THE DEVELOPMENT AND INTERACTION OF CATECHOLAMINERGIC AND PEPTIDERGIC PERIVASCULAR NERVE FIBRES IN THE RAT MESENTERIC VASCULAR BED

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The Development And Interaction Of Catecholaminergic And Peptidergic Perivascular Nerve Fibres In The Rat Mesenteric Vascular Bed.

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ABSTRACT

The hypothesis that different fibre systems interact during the development of innervation of the mesenteric vascular bed was examined. It is proposed that this interaction may be essential to the development of each innervating system and in turn affect the development of the vasculature. Immunohistochemistry was used to allow differentiation of different fibre types. Electron microscopy was used to determine relationships between axons. Although it is still premature to draw a final conclusion, the results of the present studies suggest that peptide-containing and noradrenergic nerves follow different patterns of development and that they interact during development. It appears as if peptidergic innervation limits the proliferation of catecholaminergic fibres, whereas catecholaminergic fibres are necessary for the full development of peptidergic fibres. The peptidergic fibres are normally found in large bundles whereas the catecholaminergic fibres are found in small bundles. A mixing of these catecholaminergic and peptidergic fibres can occur. Colocalization of peptidergic and catecholaminergic substances in the same fibre also appears to be possible.

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3. INTRODUCTION

Over the past few years there have been several reports concerning vascular innervation that modify and extend some of the classical concepts of organization of the autonomic nervous system proposed by Langley in 1921 (Langley, 1921). We can no longer think mainly in terms of antagonistic sympathetic adrenergic and parasympathetic cholinergic nervous control of tissues but we must recognize the involvement of a multiplicity of neurotransmitters and other control mechanisms, including co-transmission. Biochemical and molecular biological techniques have defined a large number of bioactive substances in the central and peripheral nervous systems. In addition to earlier described low molecular weight compounds such as acetylcholine, catecholamines, and certain amino acids, which are considered to act as neurotransmitters, an increasing number of peptides ranging in size from a few up to forty or so amino acids have been identified in neurons (Hokfelt *et al*, 1987). Physiological and biochemical demonstrations of peptides in well defined neuronal systems in the nervous system, indicate that in some systems these peptides may have a transmitter role.

3.1. Vascular Innervation

There is a wide variation in the pattern and density of innervation of different blood vessels, depending to a large extent on their physiological role in relation to the particular organ system they supply (Burnstock, 1975). However, some generalizations can be made. The nerve bundles supplying the vasculature travel in the outer adventitia and give off smaller bundles to a deeper plexus at the adventitial/medial border. The nerve fibre bundles are complex in that a variable number of fibres may be present and these may include fibres containing more than one transmitter or neuromodulator substance. The cells of origin of these fibres may be located in various ganglia including the sympathetic ganglia, the trigeminal ganglia, the coeliac ganglia, and the dorsal root ganglia.

As the fibres form a plexus at the adventitial/medial border, they form structures specialized for the release of neurotransmitter substances. The neuromuscular distance can be quite considerable. In the rabbit pulmonary artery, an elastic artery, the closest neuromuscular distance is 0.4 micrometers. In the rabbit ear artery the mean closest distance is 0.5 micrometers. Neuromuscular separation in the rat small mesenteric arteries averages about 0.5 micrometers and in arterioles the range is 0.1 - 0.4 micrometers. In the rabbit pulmonary vein the separation is of the order of 0.15 micrometers (Bevan and Su, 1973). Although the cleft width becomes narrower the smaller the vessel, only rarely have separations less than 0.1 micrometers approaching those in the vas deferens or at the skeletal neuromuscular junction been reported (Bevan and Su, 1973). In some situations the nerve fibres pass into the media, as in the rabbit saphenous artery (Bevan and Purdy, 1973). There is some evidence to suggest that the degree of penetration is largely related to the wall thickness. Thus in large animals such as dog, pig, sheep, cow, seal, and man, nerves commonly penetrate the media of large elastic and muscular arteries, whereas even the largest vessels in mouse, rat, guinea pig, and even rabbit are rarely penetrated. It must also be realized that many of the nerves found inside the media of very large vessels accompany the vasa vasorum (Norberg, 1967).

(a) Innervation By The Catecholaminergic (Sympathetic) System

Most large elastic arteries are sparsely innervated by the sympathetic nerve fibres. However there is considerable species variation in the density of innervation. For example, few if any adrenergic nerves can be seen in the rat aorta; in the cat, dog, calf, pig, and Rhesus monkey there is moderate innervation; while the guinea pig aorta has a relatively dense nerve supply (Burnstock, 1975). As the arteries become smaller, the density of innervation increases, with a peak in small arteries and large arterioles. In general, muscular arteries are more heavily innervated than elastic arteries. Intimal cushions, at branching sites of

arteries are also densely innervated. Most precapillary arterioles are not innervated but some precapillary sphincters are richly supplied by nerve fibres. The coronary sphincters are densely supplied. It is usually assumed that capillaries are not innervated and most collecting venules and small veins have an extremely sparse sympathetic nerve supply. Most large veins are not well supplied by the adrenergic nervous system but some medium sized muscular veins have a rich innervation.

It is generally assumed that the most important motor components in autonomic control of the vasculature are the sympathetic nerves which have their origin in the pre- or para-vertebral ganglia of the sympathetic nervous system or in central catecholaminergic neurons (Edvinsson *et al*, 1973).

The catecholaminergic innervation of the mesenteric vascular bed will be examined in this thesis. Tyrosine hydroxylase (TH) was used in this study, as a marker for catecholamines. TH is an enzyme involved in the synthesis of catecholamines, such as adrenaline and noradrenaline.

(b) Peptidergic Innervation

In this thesis, the innervation of the mesenteric vascular bed of the rat by peptidergic nerve fibres will also be examined. The literature concerning peptidergic innervation will now be reviewed. The peptides concerned are calcitonin gene-related peptide (CGRP), vasoactive intestinal polypeptide (VIP), neuropeptide Y (NPY), and substance P (SP).

3.2. Calcitonin Gene-Related Peptide (CGRP)

CGRP is a thirty-seven amino acid peptide encoded in the calcitonin gene. It was discovered by techniques of molecular genetics (Rosenfeld *et al*, 1983). The calcitonin gene was found to encode two different mRNAs with identical 5' sequences but distinct 3' sequences. The mRNAs encode either the 14,500 MW calcitonin precusor protein, which is proteolyzed to yield calcitonin and two other peptides or the 16,000 MW protein which is the predicted translation product of CGRP mRNA. The mRNA for CGRP predominates in the brain whilst calcitonin mRNA predominates in the thyroid C cells.

3.2.1. Distribution Of CGRP

(a) Distribution In The Vascular System

In the guinea pig and the rat, CGRP containing nerve fibres are present in all regions of the heart (Mulderry *et al*, 1985). In the guinea pig CGRP fibres are more numerous in the epicardium, than in the myocardium. The auricles and the atria contain a moderate number of CGRP fibres, whereas the ventricles have only few fibres. CGRP fibres are scarce around the coronary blood vessels, whereas the cartoid arteries and the thoracic aorta receive numerous CGRP nerve fibres. This supply diminishes as the aorta descends into the abdomen. The arteries to the limbs; the brachial and the femoral, contain a few CGRP fibres (Uddman *et al*, 1986).

In the human heart, relatively few fibres appear to display CGRP immunoreactivity (Wharton and Gulbenkian, 1987).

CGRP-immunoreactive nerve fibres can be found in all regions of cerebral circulation of the rat, rabbit, and guinea pig (Saito and Goto, 1986). In cerebral arteries of guinea pigs, CGRP fibres are densely distributed in the circle of Willis, middle cerebral and basilar arteries. In rabbits, a moderate number of CGRP nerve fibres were found in the circle of Willis and middle cerebral.

Whereas, a few fibres are found in the basilar artery. In rats, a moderate number of fibres can be found in the circle of Willis, basilar and middle cerebral arteries (Saito and Goto, 1986).

In the gastrointestinal tract of the guinea pig muscular arteries such as the superior and inferior mesenteric arteries are densely innervated by CGRP nerve fibres. Also, small arteries and arterioles in the gut wall are richly innervated. In the guinea pig's genito-urinary tract, a moderate supply of CGRP fibres are seen around the uterine, ovarian, and testicular arteries. Large and medium sized arteries in the uterine cervix, the urinary bladder, and small blood vessels running close to the vas deferens and seminal vesicle have a moderate supply of CGRP. Few CGRP fibres innervate the parenchyma of the liver, kidney, and pancreas of the guinea pig. A very scarce supply of CGRP fibres accompanies small blood vessels of the guinea pig in skeletal muscle (Uddman *et al*, 1986).

(b) Other Distributions In The Peripheral Nervous System

CGRP is present throughout the digestive system of the rat, guinea pig (Ghatei *et al*, 1984), monkey and cat (Goodman and Iversen, 1986). CGRP has also been found in the rat epidermis where levels are highest in the nose and lowest in the skin of the rat's back (Goodman and Iversen, 1986).

CGRP fibres can be found to be associated with human airway smooth muscle and the smooth and connective tissue below the epithelium and in the adventitia of the airways. The density of immunoreactive CGRP nerves is less in the smaller airways, such as terminal bronchioles than in the larger airways (Palmer *et al*, 1987).

Human sweat glands are well supplied with sensory axons containing

CGRP (Gibbins, Wattchow, and Coventry, 1987). It has been also localized in the cholinergic sympathetic fibres that innervate eccrine glands in the rat (Landis and Fredieu, 1986).

(c) Distribution In The Central Nervous System

In the central nervous system, a number of studies have been carried out on rat (Okimura *et al*, 1987; Venesio, Mulatero, and Fasolo, 1987; Mason *et al*, 1984; Wimalwansa, Emson, and MacIntyre, 1987), human (Inagaki *et al*, 1986; Tschopp *et al*, 1985), and several other species (Gibson *et al*, 1984).

CGRP immunoreactivity can be found throughout the entire spinal cord of man, marmoset, horse, pig, cat, guinea pig, mouse, rat, and frog (Gibson *et al*, 1984).

Wimalawansa and his co-workers reported a low density of CGRP in the trigeminal nucleus of the rat, as well as in the trigeminal, and dorsal root ganglia (Wimalawansa, Emson, and MacIntyre, 1987). Rosenfeld stated that approximately one precent of all trigeminal ganglia neurons are CGRP-producing and contain approximately 4,000 to 20,000 copies of CGRP-mRNA per cell. They found no calcitonin mRNA in any brain region of the rat (Rosenfeld *et al*, 1983).

The extensive distribution of CGRP-like immunoreactive nerve cell bodies and fibres in rat brain was first described in detail by Kawai (Kawai *et al*, 1985). CGRP-positive cell bodies were found in almost all areas of the rat's brain, including in the Purkinje cells of the cerebellum, and in sensory and motor nuclei of cranial nerves.

3.2.2. Actions On Vascular Smooth Muscle

In vitro, CGRP has been discovered to be a strong relaxant, of vascular smooth muscle (Uddman *et al*, 1986). It has been shown to dilate both pial and peripheral vessels of rabbit, cat, and pial vessels of man (Hanko *et al*, 1985). As well, it can cause vasodilatation of vessels, such as the basilar, gastroepiploic, femoral, coronary, and cerebral arteries of the guinea pig (Uddman *et al*, 1986).

There is some controversy, as to whether or not this vasodilatation depends on the presence of endothelial cells and on their capability to release an endothelial dervived factor (EDRF) involved in the relaxation (Brain *et al*, 1985). It appears as if this dependency is species and region specific. Studies on brain vessels in the rabbit, cat, and man have shown that these vessels do not appear to display an endothelium dependency. CGRP can cause a strong dilatation whether or not the endothelium is present (Hanko *et al*, 1985). However, relaxation of the rat aorta, in vitro, seems to depend on the presence of endothelial cells (Brain *et al*, 1985).

It also has been suggested that the vasodilatation seen may be a result of the activation of adenylate cyclase (Uddman *et al*, 1986) and subsequent increase in cyclic adenosine monophosphate. The increase of the second messenger system causes a relaxation of the vascular smooth muscle.

3.2.3. Actions On Non-Vascular Smooth Muscle

CGRP is a potent constrictor of human airway passages and may have an important functional effect on airway control. It has been implicated in the pathogenesis of bronchial hyperresponsiveness and asthma. It appears as if this is a direct effect and does not involve the release of other pharmacological agents (Palmer *et al*, 1987).

Both rat and human CGRP have been reported to contract the guinea pig ileum longitudinal muscle, an effect which seems to be due to the release of endogenous histamine and acetylcholine (Tippins *et al*, 1984). It has also been

suggested that this contraction is due to the activation of cholinergic neurons (Bartho, Lembech, and Holzer, 1987).

CGRP has also been reported to relax smooth muscle of the rat duodenum (Maggi *et al*, 1986). A very low threshold concentration of CGRP is required for this relaxant effect (Bartho, Lembech, and Holzer, 1987).

Systemic administration of CGRP to the rat results in a dose dependent decrease in blood pressure and an increase in heart rate. Furthermore, CGRP selectivity changes regional organ blood flows. The most prominent of which is to increase coronary blood flow. This increase could be due to a direct vasodilatation and/or due to an increase in myocardial metabolic demand (Asimakis *et al*, 1987).

An intracerebroventricular injection of CGRP will cause an increase in mean blood pressure and a rise in plasma noreprinephine levels in the rat (McEwan *et al*, 1986).

3.2.4. Other Actions

Other biological effects of CGRP include its ability to inhibit gastric acid secretion in rats and dogs (Lenz *et al*, 1985). It also has the ability to decrease circulating levels of gastrin, gastric inhibitory peptide, and enteroglucagon. It can inhibit pepsin secretion (Kraenzlin *et al*, 1985) and stimulate amylase release from dispersed rat acini (Seifert *et al*, 1985). In vivo, however, CGRP inhibits pancreatic exocrine secretion in both dogs and man. CGRP also inhibits insulin release in mice (Pettersson *et al*, 1986) but fails to do so in man (Kraenzlin *et al*, 1985).

CGRP has also been called an 'anterograde' factor important in the biogenesis and maturation of the endplate postsynaptic membrane. It has been proposed to be responsible for the accumulation of acetylcholine receptor mRNA in synaptic regions (Fontaine *et al*, 1986).

CGRP can enhance the effects of other substances. It can potentiate the effect of substance P (to be discussed later), neurokinin A, and neurokinin B in rat skin. By inducing vasodilatation, CGRP can increase vascular leakage by releasing the mediators of inflammation (Gamse and Saria, 1985).

Another example of potentiation is when CGRP is administered with substance P. Substance P, when injected alone causes caudally directed biting and scratching in mice or rats, when administered in the spinal cord. This behavior is dose dependent and lasts for a few minutes. CGRP in doses up to twenty micrograms does not cause such an effect in rats when administered alone but when injected with substance P it causes an increase in the duration of biting and scratching. It has been suggested that this potentiation may be caused by the interaction of CGRP with an enzyme involved in substance P degradation (Le Greves *et al*, 1985).

Other actions of CGRP include its ability to inhibit bone resorption (Tippins *et al*, 1984), cause relaxation of the spleen, and to stimulate adenylate cyclase activity and plasminogen activator production in renal membranes and some renal cells (Tippins *et al*, 1984).

3.3. Vasoactive Intestinal Polypeptide (VIP)

VIP is a twenty-eight amino acid isolated orginally from the small intestine of hogs in 1970, by Said and Mutt (Said and Mutt, 1970). The peptide was prepared from a methanol extract of the intestine used for the preparation of secretin. Glycine and proline residues were found to be absent from the polypeptide. This distinguishes this isolated peptide from other vasoactive peptides, the kinins, and substance P. Like secretin and glucagon, VIP has a n-terminal histidine residue, but the lack of glycine and the presence of isoleucine clearly set it apart from these two hormones (Said and Mutt, 1970).

When VIP was first discovered it was thought to be limited to the

gastrointestinal tract. However, it has since been discovered, in high levels, in neural cell lines, and in mammalian brain (Giachetti *et al*, 1977).

3.3.1. Distribution Of VIP

(a) Distribution In The Vascular System

In the peripheral vascular bed, VIP immunoreactive nerve fibres are located in the adventitia, close to the media, and in the wall of large blood vessels, including the aorta and its main branches. Small vessels, such as vasa vasorum, found in the connective tissue surrounding main arteries, have a numerous supply of VIP fibres. Blood vessels of a larger size have a less dense innervation than smaller vessels. Vessels in the liver, spleen, and kidney are devoid of VIP fibres. The same is true for the coronary arteries. VIP fibres are rare around veins.

At first VIP was thought to be absent from heart tissue but it has since been found there (Said, 1984). It has been localized in nerves in the atria, in the sino-atria node and conducting tissues, and in the walls of coronary vessels.

(b) Distribution In The Peripheral Nervous System

VIP immunoreactive neurons in the peripheral nervous system were first described by Bryant and co-workers (Bryant *et al*, 1976), and Larsson and workers (Larsson *et al*, 1976). Both groups described VIP immunoreactive nerve terminals in the gastrointestinal tract. VIP immunoreactive fibres are present in all layers of the gastrointestinal tract in the rat and guinea pig (Hokfelt *et al*, 1982). Of particular significance are the dense networks of VIP immunoreactive fibres in the lamina propria. These fibres extend into the most superficial parts of this layer, for example, into the tips of the villi. In addition, high or moderate numbers of VIP immunoreactive fibres are found in the myenteric plexus, the submucous plexus, the circular muscle layer, the lamina muscularis mucosae.

These fibres often come very close to the surface, seemingly innervating the epithelium. Dense networks of VIP immunoreactive fibres are also present around Brunner's glands. The VIP immunoreactive fibres are sparse in the longitudinal muscle layers, except for the stomach, where larger numbers are found (Hokfelt *et al*, 1982).

VIP immunoreactive structures are present in numerous peripheral ganglia. In the guinea pig very dense networks of VIP immunoreactive fibres are observed in the prevertebral ganglia, particularly the coeliac-superior mesenteric ganglion complex and the inferior mesenteric ganglion (Hokfelt *et al*, 1977).

In the rat, VIP immunoreactive fibres are found in the same ganglia, as found in guinea pigs, with approximately the same distribution patterns, although their numbers are lower.

Numerous VIP immunoreactive fibres are present in all exocrine glands including salivary glands, sweat glands, pancreas, glands in the trachea and gastrointestinal wall, lacrimal glands, Harderian glands, and others (Hokfelt *et al*, 1982). In the pancreas, VIP immunoreactive fibres are seen in the exocrine part along blood vessels and around acini with very high concentrations, around the main pancreatic duct, as well as in local ganglia, which also contain VIP immunoreactive cell bodies (Larsson *et al*, 1976).

VIP immunoreactive fibres are found in the female and male urogenital tracts of animals in association with blood vessels and smooth muscle tissue. However, in the uterus and the urinary bladder, nerves sometime seem to penetrate the epithelium. The trigone area of the bladder is particularly rich in VIP immunoreactive fibres. In the renal cortex, VIP immunoreactive fibres follow blood vessels, whereas in the pelvis of the kidney the nerves are seen close to smooth muscle cells.

In the human male urogenital tract, VIP immunoreactive nerves are

found close to the epithelium in the prostate and seminal vesicles and in the female close to the epithelium and around cervical glands of the uterus and in the lamina propria of the vagina (Hokfelt *et al*, 1982).

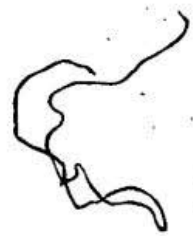
VIP immunoreactive fibres and cell bodies are present in the smooth muscle and submucosal layers and in small ganglia of the gallbladder wall (Sundler *et al*, 1977). A fairly dense plexus of VIP fibres are present in the carotid body (Lundberg *et al*, 1979). VIP immunoreactive fibres are present in the thyroid gland (Adren *et al*, 1980) and in skeletal muscles mostly following blood vessels (Lundberg *et al*, 1979).

In the lung, VIP-immunoreactive nerves have been localized in the tracheobronchial tree, especially in the smooth muscle layer, around submucosal glands, and in the walls of blood vessels (Said, 1984).

(c) Distribution In The Central Nervous System

The regional distribution of VIP in the central nervous system is relatively constant from species to species (Fahrenkrug and Schaffalitzky de Mackadele, 1978). The concentrations are highest in rodents.

In the rat and mouse, the highest concentrations of VIP are found in the cerebral cortex and in certain limbic structures, whereas the concentrations in the basal ganglia, thalmus, lower brain stem, cerebellum, and spinal cord are low. VIP containing cell bodies are mainly found in the cerebral cortex and the limbic system, with the majority of them in neo- and allocortical areas. VIP fibres have a distribution that parallels that of the cell bodies. The cerebellum is devoid of VIP fibres (Loren *et al*, 1979).



3.3.2. Actions On Vascular Smooth Muscle

VIP induces vasodilatation in most vascular beds including the peripheral systemic vessels, as well as the splanchnic, coronary, cerebral, pial, extracranial, salivary gland, and pulmonary vessels (Said, 1982). The magnitude of this vasodilatation varies in different sites and causes a decrease in diastolic and mean arterial blood pressure and an increase in cardiac output.

This relaxation of vascular smooth muscle is independent of adrenergic, cholinergic, serotonergic, or histaminergic receptors. The vasodilator effect on pial vessels appears to be mediated by vasodilator products of the cyclo-oxygenase enzyme system, as it can be abolished by indomethacin and other inhibitors of this enzyme (Wei, Kontos, and Said, 1980). However, this does not explain the vasodilator action of VIP in the femoral vascular bed of the dog nor its relaxation of the isolated guinea pig trachea, isolated rat aorta, or rat gastric fundus (Wei, Kontos, and Said, 1980).

Recently, a number of studies have been performed to investigate the importance of endothelium in the vascular relaxation induced by VIP. Sata and his co-workers investigated this question of dependence on endothelium in VIP-induced vasodilation of guinea pig, rat, and rabbit pulmonary artery and rat thoracic aorta (Sata *et al*, 1986). They found that VIP relaxed the pulmonary artery of all species by an endothelium-independent mechanism but relaxed thoracic aorta only in the presence of intact endothelium. These researchers found that VIP induced relaxation of guinea pig pulmonary artery could be abolished by methylene blue, which also prevents the relaxation by acetylcholine or sodium nitroprusside. The inhibition of vascular relaxation by methylene blue has been linked to the stimulation of cyclic GMP production as a mechanism of this relaxation (Martin *et al*, 1984. VIP has been shown to stimulate the production of cyclic AMP in many cells and tissues but its effects on cyclic GMP levels have not been thoroughly investigated (Schoeffter and Stoclet, 1985).

3.3.3. Actions On Non-Vascular Smooth Muscle

VIP relaxes isolated segments of guinea pig trachea independently of adrenergic or cholinergic receptors (Said, Geumei, and Hara, 1982). VIP is also present in pulmonary mast cells and as a bronchodilator, it may serve to modulate the influence of histamine and other mediators.

VIP has also been shown to relax circular smooth muscle of the lower esophageal sphincter of the gut, the gallbladder, the stomach , and the intestine. It also relaxes isolated gastric smooth muscle cells (Makhlouf, 1982).

3.3.4. Other Actions

VIP is a potent stimulant of intestinal fluid secretion and a putative mediator of certain watery diarrhoea syndromes (Gaginella, Hubel, and O'Dorisio, 1982). The normal pattern of distribution of VIP, in a network of intramural nerves and panacrine cells, suggests that the peptide may serve as a physiological mediator of chloride and fluid transport in the small and large bowel. Calcium and cyclic AMP may be involved in the action of VIP to bring about inhibition of coupled sodium chloride absorption and attenuation of ongoing secretion (Gaginella, Hubel, and O'Dorisio, 1982).

Experimental studies have also established that VIP has a lipolytic effect on adipose cells which leads to the liberation of free fatty acids. VIP also stimulates the release of glucose from the liver, probably through glycogenolytic and glucogenetic activities (Co and Korinek, 1982).

VIP has also been found to increase plasma renin activity. It appears as if VIP acts directly on the juxtaglomerular cells to stimulate renin secretion (Porter and Ganong, 1982).

3.4. Neuropeptide Y (NPY)

NPY was isolated and sequenced from extracts of pig brain using chemical assay for the C-terminal amide fragments (Tatemoto, 1982). It is a tyrosine rich, thirty-six amino acid peptide which shares considerable sequence homology with avian and bovine pancreatic polypeptide and polypeptide YY. This similarity probably explains early reports of avian pancreatic polypeptide in adrenergic neurons (Lundberg *et al*, 1980).

From molecular genetic studies it was discovered that the precursor molecule for NPY consists of a twenty-eight amino acid signal peptide, a thirty-eight amino acid NPY, and a thirty amino acid C-flanking peptide of NPY (Minth *et al*, 1984).

3.4.1. Distribution Of NPY

(a) Distribution In The Vascular System

NPY fibres are numerous in the heart of the guinea pig (Uddman *et al*, 1985), rat (Ballesta *et al*, 1987), cat, and man (Lundberg *et al*, 1983). The auricles and atria of the heart receive a dense supply of NPY fibres while the ventricles and ventricular septum receive fewer (Lundberg *et al*, 1983). Fibres can be seen in all layers of the heart with some predominance in the endo- and epicardium. Very few NPY fibres are seen in the valvular leaflets. The coronary vessels receive numerous NPY fibres. The major arteries are surrounded by a network of NPY fibres in the adventitia or at the junction between the adventitia and the media. A dense supply of NPY fibres are present in the thoracic aorta, the common carotid artery and the external carotid arteries. The density of NPY fibres decreases as the aorta descends into the abdominal cavity. The major veins are surrounded by NPY fibres, as well (Uddman *et al*, 1985).

In the respiratory tract of the guinea pig, fibres are numerous in the nasal muscosa and tracheal wall. The fibres surround large and medium sized

arteries. Veins and sinusoids have a scarce supply. The large pulmonary arteries receive numerous NPY fibres. In the peripheral parts of the lung, NPY fibres can not be found (Uddman *et al*, 1985).

In the genito-urinary tract numerous NPY fibres can be found. In the guinea pig, large and medium sized arteries in the uterine cervix receive a dense supply of NPY fibres. The ovarian artery, the testicular artery, and small blood vessels running close to the vas deferens and seminal vesicles, all receive a rich supply of NPY fibres (Uddman *et al*, 1985). In the rat, the most prominent supply of NPY fibres is found in the cervix, where they are associated with both vascular and non-vascular smooth musculature (Stjernquist *et al*, 1983).

A rich distribution of NPY has been found in the detrusor muscle and around arteries and arterioles of the bladder wall. A sparse distribution of NPY fibres is seen in the submuscosa (Feher and Burnstock, 1986).

Plexuses of NPY fibres have been shown to surround the portal vein of the guinea pig and the large hepatic arteries. In the parenchyma of the liver, NPY fibres are found around the small branches of the hepatic artery and around the central arteries. Interlobular veins do not have NPY fibres. The splenic artery has numerous NPY fibres whereas the splenic veins have only a few.

NPY fibres can be found around blood vessels in the kidney of the rat (Ballesta *et al*, 1987) and the guinea pig (Uddman *et al*, 1985). The nerve fibres form a dense plexus at the vascular pole of the glomerulus in the area of the juxtaglomerular apparatus. The renal artery has a moderate supply of NPY fibres (Uddman *et al*, 1985).

In the cat (Edvinsson *et al*, 1983) and the rat, NPY has been shown to form a dense plexus of fibres around cerebral arteries. NPY fibres are particularly abundant around arteries forming the circle of Willis (Edvinsson *et al*, 1984).

In the mesenteric vascular bed the density of NPY-immunoreactive fibres follows the pattern of catecholaminergic innervation (Scott and Pang, 1983). NPY-immunoreactive nerve fibres have been localized in mesenteric blood vessels in perivascular plexuses around arteries and veins (Wharton and Gulbenkian, 1987).

(b) Other Distributions In The Peripheral Nervous System

NPY immunoreactivity is widespread in nerve fibres of the anterior and posterior uvea (Terenghi *et al*, 1983). There is a dense network of fibres containing NPY in the iris and an irregular plexus in the dilator pupillae muscles. NPY fibres can be found in the choroid and these extend to the ciliary body (Terenghi *et al*, 1983).

NPY immunoreactivity has also been found in autonomic nerve terminals and fibres of the human dermis. A granular immunoreactivity has also been observed in the basal cells of the outer root sheath of hair follicles (Johansson, 1986).

NPY containing nerve cell bodies and processes in the guinea pig small intestine are numerous in the myenteric plexus. A few fibres can be found in the layers of the small intestine. Some nerve processes are close to the epithelial cells of the cyrpts of Liekerkuhn and to endothelial and smooth muscle cells (Feher and Burnstock, 1986).

(c) Distribution In The Central Nervous System

NPY is widely distributed within neurons of the central and peripheral nervous systems. It occurs in the mammalian brain in higher concentrations than all other peptides studied to date (Gray and Morley, 1986).

In the rat brain, areas of highest concentration of NPY

immunoreactivity are within the periventricular, dorsomedial, suprachiasmatic and medial preoptic hypothalmus, the paraventricular thalamic nucleus, the nucleus accumbens, periaqueductal gray, septum and medial amydala (Gray and Morley, 1986).

In the spinal cord of the rat, the highest density of NPY in terminals is within lamina 1 and 2. Cell bodies of NPY have not been observed in the spinal cord (Gray and Morley, 1986).

3.4.2. Actions On Vascular Smooth Muscle

NPY effects on arterial smooth muscle vary considerably from one artery to another. In some arteries it is a potent vasoconstrictor but in others it has been found to cause contraction only at high concentrations (Neild, 1987).

In vitro, NPY has been found to elicit two types of responses. It causes contractile responses in peripheral veins and cerebral arteries and veins. It also has been found to potentiate contractile responses in peripheral arteries (Edvinsson *et al*, 1983). Isolated peripheral arteries respond to electrical stimulation with a contraction that is sensitive to phentolamine and guanethedine. In some of these peripheral arteries nanomolar concentrations of NPY enhance the electrically evoked response. Also, contractile reponses to noradrenaline are enhanced in the presence of NPY in arteries from several different species (Edvinsson *et al*, 1984). The noradrenaline concentration response curve is shifted to the left in the presence of NPY without any change on the maximum contractile effect. Veins do not respond to NPY with enhancement of noradrenaline evoked contraction. This potentiating effect is not restricted to noradrenaline but can also be observed with histamine (in arteries and veins). The mode of action behind this potentiating effect is obscure (Edvinsson *et al*, 1983). Nifedipine, a calcium anatagonist, has been reported to abolish the NPY evoked potentiation of the noradrenaline response in rat blood vessels, whereas nifedipine, verapamil, and diltiazem have only marginal effects on this phenomenom in rabbit vessels (Edvinsson *et al*, 1987). In the rabbit femoral

artery, active removal of calcium has little effect on the NPY evoked potentiation whereas prolonged exposure to a calcium free medium containing EGTA abolishes the potentiation (Wahlestedt, Yanaihara, and Hakanson, 1986). This suggests that while intracellular calcium pools have to be intact, influx of extracellular calcium is not necessary for the potentiation.

In the heart, and in certain blood vessels of the rat (femoral artery and portal vein), it has been shown that NPY inhibits the release of noradrenaline evoked by electrical field stimulation. This may be linked to a NPY evoked inhibition of adenylate cyclase (Fredholm, Jansen, and Edvinnson, 1985).

Intravenous administration of NPY causes increased arterial blood pressure, local vasoconstiction, for example in the salivary glands, and reduced heart rate (Lundberg *et al*, 1982).

Intracisternal administration of NPY to anesthetized rats results in a lowering of blood pressure without a significant reduction in heart rate (Fuxe *et al*, 1983). The rats also develop a marked bradypnea. NPY induces behavioral and EEG signs of sedation with increased synchronized activity and reduced desynchronized activity.

3.4.3. Actions On Non-Vascular Smooth Muscle

Electrical stimulation of the vas deferens results in smooth muscle contraction secondary to norepinephine release from the intramural nerves. NPY inhibits the contractile response produced by electrical field stimulation and reduces the secretion of radiolabelled norepinephine from the vas deferens. NPY does not reduce the norepinephine evoked contraction. This suggests that its effect is secondary to its inhibition of norepinephine release. (Gray and Morley, 1986)

In the cervix, NPY inhibits neurally evoked, tetrodotoxin sensitive contractions (Stjernquist *et al*, 1983).

3.4.4. Other Actions

NPY has been found to reduce luteinizing hormone secretion in ovariectomized rats after administration of NPY into the third ventricle (Kalra and Crowley, 1984). It appears as if NPY produces its effects of luteinizing hormone secretion by modulating the release of gonadotrophin releasing hormone. NPY has also been found to suppress lordosis in the estradiol and progesterone primed ovarectomized rat (Clark, Kalra, and Kalra, 1985).

NPY also also been reported to produce a mild hypothermia in dogs (Morioka *et al*, 1984).

3.5. Substance P (SP)

von Euler and Gaddum first described SP (von Euler and Gaddum, 1931). They were studying the tissue distribution of acetylcholine and detected the presence of a substance in alcoholic extracts of equine brain and intestine. The active component was distinguishable from acetylcholine by the fact that its effects were not inhibited by atropine. The isolation of SP was accomplished subsequent to the discovery of a sialogogic peptide in hypothalamic extracts (Leeman and Hammerschlag, 1967) which was shortly characterized as SP. The name Substance P (for preparation) had been used in the von Euler and Gaddum laboratory to designate the active agent in a particular preparation of tissue extracts. This term entered the literature in 1934 and has persisted (Gaddum and Schildh, 1934).

The amino acid sequence of SP was established in 1971 (Chang, Leeman, and Niall, 1971) and shortly afterwards a synthetic peptide was prepared (Tregear *et al*, 1971).

It is now clear that SP is only one member of a family of closely related peptides known as the tachykinins. All of these peptides share a common spectrum of biological activities and possess a very similar amino acid sequence at the carboxyl terminus (Erspamer, 1981).

There have been many suggestions that the non-commital name Substance P be replaced. However, the precise physiological role of this substance has not yet been determined. Once this is known, another name may be coined which best describes this role.

3.5.1. Distribution Of SP

(a) Distribution In The Vascular System

SP immunoreactive nerve fibres can be observed throughout the heart. In the guinea pig heart SP nerve fibres can be seen running parallel to the myocardial fibres in both ventricles. As well, SP fibres can be seen running parallel to blood vessels (Wharton *et al*, 1981). Numerous SP endocardial nerve fibres, particularly around the ventricular trabeculae and papillary muscles can be found.

SP fibres can be observed in the mitral and tricuspid valves, as well as in the pulmonary and aortic valve and in the myocardium surrounding the valve orifices.

SP immunoreactive fibres are found in the interventricular septum of the heart, as well as around the bundle of His. Both atria contain SP nerve fibres, these are particularly abundant in the endocardium but SP fibres are also seen around the branches of the coronary blood vessels running in the epicardium.

An extensive network of SP immunoreactive nerve fibres can be observed around the pulmonary vessels and the aorta. SP containing nerves can be found in the regions of the sino-atrial and atrio-ventricular nodes and at the base of the aorta and the pulmonary artery (Barja, Mathison, and Huggel, 1983).

No SP containing fibres can be found around the carotid artery of the rat (Barja, Mathison, and Huggel, 1983).

SP immunoreactive nerve fibres appear to be localized mainly in the

adventitia and at the adventitial-medial border of large peripheral arteries and veins of the rat (Barja, Mathison, and Huggel, 1983). A very dense innervation of SP immunoreactive nerve fibres can be found in the hepatic artery of the rat. A moderate innervation is found in the coeliac artery, splenic artery, and spermatic artery. A small amount of SP nerve fibres can be found in the brachial, caudal, common iliac, and femoral artery, as well as the jugular, brachial, posterior vena cava, hepatic, portal, splenic, saphenous, common iliac, and femoral vein of the rat. No SP nerve fibres can be found in the renal vein of the rat.

The vessels of the mesenteric vascular bed have an extensive SP immunoreactive innervation (Scott, Robinson, and Foote, 1987). The jejunal artery has the highest density of innervation. The innervation of the mesenteric vein and mesenteric artery have a greater density of SP fibres as compared to the sympathetic innervating fibres (Scott, Robinson, and Foote, 1987).

Cerebral arteries (anterior, middle, and posterior cerebral, basilar, and communicating arteries) are innervated by a fine network of SP nerve fibres, in the guinea pig (Furness *et al*, 1982). Small arterioles in the dura, and the meningeal arteries have relatively few SP nerve fibres. There are also a small number of single SP immunoreactive fibres that traverse avascular parts of the dura. SP immunoreactivity can be found in nerve fibres following the superior sagital sinus.

The main uterine arteries have a moderately dense supply of SP nerve fibres (Furness *et al*, 1982). The testicular artery of the guinea pig and smaller arteries running to the vas deferens and seminal vesicle have a moderate supply of nerve fibres. The veins draining these reproductive organs have very few or no SP immunoreactive fibres associated with them.

Small arteries supplying skeletal muscles of the limb and other regions (abdominal wall, diaphragm, and tongue) are sparsely innervated by SP nerve fibres (Furness *et al*, 1982). There are no SP immunoreactive nerves associated with veins in skeletal muscle of the guinea pig (Furness *et al*, 1982).

(b) Other Distributions In The Peripheral Nervous System

Immunohistochemical studies have revealed the presence of SP in a scattered system of endocrine cells in the muscosal lining of the small and large intestine, and also within intrinsic neurons of the enteric nervous system (Costa *et al*, 1982).

SP containing nerve fibres also occur in the dental pulp and periodontum of various mammals (Wakisaka *et al*, 1987).

(c)Distribution In The Central Nervous System

SP-like immunoreactivity has also been detected in several regions of the central nervous system of the rat (Cuello and Karazawa, 1978). However, no SP fibres can be found in the median eminence of the hypothalmus, in the cerebellar folia, deep cerebellar nuclei, or in the hippocampus of the rat's brain (Cuello and Kanazawa, 1978).

Several anatomical defined pathways of SP-containing neurons have been identified. A particularly prominent projection originates from SP cells in the anterior striatum whose fibres descend to give rise to a very dense terminal innervation of the substantia nigra, which contains the highest concentration of SP immunoreactivity of any brain region. Another defined pathway arises from SP cells in the medial habenula nucleus whose fibres project in the fasciculus retroflexus to the region around the interpedunular nucleus (Erspamer, 1981).

SP immunoreactive neuron cell bodies are found in the nucleus habenularis medialis, and nucleus interpeduncularis. Some cells can also be found in the caudoputamen and in the globus pallidus (Cuello and Kanazawa, 1978).

3.5.2. Actions On Vascular Smooth Muscle

SP is one of the most potent vasodilating compounds so far known (Pernow, 1983). Close arterial injections of SP in doses from ten nanomolar cause a dose related vasodilatation in adipose tissue and skeletal muscle of the dog (Pernow and Rosell, 1975). Close arterial infusion of SP increases blood flow in the hepatic, mesenteric, and iliac arteries of the dog (Pernow, 1983). Intravenous infusions of SP at rates of 0.4 pmol/kgmin or higher can increase blood flow of most vascular beds such as the skin, skeletal muscle, small intestine, as well as the hepatic, carotid, mesenteric, and portal beds. SP also effects isolated strips of arteries.

Intravenous infusions of SP at rates higher than 0.4 pmol/Kgmin can cause a transient fall in systolic, diastolic, and mean arterial blood pressure in the dog (Burcher *et al*, 1977).

In man SP is also a potent vasodilator. Intradermal injections of SP can induce flare, wheal, and itching.

The effects of SP on blood flow and blood pressure are generally not influenced by atropine, alpha, or beta adrenergic blocking agents or antihistamines (Burcher *et al*, 1977). This indicates that the vascular effects of SP generally are not due to cholinergic, adrenergic, or histaminergic receptor stimulation but to a direct effect on the smooth muscle of the vascular wall. There are regional differences. The vasodilatation induced by SP in the hypothalmus is completely abolished by atropine. Similarily, blockade of alpha adrenoceptors by phenoxybenzmine or beta adrenoceptors by propranonol or destruction of adrenergic hypothalamic nerves with 6-hydroxydopamine completely inhibits SP vasodilatation (Klugman *et al*, 1980).

3.5.3. Actions On Non-Vascular Smooth Muscle

In the isolated guinea pig trachea, SP in seven to fifteen nanomolar concentrations induces an increase in muscular tone (Pernow, 1983). This effect is not influenced by atropine, adrenergic blocking agents, antihistamine, or 5-HT anatagonists.

On most smooth muscle layers in the gastrointestinal tract, SP has a powerful stimulating effect. All segments of the small intestine are contracted by SP in vitro and in vivo (Pernow, 1983). In the isolated large intestine, SP has a similar effect as in the small intestine.

Injection of SP into the anterior chamber of the eye produces miosis in the rabbit (Bill *et al*, 1979). Also, in the isolated iris sphincter muscle of the rabbit a dose-dependent miosis can be produced (Pernow, 1983).

3.5.4. Other Actions

SP is a potent stimulator of salivation in the dog, rat, guinea pig, hamster, and hen but not in the cat or rabbit (Pernow, 1983). The spontaneous release of amylase from the parotid gland is also stimulated by SP.

SP stimulates exocrine pancreatic secretion both in vivo and in vitro. It can also affect the endocrine pancreatic secretion. Both in vivo and in vitro studies have shown that SP inhibits the release of insulin induced by glucose and arginine, as well as the basal insulin plasma level. Large doses of SP inhibits the release of glucagon from the perfused rat pancreas (Pernow, 1983).

SP is one of the most potent natriuretic and diuretic compounds so far described (Pernow, 1983). It appears as if SP may exert its effects on water reabsorption by a cAMP dependent mechanism (Pernow, 1983).

3.6. Colocalization

For many years it was accepted that one nerve fibre makes and releases only one transmitter. This concept was known as Dale's Principle. However, the history behind this nomenclature is curious (Burnstock, 1976). In the Northnagel Lecture in 1934, Dale speculated in relation to the axon reflex that different endings of a sensory neuron probably released the same transmitter. In the mid 1950s, it was Eccles who coined the words Dale's Principle (apparently not entirely with the agreement of Dale) and defined it as "at all the axonal branches of a neuron, there is a liberation of the same transmitter substance or substances."

On the basis of studies of the evolution of the autonomic nervous system and on the evidence for coexistence of transmitters in invertebrate nerves, Dale's Principle was questioned. It is only recently that the idea that a neuron may contain more than one neuroactive substance has found general acceptance, and this has been largely due to the discovery of peptides in the nervous system and to the application of immunohistochemical techniques for their localization (Priestley and Cuello, 1982).

The presence of more than one peptide in a neuron was first described in certain hypothalmic neurons containing both B-endorphin and adrenocortiotrophic hormone (ACTH) (Watson *et al*, 1978). Over the last few years an increasing number of neurons containing coexisting molecules have been described both in the central and peripheral nervous systems. Different types of combinations have been encountered: 1)Classical transmitter and peptide(s) 2)more than one classical transmitter and 3)more than one peptide (Hokfelt *et al*, 1987).

3.6.1. Classical Transmitter And Peptide

It is well known that endocrine cells, particularly in the gastrointestinal tract, contain not only a peptide hormone but also a biogenic amine. Recent evidence suggests that such a coexistence may occur also in nerve cells and that, perhaps, this situation is not limited to a small number of neurons but may be rather common (Hokfelt *et al*, 1980).

In certain sympathetic ganglia, particularly in the prevertebral ganglion of the guinea pig, a majority of the noradrenergic sympathetic principle ganglion cells contain a somatostatin-like peptide (Hokfelt *et al*, 1977). In other peripheral amine cell systems immunoreactive peptides have also been observed. A small population of the principle ganglion cells of the rat superior cervical ganglion contain an enkephalin-like peptide (Schultzberg *et al*, 1979). Also adrenal gland cells in several species such as rat, guinea pig, cat, cow, monkey, and humans contain this peptide.

In the central nervous system examples of coexistence of a peptide and a transmitter has also been described. For example, in both the raphe nuclei of the medulla oblongata and adjacent nuclei such as the pars alpha of the gigantocellular reticular nucleus, numerous neurons contain both serotonin and a SP-like peptide (Chan-Palay, Johsson, and Palay, 1978).

3.6.2. More Than One Transmitter

One of the earliest proposals for coexistence of two transmitters was made by Burn and Rand (1959). They suggested that acetylcholine is present together with noradrenaline in noradrenergic sympathetic neurons.

Studies on invertebrates have been of particular importance for the question of multiple transmitters in a neuron. In species such as snail and related aplysia, extremely large neurons exist, which are easily identified and accessible for study.

More recent evidence suggests that ATP may be a transmitter and several possibilities exist where this nucleotide may coexist with other transmitters (Burnstock, 1976).

3.6.3. Several Peptides In One Neuron

Although numerous neuronal peptide systems have been defined and described both in the peripheral and central nervous systems, there are only a few examples for coexistence of two peptides in the same neuron (Hokfelt *et al*, 1980). An example of this type of coexistence occurs in the gastrointestinal tract. It has been found that certain cells in the submucous plexus of the guinea pig colon contain both somatostatin and gastrin cholecystokinin-like immunoreactivity. However, whether these peptides are released together or belong to the same precursor molecule is not known (Hokfelt *et al*, 1980).

The functional significance of coexistence of multiple messengers is difficult to evalulate. Classical transmitters and peptides may be co-released and interact in a cooperative way on effector cells or the peptides may inhibit the release of the coexisting transmitter. Thus, multiple messengers may provide a mechanism for relaying differential responses and for increasing the amount of information transmitted at synapses (Hokfelt *et al*, 1987).

Coexisting messengers may have trophic effects or induce other types of actions or longterm events on the neuron or effector cells. For example, SP has been found to exert growth-stimulating effects on smooth muscle cells. (Nilsson, von Euler, and Dalsgaard, 1985).

One important question that may be asked is why is it necessary to have one transmitter at each synapse when there are so many nerve cells? Why is there redundancy of neurons and of neuronal systems? Redundancy is also present at the level of the individual synapse. It may be that differentiated transmission process is needed to achieve the enormous operational capacity of our brain, which includes the transfer of messages for long term effects (Hokfelt *et al*, 1987).

3.6.4. Colocalization Of Substance P (SP) And Other Substances

In the central nervous system SP is colocalized with 5-hydroxytryptamine. In fact quantitative estimates indicate that up to seventy percent of all 5-hydroxytryptamine neurons, depending on their exact locations in the ventral medulla oblongata, may contain SP. Both substances appear to be stored in the same 60-90 nm dense cored vesicles (Pelletier, Steinbusch, and Verhofstad, 1981).

In the peripheral nervous system, such as in varicose sensory axons supplying the skin, viscera, and the cardiovascular system in guinea pig, in sensory endings in the rat, cat, monkey eyes (Terenghi *et al*, 1985) and in the central nervous system, SP and CGRP can be found to coexist. In the trigeminal ganglia of the rat, guinea pig, and monkey, SP can be found in 20-30 % of the cells. CGRP can be found in these cells as well (Terenghi *et al*, 1985). In the rat and cat dorsal root ganglia, CGRP can be found in SP containing cells, but also in many small and intermediate size sensory neurons which do not appear to contain SP. Several other studies have confirmed that most SP immunoreactive cells show the presence of CGRP immunoreactivity whereas only a proportion of CGRP immunoreactive cells are SP positive (Goodman and Iversen, 1986).

In addition to the extensive colocalization of SP with CGRP, SP has been found to exist with other substances. In the mammalian central nervous system SP has been found to coexist with epinephrine, acetylcholine, and GABA.

3.6.5. Colocalization Of Calcitonin Gene-Related Peptide (CGRP) With Other Substances

In addition to the coexistence of SP and CGRP, CGRP has been found to coexist with VIP. This coexistence has been found in the paravertebral sympathetic ganglia of the sympathetic stellate ganglia of the cat (Lindh *et al*, 1987).

3.6.6. Colocalization Of Neuropeptide Y (NPY) With Other Substances

NPY immunoreactive sympathetic neurons contain the catecholamine synthesizing enzymes tyrosine hydroxylase (TH) and dopamine B-hydroxylase; which indicates that NPY is colocalized with norepinephine in these neurons (Leblanc, Trimmer, and Landis, 1987).

NPY immunoreactive neurons are also abundant in three cranial parasympathetic ganglia, the otic, sphenopalatine, and ciliary, in the rat (Leblanc, Trimmer, and Landis, 1987). This may mean that NPY is a neuromodulator in the parasympathetic nervous system. It appears as if NPY can occur together with norepinephrine, VIP, or acetylcholine in the sympathetic nervous system.

The co-distribution of NPY with noradrenaline in the sympathetic nervous system is consistent with functional studies that have shown that NPY is a potent vasoconstrictor. However, the functional consequences of the colocalization of NPY with VIP and acetylcholine in cranial parasympathetic neurons remains to be determined (Leblanc, Trimmer, and Landis, 1987).

NPY has also been found to be stored with enkephalin in large dense cored vesicles in unmyelinated axons of bovine splenic nerve (Fried *et al*, 1986).

3.6.7. Colocalization Of Vasoactive Intestinal Polypeptide (VIP) With Other Substances

On the basis of acetylcholinesterase staining of tissues from cat it was first discovered by Lundberg and co-workers that VIP is present in certain populations of cholinergic neurons, including those present in sympathetic ganglia (Lundberg *et al*, 1979). It appears as if VIP is stored in large, 100 nm diameter dense cored vesicles in 'cholinergic type' nerve fibres (Lundberg, Fried, and Fakrenkrug, 1981).

VIP has also been found to coexist with enkephalins and catecholamines, in adrenal granules (Said, 1984).

3.6.8. Summary

The different types of nerve fibres examined in this thesis can occur singularly or they may occur together and innervate the vascular-smooth muscle of the mesenteric vascular bed. If coexistence of these fibres occurs then interaction between these fibres may occur. This interaction may take place during development. In this thesis the question of interaction between fibres is explored during the development of the mesenteric vascular bed.

3.7. Nerve Growth

It is not known if there is a definite stage of development at which a neuron becomes committed to a specific developmental program, nor is it known whether the neuron, once committed to one program, is able to change to another pathway of differentiation (Jacobson, 1978 - a review). There is also a large amount of conflict regarding the ideas about the development of the nerve fibre. There are three main theories of its development. The cell-chain theory originated by Theodor Schwann, which was the first theory to be proposed, was based on the concept that the axon is formed by fusion of Schwann cells. This theory was disproved and replaced by the plasmoderm or syncytial theory originated by Viktor Hensen. This theory was founded on the concept that the nerve fibre differentiates from pre-established filaments that connect all the cells of the nervous system into a "neurosyncytium". This theory was founded on various misinterpretions of histology and was finally replaced by the outgrowth theory proved by R. Harrison. Harrison described the outgrowth of a nerve fibre or exporatory fibre that preceded the rest of the development of a fibre pathway. This theory was elaborated on by Ramory Caja who described the movement of a growth cone. Only the growth cone moves, the remainder of the nerve cell is stationary. Thus, as the axon or dendrite elongates, the cell body and proximal segments of the axon or dendrite are stationary (Jacobson, 1978 - a review).

Several chemical agents have been shown to induce or increase the outgrowth of an axon or dendrite. The chief agent among these chemicals is

nerve growth factor (NGF). Several lines of evidence indicate that NGF stimulates the assembly of neurotubules. Neurotubules are required for the normal outgrowth of the axon, to provide mechanical support for the axon, and to play a role in axoplasmic flow.

In this thesis, the normal development of a particular type of neuronal system has been interfered with in order to discover the importance of this system to other neuronal systems.

3.8. Objectives Of Thesis And Review Of Methods

The objective of this thesis was to test the hypothesis that different fibre systems interact during the development of innervation of the mesenteric vascular bed. It is proposed that this interaction may be essential to the development of each innervating system and in turn affect the development of the vasculature.

From this experimental work, it is hoped that a structural relationship between the nerve networks innervating the mesenteric vascular bed, will be established. From this structural relationship it is hoped that important aspects of the functional relationships between fibre systems can be deduced.

The hypothesis was tested using the rat mesenteric vascular bed. It was proposed that interaction between perivascular nerve fibres in this bed could be determined by examining the system during development, following neonatal removal of the catecholaminergic fibres and following neonatal or prenatal removal of the peptidergic fibres.

The rat mesenteric bed was chosen because a large data base concerning this system is available, it is easily accessible, and provides a large amount of tissue.

To follow perivascular nerve development it was decided to use techniques of immunohistochemistry which would allow differentiation of different

fibre types, and electron microscopy which would allow the relationships between axons and between axons and smooth muscle cells to be determined.

The immunohistochemical method used to examine the innervation patterns is a three step method using diaminobenzidine, which allows us to easily visualize the reaction product with light microscopy (Scott, Robinson, and Foote, 1987). This method was chosen because of its simplicity and specificity. As well, primary and linking antibodies are readily available for this type of procedure. There are other methods that could have been used. Induced fluorescence for catecholamines was excluded because there are other peptides examined in this study which could not be induced to fluoresce. It was decided that it was best to use a single method for all the peptides. Fluorescence using primary antibodies could have been used, however, because of the expense of fluorescence tagged primary antibodies and the instability of the reaction product, it was decided that a three step immunohistochemical procedure using diaminobenzidine would be used. The reaction product obtained using diaminobenzidine is stable allowing specimens to be collected and examined later.

The extent of each plexus of immunoreactive fibres on the vessels of the mesenteric bed was determined by stereology (Weibel, 1980). This allowed a quantitation of the length of nerve fibres or of the plexus length on the vessels. Whole mounts of the vessels were collected and the plexus on each vessel was drawn with a camera lucida. The extent of the plexus on each vessel was determined from the drawings using a superimposed test grid with lines of known lengths. The number of intersections between the nerve fibre plexus and the lines were recorded, the plexus length ($um/1x10^5\ um^2$) was then calculated (Scott, Robinson, and Foote, 1987). There are other methods available for quantitation. Automated image analysis (Cowen *et al*, 1982) is one such method. However, with the type of preparation used in this research project, an image analysis program, may have been difficult to use. A lot of focusing would have had to be performed with the whole mount vessel preparations. At times blood cells were

present and reacted with the peroxidase anti-peroxidase, and diaminobenzidine. The image analysis program may not differentiate between blood cells and fibres.

With the knowledge obtained with this stereology method plexus lengths of different peptides can be compared from different animal models.

Electron microscopy was used to study the ultrastructural development of the mesenteric vascular bed, as well as to examine the innervating nerve fibres ultrastructurally. In many cases a chromaffin reaction was used (Hopwood, 1971). This method of fixation was chosen in order to examine the catecholaminergic content of the perivascular neurons. This is a method of fixation whereby biogenic amines react and are precipitated. In the chromaffin reaction the oxidizing agent is the dichromate ion. Under mildly acidic conditions oxidation occurs and the dichromate ion predominants. ($6e + Cr_2O_7^{2-} + 14H^+ \rightarrow 2Cr^{3+} + 7H_2O$). The dichromate ion is in equilibrium with the chromate ion. The chromate ion can also act as an oxidizing agent under mildly acidic conditions ($3e + CrO_4 + 8H^+ \rightleftharpoons Cr^{3+} + 4H_2O$). When both chromate and dichromate are reduced to Cr^{3+}, the Cr^{3+} can readily enter into complexes with catecholamines and their oxidation products.

Fixation with Karnovsky's fixative was also performed. This is a fixative containing paraformaldyde and glutaraldehyde. These compounds form strong cross links with proteins, thus allowing for a good fixation of the tissue to be studied. With the use of this fixative the structure of each vessel was examined in order to find any ultrastructural changes during normal development and during development after a particular type of nerve fibre had been removed.

Removal of some peptidergic fibre systems was carried out by capsaicin treatment. Capsaicin (8-methyl-N-vanillyl-6-nonenamide) is the major pungent ingredient of hot peppers of the plant genus *Capsicum*. It has been used for hundreds of years as a food preservative, as an ingredient in certain social rituals, and as a herbal medicine for many different types of health problems. Jancso, in

the late 1940's, became intrigued by this substance and began experimenting with capsaicin and it's pharmacological actions. Other researchers in Europe began to discover some interesting facts about the substance. Gasparovic and co-workers (1964) observed that the amount of bioassayable substance P (SP) was reduced in the spinal cord, but not in the brain, of rats treated systemically with capsaicin. Jancso and Knyihar (1975) determined that capsaicin depleted fluoride resistant acid phosphatase activity, known to be associated with central terminals of some primary afferent neurons, from the dorsal horn of the spinal cord. The neurophysiological and neurochemical effects of capsaicin were specific for primary afferent neurons, in animals treated neonatally, but not in those treated while adults. Capsaicin administered to adult animals produced only limited intracellular changes in small (type B) sensory neurons without affecting large (type A) sensory neurons or neurons in the sympathetic ganglia. Administration to neonatal rats results in actual degeneration of small sensory neurons (Buck and Burks, 1983).

Treatment of neonatal rats with capsaicin has a neurotoxicity for primary afferent neurons greater than that to adult rats and it is less limited to SP containing cells, since several other sensory neuron peptides are also affected (Buck and Burks, 1986). There is also a reduction in the number of unmyelinated fibres in the neonates (Nagy *et al*, 1981).

The evidence now available seems to indicate that there are three major actions of capsaicin on certain sensory neurons. The first effect is an excitation and this is followed by a desensitization of sensory neurons mediating certain kinds of peripheral stimuli. These actions appear to originate at the endings of and/or along the peripheral processes of the capsaicin sensitive afferents. In addition to these actions, capsaicin produces a depletion of most of the SP contained in primary afferent neurons. This depletion is never total, there may be a sub-population of SP containing sensory neurons that are capsaicin insensitive, possibly myelinated SP containing neurons.

Dorsal root ganglia taken from animals treated neonatally with capsaicin exhibit a markedly decreased rate of incorporation of labelled amino acids into SP. In adult guinea pigs treated systemically with capsaicin the overall incorporation of amino acids into proteins in the dorsal root ganglia is not depressed. This is consistent with the absence of neuronal death after capsaicin treatment of adults (Buck and Burks, 1983).

It is not clear if capsaicin produces its effects by more than one mechanism of action. However, it is thought that the biochemical make up of the compound contributes to its action on sensory membranes. Capsaicin is a relatively lipophilic molecule and is able to affect membrane fluidity and/or ion permeability such that Ca^{2+} and other cations stream across the membrane. At a free ending this will result in the initiation of an impulse and cause the release of SP.

Capsaicin is also able to interfere with the retrograde transport of trophic factors in the capsaicin sensitive primary afferents. One of these molecules is nerve growth factor. SP containing sensory neurons are highly dependent on nerve growth factor for development and maintenance. Therefore, the reduction in the transport of this factor causes a decrease in SP production in the cell body which hinders the cell's ability to replenish the depleted peptide in its more distal portions.

There is much inconsistency in reports dealing with how effective capsaicin is in removing various types of neurons. For example, in the digestive tract, there are a large number of discrepancies dealing with the gastric effects of capsaicin in various species (Holzer, 1987). The discrepancies are probably due to differences in the dosage of capsaicin, uncertainty in the dose when only crude extracts of capsaicin are given, or they may be due to species differences.

The sensory neuron specificity of capsaicin made this compound an extremely valuable tool for investigating the importance of sensory neurons in the

peripheral nervous system. When appropriate doses are used, capsaicin's actions seem to be limited to primary afferents with c-type fibres, which mostly contain SP (Buck and Burks, 1986). Capsaicin provides the opportunity to investigate the link between SP and other neurosubstances. In this thesis the link between several neurotransmitters, peptides and catecholamines, was investigated in the rat's mesenteric vascular bed.

In this thesis, the use of 6-OHDA provided an opportunity to examine the link between neurons containing catecholamines and those with other neurotransmitters. By destroying the catecholaminergic system, the influence of this system on other systems will be examined.

In 1967 Tranzer and Thoenen made the discovery that an isomer of norepinephrine, namely 6-OHDA produced a destruction of the terminal ground plexus of peripheral sympathetic (noradrenergic) neurons. This phenomenon has since been termed a 'chemical sympathectomy' (Kostrzewa and Jacobwitz, 1974). It is a relatively selective effect, with cholinergic neurons, Schwann cells, glia, endothelial, and other cell types remaining virtually unaffected at the ultrastrucural level (Kostrzewa and Jacobwitz, 1974).

In the peripheral nervous system, 6-OHDA appears to be taken up with a high degree of selectivity by noradrenergic neurons (Kostrzewa and Jacobwitz, 1974). Once inside the neuron, 6-OHDA molecules accumulate in the amine storage granules and in the cytoplasmic fraction.

When 6-OHDA is administered to newborn animals its neurotoxic effects appear to be accentuated. The destructive lesions extend to axonal processes and to the perikaya. In the newborn an irreversible chemical sympathectomy occurs whereas in the adult there is a reversible chemical sympathectomy. In newborn rats, 6-OHDA produces its initial lesions on the sympathetic ganglia within an eight hour period. After twenty-four hours, nucleur pyknosis is observed and the axonal processes appear distorted and swollen. By two to three days, virtually all sympathetic cell bodies are destroyed (Kostrzewa and Jacobwitz, 1974).

Nielsch and Keen (1987) treated neonatal rats with 6-OHDA and found that at adulthood there was a 93% depletion of noradrenaline content of all the tissues they examined. They also found that 6-OHDA administration caused an increase in the substance P content of cell bodies, axons, and terminals of sensory neurons in both the trigeminal, and sciatic systems (Nielsch and Keen, 1987).

Immediately after 6-OHDA is administered and comes in contact with catecholamine containing nerves, a chain of events is initiated which may be interrupted at any time. There is an active uptake of the substance into the neurons and it is then transported to the intraneuronal sites where it can displace norepinephrine and act as a false neurotransmitter. When critical intraneuronal concentrations of 6-OHDA or metabolites are attained then destructive processes begin and cellular enzymes and energy producing cytochromes or related elements of the respiratory transport chain are destroyed. At about this point, the nerve terminals lose their ability to conduct action potentials, but still have a relatively intact monoamine uptake mechanism. Because of the internal destructive processes norepinephrine is released into the synaptic cleft. After a period of time, if the nerve terminals are completely destroyed, there will be a marked reduction of norepinephrine.

There is some controversy, as to the usefulness of 6-OHDA. Some researchers declare that it does not produce a complete and generally distributed sympathectomy (Kurnjek, Basso, and Taquini, 1984). However, it should be noted that the degree of sympathectomy depends upon many important factors, such as the dose of drug given, the route of administration, and upon the tissue examined.

4. METHODS AND MATERIALS

In each part of this study, for example development, capsaicin treatment, 6-hydroxydopamine treatment, and vehicle treatment, light and electron microscopy was performed. Light microscopic examination was carried out using antibodies to substance P (SP), neuropeptide Y (NPY), vasoactive intestinal polypeptide (VIP), calcitonin gene related peptide (CGRP) and tyrosine hydroxylase (TH). For each of these peptides six rats were sacrificed at each time period. For electron microscopic analysis four rats were sacrificed at each time period.

4.1. Developmental Study

Male Sprague-Dawley rats, bred from stocks maintained at the Memorial University animal care facility (origin Charles River of Canada). Animals were kept in plastic cages; food (Purina rat chow) and tap water were provided *ad libitum*. The rats were sacrificed at 1, 2, 4, 6, 8, 10, 12, and 14 days and 12 weeks of age for light and electron microscopic analysis.

4.2. Capsaicin Study

(a) Capsaicin Treated Rats

Sprague-Dawley rats, two days of age were obtained from rats bred in the animal care facility at Memorial University. For capsaicin treatment it was decided to follow the method followed by Scott and Pang (1982), however it was soon discovered that this was unsatisfactory (see Appendix) and a new method was devised. The rats were injected, subcutaneously, with 25 mg/kg of capsaicin (Sigma Chemical Company, U.S.A.). The capsaicin was dissolved in 10% alcohol. This was then mixed with a solution of 10% Tween-80 (Fisher Scientific, Canada) in 0.9% saline. A dose of 50 mg/kg of capsaicin was given to the rats on days 3

and 4 after birth. This dose was repeated at 1 week, 2 weeks, 4 weeks, and 8 weeks after birth. Rats were sacrificed for light and electron microscopic analysis at 4 weeks and 12 weeks of age. It was discovered, in practice, that doses greater than 50mg, resulted in a larger number of animal deaths.

In addition, pregnant Sprague-Dawley rats, obtained from Charles River, Canada, were injected subcutaneously with 25 mg/kg of capsaicin (Sigma Chemical Company, U.S.A.), on days 14, 15, 16, 17, 18, 19, 20, and 21 days after breeding. Pups were then collected and at two days after birth, they were injected subcutaneously with 25 mg/kg of capsaicin. The capsaicin was dissolved in 10% alcohol. This was mixed with a solution of 10% Tween-80 (Fischer Scientific, Canada) in 0.9% saline. A dose of 50 mg/kg of capsaicin was given to the pups on days 3 and 4 after birth. Rats were sacrificed for electron and light microscopic analysis at 4 weeks and 12 weeks of age.

(b) Vehicle Control Rats

Sprague-Dawley rats, two days of age were obtained from rats bred in the animal care facility at Memorial University. The rats were treated subcutaneously with a solution of 10% alcohol, and 10% Tween-80 in 0.9% saline. This solution was given in the same volume as was given for capsaicin injections. The injections were repeated at days 3 and 4 after birth and 1 week, 2 weeks, 4 weeks, and 8 weeks after birth. Rats were sacrificed for light and electron microscopic analysis at 4 weeks and 12 weeks of age.

4.3. 6-Hydroxydopamine Study

(a) 6-Hydroxydopamine Treated Rats

Sprague-Dawley rats , were obtained from rats bred in the animal care facility at Memorial University. It was first decided to follow the method

described by Nielsch and Keen (1987). It was discovered that this method was unsatisfactory (see Appendix). A new method was devised. The rats were treated subcutaneously with 60 mg/kg of 6-hydroxydopamine (Sigma Chemical Company, U.S.A.) on days 0, 1, 3, 5, 7, 9, 11 days and weekly until 12 weeks of age. The 6-hydroxydopamine was dissolved in 1 millilitre of ascorbic acid in phoshate buffered saline (50 mg of ascorbic acid (Aldrich Chemical Company, U.S.A.) in 10 mls of phosphate buffered saline) that had been boiled and bubbled with nitrogen to remove oxygen from the solution. The pH of the ascorbic acid phoshate buffered solution was adjusted to approximately 6.

The Sprague-Dawley rats were sacrificed for electron and light microscopic analysis at 4 and 12 weeks of age.

(b) Vehicle Control Rats

Sprague-Dawley rats were obtained from rats bred in the animal care facility at Memorial University of Newfoundland. The rats were treated with a solution of ascorbic acid (50 mg)(Aldrich Chemical Company, U.S.A) in 10 mls of phosphate buffered saline. Injections were performed on days 0, 1, 3, 5, 7, 9, 11 days after birth and weekly until 12 weeks of age. Injections were given in the same volume as given for the 6-hydroxydopamine injections.

The rats were sacrificed for light and electron microscopic analysis at 4 and 12 weeks of age.

4.4. Perfusion And Fixation For Light Microscopy

4.4.1. Routine Fixation

Animals up to 4 weeks of age were weighed and anesthetized with diethyl ether (BDH Chemicals, Canada). 12 week animals were also weighed and anesthetized with 35 mg/kg of Somnotol (sodium pentobartital, M.T.C. Pharmaceuticals, U.S.A.).

The anesthetized animals were placed in a supine position on a metal rack over a sink for drainage. A longitudinal midline incision was made through the skin of the abdominal and thoracic areas and following separation of this tissue from the underlying musculature and linea alba, a further incision was made midline, along the linea alba extending from the level of the bladder exposing the visceral organs. The abdominal contents were next carefully exteriorized from the abdominal cavity and the mesenteric vascular bed of jejunal arteries was arranged such that they fanned out from the mesenteric artery to jejunum. The right atrium of the heart was cut, to allow clearing of the animal's blood and the animal was perfused through the left ventricle. The animal was perfused, at room temperature, with 50 mls of 0.9% saline (BDH Chemicals, Canada) and then with 50 mls of Zamboni's fixative (Zamboni and De Martinoc, 1967). The mesenteric bed was then removed from the animal and placed in a dish containing fixative.

The superior mesenteric artery and attached jejunal arteries and mesenteric vein were dissected from the perivascular fat with the use of a dissection microscope (Wild Leitz) and were then placed in Zamboni's fixative (Zamboni and De Martinoc, 1967) for two hours. The tissues were rotated on a rotary spinner. The tissues were then placed in phosphate buffered saline, in preparation for immunohistochemistry.

4.5. Immunohistochemistry

Tissues were washed for one hour in a prewash solution with the following composition : 1% normal goat serum (ICN Immunobiologicals, U.S.A.), 1% Triton-X (Fischer Scientific, U.S.A.) and phosphate buffered saline, pH 7.2. The tissues were rotated on a rotary spinner. After washing, the tissues were placed into a primary antibody for 48 hours at 0-4 degrees Celsius. Five different primary antibodies were used. They were Substance P (SP), diluted 1:2,000 (Instar Corporation, U.S.A.), Calcitonin Gene-Related Peptide (CGRP), diluted 1:20,000 (Amersham, U.S.A.), Neuropeptide Y (NPY), diluted 1:400 (Amersham, U.S.A.), Tyrosine Hydroxylase (TH), diluted 1:10,000 (Eugene Tech International, U.S.A.), and Vasoactive Intestinal Polypeptide (VIP), diluted 1:1000 (Instar Corporation, U.S.A.). The tissues were then washed three times in the prewash solution and rotated on a rotary spinner. Each wash had a duration of 15 minutes. The tissues were then transferred into a linking antibody, goat anti-rabbit, diluted 1:150 (Boehringer Mannheim Biochemicals, U.S.A.) and rotated on a rotary spinner for two hours. Washing then followed with the prewash solution. This was repeated twice. Each wash had a duration of 15 minutes. The tissues were then placed into peroxidase anti-peroxidase, diluted 1:300 (Sternberger Meyer, U.S.A.) and were rotated on the rotary spinner. Washing with phosphate buffered saline, pH 7.2, followed. The washing was repeated twice. Each wash had a duration of 15 minutes. The tissues were then placed into a diaminobenzidine solution, having the following composition: 50 mgs of diaminobenzidine (Sigma Chemical Company, U.S.A.), 50 mls of distilled water, 50 mls of 0.2M phosphate buffer pH 7.2, 33 ul of 30% hydrogen peroxide (Fischer Scientific Company, U.S.A.) The immunoreactive nerve fibres reacted to give a brown colour. The reaction was complete in thirty to sixty seconds. After reaction with this solution, the tissues were washed in phosphate buffered saline twice and dehydrated through a series of alcohols and placed in xylene. They were then squashed between two slides and whole mounted in Eukit mounting medium. The nerve fibres were examined by light microscopy.

4.5.1. Controls For Immunohistochemical Procedure

The immunohistochemical procedure was performed as described above, however, a particular step was omitted during separate experiments. Four experiments were performed where either the primary antibody was omitted, the antibody was preabsorbed with excess antigen, the linking antibody was omitted or the final antibody steps were omitted.

4.6. Photographic Techniques For Light Microscopy

Tissues were examined and photographed with a Leitz Diaplan microscope, using a blue filter. Kodak Plus X pan 125 film was used. The film was developed with Kodak Microdol developer for 7 minutes, rinsed with tap water, and fixed for three minutes in Kodak Fixer. The film was then rinsed in running tap water for twenty minutes and then allowed to dry.

After drying, light micrographs were printed from negatives using an enlarger. Prints were made using Polycontrast Rapid II Rc paper from Kodak. The paper was developed with Kodak HC-110 Developer for approximately one minute and then rinsed in running tap water and fixed in Kodak Fixer. The prints were then washed in running water for approximately ten minutes and allowed to dry.

4.7. Quantitation Method For Immunohistochemical Treated Tissues

Nerve fibres on vessels were examined and drawn with a camera lucida. The extent of the plexus on each vessel was determined from the drawings by stereology (Weibel, 1980) using a superimposed test grid.

One drawing was made from each vessel and each rat examined (at 300X magnification), for all five peptides studied. From each drawing, the number of intersections between the test grid and the plexus was determined. Four different counts from each drawing was made. An average was then calculated from these four counts for each drawing.

4.7.1. Statistical Methods

In order to perform comparisions between sets of data from different treatment rats and different peptides, a statistical method was used. The statistics package (SPSS-X) was utilized. Data was compared using analysis of variance, Tukey's test, Scheffe's Test, and the Student Newman Keuls procedure.

4.8. Perfusion and Fixation for Electron Microscopy

4.8.1. Routine Fixation

The same method of perfusion was used for this procedure, as was used in the preparation of tissues for light microscopy. Karnovsky's fixative (Karnovsky, 1965) was used in place of Zamboni's fixative.

4.8.2. Chromaffin Reaction Fixation

The same procedure for anesthesia and for the preparation of the animals were performed for routine fixation. A primary fixative was perfused, composed of 1 % glutaraldehyde (TAAB, England) and 0.4 % formaldehyde (TAAB, England) in 0.1 M sodium chromate/potassium dichromate (J.T. Baker Chemical Company, U.S.A.) buffer, pH 7.2, followed by a solution of sodium chromate and potassium dichromate, pH 6.0. This was followed by processing for electron microscopy.

4.9. Processing For Electron Microscopy

The superior mesenteric artery with attached jejunal arteries and the mesenteric vein was dissected from the perivascular fat under a dissection micrscope (Wild Leitz) and placed in the same fixative for an additional 2 hours fixation. The tissues were then processed as follows : rinsed in 0.1 M sodium cacodylate (Marivac Limited, Canada) buffer ; postfixed with 1% osmium tetroxide (Marivac Limited, Canada) in the same buffer; stained *en block* with 50% ethanol saturated uranyl acetate (TAAB, England); dehydrated in a series of graded ethanols; cleared using acetone; infiltrated with a 1:1 mixature of acetone

and araldite for 12 hours; polymerized in unadulterated Araldite for 24 hours at 70 degrees Celsius. Prior to polymerization the vessels were cut and arranged in embedding molds.

After at least 24 hours of polymerization thick sections (0.5 um) were cut on a Huxley Ultramicrotome. The sections were collected on slides and stained with toluidine blue. Thin sections (70-85 nm) were cut on a Reichert-Jung Ultracut microtome and collected onto copper grids (mesh size G300). Grids were stained with saturated uranyl acetate for ten minutes and rinsed with distilled water. They were then stained *en face* with lead citrate (TAAB, England) for 3 minutes and then rinsed with distilled water. The grids were observed using a Phillips EM300 electron microscope at 60 KV.

4.10. Processing For Chromaffin Reaction Fixed Tissue

The superior mesenteric artery with attached jejunal arteries and the mesenteric vein was dissected from the perivascular fat under a dissection microscope (Wild Leitz) and placed in the sodium chromate/potassium dichromate solution for 12 hours at 4 degrees Celsius. The tissues were then processed as follows: postfixation in 2% osmium tetroxide (Marivac, Canada) in 0.1 M sodium chromate/potassium dichromate at pH 7.2 for one hour at 0-4 degrees Celsius; stained *en block* with 50% ethanol saturated uranyl acetate (TAAB, England); dehydrated in a series of graded ethanols; cleared using acetone; infiltrated with 1:1 mixature of acetone and Aradilate for 12 hours; and polyermized in unadulterated Araldite for 24 hours at 70 degrees Celsius. Prior to polymerization the vessels were cut and arranged in embedding molds.

The same procedure for cutting of electron microscopy blocks and staining of grids was followed, as described above.

4.11. Photographic Techniques For Electron Microscopy

Electron micrographs were taken using pre-dessicated Kodak Electron Microscope film, 3 1/2 x 4 inches. Exposure times of two seconds at a photometer setting of 50 were used. The film was developed with Kodak D19 Developer, diluted 1:2 with water, for four minutes at 20 degrees Celsius. Following development the films were rinsed in running tap water for one minute, and then fixed in Kodak Rapid Fix for four minutes. The films were washed in running tap water for twenty minutes followed by further rinsing with double distilled water and then dried in a film dryer.

Electron micrographs were then printed from the negatives using an enlarger. Working prints were made using an Ilford stabilization printer with Ilford paper. Permanent prints were made using Ilford Ilfospeed paper. The Ilfospeed paper was developed in Ilford Bromophen developer, diluted 1:2 with water, for one minute at 20 degrees Celsius, the prints were rinsed briefly in running tap water for approximately ten seconds and then fixed with Kodak Rapid Fix, diluted 1:2 with tap water for approximately two minutes. The prints were rinsed in running tap water at 20 degrees Celsius for five minutes. The prints were then allowed to dry.

4.12. Analysis Of Data Collected From Electron Micrographs

The jejunal artery was selected to be the vessel to be examined. It has the most nerve profiles compared to the superior mesenteric vein and superior mesenteric artery. The wall of the artery was closely studied and the number of nerve profiles were counted. Counts were only taken from Karnovsky fixed tissue. It was decided that a number of counts would be taken from each vessel and the values representing the greatest number of nerve fibres would be recorded.

5. RESULTS

In general, the immunohistochemical method gave strong staining of nerve fibres on the mesenteric vessels. An immunohistochemical preparation was used for counting, if the nerve fibre plexus was uniformily distributed along the blood vessel. If a patchy result was obtained, that preparation was rejected for counting. Because of the experimental design, a loss of the specimen preparation, due to rejection, resulted in the loss of a value and peptide examined in that preparation. The rejected counts are shown in Table 2a as (-).

The controls for the immunohistochemical procedure all gave negative results. No nerve fibre plexus was found on the mesenteric vessels when the primary antibody was omitted, when the antibody had been pre-absorbed by excess antigen, or when the linking antibody steps had been omitted.

The electron microscopic preparations were well fixed. Tissue fixed in Karnovsky showed good preservation of cell membranes and intracellular organelles. Nerve fibre bundles showed good preservation with a range of neurotransmitter containing vesicles. In the chromaffin fixed tissue, catecholaminergic axons were plainly visible, containing dense granules. In this tissue, however, general fixation was not as good as for the Karnovsky fixed material, and in some instances mitochondria damage had occurred.

5.1. Developmental Study

5.1.1. Light Microscopic Analysis By Immunohistochemistry

At the early stages of development, the jejunal artery was found to be the easiest mesenteric vessel to examine. A clean dissection of this vessel was possible and the nerve fibres on the vessel were very prominent. CGRP-, SP-, NPY-, and TH-immunoreactive nerve fibres were present on the jejunal artery at one day after birth. CGRP-immunoreactive fibres appeared to be developing a plexus at this age. Beads on the fibres were noticeable. NPY-, TH-, and SP-

immunoreactive were not as well developed as the CGRP-immunoreactive fibres. On a small number of the jejunal arteries examined, some VIP-immunoreactive fibres were found. However, they were very scarce. The NPY-, TH-, and SP-immunoreactive nerve fibres were not photographed at one day after birth because focusing of single fibres was difficult to perform. Drawings of these fibres on the jejunal artery were made instead (Figure 1).

By day ten, all five different types of immunoreactive fibres had formed a plexus on the vessels. By day fourteen, the plexus had matured (Figure 2) and with the aid of the quantitation method it was discovered that the density of the nerve fibre plexus on all the mesenteric vessels examined, for fourteen day old rats, was comparable to that for twelve week rats (Tables 1a-1c). Typical examples of the fibre network drawings made from the twelve week animals are shown in Figure 3. As can be seen from these drawings, the form of the nerve fibre plexus is not the same for the three vessels and for the five peptides. In general, the jejunal artery and the superior mesenteric vein have the densest fibre plexus. The nerve fibres on the vein are in a parallel orientation whereas on the jejunal artery the nerve fibres are tightly clustered. On the mesenteric artery the fibres were disperse and spider like. However, the plexus also differs somewhat depending on the peptide examined. The SP- and CGRP-nerve fibre plexuses appear to be the same type of plexus, whereas the NPY-, TH-, and VIP-nerve plexuses are similar. Photographs of the nerve fibre plexuses on the vessels of the mesenteric bed at twelve weeks of age are presented in Figure 4.

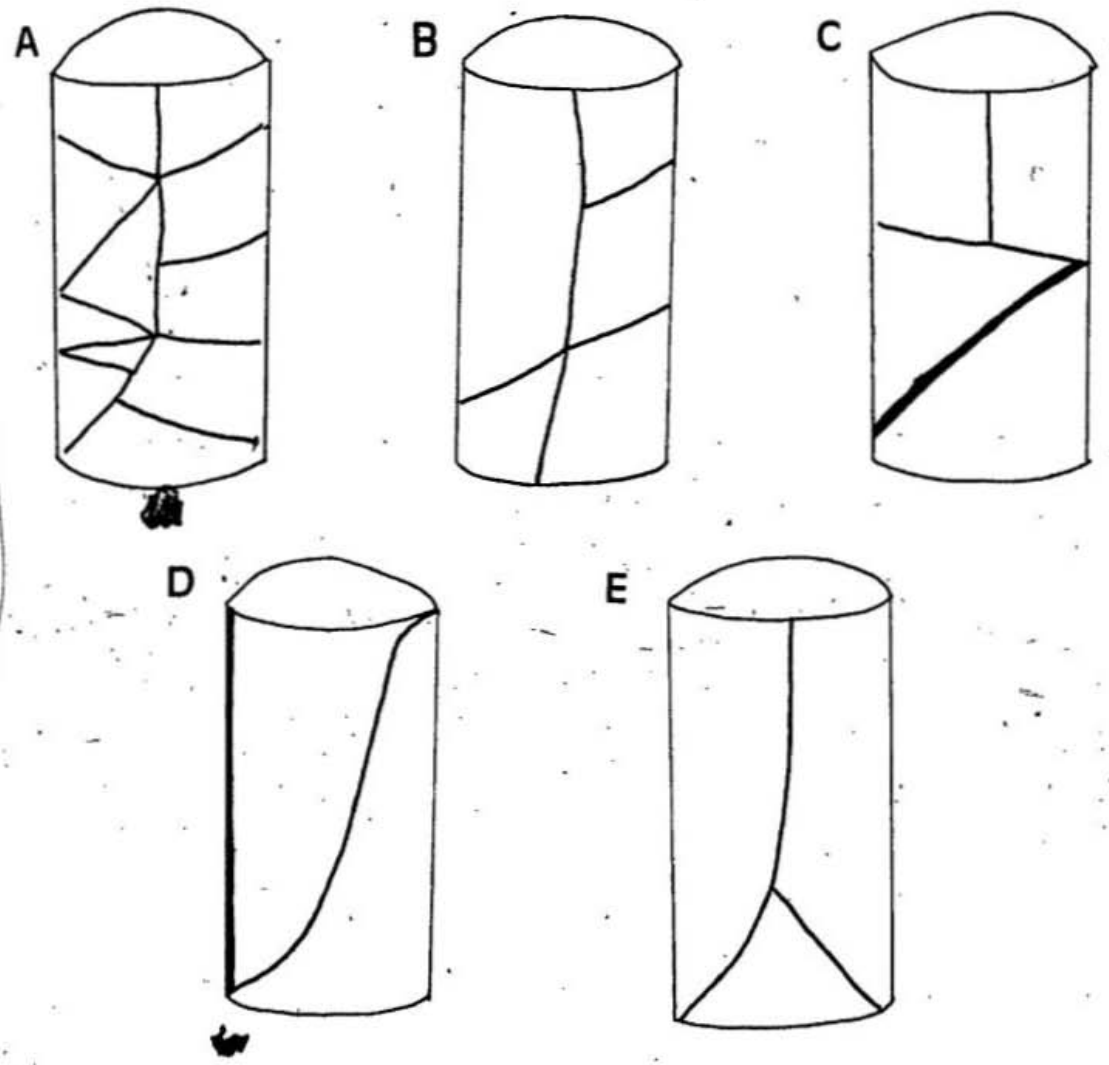


Figure 1. A drawing illustrating nerve fibre innervation on the jejunal artery one day after birth by (A) CGRP-, (B) SP-, (C) TH-, (D) NPY-, and (E) VIP-immunoreactive nerve fibres.

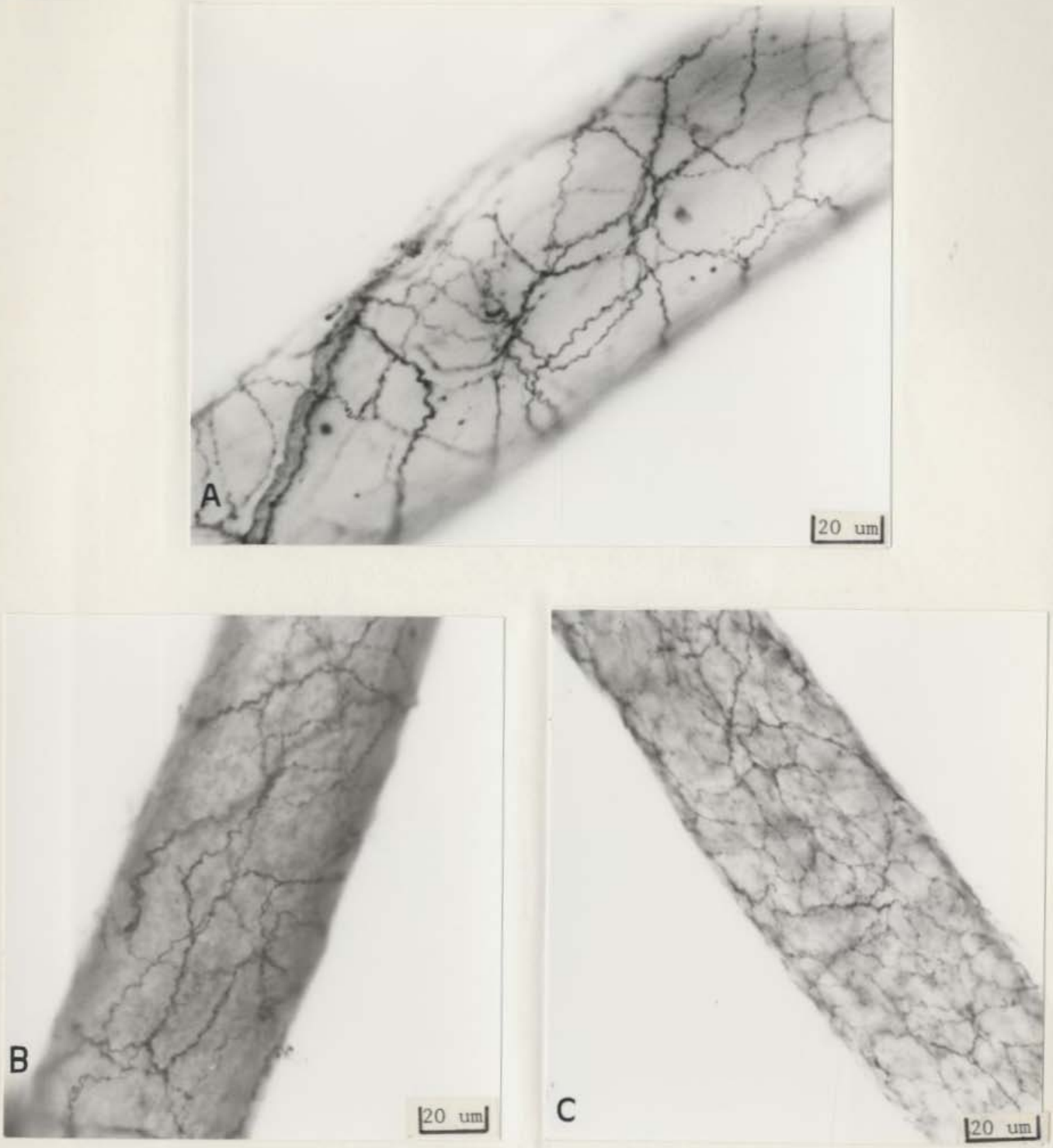


Figure 2. Innervation of the jejunal artery fourteen days after birth by (A) CGRP-, (B) SP- (C) TH-immunoreactive nerve fibres. (Magn. 600X)

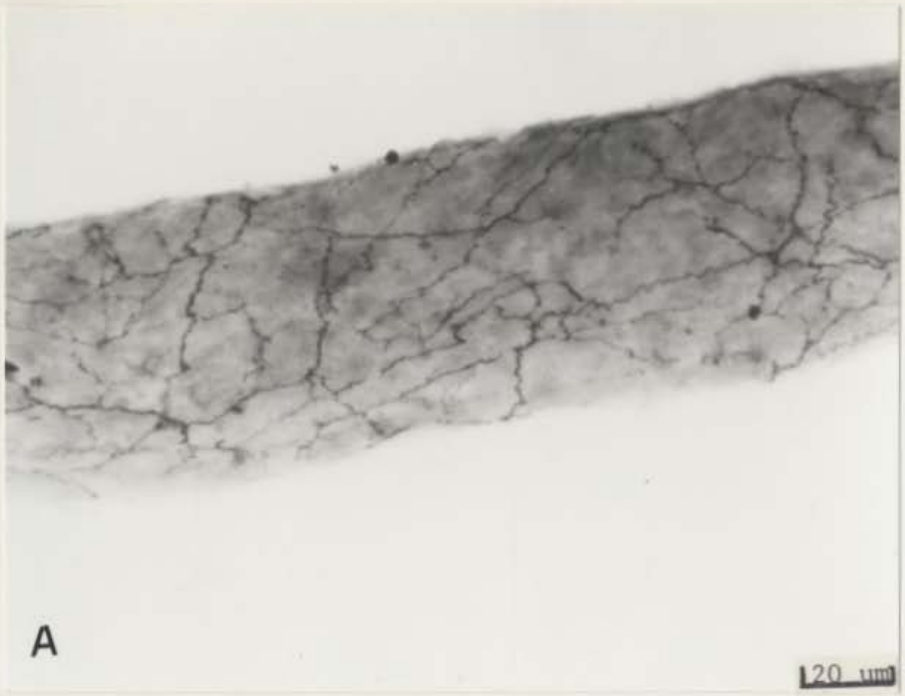


Figure 2(cont.). Innervation of the jejunal artery fourteen days after birth by (A) NPY-, and (B) VIP-immunoreactive nerve fibres. (Magn. 600X)

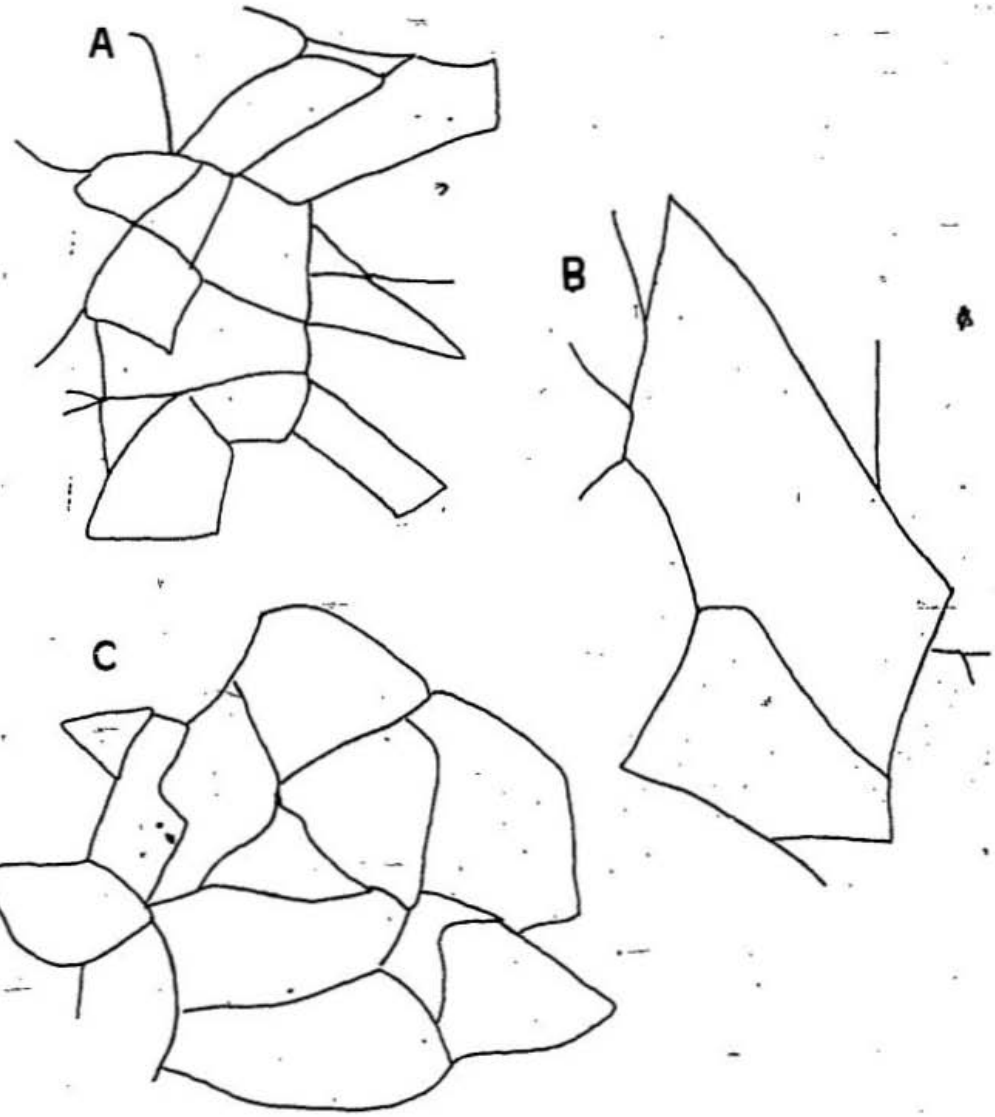


Figure 3a. Typical drawings made from immunohistochemical preparations for CGRP immunoreactive nerve fibres on the (A) mesenteric artery, (B) mesenteric vein, and (C) jejunal artery at twelve weeks of age.

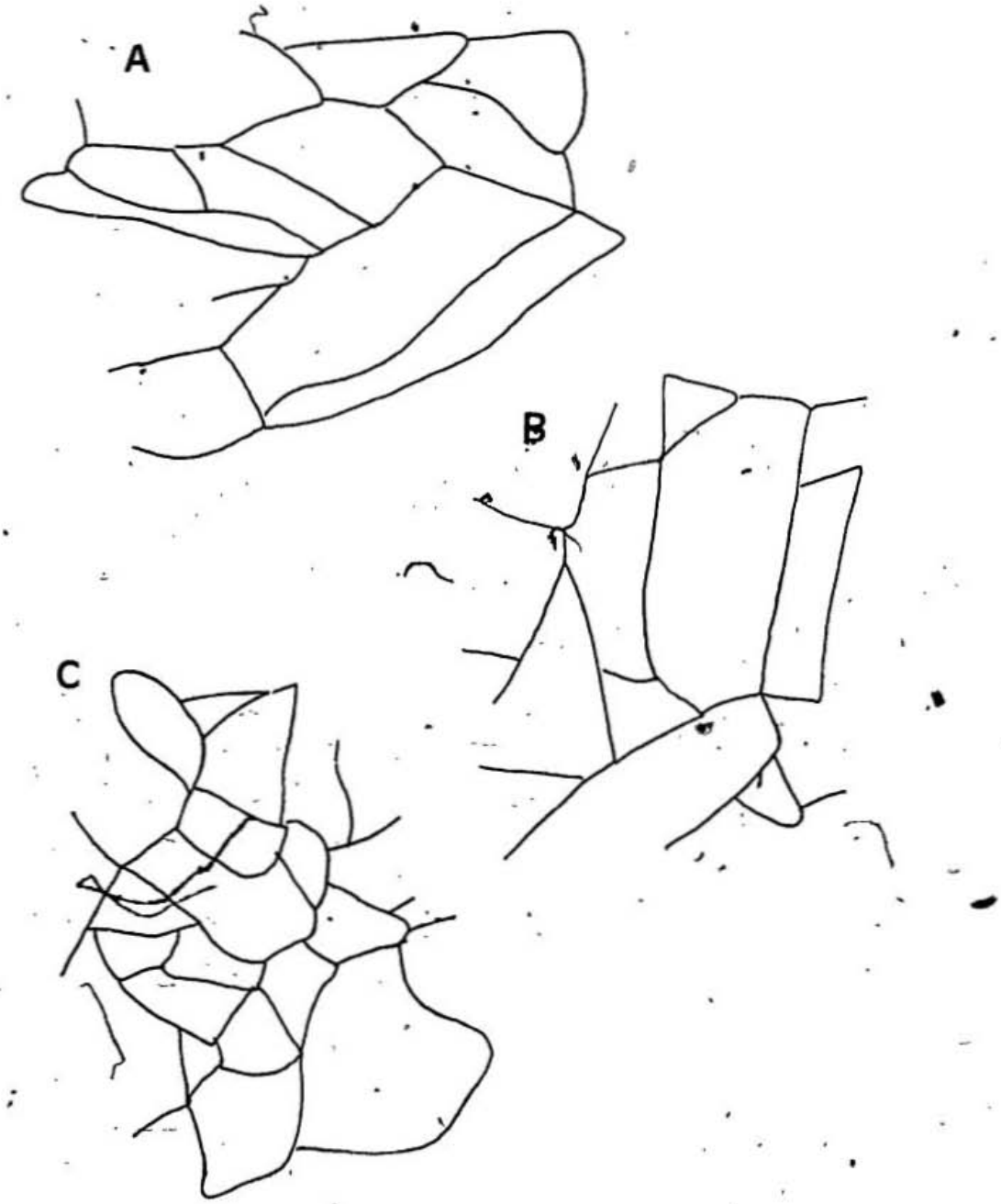


Figure 3b. Typical drawings made from immunohistochemical preparations for SP-immunoreactive nerve fibres on the (A) mesenteric artery, (B) mesenteric vein, and (C) jejunal artery at twelve weeks of age.

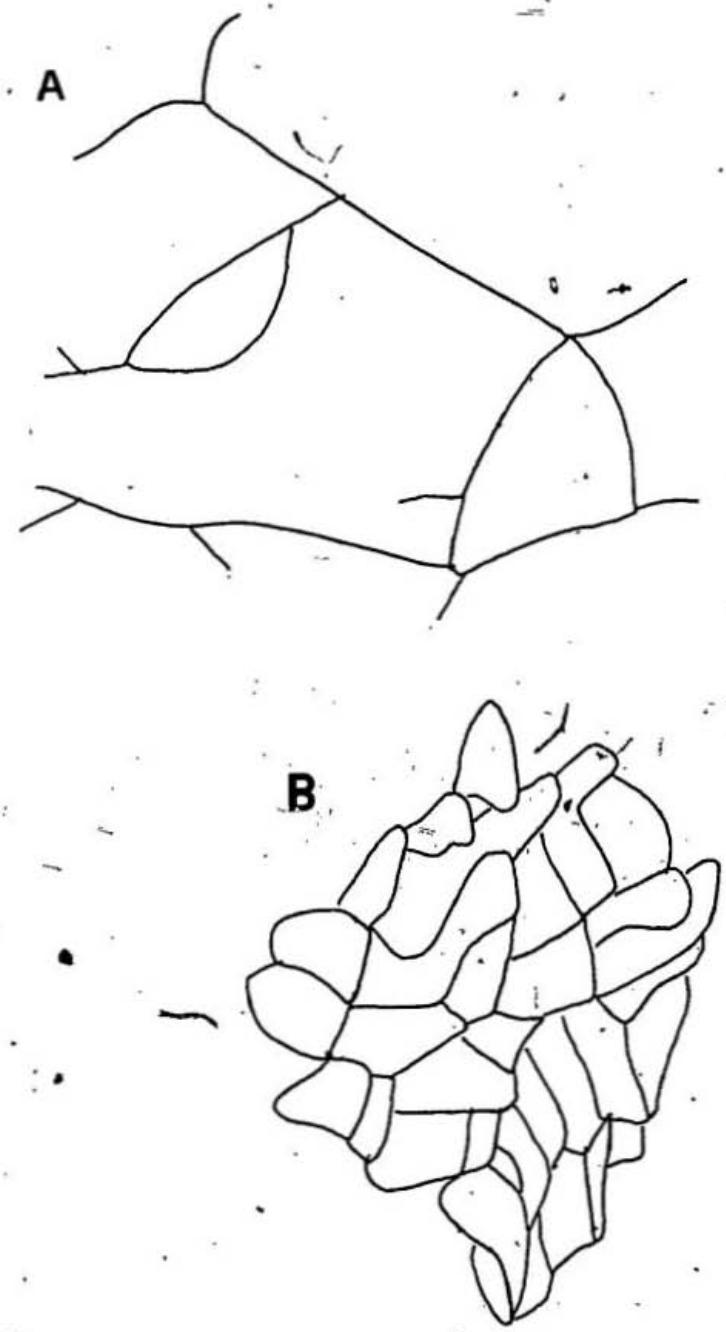


Figure 3c. Typical drawings made from immunohistochemical preparations for VIP-immunoreactive nerve fibres on the (A) mesenteric artery and (B) jejunal artery at twelve weeks of age.

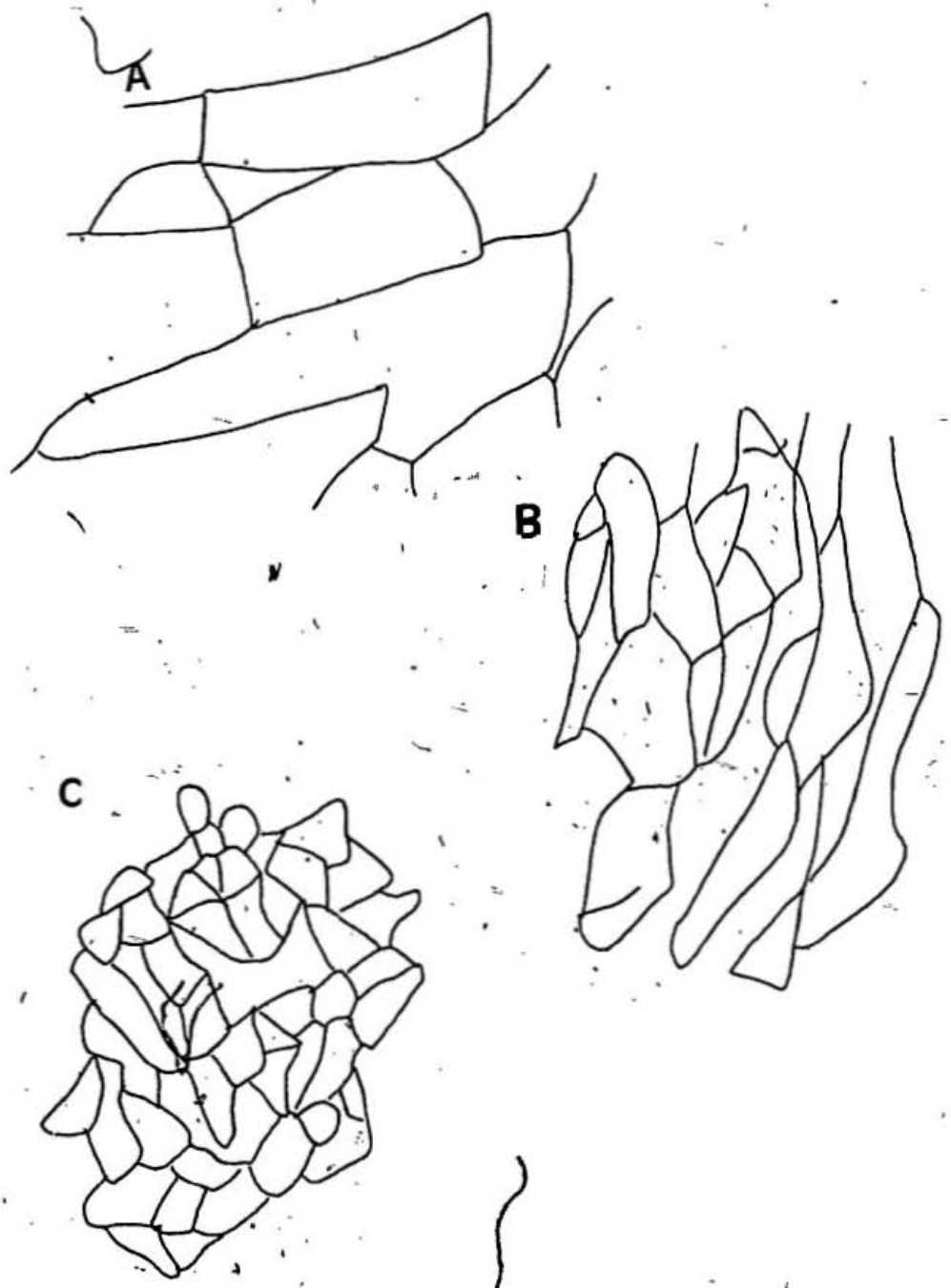


Figure 3d. Typical drawings made from immunohistochemical preparations for TH-immunoreactive nerve fibres on the (A) mesenteric artery, (B) mesenteric vein, and (C) jejunal artery at twelve weeks of age.

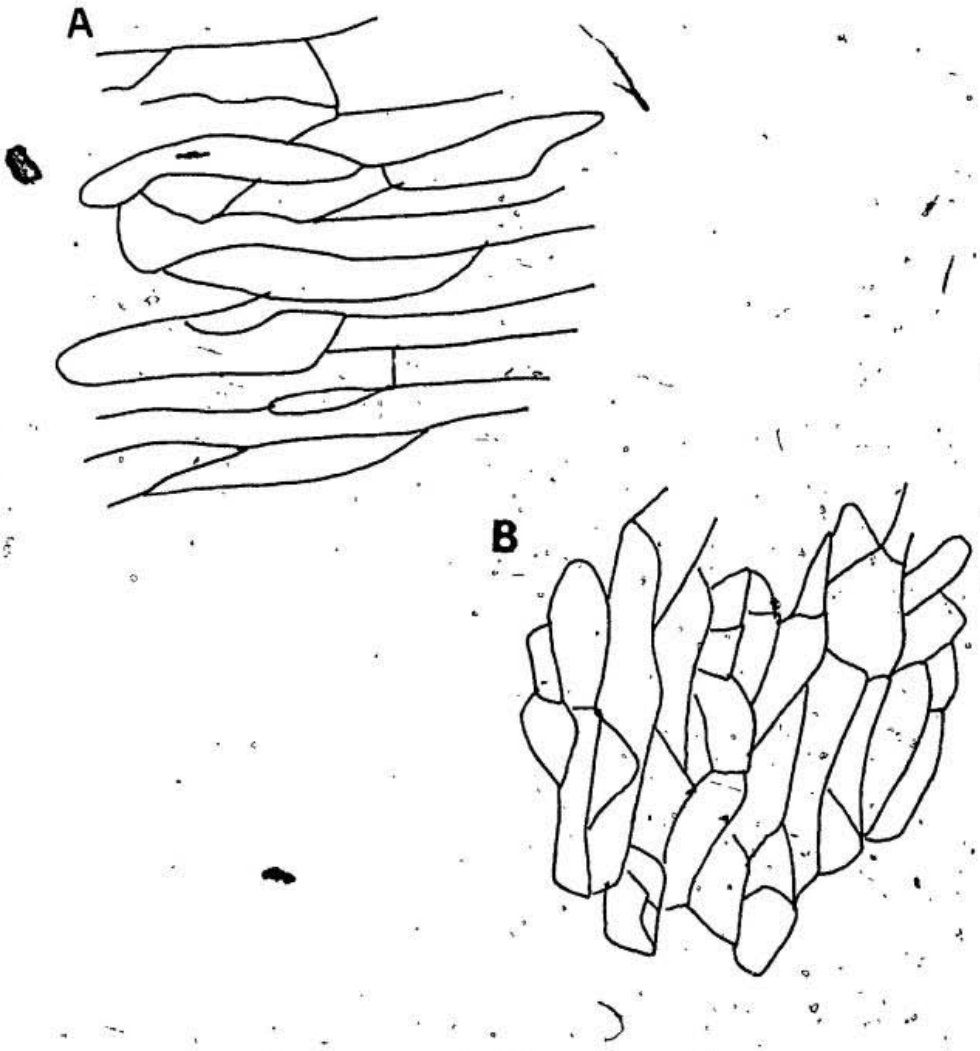


Figure 3e. Typical drawings made from immunohistochemical preparations for NPY-immunoreactive nerve fibres on the (A) mesenteric vein and (B) jejunal artery at twelve weeks of age.

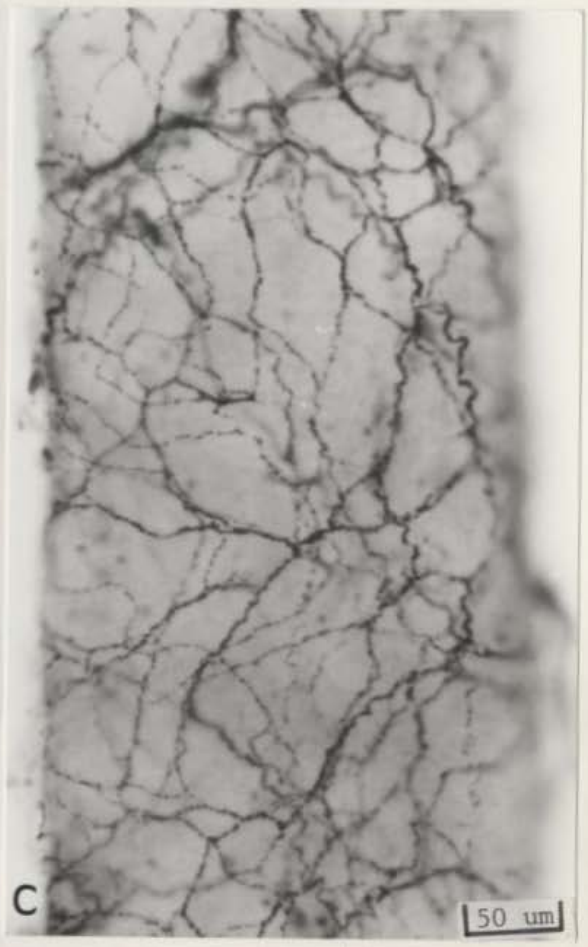


Figure 4a. Innervation by CGRP-immunoreactive nerve fibres on the (A) mesenteric artery (B) mesenteric vein, and (C) jejunal artery at twelve weeks of age. (Magn. 260X)

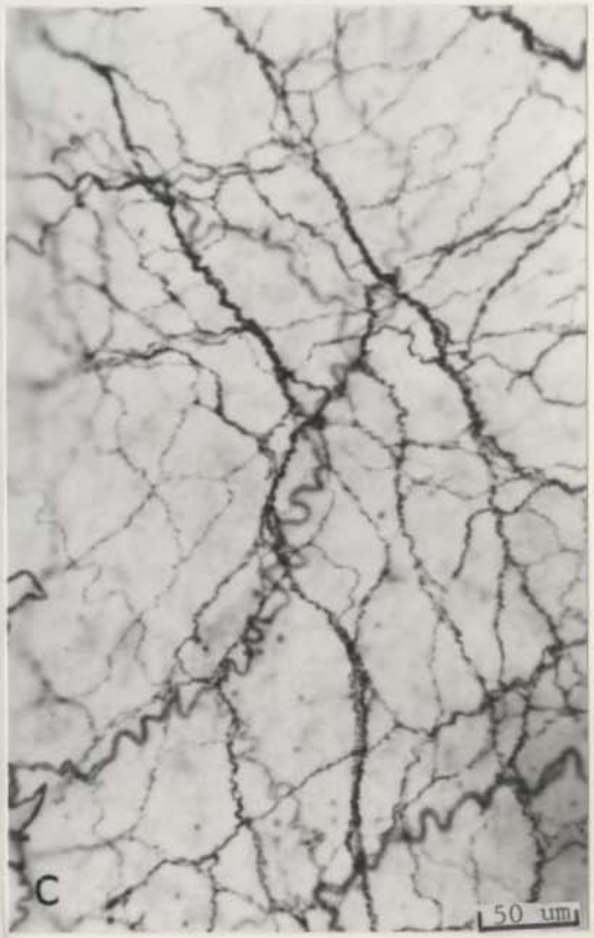


Figure 4b. Innervation by SP-immunoreactive nerve fibres on the (A) mesenteric artery (B) mesenteric vein, and (C) jejunal artery at twelve weeks of age. (Magn. 260X)

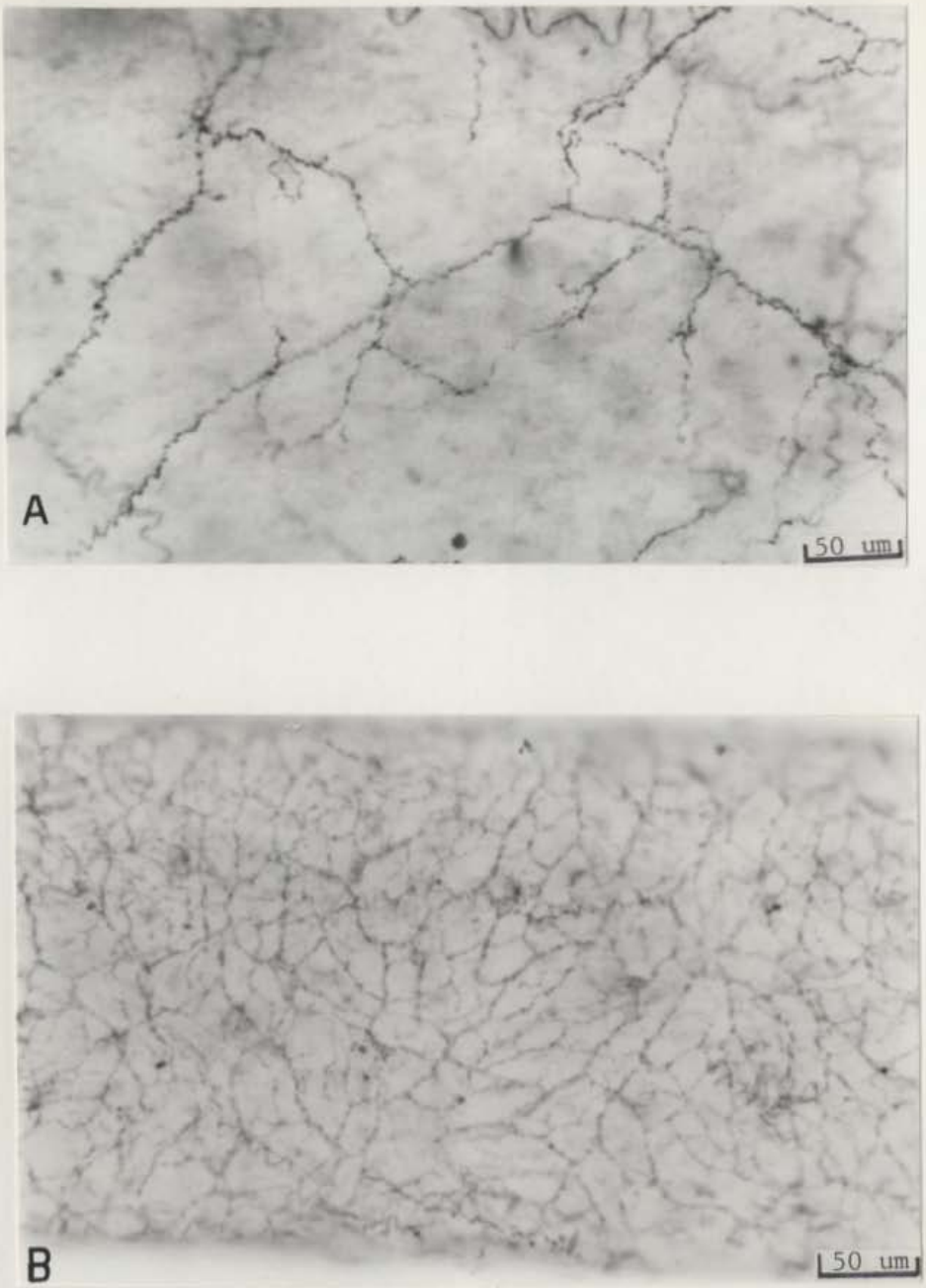


Figure 4c. Innervation by VIP-immunoreactive nerve fibres on the (A) mesenteric artery and (B) jejunal artery at twelve weeks of age. (Magn. 260X)

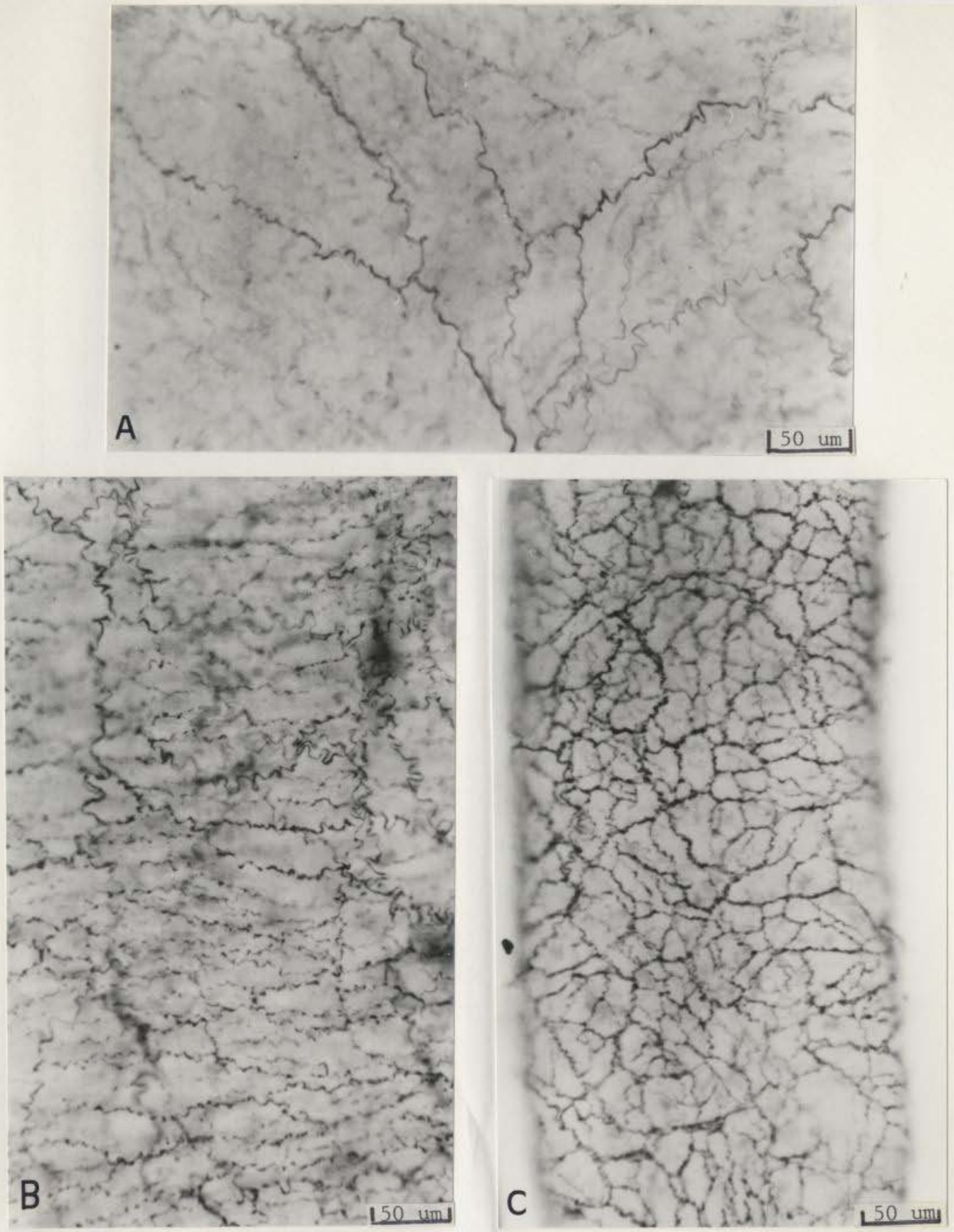
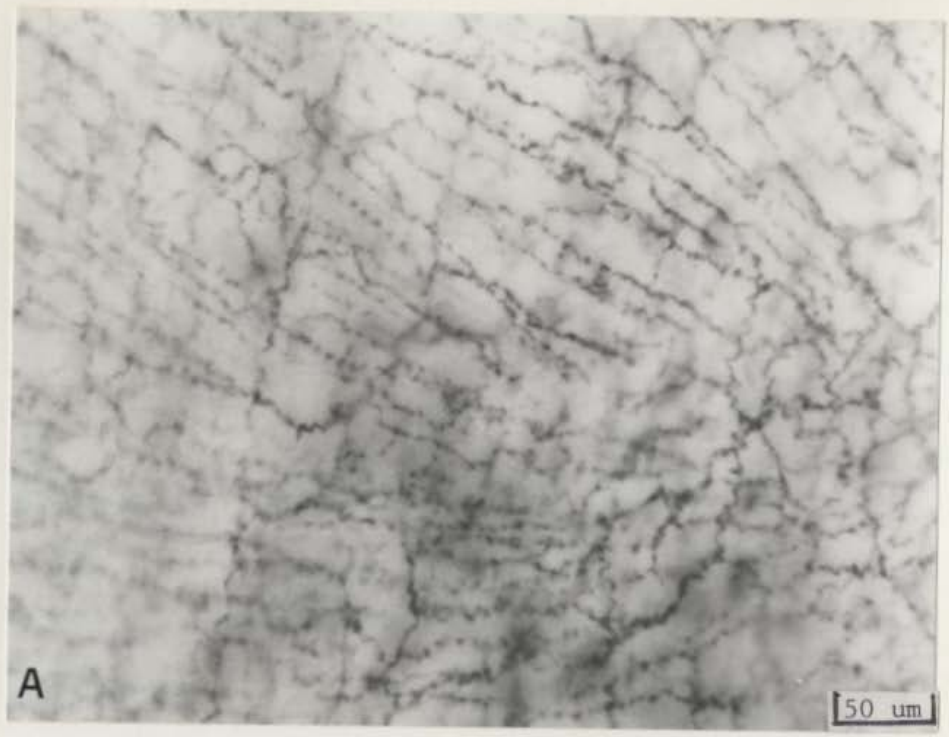
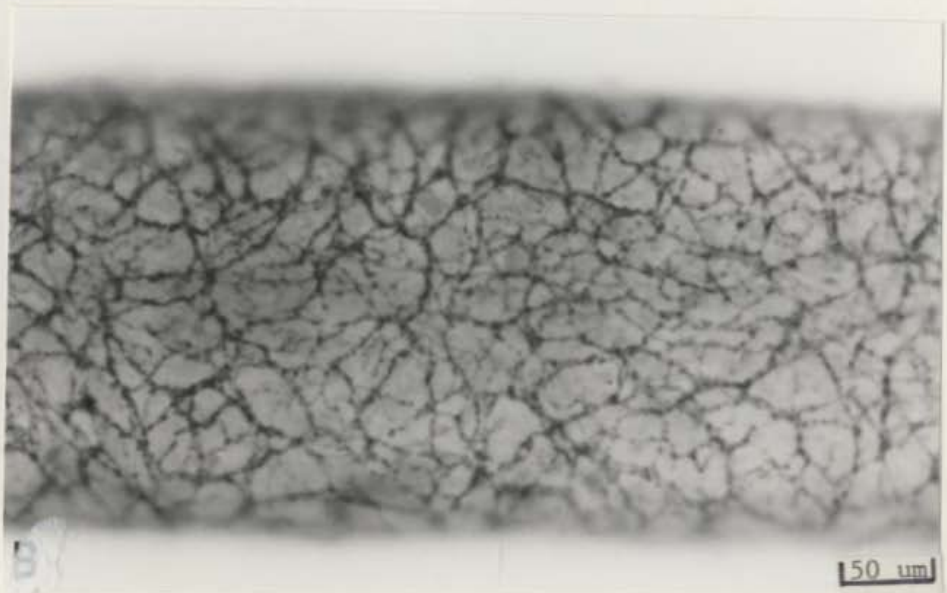


Figure 4d. Innervation by TH-immunoreactive nerve fibres on the (A) mesenteric artery (B) mesenteric vein, and (C) jejunal artery at twelve weeks of age. (Magn. 260X)





.gure 4e. Innervation by NPY-immunoreactive nerve fibres on the (A) mesenteric vein, and (B) jejunal artery at twelve weeks of age. (Magn. 260X)

TABLE 1A. The length (in microns) of the nerve fibre plexus per $1x10^5 \mu m^2$ of the mesenteric arterial wall +/- the standard error for rats at fourteen days and twelve weeks of age. (N=6)

PEPTIDES	AGE OF RATS	
	14 DAYS	12 WEEKS
Neuropeptide Y	360.6 +/-11.3	391.5 +/- 7.8
Vasoactive Intestinal Polypeptide	246.3 +/- 6.5	229.8 +/-16.9
Calcitonin Gene Related Peptide	435.1 +/-21.1	392.7 +/-20.8
Substance P	360.4 +/-20.4	304.8 +/- 4.3
Tyrosine Hydroxylase	333.7 +/-11.0	301.0 +/-25.7

Table 1B. The length (in microns) of the nerve fibre plexus per $1x10^5 \mu m^2$ of the mesenteric vein wall +/- the standard error for rats at fourteen days and twelve weeks of age. (N-6)

PEPTIDES	AGE OF RATS	
	14 DAYS	12 WEEKS
Neuropeptide Y	599.1 +/-8.9	617.7 +/-37.4
Vasoactive Intestinal Polypeptide	*	*
Calcitonin Gene Related Peptide	411.2 +/-34.6	354.8 +/-12.2
Substance P	309.0 +/-9.3	384.7 +/-3.8
Tyrosine Hydroxylase	455.9 +/-34.6	618.8 +/-63.6

(*)indicates the absence of a nerve fibre plexus.

Table 1C. The length (in microns) of the nerve fibre plexus per $1x10^5 \mu m^2$ of jejunal arterial wall +/- the standard error for rats at fourteen days and twelve weeks of age. (N=6)

PEPTIDES	AGE OF RATS	
	14 DAYS	12 WEEKS
Neuropeptide Y	790.9 +/-21.5	663.5 +/-26.9
Vasoactive Intestinal Polypeptide	685.9 +/-29.0	716.2 +/-16.2
Calcitonin Gene Related Peptide	414.6 +/-35.7	357.5 +/-14.8
Substance P	403.3 +/-1.3	349.7 +/-8.7
Tyrosine Hydroxylase	820.0 +/-54.5	778.8 +/-13.8

5.1.2. Electron Microscopic Analysis

The vessels of the mesenteric bed were found to be quite immature one day after birth. The jejunal artery had the greatest nerve supply, and therefore it was chosen to be examined more closely. At one day after birth, the jejunal artery consisted of six cellular layers (Figure 5). The first layer was an endothelial layer. The endothelial cells contained many different organelles. A large number of ribosomes and vesicular organelles were observed. Simple junctions between endothelial cells were found. Underneath the endothelial layer there was an elastic layer. This was a thin layer and was fenestrated in areas. The third layer was an immature smooth muscle layer. The smooth muscle cells contained few filaments. They did contain a large number of other organelles. Mitochondria, ribosomes, rough endoplasmic reticulum and a large number of vesicles were seen. Lying under the smooth muscle, a small amount of elastin was found. The fifth layer was an undifferentiated cell layer. Nerve bundles, making up the sixth layer were present on the outside of the vessel. The bundles consisted of several axons ensheathed by a Schwann cell. The axons contained many different types of vesicles (Figure 6). Small dense cored, small clear, and opaque vesicles were observed.

From day one to day fourteen, the vessel matured in its structure (Figure 7). The number of smooth muscle cell layers had increased. The elastin layers had thickened and the endothelial cells had lost some of their organelles and had formed complex junctions between them. No difference in the structure of the nerve bundles was observed, but they were found closer to the differentiated smooth muscle layer (Figure 8) and the number of bundles had increased in the vessel.

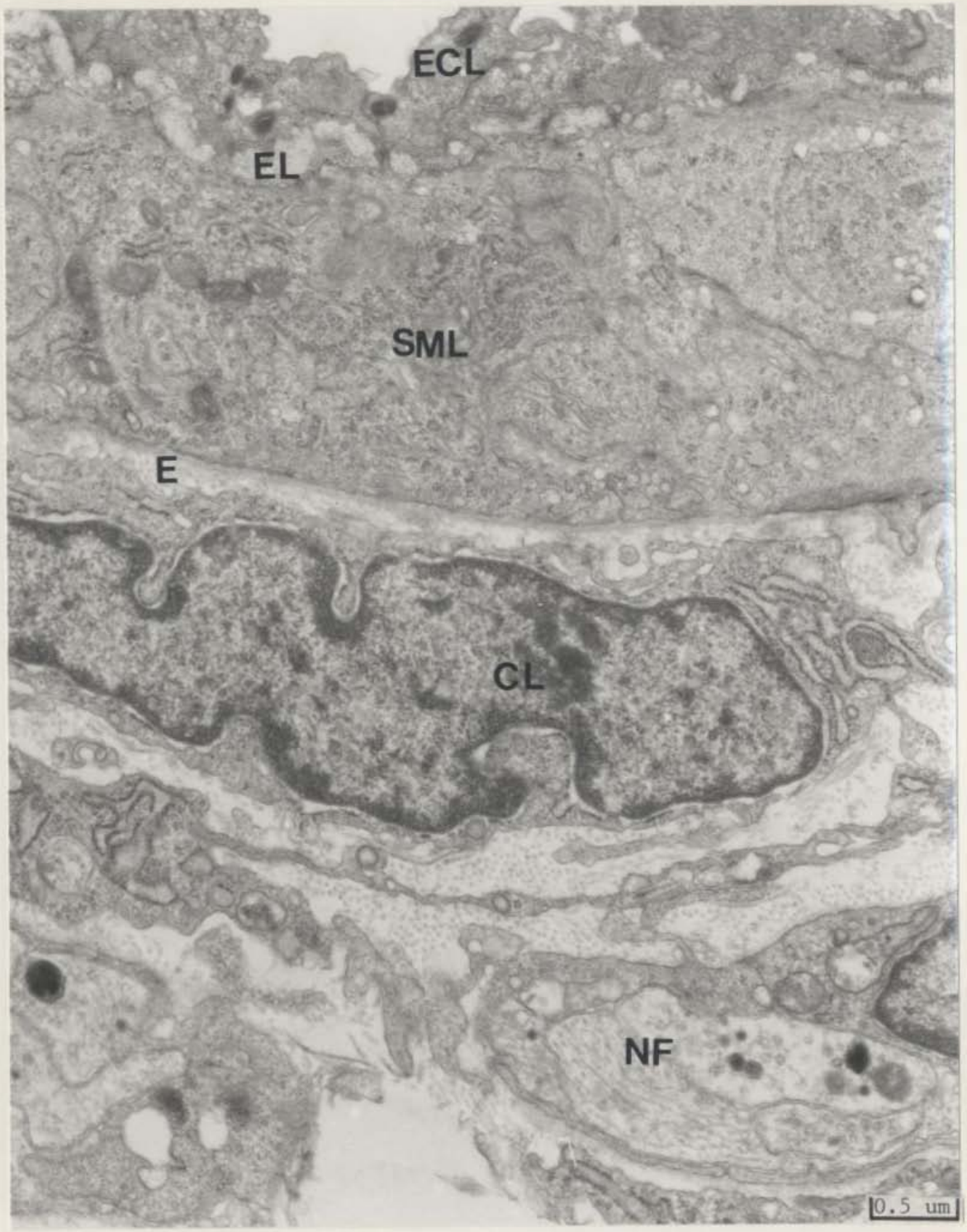


Figure 5. The jejunal artery one day after birth. It consists of six cellular layers. An endothelial cell layer (ECL), an elastic lamella (EL), a smooth muscle layer (SML), another elastic layer (E), an undifferentiated cellular layer (CL), and a layer containing nerve fibres (NF). (Magn. 28,000X)

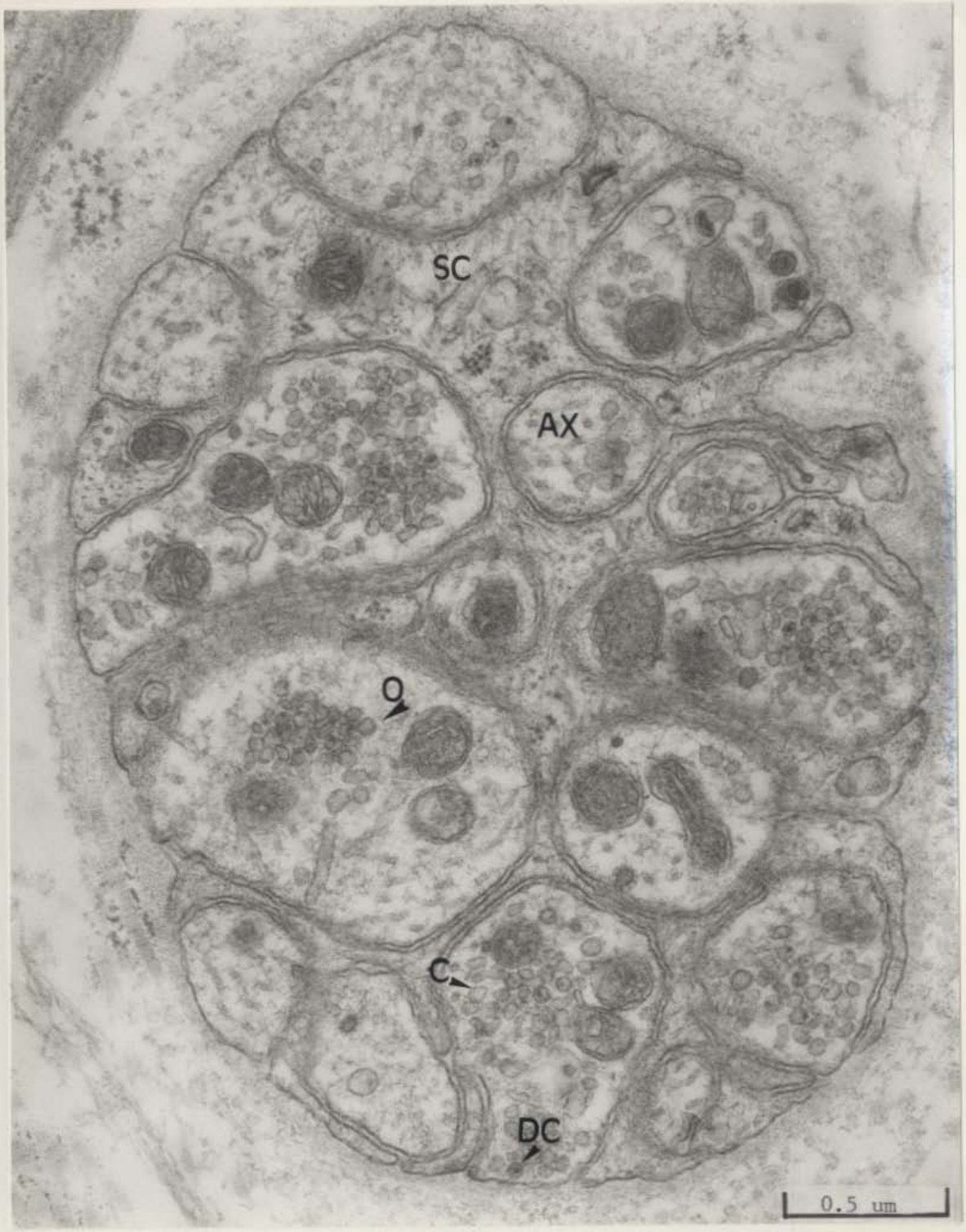


Figure 6. Nerve fibre bundles consist of several axons (AX) ensheathed by a Schwann cell (SC). The axons contain many different types of vesicles. Small dense cored (DC), small clear (C), and opaque vesicles (O) can be found. (Magn. 55,000X)

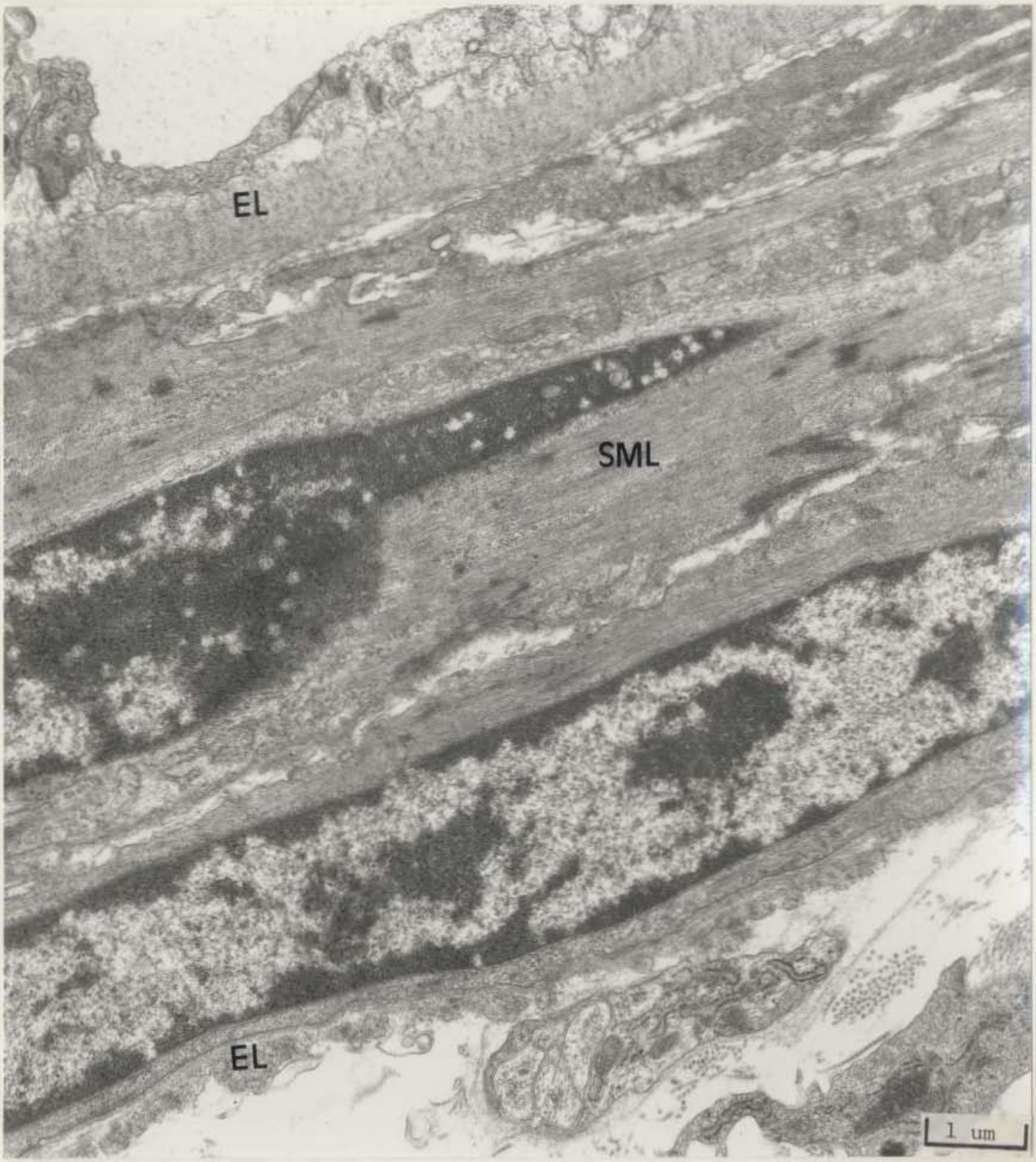


Figure 7. The jejunal artery at fourteen days after birth. The smooth muscle layers (SML) had increased and the elastin layers (EL) had thickened. (Magn. 16,800X)

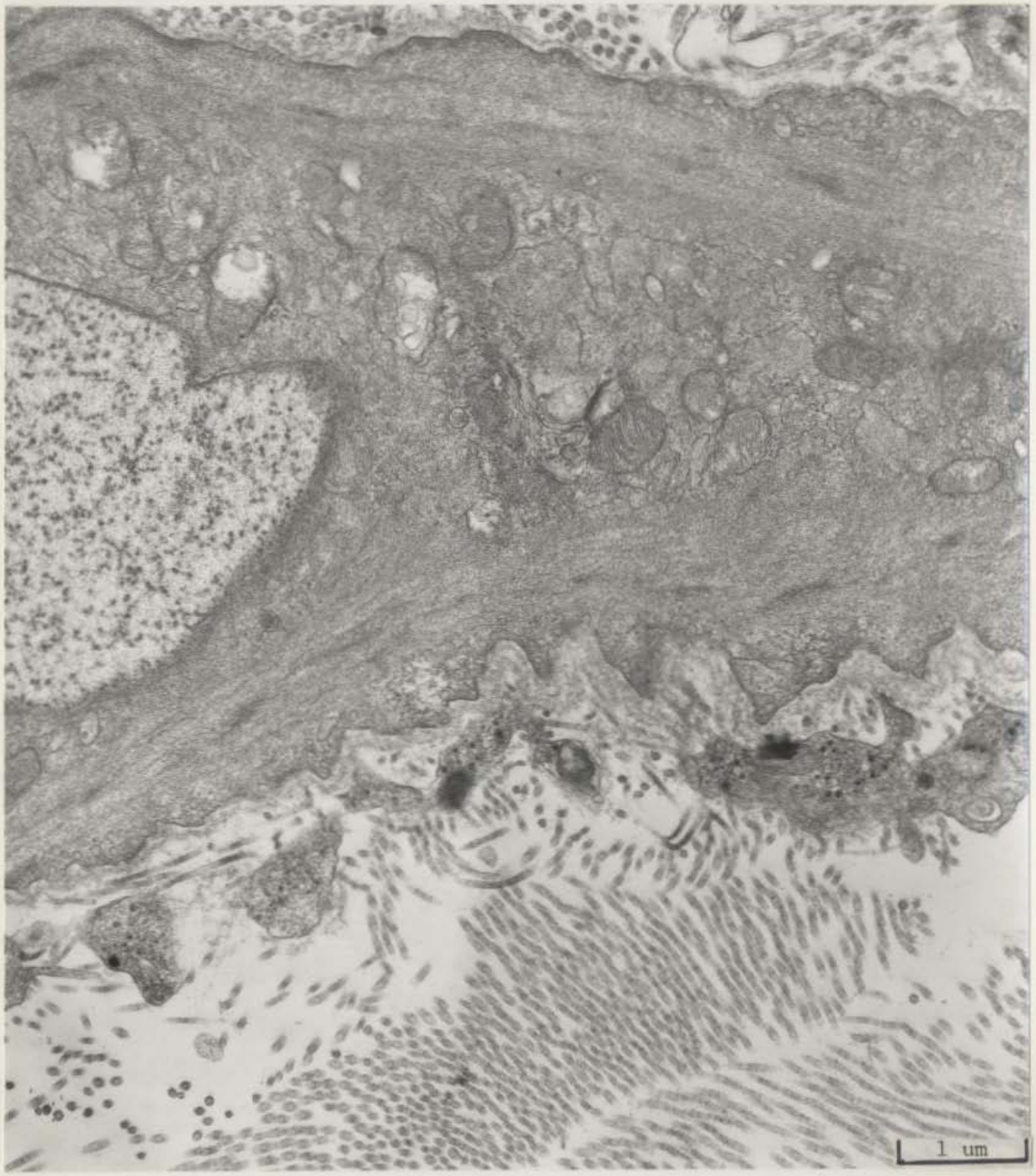


Figure 8. Innervating nerve fibre bundle. The nerve fibres are found much closer to the smooth muscle layer in the mature jejunal artery. This vessel was chromaffin fixed. (Magn. 21,000X)

5.2. Capsaicin and 6-Hydroxydopamine Studies

5.2.1. Light Microscopic Analysis By Immunohistochemistry

From the immunohistochemical preparations, values for the nerve fibre density were obtained and subjected to analysis of variance. This analysis showed that the density values obtained for the control rats and the control vehicles at four and twelve weeks of age could be grouped into one group. The 6-hydroxydopmaine rats at four and twelve weeks of age could be grouped into another group. There was some mixing of the capsaicin four and twelve week old rats into the control group and into a group of their own (see Appendix). In these groups where differences were detected, further analysis was performed using the Tukey Multiple Range test, the Student Newman Keuls test, and the Scheffe procedure in order to identify significantly different values.

In the capsaicin treated four and twelve week rats, CGRP- and SP-immunoreactive nerve fibres were absent from all three mesenteric vessels (Tables 2a-2c, Figure 9).

The TH-immunoreactive nerve fibre density was found to have increased for the mesenteric artery, in the four and twelve week treated capsaicin rats (Figure 10). As well, an increase in the density values for the TH-immunoreactive nerve plexus was found for the mesenteric vein at four weeks in the treated capsaicin rats from capsaicin treated mothers and at twelve weeks in the capsaicin treated rat group. An increase in the TH-nerve fibre density was seen on the jejunal artery for the twelve week capsaicin treated rats from capsaicin treated mothers.

The NPY-immunoreactive nerve fibre density values were found to have increased for the mesenteric vein at four and twelve weeks of age in the capsaicin treated rats. An increase was also seen in the jejunal artery at four weeks in the capsaicin treated rats and at twelve weeks for the capsaicin treated rats from capsaicin treated mothers.

The VIP-immunoreactive nerve fibre density values were found to have increased for the jejunal artery at four weeks in the capsaicin treated rats group.

For the 6-hydroxydopamine treated rats, at four and twelve weeks of age, the NPY- and TH-immunoreactive nerve fibre plexuses were absent from all three mesenteric vessels (Tables 2a-2c, Figure 11). There also appeared to be a number of significant changes in the density of the SP-, CGRP-, and VIP-nerve fibre plexuses on the mesenteric vessels at four and twelve weeks of age in the 6-hydroxydopamine treated rats.

The SP-immunoreactive nerve fibre density values were found to have decreased for the mesenteric artery at four weeks of age in the 6-hydroxydopamine treated rats. On the mesenteric vein and jejunal artery the VIP-, SP-, and CGRP-immunoreactive nerve fibre plexuses were found to have decreased at four and twelve weeks of age in the treated rat group (Figure 12).

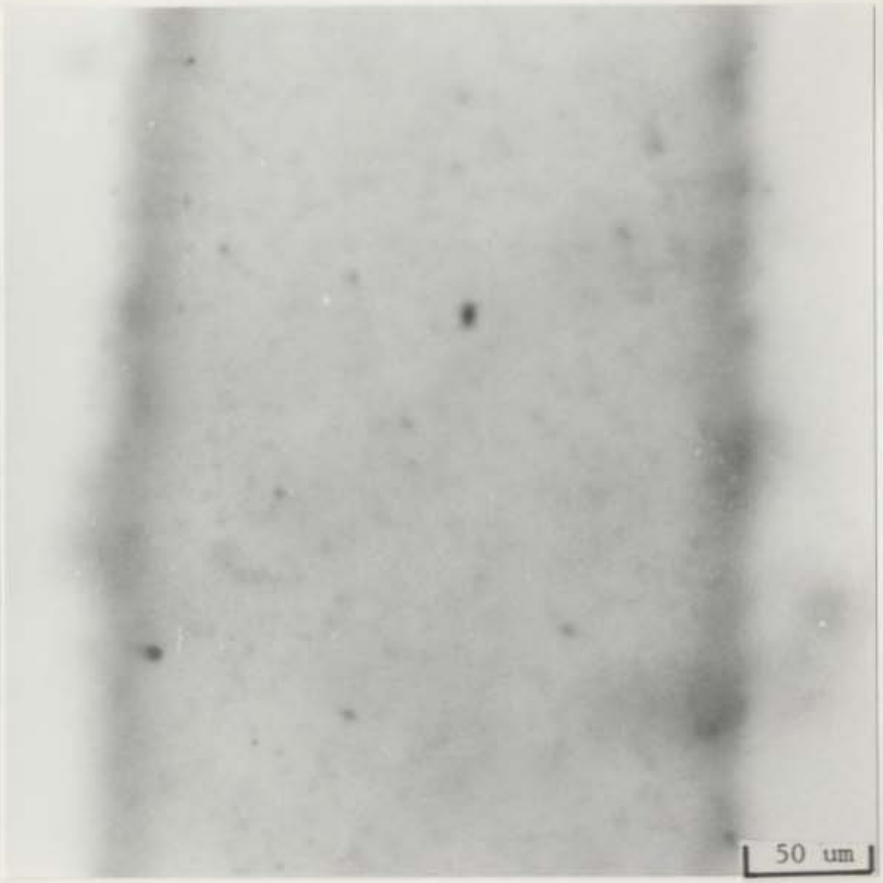


Figure 9. Jejunal artery after capsaicin treatment. The CGRP-immunoreactive nerve plexus was absent. (Magn. 350X)

Figure 10. A mesenteric artery from a capsaicin treated rats. The density of the TH-immunoreactive nerve plexus has increased (Magn. 350X)

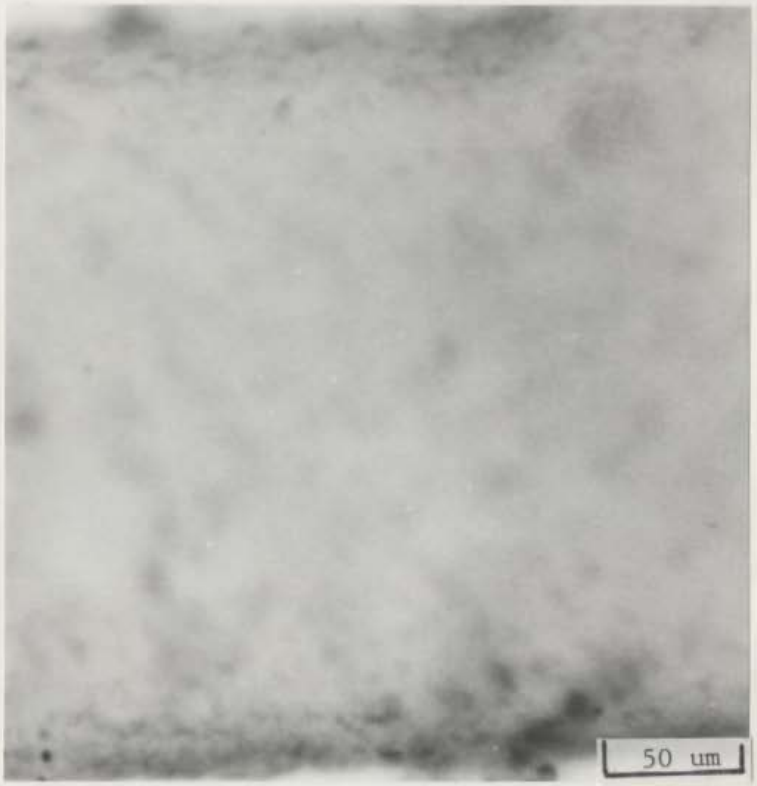


Figure 11. A jejunal artery after 6-hydroxydopamine treatment. The NPY-immunoreactive nerve fibre plexus was absent. (Magn. 350X)

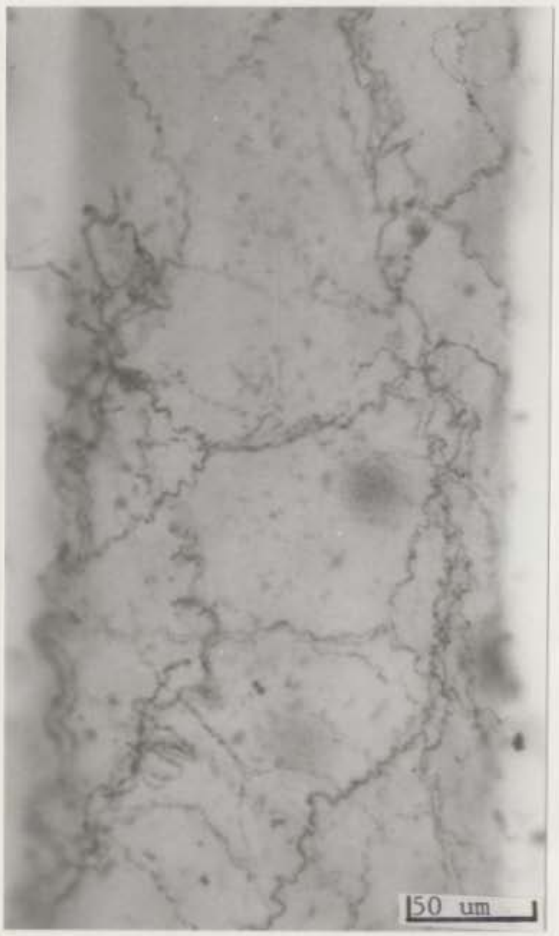


Figure 12. CGRP-immunoreactive nerve plexus on the jejunal artery after 6-hydroxydopamine treatment. The density was found to have been decreased in the 6-hydroxydopamine rat group. (Magn. 350X)

Table 2A. The nerve fibre density (length of nerve fibre plexus/1 x 10^5 μm^2 of vessel wall) +/- the standard error for five different peptides and twelve different treatments on the superior mesenteric artery. (N-6)

	PEPTIDES				
TREATMENTS	NPY	VIP	CGRP	SP	TH
4 Week Controls	-	257.5 +/-16.1	263.7 +/-11.7	306.7 +/-27.0	225.7 +/-8.7
4 Week Capsaicin	-	144.3 +/-11.6	0	0	*386.2 +/-30.3
4 Week 6-OHDA	-	202.2 +/- 9.3	264 +/-11.5	*233.7 +/-13.1	0
4 Week Capsaicin Vehicle	-	229.8 +/-18.4	437.7 +/-30.0	394.3 +/-22.2	290.2 +/-25.2
4 Week 6-OHDA Vehicle	-	236.2 +/-16.4	304.8 +/-12.5	312.7 +/-27.2	286.5 +/-28.7
4 Week Capsaicin (Injected Mothers)	-	381 +/-29.5	0	0	216.8 +/- 7.8
12 Week Controls	-	229.8 +/-16.9	392.7 +/-20.8	304.8 +/- 4.3	301 +/-25.7
12 Week Capsaicin	-	218.5 +/- 9.1	0	0	*446.8 +/-19.7
12 Week 6-OHDA	-	*170.8 +/-11.8	*208.5 +/-12.4	*207.3 +/- 9.1	0
12 Week Capsaicin Vehicle	-	273.2 +/-20.0	215 +/-28.0	330.7 +/-36.1	244.3 +/-10.5
12 Week 6-OHDA Vehicle	-	187.7 +/- 9.4	328.5 +/-11.5	314.2 +/-14.0	-
12 Week Capsaicin (Injected Mothers)	-	198.3 +/-10.6	0	0	258.3 +/-25.7

(*)indicates a value significantly different from the control values.
(-)indicates a rejected preparation

Table 2B. The nerve fibre density (length of nerve fibre plexus/1x10^5 μm^2 of vessel wall) +/- the standard error for five different peptides and twelve different treatments on the superior mesenteric vein. (N-6)

	PEPTIDES				
TREATMENTS	NPY	VIP	CGRP	SP	TH
4 Week Controls	513.8 +/-16.0	0	274.3 +/- 4.7	273.2 +/-23.5	594 +/-30.4
4 Week Capsaicin	*585 +/-28.1	0	0	0	662.2 +/-27.9
4 Week 6-OHDA	0	0	*208 +/-13.4	*161.7 +/-13.8	0
4 Week Capsaicin Vehicle	507.2 +/- 8.5	0	381.3 +/-26.8	342.2 +/-19.0	546.7 +/-29.9
4 Week 6-OHDA Vehicle	506 +/-10.2	0	404.8 +/-17.5	385.2 +/-14.3	642.5 +/-28.4
4 Week Capsaicin (Injected Mothers)	511 +/-27.6	0	0	0	*403.3 +/- 7.6
12 Week Controls	617.7 +/-37.4	0	354.8 +/-12.2	384.7 +/- 3.8	618.8 +/-63.6
12 Week Capsaicin	*760.7 +/-21.7	0	0	0	*860.5 +/-43.6
12 Week 6-OHDA	0	0	*181.3 +/- 8.7	*254.3 +/-1340	0
12 Week Capsaicin Vehicle	617.5 +/-42.3	0	340 +/-16.1	445.5 +/-36.0	626.8 +/-27.2
12 Week 6-OHDA Vehicle	670.5 +/-18.3	0	391 +/-22.5	354 +/-17.4	832.8 +/-22.2
12 Week Capsaicin (Injected Mothers)	650.5 +/-38.6	0	0	0	702.7 +/-39.1

(*)indicates a value significantly different from the control values.

Table 2C. The nerve fibre density (length of nerve fibre plexus/1 x 10^5 μm^2 of vessel wall) +/- the standard error for five different peptides and twelve different treatments on the jejunal artery. (N=6)

	PEPTIDES				
TREATMENTS	NPY	VIP	CGRP	SP	TH
4 Week Controls	744.3 +/-17.9	720 +/-31.4	385 +/-11.2	468.3 +/-22.5	853.3 +/-48.7
4 Week Capsaicin	*833.3 +/-30.2	*959.5 +/-15.7	0	0	782 +/-11.3
4 Week 6-OHDA	0	*0	*224.3 +/-16.2	*216.8 +/- 6.7	0
4 Week Capsaicin Vehicle	722.5 +/-19.3	857.8 +/-35.9	570 +/-19.6	404.7 +/-13.6	871.2 +/-17.9
4 Week 6-OHDA Vehicle	787.2 +/-35.6	783 +/-10.1	530.8 +/-26.8	536.2 +/-38.8	878.7 +/-19.3
4 Week Capsaicin (Injected Mothers)	718.7 +/-27.8	751.5 +/-16.1	0	0	*672 +/-14.4
12 Week Controls	663.5 +/-26.9	716.2 +/-16.2	357.5 +/-14.8	349.7 +/- 8.7	778.8 +/-13.8
12 Week Capsaicin	754.3 +/- 6.4	742.3 +/-26.9	0	0	768.5 +/-24.3
12 Week 6-OHDA	0	*325.8 +/-29.9	*281.3 +/-12.9	*262.5 +/-17.2	0
12 Week Capsaicin Vehicle	762 +/-13.3	720 +/-42.7	361.8 +/-18.4	383.3 +/-36.4	680.3 +/-14.5
12 Week 6-OHDA Vehicle	583.5 +/-27.1	693.7 +/-26.0	378 +/-16.7	373.2 +/-18.4	697.7 +/-27.6
12 Week Capsaicin (Injected Mothers)	*876.2 +/-36.7	780.7 +/-19.8	0	0	*860.7 +/-30.6

(*)indicates a value significantly different from the control values.

5.2.2. Electron Microscopic Analysis

The jejunal artery was chosen for study because it appeared to have the greatest number of nerve bundles of all three vessels examined. In this vessel, at four and twelve weeks of age, most of the nerve bundles have a small number of axons. There is a moderate number of bundles with an intermediate number of axons and a small number of bundles with a large number of axons (Figures 13-16). Figure 17 is an example of a nerve bundle with only a small number of axons whereas Figure 18 is an example of a bundle with a large number of axons. The catecholaminergic axons have dense granules when the vessel is fixed with the chromaffin Reaction (Figure 19).

In the capsaicin treated rats, at four and twelve weeks of age, the maximum number of nerve fibre bundles had decreased. This occurred in the capsaicin treated rats from capsaicin treated mothers as well (Table 3). At four weeks no change was seen in the size of the nerve fibre bundles in either the capsaicin treated rats or capsaicin treated rats from capsaicin treated mothers (Figure 13). At twelve weeks of age, for the capsaicin treated rats and capsaicin treated rats from capsaicin treated mothers, the number of bundles with a small and intermediate number of bundles had decreased (Figure 14).

In the 6-hydroxydopamine treated group, a decrease in the maximum number of nerve fibre bundles in the jejunal artery was observed (Table 3).

At four weeks of age, the number of bundles with a small number of axons (0-5) had decreased in the 6-hydroxydopamine treated rats (Figure 15). No change was observed for the number of bundles with an intermediate number of axons. At twelve weeks, the number of bundles with a small and intermediate number of axons had decreased in the 6-hydroxydopamine treated rats, whereas the number of bundles with a large number of axons had not changed (Figure 16).

In the 6-hydroxydopamine treated rat group, when the chromaffin fixed vessels were examined, no catecholaminergic axons containing dense granules were

found. It was observed that in these treated vessels there seemed to be a large amount of collagen (Figure 20).

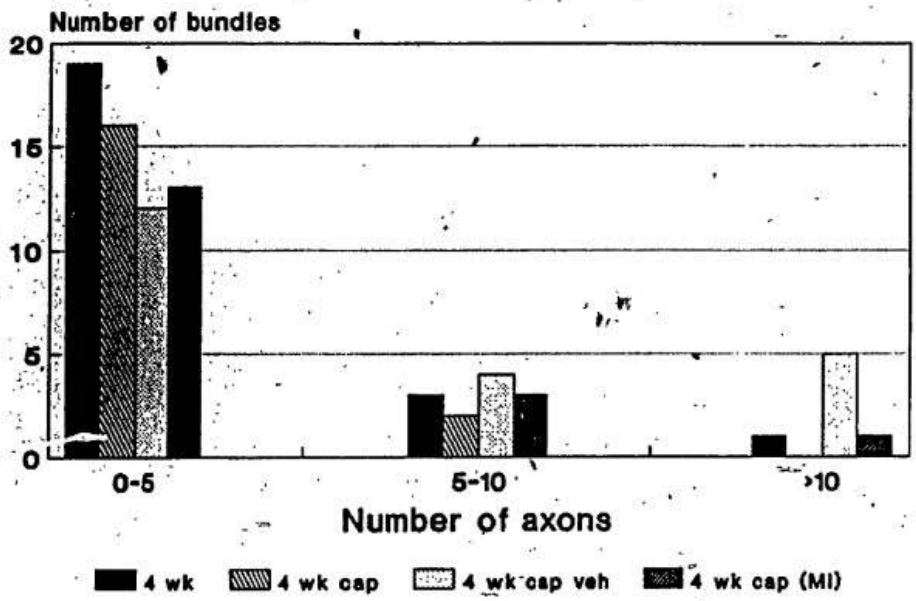


Figure 13. The number of axons per bundle in the four week old jejunal artery from capsaicin (cap) treated rats.
(wk=week, veh=vehicle, MI=mother injected)

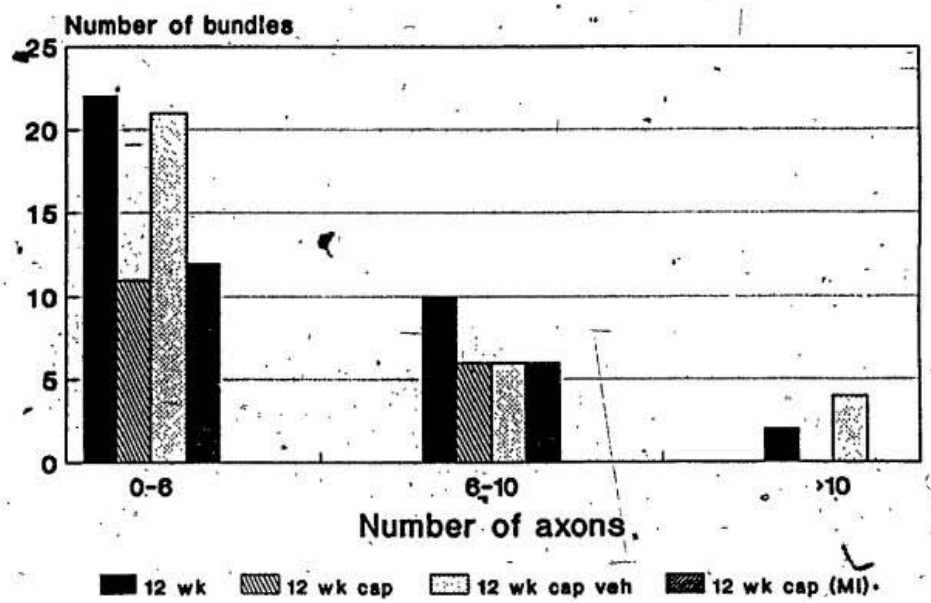


Figure 14. The number of axons per bundle in the twelve week old jejunal artery from capsaicin (cap) treated rats.
(wk=week, veh=vehicle, MI=mother injected)

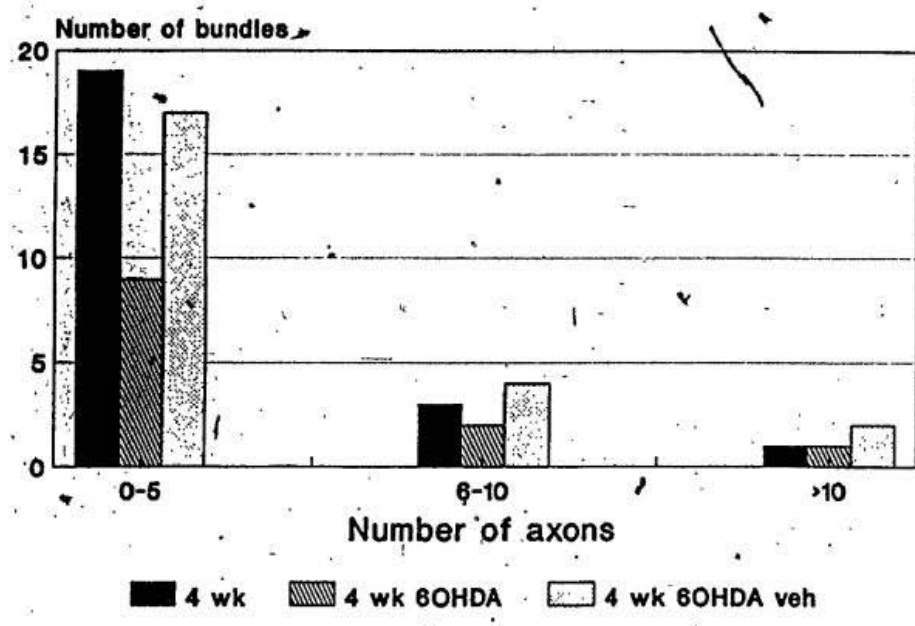


Figure 15. The number of axons per bundle in the four week old jejunal artery from 6-hydroxydopamine (6OHDA) treated rats.
(wk=week, veh=vehicle)

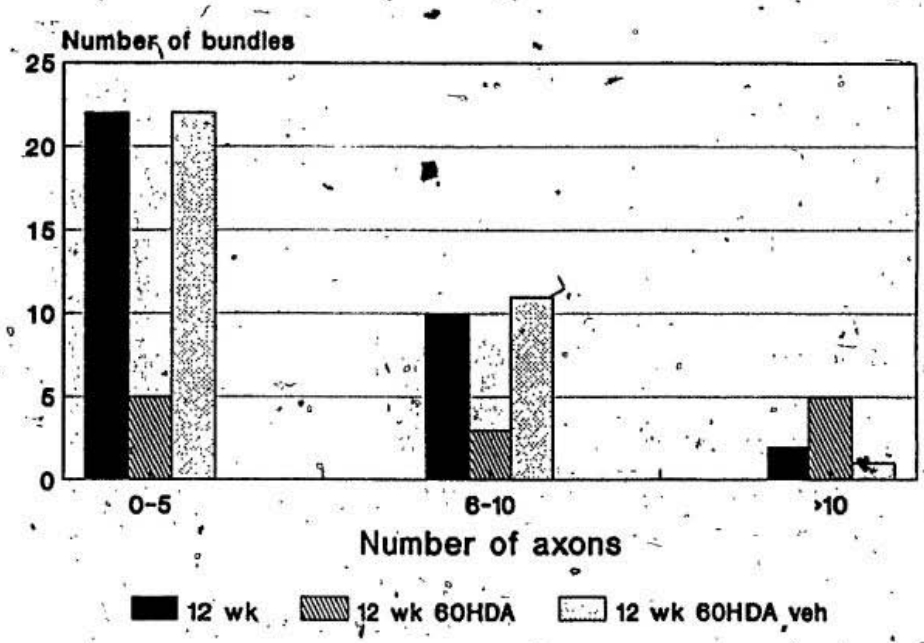


Figure 16. The number of axons per bundle in the twelve week old jejunal artery from 6-hydroxydopamine (6OHDA) treated rats.
(wk=week, veh=vehicle)

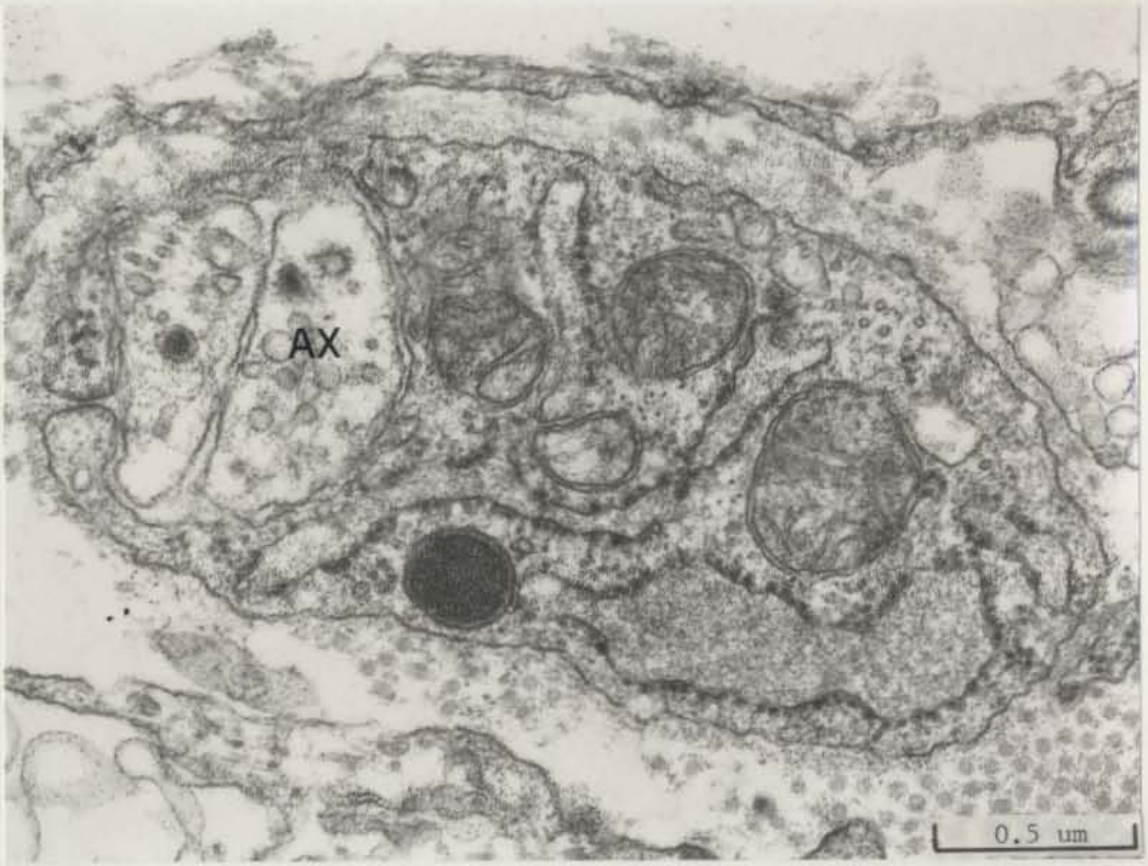


Figure 17. Small nerve fibre bundle. Nerve bundles can have a small number of axons (AX). (Magn. 55,400X)

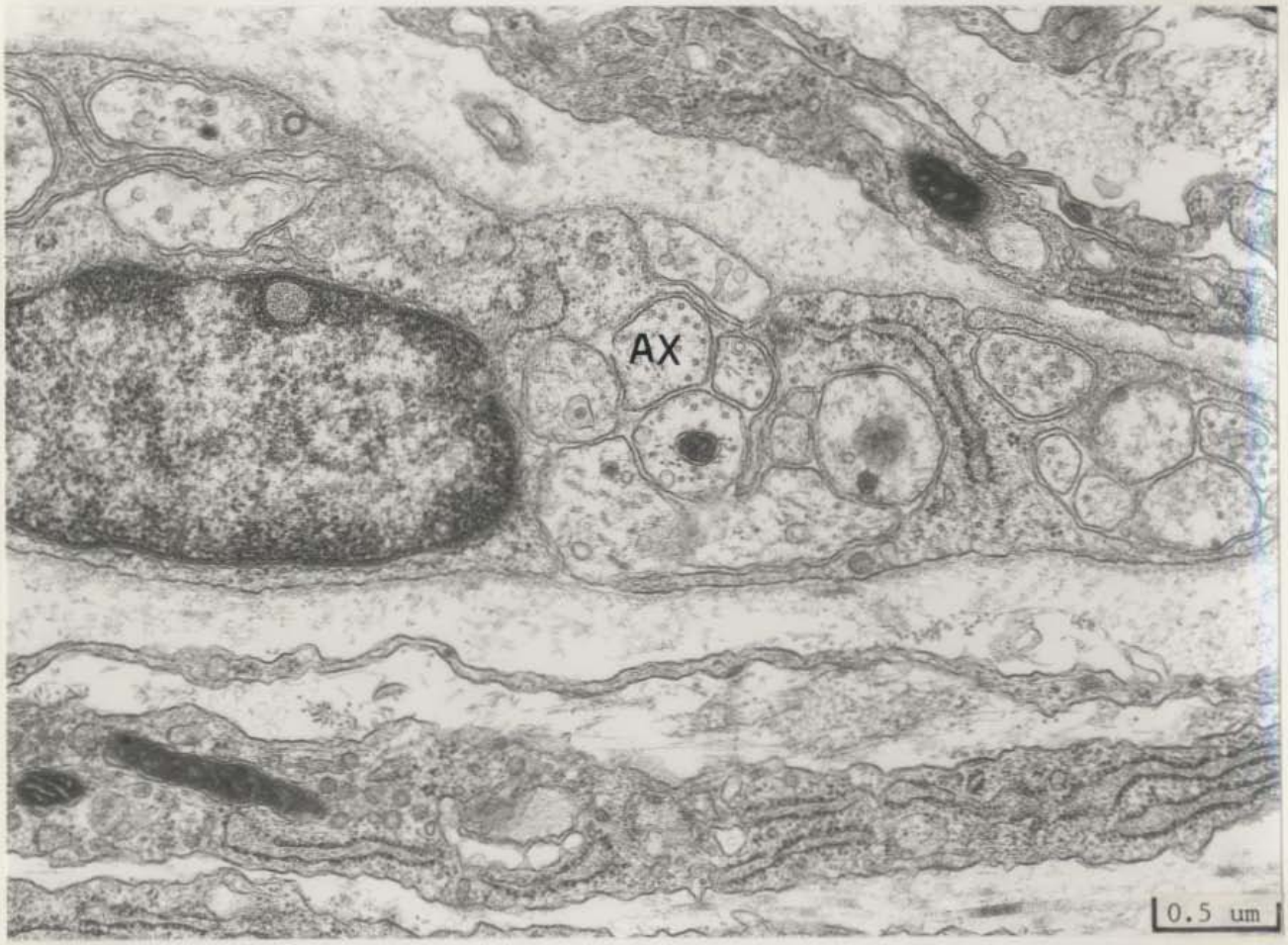


Figure 18. Large nerve fibre bundle. Nerve bundles can have a large number of axons (AX). (Magn. 37,600X)

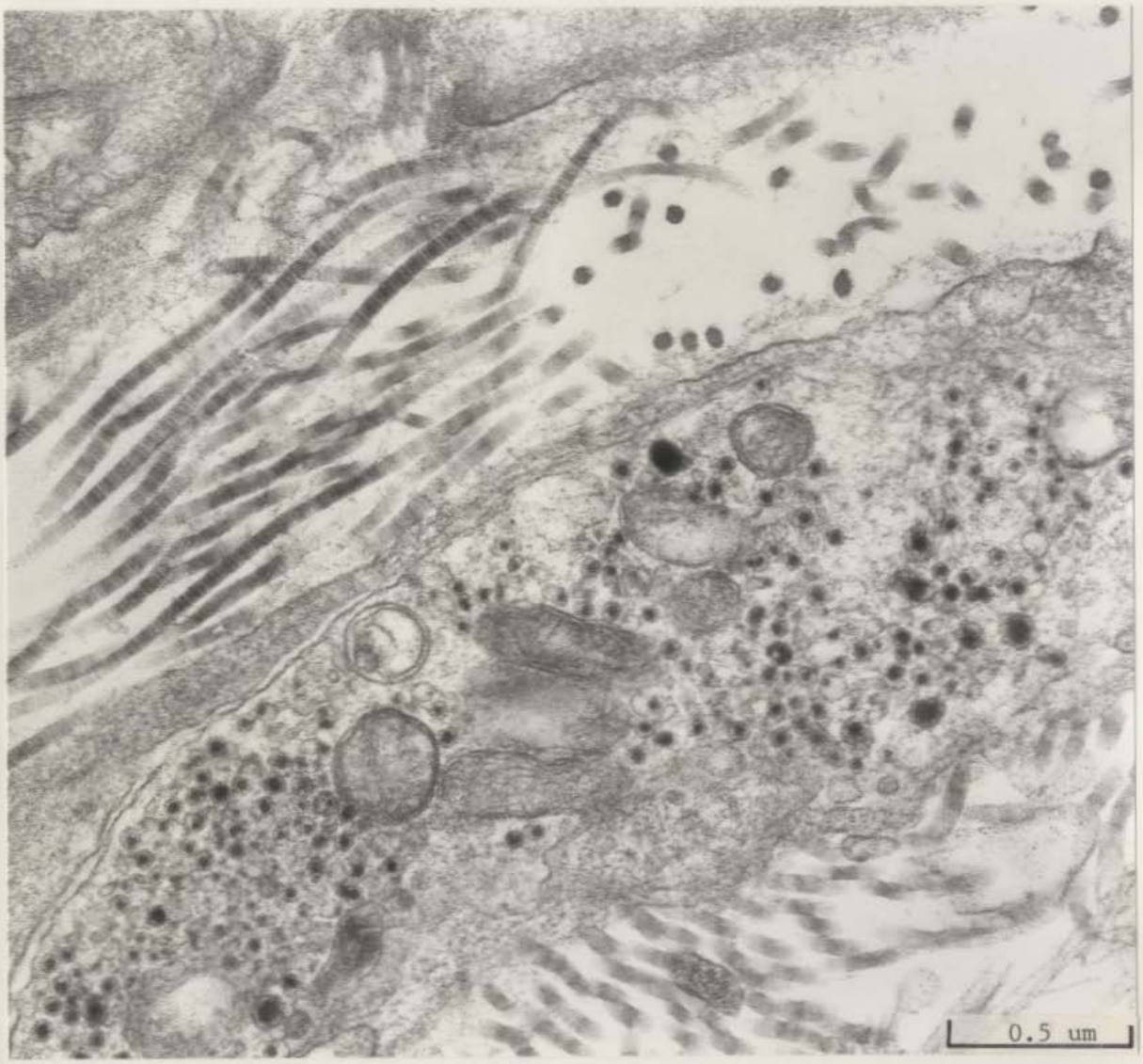


Figure 19. A vessel fixed by the chromaffin reaction. The catecholaminergic axons in the nerve fibre bundles have dense granules. (Magn. 52,600X)

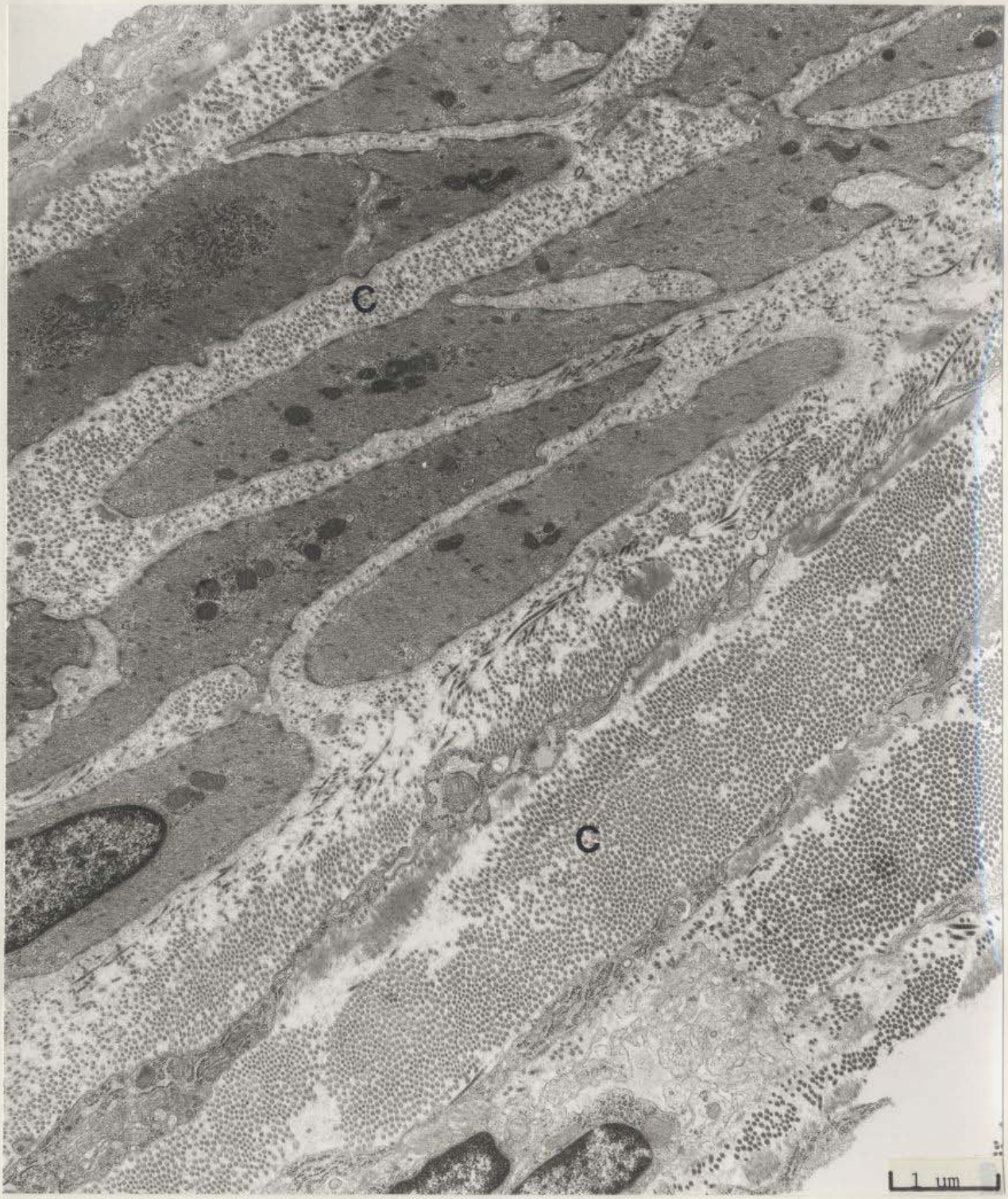


Figure 20. A 6-hydroxydopamine treated vessel. It appears to contain a large amount of collagen (C). (Magn. 10,850X)

Table 3. Maximum number of nerve fibre bundles found innervating the jejunal artery for twelve different treatments.

Treatments	Maximum Number Of Nerve Fibres
4 Week Controls	28
4 Week Capsaicin	18
4 Week 6-OHDA	12
4 Week Capsaicin Vehicle	23
4 Week 6-OHDA Vehicle	28
4 Week Capsaicin (Injected Mothers)	17
12 Week Controls	34
12 Week Capsaicin	17
12 Week 6-OHDA	13
12 Week Capsaicin Vehicle	34
12 Week 6-OHDA Vehicle	31
12 Week Capsaicin (Injected Mothers)	14

6. DISCUSSION

This study has found that peptide-containing and noradrenergic nerves follow different patterns of development and that they interact during development in the mesenteric vascular bed of the rat.

At one day after birth, in the mesenteric vascular bed, the vascular wall components of each of the vessels examined in this study were found to be immature except for the innervation. The CGRP peptidergic nerve fibres in this bed were found to be more advanced at this early age, as compared to the catecholaminergic nerve fibres. CGRP-fibres were found to be forming a plexus at one day after birth. The SP-immunoreactive fibres were beginning to branch from a single fibre and showed some signs of plexus formation. For the other fibre types (NPY, TH, and VIP) only single fibres were found.

With time, the number of nerve fibre bundles had increased and the other wall components had matured. By day fourteen all components were mature and they were comparable to the fully mature twelve week animals. It is not surprising that the vasculature has an early maturation. Vasomotor control for the regulation of blood pressure and for body temperature is important in the post-natal period (Cowen *et al*, 1982).

From the above observations, it may be concluded that CGRP peptidergic nerve fibres are pioneering nerve fibres in the mesenteric vascular bed.

It is known that peptidergic fibres immunoreactive to SP and CGRP originate from the dorsal root ganglia and trigeminal ganglia (Rosenfeld *et al*, 1983). The catecholaminergic fibres (TH and NPY) originate from the pre- and paravertebral ganglia (Edvinsson *et al*, 1973). The VIP-immunoreactive nerve fibres have been found to originate from peripheral ganglia, near the area that they innervate (Shiotani *et al*, 1986). The dorsal root ganglia develop in advance

of these other ganglia (Moore, 1982) and therefore it is reasonable to conclude that the peripheral peptidergic (CGRP and SP) nerve fibre system develops before the catecholaminergic.

CGRP nerve fibres have been discovered to be important in sensory control (Gibbins, Wattchow, and Coventry, 1987) and have been shown to enhance the effects of other substances. It can potentiate the effect of substance P, neurokinin A and B, histamine, platelet activating factor, and bradykinin (Brain and Williams, 1985). CGRP has also been proposed to play a trophic role in the synthesis of receptors on smooth muscle. It has been reported to regulate the synthesis of acetylcholine receptors in cultures of rat neonate myotubes (New and Mudge, 1986). Perhaps one of CGRP's most important roles is its ability to modulate arterial tone and control blood flow (Zaidi, Breimer, and MacIntyre, 1987). From these examples it is easy to see that CGRP may be important in the early development of the vasculature. However, the other peptides are also important at this early stage of development. NPY, for example, appears to be important in the control of the vasculature (Lundberg *et al*, 1985).

Before it can be fully concluded that CGRP peptidergic nerve fibres are pioneering fibres the immunohistochemical technique needs to be examined. The CGRP antibody purchased from Amersham, U.S.A. was found to give a strong staining of the immunoreactive fibres on the mesenteric vessels. It was diluted 1:20,000 to give this reaction. This primary CGRP antibody appears to have a high affinity and high specificity. The other primary antibodies may not have had such a high affinity and specificity.

In conclusion, it may be stated that the nerve supply to the mesenteric vessels is partially developed at one day after birth. The catecholaminergic and peptidergic nerve fibres are present on the mesenteric vessels and the CGRP peptidergic nerve fibre innervation appears to be more advanced than other fibre types. More work, however, is necessary in order to confirm these latter points. It may be necessary to perform tests with other primary antibodies purchased

from different companies other than those utilized in this experiment. It would also be interesting to examine the vascular innervation pre-natally to determine when the first appearance of these fibres occurs.

Observations made from the capsaicin and 6-hydroxydopamine studies confirmed that there was an interaction between different types of perivascular nerve fibres in the mesenteric bed of the rat. Treatment with capsaicin resulted in the complete loss of perivascular nerve fibres immunoreactive to SP and CGRP. In some of the capsaicin treated specimens, an increase in TH, NPY, and VIP-immunoreactive nerve fibres was found. Treatment with 6-hydroxydopamine resulted in the complete loss of nerve fibres immunoreactive to TH and NPY. In these specimens a reduction in the density of fibres immunoreactive to CGRP, SP, and VIP was observed.

These findings suggest that during development there is an interaction between nerve fibres innervating blood vessels, since there is a change in some fibre densities while others are removed. It appears as if the peptidergic innervation limits the proliferation of the catecholaminergic fibres whereas the catecholaminergic fibres are necessary for the full development of the peptidergic fibres.

Once neurons are formed, the last period of growth for them involves the branching and extension of existing axons rather than development of new neurons and axons. Neuronal mitosis ceases to occur about one week after birth (Papka, 1972). However, neurons do possess morphological plasticity and can grow and regenerate if they are placed in a particular physiological situation. Some neurons have been shown to possess neuron promoting activity, as in the case of sympathetic neurons (Roufe, Johnson, and Bunge, 1983).

Neuronal growth factors can also affect the development of the nervous system (Dekker, Gispen, and de Weild, 1987). Neuronal growth factor appears to be necessary for the development and maintenance of function of both the

peripheral sympathetic and sensory nervous systems (Thoenen and Barde, 1980). Sensory and sympathetic neurons have been shown to compete for neuronal growth factor (Korsching and Thoenen, 1985). Nielsch and Keen observed an increase in the SP content of cell bodies, axons, and terminals of sensory neurons in both the trigeminal and sciatic systems of 6-hydroxydopamine treated rats (Nielsch and Keen, 1987). They observed that this increase in SP depended on the relative density of sympathetic and sensory innervations. This indicated to them that competition for nerve growth factor was occuring (Nielsch and Keen, 1987). In the cat's occipital cortex, after 6-hydroxydopamine treatment, substance P can cause noradrenergic fibres to regenerate quickly (Nakai and Kasamatsu, 1984). Intact adrenergic axons are capable of exhibiting marked collateral sprouting in response to removal of other afferents innervating the same area (Stenevi, Bjorklund, and Moore, 1973).

In this experiment, a decrease in peptidergic fibres was obtained when catecholaminergic nerve fibres were removed. This is the opposite from the above examples. This suggests that it is possible that some of the peptidergic substances may be colocalized with the catecholamines in the same fibre, and they were removed with the catecholamines during treatment with 6-hydroxydopamine, or that the fibres are independent but require the presence of catecholamines for their development. CGRP, SP, and VIP may be colocalized with TH in the same fibre. When 6-hydroxydopamine treatment was given the TH- and NPY-immunoreactive nerve fibre plexuses disappeared. This indicated that all TH-immunoreactive fibres contain NPY. When capsaicin treatment was given the SP- and CGRP-immunoreactive fibre plexuses disappeared. This suggests that all SP-immunoreactive fibres contain CGRP.

Before making any conclusions, the selectivity of the drugs must be considered. For example, the 6-hydroxydopamine may be removing some SP- and CGRP-immunoreactive fibres. In the peripheral nervous system, 6-hydroxydopamine appears to be taken up with a high degree of selectivity by

noradrenergic neurons (Kostrzewa and Jacobwitz, 1974). Numerous electron microscopic studies have indicated that cholinergic and nonadrenergic neurons appear normal while noradrenergic neurons are in the process of degeneration (Siggins and Bloom, 1970; Thoenen and Tranzer, 1968). Capsaicin is also highly selective in causing degeneration of sensory ganglion neurons (Jancso *et al*, 1987).

By examining the nerve fibre plexus patterns for each of these peptides, it appears as if the TH-, NPY-, and VIP-immunoreactive fibre plexuses are similar. The CGRP- and SP-immunoreactive fibre plexus patterns are similar to each other. This provides evidence for the idea that TH, VIP, and NPY-immunoreactive substances are found in the same fibre, and CGRP- and SP-immunoreactive substances are found together.

All of these observations suggest that CGRP- and SP-immunoreactivity is associated with the same fibre. This was also discovered by many other researchers. It appears as if most SP immunoreactive cells show the presence of CGRP immunoreactivity (Goodman and Iversen, 1986). In this present study, TH- and NPY-immunoreactive substances also appeared to be found together. This has also been found in many other studies (Leblane, Trimmer, and Landis, 1987). It appears as if VIP can sometimes be found within NPY- and TH-immunoreactive fibres. In adrenal granules this co-existence can be found (Said, 1984). In this study there also appears to be cases where CGRP and SP appear to be associated with TH-immunoreactive fibres.

In the twelve week capsaicin injected rats that were from capsaicin injected mothers, a large increase in the TH-immunoreactive nerve fibre density was seen in the jejunal artery. It was decided to include this capsaicin group after it was discovered that CGRP-like immunoreactive nerve fibres were well developed at one day after birth. Normally capsaicin injections would have been started two days after birth but after considering the fact that CGRP-like immunoreactive nerve fibres were already present by two days and well developed, it was decided to treat mothers with capsaicin as early as fourteen

days after conception. If CGRP-like immunoreactive fibres were removed pre-natally, no interaction or competition between the CGRP-like immunoreactive fibres and the other fibres could occur, and therefore the result obtained is a consequence of the absence of CGRP-like immunoreactive fibres.

After discovering a large increase in TH-immunoreactive nerve fibres, in these animals, it may be concluded that peptidergic nerve fibre innervation has a strong influence on the catecholaminergic nerve fibre innervation early in development. In fact, this influence is inhibitory and occurs pre-natally. It is not surprising that this occurs so early in development. At this early age, nerve contacts are beginning to be made. Later on in development, it would be more difficult to influence the growth of nerve fibres for they have matured.

In this study, VIP-immunoreactive fibres were not found in the superior mesenteric vein. Feher and Burnstock (1986) discovered that VIP-immunoreactive nerve fibres were sparse or in most cases no VIP-immunoreactive nerve fibres were present in the walls of veins. There are also some specimens (NPY-immunoreactive nerves on the superior mesenteric artery) where no values are present for nerve densities. Some NPY-immunoreactive nerve fibres were found on the superior mesenteric artery, however they were patchy and not consistent throughout all of the specimens. These samples were rejected. It is believed that this is a consequence of the specificity of the primary antibody. There was a large amount of background staining in these samples and it was difficult to differentiate nerve fibres from background tissue.

Electron microscopic examination of the jejunal artery from both types of treated rats showed that the number of fibre bundles had been reduced. From the loss of fibre bundles following either capsaicin or 6-hydroxydopamine treatment it appears that most axons travel in bundles made up largely of one type of axon. If bundles were always made up of mixed types of axons, then no change in the number of nerve fibres would have been found. However, the change in plexus density following treatment suggested that a loss of specific fibre

types occurred. The fibres that were lost must have been in mixed fibre types. The peptidergic fibres that travel in mixed bundles with catecholaminergic fibres depend on the development of the catecholaminergic fibres for their development. When the catecholaminergic fibres are absent, there is a decrease in the number of peptidergic fibres. When the peptidergic nerve fibres are absent, the catecholaminergic fibres proliferate.

There is a large amount of evidence suggesting that neurons may contain a classical transmitter and one or more biologically active peptides (Hokfelt *et al*, 1980a). Multiple messengers may provide a mechanism for relaying differential responses and for increasing the amount of information transmitted at synapses (Hokfelt *et al*, 1987). In the peripheral nervous system, there are many examples demostrating the coexistence of SP and CGRP containing nerve fibres (Terenghi *et al*, 1985). There are also many locations where CGRP and SP are colocalized with VIP (Lindh *et al*, 1987). VIP-immunoreactive nerve fibres have been discovered to be localized with NPY-immunoreactive fibres especially around blood vessels (Hokfelt *et al*, 1986). Therefore, it is not unreasonable to assume that these peptide containing immunoreactive fibres can occur together in the same fibre bundle. Molyneux and Haller (1988) demonstrated that peptidergic, cholinergic, and noradrenergic nerve fibres could be found in the same fibre bundle.

It may be speculated that in the mesenteric vascular bed of the rat, catecholaminergic and peptidergic nerve fibres can be found together in the same bundle and these peptidergic fibres depend upon the maturation of the catecholaminergic fibres for their own maturation.

A change in the composition of nerve fibre bundles was also discovered after capsaicin and 6-hydroxydopamine treatment. After capsaicin treatment, it was discovered that bundles with greater than ten axons per bundle were absent. After 6-hydroxydopamine treatment, very few bundles with less than five axons per bundle were found.

From these observations, it can be concluded that peptidergic fibres found are normally found in large bundles, whereas catecholaminergic fibres are found in small fibre bundles.

In summary, this project has demonstrated or suggested, that the nerve supply to the mesenteric vascular bed is well developed at a rather early age. The CGRP peptidergic nerve fibres appeared to be more advanced than the other nerve fibre types examined in this study. During development there is an interaction between nerve fibres innervating blood vessels. It appeared as if the peptidergic innervation limits the proliferation of the catecholaminergic fibres whereas the catecholaminergic fibres are necessary for the full development of the peptidergic fibres. Catecholaminergic and peptidergic nerve fibres can be found in the same nerve bundle. It also appeared that catecholaminergic and peptidergic substances can be colocalized in the same nerve fibre, however more work has to be performed to confirm that point.

7.

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8. APPENDIX

8.1. Capsaicin Treatment

For capsaicin treatment, first it was decided to use the method followed by Scott and Pang (1982). When rats were sacrificed at twelve weeks after birth, fibres immunoreactive to CGRP and SP were found (see figure 1). These fibres should have been removed by the capsaicin treatment.

8.2. 6-Hydroxydopamine Treatment

For 6-hydroxydopamine treatment, it was decided to follow the method devised by Nielsch and Keen (1987). When rats were sacrificied at four weeks after birth, fibres immunoreactive to TH and NPY were found (see figure 2). These fibres should have been removed by the 6-hydroxydopamine treatment.

Figure 1: CGRP-immunoreactive fibres on the jejunal artery after capsaicin treatment. magn. 260X

Figure 2: TH-immunoreactive fibres on the jejunal artery after 6-hydroxydopamine treatment. magn. 260X

8.3. Statistical Methods for Light Microscopy

Control and vehicle control rats were subjected to statistical methods to see if any differences could be found (see table). The student Newman Keuls test, Scheffe procedure, and Tukey's test were used. Experimental rats were also subjected to these tests to find differences. The table given here is an example of the student Newman Keuls test. The student Newman Keuls test groups homogenous subsets by their means. A homogenous subset is a group whose highest and lowest means does not differ by more than the shortest significant range for a subset of that size. In the example given below there was no significant difference at the 0.05 level. The means given here are grouped means. All values for vessels and peptides were grouped for each animal group examined. All the controls and vehicle controls fell into one subset.

Subset 1

GROUP	MEAN
12 week rats	461.96
12 week capsaicin vehicle rats	384.42
12 week 6-hydroxydopamine vehicle rats	375.32
4 week rats	391.99
4 week capsaicin vehicle rats	452.27
4 week 6-hydroxydopamine vehicle rats	451.63