert et al., 1977) are present on the plasmamembrane prior to the onset of innervation. However, they are unresponsive to the agonists, because they are uncoupled to the G-protein that mediates their transduction pathway. The arrival of nerve fibres suddenly activates

this coupling, making the receptor complex functional (Lunford and Talamo, 1983; Galpert et al., 1984; Feng et al., 1989). The expression of NGF receptors in developing sensory neurons of the mouse trigeminal ganglion is upregulated by the availability of NGF molecules, and develops in parallel with the target field innervation (Wyatt et al., 1990). In rat sweat glands, ACh released from cholinergic sympathetic neurons, triggers the development of secretory responsiveness, through the activation of muscarinic receptors (Stevens and Landis, 1987). Also in this case, muscarinic binding sites in sweat glands are present before the onset of the innervation, as well as in non-innervated or denervated glands (Stevens and Landis, 1988; Grant and Landis, 1988; 1989). However these glands are not responsive to ACh. The explanation for this non-responsiveness proposed by Grant and Landis, 1991, is that of an agonist-mediated expression of a specific molecular subtype of the muscarinic receptor, which is more efficiently coupled to intracellular transducers. At the present time, five different subtypes of muscarinic receptors encoded by different genes have been described (Bonner et al., 1987; 1988). This mechanism also resembles the effects of motor innervation on the molecular forms of the nicotinic AChR at the neuromuscular junction (Mishina et al., 1986; Gu and Hall, 1988). In the latter case, motor nerve activity and/or ACh release causes the shift from the foetal to the mature form of AChR, by replacing the ε subunit with the fast channel-opener γ subunit.

Agonist regulation of transmitter expression and function not only

occurs during development. The most throughly studied effect of agonists on their receptors is desensitization. Prolonged exposure to the agonist diminishes the responsivity of the effector cells. This has been studied at depth for the β-adrenergic receptor (see Collins et al., 1991, for review). Three processes appear to be associated with the development of desensitization: 1) uncoupling of the receptor from the effector units (G-protein), which occurs after seconds to minutes of exposure and is mediated by phosphorylation; 2) temporary sequestration of the receptor from the cell surface, mediated by an unkown conformational change of the receptor molecule and occurs for a more prolonged exposure to the agonist; 3) down regulation of receptor gene expression, that takes place after hours of exposure to the agonist, and results in a decrease of the number of receptors. Recently, down regulation of α_1-adrenergic receptor mRNA in rabbit aortic smooth muscle cells has been shown to be mediated by similar mechanisms (Izzo et al., 1990). In contrast to the β-adrenergic receptor and others, the superfamily of growth factor receptors (including those for epidermal growth factor, NGF, BDNF and NT3) appears to be upregulated by the presence of the agonist (Clark et al., 1985). Expression and activity of neurotransmitter receptors can also be regulated by neurotransmitters other than the agonist. The classical example is the upregulation of AChR expression at the neuromuscular junction induced by CGRP (New and Mudge, 1986), a cotransmitter in motor nerve fibres.

From this brief overview, it is clear that neurotransmitters can influence the responsiveness of their effector organs by directly

changing both number and activity of their own, and other neurotransmitter receptors. These new insights also suggest that changes in the expression of neurotransmitters are likely to be accompanied by profound modifications of the populations of membrane receptors and of the effects of nerve activity and neurotransmitter release on the target organs.

Studies relating plasticity of neurotransmitter expression to subsequent changes in receptor expression are much needed to advance this field not only in terms of an understanding of the basic mechanisms, but also if plasticity in ageing and pathological conditions is to be recognized in the clinical context and considered in the development of therapeutic strategies.

REFERENCES

Abbound, F.M. (1982). The sympathetic system in hypertension. State-of-the-art review. Hypertension 4, 11208-11225.

Abel, P.W., Han, C., Noe, B.D. & McDonald, J.K. (1988). Neuropeptide Y: vasoconstrictor effects and possible role in cerebral vasospasm after experimental subarachnoid hemorrhage. Brain Res. 463, 250-258.

Aberdeen, J., Corr, L., Milner, P., Lincoln, J. & Burnstock, G. (1988). Increases in calcitonin gene-related peptide (CGRP) after guanethidine sympathectomy in the rat. Regul. Pept. 22, 387.

Aberdeen, J., Corr, L., Milner, P., Lincoln, J. & Burnstock, G. (1990). Marked increases in calcitonin gene-related peptide-containing nerves in the developing rat following long-term sympathectomy with guanethidine. Neuroscience 35, 175-184.

Abrahams, V.C., Koelle, G.B. & Smart, P. (1957). Histochemical demonstration of cholinesterases in the hypothalamus of the dog. J. Physiol. (Lond.) 139, 137-144.

Adamson, E.D. (1987). Oncogenes in development. Development 99, 449-471.

Adler, J.E., Schleifer, L.S. & Black, I.B. (1989). Partial purification and characterization of a membrane- derived factor regulating neurotransmitter phenotypic expression. Proc. Natl. Acad. Sci. U. S. A. 86, 1080-1083.

Adler, R., Lauda, K., Manthorpe, M. & Varon, S. (1979). Cholinergic neuronotrophic factors: II. Intraocular distribution of trophic activity for ciliary neurons. Science 204, 1434-1436.

Adrian, T.E., Gu, J., Allen, J.M., Tatemoto, K., Polak, J.M. & Bloom, S.R. (1984). Neuropeptide Y in the male genital tract. Life Sci. 35, 2643-2648.

Agnati, L.F., Fuxe, K., Benfenati, F., Harfstrand, A., Tatemoto, K., Hökfelt, T. & Mutt, V. (1983). Neuropeptide Y in vitro selectively increases the number of adrenergic binding sites in membranes of the medulla oblongata of the rat. Acta Physiol. Scand. 118, 292-298.

Akagi, H., Konishi, S., Otsuka, M. & Yanysawa, M. (1985). The role of substance P as a neurotransmitter in the reflexes of slow time courses in the neonatal rat spinal cord. Br. J. Pharmacol. 84, 663-673.

Alafaci, C., Cowen, T., Crockard, H.A. & Burnstock, G. (1986a). Cerebral perivascular serotonergic fibres have a peripheral origin in the gerbil. Brain Res. Bull. 16, 303-304.

Alafaci, C., Cowen, T., Crockard, H.A. & Burnstock, G. (1986b). Perivascular nerve types supplying cerebral blood vessels of the gerbil. Acta Physiol. Scand. 127 (Suppl. 552), 9-12.

Allen, J.M., Bircham, P.M.M., Edwards, A.V., Tatemoto, K. & Bloom, S.R. (1983). Neuropeptide Y (NPY) reduces myocardial perfusion and inhibits the phase of contraction of the isolated perfused rabbit heart. Regul. Pept. 6, 247-253.

Allen, J.M., Gu, J., Adrian, T.E., Polak, J.M. & Bloom, S.R. (1984). Neuropeptide Y in the guinea-pig biliary tract. Experientia 40, 765-767.

Allen, J.M., Polak, J.M. & Bloom, S.R. (1985a). Presence of the predicted C-flanking peptide of neuropeptide Y (C-PON) in tissue extract. Neuropeptides 6, 95-105.

Allen, J.M., Polak, J.M., Rodrigo, J., Darcy, K. & Bloom, S.R. (1985b). Localisation of neuropeptide Y (NPY) in nerves of the rat cardiovascular system and effect of 6-hydroxydopamine. Cardiovasc. Res. 19, 570-577.

Alm, P. & Lundberg, L.M. (1988). Co-existence and origin of peptidergic and adrenergic nerves in the guinea pig uterus. Retrograde tracing and immunocytochemistry effects of chemical sympathectomy, capsaicin treatment and pregnancy. Cell Tissue Res. 254, 517-530.

Alm, P., Owman, C., Sjöberg, N.-O. & Thorbert, G. (1979). Uptake and metabolism of [^{3}H]norepinephrine in uterine nerves of pregnant guinea pig. Am. J. Physiol. 136, C277-C285.

Alm, P., Björklund, A., Owman, C.h. & Thorbert, G. (1979b). Tyrosine hydroxylase and dopa decarboxylase activities in the guinea pig uterus: further evidence for a functional adrenergic denervation in association with pregnancy. Neuroscience 4, 145-154.

Alm, P., Håkanson, R., Owman, C., Sjöberg, N.-O., Sundler, F. & Walles, B. (1980). Origin and distribution of VIP (vasoactive intestinal polypeptide) nerves in the genito-urinary tract. Cell Tissue Res. 205, 337-347.

Altura, B.M. & Altura, B.T. (1977). Vascular smooth muscle and neurohypophyseal hormones. Fed. Proc. 36, 1853-1860.

Amara, S.G., Jonas, V., Rosenfeld, M.G., Ong, E.S. & Evans, R.M. (1982). Alternative RNA-processing in calcitonin gene expression generates mRNAs encoding different polypeptide products. Nature 298, 240-244.

Amara, S.G., Arriza, J.L., Leff, S.E., Swanson, L.W., Evans, R.M. & Rosenfeld, M.G. (1985). Expression in brain of a messenger RNA encoding a novel neuropeptide homologous to calcitonin gene-related peptide. Science 229, 1094-1097.

Ambar, I., Kloog, Y., Schvartz, I., Hazum, E. & Sokolovsky, M. (1989). Competitive interaction between endothelin and sarafotoxin:

binding and phosphoinositides in rat atria and brain. Biochem. Biophys. Res. Commun. 158, 195-201.

Amenta, F., Cavallotti, C. & Collier, W.L. (1985). The adrenergic innervation of the rat uterine tubes in old age: a fluorescent histochemical study. Arch. Gerontol. Geriatr. 4, 37-42.

Amezcua, J.L., Dusting, G.J., Palmier, R.MJ. & Moncada, S. (1988). Acetylcholine induces vasodilation in the rabbit isolated heart through the release of nitric oxide, the endogenous vasodilator. Br. J. Pharmacol. 95, 830-834.

Anderson, J.V., Christofides, N.D., Vinas, P., Wharton, J., Varndell, I.M., Polak, J.M. & Bloom, S.R. (1986). Radioimmunoassay of alpha rat atrial natriuretic peptide. Neuropeptides 7, 159-173.

Andersson, P.O., Bloom, S.R. & Jarhult, J. (1983a). Colonic motor and vascular responses to pelvic nerve stimulation and their relation to local peptide release in the cat. J. Physiol. (Lond.) 334, 293-307.

Andersson, P.O., Bloom, S.R., Edwards, A.V., Jarhult, J. & Mellander, S. (1983b). Neural vasodilator control in the rectum of the cat and its possible mediation by vasoactive intestinal polypeptide. J. Physiol. (Lond.) 344, 49-67.

Andres, K.H., von Dring, M., Muszynski, K. & Schmidt, R.F. (1987). Nerve fibres and their terminals of the dura mater encephali of the rat. Anat. Embryol. 175, 289-301.

Andrews, P.C., Brayton, K. & Dixon, J.E. (1987). Precursor to regulatory peptides: their proteolytic processing. Experientia 43, 784-790.

Andriantsitohaina, R. & Stoclet, J.C. (1988). Potentiation by neuropeptide Y of vasoconstriction in rat resistance arteries. Br. J. Pharmacol. 95, 419-428.

Angel, P., Inagawa, M., Chin, R., Stein, B., Imbra, R.J., Rahmsdorf, H.J., Jonat, C., Herrlich, P. & Karin, M. (1987). Phorbol ester-inducible genes contain a common cis element recognized by a TPA-modulated transacting factor. Cell 49, 729-739.

Angel, P., Hattori, K., Smeal, T. & Karin, M. (1988). The jun proto-oncogene is positively autoregulated by its product, jun/AP-1. Cell 55, 875-885.

Angus, J.A., Campbell, G.R., Cocks, T.M. & Manderson, J.A. (1983). Vasodilation by acetylcholine is endothelium-dependent: a study by sonomicrometry in canine femoral artery in vivo. J. Physiol. (Lond.) 344, 209-222.

Ann, H.B. & Ku, D.D. (1986). Magnesium inhibits basal release of endothelium-derived relaxing factor in canine coronary arteries. Eur. J. Pharmacol. 130, 353-355.

Appenzeller, O., Dhital, K.K., Cowen, T. & Burnstock, G. (1984). The nerves to blood vessels supplying blood to nerves: the innervation of vasa nervorum. Brain Res. 304, 383-386.

Arbab, M.A-R., Wiklund, L., Delgado, T. & Svendgard, N.A. (1988). Stellate ganglion innervation of the vertebro-basilar system demonstrated in the rat with anterograde and retrograde WGA-HRP tracing. Brain Res. 445, 175-180.

Atkinson, M.E. & Shehab, S.A.S. (1986). Peripheral axotomy of the rat mandibular trigeminal nerve leads to an increase in VIP and decrease of other primary afferent neuropeptides in the spinal trigeminal nucleus. Regul. Pept. 16, 69-81.

Audigier, S.M.P., Wang, J.K.T. & Greengard, P. (1988). Membrane depolarization and carbamoylcholine stimulate phosphatidylinositol turnover in intact nerve terminals. Proc. Natl. Acad. Sci. U. S. A. 85, 2859-2863.

Azanza, M.J. & Garin, P. (1986). The autonomic innervation of the rat diaphragm. Gen. Pharmacol. 17, 15603-15603.

Baetge, G., Pintor, J.E. & Gershon, M.D. (1990). Transiently catecholaminergic (TC) cells in the bowel of the fetal rat: precursors of non catecholaminergic enteric neurons. Dev. Biol. 141, 353-380.

Baker, D.M. & Santer, R.M. (1988). Morphometric studies on pre- and paravertebral sympathetic neurons in the rat: changes with age. Mech. Ageing Dev. 42, 139-145.

Ballesta, J., Polak, J.M., Allen, J.M., Pals, D.T., Lawson, J.A., Ludens, J.H., Slizgi, G.R. & Bloom, S.R. (1984). NPY immunoreactivity in experimental hypertension (aorta ligated and DOCA treated rats). Regul. Pept. 9, 323-323.

Baluk, P. & Gabella, G. (1989). Some intrinsic neurons of the guinea-pig heart contain substance P. Neurosci. Lett. 102, 191-196.

Bannister, R., Crowe, R. & Burnstock, G. (1981) Adrenergic innervation in autonomic failure. Neurology 31, 1501-1506

Barja, F. & Mathison, R. (1982). Adrenergic and peptidergic (substance P and vasoactive intestinal polypeptide) innervation of the rat portal vein. Blood Vessels 19, 263-272.

Barja, F., Mathison, R. & Huggel, M. (1983). Substance P-containing nerve fibres in large peripheral blood vessels of the rat. Cell Tissue Res. 229, 411-422.

Barnes, P.J., Cadieaux, A., Carstairs, J.R., Greenberg, B., Polak, J.M. & Rodhen, K. (1986). VIP in bovine pulmonary artery: localisation, function and receptor autoradiography. Br. J.

Pharmacol. 89, 157-162.

Bartel, D.P., Sheng, M., Lau, L.F. & Greenberg, M.E. (1989). Growth factors and membrane depolarization activate distinct programs of early response gene expression: dissociation of fos and jun induction. Genes Dev. 3, 304-313.

Bartfai, T., Iverfeldt, K. & Fisone, G. (1988). Regulation of the release of coexisting neurotransmitters. Ann. Rev. Pharmacol. Toxicol.28, 285-310

Batten, T.F.C., Appenteng, K. & Saha, S. (1988). Visualization of CGRP and ChAT-like immunoreactivity in identified trigeminal neurons by combined peroxidase and alkaline phosphatase enzymatic reaction. Brain Res. 447, 314-324.

Beal, M.F., Kowall, N.W., Ellison, D.W., Mazurek, M.F., Swartz, K.J. & Martin, J.B. (1986). Replication of the neurochemical characteristics of Huntington's disease by quinolinic acid. Nature 321, 168-171.

Belai, A. & Burnstock, G. (1987). Selective damage of intrinsic calcitonin gene-related peptide-like immunoreactive enteric nerve fibers in streptozotocin-induced diabetic rats. Gastroenterology 92, 730-734.

Belai, A., Lincoln, J., Milner, P., Crowe, R., Loesch, A. & Burnstock, G. (1985). Enteric nerves in diabetic rats: increase in vasoactive intestinal polypeptide but not substance P. Gastroenterology 89, 967-976.

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

Bell, C. (1968). Dual vasoconstrictor and vasodilator innervation of the uterine arterial supply in the guinea pig. Circ. Res. 23, 269-279.

Bell, C. (1969) Fine structural localization of acetylcholinesterase at a cholinergic vasodilatory nerve-arterial smooth muscle synapse. Circ. Res. 24, 61-70

Bell, C. (1974). Selective cholinergic denervation of the uterine artery in the guinea pig. Experientia 30, 257-258.

Bell, C. & Malcolm, S.J. (1978). Observation on the loss of catecholamine fluorescence from intrauterine adrenergic nerves during pregnancy in the guinea pig. J. Reprod. Fertil. 53, 51-58.

Bell, C. & Malcolm, S.J. (1988). Neurochemistry of the sympathetic innervation to the uterus. Clin. Exp. Pharmacol. Physiol. 15, 667-674.

Benjamin, N., Dollery, C.T., Fuller, R.W., Larkin, S. & McEwan, J. (1987). The effects of calcitonin gene-related peptide and substance P on resistance and capacitance vessels. Br. J. Pharmacol. 90, 39P.

Beny, J.L., Brunet, P.C. & Huggel, H. (1986). Effect of mechanical stimulation, substance P and vasoactive intestinal polypeptide on the electrical and mechanical activities of circular smooth muscles from pig coronary arteries contracted with acetylcholine: role of the endothelium. Pharmacology 33, 61-68.

Bevan, J.A. & Brayden, J.E. (1986). In vitro demonstration that vasoactive intestinal peptide is a cerebrovascular neurovasodilator: technical difficulties and limitations of in vitro methodology. In: Neuroregulation of Brain Circulation. Eds. C. Owman & J.E. Hardebo, Elsevier, Amsterdam. pp. 383-391.

Bevan, J.A. & Joyce, E.H. (1988). Flow-dependent dilation in myograph-mounted resistance artery segments. Blood Vessels 25, 101-104.

Bevan, J.A., Moskowitz, M.A., Said, S.I. & Buga, G. (1984). Evidence that vasoactive intestinal polypeptide is a dilator transmitter to some cerebral and extracerebral arteries. Peptides 5, 385-388.

Björklund, H., Hökfelt, T., Goldstein, M., Terenius, L. & Olson, L. (1985). Appearance of the noradrenergic markers tyrosine hydroxylase and neuropeptide Y in cholinergic nerves of the iris following sympathectomy. J. Neurosci. 5, 1633-1644.

Black, I.B. (1986). Trophic molecules and evolution of the nervous system. Proc. Natl. Acad. Sci. U. S. A. 83, 8249-8252.

Blakely, R.D., Clark, J.A., Pacholczyk, T. & Amara, S.G. (1991). Distinct, developmentally regulated brain mRNAs direct the synthesis of neurotransmitter transporter. J. Neurochem. 56, 860-871.

Blau, H.M. & Baltimore, D. (1991). Differentiation requires continuous regulation. J. Cell Biol. 112, 781-783.

Bloom, S.R., Bryant, M.G., Polak, J.M., Van Noorden, S. & Wharton, J. (1979). Vasoactive intestinal peptide-like immunoreactivity in salivary glands of the rat. J. Physiol. (Lond.) 289, 23P.

Bodner, M., Fridkin, M. & Gores, I. (1985). Coding sequences for vasoactive intestinal polypeptide and PHM peptide are located on two adjacent exons in the human genome. Proc. Natl. Acad. Sci. U. S. A. 82, 3548-3555.

Bolton, T.B. & Clapp, L.H. (1986). Endothelial-dependent relaxant actions of carbachol and substance P in the arterial smooth muscle. Br. J. Pharmacol. 87, 713-723.

Bonner, T.I., Buckley, N.J., Young, A. & Brann, M.R. (1987).

Identification of a family of muscarinic receptor genes. Science 237, 527-532.

Bonner, T.I., Young, A.C., Brann, M.R. & Buckley, N.J. (1988). Cloning and expression of the human and rat m5 muscarinic acetylcholine receptor genes. Neuron 1, 403-410.

Brain, S.D. & Williams, T.J. (1988). Substance P regulates the vasodilatory activity of calcitonin gene-related peptide. Nature 355, 73-75.

Brain, S.D., Williams, T.J., Tippins J.R., Morris H.R., MacIntyre, I. (1985) Calcitonin gene-related peptide is a potent vasodilator. Nature 313, 54-56.

Brain, S.D., Tippins, J.R. & Williams, T.J. (1988) Endothelin induces potent microvascular constriction. Br. J. Pharmacol. 95, 1005-1007.

Bray, D. (1984). Axonal growth in response to experimentally applied mechanical tension. Dev. Biol. 102, 379-389.

Brayden, J.E. & Conway, M.A. (1988). Neuropeptide Y and vasoactive intestinal polypeptide in cerebral arteries of the rat: relationship between innervation pattern and mechanical response. Regul. Pept. 22, 253-265.

Breathnach, R. & Chambon, P. (1981). Organization and expression of eukaryotic split genes coding for proteins. Ann. Rev. Biochem. 50, 349-383.

Buck, C.R., Martinez, H.J., Black, I.B. & Chao, M.V. (1987). Developmentally regulated expression of the nerve growth factor receptor gene in the periphery and brain. Proc. Natl. Acad. Sci. U. S. A. 84, 3060-3063.

Burcher, E., Atterhog, J.H., Pernow, B. & Rosell, S. (1977). Cardiovascular effects of substance P: effects on the heart and regional blood flow in the dog. In: Substance P. Eds. U.S. Von Euler & B. Pernow, Raven Press, New York. pp. 261-268.

Burn, J.H. & Rand, M.J. (1959). Sympathetic postganglionic mechanisms. Nature 184, 163-165.

Burnstock, G. (1970). Structure of smooth muscle and its innervation. In: Smooth muscle. Eds. E. Bülbring, A. Brading, A. Jones & T. Tomita, Edward Arnold, London. pp. 1-69.

Burnstock, G. (1974). Degeneration and oriented growth of autonomic nerves in relation to smooth muscle in joint tissue cultures and anterior eye chamber transplants. In: Dynamics of Degeneration and Growth in Neurons. Eds. K. Fuxe, L. Olson & Y. Zotterman, Pergamon Press, Oxford. pp. 509-519.

Burnstock, G. (1975). Innervation of vascular smooth muscle:

histochemistry and electron microscopy. In: Physiological and Pharmacological Control of Blood Pressure. Clin. Exp. Pharmacol. Physiol. Suppl. 2, 7-20.

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

Burnstock, G. (1981a). Pathophysiology of migraine: a new hypothesis. Lancet i, 1397-1399.

Burnstock, G. (1981b). Development of smooth muscle and its innervation. In: Smooth Muscle: An Assessment of Current Knowledge. Eds. E. Bülbring, A.F. Brading, A.W. Jones & T. Tomita, Edward Arnold, London. pp. 431-457.

Burnstock, G. (1982). Neuropeptides as trophic factors. In: Systemic Role of Regulatory Peptides. Symposia Medica Hoechst. Eds. S. Bloom, J.M. Polak & E. Lindenlaub, Schattauer Verlag, Stuttgart/New York. pp. 423-441.

Burnstock, G. (1983). Recent concepts of chemical communication between excitable cells. In: Dale's Principle and Communication between Neurones. Ed. N.N. Osborne, Pergamon Press, Oxford. pp. 7-35.

Burnstock, G. (1985). Neurogenic control of cerebral circulation. Cephalalgia Suppl. 2, 25-33.

Burnstock, G. (1986a). The changing face of autonomic neurotransmission. (The First von Euler Lecture in Physiology). Acta Physiol. Scand. 126, 67-91.

Burnstock, G. (1986b). Autonomic neuromuscular junctions: current developments and future directions. J. Anat. 146, 1-30.

Burnstock, G. (1987). Mechanisms of interaction of peptide and nonpeptide vascular neurotransmitter systems. J. Cardiovasc. Pharmacol. 10, Suppl.12, S74-S81.

Burnstock, G. (1988). Regulation of local blood flow by neurohumoral substances released from perivascular nerves and endothelial cells. Acta Physiol. Scand. 133, Suppl. 571, 53-59.

Burnstock, G. (1989). Vascular control by purines with emphasis on the coronary system. Eur. Heart J. 10, Suppl. F, 15-21.

Burnstock, G. & Iwayama, T. (1971b). Fine structural identification of autonomic nerves and their relation to smooth muscle. In: Progress in Brain Research, 34, Histochemistry of Nervous Transmission. Ed. O. Eränkö, Elsevier, Amsterdam. pp. 389-404.

Burnstock, G. & Griffith, S.G.(Eds.) (1988). Nonadrenergic Innervation of Blood Vessels, Vols.I (pp. 1-149) and II (pp. 1-233). CRC Press, Boca Raton, FL.

Burnstock, G. & Kennedy, C. (1986). A dual function for adenosine 5'-triphosphate in the regulation of vascular tone. Excitatory cotransmitter with noradrenaline from perivascular nerves and locally released inhibitory intravascular agent. Circ. Res. 58, 319-330.

Burnstock, G., Evans, B., Gannon, B.J., Heath, J.W. & James, V. (1971a). A new method of destroying adrenergic nerves in adult animals using guanethidine. Br. J. Pharmacol. 43, 295-301.

Burnstock, G., Chamley, J. & Campbell, G.R. (1980). The innervation of the arteries. In: Structure and Function of the Circulation, Vol. I. Eds. C.J. Schwartz, N.T. Werthessen & S. Wolf, Plenum Press, New York. pp. 729-767.

Burnstock, G., Lincoln, J., Fehér, E., Hopwood, A.M., Kirkpatrick, K., Milner, P. & Ralevic, V. (1988). Serotonin is localized in endothelial cells of coronary arteries and released during hypoxia: a possible new mechanism for hypoxia-induced vasodilatation of the rat heart. Experientia 44, 705-707.

Bülbring, E. (1955). Correlation between membrane potential, spike discharge and tension in smooth muscle. J. Physiol. (Lond.) 128, 200-221.

Cadieaux, A., Springall, D.R., Mulderry, P.K., Rodrigo, J., Ghatei, M.A., Terenghi, G., Bloom, S.R. & Polak, J.M. (1986). Occurrence, distribution and ontogeny of CGRP-immunoreactivity in rat lower respiratory tract: effect of capsaicin treatment and surgical denervation. Neuroscience 19, 605-627.

Cannon, B., Nedergaard, J., Lundberg, J.M., Hökfelt, T., Terenius, L. & Goldstein, M. (1986). Neuropeptide tyrosine (NPY) is costored with noradrenaline in vascular, but not in parenchymal sympathetic nerves of brown adipose tissue. Exp. Cell Res. 164, 546-550.

Cantin, M., Gutkowska, J., Thibault, J., Milne, R.W., Ledoux, S., Minli, S., Chapeau, C., Garcia, R., Hamet, P. & Genest, J. (1984). Immunocytochemical localization of atrial natriuretic factor in the heart and salivary glands. Histochemistry 80, 113-127.

Carraway, R. & Leeman, S.E. (1973). The isolation of a new hypotensive peptide, neurotensin, from bovine hypothalami. J. Biol. Chem. 248, 6854-6861.

Cavanagh, J.F.R., Mione, M.C. & Burnstock, G. (1988). The peptidergic innervation of the uterine artery during pregnancy. Regul. Pept. 22, 395.

Cavanagh, J.F.R., Mione, M.C. & Burnstock, G. (1990). Use of enhanced silver staining combined with electron microscopical immunolabelling to demonstrate the colocalization of neuropeptide Y and vasoactive intestinal polypeptide in cerebrovascular nerves. Neuroscience 39, 775-785.

Cavanagh, J.FR., Mione, M.C. & Burnstock, G. (1989). Colocalization of NPY and VIP in the cerebrovascular nerves originating from the pterigopalatine ganglion in the rat. J. Cereb. Blood Flow Metab. 9, Suppl. 1, S34.

Chabrier, P.E., Roubert, P. & Braquet, P. (1987). Specific binding of atrial natriuretic factor in brain microvessels. Proc. Natl. Acad. Sci. U. S. A. 84, 2078-2081.

Chan-Palay, V. (1977). Innervation of cerebral blood vessels by norepinephrine, indoleamine, substance P and neurotensin fibers and the leptomeningeal indoleamine axons: their roles in vasomotor reactivity and local alterations of brain blood composition. In: Neurogenic Control of Brain Circulation. Eds. C. Owman & L. Edvinsson, Pergamon Press, Oxford. pp. 39-53.

Chan-Palay, V. & Palay, S.L.(Eds.) (1984). Co-existence of Neuroactive Substances in Neurones. Wiley, New York, pp. 433.

Chang, M.M. & Leeman, S.E. (1970). Isolation of sialogogig peptide from bovine hypothalamic tissue and its characterization as substance P. J. Biol. Chem. 245, 4784-4790.

Chang, M.M., Leeman, S.E. & Niall, H.D. (1971). Aminoacid sequence of substance P. Nature,New Biol. 232, 86-87.

Changelion, P.S., Feng, P., King, T.C. & Milbrandt, J. (1989). Structure of the NGF1-A gene and detection of upstream sequences responsible for its transcriptional induction by nerve growth factor. Proc. Natl. Acad. Sci. U. S. A. 86, 377-381.

Chen, F.M., Yamamura, H.I. & Roeske, W.R. (1979). Ontogenity of mammalian myocardial beta-adrenergic receptors. Eur. J. Pharmacol. 58, 255-264.

Cherry, P.D., Furchgott, R.W., Zawadzki, J.V. & Jothianandan, D. (1982). Role of endothelial cells in relaxation of isolated arteries by bradykinin. Proc. Natl. Acad. Sci. U. S. A. 79, 2106-2110.

Cheung, A., Polak, J.M., Bauer, F.E., Cadieaux, A., Christofides, N.D., Springall, D.R. & Bloom, S.R. (1985). Distribution of galanin-immunoreactivity in the respiratory tract of pig, guinea-pig, rat and dog. Thorax 40, 889-896.

Christofides, N.D., Polak, J.M. & Bloom, S.R. (1984). Studies on the distribution of PHI in mammals. Peptides 5, 261-266.

Christy, B.A., Lau, L.F. & Nathans, D. (1988). A gene activated in mouse 3T3 cells by serum growth factors encodes a protein with "zinc finger" sequences. Proc. Natl. Acad. Sci. U. S. A. 85, 7857-7861.

Clague, J.R., Sternini, C. & Brecha, N.C. (1985). Localization of calcitonin gene-related peptide-like immunoreactivity in neurons of the rat gastrointestinal tract. Neurosci. Lett. 56, 63-68.

Clark, A.J., Ishii, S., Richert, N., Merlino, G.T. & Postan, I. (1985) Epidermal growth factor regulates the expression of its own receptor. Proc. Natl. Acad. Sci. U. S. A. 82, 8374-8378.

Clark, K.E., Mills, E.G., Stys, S.J. & Seeds, A.E. (1981). Effects of vasoactive polypeptides on uterine vasculature. Am. J. Obstet. Gynecol. 139, 182-188.

Claude, P., Hawrot, E., Dunis, D.A., Campenot, R.B. (1982) Binding, internalization, and retrograde transport of ^{125}I-nerve growth factor in cultured rat sympathetic neurons. J. Neurosci. 2, 431-442.

Cocks, T.M. & Angus, J.A. (1983). Endothelium-dependent relaxation of coronary arteries by noradrenaline and serotonin. Nature 305, 627-630.

Cole, D.F., Bloom, S.R., Burnstock, G., Butler, J.M., McGregor, G.P., Saffrey, M.J., Unger, W.G. & Zhang, S.Q. (1983). Increase in SP-like immunoreactivity in nerve fibres of rabbit iris and ciliary body one to four months following sympathetic denervation. Exp. Eye Res. 37, 191-197.

Collins, P., Chappel, S.P., Griffith, T.M., Lewis, M.J. & Henderson, A.H. (1986). Differences in basal endothelium-derived relaxing factor activity in different artery types. J. Cardiovasc. Pharmacol. 8, 1158-1162.

Collins, S., Caron, M.G. & Lefkowitz, R.J. (1991). Regulation of adrenergic receptor responsiveness through modulation of receptor gene expression. Ann. Rev. Physiol. 53, 497-508.

Comb, M., Birnberg, N.C., Seasholtz, A., Herbert, E. & Goodman, H.M. (1986). A cyclic AMP- and phorbolester-inducible DNA element. Nature 323, 353-355.

Comb, M., Hyman, S.E. & Goodman, H.M. (1987). Mechanisms of trans-synaptic regulation of gene-expression. Trends Neurosci. 10, 473-478.

Comb, M., Mermod, N., Hyman, S.E., Pearlberg, J., Ross, M.E. & Goodman, H.M. (1988). Proteins bound at adjacent DNA elements act synergistically to regulate human proenkephalin cAMP inducible transcription. EMBO J. 7, 3793-3805.

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

Costa, E., Guidotti, A. & Hanbauer, I. (1974). Do cyclic nucleotides promote the transynaptic induction of tyrosine hydroxylase. Life Sci. 14, 1169-1188.

Costa, M. & Furness, J.B. (1973). Observations on the anatomy and

Clark, A.J., Ishii, S., Richert, N., Merlino, G.T. & Postan, I. (1985) Epidermal growth factor regulates the expression of its own receptor. Proc. Natl. Acad. Sci. U. S. A. 82, 8374-8378.

Clark, K.E., Mills, E.G., Stys, S.J. & Seeds, A.E. (1981). Effects of vasoactive polypeptides on uterine vasculature. Am. J. Obstet. Gynecol. 139, 182-188.

Claude, P., Hawrot, E., Dunis, D.A., Campenot, R.B. (1982) Binding, internalization, and retrograde transport of ^{125}I-nerve growth factor in cultured rat sympathetic neurons. J. Neurosci. 2, 431-442.

Cocks, T.M. & Angus, J.A. (1983). Endothelium-dependent relaxation of coronary arteries by noradrenaline and serotonin. Nature 305, 627-630.

Cole, D.F., Bloom, S.R., Burnstock, G., Butler, J.M., McGregor, G.P., Saffrey, M.J., Unger, W.G. & Zhang, S.Q. (1983). Increase in SP-like immunoreactivity in nerve fibres of rabbit iris and ciliary body one to four months following sympathetic denervation. Exp. Eye Res. 37, 191-197.

Collins, P., Chappel, S.P., Griffith, T.M., Lewis, M.J. & Henderson, A.H. (1986). Differences in basal endothelium-derived relaxing factor activity in different artery types. J. Cardiovasc. Pharmacol. 8, 1158-1162.

Collins, S., Caron, M.G. & Lefkowitz, R.J. (1991). Regulation of adrenergic receptor responsiveness through modulation of receptor gene expression. Ann. Rev. Physiol. 53, 497-508.

Comb, M., Birnberg, N.C., Seasholtz, A., Herbert, E. & Goodman, H.M. (1986). A cyclic AMP- and phorbolester-inducible DNA element. Nature 323, 353-355.

Comb, M., Hyman, S.E. & Goodman, H.M. (1987). Mechanisms of trans-synaptic regulation of gene-expression. Trends Neurosci. 10, 473-478.

Comb, M., Mermod, N., Hyman, S.E., Pearlberg, J., Ross, M.E. & Goodman, H.M. (1988). Proteins bound at adjacent DNA elements act synergistically to regulate human proenkephalin cAMP inducible transcription. EMBO J. 7, 3793-3805.

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

Costa, E., Guidotti, A. & Hanbauer, I. (1974). Do cyclic nucleotides promote the transynaptic induction of tyrosine hydroxylase. Life Sci. 14, 1169-1188.

Costa, M. & Furness, J.B. (1973). Observations on the anatomy and

amine histochemistry of the nerves and ganglia which supply the pelvic viscera and on the associated chromaffin tissue in the guinea-pig. Z. Anat. EntwGesch. 140, 85-108.

Costa, M. & Furness, J.B. (1983). The origins, pathways and terminations of neurones with VIP-like immunoreactivity in the guinea-pig small intestine. Neuroscience 8, 665-676.

Costa, M. & Furness, J.B. (1984). Somatostatin is present in a subpopulation of noradrenergic nerve fibres supplying the intestine. Neuroscience 13, 911-919.

Costa, M., Furness, J.B., Pullin, C.O. & Bornstein, J. (1985). Substance P enteric neurons mediate non-cholinergic transmission to the circular muscle of the guinea-pig intestine. Naunyn Schmiedebergs Arch. Pharmacol. 328, 446-453.

Coulombe, J.N. & Bronner-Fraser, M. (1986). Cholinergic neurones acquire adrenergic neurotransmitters when transplanted into an embryo. Nature 324, 569-572.

Coulombe, J.N. & Nishi, R. (1991). Stimulation of somatostatin expression in developing ciliary ganglion neurons by cells of the choroid layer. J. Neurosci. 11, 553-562.

Couture, R., Gandreau, R., St-Pierre, S. & Regoli, D. (1980). The dog common carotid artery: a sensitive bioassay for studying vasodilatory effects of substance P and kinins. Can. J. Physiol. Pharmacol. 58, 1234-1244.

Cowen, T. (1984). An ultrastructural comparison of neuromuscular relationship in blood vessels with functional and "non functional" neuromuscular transmission. J. Neurocytol. 13, 369-392.

Cowen, T. & Burnstock, G. (1986a). Development, aging and plasticity of perivascular autonomic nerves. In: Developmental Neurobiology of the Autonomic Nervous System. Ed. P.M. Gootman, Humana Press, Clifton, NJ. pp. 211-232.

Cowen, T. & Burnstock, G. (1986b). Image analysis of catecholamine fluoresence and immunofluorescence in studies on blood vessel innervation. In: Wenner-Gren International Symposium Series: Vol. 42. Quantitative Neuroanatomy in Transmitter Research. Eds. L.F. Aquati & K. Fuxe, Macmillan, London. pp. 213-229.

Cowen, T., Haven, A.J., Wen-Qin, C., Gallen, D.D., Franc, F. & Burnstock, G. (1982). Development and ageing of perivascular adrenergic nerves in the rabbit. A quantitative fluorescence histochemical study using image analysis. J. Auton. Nerv. Syst. 5, 317-336.

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

Cowen, T., Alafaci, C., Crockard, H.A. & Burnstock, G. (1986). 5-HT-containing nerves to major cerebral arteries of the gerbil originate in the superior cervical ganglia. Brain Res. 384, 51-59.

Cowen, T., Alafaci, C., Crockard, H.A. & Burnstock, G. (1987). Origin and postnatal development of nerves showing 5-hydroxytryptamine-like immunoreactivity supplying major cerebral arteries of the rat. Neurosci. Lett. 78, 121-126.

Crossman, D., Dollery, C.T., MacDermot, J., Macintyre, I. & MacEwan, J. (1987). Effects of human calcitonin gene-related peptide on human endothelial cells. Br. J. Pharmacol. 90, 38P.

Crowe, R. & Burnstock, G. (1988). An increase of vasoactive intestinal polypeptide-, but not neuropeptide Y-, substance P- or catecholamine-containing nerves in the iris of the streptozotocin-induced diabetic rat. Exp. Eye Res. 47, 751-759.

Crowe, R., Lincoln, J., Blacklay, P.F., Pryor, J.P., Lumley, J.S.P. & Burnstock, G. (1983). Vasoactive intestinal polypeptide-like immunoreactive nerves in diabetic penis. A comparison between streptozotocin-treated rats and man. Diabetes 32, 1075-1077.

Crowe, R., Haven, A.J. & Burnstock, G. (1986). Intramural neurons of the guinea-pig urinary bladder: histochemical localization of putative neurotransmitters in cultures and newborn animals. J. Auton. Nerv. Syst. 15, 319-339.

Cuello, A.C.(Ed.) (1982). Co-transmission. Proc. Symp. 50th Anniversary Meet. Brit. Pharmacol. Soc., Oxford. Macmillan Press, London, pp. 264.

Cuello, A.C., Gamse, R., Holzer, P. & Lembeck, F. (1981). Substance P immunoreactive neurons following neonatal administration of capsaicin. Naunyn Schmiedebergs Arch. Pharmacol. 315, 185-194.

D'Orleans-Juste, P., Dion, S., Mizrahi, J. & Regoli, D. (1985). Effects of peptides and non-peptides on isolated arterial smooth muscle: role of endothelium. Eur. J. Pharmacol. 114, 9-21.

Dahlström, A., Nilsson, O., Lundgren, O. & Ahlman, H. (1988). Non-adrenergic, non-cholinergic innervation of gastrointestinal vessels: morphological and physiological aspects. In: Nonadrenergic Innervation of Blood Vessels, Vol. II. Eds. G. Burnstock & S.G. Griffith, CRC Press, Boca Raton, FL. pp. 143-172.

Dahlöf, C., Dahlöf, P. & Lundberg, J.M. (1985a). Neuropeptide Y (NPY): enhancement of blood pressure increase upon α-adrenoceptor activation and direct pressor effects in pithed rats. Eur. J. Pharmacol. 109, 289-292.

Dahlöf, C., Dahlöf, P., Tatemoto, K. & Lundberg, J.M. (1985b). Neuropeptide Y (NPY) reduces field stimulation evoked release of

noradrenaline and enhances force of contraction in the rat portal vein. Naunyn Schmiedebergs Arch. Pharmacol. 328, 327-330.

Dalsgaard, C.J., Franco-Cereceda, A., Saria, A., Lundberg, J.M., Theodorsson-Norheim, E. & Hökfelt, T. (1986). Distribution and origin of substance P- and neuropeptide Y-immunoreactive nerves in the guinea pig heart. Cell Tissue Res. 243, 477-485.

Daly, R. & Hieble, J.P. (1987). Neuropeptide Y modulates adrenergic transmission by an endothelium-dependent mechanism. Eur. J. Pharmacol. 138, 445-446.

Davis, R.L., Weintraub, H. & Lasser, A.B. (1987). Expression of a single transfected cDNA converts fibroblasts to myoblasts. Cell 51, 987-1000.

Day, S.M.G.J., Polak, J.M. & Bloom, S.R. (1985). Somatostatin in the human heart and comparison with guinea pig and rat heart. Br. Heart J. 53, 153-157.

De Camilli, P. & Jahn, R. (1990). Pathways to regulated exocytosis in neurons. Ann. Rev. Physiol. 52, 625-645.

De la Torre, J.C. & Surgeon, J.W. (1976). A morphological approach to rapid and sensitive monoamine histofluorescence using a modified glyoxylic acid technique: the SPG method. Histochemistry 49, 81-93.

De Mey, J.G. & Vanhoutte, P.M. (1981). Role of the intima in cholinergic and purinergic relaxation of isolated canine femoral arteries. J. Physiol. (Lond.) 316, 347-355.

De Mey, J.G. & Vanhoutte, P.M. (1983). Anoxia and endothelium-dependent reactivity of the canine femoral artery. J. Physiol. (Lond.) 335, 65-74.

Deacon, C.F., Agoston, D.V. & Conlon, J.M. (1986). Vesicular colocalization of substance P and neurokinin A in neurons of the guinea-pig myenteric plexus. In: Joint Meet. Br. Endocrinol. Soc., 7-10 April 1986, 53P

Delgado, T.J., Brismar, J. & Svendgaard, N.A. (1985). Subarachnoid haemorrhage in the rat: angiography and fluorescent microscopy of the major cerebral arteries. Stroke 16, 595-601.

Della, N.G., Papka, R.E., Furness, J.B. & Costa, M. (1983). Vasoactive intestinal peptide-like immunoreactivity in nerves associated with the cardiovascular system of guinea pigs. Neuroscience 9, 605-610.

Dey, R.D., Shannon, M.A. & Said, S.I. (1981). Localisation of VIP-immunoreactive nerves in airways and pulmonary vessels of dogs, cats and human subjects. Cell Tissue Res. 220, 231-238.

Dhall, U., Cowen, T., Haven, A.J. & Burnstock, G. (1986).

Perivascular noradrenergic and peptide-containing nerves show different patterns of changes during development and ageing in the guinea-pig. J. Auton. Nerv. Syst. 16, 109-126.

Dhital, K.K. & Burnstock, G. (1987). Contrôle neurogène adrénergique et non-adrénergique de la paroi artérielle. In: Les Maladies de la Paroi Artérielle. Eds. J-.P. Camilleri, C.L. Berry, J.-N. Fiessinger & J. Bariéty, Flammarion, Paris. pp. 77-105.

Dhital, K.K., Gerli, R., Lincoln, J., Milner, P., Tanganelli, P., Weber, G., Fruschelli, C. & Burnstock, G. (1988). Increased density of perivascular nerves to the major cerebral vessels of the spontaneously hypertensive rat: differential changes in noradrenaline and neuropeptide Y during development. Brain Res. 444, 33-45.

Diani, A.R., Peterson, T., Sawada, G.A., Wise, B.M., Blanks, M.C., Gerritsen, G.C., Terenghi, G., Varndell, I.M., Polak, J.M., Blank, M.A. & Bloom, S.R. (1985). Elevated level of vasoactive intestinal peptide in the eye and urinary bladder of diabetic and prediabetic clinical hamster. Diabetologia 28, 302-307.

Dimitriadou, V., Aubineau, P., Taxi, J. & Seylaz, J. (1988). Ultrastructural changes in the cerebral artery wall induced by long-term sympathetic denervation. Blood Vessels 25, 122-143.

Dolezel, S., Gerova, M. & Gero, J. (1974). Postnatal development of sympathetic innervation in skeletal muscle of the dog. Physiol. Bohemoslov. 23, 138-139.

Draper, R.L. (1920). The prenatal growth of the guinea pig. Anat. Rec. 18, 369-392.

Duckles, S.P. (1983). Age-related changes in adrenergic neuronal function of rabbit vascular smooth muscle. Neurobiol. Aging 4, 151-156.

Duckles, S.P. & Buck, S.M. (1982). Substance P in the cerebral vasculature: depletion by capsaicin suggests a sensory role. Brain Res. 245, 171-174.

Duckles, S.P., Carter, B.J. & Williams, C.L. (1985). Vascular adrenergic neuroeffector function does not decline in aged rats. Circ. Res. 56, 109-116.

Duckles, S.P. & Said, S.I. (1982). Vasoactive intestinal peptide as a neurotransmitter in the cerebral circulation. Eur. J. Pharmacol. 78, 371-374.

Dumoulin, F.L., Raivich, G., Streit, W.J. & Kreutzberg, G.W. (1991). Differential regulation of calcitonin gene-related peptide (CGRP) in regenerating rat facial nucleus and dorsal root ganglion. Eur. J. Neurosci. 3, 338-342.

Dunnet, C.W. (1964). New table for multiple comparison with a

control. Biometrics 20, 482-491.

Dwarki, V.J., Montminy, M. & Verma, I.M. (1990). Both the basic region and the "leucin zipper" domain of the cyclic AMP response element binding (CREB) protein are essential for transcriptional activation. EMBO J. 9, 225-232.

Dynam, W.S. & Tjian, R. (1985). Control of eukaryotic messenger RNA synthesis by sequence- specific DNA-binding proteins. Nature 316, 774-778.

Edvinsson, L. (1982). Sympathetic control of cerebral circulation. Trends Neurosci. 5, 425-428.

Edvinsson, L. (1985). Characterization of the contractile effect of neuropeptide Y in feline cerebral arteries. Acta Physiol. Scand. 125, 33-41.

Edvinsson, L. & Uddman, R. (1982). Immunohistochemical localization and dilatory effect of substance P on human cerebral vessels. Brain Res. 232, 466-471.

Edvinsson, L. & Ekman, R. (1984). Distribution and dilatory effect of vasoactive intestinal polypeptide (VIP) in human cerebral arteries. Peptides 5, 329-341.

Edvinsson, L. & McCulloch, J. (1985). Distribution and vasomotor effects of peptide HI (PHI) in feline cerebral blood vessels in vitro and in situ. Regul. Pept. 10, 345-356.

Edvinsson, L. & Jansen, I. (1987). Characterization of tachykinin receptors in isolated basilar arteries of guinea-pig. Br. J. Pharmacol. 90, 553-559.

Edvinsson, L. & Uddman, R. (1988). Vasoactive intestinal polypeptide (VIP): a putative neurotransmitter in the cardiovascular system. In: Nonadrenergic Innervation of Blood Vessels, Vol. I. Eds. G. Burnstock & S.G. Griffith, CRC Press, Boca Raton. pp. 101-125.

Edvinsson, L., Hardebo, J.E. & Owman, C. (1978). Influence of the cerebrovascular innervation of regional blood flow, autoregulation and blood-brain barrier function. In: Cerebral Vascular Smooth Muscle and its Control. Ciba Foundation Symposium No. 56. North Holland/ Elsevier Excerpta Medica, Amsterdam. pp. 69-86.

Edvinsson, L., Fahrenkrug, J., Hanko, J., Owman, C., Sundler, F. & Uddman, R. (1980a). VIP (vasoactive intestinal polypeptide)-containing nerves of intracranial arteries in mammals. Cell Tissue Res. 248, 135-142.

Edvinsson, L., McCulloch, J. & Uddman, R. (1981). Substance P: immunohistochemical localization and effect upon cat pial arteries in vitro and in situ. J. Physiol. (Lond.) 318, 251-258.

Edvinsson, L., Egund, N., Owman, C., Sahlin, L.H. & Svendgaard, N.A. (1982). Reduced noradrenaline uptake and retention in cerebrovascular nerves associated with angiographically visible vasoconstriction following experimental subarachnoid haemorrhage in rabbits. Brain Res. Bull. 9, 799-805.

Edvinsson, L., Emson, P.C., McCulloch, J., Tatemoto, K. & Uddman, R. (1983). Neuropeptide Y: cerebrovascular innervation and vasomotor effects in the cat. Neurosci. Lett. 43, 79-84.

Edvinsson, L., Ekblad, E., Håkanson, R. & Wahlestedt, C. (1984). Neuropeptide Y potentiates the effect of various vasoconstrictor agents on rabbit blood vessels. Br. J. Pharmacol. 83, 519-525.

Edvinsson, L., Edman, R., Ottoson, A. & Uddman, R. (1985a). Distribution, concentration and effects of neuropeptide Y (NPY), substance P (SP) and vasoactive intestinal polypeptide (VIP) in human cerebral blood vessels. J. Cereb. Blood Flow Metab. 5, Suppl. 1, S545-S546.

Edvinsson, L., Håkanson, R., Steen, S., Sundler, F., Uddman, R. & Wahlestedt, C. (1985b). Innervation of human omental arteries and veins and vasomotor responses to noradrenaline, neuropeptide Y, substance P and vasoactive intestinal polypeptide. Regul. Pept. 12, 67-79.

Edvinsson, L., Fredholm, B.B., Hamel, E., Jansen, I. & Verrecchia, C. (1985c). Perivascular peptides relax cerebral arteries concomitant with stimulation of cyclic adenosine monophosphate accumulation or release of an endothelium-derived relaxing factor in the cat. Neurosci. Lett. 58, 213-217.

Edvinsson, L., Edman, R. & Ottoson, A. (1986). Demonstration of perivascular peptides and changes in concentration with age in man. Gerontology 32, 50-52.

Edvinsson, L., Ekman, R., Jansen, I., McCulloch, J. & Uddman, R. (1987). Calcitonin gene-related peptide and cerebral vessels: distribution and vasomotor effects. J. Cereb. Blood Flow Metab. 7, 720-728.

Edvinsson, L., Copeland, J.R., Emson, P.C., McCulloch, J. & Uddman, R. (1988). Nerve fibres containing neuropeptide Y in the cerebrovascular bed: immunocytochemistry, radioimmunoassay and vasomotor effects. J. Cereb. Blood Flow Metab. 7, 45-47.

Ekblad, E., Edvinsson, L., Wahlestedt, C., Uddman, R., Håkanson, R. & Sundler, F. (1984). Neuropeptide Y co-exists and co-operates with noradrenaline in perivascular nerve fibres. Regul. Pept. 8, 225-235.

Ekblad, E., Rökaeus, A., Håkanson, R. & Sundler, F. (1985). Galanin nerve fibres in the rat gut: distribution, origin and projections. Neuroscience 16, 355-365.

Ekström, J., Ekman, R., Håkanson, R., Sjögren, C. & Sundler, F. (1988). Calcitonin gene-related peptides in rat salivary glands: neuronal localization, depletion upon nerve stimulation and effects on salivation in relation to substance P. Neuroscience 26, 933-949.

Ernfors, P., Ibàñer, C.F., Ebendal, T., Olson, L. & Persson, H. (1990). Molecular cloning and neurotrophic activities of a protein with structural similarities to nerve growth factor: developmental and topographical expression in the brain. Proc. Natl. Acad. Sci. U. S. A. 87, 5454-5458.

Ernsberg, U., Sendtner, M. & Rohrer, H. (1989). Proliferation and differentiation of embryonic chick sympathetic neurons: effects of ciliary neurotrophic factor. Neuron 2, 1275-1284.

Esparmer, V., Melchiorri, P. & Sopranzi, N. (1972). The action of bombesin on the systemic arterial blood pressure of some experimental animals. Br. J. Pharmacol. 45, 442-450.

Ewald, D.A., Sterweis, P.C., Miller, R.J. (1988). Guanine nucleotide binding protein G 6 induced coupling of neuropeptide Y receptors to Ca^{2+} channels in sensory neurones. Natl. Acad. Sci. U.S.A. 85, 3633-3637

Fallgren, B., Bjellin, L. & Edvinsson, L. (1988). Effect of pregnancy and sex-steroids on α-adrenoceptor mechanisms in the guinea-pig uterine vascular bed. Pharmacol. Toxicol. 63, 375-381.

Faucon-Biguet, N., Rittenhouse, A.R., Mallet, J. & Zigmond, R.E. (1989). Preganglionic nerve stimulation increases mRNA levels for tyrosine hydroxylase in the rat superior cervical ganglion. Neurosci. Lett.104, 189-194

Fehér, E. & Burnstock, G. (1988). Galanin-like immunoreactive nerve elements in the small intestine of the rat. An electron microscopic immunocytochemical study. Neurosci. Lett. 92, 137-142.

Feng, Z.P., Dryden, W.F. & Gordon, T. (1989). Postnatal development of adrenergic responsiveness in rabbit heart. Can. J. Physiol. Pharmacol. 67, 883-889.

Ferrante, R.J., Kowall, N.W., Beal, M.F., Richardson, E.P., Jr., Bird, E.D. & Martin, J.B. (1985). Selective sparing of a class of striatal neurons in Huntington's disease. Science 230, 561-564.

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

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

Foote, J., Robinson, J. & Scott, T.M. (1986). A comparison of

substance P innervation of the mesenteric vascular bed in hypertensive and normotensive rats. J. Physiol. (Lond.) 377, 55P.

Forssman, B.B., Hock, D. & Metz, J. (1982). Peptidergic innervation of the kidney. Neurosci. Lett. Suppl. 10, S183.

Franco-Cereceda, A., Lundberg, J.M. & Dahlöf, C. (1985). Neuropeptide Y and sympathetic control of heart contractility and coronary vascular tone. Acta Physiol. Scand. 124, 361-369.

Franco-Cereceda, A., Henke, H., Lundberg, J.M., Petermann, J.B., Hökfelt, T. & Fischer, J.A. (1987). Calcitonin gene-related peptide (CGRP) in capsaicin-sensitive substance P-immunoreactive sensory neurons in animals and man: distribution and release by capsaicin. Peptides 8, 399-410.

Freidin, M. & Kessler, J.A. (1991). Cytokine regulation of substance P expression in sympathetic neurons. Proc. Natl. Acad. Sci. U. S. A. 88, 3200-3203.

Fried, G., Terenius, L., Hökfelt, T. & Goldstein, M. (1985a). Evidence for differential localization of noradrenaline and neuropeptide Y in neuronal storage vesicles isolated from rat vas deferens. J. Neurosci. 5, 450-458.

Fried, G., Hökfelt, T., Terenius, L. & Goldstein, M. (1985b). Neuropeptide Y (NPY)-like immunoreactivity in guinea pig uterus is reduced during pregnancy in parallel with noradrenergic nerves. Histochemistry 83, 437-442.

Fried, G., Lundberg, J.M. & Theodorsson-Norheim, E. (1985c). Subcellular storage and axonal transport of neuropeptide Y (NPY) in relation to catecholamines in the cat. Acta Physiol. Scand. 125, 145-152.

Fronek, K. & Alexander, M. (1986). Sympathetic activity, lipids accumulation and arterial wall morphology in rabbits at high altitude. Am. J. Physiol. 250, R485-R492.

Fuji, K., Senba, E., Ueda, Y. & Tohyama, M. (1983). Vasoactive intestinal polypeptide (VIP) containing neurons in the spinal cord of the rat and their projections. Neurosci. Lett. 37, 51-55.

Fukuda, S., Su, C. & Lee, T.J.-F. (1986). Mechanisms of extraneuronal serotonin uptake in the rat aorta. J. Pharmacol. Exp. Ther. 232, 264-269.

Fuller, R.W., Conradson, T.B., Dixon, C.MS., Crossman, D.C. & Barnes, P.J. (1987). Sensory neuropeptide effects in human skin. Br. J. Pharmacol. 92, 781-788.

Furchgott, R.F. (1984). Role of endothelium in responses of vascular smooth muscle. In: Frontiers in Physiological Research. Eds. D.G. Garlick & P.I. Korner, Cambridge University Press, Cambridge. pp.

116-133.

Furchgott, R.F. & Zawadski, J.V. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature 288, 373-376.

Furchgott, R.F., Cherry, P.D., Zawadski, J.V. & Jothianandon, D. (1984). Endothelial cells as mediators of vasodilation of arteries. J. Cardiovasc. Pharmacol. 6, 336-343.

Furness, J.B. & Costa, M. (1982). Identification of gastrointestinal neurotransmitters. In: Handbook of Experimental Pharmacology, Vol. 59. Ed. G. Bertaccini, Springer, Berlin. pp. 383-460.

Furness, J.B. & Costa, M. (1987). The Enteric Nervous System. Churchill Livingstone, Edinburgh.

Furness, J.B., Costa, M., Franco, R. & Llewellyn, S.I.J. (1980). Neuronal peptides in the intestine: distribution and possible functions. In: Neural Peptides and Neural Communications. Eds. M. Costa & M. Trabucchi, Raven Press, New York. pp. 607-617.

Furness, J.B., Papka, R.E., Della, N.G., Costa, M. & Eskay, R.L. (1982). Substance P-like immunoreactivity in nerves associated with the vascular system of guinea-pigs. Neuroscience 7, 447-459.

Furukawa, S. & Furukawa, Y. (1990). Nerve growth factor synthesis and its regulatory mechanisms: an approach to therapeutic induction of nerve growth factor synthesis. Cerebrovasc. Brain Metab. Rev. 2, 328-344.

Fyffe, R.E.W. & Perl, E.R. (1984). Is ATP a central synaptic mediator for certain primary afferent fibres from mammalian skin?. Proc. Natl. Acad. Sci. U. S. A. 81, 6890-6893.

Förstermann, U. & Dudel, C. (1988). Endothelium-dependent dilation of resistance arteries in vivo. Blood Vessels 25, 35-37.

Förstermann, U., Hertting, G. & Neufang, B. (1986). The role of endothelial and non-endothelial prostaglandins in the relaxation of isolated blood vessels of the rabbit induced by acetylcholine and bradykinin. Br. J. Pharmacol. 87, 521-532.

Gabella, G. (1981). Structure of smooth muscles. In: Smooth Muscle: An Assessment of Current Knowledge. Eds. E. Bülbring, A.F. Brading, A.W. Jones & T. Tomita, Edward Arnold, London. pp. 1-46.

Gaddis, R.R. & Dixon, W.R. (1982). Modulation of peripheral adrenergic neurotransmission by methionine-enkephalin. J. Pharmacol. Exp. Ther. 221, 282-288.

Gallen, D.D., Cowen, T., Griffith, S.G., Haven, A.J. & Burnstock, G. (1982). Functional and non-functional nerve-smooth muscle transmission in the renal arteries of the newborn and adult rabbit

and guinea-pig. Blood Vessels 19, 237-246.

Galligan, J.J., Costa, M. & Furness, J.B. (1988). Changes in surviving nerve fibres associated with submucosal arteries following extrinsic denervation of the small intestine. Cell Tissue Res. 253, 647-656.

Gallyas, F., Görcs, T. & Merchenthaler, I. (1982). High-grade intensification of the end-product of the diaminobenzidine reaction for peroxydase histochemistry. J. Histochem. Cytochem. 30, 183-191.

Galper, J.B., Dziekan, L.C. & Smith, T.W. (1984). The development of physiological responsiveness to muscarinic agonists in chick embryo heart cultures. J. Biol. Chem. 259, 7382-7739.

Galper, J.P., Klein, W.L. & Caterall, W.A. (1977). Muscarinic acetylcholine receptors in developing chick heart. J. Biol. Chem. 252, 8692-8699.

Gamse, R., Lembeck, F. & Cuello, A. (1979). Substance P in the vagus nerve. Immunochemical and immunohistochemical evidence for axoplasmic transport. Naunyn Schmiedebergs Arch. Pharmacol. 306, 37-44.

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

Gandreau, P., Barabé, J., St-Pierre, S. & Regoli, D. (1981). Pharmacological studies of kinins on venous smooth muscle. Can. J. Physiol. Pharmacol. 59, 371-379.

Ganz, P., Sanrock, A.W., Landis, S.C., Leopold, J., Gimbrone, M.A., Jr. & Alexander, R.W. (1986). Vasoactive intestinal polypeptide: vasodilation and cyclic AMP generation. Am. J. Physiol. 250, H755-H760.

Gazelius, B., Brodin, E., Olgart, L. & Panopoulus, P. (1981). Evidence that substance P is a mediator of antidromic vasodilation using somatostatin as a release inhibitor. Acta Physiol. Scand. 113, 155-159.

Genest, J. (1986). The atrial natriuretic factor. Br. Heart J.56, 302-316.

Geppetti, P., De Rossi, M., Mione, M.C., Renzi, D. & Amenta, F. (1988). Age-related changes in vasoactive intestinal polypeptide levels and distribution in the rat lung. J. Neural Trans. 74, 1-10.

Gering, D.P., Gough, N.M., King, J.A., Hilton, D.J., Nicola, N.A., Simpson, R.J., Nice, E.C., Kelso, A. & Metcalf, D. (1987). Molecular cloning and expression of cDNA encoding a murine myeloid leukemia inhibitory factor (LIF). EMBO J. 6, 3995-4002.

Gerschenfeld, H.M., Tramezzani, J.H. & De Robertis, E. (1960).

Ultrastructure and function in neurohypophysis of the toad. Endocrinology 66, 741-762.

Gershon, M.D. (1981). The enteric nervous system. Ann. Rev. Neurosci. 5, 1-20.

Gerstheimer, F.P. & Metz, J. (1986). Distribution of calcitonin gene-related peptide immunoreactivity in the guinea pig heart. Anat. Embryol. 175, 255-260.

Giaid, A., Gibson, S.J., Ibrahim, N.B.N., Legon, S., Bloom, S.R., Yanagisawa, M., Masaki, T., Varndell, I.M. & Polak, J.M. (1989). Endothelin 1, and endothelium derived peptide, is expressed in neurones of the human spinal cord and dorsal root ganglia. Proc. Natl. Acad. Sci. U. S. A.86, 7634-7638

Gibbins, I.L. & Morris, J.L. (1987). Co-existence of neuropeptides in sympathetic, cranial autonomic and sensory neurons innervating the iris of the guinea-pig. J. Auton. Nerv. Syst. 21, 67-82.

Gibbins, I.L. & Morris, J.L. (1988). Co-existence of immunoreactivity to neuropeptide Y and vasoactive intestinal polypeptide in non-adrenergic axons innervating guinea-pig cerebral arteries after sympathectomy. Brain Res. 444, 402-406.

Gibbins, I.L., Brayden, J.E. & Bevan, J.A. (1984a). Distribution and origins of VIP immunoreactive nerves in the cephalic circulation of the cat. Peptides 5, 209-212.

Gibbins, I.L., Brayden, J.E. & Bevan, J.A. (1984b). Perivascular nerves with immunoreactivity to vasoactive intestinal polypeptide in cephalic arteries of the cat: distribution, possible origin and functional implications. Neuroscience 13, 1327-1346.

Gibbins, I.L., Furness, J.B., Costa, M., Macintyre, I., Hillyard, C.J. & Girgis, S. (1985). Co-localization of calcitonin gene-related peptide-like immunoreactivity with substance P in cutaneous, vascular and visceral sensory neurons of guinea-pigs. Neurosci. Lett. 57, 125-130.

Gibbins, I.L., Furness, J.B. & Costa, M. (1987). Pathway-specific patterns of the coexistence of substance P, calcitonin gene-related peptide, cholecystokinin and dynorphin in neurons of the dorsal root ganglia of the guinea-pig. Cell Tissue Res. 248, 417-437.

Gibbins, I.L., Morris, J.L., Furness, J.B. & Costa, M. (1988). Innervation of systemic blood vessels. In: Non-adrenergic Innervation of Blood Vessels. Eds. G. Burnstock & S.G. Griffith, Raven Press, Boca Raton,FL. pp. 1-36.

Gibson, S.J., Polak, J.M., Bloom, S.R. & Wall, P.D. (1981). The distribution of nine peptides in the rat spinal cord with special

emphasis on the substantia gelatinosa and on the area around the central canal (lamina X). J. Comp. Neurol. 201, 65-79.

Gibson, S.J., Polak, J.M., Bloom, S.R., Sabate, I.M., Mulderry, P.K., Ghatei, M.A., McGregor, G.P., Morrison, J.E.B., Kelly, J.S., Evans, R.M. & Rosenfeld, M.G. (1984). Calcitonin gene-related peptide immunoreactivity in the spinal cord of man and eight other species. J. Neurosci. 4, 3101-3111.

Gibson, S.J., McCrossan, M.V. & Polak, J.M. (1985). A subpopulation of calcitonin gene-related peptide (CGRP) immunoreactive neurons in the dorsal root ganglia also display substance P, somatostatin or galanin immunoreactivity. Proc. XII. Int. Anat. Congr. A232.

Gilbert, R.FT., Emson, P.C., Fahrenkrug, J., Lee, C.M., Penman, E. & Wass, J. (1980). Axonal transport of neuropeptides in the cervical vagus nerve of the rat. J. Neurochem. 34, 108-113.

Gillespie, M.N., Owasoyo, J.O., McMurtry, I.F. & O'Brien, R.F. (1986). Sustained coronary vasoconstriction provoked by a peptidergic substance released from endothelial cells in culture. J. Pharmacol. Exp. Ther. 236, 339-343.

Girgis, S.I., MacDonald, D.W., Stevenson, J.C., Bevis, P.J.R., Lynch, C., Wimalawansa, S.J., Morris, H.R., MacIntyre, I. (1985). Calcitonin gene-related peptide: potent vasodilator and major product of calcitonin gene. Lancet 2, 14-16

Glembotski, C.C., Wildey, G.M. & Gibson, T.R. (1985). Molecular forms of immunoreactive atrial natriuretic peptide in the rat hypothalamus and atrium. Biochem. Biophys. Res. Commun. 129, 671-678.

Glover, W.E. (1985). Increased sensitivity of rabbit ear artery to noradrenaline following perivascular nerve stimulation may be a response to neuropeptide Y released as a cotransmitter. Clin. Exp. Pharmacol. Physiol. 12, 227-230.

Goehler, L.E., Sternini, C. & Brecha, N.C. (1988). Calcitonin gene-related peptide immunoreactivity in the biliary pathway and liver of the guinea-pig: distribution and colocalization with substance P. Cell Tissue Res. 253, 145-150.

Goelet, P., Castellucci, V.F., Schacher, S. & Kandel, E.R. (1986). The long and the short of long-term memory - A molecular framework. Nature 322, 419-422.

Gonzales, G.A. & Montminy, M.R. (1989). Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. Cell 59, 675-680.

Gordon, J.C. & Rowland, H.C. (1990). Nerve growth factor induces $5HT_3$ recognition sites in rat pheochromocytoma (PC12) cells. Life Sci. 46, 1435-1442.

Gordon, J.L. (1986). Extracellular ATP: effects, sources and fate. Biochem. J. 233, 309-319.

Grace, G.C., Dusting, G.J., Kemp, B.E. & Martin, T.J. (1987). Endothelium and the vasodilator action of rat calcitonin gene-related peptide (CGRP). Br. J. Pharmacol. 91, 729-733.

Graham, J.D.P., Lever, J.D. & Spriggs, T.L.B. (1968). An examination of adrenergic axons around pancreatic arterioles of the cat for the presence of acetylcholinesterase by high resolution autoradiographic and histochemical methods. Br. J. Pharmacol. Chemother. 33, 15-20.

Gram, B.R. & Ottesen, B. (1982). Increased myometrial blood flow evoked by substance P. Pflugers Arch. 395, 347-350.

Grant, M. & Landis, S.C. (1988). Expression of muscarinic cholinergic receptors in rat sweat glands. Soc. Neurosci. Abstr. 17, 429-429.

Grant, M. & Landis, S.C. (1989). Nerve-target interactions in the developing sympathetic nervous system: development of an m3 muscarinic receptor. Soc. Neurosci. Abstr. 18, 1329-1329.

Grant, M.P. & Landis, S.C. (1991). Unexpected plasticity at autonomic junctions. Environmental regulation of neurotransmitter phenotype and receptor expression. Biochem. Pharmacol. 41, 323-331.

Green, N.M. (1966). Thermodynamics of the binding of biotin and some analogues by avidin. Biochem. J. 101, 774-780.

Greenberg, B., Rhoden, K. & Barnes, P.J. (1987). Relaxant effects of vasoactive intestinal peptide and peptide histidine isoleucine in human and bovine pulmonary arteries. Blood Vessels 24, 45-50.

Greenberg, M.E., Greene, L.A. & Ziff, E.B. (1985). Nerve growth factor and epidermal growth factor induce rapid transient changes in proto-oncogene transcription in PC12 cells. J. Biol. Chem. 260, 14101-14110.

Greenberg, M.E., Ziff, E.B. & Greene, L.A. (1986). Stimulation of neuronal acetylcholine receptors induces rapid gene transcription. Science 234, 80-83.

Greenberg, M.M., Leitao, C., Tragadis, J. & Stevens, J.K. (1990). Irregular geometries in normal unmyelinated axons: a 3D serial EM analysis. J. Neurocytol. 20, 978-988.

Greene, L.A. & Tischler, A.S. (1982). PC 12 pheochromocytoma cultures in neurobiological research. Adv. Cell Neurobiol. 3, 373-414.

Griffith, T.M., Edwards, D.H., Lewis, M.J. & Henderson, A.H. (1985). Evidence that cyclic guanosine monophosphate (cGMP) mediates endothelium-dependent relaxation. Eur. J. Pharmacol. 112, 195-202.

Grunditz, T., Ekman, R., Håkanson, R., Sundler, F. & Uddman, R.

(1988). Neuropeptide Y and vasoactive intestinal polypeptide coexist in rat thyroid nerve fibers emanating from the thyroid ganglion. Regul. Pept. 23, 193-208.

Gu, J., Huang, W.M., Blank, M., Morrison, J.F.B., Bloom, S.R. & Polak, J.M. (1983). Measurement of VIP and origin of its innervation in the rat urinary bladder. Regul. Pept. 6, 305.

Gu, J., Polak, J.M., Su, H.C., Blank, M.A., Morrison, J.F.B. & Bloom, S.R. (1984). Demonstration of paracervical ganglion origin for the vasoactive intestinal polypeptide-containing nerves of the rat uterus using retrograde tracing techniques combined with immunocytochemistry and denervation procedures. Neurosci. Lett. 51, 377-382.

Gu, Y. & Hall, Z.W. (1988). Immunological evidence for a change in subunits of the acetylcholine receptor in developing and denervated rat muscle. Neuron 1, 117-125.

Guastella, J., Nelson, N., Nelson, H., Czyk, L., Keynan, S., Miedel, M.C., Davidson, N., Lester, H.A. & Kanner, B.B.I. (1990). Cloning and expression of rat brain GABA transporter. Science 249, 1303-1306.

Guillemin, G. (1976). Somatostatin inhibits the release of acetylcholine induced electrically in the myenteric plexus. Endocrinology 99, 1653-1654.

Gulati, N., Huggel, H. & Gulati, O.P. (1983). Modulatory effects of substance P on norepinephrine induced vasoconstrictor response in the rat mesentery. Gen. Pharmacol. 14, 157-160.

Gulbenkian, S., Wharton, J., Hodson, N.P., Varndell, I.M., Bloom, S.R. & Polak, J.M. (1985). Colocalization of neuropeptide tyrosine (NPY) and its C-terminal flanking peptide (C-PON). Peptides 6, 1237-1243.

Gulbenkian, S., Merighi, A., Wharton, J., Varndell, I.M. & Polak, J.M. (1986). Ultrastructural evidence for the coexistence of calcitonin gene-related peptide and substance P in secretory vesicles of peripheral nerves in the guinea pig. J. Neurocytol. 15, 535-542.

Hadcock, J.R. & Malbom, C.C. (1991). Regulation of receptor expression by agonists: transcriptional and post-transcriptional controls. Trends Neurosci. 14, 242-224.

Hadley, R.D. (1990). Innervation and geometry of rabbit ciliary ganglion cells after preganglionic nerve regeneration. J. Comp. Neurol. 295, 268-276.

Hajdu, M.A., Heistad, D.D., Siems, J.E. & Baumbach, G.L. (1990). Effects of aging on mechanics and composition of cerebral arterioles in rats. Circ. Res. 66, 1747-1754.

Halvorson, S.W. & Nathanson, N.M. (1984). Ontogenesis of physiological responsiveness and guanine nucleotide sensitivity of

cardiac muscarinic receptors during chick embrionic development. Biochemistry 23, 5813-5821.

Hamburger, V. & Levi-Montalcini, R. (1949). Proliferation, differentiation and degeneration in the spinal ganglia of the chick embryo under normal and experimental conditions. J. Exp. Zool. 111, 457-501.

Hamel, E., Assumel-Lurdin, C., Edvinsson, L., Fage, D. & MacKenzie, E.T. (1987). Neuronal versus endothelial origin of vasoactive acetylcholine in pial vessels. Brain Res. 420, 391-396.

Hanko, J., Hardebo, J.E. & Owman, C. (1981). Effects of various neuropeptides on cerebral blood vessels. J. Cereb. Blood Flow Metab. 1, S346-S347.

Hanko, J., Hardebo, J.E. & Owman, C. (1982). Effects of various neuropeptides on cerebral blood vessels. In: Cerebral Blood Flow: Effects of Nerves and Neurotranmsitters. Eds. D.D. Heistad & M.L. Marcus, Elsevier, Amsterdam. pp. 227-234.

Hanko, J., Hardebo, J.E., Kåhrström, J., Owman, C. & Sundler, F. (1985). Calcitonin gene-related peptide is present in mammalian cerebrovascular nerve fibres and dilates pial and peripheral arteries. Neurosci. Lett. 57, 91-95.

Hanko, J.H., Törnebrandt, K., Hardebo, J.E., Kåhrström, J., Noblett, H.R. & Owman, C. (1986). Neuropeptide Y induces and modulates vasoconstriction in intracranial and peripheral vessels of animals and man. J. Auton. Pharmacol. 6, 117-124.

Hanley, M.MR., Benton, H.P., Lightman, S.L., Todd, K., Bone, E.A., Fretten, P., Palmer, S., Kir, L.J. & Mitchell, R.H. (1984). A vasopressin-like peptide in the mammalian sympathetic nervous system. Nature 309, 258-261.

Hanley, M.R. (1985). Neuropeptides as mitogens. Nature 315, 14-15.

Hara, H., Hamill, G.S. & Jacobowitz, D.M. (1985). Origin of cholinergic nerves to the rat major cerebral arteries: coexistence with vasoactive intestinal polypeptide. Brain Res. Bull. 14, 179-188.

Hara, H., Kobayashi, S., Takema, T., Sugita, K., Weir, B. & Jacobowitz, D.M. (1987). Origin of cerebral perivascular nerves containing acetylcholinesterase and vasoactive intestinal polypeptide. J. Cereb. Blood Flow Metab. 7, Suppl. 1, S247.

Hardebo, J.E., Hanko, J., Kåhrström, J. & Owman, C. (1985). Endothelium-dependent relaxation in cerebral arteries. J. Cereb. Blood Flow Metab. 5, S533-S534.

Harmar, A., Schofield, J.G. & Keen, P. (1981). Substance P biosynthesis in dorsal root ganglia. An immunochemical study of [^{38}S]methionine and [^{3}H]proline incorporation in vitro. Neuroscience

6, 1917-1922.

Harrington, C., Lewis, E., Krzemien, D. & Chikaraishi, D. (1987). Identification and cell type specificity of the tyrosine hydroxylase gene promoter. Nucleic Acid Res. 15, 2363-2384.

Harrison, D.G. (1988). Atherosclerosis alters endothelium-dependent responses to a variety of neurohumoral agents: potential mechanisms and pathophysiological consequences. In: Vasodiation, Vascular Smooth Muscle, Peptides, Autonomic Nerves and Endothelium. Ed. P.M. Vanhoutte, Raven Press, New York. pp. 2071-2076.

Hassall, C.J.S. & Burnstock, G. (1987). Immunocytochemical localisation of neuropeptide Y and 5-hydroxytryptamine in a subpopulation of amine-handling intracardiac neurones that do not contain dopamine β-hydroxylase in tissue culture. Brain Res. 422, 74-82.

Hedge, G.A., Huffman, L.J., Grunditz, L.J. & Sundler, F. (1984). Immunocytochemical studies of the peptidergic innervation of the thyroid gland in the Brattleboro rat. Endocrinology 115, 2071-2076.

Helke, C.J. & Niederer, A. (1990). Studies on the coexistence of substance P with other putative transmitters in the nodose and petrosal ganglia. Synapse 5, 144-151.

Helke, C.J., O'Donohue, T.L. & Jacobowitz, D.M. (1980). Substance P as a baro- and chemoreceptor afferent neurotransmitter: immunocytochemical and neurochemical evidence in the rat. Peptides 1, 1-9.

Helke, C.J., Charlton, C.G. & Wiley, R.G. (1986). Studies on the cellular localization of spinal cord substance P receptors. Neuroscience 19, 523-533.

Helps, S. & Reilly, P. (1985). Cerebral blood flow response to carbon dioxide after capsaicin treatment. J. Cereb. Blood Flow Metab. 5, S503-S504.

Hempstead, B.L., Martin-Zanca, D., Kaplan, D.R., Parada, L.F. & Chao, M.V. (1991). High-affinity NGF binding requires coexpression of the TRK proto-oncogene and the low-affinity NGF receptor. Nature 350, 678-683.

Hendry, I.A. (1977a). Cell division in the developing sympathetic nervous system. J. Neurocytol. 6, 299-309.

Hendry, I.A. (1977b) The effect of the retrograde axonal transport of nerve growth factor on the morphology of adrenergic neurones. Brain Res. 134, 213-223

Hendry, I.A., Stockel, K., Thoenen, H. & Iversen, L.L. (1974). The retrograde axonal transport of nerve growth factor. Brain Res. 68, 103-121.

Hendry, S.HC., Jones, E.G. & Beifeld, M.C. (1983). Cholecystokinin-immunoreactive neurons in rat and monkey cerebral cortex make symmetric synapse and have intimate association with blood vessels. Proc. Natl. Acad. Sci. U. S. A. 80, 2400-2404.

Hengerer, B., Lindholm, D., Henman, R., Rüther, V., Wagner, E.F. & Thoenen, H. (1990). Lesion-induced increase in nerve growth factor mRNA is mediated by c-fos. Proc. Natl. Acad. Sci. U. S. A. 87, 3899-3903.

Henken, D.B., Battisti, W.P., Chesselet, M.F., Murray, M. & Tessler, A. (1990). Expression of β-preprotachykinin mRNA and tachykinins in rat dorsal root ganglion cells following peripheral or cerebral axotomy. Neuroscience 39, 733-742.

Hervonen, A., Vaalasti, A., Partanen, M., Kanerva, L. & Hervonen, H. (1978). Effect of ageing on the histochemically demonstrable catecholamines and acetylcholinesterase of human sympathetic ganglia. J. Neurocytol. 7, 11-23.

Hervonen, A., Kimura, A., Sato, A. & Sato, Y. (1990). Responses of cortical cerebral blood flow produced by stimulation of cervical sympathetic trunks are well maintained in aged rats. Neurosci. Lett. 120, 55-57.

Hickey, K.A., Rubanyi, G., Paul, R.J. & Highsmith, R.F. (1985). Characterization of a coronary vasoconstrictor produced by cultured endothelial cells. Am. J. Physiol. 248, C550-C556.

Hill, C.E., Jelinek, H., Hendry, I.A., MacLennan, I.S., Rush, R.A. (1988) Destruction by anti-NGF of autonomic sudomotor neurons and subsequent hyperinnervation of the foot pad by sensory fibres. J. Neurosci. Res. 19, 474-482.

Hirata, Y., Yoshimi, H., Takata, S., Watanabe, T.X., Kumagai, S., Nakajima, K. & Sakakibara, S. (1988). Cellular mechanism of action by a novel vasoconstrictor endothelin in cultured rat vascular smooth muscle cells. Biochem. Biophys. Res. Commun. 154, 868-875.

Holtz, J., Förstermann, U., Pohl, U., Giesler, M. & Bassenge, E. (1984). Flow-dependent, endothelium-mediated dilation of epicardial coronary arteries in conscious dogs: effects of cyclooxygenase inhibition. J. Cardiovasc. Pharmacol. 6, 1161-1169.

Holzer, P., Gamse, R. & Lembeck, F. (1980). Distribution of substance P in the rat gastrointestinal tract - lack of effect of capsaicin pretreatment. Eur. J. Pharmacol. 61, 303-307.

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

Holzmann, S. (1982). Endothelium-induced relaxation by acetylcholine associated with larger rise in cyclic GMP in coronary arterial strips. J. Cyclic Nucl. Res. 8, 409-419.

Hope, B.T., Michael, G.J., Knigge, K.M. & Vincent, S.R. (1991). Neuronal NADPH diaphorase is a nitric oxide synthase. Proc. Natl. Acad. Sci. U. S. A. 88, 2811-2814.

Hsu, S.M. & Soban, E. (1982). Colour modification of diaminobenzidine (DAB) precipitation by metallic ions and its application for double immunohistochemistry. J. Histochem. Cytochem. 30, 1079-1082.

Hsu, S.M., Raine, L. & Fanger, H. (1981). Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxydase techniques: a comparison between ABC and unlabelled antibodies (PAP) processes. J. Histochem. Cytochem. 29, 577-580.

Hua, X., Theodorsson-Norheim, E., Brodin, E., Lundberg, J.M. & Hökfelt, T. (1985). Multiple tachykinins (neurokinin A, neuropeptide K and substance P) in capsaicin-sensitive sensory neurons in the guinea-pig. Regul. Pept. 13, 1-19.

Huang, W.M., Gibson, S.J., Facer, P., Gu, J. & Polak, J.M. (1983). Improved section adhesion for immunocytochemistry using high molecular weight polymers of L-Lysine as a slide coating. Histochemistry 77, 275-279.

Hughes, A., Thom, S., Martin, G. & Sever, P. (1986). Endothelial-dependent relaxation of human arteries by peptides hormones. Clin. Sci. 70, 88.

Hughes, A.D., Schachter, M., Hair, W.M. & Sever, P.S. (1988). Endothelin is a potent constrictor of isolated human resistance arteries. Br. J. Pharmacol. Proc. 95 (Suppl.), 722P.

Hull, S.S., Kaiser, L., Jaffe, M.D. & Sparks, H.V. (1986). Endothelium-dependent flow-induced dilation of canine femoral and saphenous arteries. Blood Vessels 23, 183-198.

Humphrey, C.D. & Pittman, F.E. (1974). A simple methylene blue-AzurII-basic fuchsin stain for epoxy-embedded tissue sections. Stain. Technol. 49, 9-14.

Hunt, S.P., Emson, P.C., Gilbert, R., Goldstein, M. & Kimmel, J.R. (1982). Presence of avian pancreatic polypeptide-like immunoreactivity in catecholamine and methionine-enkephaline-containing neurons within the central nervous system. Neurosci. Lett. 21, 125-132.

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

Hökfelt, T., Elde, R., Johansson, O., Luft, R. & Arimura, A. (1975b). Immunohistochemical evidence for the presence of somatostatin, a powerful inhibitory peptide, in some primary sensory neurones. Neurosci. Lett. 1, 231-235.

Hökfelt, T., Johansson, O. & Kellerth, J.O. (1977). Immunohistochemical distribution of substance P. In: Substance P. Eds. U.S. Von Euler & B. Pernow, Raven Press, New York. pp. 117-145.

Hökfelt, T., Johansson, O., Ljungdahl, A., Lundberg, J.M. & Schultzberg, M. (1980). Peptidergic neurones. Nature 284, 515-521.

Hökfelt, T., Fuxe, K. & Pernow, B.(Eds.) (1986). Coexistence of Neuronal Messengers: A New Principle in Chemical Transmission. Progress in Brain Research, Vol. 68. Elsevier, Amsterdam,

Iacovitti, L., Johnson, M.I., Joh, T.H. & Bunge, R.P. (1982). Biochemical and morphological characterization of sympathetic neurons grown in a chemically defined medium. Neuroscience 7, 2225-2239.

Ignarro, L.J., Byrns, R.E. & Wood, K.S. (1986). Pharmacological and biochemical properties of endothelium-derived relaxing factor (EDRF): Evidence that it is closely related to nitric oxide (NO) radical. Circulation 74, Suppl.II, 287.

Ignarro, L.J., Byrns, R.E., Buga, G.M. & Wood, K.S. (1987). Mechanisms of endothelium-dependent vascular smooth muscle relaxation elicited by bradykinin and VIP. Am. J. Physiol. 253, H1074-H1082.

Ignarro, L.J., Byrns, R.E. & Wood, K.S. (1988). Biochemical and pharmacological properties of endothelium- derived relaxing factor and its similarity to nitric oxide radical. In: Vasodilation, Vascular Smooth Muscle, Peptides, Autonomic Nerves, and Endothelium. Ed. P.M. Vanhoutte, Raven Press, New York. pp. 427-435.

Illes, P., Pfeiffer, N., Limberger, N. & Starke, K. (1983). Presynaptic opioid receptors in the rabbit ear artery. Life Sci. 33, 307-310.

Inyama, C.O., Wharton, J., Su, H.C., Polak, J.M. (1986). CGRP-immunoreactive nerves in the genitalia of the female rat originate from dorsal ganglia T_{11}-L_3 and L_6-S_1: a combined immunocytochemical and retrograde tracing study. NNeurosci. Lett. 69, 13-18

Ip, N.Y. & Zigmond, R.E. (1984). Pattern of presynaptic nerve activity can determine the type of neurotransmitter regulating a postsynaptic event. Nature 311, 472-474.

Ip, N.Y., Baldwin, C. & Zigmond, R.E. (1985). Regulation of the concentration of adenosine 3',5'-cyclic monophosphate and the activity of tyrosine hydroxylase in the rat superior cervical ganglion by three neuropeptides of the secretin family. J. Neurosci. 5, 1947-1954.

Isaacson, L., Saffran, B.N. & Crutcher, K.A. (1990). Intracerebral NGF infusion induces hyperinnervation of cerebral blood vessels. Neurobiol. Aging 11, 51-55.

Ishi, T., Shimo, Y. & Minematsu, S. (1982). Localisation of vasoactive intestinal polypeptide immunoreactive nerve fibres in the rat portal vein. Dokkio. J. Med. Sci. 9, 89-92.

Itakura, T., Okuno, T., Nakakita, K., Kamei, T., Nakai, K., Imai, M., Komai, N., Kimura, H. & Maeda, T. (1984). A light and electronmicroscopical immunohistochemical study of vasoactive intestinal polypeptide and substance P containing nerve fibres along the cerebral blood vessels: comparison with aminergic and cholinergic nerve fibres. J. Cereb. Blood Flow Metab. 4, 407-414.

Itoh, M., Obata, K., Yanaihara, N. & Okamoto, H. (1983). Human pre pro vasoactive intestinal polypeptide (VIP) mRNA contains the coding sequence for a novel PHI-like peptide. Nature 304, 547.

Iversen, L.L. (1967). The Uptake and Storage of Noradrenaline in Sympathetic Nerves. Cambridge University Press, London,

Izzo, N.J., Jr., Seidman, C.E., Collins, S. & Colucci, W.S. (1990). α_1-adrenergic receptor mRNA level is regulated by norepinephrine in rabbit aortic smooth muscle cells. Proc. Natl. Acad. Sci. U. S. A. 87, 6268-6271.

Jacob, M.H. (1991). Acetylcholine receptor expression in developing chick ciliary ganglion neurons. J. Neurosci. 11, 1701-1712.

Jacobowitz, D.M. & Olschowka, J.A. (1982). Bovine pancreatic polypeptide-like immunoreactivity in brain and peripheral nervous system: coexistence with catecholaminergic nerves. Peptides 3, 369-379.

Jahr, C.E. & Jessel, T.M. (1983). ATP excites a subpopulation of rat dorsal horn neurones. Nature 304, 730-733.

James, S. & Burnstock, G. (1988). Neuropeptide Y-like immunoreactivity in intramural ganglia of the newborn guinea pig urinary bladder. Regul. Pept. 23, 237-245.

Jancso, G., Kirali, E. & Jancso-Gabor, A. (1977). Pharmacologically induced selective degeneration of chemosensitive primary sensory neurons. Nature 270, 741-743.

Jaspan, J., Polonski, K., Lewis, M. & Moossa, A.R. (1979). Reduction in portal vein blood flow by somatostatin. Diabetes 28, 888-892.

Jayakody, R.L., Senaatne, M.P.J., Thomson, A.B.R. & Kappagoda, C.T. (1985). Cholesterol feeding impairs endothelium-dependent relaxation of rabbit aorta. Can. J. Physiol. Pharmacol. 63, 1206-1209.

Jessel, T.M. & Iversen, L.L. (1977). Opiates analgesics inhibit

substance P release from rat trigeminal nucleus. Nature 268, 549-551.

Jessen, K.R., Saffrey, M.J., Van Noorden, S., Bloom, S.R., Polak, J.M. & Burnstock, G. (1980). Immunohistochemical studies of the enteric nervous system in tissue culture and in situ: localization of vascoactive intestinal polypeptide (VIP), substance-P and enkephalin immunoreactive nerves in the guinea-pig gut. Neuroscience 5, 1717-1735.

Johnson, A.R. & Erdos, E.G. (1973). Release of histamine from mast cells by vasoactive peptides. Proc. Soc. Exp. Biol. Med. 142, 1252-1256.

Johnson, E.M. & O'Brien, F. (1975). Evaluation of the permanent sympathectomy produced by the administration of guanethidine to adult rats. J. Pharmacol. Exp. Ther. 196, 53-61.

Johnson, P.F. & McKnight, S.L. (1989). Eukaryotic transcriptional regulatory proteins. Ann. Rev. Biochem. 58, 799-839.

Jonakait, G.M., Markey, K.A., Goldstein, M. & Black, I.B. (1984). Transient expression of selected catecholaminergic traits in cranial sensory and dorsal root ganglia of the embryonic rat. Dev. Biol. 101, 51-61.

Jonakait, G.M., Markey, K.A., Goldstein, M., Dreyfus, C.F. & Black, I.B. (1985). Selective expression of high affinity uptake of catecholamines by transiently catecholaminergic cells of the rat embryo : studies in vivo and in vitro. Dev. Biol. 108, 6-17.

Ju, G., Hökfelt, T., Brodin, E., Fahrenkrug, J., Fisher, J.A., Frey, P., Elde, R.P. & Brown, J.C. (1987). Primary sensory neurons of the rat showing calcitonin gene- related peptide immunoreactivity and their relation to substance P-, somatostatin-, galanin-, vasoactive intestinal polypeptide- and cholecystokinin-immunoreactive ganglion cells. Cell Tissue Res. 247, 417-431.

Järhult, J., Hellstrand, P. & Sundler, F. (1984). Immunohistochemical localization and vascular effects of vasoactive intestinal polypeptide in skeletal muscle of the cat. Cell Tissue Res. 207, 55-64.

Jòjàrt, I., Joò, F., Siklos, L. & Laszlo, F.A. (1984). Immunoelectron histochemical evidence for innervation of brain microvessels by vasopressin immunoreactive neurons in the rat. Neurosci. Lett. 51, 259-264.

Kaiser, L., Hull, S.S. & Sparks, H.V. (1986). Methylene blue and EDTA block flow-dependent dilation in canine femoral artery. Am. J. Physiol. 250, H974-H981.

Kapadia, S.E. & DeLanerolle, N.C. (1984). Immunohistochemical and electron microscope demonstration of vascular innervation in the mammalian brain stem. Brain Res. 292, 33-39.

Katusic, Z.S., Shepherd, J.T. & Vanhoutte, P.M. (1984). Vasopressin causes endothelium-dependent relaxation of canine basilar arteries. Circ. Res. 55, 575-579.

Katusic, Z.S., Shepherd, J.T. & Vanhoutte, P.M. (1986a). Endothelium-dependent contractions to stretch in canine basilar arteries. Fed. Proc. 45, 289.

Katusic, Z.S., Shepherd, J.T. & Vanhoutte, P.M. (1986b). Oxytocin causes endothelial-dependent relaxation of canine basilar arteries by activating V1-vasopressinergic receptors. J. Pharmacol. Exp. Ther. 236, 166-170.

Katusic, Z.S., Shepherd, J.T. & Vanhoutte, P.M. (1987a). Potassium-induced endothelium-dependent rhythmic activity in the canine basilar artery. J. Cardiovasc. Pharmacol. 12, 37-41.

Katusic, Z.S., Shepherd, J.T. & Vanhoutte, P.M. (1987b). Endothelium-dependent contractions to stretch in canine basilar artery. Am. J. Physiol. 252, H671-H673.

Katz, D.M., Markey, K.A., Goldestein, M. & Black, I.B. (1983). Expression of catecholaminergic characteristics by primary sensory neurons in the normal adult rat in vivo. Proc. Natl. Acad. Sci. U. S. A. 80, 3526-3530.

Katz, D.M. & Black, I.B. (1986). Expression and regulation of catecholaminergic traits in primary sensory neurons: relationship to target innervation in vivo. J. Neurosci. 6, 983-989.

Kawasaki, H. & Takasaki, K. (1984) Vasoconstrictor responses induced by 5-hydroxytryptamine released from vascular adrenergic nerves by periarterial nerve stimulation. J. Pharmacol. Exp. Ther. 229, 816-822.

Kawatami, M., Lowe, I., Morgan, C., Nadelhaft, I., Erdman, S. & de Groat, W.C. (1982). Vasoactive intestinal polypeptide in primary afferent projections to the sacral spinal cord of the cat. Soc. Neurosci. London. 8, 585-585.

Keen, P., Nielsch, U. & Watts, P.A. (1988). An investigation into the mechanism of induction of vasoactive intestinal peptide gene expression in rat sensory neurones following nerve injury. Proc. Physiol. Soc. London 68P.

Keller, R., Oke, A., Mefford, I. & Adams, R.N. (1976). Liquid chromatographic analysis of catecholamines: routine assay for regional brain mapping. Life Sci. 19, 995-1004.

Kelm, M. & Schrader, J. (1988). Nitric oxide release from the isolated guinea pig heart. Eur. J. Pharmacol. 155, 317-321.

Kessler, J.A. (1985a). Parasympathetic, sympathetic and sensory interactions in the iris: nerve growth factor regulates cholinergic ciliary ganglion innervation in vivo. J. Neurosci. 5, 2719-2725.

Kessler, J.A. (1985b). Differential regulation of peptide and catecholamine characters in cultured sympathetic neurons. Neuroscience 15, 827-838.

Kessler, J.A. & Black, I.B. (1980). Nerve growth factor stimulates the development of substance P in sensory ganglia. Proc. Natl. Acad. Sci. U. S. A. 77, 649-652.

Kessler, J.A., Cochard, P.P. & Black, I.B. (1979). Nerve growth factor alters the fate of embryonic neuroblasts. Nature 280, 141-142.

Kessler, J.A., Adler, J.E., Bohn, M.C. & Black, I.B. (1981). Substance P in principal sympathetic neurons: regulation by impulse activity. Science 214, 335-336.

Kessler, J.A., Bell, W.O. & Black, I.B. (1983). Interaction between the sympathetic and sensory innervation of the iris. J. Neurosci. 3, 1301-1307.

Kimball, E.S. (1990). Substance P, cytokines and arthritis. Ann. NY. Acad. Sci. 594, 293-308.

Kimelberg, H.K. & Pelton, E.W. (1983). High-affinity uptake of ^{3}H-norepinephrine by primary astrocites cultures and its inhibition by tricyclic antidepressant. J. Neurochem. 40, 1265-1270.

Kimura, S., Okada, M., Sugita, Y., Kanazawa, I. & Munekato, E. (1983). Novel neuropeptides neurokinin A and B, isolated from porcine spinal cord. Proc. Jap. Acad. Serv. B59, 101-104.

Kley, N., Loeffler, J., Pittius, L. & Höllt, V. (1987). Involvement of ion channels in the induction of proenkephalin A gene expression by nicotine and cAMP in bovine chromaffin cells. J. Biol. Chem. 262, 4083-4089.

Kline, L. & Pang, P. (1987). Calcitonin gene-related peptide activates tail artery helical strips in vitro and in an intracellular calcium- dependent manner. Eur. J. Pharmacol. 150, 233-238.

Kobayashi, S., Kyoshima, K., Olschwowka, J.A. & Jacobowitz, D.M. (1983). Vasoactive intestinal polypeptide immunoreactive and cholinergic nerves in the whole mount preparation of the major cerebral arteries of the rat. Histochemistry 79, 377-381.

Kohzuki, M., Johnston, C.I., Chai, S.Y., Casley, D.J. & Mendelsohn, F.A.O. (1989). Localization of endothelin receptors in rat kidney. Eur. J. Pharmacol. 160, 193-194.

Korsching, S. & Thoenen, H. (1983a). Nerve growth factor in sympathetic ganglia and corresponding target organs of the rat.

Correlation with density of sympathetic innervation. Proc. Natl. Acad. Sci. U. S. A. 80, 3513-3516.

Korsching, S. & Thoenen, H. (1983b). Quantitative demonstration of the retrograde axonal transport of endogenous nerve growth factor. Neurosci. Lett. 39, 1-4.

Korsching, S. & Thoenen, H. (1985). Treatment with 6-hydroxydopamine and colchicine decreases nerve growth factor levels in sympathetic ganglia and increases them in the corresponding target tissues. J. Neurosci. 5, 1058-1061.

Korsching, S. & Thoenen, H. (1988). Developmental changes of nerve growth factor levels in sympathetic ganglia and their target organs. Dev. Biol. 126, 40-46.

Krulich, L., Dhariwal, A.PS. & McCann, S.M. (1968). Stimulatory and inhibitory effects of purified hypothalamic extracts on growth hormone release from rat pituitary in vitro. Endocrinology 83, 783-790.

Kubota, M., Moseley, J.M., Butera, L., Dusting, G.J., MacDonald, P.S. & Martin, T.J. (1985). Calcitonin gene-related peptide stimulates cyclic AMP formation in rat aortic smooth muscle cell. Biochem. Biophys. Res. Commun. 132, 88-94.

Kummer, W. & Heim, C. (1986). Correlation of neuronal size, and peptide immunoreactivity in the guinea pig trigeminal ganglion. Cell Tissue Res. 245, 657-665.

Kuwayama, Y., Grimes, P.A., Ponte, B. & Stone, R.A. (1987). Autonomic neurons supplying the rat eye and the infraorbital distribution of vasoactive intestinal polypeptide (VIP)- like immunoreactivity. Exp. Eye Res. 44, 907-922.

Kuwayama, Y., Emson, P.C. & Stone, R.A. (1988). Pterygopalatine ganglion cells contain neuropeptide Y. Brain Res. 446, 219-224.

Lagnado, M.L.J., Crowe, R., Lincoln, J. & Burnstock, G. (1987). Reduction of nerves containing vasoactive intestinal polypeptide and serotonin, but not neuropeptide Y and catecholamine, in cerebral blood vessels of the 8-week streptozotocin-induced diabetic rat. Blood Vessels 24, 169-180.

Lamano-Carvalho, T.L., Hodson, N.P., Blank, M.A., Watson, P.F., Mulderry, P.K., Bishop, A.E., Gu, J., Bloom, S.R. & Polak, J.M. (1986). Occurrence, distribution and origin of peptide containing nerves of guinea-pig and rat male genitalia and the effects of denervation on sperm characteristics. J. Anat. 149, 121-141.

Landis, S.C. (1980). Developmental changes in the neurotransmitter properties of dissociated sympathetic neurons: a cytochemical study on the effects of the medium. Dev. Biol. 77, 349-361.

Landis, S.C. (1990). Target regulation of neurotransmitter phenotype. Trends Neurosci. 13, 344-350.

Landis, S.C. & Keefe, D. (1983). Evidence for neurotransmitter plasticity in vivo: developmental change in properties of cholinergic sympathetic neurons. Dev. Biol. 98, 349-372.

Landis, S.C., Jackson, P.C., Fredieu, J.R. & Thibault, J. (1987). Catecholaminergic properties of cholinergic neurons and synapses in adult rat ciliary ganglion. J. Neurosci. 7, 3574-3587.

Landschulz, W.H., Johnson, P.F. & McKnight, S.L. (1989). The leucine zipper: a hypothetical structure common to a new class of DNA binding proteins. Science 240, 1759-1764.

Lang, R.E., Hermann, K., Dietz, R., Gaida, W., Ganten, D., Kraft, K. & Unger, T.H. (1983). Evidence for the presence of enkephalins in the heart. Life Sci. 32, 399-406.

Lansman, J.B., Hallam, T.J. & Rink, T.J. (1987). Single stretch-activated ion channels in vascular endothelial cells as mechanotransducers?. Nature 325, 811-813.

Large, T.H., Ranh, J.J., De Mello, F.G. & Klein, W.L. (1985). Two molecular weight forms of muscarininc acetylcholine receptors in the avian central nervous system: switch in predominant form during differentiation and synapses. Proc. Natl. Acad. Sci. U. S. A. 82, 8785-8789.

Larhammar, D., Ericsson, A. & Persson, H. (1987). Structure and expression of the rat neuropeptide Y gene. Proc. Natl. Acad. Sci. U. S. A. 84, 2068-2072.

Larsson, L.-I., Edvinsson, L., Fahrenkrug, J., Håkanson, R., Owman, C., Schaffalitzki de Muckadell, O.B. & Sundler, F. (1976a). Immunohistochemical localization of a vasodilatory polypeptide (VIP) in cerebrovascular nerves. Brain Res. 113, 400-411.

Larsson, L.-I., Fahrenkrug, J., Schaffalitzki de Muckadell, O.B., Sundler, F., Håkanson, R. & Rehfeld, J.F. (1976b). Localization of vasoactive intestinal peptide (VIP) to central and peripheral neurons. Proc. Natl. Acad. Sci. U. S. A. 73, 3197-3200.

Larsson, L.-I., Fahrenkrug, J. & Schaffalitzki de Muckadell, O. (1977a). Vasoactive intestinal polypeptide occurs in nerves of the female genito-urinary tract. Science 197, 1374-1375.

Larsson, L.-I., Fahrenkrug, J. & Schaffalitzki de Muckadell, O. (1977b). Occurrence of nerves containing vasoactive intestinal polypeptide immunoreactivity in the male genital tract. Life Sci. 21, 503-508.

Lawson, S.N. & McCarthy, P.W. (1988). Substance P like immunoreactivity is found in primary afferent neurones with A as well

as with C fibres, in the rat in vitro. J. Physiol. (Lond.) 396, 71P.

Le Douarin, N. & Teillet, M.A. (1974). Experimental analysis of the migration and differentiation of neuroblasts of the autonomic nervous system and of neuroectodermal mesenchymal derivatives, using a biological cell marking technique. Dev. Biol. 61, 162-184.

Le Douarin, N. (1981). Plasticity in the development of the peripheral nervous system. In: Development of the Autonomic Nervous System, Ciba Foundation Symposium No. 83. Pitman Medical, London. pp. 19-50.

Le Douarin, N.M., Renad, D., Teillet, M.A. & Le Douarin, G.H. (1975). Cholinergic differentiation of presumptive adrenergic neuroblasts in interspecific chimeras after heterotopic transplantation. Proc. Natl. Acad. Sci. U. S. A. 72, 728-732.

Le Douarin, N.M. (1986). Cell line segregation during peripheral nervous system ontogeny. Science 231, 1515-1522.

Le Greves, P., Nyberg, F., Terenius, L. & Hökfelt, T. (1985). Calcitonin gene-related peptide is a potent inhibitor of substance P degradation. Eur. J. Pharmacol. 115, 309-311.

Leah, J.D., Cameron, A.A. & Snow, P.J. (1985). Neuropeptides in physiologically identified mammalian sensory neurones. Neurosci. Lett. 56, 257-263.

Leblanc, G.C., Trimmer, B.A. & Landis, S.C. (1987). Neuropeptide Y-like immunoreactivity in rat cranial parasympathetic neurons: coexistence with vasoactive intestinal peptide and choline acetyltransferase. Proc. Natl. Acad. Sci. U. S. A. 84, 3511-3515.

Lee, R.M.K.W., Forrest, J.B., Garfield, R.E. & Daniel, E.E. (1983). Comparison of blood vessel wall dimensions in normotensive and hypertensive rats by histometric and morphometric methods. Blood Vessels 20, 245-254.

Lee, T.J. (1986). Sympathetic and nonsympathetic transmitter mechanism in cerebral vasodilation and constriction. In: Neural Regulation of Brain Circulation. Eds. C. Owman & J.E. Hardebo, Elsevier, Amsterdam. pp. 285-296

Lee, T.J.-F. & Saito, A. (1984b). Altered cerebral vessel innervation in the spontaneously hypertensive rat. Circ. Res. 55, 392-403.

Lee, T.J.-F., Saito, A. & Berezin, I. (1984). Vasoactive intestinal polypeptide-like substance: the potential transmitter for cerebral vasodilation. Science 224, 898-901.

Lee, W., Mitchell, P. & Tjian, R. (1987). Purified transcription factor AP-1 interacts with TPA-inducible enhancer elements. Cell 49, 741-752.

Lee, Y., Kawai, Y., Shiosaka, S., Takami, K., Kiyama, H., Hillyard, C.J., Girgis, S., Macintyre, I., Emson, P.C. & Tohyama, M. (1985a). Co-existence of calcitonin gene-related peptide and substance P-like peptide in single cells of the trigeminal ganglion of the rat: immunohistochemical analysis. Brain Res. 330, 194-196.

Lee, Y., Takami, K., Kawai, Y., Girgis, S., Hillyard, C.J., Macintyre, I., Emson, P.C. & Tohyama, M. (1985b). Distribution of calcitonin gene-related peptide in the rat peripheral nervous system with reference to its coexistence with substance P. Neuroscience 15, 1227-1237.

Leeman, S.E. (1987). Substance P and neurokinins. In: Substance P and Neurokinins. Eds. J.L. Henry, R. Couture, A.C. Cuello, G. Pelletier, R. Quirion & D. Regoli, Springer, London.

Leibrock, J., Lotespeich, F., Hohn, A., Hoper, M., Hengerer, B., Masiakowski, P., Thoenen, H. & Barde, Y.A. (1989). Molecular cloning and expression of brain-derived neurotrophic factor. Nature 341, 149-151.

Lembeck, F. (1953). Zür Frage der zentralen Ubertragung afferenter Impulse. III Das Vorkommen und di Bedeutung der Substanz P in den Wurzeln des Ruckenmarks. Naunyn Schmiedebergs Arch. Pharmacol. 219, 197-213.

Lembeck, F. & Holzer, P. (1979). Substance P as a neurogenic mediator of antidromic vasodilation and neurogenic plasma extravasation. Naunyn Schmiedebergs Arch. Pharmacol. 310, 175-183.

Lembeck, F., Donnerer, J. & Barthò, L. (1982). Inhibition of neurogenic vasodilation and plasma extravasation by substance P antagonists, somatostatin and [d-Met2,Pro5]-enkephalinamide. Eur. J. Pharmacol. 85, 171-176.

Levi-Montalcini, R. (1987). The nerve growth factor: thirty-five years later. EMBO J. 6, 1145-1154.

Lewis, E.J., Harrington, C.A. & Chikaraishi, D.M. (1987). Transcriptional regulation of the tyrosine hydroxylase gene by glucocorticoid and cyclic AMP. Proc. Natl. Acad. Sci. U. S. A. 84, 3550-3554.

Leys, K., Schachter, M. & Scier, P. (1987). Autoradiographic localization of NPY receptors in rabbit kidney: comparison with rat, guinea-pig and human. Eur. J. Pharmacol. 134, 233-237.

Lin, H.L., Mismer, D., Lile, J.D., Armes, G.L., Butler, E.T., Vannice, J.L. & Collins, F. (1989). Purification, cloning and expression of ciliary neurotrophic factor (CNTF). Science 246, 1023-1025.

Lincoln, J., Bokor, J.T., Crowe, R., Griffith, S.G., Haven, A.J. & Burnstock, G. (1984). Myenteric plexus in streptozotocin-treated

rats. Neurochemical and histochemical evidence for diabetic neuropathy in the gut. Gastroenterology 86, 654-661.

Lindsay, R.M. & Harmar, A.J. (1989). Nerve growth factor regulates expression of neuropeptide genes in adult sensory neurons. Nature 337, 362-364.

Lindvall, O. & Björklund, A. (1974). The glyoxylic acid fluorescence histochemical method: a detailed account of the methodology for the visualization of central catecholamine neurons. Histochemistry 39, 97-127.

Liu-Chen, L.Y., Mayberg, M.R. & Moskowitz, M.A. (1983). Immunohistochemical evidence for a substance P-containing trigeminovascular pathway to pial arteries in cats. Brain Res. 268, 162-166.

Liu-Chen, L.Y., Norregaard, T.V. & Moskowitz, M.A. (1985). Some cholecystokinin-8 immunoreactive fibres in large pial arteries originate from trigeminal ganglion. Brain Res. 359, 166-176.

Llewellyn-Smith, I.J. (1984). Neuropeptide-containing nerves associated with mesenteric arteries in neonatal rats. Proc. Aust. Physiol. Pharmacol. Soc. 15, 94.

Lobato, R.D., Marin, J., Salaices, M., Burgos, J., Rivilla, F. & Garcia, A.G. (1980). Effect of experimental subarachnoid haemorrage on the adrenergic innervation of cerebral arteries. J. Neurosurg. 53, 477-479.

Loesch, A. & Burnstock, G. (1988). Ultrastructural localisation of serotonin and substance P in vascular endothelial cells of rat femoral and mesenteric arteries. Anat. Embryol. 178, 137-142.

Long, C.J. & Stone, T.W. (1985). The release of endothelium-derived relaxant factor is calcium dependent. Blood Vessels 22, 205-208.

Low, P.A., Walsh, J.C., Huang, C.Y. & McLeod, J.G. (1975). The sympathetic nervous system in alcoholic neuropathy. A clinical and pathological study. Brain 98, 357-364.

Luff, S.E., McLachlan, E.M. & Hirst, G.DS. (1987). An ultrastructural analysis of sympathetic neuromuscular junction on arterioles of the submucosa of the guinea pig ileum. J. Comp. Neurol. 257, 578-594.

Luft, R., Efèndic, S. & Hökfelt, T. (1978). Somatostatin - both hormone and neurotransmitter?. Diabetologia 14, 1-13.

Lumford, J.M. & Talamo, B.R. (1980). Beta-adrenergic and muscarinic receptors in developing rat parotid glands. J. Biol. Chem. 255, 4619-4627.

Lumford, J. & Talamo, B.R. (1983). Independent regulation of α-adrenergic receptor and nucleotide binding proteins of adenylate

cyclase. J. Biol. Chem. 258, 4831-4838.

Lundberg, J.M. (1981). Evidence for coexistence of vasoactive intestinal polypeptide (VIP) and acetylcholine in neurons of cat exocrine glands. Morphological, biochemical and functional studies. Acta Physiol. Scand. 112, Suppl.496, 1-57.

Lundberg, J.M. & Saria, A. (1982). Vagal substance P nerves involved in control of vascular permeability and smooth muscle tone in trachea and bronchi. Br. J. Pharmacol. 77, 441P-441P.

Lundberg, J.M. & Tatemoto, K. (1982). Pancreatic polypeptide family (APP, BPP, NPY, PYY) in relation to sympathetic vasoconstriction are resistant to alpha adrenoceptor blockade. Acta Physiol. Scand. 116, 393-402.

Lundberg, J.M. & Hökfelt, T. (1983). Coexistence of peptides and classical neurotransmitters. Trends Neurosci. 6, 325-333.

Lundberg, J., Ljung, B., Stage, D. & Dahlström, A. (1976). Postnatal ontogenic development of the adrenergic innervation pattern in rat portal vein: a histochemical study. Cell Tissue Res. 172, 15-27.

Lundberg, J.M., Hökfelt, T., Ånggard, A., Kimmel, J., Goldstein, M. & Markey, K. (1980a). Coexistence of the avian pancreatic polypeptide (APP) immunoreactive substance and catecholamines in some peripheral and central neurones. Acta Physiol. Scand. 110, 107-109.

Lundberg, J.M., Hökfelt, T., Ånggard, A. & Saria, A. (1980b). Peripheral peptide neurons: distribution, axonal transport and some aspects of possible function. In: Neuronal Peptides and Neural Communication: Advances in Biochemical Psychopharmacology, Vol. 22. Eds. E. Costa & M. Trabucchi, Raven Press, New York. pp. 25-36.

Lundberg, J.M., Ånggard, A., Fahrenkrug, J., Hökfelt, T. & Mutt, V. (1980). Vasoactive intestinal polypeptide in cholinergic neurones of exocrine glands. Functional significance of coexisting transmitters for vasodilation and secretion. Proc. Natl. Acad. Sci. U. S. A. 77, 1651-1655.

Lundberg, J.M., Ånggard, A. & Fahrenkrug, J. (1981). Complementary role of vasoactive intestinal polypeptide (VIP) and acetylcholine for cat submandibulary gland blood flow and secretion. Acta Physiol. Scand. 113, 317-327.

Lundberg, J.M., Hedlund, B., Ånggard, A., Fahrenkrug, J., Hökfelt, T., Tatemoto, K. & Bartfai, T. (1982a). Costorage of peptides and classical transmitters in neurones. In: Systemic Role of Regulatory Peptides. Eds. S.R. Bloom, J.M. Polak & E. Lindenlaub, Schattauer, Stuttgart. pp. 93-119.

Lundberg, J.M., Terenius, L., Hökfelt, T., Martling, C.R., Tatemoto, K., Mutt, V., Polak, J. & Bloom, S.R. (1982b). Neuropeptide Y (NPY)-like immunoreactivity in peripheral noradrenergic neurons and effects

of NPY on sympathetic function. Acta Physiol. Scand. 116, 477-480.

Lundberg, J.M., Terenius, L., Hökfelt, T. & Goldstein, M. (1983). High levels of neuropeptide Y in peripheral noradrenergic neurons in various mammals including man. Neurosci. Lett. 42, 167-172.

Lundberg, J.M., Fahrenkrug, J., Larsson, O. & Ånggard, A. (1984a). Corelease of vasoactive intestinal polypeptide and peptide histidine isoleucine in relation to atropine resistant vasodilation in cat submandibular salivary gland. Neurosci. Lett. 52, 37-45.

Lundberg, J.M., Terenius, L., Hökfelt, T. & Tatemoto, K. (1984b). Comparative immunohistochemical and biochemical analysis of pancreatic polypeptide-like peptides with special reference to presence of neuropeptide Y in central and peripheral neurons. J. Neurosci. 4, 2376-2386.

Lundberg, J.M., Saria, A., Franco-Cereceda, A., Hökfelt, T., Terenius, L. & Goldstein, M. (1985a). Differential effects of reserpine and 6-hydroxydopamine on neuropeptide Y (NPY) and noradrenaline in peripheral neurons. Naunyn Schmiedebergs Arch. Pharmacol. 328, 331-340.

Lundberg, J.M., Ånggård, A., Pernow, J. & Hökfelt, T. (1985b). Neuropeptide Y-, substance P- and VIP-immunoreactive nerves in relation to autonomic vascular and volume control. Cell Tissue Res. 239, 9-18.

Lundberg, J.M., Franco-Cereceda, A., Hua, X., Hökfelt, T. & Fischer, J.A. (1985c). Coexistence of substance P and calcitonin gene-related peptide-like immunoreactivities in sensory nerves in relation to cardiovascular and bronchoconstrictor effects of capsaicin. Eur. J. Pharmacol. 108, 315-319.

Lundberg, J.M., Rudehill, A., Sollen, A., Theodorsson-Norheim, A. & Hamberger, B. (1986a). Frequency- and reserpine-dependent chemical coding of sympathetic transmission: differential release of noradrenaline and neuropeptide Y from pig spleen. Neurosci. Lett. 63, 96-100.

Lundberg, J.M., Al-Saffar, A., Saria, A. & Theodorsson-Norheim, E. (1986b). Reserpine-induced depletion of neuropeptide Y from cardiovascular nerves and adrenal gland due to enhanced release. Naunyn Schmiedebergs Arch. Pharmacol. 332, 163-168.

Lundberg, J.M., Pernow, J., Franco-Cereceda, A. & Rudehill, A. (1987). Effects of antihypertensive drugs on sympathetic vascular control in relation to neuropeptide Y. J. Cardiovasc. Pharmacol. 10, S51-S68.

Lundberg, L.M., Alm, P. & Carlen, B. (1987). S-100 immunoreactive nerves in the guinea-pig uterus with reference to ultrastructural correlations: effect of chemical sympathectomy and pregnancy. Cell Tissue Res. 250, 241-249.

Lundberg, L.M., Alm, P., Wharton, J. & Polak, J.M. (1988). Protein gene product 9.5 (PGP 9.5). A new neuronal marker visualizing the whole uterine innervation and pregnancy-induced and developmental changes in the guinea pig. Histochemistry 90, 9-17.

Lüscher, T.F.(Ed.) (1988). Endothelial Vasoactive Substances and Cardiovascular Disease. Karger, Basel.

Lüscher, T.F. & Vanhoutte, P.M. (1986). Endothelium-dependent responses to platelets and serotonin in spontaneously hypertensive rats. Hypertension 8, 55-60.

Mabe, Y., Pèrez, R., Tatemoto, K. & Huidobro-Toro, J.P. (1987). Chemical sympathectomy reveals pre- and postsynaptic effects of neuropeptide Y (NPY) in the cardiovascular system. Experientia 43, 1018-1020.

Macrae, I.M., Furness, J.B. & Costa, M. (1986). Distribution of sub-groups of noradrenaline neurons in the coeliac ganglion of the guinea-pig. Cell Tissue Res. 244, 173-180.

Maegawa, M., Hisano, S., Tsuro, Y., Katoh, S., Nakanishi, J., Chikamori-Aoyama, M. & Daikoku, S. (1987). Differential immunolabelling for electron microscopy of diverse peptidergic neurons. J. Histochem. Cytochem. 35, 251-255.

Maggi, C.A. & Meli, A. (1988). The sensory-efferent function of capsaicin-sensitive sensory nerves. Gen. Pharmacol. 19, 1-43.

Magnoni, M.S., Govoni, S., Battaini, F. & Trabucchi, M. (1991). The aging brain: protein phosphorylation as a target of changes in neuronal function. Life Sci. 48, 373-385.

Maisonpierre, P.C., Belluscio, L., Squinto, S., Ip, N.Y., Furth, M.E., Lindsay, R.M. & Yancopoulos, G.D. (1991). Neurotrophin-3: a neurotrophic factor related to NGF and BDNF. Science 247, 1446-1451.

Maki, M., Takayanagi, R., Misona, K.S., Tanday, K.N., Tibbets, C. & Inagami, T. (1984). Structure of rat atrial natriuretic factor precursors deduced from cDNA sequence. Nature 309, 722-724.

Malcolm, S.J. (1986). Mechanism of catecholamine depletion by progesterone in guinea pig uterus. Proc. Austr. Physiol. Pharmacol. Soc. 17, 118P.

Maldonado, C.A., Saggon, W. & Forssman, W.G. (1986). Cardiodilatin-immunoreactivity in specific atrial granules of human heart by the immuno-gold stain. Anat. Embryol. 173, 295-298.

Malmfors, T., Furness, J.B., Campbell, G.R. & Burnstock, G. (1971). Re-innervation of smooth muscle of the vas deferens transplanted into the anterior chamber of the eye. J. Neurobiol. 2, 193-207.

Maniatis, T., Goodbourn, S. & Fischer, J.A. (1987). Regulation of inducible and tissue specific gene expression. Science 236, 1237-1245.

Mantyh, C.R., Kruger, L., Brecha, N.C. & Mantyh, P.W. (1986). Localization of specific binding sites for atrial natriuretic factor in peripheral tissues of the guinea- pig, rat and humans. Hypertension 8, 712-721.

Marceau, F., Knaps, M. & Regoli, D. (1981). Pharmacological characterization of the vascular permeability enhancing effects of kinins in the rabbit skin. Can. J. Physiol. Pharmacol. 59, 921-926.

Marek, K.L. & Mains, R.E. (1989). Biosynthesis, development and regulation of neuropeptide Y in superior cervical ganglion cultures. J. Neurochem. 52, 1807-1816.

Marsden, P.A., Danthuluri, N.R., Brenner, B.M., Ballermann, B.J. & Brock, T.A. (1989). Endothelin action on vascular smooth muscle involves inositol triphosphate and calcium mobilization. Biochem. Biophys. Res. Commun. 158, 86-93.

Marshall, J.M. (1970). Adrenergic innervation of the female reproductive tract: anatomy, physiology and pharmacology. Rev. Physiol. Biochem. Pharmacol. 62, 5-67.

Martin, W., Villani, G.M., Jothianandan, D. & Furchgott, R.F. (1985). Selective blockade of endothelium-dependent and glycerol trinitrate-induced relaxation by hemoglobin and by methylene blue in the rabbit aorta. J. Pharmacol. Exp. Ther. 232, 708-716.

Mastrangelo, D., Mathison, R., Huggel, H.J., Dion, S., D'Orlèans-Juste, P., Rhaleb, N.E., Drapeau, G., Rovero, P. & Regoli, D. (1986). The rat isolated portal vein: a preparation sensitive to neurokinins, particularly to neurokinin B. Eur. J. Pharmacol. 134, 321-326.

Matsuda, H., Kuon, N., Holtz, J. & Busse, R. (1985). Endothelium-mediated dilations contribute to the polarity of the arterial wall in vasomotion induced by α_2-adrenergic agonists. J. Cardiovasc. Pharmacol. 7, 680-688.

Matsuyama, T., Shiosaka, S., Matsumoto, M., Yoneda, S., Kimura, K., Abe, H., Hyakawa, T., Inoue, H. & Tohyama, M. (1983). Overall distribution of vasoactive intestinal polypeptide containing nerves in the wall of the cerebral arteries: an immunohistochemical study using whole mounts. Neuroscience 10, 89-96.

Matsuyama, T., Matsumoto, M., Shiosaka, S., Mayakawa, T., Yoneda, S., Kimura, K., Abe, H. & Tohyama, M. (1984). Dual innervation of substance P-containing neuron system in the wall of the cerebral arteries. Brain Res. 322, 144-147.

Matsuyama, T., Shiosaka, S., Wanaka, A., Yoneda, S., Kimura, K., Hayakawa, T., Emson, P.C. & Tohyama, M. (1985). Fine structure of

peptidergic and catecholaminergic nerve fibres in the anterior cerebral artery and their interrelationship: an immunoelectron microscopic study. J. Comp. Neurol. 235, 268-276.

Matsuyama, T., Wanaka, A., Yoneda, S., Kimura, K., Kamada, T., Girgis, S., Macintyre, I., Emson, P.C. & Tohyama, M. (1986). Two distinct calcitonin gene-related peptide-containing peripheral nervous systems: Distribution and quantitative differences between the iris and cerebral artery with special reference to substance P. Brain Res. 373, 205-212.

Mattiasson, A., Ekblad, E., Sundler, F. & Uvleius, B. (1985). Origin and distribution of neuropeptide Y, vasoactive intestinal polypeptide- and substance P-containing nerve fibres in the urinary bladder of the rat. Cell Tissue Res. 239, 141-146.

Maycox, P.R., Hell, J.W. & Jahn, R. (1990). Aminoacid transmission: spotlight on synaptic vesicles. Trends Neurosci. 13, 83-87.

McCulloch, J. & Edvinsson, L. (1980). The effect of vasoactive intestinal polypeptide upon pial arteriolar calibre, cerebral blood flow, cerebral oxygen consumption and the electroencephalogram. Am. J. Physiol. 238, H449-H456.

McCulloch, J. & Kelly, P.A.T. (1984). Effects of cholecystokinin octapeptide on pial arteriolar diameter. J. Cereb. Blood Flow Metab. 4, 625-628.

McCulloch, J., Uddman, R., Kingman, T.A. & Edvinsson, L. (1986). Calcitonin gene-related peptide: functional role in cerebrovascular regulation. Proc. Natl. Acad. Sci. U. S. A. 83, 5371-5735.

McEwan, J., Larkin, S., Davies, G., Chierchia, S., Brown, M., Stevenson, J., Macintyre, I. & Maseri, A. (1986). Calcitonin gene-related peptide: a potent dilator of human epicardial coronary arteries. Circulation 74, 1243-1247.

McGregor, G.P., Gibson, S.J., Sabate, I.M., Blank, M.A., Christofides, N.D., Wall, P.D., Polak, J.M. & Bloom, S.R. (1984). Effect of peripheral nerve section and nerve crush on spinal cord neuropeptides in the rat. Increased VIP and PHI in the dorsal horn. Neuroscience 15, 202-216.

McKnight, S. & Tjian, R. (1986). Transcriptional selectivity of viral genes in mammalian cells. Cell 46, 795-805.

McLennan, I.S., Hill, C.E. & Hendry, I.A. (1980). Glucocorticosteroids modulate transmitter choice in developing superior cervical ganglion. Nature 283, 206-207.

McMahon, S.B. & Gibson, S. (1987). Peptide expression is altered when afferent nerves innervate inappropriate tissue. Neurosci. Lett. 73, 9-15.

Melander, T., Hökfelt, T., Rökaeus, A., Fahrenkrug, J., Tatemoto, K. & Mutt, V. (1985). Distribution of galanin-like immunoreactivity in the gastro-intestinal tract of several mammalian species. Cell Tissue Res. 239, 253-270.

Metz, J., Gerstheimer, F.P. & Herbst, M. (1986). Distribution of synaptophysin immunoreactivity in guinea pig heart. Histochemistry 86, 221-224.

Miller, F.D., Mathew, T.C. & Toma, J.G. (1991). Regulation of nerve growth factor receptor gene expression by nerve growth factor in the developing peripheral nervous system. J. Cell Biol. 112, 303-312.

Miller, M.S., Buck, S.H., Sipes, J.G., Yamamura, H.I. & Burks, T.F. (1982). Regulation of substance P by nerve growth factor: disruption by capsaicin. Brain Res. 250, 193-196.

Miller, V.M. & Vanhoutte, P.M. (1985). Endothelial α_2-adrenoceptors in canine pulmonary and systemic blood vessels. Eur. J. Pharmacol. 118, 123-129.

Milner, P., Crowe, R., Burnstock, G. & Light, J.K. (1987). Neuropeptide Y- and vasoactive intestinal polypeptide-containing nerves in the intrinsic external urethral sphincter in the areflexic bladder compared to detrusor-sphincter dyssynergia in patients with spinal cord injury. J. Urol. 138, 888-892.

Milner, P., Ralevic, V., Hopwood, A.M., Fehér, E., Lincoln, J., Kirkpatrick, K.A. & Burnstock, G. (1989). Ultrastructural localisation of substance P and choline acetyltransferase in endothelial cells of rat coronary artery and release of substance P and acetylcholine during hypoxia. Experientia 45, 121-125.

Minamino, N., Masuda, H., Kangawa, K. & Matsuo, H. (1984). Regional distribution of neuromedin K and neuromedin L in rat brain and spinal cord. Biochem. Biophys. Res. Commun. 124, 731-738.

Minth, C.D., Bloom, S.R., Polak, J.M. & Dixon, J.E. (1984). Cloning characterization and DNA sequence of a human cDNA encoding neuropeptide tyrosine. Proc. Natl. Acad. Sci. U. S. A. 81, 4577-4581.

Mione, M.C., Corr, L., Aberdeen, J., Milner, P., Lincoln, J., Cavanagh, J.F.R. & Burnstock, G. (1988b). Chemical sympathectomy does not change the density of neuropeptide Y (NPY)-containing nerves in the cerebral vessels of the rat. Regul. Pept. 22, 423.

Mione, M.C. & Gabella, G. (1991). Nerve fibres in the uterine artery increase in number in pregnant guinea-pigs. Neuroreport in press

Mione, M.C., Sancesario, G., D'Angelo, V. & Bernardi, G. (1991). Increase of dopamine β-hydroxylase immunoreactivity in non-noradrenergic nerves of rat cerebral arteries following long-term sympathectomy. Neurosci. Lett. 123, 167-171.

Miserez, B., Bastiaensen, E., De Block, J. & De Potter, W. (1988). Tissue fractionation studies on bovine ganglium stellatum: co-storage of noradrenaline, Met-enkephalin and neuropeptide Y in large dense cored vesicles. Abstr. Br. Physiol. Pharmacol. Soc. P299-P300.

Mishina, M., Takai, T., Imoto, K., Noda, M., Takahashi, T., Numa, S., Methfessel, C. & Sakman, B. (1986). Molecular distinction between fetal and adult forms of muscle acetylcholine receptor. Nature 321, 406-411.

Mitchell, B.S. & Stauber, U.V. (1990). Morphological, histochemical and immunohistochemical studies of the paracervical ganglion in prepubertal, pregnant and adult, non-pregnant guinea-pigs. J. Anat. 172, 177-189.

Miyata, A., Kangawa, K., Toshimori, T., Hatoh, T. & Matsuo, H. (1985). Molecular forms of atrial natriuretic polypeptides in mammalian tissues and plasma. Biochem. Biophys. Res. Commun. 129, 248-255.

Moll, W. & Künzel, W. (1971). Blood pressures in the uterine vascular system of anaesthetized pregnant guinea pigs. Pflugers Arch. 330, 310-322.

Moncada, S., Palmer, R.MJ. & Higgs, E.A. (1987). Prostacyclin and endothelium-derived relaxing factor: biological interactions and significance. In: Thrombosis and Haemostasis. Eds. V. Verstraete, J. Vermylen, H.R. Lijnen & J. Arnout, Leuven University Press, Leuven. pp. 597-618.

Montminy, M., Goodman, R., Horovitch, S. & Habener, J. (1984). Primary structure of the gene encoding rat preprosomatostatin. Proc. Natl. Acad. Sci. U. S. A. 81, 3337-3340.

Montminy, M.R., Sevarins, K.A., Wagner, J.A., Mandel, G. & Goodman, R.H. (1986). Identification of a cyclic-AMP-responsive element within the rat somatostatin gene. Proc. Natl. Acad. Sci. U. S. A. 83, 6682-6686.

Moore, R.H. & Dowling, D.A. (1982). Effects of enkephalins on perfusion pressure in isolated hindlimb preparations. Life Sci. 31, 1559-1566.

Morgan, J.I. & Curran, T. (1986). Role of ion flux in the control of c-fos expression. Nature 322, 552-555.

Morris, J.L. & Gibbins, I.L. (1987). Neuronal colocalization of peptides, catecholamines, and catecholamine-synthesizing enzymes in guinea pig paracervical ganglia. J. Neurosci. 7, 3117-3130.

Morris, J.L. & Murphy, R. (1988). Evidence that neuropeptide Y released from noradrenergic axons causes prolonged contractions of the guinea-pig uterine artery. J. Auton. Nerv. Syst. 24, 241-250.

Morris, J.L., Gibbins, I.L., Furness, J.B., Costa, M. & Murphy, R. (1985). Co-localization of vasoactive intestinal polypeptide and dynorphin in noradrenergic axons of the guinea-pig uterine artery. Neurosci. Lett. 62, 31-37.

Morris, J.L., Murphy, R., Furness, J.B. & Costa, M. (1986). Partial depletion of neuropeptide Y from noradrenergic perivascular and cardiac axons by 6-hydroxydopamine and reserpine. Regul. Pept. 13, 147-162.

Morris, J.L., Gibbins, I.L. & Furness, J.B. (1987a). Increased dopamine-β-hydroxylase-like immunoreactivity in non-noradrenergic axons supplying the guinea-pig uterine artery after 6-hydroxydopamine treatment. J. Auton. Nerv. Syst. 21, 15-27.

Moskowitz, M.A. (1984a). The neurobiology of vascular head pain. Ann. Neurol. 16, 157-160.

Moskowitz, M.A., Norregaard, T.V., Liu-Chen, L.-Y., Weatherwax, R., Michener, S. & Go, V.L.W. (1984b). VIP, CCK and met-enkephalin in pia arachnoid and cerebral arteries after unilateral lesion of cat trigeminal ganglia. Abstr. Soc. Neurosci. 13, 576-576.

Moskowitz, M.A., Saito, K., Brezina, L. & Dickson, J. (1987). Nerve fibres surrounding intracranial and extracranial vessels from human and other species contain dynorphin- like immunoreactivity. Neuroscience 23, 731-737.

Moss, H.E. (1988). Autonomic control of the urinogenital organs in man and animal models of diabetes. Ph.D. Thesis, University of London,

Moyer, T.P. & Jiang, N.-S. (1978). Optimized isocratic conditions for analysis of catecholamines by high performance reversed-phase paired-ion chromatography with amperometric detection. J. Chromatogr. 153, 365-372.

Mulderry, P.K., Ghatei, M.A., Rodrigo, J., Allen, J.M., Rosenfeld, M.G., Polak, J.M. & Bloom, S.R. (1985). Calcitonin gene-related peptide in cardiovascular tissues of the rat. Neuroscience 14, 947-954.

Mulderry, P.K., Ghatei, M.A., Spokes, R.A., Jones, P.M., Pierson, A.M., Hamid, Q.A., Kanse, S., Amara, S.G., Burrin, J.M., Legon, S., Polak, J.M. & Bloom, S.R. (1988). Differential expression of α-CGRP and β-CGRP by primary sensory neurons of the rat. Neuroscience 25, 195-205.

Murphy, K.MM., McLaughlin, I.L., Michener, M.L. & Needleman, P. (1985). Autoradiographic localization of atriopeptide III receptors in rat kidney. Eur. J. Pharmacol. 3, 291-296.

Nagata, M., Franco-Cereceda, A., Saria, A., Amam, R. & Lundberg, J.M. (1987). Reserpine-induced depletion of neuropeptide Y in the guinea

pig: tissue specific effect and mechanism of action. J. Auton. Nerv. Syst. 20, 257-263.

Nakano, J. (1973). Cardiovascular actions of vasopressin. Jap. Circ. J. 37, 363-371.

Napier, M.A., Sandler, L.R., Albers-Schonberg, G., Nult, R.F., Brady, S., Lyle, T., Winquist, R., Maison, E.P., Heinel, L.A. & Blaine, E.M. (1984). Specific membrane receptors for atrial natriuretic factor in renal and vascular tissues. Proc. Natl. Acad. Sci. U. S. A. 81, 5946-5949.

Narajo, J.R., Mellström, B., Achaval, M. & Sassone-Corsi, P. (1991). Molecular pathways of pain:fos/jun-mediated activation of a non-canonical AP-1 site in the prodynorphin gene. Neuron 6, 607-617.

Navone, F., Greengard, P. & De Camilli, P. (1984). Synapsin I in nerve terminals: selective association with small synaptic vesicles. Science 226, 1209-1211.

Navone, F., Reinhard, J., Di Gioia, G., Stukenbrok, H., Greengard, P. & De Camilli, P. (1986). Protein P38: an integral membrane protein specific for small vesicles of neurons and neuroendocrine cells. J. Cell Biol. 103, 2511-2527.

Nawa, H., Hirose, T., Takashima, H., Inayama, S. & Nakanishi, S. (1983). Nucleotide sequence of cloned cDNAs for two types of bovine substance P precursors. Nature 306, 32-36.

Nawa, H., Doteuchi, M., Igano, K., Inoue, K. & Nakanishi, S. (1984). Substance K: a novel mammalian tachykinin that differs from substance P in its pharmacological profile. Life Sci. 34, 1153-1160.

Nawa, H. & Patterson, P.H. (1990). Separation and partial characterization of neuropeptide- inducing factors in heart cell conditioned medium. Neuron 4, 269-277.

Nawa, H. & Sah, D.WY. (1990). Differential biological activities in conditioned media control the expression of a variety of neuropeptides in cultured sympathetic neurons. Neuron 4, 279-287.

Nelson, H., Mandiyan, S. & Nelson, N. (1990). Cloning of the human brain GABA transporter. FEBS Lett. 269, 181-184.

Nemer, M., Lavigne, J.-P., Dronin, J., Thibault, G., Gannon, M. & Autakly, T. (1986). Expression of atrial natriuretic factor gene in heart ventricular tissue. Peptides 7, 1147-1152.

New, H.V. & Mudge, A.W. (1986). Calcitonin gene-related peptide regulates muscle acetylcholine receptor synthesis. Nature 323, 809-811.

Noguchi, K., Senba, E., Morita, Y., Sato, M. & Tohyama, M. (1989). Prepro-VIP and preprotachykinin mRNAs in the rat dorsal root ganglion

cells following peripheral axotomy. Mol. Brain Res. 6, 327-330.

Nyberg, F., Le Greves, P. & Terenius, L. (1988). Modulation of endopeptidase activity by calcitonin gene-related peptide: a mechanism affecting substance P action?. Biochimie 70, 65-68.

O'Birne, E.M., Blancuzzi, V., Wilson, D.E., Wong, M. & Jeng, A.Y. (1990). Elevated substance P and accelerated cartilage degradation in rabbit knees injected with interleukin 1 and tumor necrosis factor. Arthritis Rheum. 33, 1023-1028.

O'Brien, R.F. & McMurthy, T.F. (1984). Endothelial cell supernates contract bovine pulmonary artery rings. Am. Rev. Respir. Dis. 129, 337.

O'Connor, T.P. & Van der Kooy, D. (1989). Cooperation and competition during development: neonatal lesioning of the superior cervical ganglion induces cell death of trigeminal neurons innervating the cerebral blood vessels but prevents the loss of axon collaterals from the neurons that survive. J. Neurosci. 9, 1490-1501.

O'Donohue, T.L., Millington, W.R., Handelman, G.E., Contreras, P.C. & Chronwall, B.M. (1985) On the 50th anniversary of Dale's law: multiple neurotransmitters neurons. TIPS 6, 305-309

Oikawa, S., Imai, M., Veno, A., Tanaka, S., Noguchi, T., Nakazato, H., Kangawa, K., Fukuda, A. & Matsuo, H. (1984). Cloning and sequence analysis of cDNA encoding a precursor for human atrial natriuretic polypeptide. Nature 309, 724-725.

Oku, R., Satoh, M., Fuji, N., Otaka, A., Yajima, H. & Takagi, H. (1987). Calcitonin gene-related peptide promotes mechanical nociception by potentiating release of substance P from the spinal dorsal horn in rats. Brain Res. 403, 350-354.

Olson, E.N. (1990). MyoD family: a paradigm for development. Genes Dev. 4, 1454-1461.

Oppenheim, R.W. (1981) Neuronal cell death and some related regressive phenomena during morphogenesis: a selective historical review and progress report. In: Studies in Developmental Neurobiology (eds W.M. Cowen) Oxford University Press, Oxford, pp. 74-133

Osborne, N.N. (1983). Dale's Principle and Communication between Neurones. Pergamon Press, Oxford, pp. 204.

Otsuka, M. & Konishi, S. (1976). Release of substance P-like immunoreactivity from isolated spinal cord of newborn rat. Nature 264, 83-84.

Otten, V. & Lorez, H.P. (1982). Nerve growth factor increases substance P, cholecystokinin and vasoactive intestinal polypeptide immunoreactivities in primary sensory neurons of newborn rats. Neurosci. Lett.34, 153-158

Ottesen, B. & Fahrenkrug, J. (1981). Effect of vasoactive intestinal polypeptide (VIP) upon myometrial blood flow in the non-pregnant rabbit. Acta Physiol. Scand. 112, 195-201.

Ottesen, B., Wagner, G., Viras, R. & Fahrenkrug, J. (1984). Penile erection: possible role for vasoactive intestinal polypeptide as a neurotransmitter. Br. Med. J. 288, 9-13.

Owman, C. (1983). Pregnancy induces degenerative and regenerative changes in the autonomic innervation of the female reproductive tract. In: Development of the Autonomic Nervous System. Ciba Foundation Symposium, . pp. 252-279.

Owman, C., Alm, P., Rosengren, E. & Sjöberg, N.-O. (1975). Variations in the levels of uterine norepinephrine during pregnancy in guinea pig. Am. J. Obstet. Gynecol. 122, 961-969.

Owman, C. & Sjöberg, N.-O. (1977). Influence of pregnancy and sex hormones on the system of short adrenergic neurons in the female reproductive tract. Excerpta Med. Int. Congr. Serv. 402, 205-209.

Pacholczyk, T., Blakely, R.D. & Amara, S.G. (1991). Expression cloning of a cocaine and antidepressant sensitive human noradrenaline transporter. Nature 350, 350-354.

Paddle, B.M. & Burnstock, G. (1974). Release of ATP from perfused heart during coronary vasodilatation. Blood Vessels 11, 110-119.

Palmer, R.M.J., Ferrige, A.G. & Moncada, S. (1987). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. Nature 327, 524-526.

Papka, R.E. & Traurig, H.H. (1988). Distribution of subgroups of neuropeptide Y-immunoreactive and noradrenergic nerves in the female rat uterine cervix. Cell Tissue Res. 252, 533-541.

Papka, R.E., Cotton, J.P. & Traurig, H.H. (1985). Comparative distribution of neuropeptide tyrosine-, vasoactive intestinal polypeptide-, substance P-TI - immunoreactive, acetylcholinesterase-positive and noradrenergic nerves in the reproductive tract of the female rat. Cell Tissue Res. 242, 475-490.

Parnavelas, J.G., Kelly, W. & Burnstock, G. (1985). Ultrastructural localization of choline acetyltransferase in vascular endothelial cells in rat brain. Nature 316, 724-725.

Patterson, P.H. (1978). Enviromental determination of autonomic neurotransmitter functions. Ann. Rev. Neurosci. 1, 1-17.

Pearson, J.D. & Gordon, J.L. (1984). Metabolism of serotonin and adenosine. In: Biology of Endothelial Cells. Ed. E.A. Jaffe, Martinus Nijhoff, Boston. pp. 330-342.

Penit, J., Faure, M. & Jard, S. (1983). Vasopressin and angiotensin II receptors in rat aortic muscle cells in culture. Am. J. Physiol. 244, E72-E82.

Penschow, J.D., Haralambidis, J., Darling, P.E., Darby, I.A., Wintour, E.M., Tregear, G.W. & Coghlan, J.P. (1987). Hybridization histochemistry. Experientia 43, 741-750.

Pernow, B. & Rosell, S. (1975). Effect of substance P on blood flow in canine adipose tissue and skeletal muscle. Acta Physiol. Scand. 93, 139-141.

Pernow, J. (1988). Corelease and functional interactions of neuropeptide Y and noradrenaline in peripheral vascular control. Acta Physiol. Scand. Suppl. 568, 1-39.

Pernow, J., Saria, A. & Lundberg, J.M. (1986). Mechanisms underlying pre- and postjunctional effects of neuropeptide Y in sympathetic vascular control. Acta Physiol. Scand. 126, 239-249.

Pernow, J., Kahan, T. & Lundberg, J.M. (1988). Neuropeptide Y and reserpine resistant vasoconstriction evoked by sympathetic nerve stimulation in the dog skeletal muscle. Br. J. Pharmacol. 94, 952-960.

Pincus, D.W., DiCicco-Bloom, E.M. & Black, I.B. (1990). Vasoactive intestinal peptide regulates mitosis, differentiation and survival of cultured sympathetic neuroblasts. Nature 343, 564-567.

Piotrowski, W. & Foreman, J.C. (1986). Some effects of human calcitonin gene-related peptide in human skin and on histamine release. Br. J. Derm. 114, 37-46

Polak, J.M. & Bloom, S.R. (1980). Peripheral localization of regulatory peptides as a clue to their function. J. Histochem. Cytochem. 28, 918-924.

Power, R.F., Rutherford, A.J., Salas, S.P., Wharton, J. & Polak, J.M. (1988). Calcitonin gene-related peptide (CGRP) binding sites in the female guinea pig genital tract. Regul. Pept. 22, 427.

Power, R.F., Wharton, J., Salas, S.P., Kanse, S., Ghatei, M., Bloom, S.R. & Polak, J.M. (1989). Autoradiographic localisation of endothelin binding sites in human and porcine coronary arteries. Eur. J. Pharmacol. 160, 199-200.

Purves, D. & Hume, R.I. (1981). The relation of postsynaptic geometry to the number of presynaptic axons that innervate autonomic ganglion cells. J. Neurosci. 1, 441-452.

Qu, Z., Moritz, E. & Huganir, R.L. (1990). Regulation of tyrosine phosphorylation of the nicotinic acetylcholine receptor at the neuromuscular junction. Neuron 2, 367-378.

Quirion, R., Rioux, F., St-Pierre, S. & Regoli, D. (1979). Increased sensitivity to neurotensin in fasted rats. Life Sci. 25, 1969-1973.

Randic, M., Ryu, P.D. & Urban, L. (1986). Effects of polyclonal and monoclonal antibodies to substance P on slow excitatory transmission in rat spinal dorsal horn. Brain Res. 383, 15-27.

Rao, M.S. & Landis, S.C. (1990). Characterization of a target-derived neuronal cholinergic differentiation factor. Neuron 5, 899-910.

Rapoport, R.M. & Murad, F. (1983). Endothelium-dependent and nitrovasodilator-induced relaxation of vascular smooth muscle: role of cyclic GMP. J. Cyclic Nucleotide Protein Phosphor. Res. 9, 281-296.

Raynaud, B., Faucon-Biguet, N., Vidal, S., Mallet, J. & Weber, M.J. (1988). Regulation of neurotransmitter metabolic enzymes and tyrosine hydroxylase mRNA level by nerve growth factor in cultured sympathetic neurones. Development 102, 361-368.

Rees, D.D., Palmer, R.M.J., Hodson, H.F. & Moncada, S. (1989). A specific inhibitor of nitric oxide formation from L-arginine attenuates endothelium-dependent relaxation. Br. J. Pharmacol. 96, 418-424.

Regoli, D. (1984). Neurohumoral regulation of precapillary vessels: the kallicrein-kinin system. J. Cardiovasc. Pharmacol. 6, S401-S412.

Regoli, D. & Barabè, J. (1980). Pharmacology of bradykinin and related kinins. Pharmacol. Rev. 32, 1-46.

Regoli, D., Marceau, F. & Barabè, J. (1978). De novo formation of vascular receptors for bradykinins. Can. J. Physiol. Pharmacol. 56, 674-677.

Regoli, D., Marceau, F. & Lavigne, J. (1981). Induction of β_1 receptors for kinins in the rabbit by a bacterial lipopolysaccharide. Eur. J. Pharmacol. 70, 105-115.

Regoli, D., Escher, E. & Mizrahi, J. (1984a). Substance P structure-activity studies and the development of antagonists. Pharmacology 28, 301-320.

Regoli, D., Escher, E., Drapeau, G., D'Orleans-Juste, P. & Mizrahi, J. (1984b). Receptors for substance P. III. Classification by competitive antagonists. Eur. J. Pharmacol. 97, 179-189.

Regoli, D., Drapeau, G., Dion, S. & D'Orleans-Juste, P. (1987). Pharmacological receptors for substance P and neurokinins. Life Sci. 40, 107-117.

Reinecke, M., Weihe, E. & Forssmann, W.J. (1980). Substance P-immunoreactive nerve fibres in the heart. Neurosci. Lett. 20, 265-269.

Reinecke, M., Weihe, E., Carraway, R.E., Leeman, S.E. & Forssman, W.J. (1982a). Localization of neurotensin-immunoreactive nerve fibres in the guinea-pig heart: evidence derived by immunocytochemistry, radioimmunassay and chromatography. Neuroscience 7, 1785-1795.

Reinecke, M., Vallance, C., Weihe, E. & Forssmann, W.J. (1982b). Neuropeptide (substance P, neurotensin) immunoreactive nerve fibres in the heart of higher vertebrates. Neurosci. Lett. 7, S403.

Reusch, M.K., Karasek, M.A. & Nickoloff, B.J. (1988). Effect of neuropeptides present in skin on the proliferation of human peripheral blood mononuclear cells and T cells. Arch Dermatol. Res. 280, 279-281.

Richardson, P.M. & Riopelle, R.J. (1984) Uptake of nerve growth factor along peripheral and spinal axons of primary sensory neurons. J. Neurosci. 4, 1683-1689.

Rodriguez-Tebar, A., Dechant, G. & Barde, Y.A. (1990). Binding of brain-derived neurotrophic factor to the nerve growth factor receptor. Neuron 4, 487-492.

Rogers, A.W. (1979). Techniques of autoradiography. Elsevier Biochemical Press, The Netherlands,

Rohrer, H. (1991). The role of growth factors in the control of neurogenesis. Eur. J. Neurosci. 2, 1005-1015.

Rorie, D.K. & Tyce, G.M. (1985) Uptake and metabolism of noradrenaline by endothelium of dog pulmonary artery. Am. J. Physiol. 248, H193-H197

Rosenfeld, M.G., Mermod, J.J., Amara, S.G., Swanson, L.W., Sawchenko, P.E., Rivier, J., Vale, W.W. & Evans, R.H. (1983). Production of a novel neuropeptide encoded by calcitonin gene via tissue specific RNA processing. Nature 304, 129-135.

Rossi, F. & Scevola, D. (1935). Contributo alla conoscenza della distribuzione delle fibre nervose nella dura madre encefalica. Monitore. Zool. Ital. 45, 289-289.

Rubanyi, G.M. & Vanhoutte, P.M. (1985). Hypoxia releases a vasoconstrictor substance from the canine vascular endothelium. J. Physiol. (Lond.) 364, 45-56.

Rubanyi, G.M. & Vanhoutte, P.M. (1988). Heterogeneity of endothelium-dependent responses to acetylcholine in canine femoral arteries and veins. Blood Vessels 25, 75-81.

Rubanyi, G.M., Romero, J.C. & Vanhoutte, P.M. (1986). Flow-induced release of endothelium-derived relaxing factor. Am. J. Physiol. 250, H1145-H1149.

Russel, J.T. (1987). The secretory vesicles in processing and secretion of neuropeptides. Curr. Top. Membr. Transport 31, 277-312.

Saadat, S., Sendtner, M. & Rohrer, H. (1989). Ciliary neurotrophic factor induces cholinergic differentiation of rat sympathetic neurons in culture. J. Cell Biol. 108, 1807-1816.

Saba, H., Cowen, T., Haven, A.J. & Burnstock, G. (1984). Reduction in noradrenergic perivascular nerve density in the left and right cerebral arteries of old rabbits. J. Cereb. Blood Flow Metab. 4, 284-289.

Sabol, S.L. & Higuchi, H. (1990). Transcriptional regulation of the neuropeptide Y gene by nerve growth factor: antagonism by glucocorticoids and potentiation by adenosine 3',5' monophosphate and phorbol ester. Mol. Endocrinol. 4, 384-392.

Saffran, B.N., Woo, J., Mobley, W.C. & Crutcher, K.A. (1989). Intraventricular infusion of NGF in the mature rat brain enhances sympathetic innervation of cerebrovascular targets but fails to elicit sympathetic ingrowth. Brain Res. 492, 245-254.

Saffrey, M.J., Polak, J.M. & Burnstock, G. (1982). Distribution of vasoactive intestinal polypeptide-, substance P-, enkephalin and neurotensin-like immunoreactive nerves in the chicken gut during development. Neuroscience 7, 279-293.

Said, S.I. & Mutt, V. (1970). Polypeptide with broad physiological activity: isolation from small intestine. Science 169, 1217-1218.

Saito, A. & Goto, K. (1986). Depletion of calcitonin gene-related peptide (CGRP) by capsaicin in cerebral arteries. J. Pharmacobio-Dyn. 9, 613-619.

Salt, T.E. & Hill, R.G. (1983). Excitation of single sensory neurones in the rat caudal trigeminal nucleus by iontophoretically applied adenosine 5'-triphosphate. Neurosci. Lett. 35, 53-57.

Santer, R.M. (1979). Fluorescent histochemical evidence for decreased noradrenaline synthesis in sympathetic neurons of aged rats. Neurosci. Lett. 15, 177-180.

Santer, R.M. (1982). Fluorescent histochemical observation of the adrenergic innervation of the cardiovascular system in aged rats. Brain Res. Bull. 9, 667-672.

Santer, R.M., Partanen, M. & Hervonen, A. (1980). Glyoxylic acid fluorescence and ultrastructural studies of neurones in the coeliac-superior mesenteric ganglion of the aged rat. Cell Tissue Res. 211, 475-485.

Sasaki, Y., Hayashi, N., Kashara, A., Matsuda, H., Fusamoto, H., Sato, N., Hillyard, C.J., Girgis, S., Macintyre, I., Emson, P.C., Shiosaka, S., Tohyama, M., Shitani, Y. & Kamada, T. (1986).

Calcitonin gene-related peptide in the hepatic and splanchnic vascular systems of the rat. Hepatology 6, 676-681.

Sassone-Corsi, P., Sisson, J.C. & Verma, I.M. (1988). Transcriptional autoregulation of the proto-oncogene fos. Nature 334, 314-319.

Scadding, J.W. (1980). The permanent anatomical effects of neonatal capsaicin on somatosensory nerves. J. Anat. 131, 473-484.

Schaife, R. & Margolis, R.L. (1990). Biochemical and immunochemical analysis of rat brain dynamin interaction with microtubules and organelles in vivo and in vitro. J. Cell Biol. 116, 3023-3033.

Schalling, M., Stieg, P.E., Lindquist, C., Goldstein, M. & Hökfelt, T. (1989). Rapid increase in enzyme and peptide mRNA in sympathetic ganglia after electrical stimulation in humans. Proc. Natl. Acad. Sci. U. S. A. 86, 4302-4305.

Schalling, M., Franco-Cereceda, A., Hemsén, A., Dargerling, Å., Seroogy, K., Persson, H., Hökfelt, T. & Lundberg, J.M. (1991). Neuropeptide Y and catecholamine synthetizing enzymes and their mRNAs in rat sympathetic neurons and adrenal glands: studies on expression, synthesis and axonal transport after pharmacology and experimental manipulations using hybridization techniques and radioimmunoassay. Neuroscience 41, 753-766.

Schleifer, L.S., Black, I.B. & Reid, L.M. (1989). Regulation of β-adrenergic receptor expression in rat liver. J. Cell Physiol. 140, 52-58.

Schon, F., Ghatei, M., Allen, J.M., Mulderry, P.K., Kelly, J.S. & Bloom, S.R. (1985a). The effect of sympathectomy on calcitonin gene-related peptide levels in the rat trigemino-vascular system. Brain Res. 348, 197-200.

Schon, F., Allen, J.M., Yeats, J.C., Allen, Y.S., Ballesta, J., Polak, J.M., Kelly, J.S. & Bloom, S.R. (1985b). Neuropeptide Y innervation of the rodent pineal gland and cerebral blood vessels. Neurosci. Lett. 57, 65-71.

Schontal, A., Herrlich, P., Rahmsdorf, H.J. & Ponta, H. (1988). Requirement for fos gene expression in the transcriptional activation by collagenase by other oncogenes and phorbol ester. Cell 54, 325-334.

Schultzberg, M., Hökfelt, T., Nilsson, G., Terenius, L., Rehfeld, J.H., Brown, M., Elde, R., Goldstein, M. & Said, S. (1980). Distribution of peptide and catecholamine-containing neurons in the gastrointestinal tract of rat and guinea-pig: immunohistochemical studies with antisera to substance P, vasoactive intestinal polypeptide, enkephalins, somatostatin, gastrin/cholecystokinin, neurotensin and dopamine-β-hydroxylase. Neuroscience 5, 689-744.

Schultze, S.M. & Role, L.W. (1987). Developmental regulation of

nicotinic acetylcholine receptors. Ann. Rev. Neurosci. 10, 403-457.

Schwartz, J.P. & Mishler, K. (1990). Beta-adrenergic receptor regulation, through cyclic AMP, of nerve growth factor expression in rat cortical and cerebellar astrocytes. Cell. Mol. Neurobiol. 10, 447-457.

Schwarzschild, M.A. & Zigmond, R.E. (1989). Secretin and vasoactive intestinal peptide activate tyrosine hydroxylase in sympathetic nerve endings. J. Neurosci. 9, 160-169.

Scivoletto, R. & Carvalho, M.J.C. (1984). Cardionatrin causes vasodilation in vitro which is not dependent on the presence of endothelial cells. Eur. J. Pharmacol. 101, 143-145.

Scott, T.M. & Woolgar, J.R. (1987). Development of the innervation of the rat mesenteric vascular bed. In: Proc. Austr. Physiol. Soc., 17-18 Dec. 1987. 90P.

Senapati, A., Ånand, P., McGregor, G.P., Ghatei, M.A., Thompson, R.P.H. & Bloom, S.R. (1986). Depletion of neuropeptides during wound healing in rat skin. Neurosci. Lett. 71, 61-105.

Senba, E. & Tohyama, M. (1988). Calcitonin gene-related peptide containing autonomic efferent pathways to the pelvic ganglia of the rat. Brain Res. 449, 386-390.

Shehab, S.A.S. & Atkinson, M.E. (1986). Vasoactive intestinal polypeptide increases in areas of the dorsal horn from which other neuropeptides are depleted following peripheral axotomy. Exp. Brain Res. 62, 422-430.

Shelton, D.L. & Reichardt, L.F. (1984). Expression of the β-nerve growth factor gene correlates with the density of sympathetic innervation in effector organs. Proc. Natl. Acad. Sci. U. S. A. 81, 7951-7955.

Sheng, M.M., Dougan, S.T., McFadden, G. & Greenberg, M.E. (1988). Calcium and growth factors pathways of c-fos transcriptional activation require distinct upstream regulatory sequences. Mol. Cell Biol. 8, 2787-2796.

Sikri, K.L., Hoyes, A.D., Barber, P. & Jagessar, M. (1981). Substance P-like immunoreactivity in the intramural nerve plexuses of the guinea-pig ureter: a light and electron microscopical study. J. Anat. 133, 425-442.

Silverman, J.D. & Kruger, L. (1989). Calcitonin gene-related peptide-immunoreactive innervation of the rat head with emphasis on specialized sensory structures. J. Comp. Neurol. 280, 303-330.

Simons, T. & Ruskell, G.L. (1988). Distribution and termination of trigeminal nerves to the cerebral arteries in monkey. J. Anat. 159, 57-71.

Singer, H.A. & Peach, M.J. (1982). Calcium-endothelial-mediated vascular smooth muscle relaxation in rabbit aorta. Hypertension 4, 19-25.

Sjöberg, N.-O. (1967). The adrenergic transmitter of the female reproductive tract: distribution and functional changes. Acta Physiol. Scand. Suppl. 305, 1-67.

Sjöberg, N.-O. (1968). Consideration on the cause of disappearance of the adrenergic neurotransmitter in the uterine nerves during pregnancy. Acta Physiol. Scand. 72, 510-517.

Skofitsch, G. & Jacobowitz, D.M. (1985a). Calcitonin gene-related peptide coexists with substance P in capsaicin sensitive neurons and sensory ganglia of the rat. Peptides 6, 747-754.

Skofitsch, G., Jacobowitz, D.M., Eskay, R.L. & Zamir, N. (1985b). Distribution of atrial natriuretic factor-like immunoreactive neurons in the rat brain. Neuroscience 16, 917-948.

Smith, P.G., Bruckert, J.W. & Mills, E. (1987). Reinnervation of Müller's smooth muscle by atypical sympathetic pathways following neonatal ganglionectomy in the rat: structural and functional investigations and enhanced neuroplasticity. Neuroscience 23, 781-793.

Smièsko, V., Lang, D.L. & Johnson, P.C. (1987). Dilator response of rat mesenteric arcading arterioles to increased blood flow. Fed. Proc. 46, 643-643.

Sossin, W.S., Fisher, J.M. & Scheller, R.H. (1989). Cellular and molecular biology of neuropeptide processing and packaging. Neuron 2, 1407-1417.

Sporrong, B., Alm, P., Owman, C., Sjöberg, N.-O. & Thorbert, G. (1981). Pregnancy is associated with extensive adrenergic degeneration in the uterus. An electronmicroscopic study in the guinea-pig. Neuroscience 6, 1119-1126.

Springall, D.R., Howard, L., Power, R.F., Khaghani, A., Rose, M., Yacoub, M. & Polak, J.M. (1988). Alterations in the innervation of the human respiratory tract following heart-lung trasplantation. Regul. Pept. 22, 433-433.

Steenberg, P.H., Hoppener, J.WM., Zandberg, J., Lips, C.JM. & Jansz, H.S. (1985). A second human calcitonin/CGRP gene. FEBS Lett. 183, 403-407.

Stephenson, J.A., Burcher, E. & Summers, R.J. (1986). Autoradiographic demonstration of endothelium-dependent ^{125}I-Bolton-Hunter substance P binding to dog carotid artery. Eur. J. Pharmacol. 124, 377-378.

Stephenson, J.A. & Summers, R.J. (1987). Autoradiographic analysis of receptors on vascular endothelium. Eur. J. Pharmacol. 134, 35-43.

Sternbach, Y., Greenberg-Ofrath, N., Flechner, I. & Schuldiner, S. (1990). Identification and purification of a functional amine transporter from bovine chromaffin granules. J. Biol. Chem. 265, 3961-3966.

Sternini, C. & Brecha, N. (1985). Distribution and localization of neuropeptide Y and tyrosine hydroxylase-immunoreactivity in the guinea-pig heart. Cell Tissue Res. 241, 93-102.

Stevens, L.M. & Landis, S.C. (1987). Development and properties of the secretory response in rat sweat glands: relationship to the induction of cholinergic function in sweat gland innervation. Dev. Biol. 123, 179-190.

Stevens, L.M. & Landis, S.C. (1988). Developmental interactions between sweat glands and the sympathetic neurons which innervate them: effects of delayed innervation on neurotransmitter plasticity and gland maturation. Dev. Biol. 130, 703-720.

Stevens, L.M. & Landis, S.C. (1990). Target influences on neurotransmitter choice by sympathetic neurons developing in the anterior chamber of the eye. Dev. Biol. 137, 109-124.

Stewart-Lee, A.L. & Burnstock, G. (1989). Actions of tachykinins on the rabbit mesenteric artery: substance P and [Glp^{6},L-Pro^{9}]SP$_{6-11}$ are potent agonists for endothelial neurokinin-1 receptors. Br. J. Pharmacol. 97, 1218-1224.

Stjernquist, M., Håkanson, R., Leander, S., Owman, C., Sundler, F. & Uddman, R. (1983). Immunohistochemical localization of substance P, vasoactive polypeptide and gastrin-releasing peptide in vas deferens and seminal vesicles, and the effect of these and eight other neuropeptides on resting tension and neurally evoked contractile activity. Regul. Pept. 7, 67-86.

Stjernquist, M., Alm, P., Ekman, R., Owman, C., Sjöberg, N.-O. & Sundler, F. (1985). Levels of neural vasoactive intestinal polypeptide in rat uterus are markedly changed in association with pregnancy as shown by immunocytochemistry and radioimmunoassay. Biol. Reprod. 33, 157-163.

Stjerschantz, J., Gregerson, D., Bauscher, L. & Sears, M. (1982). Enzyme-linked immuno-sorbent assay of substance P: a study of the eye. J. Neurochem. 38, 1323-1328.

Stjärne, L., Lundberg, J.M. & Åstrand, P. (1986). Neuropeptide Y - a cotransmitter with noradrenaline and adenosine 5'-triphosphate in the sympathetic nerves of the mouse vas deferens? A biochemical, physiological and electropharmacological study. Neuroscience 18, 151-166.

Stone, D.K., Crider, B.P., Südhof, P. & Xie, X.-S. (1989). Vacuolar proton pumps. J. Bioenerg. Biomembr. 21, 605-620.

Stone, R.A., Laties, A.M. & Brecha, N.C. (1982). Substance P-like immunoreactive nervesin the anterior segment of the rabbit, cat and monkey eye. Neuroscience 7, 2459-2468.

Stricker, S. (1876). Untersuchungen uber die Gefasswurzeln des Ischiadicus. Stitz. Ber. Kaiserl. Akad. Wiss. (Wein) 3, 173-185.

Suhonen, J., Koistinaho, J. & Hervonen, A. (1991). Histochemistry of sympathetic neurons allotransplanted from young and aged mice to the submandibular gland. Exp. Neurol. 112, 104-111.

Suigiura, M., Inagami, T., Hare, G.M.T. & Johns, J.A. (1989). Endothelin action: inhibition by a protein kinase C inhibitor and involvement of phosphoinositols. Biochem. Biophys. Res. Commun. 158, 170-176.

Sundler, F., Alumets, J. & Brodin, E. (1977a). Perivascular substance P-immunoreactive nerves in tracheobronchial tissue. In: Substance P. Eds. U.S. Von Euler & B. Pernow, Raven Press, New York. pp. 271-273.

Sundler, F., Alumets, J. & Håkanson, R. (1977b). VIP innervation of the gallbladder. Gastroenterology 72, 1375-1377.

Sundler, F., Moghimzadeh, E., Håkanson, R., Eklund, M. & Emson, P.C. (1983). Nerve fibres in the gut and pancreas of the rat displaying neuropeptide Y-immunoreactivity. Intrinsic and extrinsic origin. Cell Tissue Res. 230, 487-493.

Suzuki, N., Hardebo, J.E. & Owman, C. (1988). Origins and pathways of cerebrovascular vasoactive intestinal polypeptide-positive nerves in the rat. J. Cereb. Blood Flow Metab. 8, 697-712.

Suzuki, N., Hardebo, J.E. & Owman, C. (1989). Origins and pathways of cerebrovascular nerves storing substance P and calcitonin gene-related peptide in rat. Neuroscience 31, 427-438.

Suzuki, Y., McMaster, D., Lederis, K. & Rorstad, O.P. (1984). Characterization of the relaxant effects of vasoactive intestinal peptide (VIP) and PHI on isolated brain arteries. Brain Res. 322, 9-21.

Suzuki, Y., McMaster, D., Huang, M., Lederis, K. & Rorstad, O.P. (1985). Characterization of functional receptors for vasoactive intestinal peptide in bovine cerebral arteries. J. Neurochem. 45, 890-899.

Suzuki, Y., Shihuya, M., Ikegaki, I., Satoh, S., Takayasu, M. & Asona, T. (1988). Effects of neuropeptide Y on canine cerebral circulation. Eur. J. Pharmacol. 146, 271-277.

Takami, K., Kawai, Y., Uchida, S., Tohyama, M., Shiotani, Y., Yoshida, H., Emson, P.C., Girgis, S., Hillyard, C.J. & McIntyre, J. (1985a). Effect of calcitonin gene-related peptide on contraction of striated muscle in the mouse. Neurosci. Lett. 60, 227-230.

Takami, K., Kawai, Y., Shiosaka, S., Lee, Y., Girgis, S., Hillyard, C.J., Macintyre, I., Emson, P.C. & Tohyama, M. (1985b). Immunohistochemical evidence for the coexistence of calcitonin gene-related peptide and choline acetyltransferase-like immunoreactivity in neurons of the rat hypoglossal, facial and ambiguous nuclei. Brain Res. 328, 386-389.

Takeuchi, T., Gumucio, D., Meisler, M., Minth, C., Dixon, J., Shown, T. & Yamadi, T. (1985). Genes encoding pancreatic polypeptide (PP) and neuropeptide Y are on human chromosome 17 and chromosome 7. Gastroenterology 88, 1607-1607.

Tare, M., Parkington, H.C. & Coleman, H.A. (1988). Changes in the adrenergic innervation of the main uterine artery during pregnancy in guinea pigs. Proc. Austr. Physiol. Pharmacol. Soc. 19, 33P.

Tatemoto, K. (1982b). Neuropeptide Y: a complete amino acid sequence of the brain peptide. Proc. Natl. Acad. Sci. U. S. A. 79, 5485-5489.

Tatemoto, K., Carlquist, M. & Mutt, V. (1982a). Neuropeptide Y - a novel brain peptide with structural similarities to peptide YY and pancreatic polypeptide. Nature 296, 659-660.

Tatemoto, K., Lundberg, J.M., Jornvall, H. & Mutt, V. (1985). Neuropeptide K: isolation, structure and biological activities of a novel brain tachykinin. Biochem. Biophys. Res. Commun. 128, 947-957.

tenTuscher, M.P.M., Klooster, J., van der Want, J.J.L., Lamers, W.P.M.A. & Vrenson, G.F.J.M. (1989). The allocation of nerve fibres to the anterior eye segment and peripheral ganglia of rats. I. The sensory innervation. Brain Res. 494, 95-104.

tenTuscher, M.P.M., Klooster, J., Baljet, B., Van der Werf, F. & Vrensen, G.F.J.M. (1990). Pre- and post-ganglionic nerve fibres of the pterygopalatine ganglion and their allocation to the eyeball of rats. Brain Res. 517, 315-323.

Terenghi, G., Polak, J.M., Probert, L., McGregor, G.P., Ferri, G.L., Blank, M.A., Butler, J.M., Unger, W.G., Cole, D.F. & Bloom, S.R. (1982). Mapping, quantitative distribution and origin of substance P and VIP-containing nerves in the uvea of guinea pig eye. Histochemistry 75, 399-417.

Terenghi, G., Polak, J.M., Ghatei, M.A., Mulderry, P.K., Butler, J.M., Unger, W.G. & Bloom, S.R. (1985). Distribution and origin of CGRP-immunoreactivity in the sensory innervation of the mammalian eye. J. Comp. Neurol. 233, 505-516.

Terenghi, G., Zhang, S.-Q., Unger, W.G. & Polak, J.M. (1986a).

Morphological changes of sensory CGRP-immunoreactive and sympathetic nerves in peripheral tissue following chronic denervation. Histochemistry 86, 89-95.

Terenghi, G., Polak, J.M., Rodrigo, J., Mulderry, P.K. & Bloom, S.R. (1986b). Calcitonin gene-related peptide-immunoreactive nerves in the tongue, epiglottis and pharynx of the rat: Occurrence, distribution and origin. Brain Res. 365, 1-14.

Thayer, M.J., Tapscott, S.J., Davis, R.L., Wright, W.E., Lassar, A.B. & Weintraub, H. (1989). Positive autoregulation of the myogenic determination gene myoD1. Cell 58, 241-248.

Thoenen, H. & Barde, Y.A. (1980). Physiology of nerve growth factor. Physiol. Rev. 60, 1284-1335.

Thorbert, G., Alm, P. & Rosengren, E. (1978). Cyclic and steroid-induced changes in adrenergic neurotransmitter level of guinea pig uterus. Acta Obstet. Gynecol. Scand. 57, 45-48.

Thorbert, G., Alm, P., Björklund, A., Owman, C. & Sjöberg, N.-O. (1979). Adrenergic innervation of the human uterus. Disappearance of the transmitter-forming enzymes during pregnancy. Am. J. Obstet. Gynecol. 135, 223-226.

Toda, N. (1977). Actions of bradykinin on isolated cerebral and peripheral arteries. Am. J. Physiol. 232, H267-H274.

Toda, N. & Fujita, Y. (1973). Responsiveness of isolated cerebral arteries to serotonin, norepinephrine and transmural electrical stimulation. Circ. Res. 33, 98-104.

Todd, M.E. (1980). Development of adrenergic innervation in rat peripheral vessels: a fluorescent microscopic study. J. Anat. 131, 121-133.

Todd, M.E., Laye, C.G. & Osborne, D.N. (1983). The dimensional characteristics of smooth muscle in rat blood vessels. A computer assisted analysis. Circ. Res. 53, 319-331.

Tranzer, J.P.S. & Thoenen, H. (1967). Electronmicroscopic localization of 5-hydroxydopamine (345-trihydroxy-phenyl-ethylamine) a new 'false' sympathetic transmitter. Experientia 25, 743-745.

Tregear, G., Niall, H.D., Potts, J.T., Leeman, S.E. & Chang, M.M. (1971). Synthesis of substance P. Nature,New Biol. 232, 87-89.

Tsai, S.H., Tew, J.M., McLean, J.H. & Shipley, M.T. (1988). Cerebral arterial innervation by nerve fibres containing calcitonin gene-related peptide (CGRP): I. Distribution and origin of CGRP perivascular innervation in the rat. J. Comp. Neurol. 271, 435-444.

Tsukada, T., Fink, J.S., Mandel, G. & Goodman, R.H. (1987). Identification of a region in the human vasoactive intestinal

polypeptide gene responsible for regulation by cyclic AMP. J. Biol. Chem. 262, 8743-8747.

Tsukada, T., Horovitch, S., Montminy, M., Mandel, G. & Goodman, R.H. (1985). Structure of the human vasoactive intestinal polypeptide gene. DNA 4, 293-300.

Törnebrandt, K., Nobin, P. & Owman, C. (1987). Contractile and dilatory action of neuropeptides on isolated human mesenteric blood vessels. Peptides 8, 251-256.

Uddman, R. & Sundler, F. (1979). Vasoactive intestinal polypetide nerves in human upper respiratory tract. Otorhinolaryngol. 41, 221-226.

Uddman, R., Alumets, J., Håkanson, R., Lorèn, I. & Sundler, F. (1980a). Vasoactive intestinal peptide (VIP) occurs in nerves of the pineal gland. Experientia 36, 1119-1120.

Uddman, R., Malm, L. & Sundler, F. (1980b). The origin of vasoactive intestinal polypeptide (VIP) nerves in the feline nasal mucosa. Acta Otolaryngol. 89, 152-156.

Uddman, R., Alumets, J., Edvinsson, L., Håkanson, R. & Sundler, F. (1981). VIP nerve fibres around peripheral blood vessels. Acta Physiol. Scand. 112, 65-70.

Uddman, R., Ninoyu, O. & Sundler, F. (1982). Adrenergic and peptidergic innervation of cochlear blood vessels. Arch Otorhinolaryngol. 236, 7-14.

Uddman, R., Edvinsson, L., Owman, C. & Sundler, F. (1983). Nerve fibres containing gastrin-releasing peptide around pial vessels. J. Cereb. Blood Flow Metab. 3, 386-390.

Uddman, R., Sundler, F. & Emson, P.C. (1984). Occurrence and distribution of neuropeptide Y-immunoreactive nerves in the respiratory tract and middle ear. Cell Tissue Res. 237, 321-327.

Uddman, R., Edvinsson, L., Ekman, R., Kingman, T. & McCulloch, J. (1985). Innervation of the feline cerebral vasculature by nerve fibres containing calcitonin gene-related peptide: trigeminal origin and co-existence with substance P. Neurosci. Lett. 62, 131-136.

Uddman, R., Ekman, E., Edvinsson, L., Håkanson, R. & Sundler, F. (1986). Calcitonin gene-related peptide (CGRP): perivascular distribution and vasodilatory effects. Regul. Pept. 15, 1-23.

Ueda, N., Muramatsu, J. & Fujiwara, M. (1985). Dual effects of dynorphin (1-13) on cholinergic and substance Pergic transmission in the rabbit iris sphincter muscle. J. Pharmacol. Exp. Ther. 232, 545-550.

Uemura, Y., Sugimoto, T., Okamoto, S., Handa, H. & Mizuno, N. (1986).

Changes of vasoactive intestinal polypeptide-like immunoreactivity in cerebrovascular nerve fibres after subarachnoid hemorrhage: an experimental study in the dog. Neurosci. Lett. 71, 137-141.

Untaberg, A., Wahl, M. & Baethmann, A. (1984). Effects of bradykinin on permeability and diameter of pial vessels in vivo. J. Cereb. Blood Flow Metab. 4, 574-585.

Vaalasti, A., Linnoila, I. & Hervonen, A. (1980). Immunohistochemical demonstration of VIP, [Met^5]- and [Leu^5]-enkephalin immunoreactive nerve fibres in the human prostate and vesicles. Histochemistry 66, 89-98.

Vanhoutte, P.M. & Rimele, T.J. (1983). Role of endothelium in the control of vascular smooth muscle function. J. Physiol. (Paris) 78, 681-686.

Vanhoutte, P.M., Cohen, R.A. & Van Nueten, J.M. (1984). Serotonin and arterial vessels. J. Cardiovasc. Pharmacol. 6, S421-S428.

Vanhoutte, P.M. & Miller, V.M. (1985). Heterogeneity of endothelium-dependent responses in mammalian blood vessels. J. Cardiovasc. Pharmacol. 7, Suppl.3, S12-S23.

Vernadakis, A. & Kentroti, S. (1990). Opioids influence neurotransmitter phenotypic expression in chick embryonic neuronal cultures. J. Neurosci. Res. 26, 342-348.

Vincent, S.R., Johansson, O., Hökfelt, T., Skirboll, L., Elde, R.P., Terenius, L., Kimmel, J. & Goldstein, M. (1983). NADPH-diaphorase: an elective histochemical marker for striatal neurons containing both somatostatin- and avian pancreatic polypeptide (APP)-like immunoreactivities. J. Comp. Neurol. 217, 252-263.

von Euler, U.S. & Gaddum, J.H. (1931). An unidentified depressor substance in certain tissue extracts. J. Physiol. 72, 74-87.

Voyvodic, J.T. (1989). Peripheral target regulation of dendritic geometry in the rat superior cervical ganglion. J. Neurosci. 9, 1997-2010.

Wahl, M., Young, A.R., Edvinsson, L. & Wagners, F. (1983). Effects of bradykinin on pial arteries and arterioles in vitro and in situ. J. Cereb. Blood Flow Metab. 3, 231-237.

Wahlestedt, C., Edvinsson, L., Ekblad, E. & Håkanson, R. (1985). Neuropeptide Y potentiates noradrenaline-evoked vasoconstriction. J. Pharmacol. Exp. Ther. 234, 735-745.

Wahlestedt, C., Yanaihara, N. & Håkanson, R. (1986). Evidence for different pre- and postjunctional receptors for neuropeptide Y and related peptides. Regul. Pept. 13, 307-318.

Wallace, B.G. (1990). Inhibition of agrin-induced acetylcholine-

receptor aggregation by heparin, heparan sulphate, and other polyanions. J. Neurosci. 10, 3576-3582.

Wallace, B.G., Qu, Z. & Huganir, R.L. (1991). Agrin induces phosphorylation of the nicotinic acetylcholine receptor. Neuron 6, 869-878.

Wallengren, J. & Håkanson, R. (1987). Effects of substance P, neurokinin A and calcitonin gene-related peptide in human skin and their involvement in sensory nerve-mediated responses. Eur. J. Pharmacol. 123, 267-273.

Wanaka, A., Matsuyama, T., Yoneda, S., Kimura, K., Kamada, T., Girgis, S., Macintyre, I., Emson, P.C. & Tohyama, M. (1986). Origins and distribution of calcitonin gene-related peptide containing nerves in the wall of cerebral arteries of the guinea pig with special reference to the coexistence with substance P. Brain Res. 369, 185-192.

Warner, T., de Nucci, G. & Vane, J.R. (1988). Release of EDRF by endothelin in the rat isolated perfused mesentery. Br. J. Pharmacol. Proc. 95 (Suppl.), 723P.

Watanabe, M., Yanaihara, N. & Kondo, H. (1991). Partial down-regulation at post-transcriptional level of the gene expression for prepro-enkephalin in the superior cervical ganglion of the maturing rat. Dev. Brain Res. 59, 113-116.

Weihe, E. (1991). Peripheral innervation of the heart. In: Silent Ischemia. Eds. Th. Von Armin & A. Maseri, Steinkorff, Darmstadt. pp. 7-18.

Weihe, E., Reinecke, M. & Forsmann, W.G. (1984). Distribution of vasoactive intestinal polypeptide-like immunoreactivity in the mammalian heart. Interrelation with neurotensin and substance P-like immunoreactive nerves. Cell Tissue Res. 236, 527-537.

Westfall, T.C., Carpentier, S., Chen, X., Beinfeld, M.C., Naes, L. & Meldrum, M.J. (1987). Prejunctional and postjunctional effects of neuropeptide Y at the noradrenergic neuroeffector junction of the perfused mesenteric arterial bed of the rat. J. Cardiovasc. Pharmacol. 10, 716-722.

Westlind-Danielsson, A., Undén, A., Abens, J., Andell, S. & Bartfai, T. (1987). Neuropeptide Y receptors and the inhibition of adenylate cyclase in the human frontal and temporal cortex. Neurosci. Lett. 74, 237-242.

Wexler, B.C. & True, C.W. (1963). Carotid and cerebral arteriosclerosis in the rat. Circ. Res. 12, 659-666.

Whalley, E.T., Fritz, H. & Geiger, R. (1983). Kinin receptors and angiotensin converting enzyme in rabbit basilar arteries. Naunyn Schmiedebergs Arch. Pharmacol. 234, 296-301.

Whalley, E.T., Lye, R. & Amure, Y.O. (1984). Effects of kinins on human cerebral vessels. In: Proceedings of the 9th IUPHAR Congress, London. P657.

Whalley, E.T., Amure, Y.O. & Lye, R.H. (1987). Analysis of the mechanism of action of bradykinin on human basilar artery in vitro. Naunyn Schmiedebergs Arch. Pharmacol. 355, 433-437.

Wharton, J. & Gulbenkian, S. (1987). Peptides in the mammalian cardiovascular system. Experientia 43, 821-832.

Wharton, J., Polak, J.M., McGregor, G.P., Bishop, A.E. & Bloom, S.R. (1981). The distribution of substance P-like immunoreactive nerves in the guinea-pig heart. Neuroscience 6, 2193-2204.

Wharton, J., Gulbenkian, S., Varndell, I.M., Merighi, A. & Polak, J.M. (1986a). Calcitonin gene-related peptide and substance P coexist in secretory vesicles of guinea-pig sensory neurons. Soc. Neurosci. Abstr. 12, 302.

Wharton, J., Gulbenkian, S., Mulderry, P.K., Ghatei, M.A., McGregor, G.P., Bloom, S.R. & Polak, J.M. (1986b). Capsaicin induces a depletion of calcitonin gene-related peptide (CGRP)-immunoreactive nerves in the cardiovascular system of the guinea-pig and rat. J. Auton. Nerv. Syst. 16, 289-309.

Wharton, J., Su, H.C. & Polak, J.M. (1986c). CGRP-immunoreactive nerves in the genitalia of the female rat originate from dorsal root ganglia T11-L3 and L6-S1: a combined immunocytochemical and retrograde tracing study. Neurosci. Lett. 69, 13-18.

Wiesenfeld-Hallin, Z., Hökfelt, T., Lundberg, J.M., Forsmann, W.G., Reinecke, M., Tschopp, F.A. & Fischer, J.A. (1984). Immunoreactive calcitonin gene-related peptide and substance P coexist in sensory neurons of the spinal cord and interact in spinal behavioural responses of the rat. Neurosci. Lett. 52, 199-204.

Wikkelsö, C., Fahrenkrug, J., Blomstrand, C. & Johansson, B.B. (1985). Dementia of different etiologies: vasoactive intestinal polypeptide in CSF. Neurology 35, 592-601.

Wilson, S.P., Abou-Doma, M.M., Chang, K.J. & Viveros, O.H. (1981). Reserpine increases opiate-like peptide content and tyrosine hydroxylase activity in adrenal medullary chromaffin cells in culture. Neuroscience 6, 71-79.

Winquist, R.J., Bunting, P.B. & Schofield, T.L. (1985). Blockade of endothelium-dependent relaxation by the amiloride analog dichlorobenzamil: possible role of endothelium-derived relaxing factor. J. Pharmacol. Exp. Ther. 235, 644-650.

Wong, J. & Oblinger, M.M. (1991). NGF rescues substance P expression but not neurofilament or tubulin gene expression in axotomized

sensory neurones. J. Neurosci. 11, 543-552.

Wong, V. & Kessler, J.A. (1987). Solubilization of a membrane factor that stimulates level of substance P and choline acetyltransferase in sympathetic neurons. Proc. Natl. Acad. Sci. U. S. A. 84, 8726-8729.

Wright, L.L. & Luebke, J.I. (1989). Somatostatin-, vasoactive intestinal polypeptide- and neuropeptide Y-like immunoreactivity in eye- and submandibular gland-projecting sympathetic neurons. Brain Res. 494, 267-275.

Wu, R., McKenna, P.G. & McAfee, D.A. (1991). Age-related changes in the synaptic plasticity of rat superior cervical ganglia. Brain Res. 542, 324-329.

Wyatt, S., Shooter, E.M. & Davies, A.M. (1990). Expression of the NGF receptor gene in sensory neurons and their cutaneous targets prior to and during innervation. Neuron 4, 421-427.

Yaksh, T.L., Jessel, T.M., Gamse, R., Mudge, A.W. & Leeman, S.E. (1980). Intrathecal morphine inhibits substance P release from mammalian spinal cord in vivo. Nature 286, 155-157.

Yamamori, T., Fukada, K., Aebersold, R., Korsching, S., Fann, M. & Patterson, P.H. (1990). The cholinergic neuronal differentiation factor is identical to leukemia inhibitory factor. Science 246, 1412-1416.

Yamamoto, K., Matsuyama, T., Shiosaka, S., Inagaki, S., Senba, E., Shimizu, Y., Ishimoto, I., Hayakawa, T., Matsumoto, M. & Tohyama, M. (1983). Overall distribution of substance P-containing nerves in the wall of cerebral arteries of the guinea-pig and its origin. J. Comp. Neurol. 215, 421-426.

Yanagisawa, A., Osborne, J.A., Stahl, G.L. & Lefer, A.M. (1987). Coronary vascular reactivity of synthetic atrial natriuretic factor in isolated vascular preparations. J. Cardiovasc. Pharmacol. 10, 320-326.

Yanagisawa, M., Kurihara, H., Kimura, S., Tomobe, Y., Kobayashi, M., Mitsui, Y., Yazaki, Y., Goto, K. & Masaki, T. (1988). A novel potent vasoconstrictor peptide produced by vascular endothelial cells. Nature 332, 411-415.

Yodlowski, M.L., Fredieu, J.R. & Landis, S.C. (1984). Neonatal 6-hydroxydopamine treatment eliminates cholinergic sympathetic innervation and induces sensory sprouting in rat sweat glands. J. Neurosci. 4, 1535-1548.

Zawadzki, J.V., Furchgott, R.F. & Cherry, P. (1981) The obligatory role of endothelial cells in the relaxation of arterial smooth muscle. Fedn. Proc. 40, 689.