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NITRERGIC INNERVATION OF THE GUT IN HEALTH AND DISEASE

by

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A thesis submitted to the School of Graduate Studies of the University of Ottawa in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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September 1995

ISBN 0-612-04968-X

UNIVERSITÉ D'OTTAWA
UNIVERSITY OF OTTAWA

ABSTRACT

Nitric oxide (NO) is a recently identified neurochemical and putative neurotransmitter/neuromodulator of the gut. At the outset of this study anatomical and physiological findings indicated that NO was a putative inhibitory transmitter of at least a subpopulation of enteric non-adrenergic, non-cholinergic (NANC) myenteric motor neurons. However comprehensive information about the neural disposition and distribution of NO synthesizing sites in the mammalian intestine was lacking. NO is co-produced with L-citrulline from L-arginine in the presence of NADPH and oxygen. This reaction is catalyzed by both calcium (Ca^{2+})-dependent and Ca^{2+}-independent isoforms of NO synthase. Biochemical and molecular analyses confirmed that NO synthase, under appropriate conditions of pH and fixation, could be histochemically identified using an NADPH-dependent diaphorase technique. In this work, this histochemical technique was applied to whole mounts and tissue sections of the guinea-pig, rat and human intestine in order to provide a complete report on the distribution of sites of NO synthase activity in these species. NO synthase reactive neural elements were found to occur within the myenteric plexus as well as the submucosa throughout all regions of the intestine. In each species stained ganglion cells within the myenteric and submucous nerve plexi consistently displayed both type I and type II morphologies. In the guinea-pig and human intestine type IV morphologies were also observed. Since NANC neurons are distinguished as having a type I morphology these findings were consistent with NO being a transmitter of this functional subpopulation of enteric neurons. Moreover, since NO was found in other morphological subtypes and a profusion of fiber types, NO must also be produced by other populations of enteric neurons functionally distinct from NANC motor neurons. Quantitative analysis revealed a range of 10 to 30% of myenteric neurons contain NADPH diaphorase activity. Taken together, these findings indicate an elaborate neural localization of NO generation potential throughout the wall of the guinea-pig, rat and human intestine.

In addition to the neural actions of NO in the gut, it was becoming apparent that, as in other sites in the periphery, NO could exert local vasodilatory actions in the gut microvasculature and as such was proposed to play a role in enteric vasomotor regulation. However, the potential for blood vessels in the gut to synthesize NO had never been anatomically shown. Hence, an endothelium-like factor role for NO, typical of its function in the cardiovascular system, could not be confirmed. The potential for blood vessels of the gut to synthesize NO was examined using NADPH diaphorase histochemistry and endothelial cell immunohistochemistry in laminar preparations and cryosectioned tissue of the guinea-pig, rat and human intestine. This aspect of the study provided the first evidence to indicate that (1) both the endothelial and vascular smooth

muscle cells of the microvessels irrigating the intestinal wall of these species possess NO synthesis potential; (2) high endothelial venules in both the perifollicular area of Peyer's patches and extralymphoid regions of the submucosa and lamina propria also display NO synthase activity and (3) NO synthase activity is consistently and predominantly localized to discrete endothelial cell subcellular patches.

The functional significance of the nitrergic innervation in the human intestine was addressed by examining whether this innervation could be distinguished on the basis of existing peptidergic neurons, such as those utilizing neuropeptide Y (NPY). In addition to NO and NPY exerting similar biological actions in the mammalian intestine including modulation of food intake, blood flow, motility, and secretion, these substances coexist in submucosal secretomotor neurons of the rodent intestine. This study examined the relative disposition of elements displaying NPY immunoreactivity and NADPH diaphorase activity in the nerve networks of the infant human colon. Neural elements containing NPY immunoreactivity and NADPH diaphorase activity were identified in the external muscle layers, myenteric plexus, and all nerve layers of the submucosa, including Henle's plexus, the Intermediate nerve layer, and Meissner's plexus. Perivascular NPY-immunoreactive nerve fibers did not contain NO synthase activity. These findings not only display an extensive codistribution of NPY and NO synthesizing innervations but also provide the first anatomical evidence in support of the proposal that NO is a transmitter of enteric secretomotor neurons in the human colon.

Enteric NANC inhibitory motor nerves including NO neurons, have been shown pharmacologically to be targeted by GABAergic and cholinergic interneurons. Since NO is synthesized by a variety of intrinsic nerves throughout the gut it has the potential to be a transmitter of many nerve types, including interneurons. Enteric GABAergic nerve cells are exclusively interneurons. Therefore, this study also sought to determine whether some populations of nitrergic neurons and GABAergic interneurons represent the same subset of neurons in the human colon by assessing the colocalization of NADPH diaphorase activity in GABAergic nerve cells. GABA-T immunohistochemistry was used to identify GABAergic enteric neurons in specimens of infant human colon. A subpopulation of GABA-T immunoreactive myenteric and submucosal neurons display NO synthase activity. In the myenteric plexus this colocalization is extensive, with almost 40% of GABA-T immunoreactive myenteric neurons displaying NADPH diaphorase reactivity. These findings provide direct anatomical evidence in support of the proposal that NO is a transmitter of enteric interneurons in the human colon.

Pharmacologic manipulation of specific GABAergic receptor sites significantly alters the development of cysteamine (CSH)-induced duodenal ulceration. CSH interacts with

enteric neural mechanisms including the NANC inhibitory neurons targeted by the intrinsic enteric GABAergic neurons and therefore may be acting on the GABAergic-nitrergic NANC inhibitory pathway. In order to address this question, NO production was assessed from the duodenum of rats treated with CSH by NADPH diaphorase histochemistry and the formation of ^{14}C-L-citrulline from ^{14}C-L-arginine. The aim of this study was to assess the site, enzyme source and magnitude of NO production in the cysteamine-HCl rat model of duodenal ulcer. NO synthase-related NADPH diaphorase innervation of the muscularis, Brunner's glandular submucosa and mucosa in the duodenum of CSH treated rats was disrupted compared to control animals, but the intensity of staining was similar in both groups. CSH-induced ulceration was found to be coincident with a 3-fold increase in Ca^{2+}-dependent NO synthase activity of the proximal duodenum. Treatment with nitrergic compounds that inhibit NO synthase activity, significantly represses duodenal ulcerogenesis, whilst combined pharmacologic manipulation of the nitrergic and $GABA_B$-receptor related systems completely prevented ulcer formation. These findings indicate that: (1) excess NO formed from Ca^{2+}-dependent NO synthase contributes to the development CSH induced duodenal ulceration and (2) treatment with a $GABA_B$ receptor agonist in combination with the inhibition of NO synthase activity completely prevents experimental duodenal ulceration.

Typical of a gut disorder where disruption of enteric innervation can give rise to dysfunctional intestine, is Hirschsprung's disease (HSCR). This disease is characterized histologically by the absence of enteric ganglion cells (aganglionosis) and the consequent failure of innervation in a variable segment of the large bowel, and functionally by impaired relaxation of the musculature resulting in obstruction of propulsive movements. A major factor underlying obstruction in HSCR, may be a normal cholinergic fibre innervation acting within the context of a functional defect in the intrinsic NANC inhibitory transmission in the so called 'aganglionic' region of the colon. Since NO is a NANC transmitter it was of interest to investigate the nature of the nitrergic innervation in the pathological human gut. Biochemical and histochemical assessment of NO synthase activity was used to assess NO synthase activity levels and distribution in the colon of ten patients with HSCR and three age-matched controls. The results show a reductions of NO synthase activity and loss of nitrergic innervation in the diseased bowel. However, in a subpopulation of the patients examined survival of at least one population of (NO synthesizing) neurons in the so-called 'aganglionic' portion of the HSCR intestine was observed. This unexpected finding of a selective survival of NO synthesizing neurons under conditions of neurodegeneration is not unique, since it also occurs in CNS lesions such as Huntington's chorea.

NO, formed from the Ca^{2+}-dependent NO synthase isoforms, may be implicated in the pathogenesis of certain gut disorders.

ACKNOWLEDGEMENTS

It would not have been possible to complete the graduate program in such a satisfying way if not for the nurturing guidance and interest given by Dr. Anthony Krantis. In his laboratory my enthusiasm for scientific research was fostered as was my ability to compose and critique publications. For this my sincerest gratitude is extended. I must admit my amazement at his ability to motivate and push me above my viewed personal best. A better mentor, I will never meet again. Thank you for 150%!

A special thanks goes to Dr. William Staines for continuous coaching in experimental approach and composition to no avail! Hindsight has perfect vision, and keeping the momentum going is a great philosophy as is keeping a sense of humor. Thank you for the tremendous support, effort and time in training not to mention microscope and laboratory use at any time! I feel very fortunate to have had a second mentor.

A very special thanks goes to my husband, Brent Hodgkinson for his continual love, inspiration and support which provided the needed impetus to complete the graduate program. This degree is as much his as it is mine. A second very special thank you goes to my son, Liam. His unconditional love and happy demeanor gave me confidence.

Special thanks go to my mother, Brenda Nichols, brother, Kevin Nichols and his wife Ginette for their continual support and enthusiasm about my education.

I would also like to thank several friends who assisted in keeping my attitude a positive one. These include, my childhood friend, Katherine Boisvert as well as Rouhi Ahmed, Allison and Andrea Mckay, Annie Aria and Nicola Wigle.

Gratitude is also expressed to Kim Wong and Anu Pendeya for their invaluable assistance in technical aspects during the latter stages of this work. Special thanks to Kim Wong for her encouragement.

DEDICATION

I would like to dedicate this thesis to Brent, my husband and my son Liam.

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To gain insight into the complexity of the Enteric Nervous System (ENS), a detailed description of the structural organization of the gut wall and the integration of enteric neural elements with respect to the transmural constituents is necessary. These are described in the following pages and illustrated in Figure 1.1.

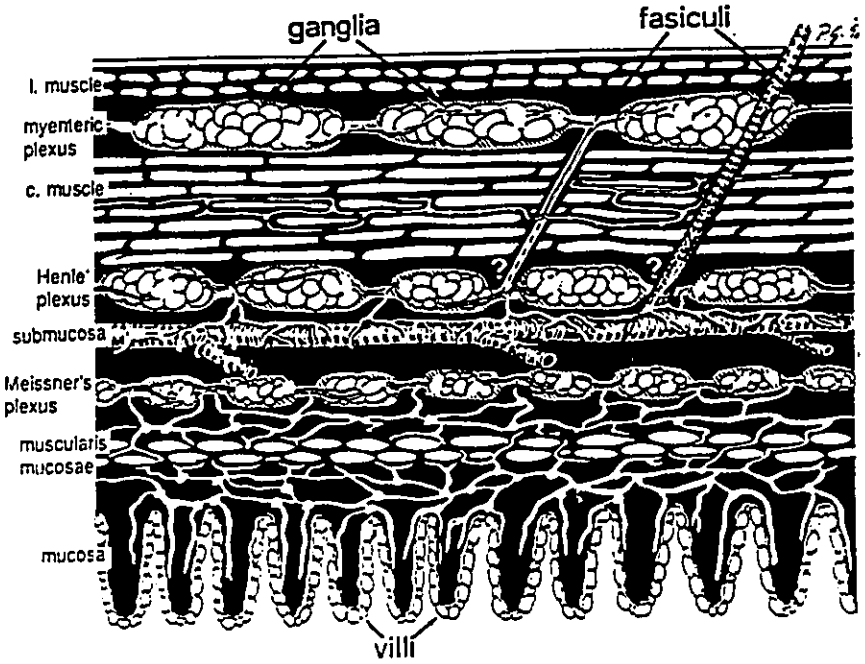
In 1921 Langley (1921) recognized that many of the intrinsic neurons of the GI tract do not receive input from the CNS. The works of Bayliss and Starling in 1899 and Trendelenburg in 1917 demonstrated that reflex motor activity could be exhibited in isolated gut segments in vitro (in the absence of CNS input) and was referred to as the "Law of the Intestine". This anatomical and physiological independence from the CNS suggested that there was an intrinsic nerve network(s) in the GI tract that functioned autonomously. In 1921 Langley coined the term Enteric Nervous System (ENS) for the intrinsic nerve networks of the gut, and proposed that it represented the third arm of the mammalian autonomic nervous system the other two being the sympathetic and parasympathetic divisions (Langley 1921).

The wall of the GI tract possesses a planar arrangement of component layers both neural and non-neural, frequently described in order from the outside layer of the tube, the serosa, to the inside layer or mucosa (fig. 1.1). From the serosa, the major layers (in order) include the longitudinal smooth muscle, the myenteric nerve plexus, circular smooth muscle, the submucosa, muscularis mucosae and the mucosal layer. Although the anatomical arrangement of the enteric layers is simplistic there is a great deal of complexity in terms of the integral components of each layer (particularly within the neural layers) and the manner in which they communicate with one another to exert a particular gut function. The neural layers of the gut wall are collectively referred to as the ENS. Within the ENS are the necessary fundamental components which enables the gut to respond to various stimuli with reflex behavior. These include sensory, integrative and motor neurons as well as effector cells. The complexity of the ENS is also evidenced by the extent of GI functions it controls. These include transport of digesta from the pharynx to the anal canal, the transit of contents through the different regions of the digestive tract, secretion of acid, transport of ions, release of chemicals and blood flow through the gut wall; all requiring some level of reflex behavior. In general some several million enteric neurons are involved in reflex regulation of gut function as well as in communication between the gut, sympathetic ganglia and the CNS.

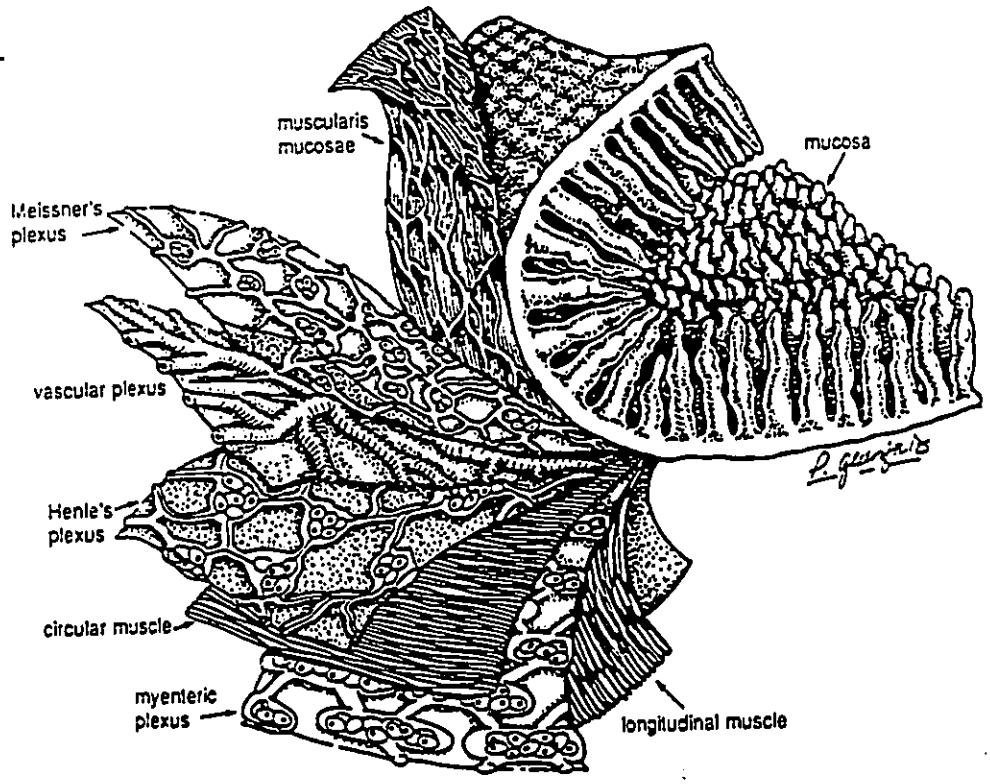
Figure 1.1. (A) A schematic illustration of a typical cross-section through the gut wall showing nerve layers consisting of grouped nerve cells in ganglia interconnected by nerve fibre bundles (fasciculi). Three such nerve networks are illustrated including the myenteric plexus situated between the longitudinal (l.) and circular (c.) muscle, and in Henle's and Meissner's nerve networks of the submucosa. Varicose fibres can be seen emerging from myenteric ganglia and ramifying within the deep muscular plexus of the circular muscle layer. Similar varicose fibre labeling of the muscularis mucosae and further projections within the mucosa are also evident. The origins of these fibres arise from efferent processes from submucosal ganglion cells. Blood vessels supplying the intestine enter the mesenteric border and penetrate through the external muscle layers to give rise a vascular network within the submucosa.

(B) The individual layers or laminae of the gut wall separated to reveal the organization of elements within each layer. The myenteric plexus sandwiched between the external muscle layers possesses larger ganglia and interconnecting fasciculi than either Henle's or Meissner's nerve layers. Although not shown the Intermediate nerve layer is situated between Henle's and Meissner's nerve networks in the plane of the vascular plexus. Together these constitute the neural and vascular components of the submucosal layer.

A


ganglia
fasiculi
l. muscle
myenteric plexus
c. muscle
Henle' plexus
submucosa
Meissner's plexus
muscularis mucosae
mucosa
villi

B


muscularis mucosae
mucosa
Meissner's plexus
vascular plexus
Henle's plexus
circular muscle
myenteric plexus
longitudinal muscle

As in the central nervous system (CNS), the ENS contain neurons of multiple chemical types. Moreover, the range of peptides and other putative neurotransmitters in the ENS is comparable to that in the CNS (Table 1.1). Also like the CNS, neurons of the ENS are plurichemical in that there is coexistence of transmitter candidates in the same neuron (Costa et al 1991a; Furness et al 1992). The concept of cotransmission (Burnstock 1976),where the same neuron releases more than one transmitter also applies to enteric neurons. However in contrast to coexistence, this phenomenon refers to particular instances where substances released from nerve terminals have no direct actions on effector cells, but rather exert pre- and/or postjunctional modulatory effects on neurotransmission. Therefore, each colocalized neurochemical could be a potential neurotransmitter/neuromodulator. When cotransmission does occur, it usually involves synergistic actions of the cotransmitters. Toward unraveling the complexity of this nervous system, enteric neurons have begun to be characterized based on neurochemical content, morphology, physiology and topographical location within the gut wall (ibid). These efforts are aided by the laminar arrangement of the gut wall layers which can be dissected out, isolated and maintained intact for anatomical, physiological and developmental investigations (Furness et al. 1980). The anatomical, neurochemical and functional nature of the gut wall with respect to its neural and endocrine elements is described in Chapter 1 and should be used by way of introduction to help understand basic enteric control systems.

1.1 Structural Organization of the Gut Wall

For the most part the organization of the component layers of the gut wall follows a similar pattern along the digestive tract and unless otherwise noted is assumed in the following descriptions. Several detailed reviews discuss the general organization of enteric neural innervation (Gabella 1979; Cooke 1986; Furness et al. 1987; Keast 1987) and the neurochemical and functional coding of enteric neurons (Keast et al. 1985; Costa et al. 1986a; Costa et al. 1992; Furness et al. 1992; Bornstein 1994; Costa et al. 1994). In addition to these reviews, the contribution of the neural innervation of the gut from extrinsic sources is also described.

Table 1.1: Neuropeptides and other putative enteric neurotransmitters, their location and some possible functions. Modified from Furness et al 1992.

SUBSTANCE	LOCATION AND PUTATIVE ROLE(S)
Acetylcholine (ACh)	Present in excitatory motor neurons, interneurons, putative enteric sensory neurons and secretomotor neurons Stimulates epithelial secretion, parietal cell secretion and some endocrine cells
Adenosine triphosphate (ATP)	A cotransmitter of enteric inhibitory muscle motor neurons
Gamma-Aminobutyric acid (GABA)	Present in interneurons and some endocrine cells Modulates transmitter release, motility and acid secretion
Calcitonin gene-related peptide (CGRP)	Present in some secretomotor neurons and interneurons Mediator of axon reflexes at sensory neuron terminals Induces secretion of water and electrolytes
Cholecystokinin (CCK)	Present in some secretomotor neurons, interneurons where it plays a role in excitatory neurotransmission Present in epithelial cells where it detects luminal contents and transmits paracrine signal to vagal afferent terminals
Galanin (GAL)	Present in secretomotor neurons, descending interneurons and inhibitory motor neurons in human intestine Induces secretion of water and electrolytes
Gastrin-releasing peptide (GRP)	Present to nerve fibers in muscle and interneurons Excitatory transmitter to gastrin cells
Peptide Y family [neuropeptide Y (NPY), peptide YY (PYY) and pancreatic polypeptide (PP)]	Present in interneurons, inhibitory motor neurons to muscle, in secretomotor neurons Inhibits ACh release prejunctionally, and inhibits secretion of water and electrolytes
Nitric Oxide (NO)	A cotransmitter of enteric inhibitory muscle motor neurons and interneurons Modulates motility and transmitter release, inhibits chloride secretion and modulates acid and bicarbonate secretion

Table 1.1 continued: Neuropeptides and other putative enteric neurotransmitters, their location and some possible functions. Modified from Furness et al 1992.

SUBSTANCE	LOCATION AND PUTATIVE ROLE(S)
Opioids [met- and leu-enkephalin (ENK), dynorphins (DYN)]	Present in interneurons and motor neurons to muscle Role in prejunctional inhibition of transmitter release
Serotonin (5-HT)	Present to inhibitory interneurons. Modulator of transmitter release
Somatostatin (SOM)	Present to inhibitory interneurons Modulator of transmitter release
Tachykinins (substance P (SP), neurokinins A, K and γ)	Present in excitatory motor neurons and interneurons and putative enteric sensory neurons (SP); Co-transmitters with ACh
Vasoactive intestinal peptide (VIP) and [peptide histodine isoleucine (PHI) and pituitary adenylate cyclase activating peptides (PACAPs)]	Present in interneurons, cotransmitter of enteric inhibitory motor neurons Candidate transmitter of enteric vasodilator neurons and of secretomotor neurons Modulates motility, stimulates blood flow and epithelial secretion

NOTE: The functions of the putative enteric transmitters listed in this table is a composite of information and does not consider subtle inter-species or inter-regional variations. Extensive reviews include Burks (1994), Dockray (1994) and Cooke and Reddix (1994).

1.1.1. Longitudinal Muscle and Associated Neural Innervation:

Within the longitudinal muscle, fine nerve fibre bundles run parallel to the muscle cells in cases where the muscle is thicker and form muscle fiber bundles, as in the taenia coli of the colon of man and guinea-pig. Apart from these, there are few or no fibers within the thickness of the longitudinal muscle with the exception of the human intestine which possesses a longitudinal muscle plexus (Llewellyn-Smith et al. 1993).

Immunohistochemical studies reveal that nerve fibers immunoreactive for ENK, SP, DYN and less frequent are fibers with VIP, NPY, galanin and gastrin releasing peptide are part of the innervation of this muscle. These fibers may originate from intrinsic neurons of the gut wall but their functional significance is unknown (Brookes et al. 1992). This muscle layer is also supplied by excitatory motor neurons containing acetylcholine and SP as well as inhibitory motor neurons containing VIP, NO, DYN and GAL (ibid).

1.1.2. The External Muscle Layers and Associated Innervation from the Myenteric Plexus:

The muscularis externa consists of two layers of smooth muscle. The outer longitudinal smooth muscle runs parallel to the axis of the intestine, while the inner thicker circular smooth muscle has a perpendicular orientation (running in the circumferential axis of the intestine). These two muscle layers mediate the propulsive and mixing movements of the gut via innervation by the intrinsic (enteric) neurons. This innervation arises largely from a ganglionated plexus lying in the plane between the longitudinal and circular muscle layers discovered in 1864 by Auerbach. Referred to as Auerbach's plexus, and otherwise known as the myenteric plexus, this nerve layer is coexistent and communicates with the gut smooth muscle, without interruption through the various regions of the GI tract. Analyses of neuronal projections using lesion experiments and tracing of terminals from their cell bodies of origin indicate that in large part the cell soma of neurons that project to the external muscle layers lie in the myenteric plexus (Wilson et al. 1987; Brookes et al. 1991a).

The primary function of the myenteric plexus is to coordinate contractions and relaxations of the longitudinal and circular smooth muscle layers along the gut to permit mixing (segmentation) and propulsion of contents (peristalsis), events necessary for efficient absorption of nutrients and elimination of waste to occur. These motor reflex activities require communication between regions of the GI tract such that contraction of the musculature occurs behind the contents to force them into the relaxed region ahead of the contents. Interruption of the

myenteric plexus, prevents the propagation of this reflex activity down the intestine (Smith et al. 1988; Smith et al. 1990). In addition, reflex activity can be elicited by distension of guinea-pig intestinal segments following removal of the mucosa, submucous plexus and much of the circular muscle layer (Smith et al. 1990). Functionally defined myenteric neurons and their projection targets within the gut wall, include putative sensory neurons projecting to the mucosa (Furness et al. 1990; Song et al. 1991); interneurons projecting to other myenteric ganglia (Costa et al. 1980; Steele et al. 1991; Brookes et al. 1991b; Costa et al. 1992) or submucosal ganglia (Bornstein et al. 1986), and motor neurons projecting to the smooth muscle (Bornstein et al. 1986; Brookes et al. 1991a; Costa et al. 1992). The longitudinal and circular muscle layers are supplied by separate populations of motor neurons as revealed by retrograde tracing and neurochemical analyses (Brookes et al. 1991a; Brookes et al. 1992).

The myenteric plexus possesses a unique structure consisting of three interconnecting meshworks of nerve cell soma and/or fibres enclosed in a basal lamina. The largest meshwork, or 'primary plexus', was described as being composed of large bundles (fasciculi) of nerve fibres that form interconnections between variable sized aggregations of cell soma (ganglia). A secondary meshwork is formed by the emergence of thinner fasciculi from the primary nerve fiber bundles and runs in the circumferential axis of the intestine. This secondary component of the plexus is proposed to provide a pathway by which nerve fibres enter the circular muscle (Wilson et al. 1987). Auerbach's findings were confirmed by many others by 1930 Schabadasch and Stohr describe a third component to the plexus, a tertiary meshwork of fasciculi. This third component possesses even finer caliber nerve fibres which are offshoots of the secondary strands that make up a network in the gaps formed by the meshwork of the primary plexus. While the primary and secondary meshworks are always seen, the tertiary plexus is reported to exist in most species with the exception of the dog and human intestine (Furness et al. 1987; Llewellyn-Smith et al. 1993). It is now proposed that in those species where the longitudinal muscle is thin (guinea-pig and rat) the tertiary component of the myenteric plexus provides the fibre innervation juxtaposed to the muscle and in those species where it is thicker (human and dog) there is a plexus of nerve fibres that penetrates this muscle coat (Llewellyn-Smith et al. 1993).

The ganglia of the myenteric plexus are typically elongate tetrahedral-like structures in the small intestine and stout symmetrical tetrahedral-like structures in the large intestine containing between 5-200 neurons (Furness et al. 1986; Furness and Costa 1987; Young et al 1993; Parr and Sharkey 1994). Forming the tetrahedron are the large fasciculi of the primary

plexus. Each myenteric ganglion contains a heterogeneous population of neurons, sensory, integrative and motor neurons rather than individual ganglia being sensory, integrative or motor in nature (Takaki et al. 1985). Rather than having Schwann cells like the rest of the peripheral nervous system; the supporting cells (glial cells) closely resemble astroglia of the CNS. These glial cells form a protective sheath about the plexus and separate the neural elements from the surrounding connective tissue space (Gershon et al. 1978; Gabella 1979).

The myenteric plexus is avascular, however the ganglia receive nutrients by diffusion from the blood vessels irrigating the connective tissue that surrounds them. The neural tissue lying outside of the glial sheath is supplied by capillaries, closely resembling cerebral capillaries, being non-fenestrated and impermeable to large macromolecules (Gershon et al. 1978). This represents the blood-myenteric barrier much like the blood-brain barrier.

1.1.3. Deep Muscular Plexus:

Situated deep within the circular smooth muscle layer is a deep muscular plexus named and discovered by Cajal in 1895. This plexus consists of small meshworks of nerve fibre bundles which run in a parallel fashion within the circular muscle. The great majority of nerve fibers in deep muscular plexus are 'varicose' (Gabella 1979; Furness et al. 1987). Varicosities are swellings which occur at intervals along the length of the axon and while they are referred to as 'terminals' of enteric neurons they are not the true anatomical ending of a fiber. Varicosities are 1-2μm in diameter and spaced anywhere from 250 to 300 per μm of axon (see Gabella 1979). Each varicosity possesses the necessary machinery for storage and release of transmitter such as mitochondria and so-called synaptic vesicles. These varicosities, often bare of glial cells and facing the muscle, are presumed sites of neuromuscular transmission and represent efferent processes of myenteric neurons innervating the smooth muscle or fibers coursing through the muscle layer to connect with the submucous plexus.

1.1.4. Circular Muscle - Submucosal Interface Plexus:

At the interface between the external circular smooth muscle and the underlying submucosal layers of the gut wall is a plexus of interconnecting nerve fibers and specialized cells known as interstitial cells of Cajal (ICCs) (Christensen et al. 1987). This plexus is believed to be the source of the electrical slow waves that pace peristalsis. The source of the nerve fibers within this plexus is believed to be the myenteric tertiary plexus since slow wave signals arise from this level (Christensen et al. 1987). ICCs are proposed to be specialized cells situated between nerve

endings and effector cells that are involved in the development of nerve to muscle signals; and/or an intestinal smooth muscle pacemaker since neurotransmission and ICC cells are concentrated in pacemaker regions (Publicover et al 1993). These cells are neuronal-like displaying prominent nuclei and two to five long similar processes making it difficult to consider one an axon and the others dendrites and form networks at various levels of the gut wall. Ultrastructural analysis indicates that these cells contain no structures that might be regarded as synaptic vesicles (see Furness and Costa 1987).

1.1.5. Submucosa and Associated Neural Innervation

Lying below the circular muscle layer, the submucosa consists primarily of loose connective tissue. In some regions this layer may contain glands such as the Brunner's glands of the duodenum. A distinctive characteristic is the presence of the fine blood vessels of the intestinal wall that travel via the mesentery through this layer and give rise to a profuse network of arterioles, venules and unfenestrated capillary meshworks. Also lying within the connective tissue of the submucosa, is a network of ganglia and interconnecting fasciculi, less prominent than those of the myenteric plexus. In species other than the guinea-pig, the submucosa possesses two such nerve networks (fig. 1.1). These plexuses are interconnected but situated in distinct planes, either closer to the overlying circular muscle layer, or closer to the underlying mucosal layer and are referred to as Henle's plexus and Meissner's plexus respectively since they discovered these individual nerve layers. Henle's plexus innervates the circular muscle layer (Smith et al 1989; Furness et al. 1990). In the porcine and human intestine there is a third layer more centrally located within the submucosa but closer to Meissner's plexus in the vicinity of the fine submucosal blood vessels termed the Intermediate plexus (Hoyle et al. 1989). Variability in size among these submucosal nerve layers also exists with Henle's plexus being the largest meshwork. The ganglia of Meissner's plexus are the smallest of the three nerve layers and the interconnecting fasciculi are much finer. As the name implies, the Intermediate plexus, with respect to ganglia and interconnecting fasciculi, is intermediate in size with respect to the other two nerve layers (ibid).

The neural component of the submucosa is presumed to contain separate sensory, integrative and motor neurons (Keast 1987). Moreover, since intestinal transit has been shown to proceed following destruction of the myenteric plexus in the rat jejunum (Luck et al. 1993) it has been proposed that the neural component of the submucosa may, under certain conditions, be an ectopic motility control center for the gut. Submucous neurons project to the mucosa and regulate

differing functions including control of blood flow and vascular permeability, as well as control of exocrine secretion, transport across the intestinal lining and immune response (see Cooke 1986; Keast 1987). Submucosal motor neurons involved in mediating these functions are collectively referred to as secretomotor and vasomotor neurons.

It is important to note that although there appears to be a separation of functions, eg. the motor neurons to muscle are in the myenteric ganglia and most secretomotor-vasomotor neurons are in the submucous ganglia, it is probable that coordination of motor, secretory and circulatory functions occurs. Indeed, electrophysiological data indicates that submucous neurons receive cholinergic and non-cholinergic excitatory input and non-cholinergic inhibitory input from myenteric ganglia (Bornstein et al. 1986). Nerve fibers from the submucosa project to the myenteric plexus, to the mucosa, and into paravascular nerve bundles (described below). In addition a subpopulation of cholinergic submucous sensory neurons send processes to the myenteric ganglia (Bornstein et al. 1988; Brookes et al 1995).

1.1.6. Submucosal Vascular Plexus:

The arteries which run into the submucosa from the mesentery and branch into arteriolar networks have two sets of accompanying nerves. These are the fine *perivascular* nerve strands surrounding the arteries and arteriolar network of the submucosa, and the *paravascular* nerves which follow in a parallel fashion the arteries and arterioles in the mesentery and within the submucosal vascular plexus (fig. 1.1). The microvasculature of the gut collectively receives intrinsic innervation from neurons of the submucosa as well as extrinsic, sympathetic efferent and visceral afferent innervation. A population of intrinsic vasodilatory nerve fibers which originate from submucous neurons contain DYN, GAL and VIP and give rise to the perivascular plexus. Sympathetic nerve fibres originating from the prevertebral ganglia containing noradrenaline and NPY and sensory fibers originating from the dorsal root ganglia containing SP, CGRP, VIP and CCK give rise to both para- and perivascular innervation of submucosal vessels. The intrinsic vasodilatory neurons play a role in the reflex vasodilation elicited by mechanical stimulation of the intestinal mucosa from oncoming digesta (Vanner et al 1993). The sympathetic innervation of submucosal arterioles causes local vasoconstriction. The sensory innervation of the these vessels regulate resting blood flow and are proposed to play a role in mucosal defense by modulating blood flow in response to toxic luminal agents (Vanner 1994). There are very few nerves supplying veins.

1.1.7. Innervation of the Muscularis Mucosae:

The muscularis interna or 'muscularis mucosae' is a thin layer of muscle cells which separates the submucosa and mucosa. Like the muscularis externa, it has smooth muscle cells running in the longitudinal and circumferential axis of the intestine. This layer contains nerve fibre bundles traversing between submucosa and mucosa, as well as a plexus of fine nerve fibres that run parallel to the muscle. Bundles of smooth muscle that are considered part of the muscularis mucosae make finger-like projections into the cores of the intestinal villi and possess accompanying nerve fibers (fig. 1.1).

1.1.8. Mucosa and Associated Neural Innervation:

The organization of the mucosa is highly variable from one region of the gut to another. In the small intestine, it consists of an epithelial surface, the connective tissue lamina propria, into which simple tubular glands protrude and the villi. The large intestine possesses a glandular mucosa rather than villi. The lamina propria is a distinctive layer containing not only several gland types but also lymph nodules and capillary networks. The mucosal layer mediates several functions of the gut including immunity, absorption of nutrients and secretion (see Cooke 1986).

The mucosal plexus was first described by Billroth in 1858 as a dense network of fine interconnecting nerve bundles found throughout the lamina propria, along with processes connecting with the submucous plexus. The neural innervation of this layer is made up in part by a subglandular plexus, a periglandular plexus, a villous subepithelial plexus, and a plexus of the villous core as well as efferent processes from the myenteric plexus, submucous plexus and nerve fibres of extrinsic origin. Interestingly, intramucosal nerve cells have recently been identified in the rat and human small intestine (Fang et al. 1993). These cells resemble neurons of the submucous plexus but are not grouped in ganglia and are found subjacent to or further away from the muscularis mucosae lying within the lamina propria. *The functional properties of these neurons are unknown .*

1.2 The Extrinsic innervation of the gut

The parasympathetic and sympathetic nervous systems modulate ENS function through efferent projections to the nerve networks in the gut wall. In Langley's original descriptions of the autonomic nervous system (1921) he did not discuss the afferent components

of the parasympathetic and sympathetic divisions. However, there is now compelling evidence that these systems also possess an afferent component (for review see Szurszewski and Miller 1994). In fact afferent feedback to these systems is achieved through communication at the level of the prevertebral ganglia and centrally in the spinal cord and brainstem (for review see Sharkey and Pittman 1994; also see Szurszewski and Miller 1994).

1.2.1. Parasympathetic innervation:

The parasympathetic supply to the gut is divided into cranial and sacral divisions (fig. 1.2). The cell bodies of the parasympathetic preganglionic efferent nerve fibers originate from the brain stem and the sacral portion of the spinal cord. Vagal motor neurons in the dorsal root ganglia supply fibers down to the proximal portion of the large intestine targeting both smooth muscle and enteric neurons, giving rise to modulation of motility, and acid secretion. The effects of parasympathetic transmission on intrinsic neurons of the gut is mediated via acetylcholine through nicotinic receptors. The sacral parasympathetic fibers innervating the gastrointestinal tract originate specifically from the second, third and fourth sacral segments of the spinal cord and proceed to form the pelvic nerves. The pelvic nerves (of sacral origin) that project to the rat large intestine have been shown to contain several peptides including NPY, SOM, CCK and ENK (Keast 1994).

Coursing with the parasympathetic efferent fibers are the visceral primary afferents of the afferent parasympathetic division. The majority of these visceral afferents contain calcitonin gene-related peptide. The cell bodies of vagal afferent fibers are found in the cranial vagal (nodose and jugular) ganglia and those of the pelvic afferents are situated in the dorsal root ganglia. Visceral afferents have several functions including the detection of environmental changes (stimuli), conveying them into a signal and transmitting the signal (see Sharkey and Pittman 1994).

1.2.2. Sympathetic innervation:

The sympathetic efferent fibers innervating the gut originate from the mid portion of the spinal cord (the thoracic and lumbar segment). After leaving the cord, the preganglionic fibers enter the paravertebral ganglia to the outlying prevertebral (celiac, superior and inferior mesenteric and pelvic-hypogastric) ganglia. The prevertebral ganglia contain the majority of the postganglionic nerve cell bodies, and from here, postganglionic fibers spread along with the blood vessels to innervate the gut. Fibers originating from the celiac ganglia supply fibers down to the

proximal colon; the superior mesenteric ganglia supply fibers innervating the small intestine and proximal colon; the inferior mesenteric ganglia supply fibers to the middle and distal colon and pelvic ganglia supply fibers innervating the distal colon and rectum (fig. 1.2) (Luckensmeyer and Keast 1994). Some fibers make synaptic connections with the intrinsic nerve plexuses in the wall of the GI tract, while others end directly on blood vessels. These sympathetic postganglionic innervations serve to modulate gut motility and mucosal transport via prejunctional inhibition of acetylcholine release from enteric excitatory cholinergic motor neurons; and these functions are mediated, at least in part, through the action of noradrenaline at α-adrenoreceptors. Noradrenergic axons containing NPY as a cotransmitter also form a perivascular neural network around intramural arteries which cause vasoconstriction (see Furness et al. 1987). In addition afferent fibers from the gut containing VIP, CCK, DYN and some containing ACh as well as collaterals of visceral afferent fibers containing CGRP and SP project to prevertebral ganglia (see Szursewski and Miller 1994). These afferent and efferent neurons in the prevertebral ganglia mediate peripheral reflex activity. As such, the prevertebral ganglia are known as integrative and coordinating centers for gut function (ibid; also see Sharkey and Pittman 1994).

The afferent sympathetic division contains spinal visceral afferents which have their cell bodies in the dorsal root ganglia. These visceral afferents also project and terminate in the substantia gelatinosa (lamina I and V) within the dorsal horn of the spinal cord (see Sharkey and Pittman 1994). There appears to be a differential distribution of peptides in subsets of spinal visceral afferents innervating the gut. For example, of the visceral afferents innervating the rat stomach, a larger proportion have been found to contain calcitonin gene-related peptide than SP (Sharkey 1992). The function of these visceral afferents has been discussed in section 1.2.1.

In conclusion, all parts of the GI tract are innervated by the autonomic nervous system and in turn visceral afferents are sent to the central nervous system (CNS).

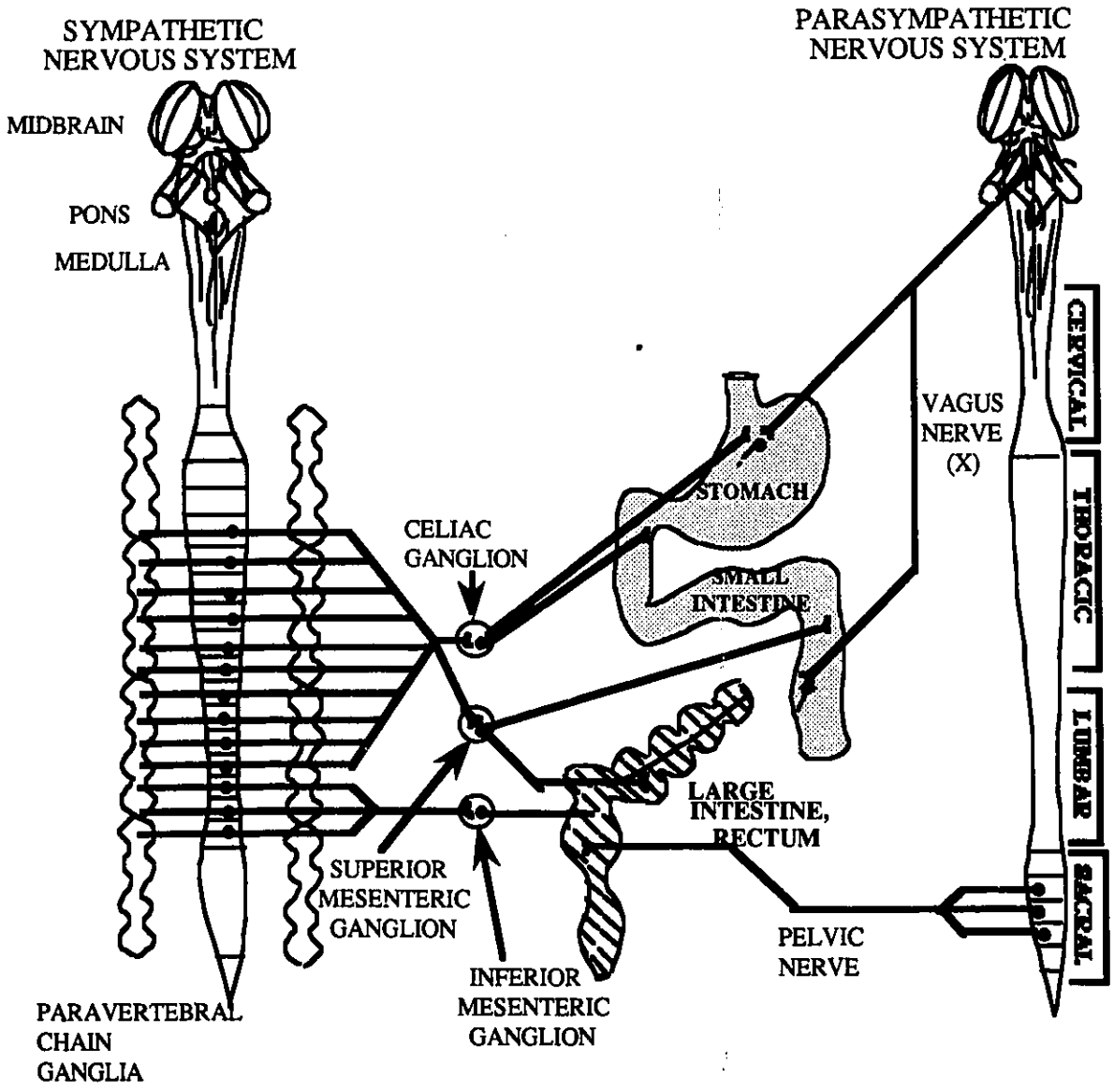


FIGURE 1.2: The extrinsic innervation of the gut (modified from Sharkey and Pittman, 1994)

1.3. The Characteristics of Enteric Neurons

The ENS contains a diversity of enteric neuronal types and this is revealed by the attempts to classify them based on morphology, ultrastructure, histochemistry and physiology.

The first classification system for enteric neurons was described by Dogiel (1899) which included three classes of enteric nerve cells distinguished by their morphology . Type I cells displayed flattened, angular forms with several short, stout dendrites and a lengthy axon which courses through several ganglia before ramifying within the circular muscle. Type II cells were described as elongate, angular forms with fewer dendrites and axons arising from the conical portion of the cell and projecting to adjacent ganglia. Type III cells, similar in shape to type II cells, possess shorter dendritic processes and axons which are confined to the ganglia of origin or adjacent ganglia. All three Dogiel-type neurons are found in the myenteric plexus, but type II nerve cells predominate in the submucous plexus. Dogiel proposed that the differing morphologies had a functional significance (eg. type I cells were motor neurons because their axons go to the muscle, and type II cells were sensory because some could be seen projecting to the mucosa). Several cell types whose shapes do not readily fit Dogiel's classification have been identified. Therefore the three morphologically defined types of neurons described by Dogiel were further differentiated, enlarging this classification of neurons based on shape, by eight neuron populations (Stach 1989) (see table 1.2).

Intracellular recording electrodes containing dyes (Lucifer Yellow, Neurobiotin and biocytin) have been used in electrophysiological studies to record from enteric neurons and determine cellular morphology (North, 1972; Hirst et al. 1974). Two distinct classes of electrophysiological cell types distinguished by Hirst et al (1974) and Wood (1989) are AH/type 2 and S/type 1 neurons. AH/type 2 neurons are defined as neurons with Dogiel type II morphology in which action potentials are followed by prolonged (up to 25s) after-hyperpolarizations (AH) while S/type 1 displayed Dogiel type I morphology and lacked such hyperpolarizations. Moreover, electrical stimulation of interconnecting strands within the myenteric plexus evoked fast excitatory post-synaptic potentials (EPSPs) in S/type 1 and many AH/type 2 neurons. The majority (80 - 90%) of AH/type 2 neurons display calbindin immunoreactivity, but are never immunoreactive for ENK, VIP, DYN or calretinin. Conversely, S/type 1 neurons never display calbindin immunoreactivity but display immunoreactivity for VIP, DYN or calretinin (see Bornstein 1994). Most submucosal neurons exhibit the electrical behavior of S/type 1 or AH/type 2 neurons (see Cooke 1986).

The variety of classification schemes created in order to encompass and distinguish all neuronal types indicates that the ENS is a complex system. They also indicate that each nerve cell whether characterized based on its morphology, projection, neurochemistry or physiology and all types of nerve fibers are scattered among the ganglia of all the enteric plexuses. In other words, enteric ganglia are not distinctly motor, sensory or integrative but appear to possess a grouping of various nerve cell types or a profuse distribution of fiber types. This characteristic of enteric neurons was confirmed by an in vitro study of the guinea-pig small intestine using combined lesion and retrograde tracer analyses on myenteric ganglia (Takaki et al. 1985). Efferent fibers from any one ganglion was seen to project in the anal, oral or circumferential axes and any one ganglion displayed varicosities of neurons from distant ganglia situated in the oral, anal or circumferential direction.

Functionally distinct populations of enteric neurons have been revealed primarily through electrophysiological and pharmacological investigations (see sections 1.4 to 1.6). There are three functional groups of enteric neurons including (i) sensory neurons, which monitor transmural tension of the intestine or the chemical composition of intestinal contents, (ii) interneurons forming connections between enteric neurons and relaying sensory information to (iii) motor neurons that change the activity of the intestine (i.e. evoke muscle contraction, dilation of blood vessels, or transport of water and electrolytes across the mucosa) (see table 1.3).

Enteric neurons are continuing to be classified by correlating their functional and neurochemical status. In table 1.2 and 1.3 a summary of the latest classifications of neuron populations in the ENS is provided based on anatomical, histochemical, immunohistochemical, electrophysiological and pharmacological studies in the guinea-pig small intestine. Each neuron type is described on the basis of cell structure (morphology), dendritic structures, the projections of nerve processes (neurites) along the axis of the intestine (ascending-orad or descending-aborad), or within the wall of the intestine (to the ganglionated or nonganglionated plexuses); the organization of the cells within ganglia, topographical distribution (intramural location), regional disposition (in ganglionated or nonganglionated plexuses; which intestinal region), synaptic inputs to these cells, the electrophysiological type, the neurochemistry (based on neurochemical localization) and the proposed functional roles.

Table 1.2. Summary of morphological types and correlated distributions, dispositions, neurochemistries and proposed functions as determined from guinea-pig and porcine intestine. See text for general descriptions. Modified from Stach (1989).

	TYPE I:	TYPE II:	TYPE III:
MORPHOLOGY:	- elongate, stellate, flattened form ("cogwheel" shape) - radially multidendritic, uniaxonal	- angular, oval, sometimes elongated form - adentritic, pseudo-uniaxonal, multi-axonal - may have 1* or 2* branching of neurites near cell body	- irregular elongate, stellate forms - central nucleus - radially multidendritic, uniaxonal
DENDRITES:	- short, lamellar and/or longer infrequent lamellar dendrites with branchings - may have lamellar axon dendrites	- rare or absent	- long, slender, little to no brandhing
PROJECTIONS:	- orad up to 8mm - aborad 1-20mm	- predominantly circumferential in circular muscle), verticle (via submucosa / mucosa), and longitudinal (within Myenteric plexus and longitudinal muscle)	- orad (about 15%), aborad (about 85%)
CELL RELATIONSHIPS:	- isolated, or in closely associated pairs, with neurites running together	- in aggregates	- predominantly in aggregates
TOPOGRAPHY:	- predominantly orad portions of ganglia - superficial	- at the periphery of ganglia / extraganglionic - embedded in the ganglia	- central, aborad portions of ganglia
DISTRIBUTION:	- in plexuses - in all regions	- in all plexuses - in all regions	- Myenteric and Henle's Plexuses - decreasing frequency from duodenum to ileum
INPUTS:	- somata, axon initial segments - cholinergic, adrenergic, peptidergic, GABAergic, serotonergic, purinergic (ACH, NA, NPY, ENK, SOM, GABA, 5-HT, ATP)	- cholinergic, peptidergic (ACH, CGRP, SP)	- somata, dendrites - adrenergic, serotonergic, peptidergic (ENK)
CHEMISTRY:	-orad: ChAT, SP, calretinin, NF - aborad: VIP, NO synthase, ATP, GABA, NPY, ENK, NF, calretinin SOM, 5-HT	- ChAT, SP, NO, GABA, VIP, CGRP	- serotonin, bombesin
ELECTROPHYSIOLOGY:	- S/type 1	- AH/type 2	- S/type 1, AH/type 2
FUNCTIONAL ROLE:	- ascending excitatory motor neurons - descending inhibitory motor neurons - ascending excitatory interneurons - descending inhibitory interneurons - longitudinal muscle motor neurons	- sensory neurons - secretomotor/vasomotor neurons	- not known

Table 1.2 continued. Summary of morphological types and correlated distributions, dispositions, neurochemistries and proposed functions as determined from guinea-pig and porcine intestine. See text for general descriptions. Modified from Stach (1989).

	TYPE IV	TYPE V	TYPE VI
MORPHOLOGY:	- irregular forms, predominantly round to oval, infrequently elongate - eccentric nucleus - polar to radially multidentritic, uniaxonal, axon hillock visible	- shape similar to type IV, some angular forms - extremely eccentric nuclei - polar multidendritic, uniaxonal	- predominantly flattened elongate form, few with type I shape - uniaxonal, axon - dendritic, long short, tapering, branched, partly dendrites arise from initial part of axon
DENDRITES:	- short / medium lengyh, tapering polar (tail dendrites) that are slightly branched, frequently short dendrites at nuclear pole, opposite the axon (head dendrites)	- long, short, tapering, branched few in number, mostly long - fequently in pair formations	- short soma dendrites predominate longer than type I and more slender - axonal dendrites frequently, spear tip-shaped dendrites on anti-axonal pole
PROJECTIONS:	- vertical, towards submucosa / mucosa	- predominantly aboral	- aboral
CELL RELATIONSHIPS:	- aggregates, centralized / decentralized	- detached and in aggregates	- detached and in aggregates
TOPOGRAPHY:	- ganglionic, extraganglionic, in type II neuron areas	- ganglionic, localized non-specifically	- aboral portions of ganglia
DISTRIBUTION:	- all plexuses - increasing in frequency from duodenum to colon	- Myenteric and Henle's plexuses from terminal jejenum to terminal ileum, increasing in frequency	- Myenteric and Henle's plexuses - increasing frequency from duodenum to ileum
INPUTS:	- somata, dendrites - chemistry unknown	- somata, dendrites, - chemistry not determined	- somata, dendrites - chemistry not known
CHEMISTRY:	- CGRP, CCK, ChAT, SOM, NPY, GAL, NO	- not known	- NO
ELECTROPHYSIOLOGY	- not known	- not known	- not known
FUNCTIONAL ROLE:	- not known	- not known	- not known

Table 1.2 continued. Summary of morphological types and correlated distributions, dispositions, neurochemistries and proposed functions as determined from guinea-pig and porcine intestine. See text for general descriptions. Modified from Stach (1989).

	DENDRITIC TYPE II	[VIP] MININEURON
MORPHOLOGY:	- typeII smooth cell shape - multidendritic, pseudo-uniaxonal, may be 1* or 2* branching of neurite close to cell body	- small, oval cell form - multidendritic, uniaxonal
DENDRITES:	- short, long, tapering, from sparse to frequent, may be polar or radially arranged, infrequent branching of dend-rites	- very slender, predominantly radial arrangement, some long with prominant branching
PROJECTIONS:	- circumferential (in circular muscle), verticle (to mucosa) - orad 2mm - aboral up to 100mm - to short descending inhibitory motor neurons	- not determined
CELL RELATIONSHIPS:	- detached and in small aggregates	- frequently in aggregates
TOPOGRAPHY:	- ganglionic, without order	- ganglionic without order in connective strands, frequently close to type II
DISTRIBUTION:	- in all plexuses - small intestine, otherwise unknown	- in all plexuses - from duodenum to colon
INPUTS:	- none	- somata, dendrites - peptidergic
CHEMISTRY:	- ChAT, SP, calbindin	- VIP, GAL, NO
ELECTROPHYSIOLOGY:	- AH/type 2	- not known
FUNCTIONAL ROLE:	- putative sensory neurons	- vasoactive inhibitory neurons, NANC motor neurons

TABLE 1.3: Functionally defined types of enteric neurons and their neurochemistries in the guinea-pig intestine. See text for neurochemical abbreviations.

FUNCTIONAL TYPE:	NEUROCHEMISTRY:
1) INTRINSIC SENSORY NEURONS	
- myenteric dendritic type II	Ach, SP, DYN, NMU (calbindin)
- submucosal cholinergic sensory	Ach, SP, DYN, NMU (calbindin)
2) INTERNEURONS	
- 1 ascending cholinergic excitatory	Ach, ENK
- 3 cholinergic descending inhibitory	Ach, SOM
	Ach, VIP
	Ach, 5-HT
- 1 non-cholinergic descending inhibitory	VIP, NO, GRP
3) MOTOR NEURONS:	
- intrinsic motor neurons to the muscle:	
- short orally projecting excitatory cholinergic	Ach, SP, TK (ENK)
- long orally projecting excitatory non-cholinergic	Ach, SP, TK (DYN,ENK)
- short anally projecting NANC inhibitory	NO, VIP, ATP(DYN, GAL, NPY)
- long anally projecting NANC inhibitory	NO, VIP, ATP (DYN, GRP)
- intrinsic secretomotor/vasomotor neurons:	
- cholinergic secretomotor	Ach, (CCK, CGRP, DYN, NPY, SOM, NMU, GAL)
- cholinergic secretomotor (interneuron)	Ach, (DYN)
- cholinergic vasodilator sensory	Ach, (SP, DYN, NMU)
- non-cholinergic secretomotor and vasodilator	VIP (DYN, GAL, NMU, PHI)

1.4. Enteric Sensory Neurons:

The notion that enteric sensory neurons exist was initially suggested by the studies of Trendelenberg in 1917 which displayed that motility reflexes could be evoked in isolated segments of intestine, *in vitro*. In addition, following surgical extrinsic denervation of the intestine, or treatment with capsaicin (a neurotoxin which results in the loss of much of the extrinsic sensory innervation to the gut), animals survived for substantial periods without observable distress (Bornstein 1994). Finally, mechanical stimulation of the mucosa can evoke polarized motor reflexes (contraction oral to and relaxation distal to the stimulus) and these reflex responses are observed following denervation of extrinsic nerve fibers (Smith et al. 1988).

There is considerable circumstantial evidence that enteric sensory neurons exist (see Bornstein 1994; Brookes et al. 1995). Mechanical stimulation of the mucosa induces Fos protein expression in a subpopulation of submucous neurons in the guinea-pig intestine even when hexamethonium (to block cholinergic transmission) is present (Kirchgessner et al. 1992). The expression of Fos was found to be associated with serotonin-induced excitation of neurons, and it was concluded that enteric sensory neurons had been identified. Assuming the existence of enteric sensory neurons, they are proposed to be AH-type 2 neurons as determined by combined electrophysiological and morphological experiments. Most electrophysiologically identified AH cells display Dogiel type II morphological features (Hendriks et al. 1990; Bornstein 1994) and make synaptic contact with all other classes of myenteric neurons (Pompolo and Furness 1989). Using silver impregnation for nerve cell visualization in the wall of the porcine small and large intestines, Stach (see Stach 1989) identified a subpopulation of Dogiel type II cells which displayed multiple short dendritic processes and named them 'dendritic type II' cells which account for 10% of all Dogiel type II neurons (Brookes et al. 1995). Retrograde tracer studies using dyes capable of retrograde axonal transport such as DiI, applied to the muscularis or mucosa in isolated preparations of the guinea-pig small intestine, have demonstrated that the efferent processes of these 'dendritic' type II cells project anally (Brookes et al. 1995) circumferentially (Furness et al. 1990; Hendriks et al. 1990); branch extensively within the myenteric plexus (Bornstein 1994) and further extend to the submucosa and mucosa (Furness et al. 1990; Song et al. 1991). This morphological subtype of Dogiel type II neurons cannot be motor neurons since they cannot be retrogradely labeled from the circular or longitudinal muscle layers (Brookes and Costa 1991a; Brookes et al. 1991a; Brookes et al. 1992). 70% of these neurons display immunoreactivity for the calcium binding protein calbindin, of which negligible levels are found in the muscle layers (Furness et al. 1990). All dendritic type II neurons with calbindin

immunoreactivity can be retrogradely labeled from the mucosa (Song et al. 1994) and therefore are not likely to be interneurons in the myenteric plexus. These cells possess very long projections which run anally within the myenteric plexus for up to 100 mm (Brookes et al. 1995). These neurons do not exhibit fast excitatory synaptic potentials (expected of motor neurons or interneurons) (Hirst et al. 1974) and this suggests that they are not directly stimulated by other presynaptic neurons, reminiscent of a sensory role (see Brookes et al. 1995). There is one neurochemically defined class of sensory neuron identified in the myenteric plexus containing ACh, DYN, neuromedin U, SP and probably calbindin (Table 1.3, fig. 1.3). This class of sensory neuron represents the largest single class of nerve cells with long projections in the guinea-pig small intestine constituting 10% of the total neuron population of the submucosa.

1.5. Enteric Interneurons:

Interneurons relay sensory information into motor (effector) functions. Studies of distension- or mechanically- evoked stimulation of ascending (orally projecting) excitatory reflexes in isolated preparations of the guinea-pig small intestine, demonstrated that the ascending excitatory reflex involves a chain of ascending cholinergic interneurons (Smith et al. 1990) as well as excitatory motor neurons. Reflex excitation has been recorded at least 30 mm oral (proximal) to the site of mucosal stimulation (see Smith et al. 1990) It was concluded that this ascending excitatory reflex is conducted via transmission at a nicotinic synapse since it is completely blocked by the nicotinic receptor antagonist hexamethonium (ibid) and that it must involve ascending interneurons since the maximum length that can be attributed to this response by excitatory motor neurons is 8 mm (Brookes et al. 1991a) and the maximum possible length of projection in the oral direction of the presumed enteric sensory neurons is 2 mm (Bornstein 1994). Ascending interneurons are all cholinergic as shown immunohistochemically and electrophysiologically. These neurons display immunoreactivity for choline acetyltransferase (ChAT), SP, ENK, calretinin (a calcium binding protein) and neurofilament protein triplet (Brookes et al. 1991b) and project to other myenteric ganglia up to 13 mm orally (Table 1.3, fig. 1.3).

There are at least three classes of anally-projecting myenteric interneurons both cholinergic and non-cholinergic identified in the guinea-pig small intestine (Costa et al. 1992). These classes are neurochemically characterized by different combinations of markers. The cholinergic marker ChAT is present in most of these descending interneurons (Steele et al. 1991), but one subpopulation is characterized by VIP immunoreactivity and represents about 3-4% of total myenteric neurons. A second class contains SOM (about 4% of all myenteric neurons) and a

third class also contains 5-HT (less than 2% of myenteric neurons). The SOM- and 5-HT-immunoreactive interneurons contact more anally located interneurons with the same neurochemical phenotype so that descending interneurons form chains much like the ascending interneurons. Non-cholinergic interneurons are immunoreactive for VIP, NO synthase (the synthesizing enzyme for NO) and GRP (Table 1.3, fig. 1.3). The roles of these different populations of interneurons are unclear but they are proposed to make specific connections with excitatory and inhibitory motor neurons to the circular and longitudinal muscle layers and to other interneurons.

Therefore in the guinea-pig small intestine five distinct populations of interneurons, consisting of one population of ascending and four of descending interneurons, have been neurochemically identified.

1.6. Enteric motor neurons to the muscle

The motor neurons are the final outputs of neuronal control circuits in the ENS. Both excitatory (orally directed) and inhibitory (orally and anally directed) motor neurons to the muscle (circular and longitudinal) are found with their cell bodies localized to the myenteric plexus. In addition, the cell bodies of enteric vasodilator neurons and secretomotor neurons to the mucosa reside within the submucosa. There are 2 classes each of excitatory and inhibitory motor neurons and 3 classes of secretomotor/vasomotor neurons based on neurochemical content and in some cases length of projection. It must be considered that the identification and characterization of these neurons has been derived by anatomical and physiological studies of the guinea-pig small intestine and does not apply to other regions or to other species. Sections 1.6.1 to 1.6.4 provide a description of these different motor neurons.

1.6.1. Excitatory Circular Muscle Motor Neurons

The cell bodies of excitatory circular muscle motor neurons reside in myenteric ganglia and represent 12 % of myenteric neurons These motor neurons display Dogiel type I morphology and project locally (directly innervating the circular muscle) or orally (up to 8mm) within the myenteric plexus before projecting to the circular muscle. Both populations contain ChAT and SP, but the long orally projecting population also contain neurofilament protein triplet and ENK (Table 1.3, fig. 1.3). These neurons release ACh and tachykinins (TKs) which evoke contraction of the circular smooth muscle. ACh mediates excitatory junction potentials (EJPs) via

its actions on muscarinic receptors while TKs mediate slow EJPs via their actions on NK1 and NK2 and NK3 receptors (see Dockray 1994; Costa et al. 1994).

1.6.2. Inhibitory Circular Muscle Motor Neurons

The first evidence for the presence of enteric inhibitory muscle motor neurons was demonstrated in isolated preparations of guinea-pig taenia coli when anticholinergic and noradrenergic blocking drugs were found to be ineffective in blocking nerve-mediated inhibition of the intestinal muscle (see Burnstock 1972). Since these inhibitory motor neurons were neither noradrenergic nor cholinergic in nature Burnstock and his colleagues referred to them as nonadrenergic, noncholinergic (NANC) inhibitory motor neurons (ibid). The initial chemical characterization of the NANC inhibitory transmission came from Burnstock who put forward the 'Purinergic hypothesis' where adenosine triphosphate (ATP) was proposed to be the transmitter (Burnstock 1972) since it mimicked the relaxation elicited by electrical stimulation of motor nerve fibers. In 1970 vasoactive intestinal peptide (VIP) was discovered and subsequently shown not only to be released by enteric nerves (Fahrenkrug 1979) but also to cause relaxation of enteric smooth muscle. Subsequently, there was a long period of debate as to whether ATP or VIP was the inhibitory neurotransmitter of NANC motor neurons until Costa et al (1986b) demonstrated in isolated preparations of guinea-pig intestine at least two mechanisms of transmission from these inhibitory motor neurons. Using the bee venom apamin they identified inhibitory responses which were apamin-sensitive (which blocked relaxation caused by ATP, but not VIP) and those that were apamin-insensitive. These inhibitory responses were reduced by apamin in the circular muscle of the stomach and ileum and in the longitudinal muscle of the ileum, taenia coli, and distal colon. However, apamin was ineffective on distension-induced reflex relaxation of the circular muscle of the ileum or colon. From this work, it was suggested that ATP and VIP, either separately or together, were insufficient to account for all forms of inhibitory neurotransmission to gut smooth muscle. With the identification of NO as a transmitter of NANC motor neurons (see Sanders et al. 1992; Stark et al. 1992; Brookes et al. 1993) a third candidate transmitter had arrived.

Retrograde and physiological tracing studies (Bornstein et al. 1986; Smith et al. 1988; Brookes et al. 1991b) have shown that NANC inhibitory motor neurons may project 1-20mm orally within the myenteric plexus before innervating the circular muscle. These neurons display Dogiel type I morphology, constitute roughly 16% of myenteric neurons and contain NO synthase and VIP (Table 1.3, fig. 1.3). The shorter anally projecting (5-6mm) population also

contain ENK and NPY while the longer ones contain DYN, GRP, neurofilament protein, and alkaline phosphatase (Table 1.3, fig. 1.3).

1.6.3. Secretomotor/Vasomotor Neurons

Within the mucosal plexus are the fibers of secretomotor neurons which modulate secretion of water and electrolytes in the intestine. In addition some submucosal neurons also project to the submucosal and mucosal microvessels and are proposed to modulate blood flow. Three classes of secretomotor/vasomotor neurons have been identified. These include the two cholinergic secretomotor/vasomotor neurons and one non-cholinergic secretomotor/vasomotor class of neurons. Within the cholinergic component one class contains ACh, SOM, NPY, neuromedin U, DYN, CGRP, CCK and GAL (Table 1.3, fig. 1.3). This class of secretomotor neurons represents 30% of the submucosal neurons in the guinea-pig small intestine and has the greatest number of transmitters ever attributed to any neuron. The principal transmitter of these neurons is ACh and also CCK (Table 1.3, fig. 1.3). The second class of secretomotor neuron representing 16% of the total submucosal neuron population contains ACh, calretinin and DYN. There is one class of non-cholinergic secretomotor/vasomotor neuron which contain VIP as their primary transmitter and also contain DYN, GAL, neuromedin U and PHI. This class of secretomotor/vasomotor neuron represents 10% of the total submucosal neuron population (Furness et al. 1985; O'Brien et al. 1995).

In summary at least 12 classes of neurons have been identified in the myenteric plexus of the guinea-pig small intestine (summarized in fig. 1.3) including, motor neurons to the circular muscle (2 excitatory and 2 inhibitory), ascending (projecting orally) interneurons (one class), descending (projecting anally) interneurons (4 classes), sensory neurons (one main class), secretomotor/vasomotor neurons (2 classes). In the submucous plexus of the guinea-pig there are 3 classes of secretomotor/vasomotor neurons and one class of sensory neuron. This classification of enteric neurons in the guinea-pig intestine has been attained by studies involving multiple labeling immunohistochemistry combined with retrograde tracing of nerve pathways within the gut wall (see Costa et al. 1992; Furness et al. 1992; Bornstein 1994; Costa et al. 1994).

1.7. Enteric neurotransmitters/neuropeptides:

Almost all of the neuropeptides localized in the brain have also been found in the ENS and vice versa, many of which are putative neurotransmitters. Established enteric neurotransmitters include ACH, 5-HT, GABA, NO, SOM, opioids (leu- and met-enkephalin) and

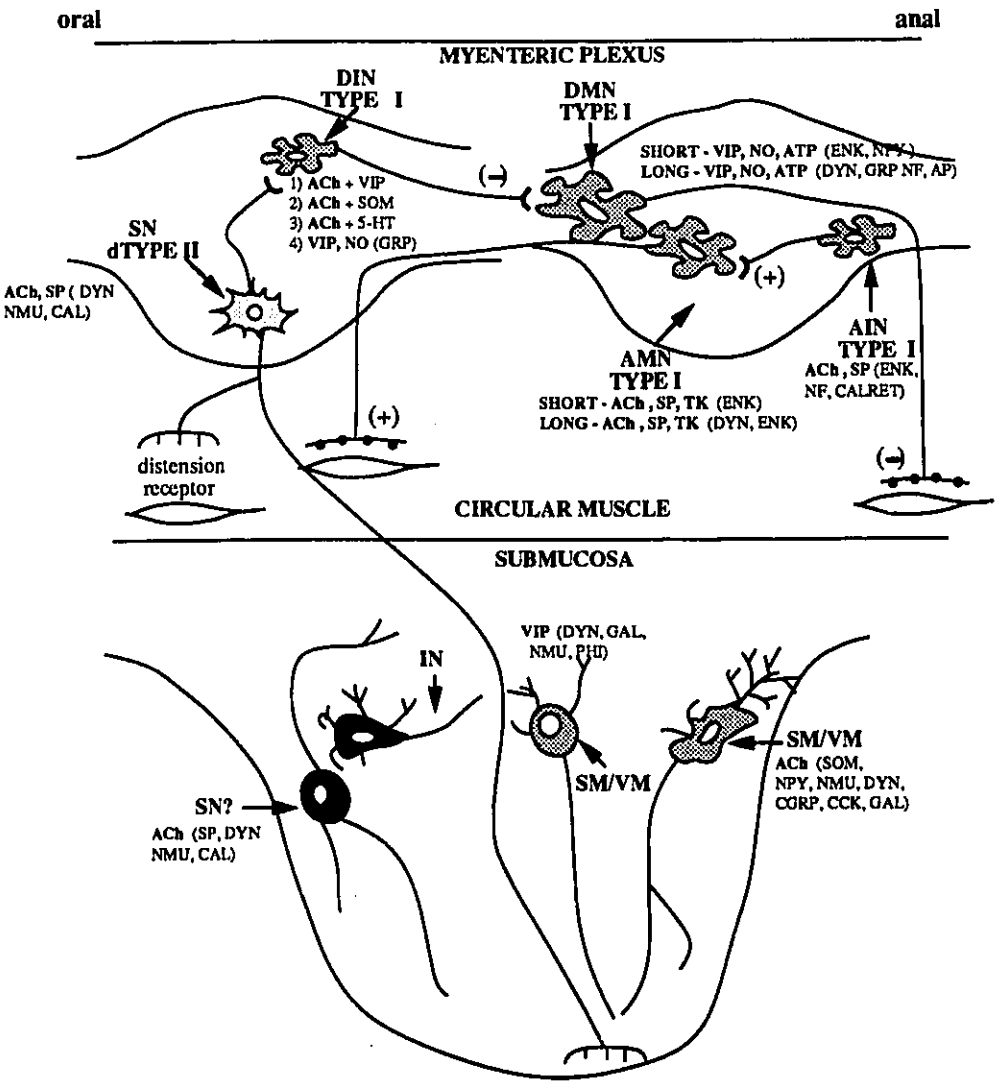


Figure 1.3. Schematic diagram showing the major classes of myenteric and submucosal neurons and there chemical code in the guinea-pig intestine. The proposed primary transmitters are in bold, and the substances listed in brackets are also contained in the neurons and may play a role in transmission. In the myenteric plexus are short and long ascending excitatory (+) motor neurons (AMN) as well as short and long descending inhibitory (-) motor neurons (DMN). The AMN have ACh, substance P (SP) and three tachykinins (neurokinin A, γ and K) as their primary transmitters while the IMN have nitric oxide (NO) and vasoactive intestinal peptide (VIP) as primary transmitters. The primary transmitters of the ascending excitatory interneurons (AIN) are ACh and SP. There are three populations of descending inhibitory interneurons (DIN) all containing ACh as their primary transmitter. There is one population of DIN with NO and VIP as their primary transmitters. Dogiel type II (dTYPE II) sensory neurons (SN) which contain ACh and SP and characterized by calbindin immunoreactivity. Within ganglia of the submucosa there are four major types of neuron. Neurons with ACh that are proposed to be sensory (SN?) based on their physiology and projections. Neurons with ACh that are proposed to be interneurons (IN) based on projections. Non-cholinergic secretomotor/vasomotor (SM/VM) neurons with VIP as their primary transmitter. Cholinergic SM/VM with ACh as their primary transmitter but also display immunoreactivity for up to seven other peptides. Other abbreviations: ENK, enkephalin, DYN, dynorphin, GAL, galanin, NPY, neuropeptide Y, NMU, neuromedin U, GRP, gastrin releasing peptide, PHI, peptide histidine isoleucine, CALRET, calretinin, CAL, calbindin. Modified from Furness et al (1992).

VIP. Proposed transmitters include peptide histidine isoleucine (PHI) (the VIP/PHI secretin group, bombesin, gastrin, cholecystokinin (CCK), gastrin releasing peptide the peptide Y family [neuropeptide Y (NPY), peptide YY (PYY) and pancreatic polypeptide (PP)], neurotensin, neuromedin U (NMU), the tachykinins [substance P (SP) and substance K(SK)], dynorphin (DYN), calcitonin gene-related peptide (CGRP), and motilin.

Before a substance can be considered a neurotransmitter it must meet with several criteria: (1) both the substance and mechanism for its synthesis must exist in neurons; (2) the nerve terminal concentrates the substance and releases it by depolarizing stimuli through a calcium-dependent mechanism; (3) the effect exerted by exogenous application of the substance can be mimicked when the endogenous material is released; (4) antagonists elicit similar effects on endogenous and exogenous material and (5) mechanisms for breakdown, reuptake, or removal of the substance exist. There are some views that criteria (1) to (3) are sufficient to establish a transmitter role (Werman 1966; Orrego 1979). Within this framework many of the gut neuropeptides have satisfied the criteria. Much caution has been exercised when interpreting whether an enteric substance is a transmitter since peptides and some recently discovered neural substances (i.e. nitric oxide) have different mechanisms of synthesis or release from those of conventional transmitters. In addition, since coexistence of two or more candidate transmitters occurs in enteric neurons the postjunctional responses may be elicited by combined actions of one or all of the substances (cotransmission). In this case one substance may have a direct action on the target cell while the other exerts a pre- and/or postjunctional modulatory effect on neurotransmission. Therefore, each colocalized neurochemical could be a potential neurotransmitter/neuromodulator. Finally, many of the gut peptides are members of families (i.e. tachykinins) with some behaving as transmitters and yet others as hormones. Therefore structurally similar molecules released from different sites may exert their actions at the same target cell, or evoke the same response from distinct sites of action. These caveats and the lack of specific antagonists have hampered the establishment of a 'transmitter' identity of some enteric substances (see Dockray 1994).

The research described in this thesis has focused on three of these neurochemicals; NO, GABA and NPY.

1.8 Nitric Oxide

NO synthesis is catalyzed by a family of NO synthase isoforms. The substrate for this biosynthetic pathway is L-arginine and a byproduct is L-citrulline. It is now known that

mammalian cells possess at least three NO synthase genes (encoding distinct isoforms) as determined by southern blot analysis (Nathan 1992). NO synthase isoforms have been classified based on the cell type they were originally discovered in, molecular characteristics and subcellular distribution and their mechanism of expression. (Table 1.3). These NO synthase isoforms, in order of discovery and referred to by the cell type they were discovered in, include the neuronal-type (nNOS or type I), macrophage-type (mNOS or type II) and endothelial-type (eNOS or type III). The cDNAs of these NO synthase isoforms have been cloned, sequenced, and share 50-60% homology with one another at the nucleotide and amino acid levels (Nathan 1992). The genes for the NO synthase isoforms have been mapped to chromosome 12 (nNOS), 17 (mNOS) and 7 (eNOS) (Table.1.3) (ibid). nNOS is a cytosolic 150 kD protein monomer that was originally isolated from rat cerebellum (Bredt et al 1990) . eNOS originally isolated from the membrane fraction of bovine aortic endothelial cells (Pollock et al. 1991), has a molecular weight of 133 kDa (Janssens et al. 1992). Cloning studies reveal that nNOS and eNOS have recognition sites for a number of factors including calmodulin, NADPH, flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and tetrahydrobiopterin, as well as multiple phosphorylation sites (Bredt et al. 1990). mNOS, originally isolated (Stuehr et al. 1991) and sequenced (Xie et al. 1992) from murine macrophages, has a molecular weight of 130 kDa (Xie et al. 1992) and has recognition sites for FAD, FMN, NADPH but not for calmodulin. mNOS, like nNOS is found in the soluble fraction of cell homogenates.

The two NO synthase gene products eNOS and nNOS are constitutively expressed in multiple cell types and have been classed together as constitutive NO synthase isoforms (Table 1.3). Small amounts (pmol) of NO are produced by these enzymes in response to physiochemical (altered PO_2), mechanical (i.e. eNOS) (Pohl et al. 1986; Pohl et al. 1989; Lamontage et al. 1992) or receptor (i.e. nNOS) (Garthwaite et al. 1988; Garthwaite 1991) stimulation of the cell, resultant transient elevations of intracellular calcium and activation and binding of calmodulin (Bredt and Snyder 1990a). The NO synthesized by these enzyme isoforms acts as an intra- and intercellular messenger effecting its target cells through activation of soluble guanylate cyclase, and resultant stimulation of cGMP production (ibid). The expression of mNOS has been found to be induced by certain cells following exposure to endotoxin or some cytokines (e.g. interleukin-1, interleukin-2) (see Moncada et al 1989; Moncada et al 1991) and mediated by the intracellular nuclear factor κB (Schreck et al 1992). NO produced by activated macrophages following activation mediates the cytotoxic actions of these cells against microorganism, bacteria, protozoa and against tumour cells (Hibbs et al 1988; Granger et al 1990; Liew et al 1990). Since

expression of mNOS is induced in many cell types, inducible NO synthase was subsequently used as a descriptor. It was originally thought that inducible NO synthase is not expressed in healthy quiescent cells however this isoform is present in epithelium of fetal and adult lung (Kobzik et al. 1993) and cells of the juxtaglomerular apparatus (Tojo et al 1994) of normal animals. mNOS is Ca^{2+}-independent and once expressed, continues to produce large (nmolar) quantities of NO until substrate becomes limiting (see Griffith and Stuehr 1995). However since mNOS is present under normal physiological conditions (Kobzik et al 1993, Tojo et al 1994), there is some speculation that mNOS may be constitutively or chronically induced by inflammatory mediators present in normal tissues and that NO, produced from this basal mNOS, plays a role in host-defense (Kobzik et al 1993). Since mNOS can produce such large quantities of NO that is cytoxic, it is thought to be the isoform that is most likely involved in tissue damage or death. However, it has been determined that a high enough level of intracellular calcium may lead to production of cytotoxic quantities of the constitutively expressed NO synthase isoforms following tissue ischemia/reperfusion (Matheis et al 1992, Patel et al 1993). nNOS, originally immunolocalized in central and enteric neurons (Bredt and Snyder 1990b) has also been identified in skeletal muscle (Nakane et al 1993), kidney macula densa cells (Wilcox et al 1992), ß-pancreatic cells (Schmidt et al 1992), and epithelial cells of the lung (Kobzik et al 1993), stomach and uterus (Brown et al 1992; Schmidt et al 1992). In addition to the expression of eNOS in the vascular endothelium, it has been displayed immunohistochemically in kidney tubular epithelial cells (Tojo et al 1994). mNOS expression has been identified following exposure to inflammatory cytokines in several tissue and cell types including vascular endothelial cells and smooth muscle cells, myocytes, immune cells, and astrocytoma cells in the brain (for review see Moncada et al 1991).

Since NO is potentially toxic, NO synthase is one of the most tightly regulated enzymes in the mammalian system. NO may affect NO synthase activity through feedback inhibition; which involves binding to heme within the active site (Griscavage et al. 1993). The constitutively expressed NO synthase isoforms may also be downregulated by phosphorylation (Michel et al. 1993) via cGMP-dependent activation of protein kinases. Finally, the availability of substrate and cofactor also regulate NO synthase activity.

Table 1.4: A summary of identified NO synthase isoforms and their distinguishing characteristics.

	nNOS/TYPE I	mNOS/TYPE II	eNOS/TYPE III
GENE LOCATION	Chromosome 12	Chromosome 17	Chromosome 7
SUBCELLULAR DISTRIBUTION	Cytosolic	Cytosolic	Membrane bound
EXPRESSION	Constitutive	Inducible	Constitutive
LOCATION	- Central neurons - Peripheral neurons - Skeletal muscle	- Immune cells - Vascular endothelial and smooth muscle cells - Astrocytes - Endocardium Fibroblasts	- Vascular endothelial cells - Kidney tubular epithelial cells

NO is a free radical in the form of a highly diffusible gas. In contrast to other signaling molecules in the mammalian system, NO has a unique chemical nature since it can diffuse rapidly through both lipid (membrane) and aqueous environments (see Gross and Wolin 1995). This property of NO permits it to spread three-dimensionally, the extent of which is limited only by its half-life (ibid) which in vivo may be as short as a few seconds or longer if it is transported by a carrier molecule (i.e. cysteine or glutathione) (see Gross and Wolin 1995). Interestingly, based on the half-life of NO and ability for diffusion it has been estimated that NO may spread across as many as 2 million synapses in the CNS (Bruhwyler et al. 1993).

As a free radical gas NO is a highly reactive molecule and accordingly has many receptors or target sites of action. NO reacts with hemoproteins such as soluble gunaylyl cyclase and exerts cGMP-dependent actions. These include stimulation of cGMP-dependent protein kinases which regulates protein phosphorylation and intracellular calcium levels (Nairn and

Greengard 1983), activation of phosphodiesterases which regulate cAMP levels and cGMP levels (and provide feedback inhibition of NO actions) (Nicholson et al 1991; Mayer et al 1992) and activation of ion channels (Ahmad et al 1990). NO also exerts cGMP-independent actions targeting other aspects of cellular function. NO activates nuclear poly-ADP ribosyl transferase which leads to inhibition of the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (see Gross and Wolin 1995). NO also inhibits mitochondrial enzymes such as aconitase, critical to the Krebs cycle, and succinate-ubiquinone oxidoreductase involved in the electron transport chain. If the concentration of NO is sufficiently high such that glycolysis and aerobic mitochondrial respiration are both inhibited, intracellular ATP synthesis would be compromised leading to cell death (ibid).

1.9 NO: As an Endothelium-Derived Relaxing Factor

Although NO was known since the late 1970's to be a ligand that causes vascular smooth muscle relaxation (Gruetter et al. 1979), the endogenous vasodilator substance, endothelium-derived relaxing factor (EDRF) was first discovered in 1980 (Furchgott et al. 1980) when acetylcholine was shown to require the presence of an intact endothelium to evoke relaxant responses in isolated arteries. From 1987, when NO was proposed to be EDRF, (Ignarro et al. 1987) to the present, biochemical and pharmacological investigations have provided convincing evidence that NO is synthesized by the vascular endothelium (Palmer et al. 1987; Palmer et al. 1988; Myers et al. 1989; Janssens et al. 1992) and that this free radical gas and EDRF have similar biological and chemical properties (Ignarro et al. 1987; Ignarro et al. 1987; Palmer et al. 1987; Ignarro et al. 1991). NO has been shown to mediate a vasodilator response in both arteries and veins (Ignarro et al. 1987; Palmer et al. 1988; Vallance et al. 1989). In addition, the capacity to convert L-arginine to NO has been demonstrated in arterial smooth muscle cells (Wood et al. 1990; Schini et al. 1991).

NO release from the vascular endothelium occurs under basal conditions (Ignarro et al. 1991), in response to several vasodilator substances (such as ACH, ATP and bradykinin) (Busse et al. 1991; Busse et al. 1993) and following physiochemical (altered PO_2) (Pohl et al. 1989) and mechanical (shear stress) (Pohl et al. 1986; Lamontage et al. 1992) stimulation of the vasculature. The vasodilatory response to these stimuli is effected through binding of NO to the heme moiety of soluble guanylyl cyclase in the vascular smooth muscle causing an allosteric modulation of the enzyme and resulting in elevated levels of cyclic guanosine monophosphate (cGMP). This increase in cGMP production leads to a reduction in intracellular Ca^{2+} levels

through a multitude of proposed cascade events (see Moncada et al. 1991) resulting in myosin light-chain kinase phosphorylation and vascular smooth muscle relaxation. Noncompetetive inhibitors of the synthesizing enzyme NO synthase, enantiomeric analogues of L-arginine, [N^G-nitro-L-arginine (L-NNA) and N^G-monomethyl-L-arginine (L-NMMA)] inhibit ACh induced vasodilation and the resultant effect is restored by excess arginine (see Moncada et al. 1991). When referring to the endogenous vasodilator substance the names NO and EDRF are now used interchangeably. To differentiate NO synthesized within the endothelium from other sources the term endothelium derived nitric oxide (EDNO) has proven useful.

In the gastrointestinal tract, EDNO has recently been considered to play an important role in the control of blood flow (Walder et al. 1990; Pique et al. 1992; Poeggel et al. 1992).

1.10 Nitric Oxide: As a Neurotransmitter

As a neural messenger, NO was first discovered in the brain where its synthesis in some cells is stimulated by Ca^{2+} entry subsequent to excitatory amino acid (N-methyl-D-aspartate) receptor stimulation (Garthwaite 1991). Upon synthesis, NO diffuses to neighboring cells where it interacts with ferrous heme of soluble guanylate cyclase and stimulates formation of cyclic GMP. This process has been seen to carry signals between neurons, and between neurons and glial cells (Moncada et al. 1991) Proposed roles for NO in CNS function include; synaptic plasticity and long term potentiation, and the activity-dependent determinant of neural development (Garthwaite et al. 1988; Gally et al. 1990). In addition, it is proposed that non-adrenergic, non-cholinergic (NANC) vasodilator nerves release NO and therefore are involved in the neural control of cerebral blood flow (Toda et al. 1990b) .

Neuronal NO synthase may synthesize NO whenever the local calcium concentration exceeds approximately 500nM (Schmidt et al. 1992). NO is proposed to diffuse from these sites when synthesized to act on its target, soluble guanylyl cyclase. It is still not entirely certain whether NO is released mainly from somata (thereby controlled by release of intracellular calcium stores), from axon terminals or varicosities (thus controlled via voltage-gated calcium channels) or from dendrites (thereby controlled by transmitter-gated channels). Electron microscopic reports on enteric neurons (Llewellyn-Smith et al. 1992) suggest that NO synthase may be concentrated immediately beneath the plasma membrane (associated with subcellular organelles including mitochondria, endoplasmic reticulum and Golgi apparatus), where it would be directly exposed to calcium entering through transmitter-gated channels. *These findings*

indicate that the classical concept of storage and quantal release of NO as a transmitter may not be applicable to NO releasing neurons. Unlike the classical transmitters like ACh and putative peptide transmitters like VIP, NO is not prestored and released by directed vesicular exocytosis but is produced on demand (Bredt and Snyder 1992; Sanders and Ward 1992).

Hope et al (1991) discovered that NO synthase displays reduced nicotinamide adenine dinucleotide phosphate (NADPH) diaphorase activity (can reduce a tetrazolium dye to a visible blue formazan precipitate in an NADPH dependent manner) in fixed tissue. In fact several findings indicated that sites of NO synthase reactivity could be detected by NADPH diaphorase histochemistry. Purified NO synthase exhibits NADPH diaphorase activity while its own activity is competitively inhibited by NBT (Hope et al. 1991). NO synthase immunoreactivity and NADPH diaphorase histochemical reactivity have a nearly identical codistribution in brain and peripheral tissues (Dawson et al. 1991a). In the enteric system, neurons that are NADPH-diaphorase positive are also NO synthase immunoreactive in a one-to-one relation (Belai et al. 1992; Schmidt et al. 1992; Ward et al. 1992; Young et al. 1992; Timmermans 1994b). In addition this reaction has been shown to identify endothelial NO synthase reactive sites (O'Brien et al. 1995). NO synthase is the only enzyme in the ENS known to retain NADPH diaphorase activity after aldehyde fixation since in NO synthase gene knockout mice there is a lack of NO synthase immunoreactive or NADPH-diaphorase staining in the gut (Huang et al. 1993). Therefore, the NADPH-diaphorase reaction can be used as a marker for the presence of NO synthase activity. Calcium chelators and NO synthase inhibitors do not affect the level of NADPH diaphorase staining which indicates that arginine and NADPH interact at separate sites of the NO synthase molecule . The diaphorase activity of NO synthase appears to due to the reduction by NADPH of a flavin associated with NO synthase that in turn directly reduces the tetrazolium dye, since diphenyleneiodonium (DPI) which binds the flavin moiety of NO synthase, inhibits diaphorase staining. Therefore the NADPH-dependent diaphorase reactive portion of NO synthase enzyme activity is independent of arginine, calcium and calmodulin (see Griffith and Stuehr 1995). This histochemical method is an easy and inexpensive protocol for localizing nitric oxide synthase activity (Hope et al. 1989; Hope et al. 1991). The principal advantage of this method is that it does not limit the localization of NO synthase activity to alterations in the enzymes' conformation, or steric hindrance as is the case with an immunohistochemical approach.

NADPH diaphorase histochemistry and NO synthase immunohistochemistry have been used to investigate the anatomy NO synthesizing or nitrergic innervation of the enteric nervous system in multiple species, including the guinea-pig (Costa et al. 1992; Nichols et al.

1992; Young et al. 1992; Llewellyn-Smith 1992; McConalogue and Furness 1993), porcine (Timmermans et al. 1993; Timmermans et al 1994b), rat (Bredt and Snyder 1990b; Belai et al. 1992; Aimi et al 1993; Nichols et al 1993) , canine (Ward et al. 1992; Berezin et al. 1994), mouse (Grozxdanovic et al. 1992) and human (Timmermans et al 1993; Timmermans, 1994a) intestine using both laminar preparations and tissue cross sections and under light and electron microscopic conditions. Table 1.5 provides a collective summary of these findings. The studies reviewed in table 1.5 reveal that the nitrergic innervation patterns in the gut wall display considerable similarities across species with the exception of the minor variations in anatomy of the ENS. Since a complete description and species comparison of the nitrergic innervation of the guinea-pig, rat and human intestine (as determined from studies included in this thesis) is described and compared to the findings of other studies in subsequent chapters of this thesis, a brief description along with Table 1.5 is provided by way of introduction. Nitrergic neurons localized to both myenteric and submucosal nerve layers exhibit shapes and projections of Dogiel type I and II nerve cell morphologies. Efferent processes of myenteric ganglionic neurons emerge within primary, secondary and tertiary nerve fiber bundles of the plexus and within the circular smooth muscle layer, suggesting that NO synthase is found in circular muscle motor neurons. Lesion and retrograde tracer studies indicate that NO synthesizing neurons project anally within the myenteric plexus (Costa et al 1992), a proportion of which innervate the circular muscle. The anal projection and type I morphology of nitrergic neurons is consistent with a NANC neuron innervation. Both light and electron microscopic localization of NO synthase has revealed that within the myenteric plexus of the guinea-pig intestine nitrergic varicose nerve fibers appear to innervate non-nitrergic myenteric ganglionic cells (Llewellyn-Smith et al. 1992) suggesting that NO synthase is found in some interneurons. Within the submucosa fine nitrergic fibers have been localized to Henle's nerve layer of all species examined, Meissner's nerve layer of the rat and human intestine, the Intermediate nerve layer of the human intestine, the paravascular innervation and within the muscularis mucosae. In addition a network of nitrergic nerve fibers have been localized underlying the base of the mucosal crypts. Ultrastructurally, NO synthase immunoreactivity is found in enteric nerve varicosities and over electron-dense cytoplasmic material associated with the Golgi apparatus, mitochondria and endoplasmic reticulum (Llewellyn-Smith et al. 1992). *This nonvesicular localization of NO synthase suggests that in contrast to classical transmitters like ACh and peptides such as VIP, NO may not be stored and released by exocytosis but is produced on demand (Sanders and Ward 1992). However, the distribution of*

NO synthase in the ENS of multiple species and in multiple nerve types supports a transmitter role for NO in the mammalian gut.

Pharmacological studies investigating the action of NO in the gut have been aided by the use of enantiomeric analogues of the NO synthase substrate L-arginine, to assess whether certain gut responses are mediated by NO or whether they are NO synthase dependent. The NO synthase inhibitors that have been used to study NO in gut function include N^G-nitro-L-arginine methyl ester (L-NAME), nitro-L-nitro arginine (L-NNA) and N^G-iminoethyl-L-ornithine (L-NIO) (Sanders et al. 1992; Stark et al. 1992; Brookes 1993). L-NAME is a reversible inhibitor of NO synthase since inhibition can be reversed by L-arginine. L-NIO is an irreversible inhibitor of NO synthase but its mechanism of action is unknown. While L-NNA is a reversible inhibitor, it is not a mechanism-based inhibitor like L-NAME, but a tight-binding, slow-dissociating ligand of the arginine binding site. These inhibitors show modest selectivity for the different NO synthase isoforms. Both L-NAME and L-NNA preferentially inhibit the constitutive over the inducible isoforms of NO synthase (Pollock et al. 1991). L-NNA, however, is a more potent inhibitor of neuronal NO synthase (Buisson et al. 1992; Buisson et al. 1993; Klatt et al. 1994). NO synthase inhibitors are not completely NO synthase specific or entirely resistant to being metabolized to other biologically active products. NO synthase inhibitors are metabolized by NO synthase to citrulline (see Griffith et al. 1995), which is recycled to L-arginine and therefore supports NO production as well as reducing NO synthase inhibition. Finally, L-NAME is a muscarinic receptor antagonist (Buxton et al 1993).

The first studies reporting a role of NO in the gut indicated that this molecule was a mediator of NANC inhibitory motor neuron transmission (Bult et al 1990; Boeckxstaens et al 1990). These reports and the finding of Bredt et al (1990b) and Costa et al (1992) that NO synthase is localized to Dogiel type I motor neurons and associated innervation of the circular muscle (consistent with a NANC innervation) in the rat small intestine, suggested that NO might be an inhibitory transmitter of NANC motor neurons in the gut. Table 1.6 provides a listing of the methods used to establish a role for NO in inhibitory motor transmission, including these early studies, in several different gut regions and from a variety of species. The findings of these studies have been recently reviewed in some detail (Sanders et al. 1992; Stark et al. 1992; Brookes 1993) and are summarized here. Pharmacological analyses in combination with simultaneous mechanical and electrical recordings of isolated gut preparations, have shown that NO synthase inhibitors reduce electrically-evoked NANC relaxations of the mammalian intestine. This inhibition of relaxation responses is reversed with L-arginine (conceivably by displacing the

SPECIES	REGION	MYENTERIC PLEXUS	SUBMUCOUS PLEXUS	TISSUE PREPS	METHOD	REFERENCES
Pig	small intestine	neurons/ fibers	fibres/neurons of Henle's and Meissner's nerve layers	laminar preps	NOS-imm NADPH-d (L-M)	Timmermans et al. 1993 Timmermans et al 1994b
Guinea-pig	ileum colon	neurons/ fibers neurons/ fibers	-fibers only, no cells found -fibres/neurons	laminar preps	NOS-imm NADPH-d (L-M)	Young et al. 1992 Costa et al 1992 McConalogue and Furness 1993
Guinea-pig	small intestine	neurons/ fibers	ND	laminar preps	NOS-imm (E-M)	Llewellyn-Smith et al. 1992
Rat	esophagus stomach duodenum ileum jejunum colon	neurons/fibers	-fibres/neurons all regions -which nerve layer Henle's or Meissner's not specified -blood vessels not labeled	laminar preps and tissue sections	NADPH-d (L-M)	Aimi et al (1993)
Rat	stomach duodenum ileum coecum colon	neurons/ fibers in all regions	ND	laminar preps	NOS-imm NADPH-d (L-M)	Schmidt et al. (1992)
Dog	ileum colon	varicosities and CM fibers	-fibers in submucosa and CM interface	tissue sections	NOS-imm (EM)	Berezin et al. (1994)
Dog	colon	neurons/ fibers LM/ CM fibers	fibres/neurons Henle's	laminar preps and tissue sections	NOS imm NADPH-d (L-M)	Ward et al. (1992)
Mouse	all regions	neurons/ fibers	-fibres/neurons -mucosal epithelial cells -perivascular fibres about blood vessels	laminar preps	NADPH-d (L-M)	Groxzdanovic et al.
Human	small intestine large intestine	neurons/fibres	-fibres/neurons of Henle's and Meissner's - no blood vessel labeling	laminar preps and tissue sections	NOS-imm NADPH-d (L-M)	Timmermans et al. (1993)

Table 1.5: A review of some of the methods employed to localize NO synthase in various species and regions and their findings. ND = not determined. CM = circular muscle, LM = longitudinal muscle, NOS-imm = NO synthase immunoreactivity, NADPH-d = NADPH diaphorase activity, L-M=light microscopy, E-M=electron microscopy. Note that findings embodied in this thesis are not included.

Table 1.6 Summary of physiological studies on enteric NO function in different species and in various regions. CM = circular muscle, LM = longitudinal muscle, MM = muscularis mucosae. Most studies were performed on isolated preparations of the gut and carried out simultaneous mechanical and electrical recordings using force transducers and intracellular microelectrodes. There are fewer instances where electrophysiological recordings of junction potentials were carried out on isolated preparations. These findings are summarized in the text. From Brookes et al (1993).

REGION OF GUT	SPECIES	PREPARATION	RECORDING METHOD	STIMULATION TECHNIQUE	IN VITRO/ IN VIVO	REFERENCES
Oseophagus	Opossum	CM	Electrophysiological	Electrical	in vitro	Christinck et al 1991
	Opossum	CM	Mechanical	Electrical	in vitro	Knudsen et al 1991
	Opossum	CM	Mechanical	Reflex/Electrical	in vitro/ in vivo	Yamato et al 1992a
	Rat	MM	Mechanical	Electrical	in vitro	Will et al 1990
Lower oesophageal sphincter	Dog	CM	Mechanical	Electrical	in vitro	Pelckmans et al 1991
	Human	CM	Mechanical	Electrical	in vitro	McKirdy et al 1992
	Opossum	CM	Mechanical	Electrical	in vitro	Knudsen et al 1992
	Opossum	CM	Mechanical	Electrical	in vitro	Tottrup et al 1991
	Opossum	CM	Mechanical	Reflex/ Electrical	in vitro/ in vivo	Yamato et al 1992b
Stomach	Dog	Antrum	Electrophys/ Mechanical	Electrical	in vitro	Ozai et al 1992
	Guinea-pig	Whole stomach	Mechanical	Reflex	in vitro	Desai et al 1991a
	Guinea-pig	Whole stomach	Mechanical	Electrical	in vitro	Desai et al 1991b
	Guinea-pig	Fundus	Mechanical	Electrical	in vitro	Lefebvre et al 1992a
	Rabbit	Corpus	Mechanical	Pharmacol	in vitro	Baccar et al 1992
	Rat	Fundus	Mechanical	Electrical/ Pharmacol	in vitro	Barbien and Lefebvre 1992
	Rat	Fundus	Mechanical	Electrical	in vitro	Li and Rand 1990
	Rat	Fundus	Mechanical	Electrical	in vivo	Lefebvre et al 1992b
Pylorus	Dog	CM	Mechanical	Electrical/ Pharmacol	in vitro	Allescher et al 1992

Table 1.6 continued on next page.

Table 1.6 continued. Summary of physiological studies on enteric NO function in different species and in various regions. CM = circular muscle, LM = longitudinal muscle, MM = muscularis mucosae. Most studies were performed on isolated preparations of the gut and carried out simultaneous mechanical and electrical recordings using force transducers and intracellular microelectrodes. There are fewer instances where electrophysiological recordings of junction potentials were carried out on isolated preparations. These findings are summarized in the text. From Brookes et al (1993).

REGION OF GUT	SPECIES	PREPARATION	RECORDING METHOD	STIMULATION TECHNIQUE	IN VITRO/ IN VIVO	REFERENCES
Small Intestine	Dog	LM	Mechanical	Electrical	In vitro	Toda et al 1991
	Dog	CM	Mechanical	Electrical/ Pharmacol	in vitro	Boeckxstaens et al 1991a
	Dog	CM	Electrophys/ Mechanical	Electrical	in vitro	Christinck et al 1991
	Guinea-pig	Whole wall	Mechanical	Reflex	in vitro	Costa et al 1991
	Guinea-pig	CM	Mechanical	Electrical	in vitro	Humphreys et al 1991
	Guinea-pig	CM	Electrophysiological	Electrical	in vitro	Bywater et al 1993
	Human	CM	Mechanical	Electrical	in vitro	Maggi et al 1991
Ileocolonic junction	Dog	CM	Mechanical	Electrical	In vitro	Boeckxstaens et al 1991b
Caecum	Guinea-pig	Intertaenial	Mechanical	Electrical	In vitro	Boeckxstaens et al 1991b
	Guinea-pig	Taenia	Mechanical	Electrical	In vitro	Ward et al 1992
	Guinea-pig	Intertaenia	Mechanical	Electrical	In vitro	Shuttleworth et al 1991
Colon	Dog	CM	Electrophysiological	Electrical	In vitro	Knudsen and Tottrup 1992
	Dog	CM	Electrophysiological	Electrical/Pharmacol	In vitro	Ward et al 1992b
	Guinea-pig	CM	Mechanical	Electrical	In vitro	Huizinga et al 1992
	Guinea-pig	LM	Mechanical	Electrical	In vitro	Humphreys et al 1991
	Rat	CM	Mechanical	Reflex	In vitro	Kojima et al 1992
	Rat	CM	Mechanical	Electrical	In vitro	Bult et al 1990
	Human	CM	Mechanical	Electrical	In vitro	Niklasson et al 1992
Sphincter of Oddi	Human	CM	Mechanical	Electrical	In vitro	Burleigh et al 1992
	Opossum	CM	Mechanical	Electrical	In vitro	Tottrup et al 1992
	Opossum	CM	Mechanical	Reflex/Pharmacol	In vitro	Rattan et al 1992

inhibitor from the active site of NO synthase). Exogenously applied NO mimics the responses to NANC inhibitory nerve stimulation, which include hyperpolarization of the membrane potential, and produces inhibitory junction potentials (IJPs) similar in amplitude to NANC induced IJPs. These responses, which are evoked following applied NO and NANC inhibitory nerve stimulation, are reduced by oxyhemoglobin which binds and inactivates NO. NANC nerve stimulation results in the release of NO (Boeckxstaens et al. 1991a; Boeckxstaens et al. 1991b).

Anatomical studies taken together with physiological and pharmacological findings as summarized in tables 1.5 and 1.6 respectively, have provided compelling evidence that NO is a transmitter of enteric NANC motor neurons. Moreover, these studies provide considerable experimental evidence that NO is involved in the control of gut motility in several regions through mediation of NANC relaxation responses, either alone or in combination with other cotransmitters such as VIP, as well as playing a role in modulation of spontaneous motility and peristalsis. Currently, the source of NO either from a neuronal origin exclusively, or from smooth muscle, or other cells is under critical debate. This has come about due in part to the studies of Grider et al (1992; 1994) on the action of NO on guinea-pig gastric smooth muscle and rat colonic smooth muscle. Electrical stimulation of smooth muscle strips elicited a relaxation response, caused the release of VIP and an increase in NO synthase activity. VIP evoked a relaxation response in isolated smooth muscle cells and also lead to an increase in NO synthase activity but inhibition of NO synthase only partially attenuated the motor response. Moreover, NO caused the release of VIP from isolated myenteric ganglia (Grider and Jin 1993). From these findings Grider and colleagues proposed that in vivo neurally-derived VIP stimulates NO synthase in smooth muscle cells to produce NO, which then in turn enhances neural release of VIP and amplifies the relaxant response. Amplification of relaxation of smooth muscle is due to the independent actions of VIP and NO on intracellular second messenger systems responsible for muscle relaxation (Jin et al 1993). NO stimulates a cGMP-dependent protein kinase G pathway and VIP stimulates a cAMP-dependent protein kinase A pathway that both lead to smooth muscle relaxation. From these studies it was evident that NO is not only a cotransmitter of NANC neurons but may also behave as a neuromodulator. Further evidence that NO could modulate transmitter release has come from electrophysiological assessment of the actions of exogenous NO on enteric AH/type 2 neurons which indicated that NO caused presynaptic suppression of noncholinergic slow EPSPs that occurred in these cells by suppressing the ongoing release of a slow excitatory synaptic mediator such as substance P (SP) (Tamura et al 1993). In vitro studies on isolated guinea-pig (Wiklund et

al 1993) and canine (Larysa et al 1994) ileum indicate that NO may also modulate pre- and postjunctional release of ACh.

1.11 Gamma - Aminobutyric acid

γ-aminobutyric acid (GABA) synthesis and degradation is effected by L-glutamate decarboxylase (GAD) and 4-aminobutyrate-2-ketoglutarate transaminase (GABA-T) respectively. The substrate for this biosynthetic pathway is glutamate. In addition GABA can be derived from other precursors (eg. ornithine, putrescine) and degraded via routes other than transamination. In fact there are at least five different metabolic pathways of GABA formation (Erdo et al. 1986). The major metabolic pathway in the CNS and ENS is the GAD-catalyzed reaction. GAD activity has been demonstrated in the guinea-pig and cat intestinal tract as well as the human colon (Miki et al. 1983; Erdo et al. 1986). The highest transmural level of GAD activity in the colon was found in the myenteric plexus (ibid).

Anatomical, pharmacological and physiological studies indicate that GABA is a transmitter of enteric interneurons in a variety of mammals where it is proposed to modulate motility. A widely used descriptive of this substance is the term 'GABAergic'.

GABAergic innervation of the mammalian gastrointestinal tract is extensive, comprising neuronal cell bodies and fiber networks in the myenteric and submucosal nerve layers as well as fiber innervation of the mucosa. The anatomy of this system has been investigated using autoradiographical (Krantis et al. 1981; Jessen et al. 1983; Krantis et al. 1986; Krantis et al. 1989; Krantis et al. 1991a; Krantis et al. 1991b) and immunohistochemical (Hills et al. 1987; Saito et al. 1987; Furness et al. 1989) approaches which collectively demonstrated emergent ganglionic GABAergic processes within primary, secondary and tertiary fasciculi of the rodent myenteric plexus and associated innervation of the enteric circular smooth muscle layer. In addition GABAergic neurons were localized to both myenteric and submucosal nerve layers displaying Dogiel Type I and II nerve cell morphologies. Within the myenteric plexus of the rodent intestine GABAergic varicose nerve fibers appear to innervate non-GABAergic myenteric ganglionic cells (Krantis et al. 1981; Jessen et al. 1983). Within the rat submucosa fine GABAergic fibers have been localized to Henle's and Meissner's nerve layers, the perivascular innervation and within the muscularis mucosae (Krantis et al. 1991a). GABA immunoreactivity has also been found in endocrine cells (Davenger et al. 1989; Krantis et al. 1993) suggesting an endocrine role for GABA. In addition a network of GABAergic nerve fibers have been localized

underlying the base of the mucosal crypts (Krantis et al. 1991b). These fibers could potentially contribute to the plexus of the villus core.

As in the CNS, GABA exerts its actions via $GABA_A$ receptors. These receptors are found on enteric motor nerves and display a pharmacology analogous to the $GABA_A$ receptors of the CNS (Johnston 1978; Tanaka 1985; Erdo et al. 1986). Recently, immunohistochemical demonstration of $GABA_A$ receptors was carried out in the rat intestine where they were localized to a subpopulation of myenteric and submucosal neurons (Krantis et al. 1994; Krantis et al. 1995). Applied GABA or $GABA_A$ receptor agonists (bicuculline, picrotoxin) stimulate enteric cholinergic excitatory and the NANC inhibitory motor neurons (Krantis et al. 1980; Krantis et al. 1981; Maggi et al. 1984; Krantis et al. 1987). These GABA mediated excitatory and inhibitory effects elicit contractile and relaxant responses respectively in enteric smooth muscle. $GABA_A$ receptor agonists and chloride channel blockers dose-dependently inhibit these responses (Krantis et al. 1981) indicating that they are elicited through stimulation of $GABA_A$ receptors and that this receptor is a ligand binding chloride channel like that of the CNS. Since electrical stimulation of cholinergic or NANC neurons could not be blocked by $GABA_A$ antagonists, GABAergic neurons appeared to be interneurons. Electrophysiological studies indicate that, unlike the brain where $GABA_A$ mediates hyperpolarization of neurons, enteric GABA mediates depolarization of AH/type II and S/type I myenteric neurons (Mayer et al. 1982; Cherubini et al. 1984). The chloride-dependent, bicuculline-sensitive, $GABA_A$ receptor-mediated depolarization of AH/type 2 cells resembles the depolarizing action of GABA on dorsal root ganglion cells (Erdo et al. 1986) and some enteric AH/type 2 cells are afferent neurons (ibid). Taken together, this shows that GABA is a transmitter of gut interneurons and the enteric 'A-GABAergic' system functions to modulate the contractile and relaxant phases of peristalsis.

Enteric GABA also elicits actions via a separate population of receptors, the $GABA_B$ receptor sites. This receptor was found to be insensitive to $GABA_A$ agonists and antagonists as well as chloride channel blockers but sensitive to ß-p-chlorophenyl GABA (baclofen) (Bowery et al. 1981). Physiological studies determined that $GABA_B$ receptor activation does not affect membrane conductance (Erdo et al. 1986) which indicated that this receptor is a metabotropic receptor rather than a ligand binding channel receptor. In the CNS this receptor appears to act via prejunctional potassium channels coupled to inhibitory G-proteins (Sivilotti et al. 1991). The pharmacological profile of the $GABA_B$ receptor indicates that it is involved in prejunctional inhibition of cholinergic neurons. In isolated preparations of guinea-pig ileum, applied baclofen inhibits both acetylcholine induced and electrically stimulated contractions

of the muscularis whilst having no effect on unstimulated preparations (Giotti et al. 1983; Ong et al. 1983; Kerr et al. 1986). Hence the 'B-GABAergic' system modulates the intensity of enteric smooth muscle contraction.

1.12 Neuropeptide Y

Neuropeptide Y (NPY) is part of the 'regulatory peptide' group, a term used to describe active peptides, discovered in the brain and/or gut, that function as neurotransmitters, circulating hormones or local regulators. These peptides usually belong to chemically related families. NPY belongs to the YY peptide family of 36 amino acid peptides containing terminal tyrosine (Y) residues and was originally isolated from porcine brain tissue (Tatemoto et al. 1982). In the gut, this family of peptides are found in nerves (referred to as NPY) and enterochromaffin cells (referred to as peptide YY, or PYY).

In the periphery, the richest source of NPY is found in the gastrointestinal tract (Lundberg et al. 1983). In the guinea-pig, rat and human intestine, there are NPY-positive intrinsic neurons within the myenteric and submucosal nerve layers as well as nerve fibers within the external muscle layers (Furness et al. 1983; Sundler et al. 1983; Hughes et al. 1987; Wattchow et al. 1988; Kawana et al. 1990; Costa et al. 1991a). In addition, NPY is proposed to be localized to the short anally projecting inhibitory motor neurons along with NO and VIP (see (Furness et al. 1992). They are distinct from the rich (extrinsic in origin) sympathetic NPY perivascular nerve fiber innervation of the submucosal microvessels (Lundberg et al. 1985) in which NPY coexists with noradrenaline (Gray et al. 1986). Fine varicose NPY nerve fibers have been found in the intestinal mucosa of guinea-pigs, rats, mice, cats, dogs, rabbits, and humans (see (Keast 1987) where they appear to form a network within the lamina propria and occasionally extend to the epithelium of the villi. Most of the mucosal NPY nerve fibers arise from submucosal ganglia (Keast et al. 1985) and the remaining smaller proportion come from myenteric ganglia (Furness et al. 1985). Following sympathectomy, the remaining intrinsic NPY neurons represent 5% of the entire neuron population of the myenteric plexus and 26% of the submucous ganglia neurons in the guinea-pig (Holst et al. 1989). A subpopulation of these NPY-positive intrinsic neurons have been shown to colocalize vasoactive intestinal popypeptide (VIP); in the myenteric plexus of the rat small intestine (Ekblad et al. 1989), cholecystokinin; in two subpopulations of cholinergic secretomotor neurons in the submucosa of the guinea-pig small intestine (Furness et al. 1987; Costa et al. 1991a), and somatostatin in submucosal neurons of the human colon (Hirose et al. 1989).

In the mammalian gut, one of the proposed actions of NPY is to modulate smooth muscle contractions by prejunctional inhibition of intrinsic cholinergic motor nerves (Hellström et al. 1985; Garzon et al. 1986; Holzer et al. 1987). NPY may also inhibit motility by a direct action on enteric smooth muscle (Hellström 1987). Fluid and electrolyte secretion from the small intestine are also inhibited by NPY (Friel et al. 1986; Cox et al. 1988) and this antisecretory effect results from direct actions on the mucosa cells. Finally, NPY released from the extrinsic sympathetic noradrenergic neurons is a potent inhibitor of intestinal blood flow (Hellström et al. 1985; Sheikh 1991).

Taken together these anatomical and functional characteristics form the basis of the proposal that NPY-containing intrinsic enteric neurons may be interneurons or motor neurons.

1.13 Summary

In order to reach an understanding of how particular gut behaviors result from the integration of all components of the ENS, research must eventually equate the disposition and neurochemical content of, and communication links between enteric neurons to the workings carried out by enteric neuronal circuits. The pursuit of this goal is aided by the fact that the ENS can be separated and studied in isolation from the rest of the nervous system since it contains all the necessary fundamental elements for autonomous regulation of integrative behavior. Functionally defined populations of enteric neurons are being classified based on their shapes and projections. The earliest reports proposing correlations between morphology and function including those of Dogiel, have since been shown to have a possible association by combining anatomical and physiological analyses.

Currently we are still in the phase where the physiology and circuitry of the ENS are still being deciphered. While chemical coding may yet provide a comprehensive picture of this system, new chemical substances continue to be identified and how these fit into the established scheme awaits consideration. Interestingly, some established enteric transmitters such as GABA are not yet included in the coding systems. Clearly much remains to be determined. Conceivably, when sufficient current information let alone new discoveries is integrated into neurochemical schemes of the ENS, then we well be better able to consider the ENS in the context of health and disease. Contributions to the classification of enteric neurons have been made by several groups, and considerable effort toward determining the neurons underlying distinct GI functions has been made in the guinea-pig small intestine. This has occurred because of the relative ease in isolating and analyzing the enteric nerve layers of the guinea-pig. Unfortunately, this classification system

does not appear to apply to other regions of the gut or even other species, particularly human. Since all GI functions are either controlled or modified by the ENS, neuropathology associated with this system may contribute to the development of many gut disorders. Therefore, a base classification system in the human intestine would be particularly useful in allowing us to identify which class of neurons might be involved in a given pathology. In order to do this, a complete description of the disposition and distribution of enteric substances in various species other than the guinea-pig, including man is required. This holds the prospect of more rational therapies for conditions such as those that occur due to abnormalities in gut motility.

1.14 General Aims

At the outset of the studies embodied in this thesis, NO, in addition to its neural actions in the CNS (Garthwaite et al. 1988; Garthwaite 1991), was proposed to be a putative neurotransmitter of enteric motor neurons. The synthesizing enzyme was isolated and immunolocalized to a subpopulation of myenteric ('NO producing') neurons and associated fiber innervation of the muscularis in the rat small intestine (Bredt et al. 1990b). Pharmacological studies indicated that NO could relax enteric smooth muscle in several species (Boeckxstaens et al. 1990; Bult 1990; Li et al. 1990; D'Amato et al. 1991; Dalziel et al. 1991; Du et al. 1991; Knudsen et al. 1991; Pelckmans et al. 1991; Tottrup et al. 1991; Boeckxstaens et al. 1991a; Desai et al. 1991a; Boeckxstaens et al. 1991b) and taken together with the scant anatomical findings, the data was consistent with a non-adrenergic non-cholinergic (NANC) inhibitory neuron distribution and function. In addition to its neural localization, NO was established to be an endothelium-derived relaxing factor effecting local vasodilation in the central- and cardio- vascular systems. However, little was known about the neural or vascular distribution and disposition of NO producing elements in the rat intestine or other species for that matter or even how this system is affected in the pathological gut. This formed the basis of the rationale behind the research embodied in this thesis. *The overall objective of my research was to investigate the enteric nitrergic system in health and disease associated with abnormalities in gut motility.*

The initial phase of my research involved the study of the nature and disposition of nitrergic elements in the gut wall from different species. A histochemical method had been developed to localize NO synthase activity (Hope et al. 1991) and in Chapter 2 I describe the modification and application of this NADPH diaphorase-dependent technique to dissected laminae of the guinea-pig and rat intestine. The guinea-pig enteric neural networks are the most investigated and best known of any species. Since the rat intestine is less well characterized yet

represents one of the most widely used animal models of human gut disease it is deserving of special attention. Therefore I undertook histochemical studies (published in consecutive papers, (Nichols et al. 1992; Nichols et al. 1993) that sought to provide a more complete analyses of the distribution and disposition of nitrergic neural elements within the layers of the guinea-pig and rat intestinal wall. In parallel to my analysis of the enteric nerve networks, I sought to also characterize NO synthase activity in enteric vasculature. The results presented in Chapters 2 and 5 provided the first anatomical characterization of the enteric vascular nitrergic system in the guinea-pig, rat and human intestine. These findings were presented in part within the Nichols et al 92 and 93 papers as well as in Nichols et al 94, where both rodent and human vasculature were examined and compared.

The interaction of GABAergic and nitrergic neural elements in the gut wall appears to be of physiological significance. GABA is an established neurotransmitter of enteric interneurons in a number of species, and NO is now accepted as a transmitter of at least one population of enteric motor neurons. In the rodent where I have shown an extensive enteric NO innervation, there is scope for NO to be a transmitter of other nerve types including interneurons. The aim of experiments described in Chapter 3 was to assess the nitrergic system in the human intestine with particular attention on the enteric vasculature and codistribution of the GABAergic system. The goal of the latter analysis was to use transmitter identification of enteric GABAergic interneurons to determine anatomically whether NO was a transmitter of this functional population of neurons in the human gut. Very little is known about the anatomy of the GABAergic system in the human intestine or the nature of this system with respect to the nitrergic system. The strong collaborative research efforts between the Children's Hospital of Eastern Ontario (CHEO) and the University of Ottawa, Digestive Disease Research Groups afforded an ideal circumstance for me to study human enteric innervation utilizing freshly resected gut segments from infants.

With respect to the GABAergic system, localization of GABA in neurons is problematic since there is a very rapid degradation of GABA by GABA-transaminase (GABA-T) following resection. For this reason, coincident examination of the GABAergic and nitrergic systems described in Chapter 3, was carried out in human colonic tissue using immunohistochemical demonstration of GABA-transaminase to identify GABAergic neurons and histochemical demonstration of NO synthase activity.

The information obtained from the studies presented in Chapter 3 served as a base of comparison for the potential changes in the anatomy of the nitrergic innervation in disease. Hirschsprungs's disease (HSCR) is one of the few enteric conditions where the neuropathology

has a neurological origin. The disorder partly arises from the lack of ganglion cells or 'aganglionosis' of variable lengths of the large bowel. As the NANC system is non functional in this disease, it seemed rational to look for putative neurological abnormalities in the nitrergic system by histochemical and biochemical examination of NO synthase activity in colonic resections obtained from Hirschsprung's patients and age-matched controls. Ultimately I wanted to determine if this disorder was associated with a neurochemical deficit of NO. The results of this study are described in Chapter 4.

My investigation of the rat nitrergic system formed the basis for a study of the involvement of the cysteamine-HCl (CSH) rat model for duodenal ulcer. CSH duodenal ulceration in the rat has been the most extensively used model to investigate pathogenic factors associated with this disease since this animal model most closely imitates the human condition. The aim of this research (described in Chapter 6) was to ascertain whether the nitrergic system is involved in the development of duodenal ulceration. Research by our group has shown that the enteric GABAergic system plays a role in the development of CSH induced duodenal ulcers. Pharmacological manipulation of the GABAergic system through the two separate populations of GABA receptors, the $GABA_A$- and $GABA_B$-receptor sites modulates CSH induced ulcerogenesis. Stimulation of $GABA_A$ receptors aggravates CSH induced duodenal ulceration while $GABA_B$ receptor stimulation with baclofen improves ulcer formation in this animal model. Since NO mediates the neural inhibitory effects of enteric GABA and modulates other contributing factors involved in duodenal ulceration, the involvement of NO in this process deserved to be studied. The pathogenesis of duodenal ulcer disease has its origins in abnormalities of gastroduodenal motility and blood flow. Hypoxia/ischemia-hyperemia/reperfusion events are associated with duodenal ulcer formation and NO mediates hyperemic/reperfusion responses. Taken together with the involvement of nitrergic elements in inhibitory motor activity of the gut, these facts suggest the potential role of NO in duodenal ulcer formation. Therefore I sought to test whether CSH induced duodenal ulceration is associated with changes in NO production and whether pharmacologic manipulation of the nitrergic system via inhibitors to the synthesizing enzyme NO synthase could modulate ulcerogenesis. In addition, I sought to determine whether combined pretreatment of CSH treated rats with baclofen and NO synthase inhibitors (preferentially affecting Ca^{2+}-dependent NO synthase activity) would be therapeutically advantageous.

CHAPTER 2

2.1 The Nitrergic Innervation of the Guinea-pig Intestine

Prior to this study, pharmacological evidence for applied NO to specifically relax the muscularis of the rat gastric fundus and ileocecal junction, canine small and large intestine (Boeckxstaens et al. 1990; D'Amato et al. 1991; Dalziel et al. 1991; Boeckxstaens et al. 1991a; Bredt et al. 1991a; Boeckxstaens et al. 1991b) guinea-pig stomach and ileum (Desai et al. 1991a; Humphreys et al 1991) and opossum esophageal circular muscle (Du et al. 1991; Tottrup et al. 1991) was rapidly emerging in the literature. This information led to the proposal that NO mediates non-adrenergic, non-cholinergic (NANC) inhibitory motor innervation of the mammalian gastrointestinal tract. Little was known however, about the distribution of NO in the gut wall. What proportion of myenteric nerves are NO producing? What is the pattern of innervation of these neurons? Since NO synthase was localized to myenteric type I motor neurons and innervation of the circular muscle in the rat duodenum (Bredt and Snyder 1990b) consistent with a NANC innervation, *we hypothesized that NO synthase would also be localized to type I neurons and associated innervation of the muscularis in the guinea-pig intestine.* Moreover, since a vasodilator role for NO was proposed to play a role in maintaining mucosal integrity in the gut (MacNaughton et al 1989), *we hypothesized that NO synthase would also be localized to vascular sites of the guinea-pig intestine.* These questions were addressed in this study where I modified a histochemical technique for localizing NO synthase-related diaphorase activity in rat brain (Hope et al. 1989; Hope et al. 1991) and applied it to stretch preparations of laminae dissected from the wall of the guinea-pig intestine.

The guinea-pig is the most investigated and best understood species with regards to enteric nervous innervation and function. This has come about in part by the ease with which the various nerve and muscle layers can be separated by fine dissection. The histochemical reaction employed in this investigation is based on the presence of an enzyme, known as a diaphorase, which can reduce a tetrazolium dye, nitro blue tetrazolium (NBT), in the presence of the cofactor, reduced nicotinamide adenine dinucleotide phosphate (NADPH), with the resultant product being a visible formazan precipitate. NO synthase activity fully accounts for the NADPH-dependent diaphorase activity identified by this histochemical reaction in fixed tissue. NADPH diaphorase activity has been observed in human kidney cells transfected with cDNA for NO synthase (Snyder et al. 1991; Dawson et al. 1991a; Bredt et al. 1991b). In addition, purified NO synthase exhibits NADPH diaphorase activity while its own activity is competitively inhibited by

NBT (Hope et al. 1991). Finally, NADPH diaphorase activity colocalizes not only with NO synthase immunoreactivity, but also with the mRNA for NO synthase both centrally and in the periphery; the latter including myenteric neurons (Dawson et al. 1991a; Bredt et al. 1991b).

Using this histochemical technique I localized NO synthase activity in the guinea-pig intestine, and present here evidence for NO synthase activity at specific sites in various enteric neural and vascular networks. In addition, the extent of the nitrergic innervation was determined by the estimation of NO synthesizing nerve cells in the total population of myenteric plexus neurons. A portion of the findings of this study were presented at a Canadian Physiological Society (CPS) Meeting in 1991. A complete description of the findings, as described in this section, were published in 1992 (Nichols et al. 1992).

The aims of this study were to:

(1) examine the nitrergic innervation in the guinea-pig intestine. In particular, report on the morphologies and apparent projections of NO synthesizing neurons in the different nerve layers.

(2) assess the distribution of NO synthesis potential in the gut microvessels. Since NO is an EDRF-like factor it must be localized to enteric vasculature.

2.1.1 Materials and Method

Male guinea-pigs (Charles River, Quebec) weighing 300-350g were housed in wire-topped plastic cages and provided with standard chow and water *ad libitum*. The guinea-pigs were given at least a week to adjust to the housing conditions (room temperature: 22^{0}C; photoperiod: 12 hr light and 12 hr dark). Experimental procedures were carried out following the guidelines set by the Canadian Council on Animal Care.

Guinea-pigs were cervically dislocated and given an abdominal midline incision and all intestinal segments were removed. Duodenal segments were taken 2 cm distal of the pyloric sphincter, jejunal segments 5 cm distal to the ligament of Treitz, ileal segments beginning 2 cm proximal to the ileocecal junction and colonic segments from 2 cm distal to the caecum. Segments were carefully cleared of contents and immediately washed in pre-chilled 0.1M sodium phosphate buffered saline (PBS), pH 7.2. Individual intestinal segments 2.5 to 4.0 cm in length were cut along the mesenteric border, opened and pinned (mucosa up) to styrofoam. Gut segments were then subjected to a 2hr incubation period at 4^{o}C in 4% paraformaldehyde/0.4% picric acid in 0.16M sodium phosphate buffer (PB), pH 7.0. The segments were then rinsed three times in pre-chilled 0.1M PBS, pH 7.2 and stored at 4^{o}C in fresh PBS.

Dissection and isolation of the different layers (laminae) of the intestinal wall (fig. 2.1A) was carried out using a modification of the method described by Furness and Costa (1980). Individual segments were removed from the styrofoam blocks, stretched and pinned to the base of a 0.1M PBS-filled container (pH 7.2) with serosal side facing up. To obtain laminar preparations of the myenteric plexus (fig. 2.1B), a superficial incision was made along one end of the tissue segment, sufficient to allow the serosa, longitudinal muscle and myenteric plexus to be peeled away together using fine forceps. This laminar preparation was then positioned with the myenteric plexus uppermost and any adherent circular muscle stripped away.

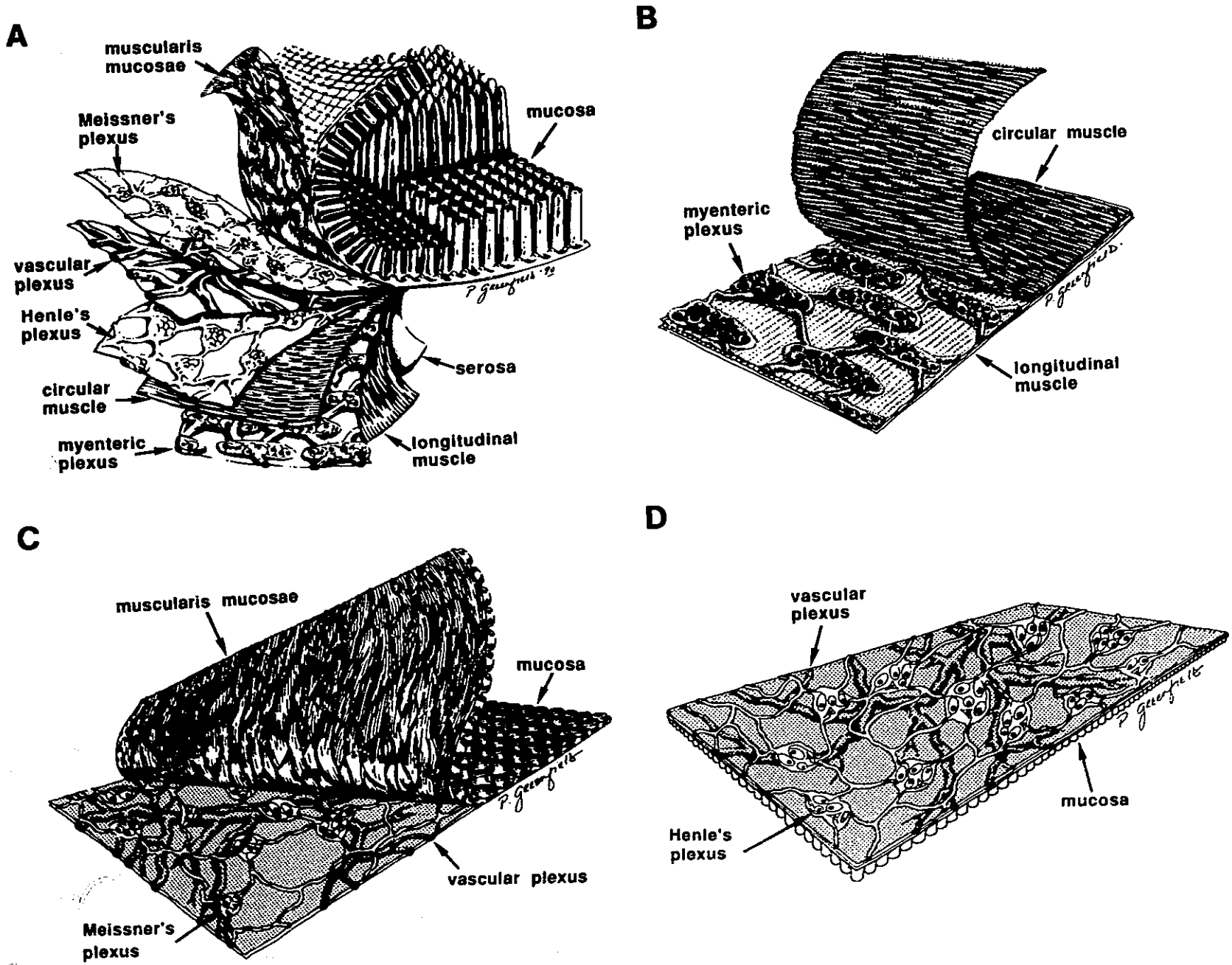
The remaining tissue segment was then further dissected to expose the submucosal layer, by first stripping away the circular muscle, then repositioning the tissue with the mucosal layer uppermost for blunt dissection of the mucosa. These laminar preparations of the (i) myenteric plexus and (ii) submucosal layer (mucosal side down) (fig. 2.1D) were then stretched onto electrostatic glass slides, allowing the outer edges of the preparations to air dry and adhere.

To enhance penetration of the histochemical staining medium an additional step was added. The air dried preparations were subjected to an overnight incubation at 4^{o}C in 3% Triton x-100 0.1M PBS pH 7.2 .

Laminar preparations were then treated for NO synthase histochemistry after the method of Hope and Vincent (1989). After washing in several changes of sodium phosphate buffer (10mM PB, pH 8.0), laminar preparations were incubated in a darkened, moist chamber for 1hr at 37^{o}C with reaction medium for NADPH diaphorase activity consisting of 1mM NADPH, 0.5 mM nitro blue tetrazolium (NBT), and 0.3% Triton x-100 in 10mM sodium phosphate buffer, pH 8.0. Control preparations were treated in the same way but without NADPH in the reaction medium. To ensure that the diaphorase activity observed was not due to DT diaphorase a second control procedure was carried out by testing the resistance to 0.1 mM dicumarol placed in the reaction medium. Tissues were given a final wash with PB (10mM, pH 8.0) air dried, coverslipped under permount and analyzed by light microscopy.

The mean frequency of NADPH diaphorase positive labeled cells per ganglion was estimated from a sample population of 100 myenteric ganglia for each region taken from six animals. Ganglia were chosen at random with specific exclusion criteria. Only ganglia from which intact primary fasciculi could be discerned were included. Ganglia at the edges of the tissue were automatically excluded since their primary strands were usually not intact. Extraganglionic cells were not counted. Although NADPH diaphorase positive cells in a particular ganglion displayed different staining intensities all were counted as a single group. Separate laminar

Figure 2.1. Schematic diagrams of the rat intestinal wall to illustrate the preparation of intestinal laminae from both the guinea-pig and rat intestine. (A) Illustration of the intestinal wall with the laminae partially dissected to display the organization of the neural and vascular elements. Note that the guinea-pig intestine does not possess Meissner's nerve layer. B and D depict the laminar preparations taken from the guinea-pig intestine whilst B, C and D display those preparations taken from the rat and human intestine. (B) A laminar preparation of the myenteric plexus with the circular muscle peeled back. (C) A laminar preparation 'mucosa up' showing the muscularis mucosae and mucosa partly separated to reveal Meissner's plexus and the underlying nerve networks. (D) A laminar preparation oriented 'mucosa down' with Henle's plexus uppermost. See text for description.


A
muscularis mucosae
Meissner's plexus
mucosa
vascular plexus
Henle's plexus
serosa
circular muscle
myenteric plexus
longitudinal muscle
B
circular muscle
myenteric plexus
longitudinal muscle
C
muscularis mucosae
mucosa
vascular plexus
Meissner's plexus
D
vascular plexus
mucosa
Henle's plexus

preparations taken from all regions of three animals were stained with standard Giemsa, a Nissl stain (cytoplasmic stain) which allowed visualization and estimation of the total number of ganglion nerve cells. The same criteria were applied to the randomly chosen ganglia of this experimental set.

2.1.2 Chemicals

Nitro blue tetrazolium (NBT) and ß-nicotinamide adenine dinucleotide phosphate, reduced form (NADPH) were purchased from Sigma, St. Louis, Missouri. The remaining reagents were purchased from BDH.

2.1.3 *Results* - Myenteric plexus

For publication purposes figures 2.2 to 2.4 were labeled from 1 to 13 rather then using an alphabetic format.

In laminar preparations of the myenteric plexus, intensely stained cells were present within *or* localized to ganglia (figs. 2.2-1 to 4 and 2.3-5 and 6) and occasionally found in the interconnecting fibre bundles (fasciculi) of the primary and secondary meshwork of this plexus (fig. 2.3-7). The relative numbers of stained cells/ganglia are shown in table 2.1. The density distribution of stained ganglion cells was not constant along the length of the intestine. Rather, the lowest proportion of cells/ganglion occurred in the duodenum and higher but similar proportions occurred in the jejunum, ileum and colon.

The cytoplasm of the NADPH diaphorase-positive cells was completely labeled. In addition, reaction product was visible within very long, fine processes enabling description of both the morphology of labeled cells and their projections. Labeled ganglionic cells displayed either weak or intense staining. The morphological types according to the classification by Stach (1989) of the weakly stained cells were difficult to discern. However, intensely stained cells encompassed a number of morphological types including types I and II (figs. 2.2-2 to 4 and 2.3-5 and 6), and type VI (fig. 2.3-5). Of these, diaphorase-positive neurons corresponding to type II were most common. In addition, the longest emergent processes were always associated with type II cells. The perikarya of these cells were usually located at the poles of the ganglia with the large emergent process projecting out into the interconnecting fasciculi. These processes could often be traced considerable distances coursing through up to 4 ganglia of the primary meshwork (Ps) on into the fine tertiary meshwork (Pt) that innervates the circular muscle and submucosa (figs. 2.2-1 and 2.3-7). There was no pattern to the projection of labeled fibres in the myenteric

meshworks, projecting as they did, circumferentially radially, aborally and orally. In many instances these labeled fibres were lost from view before any termination could be seen, suggesting that many of the labeled fibres projected considerably further than could be discerned here. Most of the labeled fibres within a ganglion appeared to originate from outside the ganglion some of which bore varicosities en passant. Others ramified within the ganglion around clearly unlabeled cells.

TABLE 2.1. Proportion of NO Synthesizing Cells Per Ganglion Estimated from Counts of NADPH Diaphorase Positive and Total Cells Per Ganglion in Various Regions of the Guinea-Pig Intestine.

	DUODENUM	JEJUNUM	ILEUM	COLON
NO CELLS/GANGLION	5 ± 1	8 ± 2	10 ± 2	9 ± 1
TOTAL CELLS/GANGLION	90 ± 4	86 ± 4	90 ± 5	108 ± 4
% NO CELLS/GANGLION	6	9	11	8

NOTE: The total number of cells per ganglion represents the mean of cell counts for 100 ganglia per region of tissue examined with the nissl stain Giemsa from 3 animals. The number of NO synthase-reactive cells per ganglion are presented as the mean ± SEM of counts from 100 ganglia per region from 6 animals. The proportion of NO synthase-reactive cells is presented as percentage of NO cells per total cells per ganglion.

Intensely stained extraganglionic cells (Schofield 1989) were rarely encountered (fig. 2.3-8) and when found were small and round (approximately 20 μm in diameter). The primary meshwork fasciculi contained many stained fibres and therefore it was difficult to distinguish whether these extraganglionic cells, gave off positive processes.

In parts of the tissue where the circular muscle remained attached to the myenteric plexus, intensely stained fibres could be seen coursing amongst the muscle cells (fig. 2.4-13). This pattern is characteristic of the meshwork of nerve fibres that ramify in the deep muscular nerve plexus within the muscularis (Gabella 1979; Furness et al. 1987). In regions of the tissue preparation where the circular muscle and myenteric plexus were stripped away leaving only the longitudinal muscle layer, only a diffuse non specific staining was evident.

Figure 2.2 (1-4): Light microscopic micrographs of laminae from the guinea-pig intestine treated for histochemical localization of NO synthase-related NADPH diaphorase activity. (1) Laminae of the myenteri plexus from the proximal. Intensely stained nerve cells and their processes are present in their ganglia (G). Intensely stained fibres (small arrowheads) can be seen within the ganglia and interconnecting primary (Pp) and secondary (Ps) meshworks of this nerve network. (2 and 3) Myenteric ganglia (G) from the proximal colon showing intensely stained nerve cells and their processes typical of type I and II cells. (4) High magnification micrograph of a myenteric ganglion from the proximal colon, showing an intensely stained type I cell. Scale bar = 100μm.

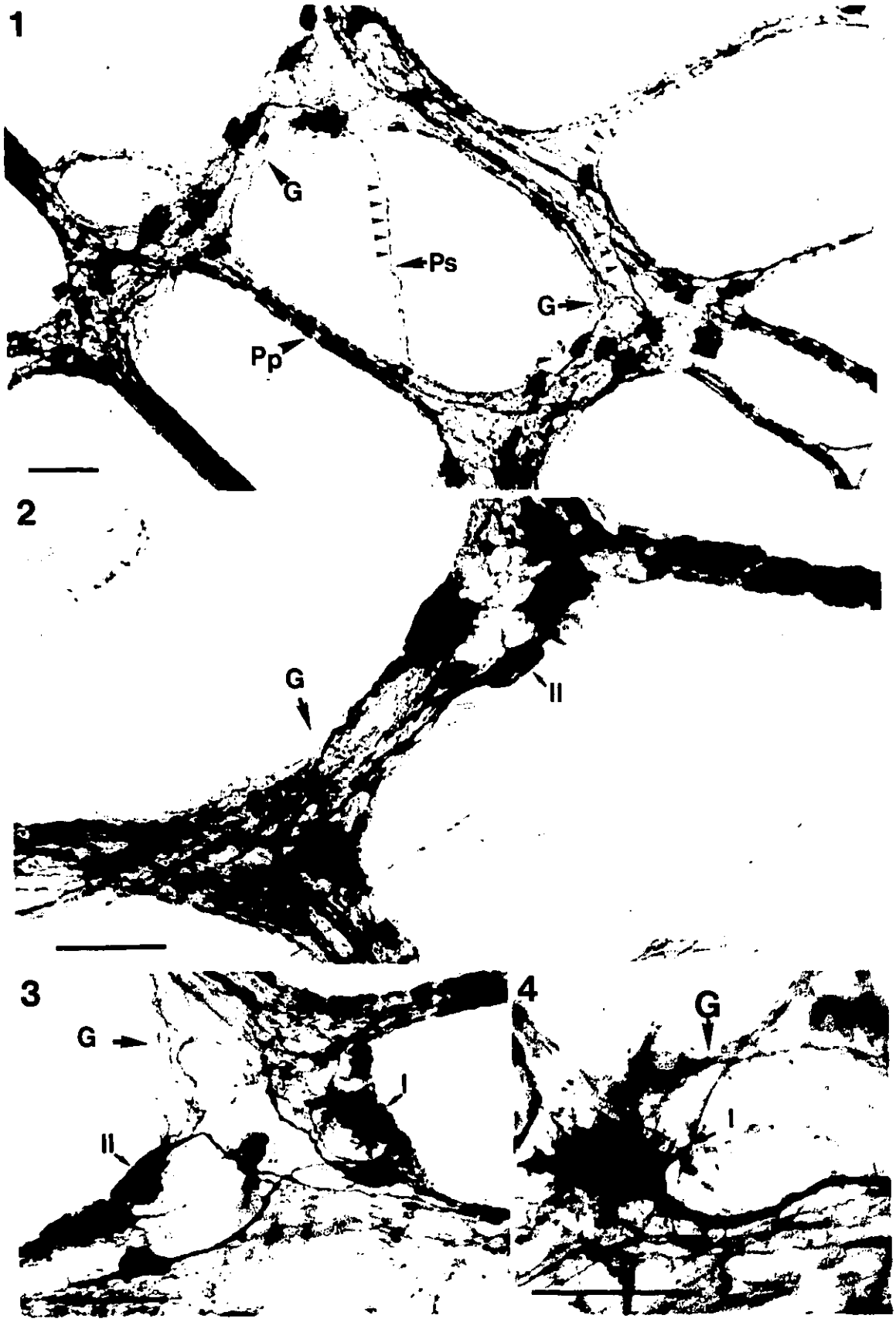
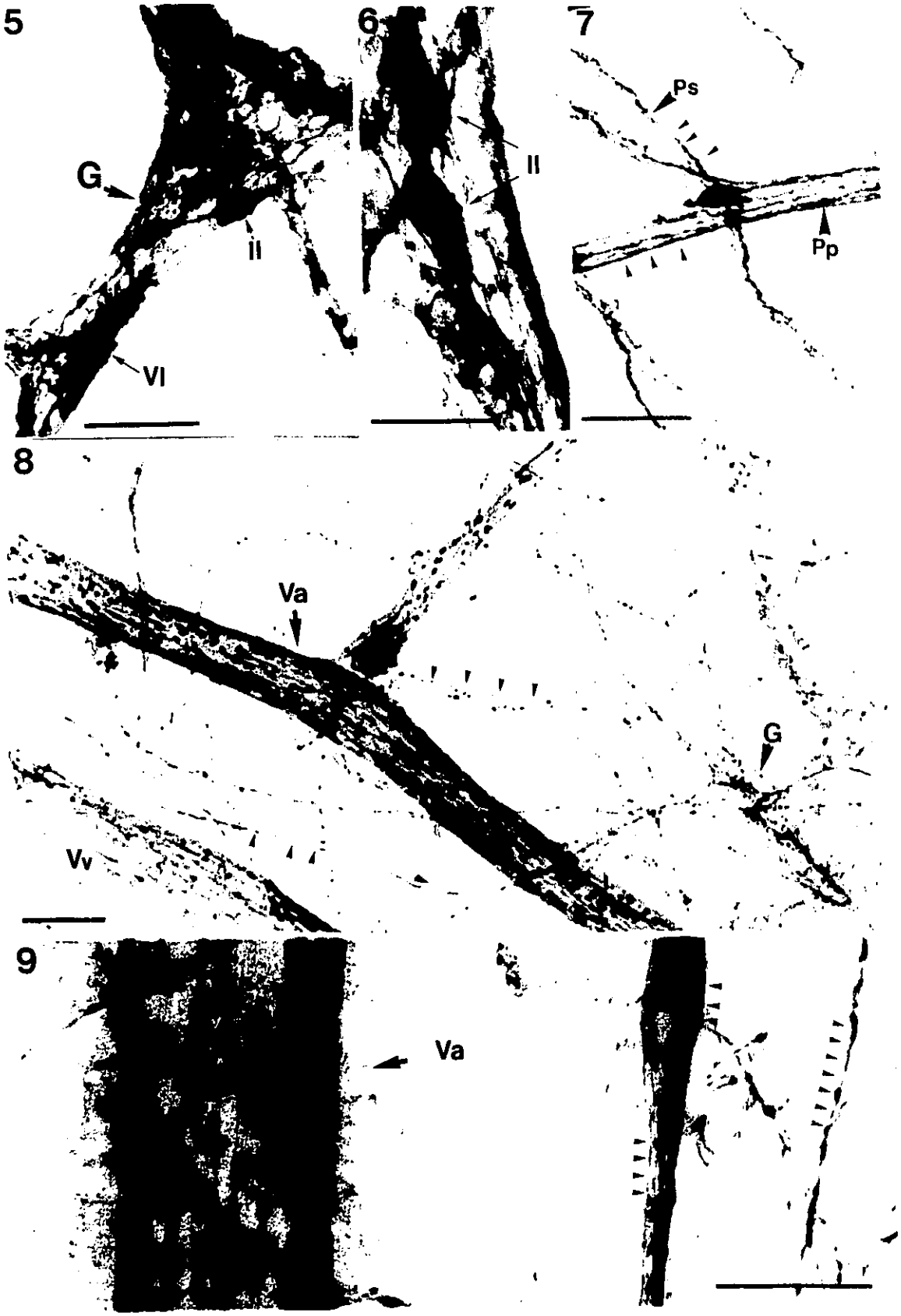

1
G
Ps
G
Pp
2
G
II
3
G
I
II
4
G
I

Figure 2.3 (5-9): (5) High magnification micrograph of a myenteric ganglion from the proximal colon, showing intensely stained type II and VI cells. (6) High magnification micrograph of a myenteric ganglion from the distal ileum, showing two type II cells. (7) Micrograph of the interconnected meshworks of the myenteric plexus from the distal ileum, showing an intensely stained extraganglionic cell in the primary (Pp) meshwork. (8) Submucosal laminae from the proximal jejunum. Intensely stained varicose fibres (small arrowheads) can be seen coursing throughout the nerve bundles of Henle's plexus. Fibres are efident in a ganglion (G), ramifying around clearly unlabeled cells. Arterioles (Va), and venules (Vv), of the underlying vascular network contain intensely stained punctate deposits over their surface. (9) High magnification of the vascular network and Henle's plexus from the distal ileum. The distinct pattern of labeling of nerve fibres (small arrowheads) vs blood vessels is evident. Scale bar = 100 μm


5
G
II
VI
6
II
7
Ps
Pp
8
Va
G
Vv
9
Va

2.1.4 *Results* - Submucosa

Henle's plexus displayed a profuse network of intensely stained varicose fibres within individual ganglia and throughout the interconnecting fasciculi. This fibre network appeared to be more profuse than in the myenteric plexus and single stained fibres were distinctly varicose in appearance (fig.2.4-9 to 11). These fibres could often be traced long distances (up to 800 μm). Within the ganglia, stained varicose fibres could be seen to ramify around clearly unlabeled cells. Few ganglia contained labeled cells. The maximum number of labeled cells/ganglia was found to be 2. These cells could be easily identified as type II cells (fig. 2.4-10 and 11). In preparations where the circular muscle was not completely stripped away, labeled elements of the deep muscular plexus could be seen, (fig. 2.4-13) as well.

2.1.5 *Results* - Blood vessels

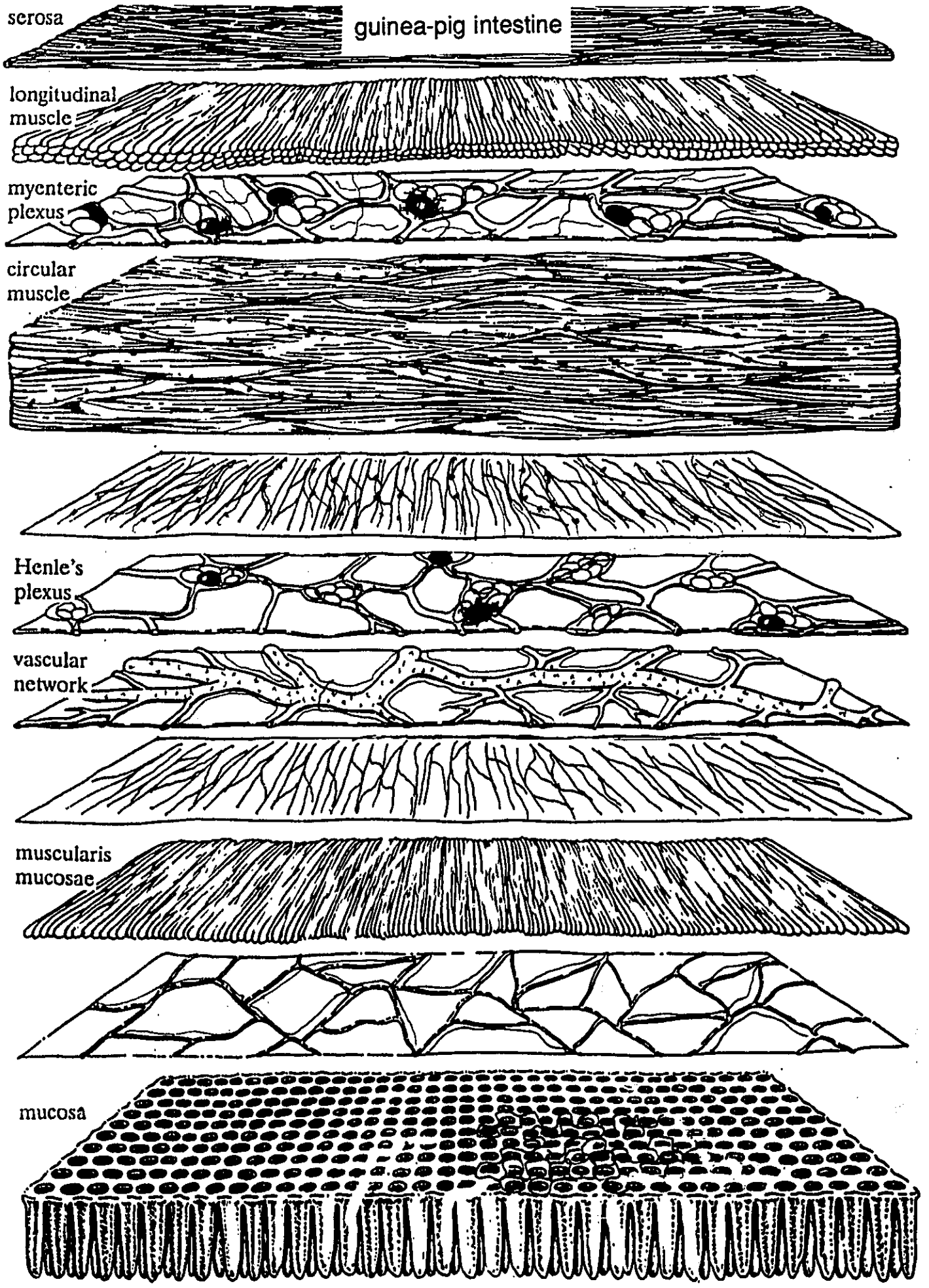
Underlying the Henle's plexus an extensive vascular network is visible (figs. 2.3-8 and 2.4-12). The blood vessels and associated paravascular nerve bundles were in close contact with and often connected to fibres of Henle's plexus. Arterioles (Va), easily distinguished by their smaller diameter and characteristic striated appearance displayed an extensive labeling over their surface, (figs. 8, 9, 12 and 13). The punctate labeling of the arterioles was comprised of uniformly large puncta distributed relatively evenly over the visible surface of the blood vessels (fig. 2.3-8 and 9). These puncta appeared to represent patches of NO synthase activity within the endothelial cells rather than NO synthase activity on approximated neurites. The venules (Vv) were not labeled and there was no obvious perivascular labeling.

Figure 2.5 provides a summary of the overall distribution and disposition of NO synthesizing neural elements and vascular sites in the guinea-pig intestine.

Figure 2.4 (10-13): (10) Henle's plexus from the distal ileum, showing two labeled ganglion cells and their processes. Intensely stained fibres can be seen coursing within the ganglion (G) and in the interconnecting nerve bundles (small arrowheads) of this plexus. (11) A ganglion (G) from Henle's plexus of the dital ileum, showing an intensely stained type II cell. (12) High magnification micrograph of the nerve and vascular networks of the proximal duodenum submucosa. Intensely stained varicose fibres can be seen coursing through a ganglion (G) and in the nerve bundles of Henle's plexus. The underlying arterioles (Va) display intense punctate labeling. (13) A lamina from the submucosa of the proximal jejunum. Intensely stained fibres (small arrowheads) can be seen coursing in the plane of the circular muscle layer (cm) still adherent to the underlying submucosal laminae. Submucosal nerve and vascular networks can be seen beneath the muscle layer. Scale bar = 100 μm.


10
G
11
II
G
12
G
Vv
Va
cm
Va

Figure 2.5. A schematic illustration of the gut wall with the component layers separated provides a summary of the observed neural and vascular elements stained (blue) with NADPH diaphorase in the guinea-pig intestine. Myenteric ganglion cells of type I, II and VI morphologies stained at varying intensities. A dense innervation of labeled varicose nerve fibres was observed in the circular muscle layer. Between the circular muscle layer and Henle's plexus, a fine meshwork of labeled fibers was also observed, reminiscent of the circular muscle submucosal interface plexus. Fine submucosal vessels displayed punctate patches of NADPH diaphorase activity and paravascular nerve fibers coursing along side these vessels were also labeled. In laminar preparations with mucosa facing up with remnants of mucosa remaining displayed a fine meshwork of fibres could be seen both at the level of the muscularis mucosae and at the base of the mucosal crypts (villi).


guinea-pig intestine
serosa
longitudinal muscle
myenteric plexus
circular muscle
Henle's plexus
vascular network
muscularis mucosae
mucosa
P.S. '91

2.1.6 Discussion

Nerve and vascular networks, and smooth muscle layers of the gut wall could be easily identified in laminar stretch preparations of the guinea-pig small and large intestine treated for histochemical demonstration of reduced nicotinamide adenine dinucleotide phosphate (NADPH) diaphorase activity under conditions specific for NO synthase-related activity. No staining of gut tissue occurred in the absence of NADPH and all the staining features described below were resistant to inhibition with dicumarol. DT diaphorase, also capable of reducing tetrazolium dyes to formazan precipitates in the presence of NADPH or NADH, is selectively inhibited by dicumarol (Hope et al. 1991). Therefore DT diaphorase does not account for the diaphorase activity exhibited here.

As understood at present, there are two forms of NO synthase; one (the constitutive form) which is found primarily in neurons and vascular endothelial cells producing NO for intercellular signaling and a second (inducible) form of NO synthase which is found in non-neural cells including macrophages and is used to synthesize NO for use as a toxin in cell mediated immune responses. The activity of the constitutive form of NO synthase generally accounts for the NADPH diaphorase reactivity of the histochemical procedure employed in this investigation (Hope et al. 1991; Bredt et al. 1991a; Dawson et al. 1991a; Bredt et al. 1991b). NADPH diaphorase activity has been reported in murine enteric neurons at certain stages of development (Branchek et al. 1989) and in rat myenteric neurons colocalized with NO synthase immunoreactivity (Dawson et al. 1991a). However, the results of this study show for the first time, the localization and distribution of NO synthase activity in different laminae of the guinea-pig intestine. Moreover, the discrete staining of enteric sites by this method allowed for morphological identification of elements staining positive for NO synthase activity. NO synthase-related diaphorase activity was found in both myenteric and submucous neurons and their emergent processes.

The frequency of labeled cells seen in this study, indicates that in any region of the guinea-pig intestine, less than 10% of myenteric nerve cells have the capacity to produce NO. This proportion is less than that reported by Furness et al (1994). Several factors may account for this difference. The NADPH-dependent diaphorase activity associated with NO synthase is proposed to be fixation-dependent in that the concentration and type of fixative used may affect the sensitivity for detection of the enzyme (Matsumoto et al 1993; Spessert and Layes 1994). According to Matsumoto et al (1993), the use of buffered paraformaldehyde at a concentration of

4% provides optimum results whereas lower concentrations may reveal non-NO synthase-related NADPH diaphorase activity. In this regard, Furness et al (1994) used 2% paraformaldehyde, but prior to this report used 4% paraformaldehyde to colocalize NADPH diaphorase activity and NO synthase immunoreactivity in the guinea-pig intestine (compare Young et al 1993 and Furness et al 1994). The reason for the change in fixation conditions from one study to the next was not discussed. However this may account for the greater estimation of diaphorase-positive cells by Furness et al (1994). In this work, and for the study by Furness et al (1994) counts for NADPH diaphorase positive cells were taken from ganglia that were selected at random. Since ganglia are variable in size containing between 5 and 200 cells, counting mainly large ganglia or conversely failure to adequately detect smaller ganglia would account for the difference in calculation of proportions. It is interesting that estimation of total cell counts in this study and by Young et al (1993) are similar and cannot account for the difference in the estimated proportion of NADPH diaphorase positive cells.

Three of the eight morphologically classified myenteric nerve cells were found to label intensely under the treatment conditions employed, suggesting that NO synthase is widely distributed in the myenteric plexus of the guinea-pig intestine. Our findings of NADPH diaphorase activity in Dogiel type II cells was not supported by the study of Furness et al (1994). Neurons with Dogiel type II morphology account for approximately one-third of all guinea-pig myenteric neurons and are proposed to have a sensory function (Pompolo et al. 1989; Costa et al. 1991a). In the present study cell types displaying NO synthase activity and Dogiel type II morphology appeared to be more numerous in the myenteric and submucosal plexi. Interestingly, Pompolo et al (1989) report that about 85% of Dogiel type II neurons have been shown to contain calcium-binding proteins (calbindins). This suggests that a major proportion of type II neurons have a potentially large capacity to buffer Ca^{2+}. We might speculate therefore that these same neurons could easily regulate Ca^{2+}/calmodulin mediated activation of NO synthase. If this were so, the findings of the present work where type II neurons stained with NADPH diaphorase activity indicates that NO may play a role in mediating sensory information in the gut. The possibility that NO may be a transmitter/modulator of enteric sensory neurons is supported indirectly by the findings that NADPH diaphorase positive cells have been localized to rodent (Aimi et al 1992) and feline (Vizzard et al 1994) lumbar and sacral dorsal root ganglia. In addition, electrophysiological assessment of the actions of exogenous NO on enteric AH/type 2 neurons indicates that NO presynaptically suppresses noncholinergic slow EPSPs that occurred in

these cells by suppressing the ongoing release of a slow excitatory synaptic mediator such as substance P (SP) (Tamura et al 1993).

In vitro and *in vivo* pharmacological studies in different species, show NO to be a powerful relaxant of gastrointestinal smooth muscle (Boeckxstaens et al. 1990; Bult 1990; Toda et al. 1990a; Toda et al. 1990b; D'Amato et al. 1991; Dalziel et al. 1991; Du et al. 1991; Knudsen et al. 1991; Tottrup et al. 1991; Boeckxstaens et al. 1991a; Desai et al. 1991a; Boeckxstaens et al. 1991b; Humphreys et al 1991) and stimulation of intestinal nerves liberate NO (Boeckxstaens et al. 1991a). These electrical stimulations induced relaxations of the muscularis that could be blocked by inhibitors of NO synthesis. Taken together, this data is strong evidence for NO to be a mediator of NANC inhibitory motor transmission. However, the identity of the neurotransmitter(s) mediating the inhibitory motor innervation of the gut wall is controversial (Westfall et al. 1982; Matusak et al. 1986). Moreover, Costa et al (1986b) provide evidence for at least two different NANC enteric inhibitory motor nerves. ATP and VIP have been proposed as candidate NANC inhibitory transmitters (Burnstock 1972; Furness et al. 1980; Furness et al. 1987; Bult 1990) and these substances have also been localized to type I, II and III cells of the rodent intestine (Furness et al. 1980). Subsequent to this study nitrergic type I myenteric motor neurons of the guinea-pig intestine were found to contain VIP, galanin and calretinin. These neurons project anally for up to 8mm and are referred to as the long descending inhibitory motor neurons (Costa et al 1992) that mediate the descending inhibitory reflex pathways of the intestine. Whether the NO-positive type I neurons identified in this study represent NANC inhibitory neurons already described, or another subset of NANC inhibitory neurons remains to be determined.

Only a small number of submucosal nerve cells localized in ganglia of Henle's plexus displayed NO synthase activity. However, a conspicuous feature of the submucosal preparations was the extensive labeling of varicose fibres within Henle's plexus. The ratio of labeled fibres to ganglion cells was high compared to the myenteric plexus. The submucosal nerves are integrated with the myenteric plexus and modulated by innervations from extrinsic autonomic ganglia (Furness et al. 1987). As such, the submucosa normally contains an extensive and complex array of nerve fibres of diverse origins. It is possible that a proportion of the nerve fibre ramifications that were shown to display NO synthase-related diaphorase activity in the submucosa are sensory afferents since a large population of diaphorase positive neurons and fibers have been found in the sensory ganglia of the rat (Aimi et al. 1991).

A prominent feature of the actions of NO in mammals is its effect on vascular smooth muscle (for review, see Long et al. 1989). NO is a powerful vasodilator of central and peripheral blood vessels in a variety of species with similar actions and properties to EDRF (Long et al. 1989; Vallance et al. 1989). This study found extensive NO synthase activity in the submucosal arterioles. The labeling of blood vessels was similar in all regions of the small and large intestine indicative of a homogenous distribution to the vasculature. The NO related labeling of the enteric vasculature, bore none of the characteristics of a perivascular distribution of NO-positive nerve fibres. This suggests there are no NO vasodilator nerves present, and therefore no direct NO-related neural modulation of vasomotor activity in the guinea-pig submucosa. Whether this NO-related labeling is associated with the endothelium or vascular smooth muscle cannot be resolved here. *However, the presence of NO synthesizing sites on enteric blood vessels may represent some intrinsic role for NO as a mediator of neurohumoral control of blood flow, similar to its EDRF-like actions at cerebral blood vessels.*

The ability of specific enteric neurons to produce and release NO together with the ability of this molecule to relax gastrointestinal smooth muscle is highly suggestive of a transmitter/modulator role for enteric NO. These results show NO synthase-related diaphorase labeling of a spectrum of enteric nerve types along the length of the intestine. This indicates that NO may be released at multiple gastrointestinal sites similar to a variety of putative enteric transmitters. Although this myenteric and submucosal nerve-related NO synthase labeling displayed similar histochemical sensitivity to that associated with the submucosal arterioles, the pattern of labeling suggests they are not functionally linked.

2.2 The Nitrergic Innervation of the Rat Intestine

Bredt and Snyder (Bredt et al. 1990a) were the first to discover NO synthase in enteric neurons using immunohistochemical analysis on tissue sections of the rat intestine. This study displayed NO synthase to be localized to a subpopulation of neurons of the myenteric plexus and to the innervation of the circular muscle layer. Subsequently, the histochemical identification of NO synthase activity in laminar preparations of the rat intestine was reported (Aimi et al. 1993). The type of neurons involved and their disposition within all the neural networks of the gut wall as well as the enteric vascular localization of NO synthase were not defined in these studies. In addition, during and subsequent to the investigation of the nitrergic innervation in the guinea-pig intestine, pharmacological evidence for NO to be an inhibitory

transmitter of NANC inhibitory motor neurons in the gut of several species was accumulating. Separate experiments in Dr. Krantis's laboratory provided initial pharmacological evidence for NO to be an inhibitory transmitter of interneurons in the rat intestine. Thus the next aim of the study was to provide a complete stuctural analysis of the nitrergic innervation in the rat intestine. Aspects of this work were awarded a student research prize and presented to a forum at an American Gastroenterology Association (AGA) Meeting in 1992. A more detailed report was subsequently published in 1993 (Nichols et al. 1993). The specific aims of this study were to: (1) assess the distribution and disposition of histochemically identified NO synthase reactive neural and vascular elements in the rat intestine. Since NO synthase was localized to myenteric type I motor neurons and innervation of the circular muscle in the rat duodenum (Bredt and Snyder 1990b) and all regions of the guinea-pig intestine (section 2.1) consistent with a NANC innervation, *we hypothesized that NO synthase would also be localized to type I neurons and associated innervation of the muscularis in other regions of the rat intestine. We also hypothesized that NO synthase would be localized to vascular sites of the rat intestine as viewed in the guinea-pig intestine*. Since an idea of the localization of enteric nitrergic elements was obtained in the guinea-pig a similar investigation could be easily carried out in the rat intestine; and (2) compare and contrast the findings in the rat intestine to those in the guinea-pig intestine.

2.2.1 Materials and Methods

Male Sprague-Dawley rats (Charles River, Quebec) weighing 250-350g were housed under the same conditions as the guinea-pigs as described in section 2.1.1. Experimental protocol for this study complied with the guidelines of the Canadian Council on Animal Care.

Segments (2-4 cm in length) of the small and large intestine were removed from freshly decapitated rats in the same manner as was done in section 2.1.1 and placed immediately in pre-chilled 0.1M sodium phosphate buffered saline, pH 7.2 (PBS) to clean the tissue of surface blood and debris. Preparation and fixation of the tissue segments were identical to that described in section 2.1.1.

Laminar preparations from the myenteric plexus and submucosa were prepared by fine dissection of fixed segments of the small and large intestine (fig. 2.1A) as described in section 2.1 (fig. 2.1) with modifications including the isolation of Meissner's plexus (not found in the guinea-pig). For this reason a full description of the isolation of the nerve layers is described herein. All dissections were carried out in a PBS-filled container at room temperature

The serosa, longitudinal smooth muscle layer and myenteric plexus were peeled back together using fine forceps, from a superficial incision made along one end of the tissue segment. The tissue was then pinned out with the myenteric plexus uppermost, and any adhering circular smooth muscle stripped away (fig. 2.1B). In this orientation, the preparation was placed onto electrostatic glass slides, and gently stretched while allowing the edges to dry and adhere. The remaining portion of the tissue segment wall consisted of some remaining circular smooth muscle, the submucosa, muscularis mucosae and mucosa. Following the removal of any remnants of circular smooth muscle from the remaining portion of the segment wall, the tissue was reoriented so that the mucosa was facing uppermost. The overlying muscularis mucosae and mucosa were peeled away using fine forceps, and this orientation revealed the aspect of the submucous layer closest to the mucosa. Submucosal preparations included; a) mucosal side up revealing Meissner's plexus (fig. 2.1C) and b) mucosal side down revealing Henle's plexus respectively (fig. 2.1D).

Laminar preparations which had sufficiently adhered to the glass slides were subjected to an overnight incubation at 4^0C in 3% Triton x-100 in a 1:1 mixture of PBS and 10% sucrose in 0.1M PB pH 7.2, Tissue preparations were then washed and incubated at room temperature for 15 minutes in 10mM sodium phosphate buffer pH 8.0. Subsequently, preparations were placed in a dark, moist chamber for 1hr at 37^0C exposed to the reaction medium for NO synthase activity as described in section 2.1.1. Control experiments were also carried out in the same manner as was done for the guinea-pig. Tissues were given an extensive final wash with 0.1M PB, pH 8.0, air dried, coverslipped under permount and analyzed by light microscopy.

Separate laminar preparations of the myenteric plexus from each intestinal region was incubated in 0.00002% ethidium bromide in distilled water (modified after the method of Schmued et al 1982 (24) for approximately 3 min. at room temperature. The stained tissue preparations were then washed briefly in 0.1M PB, pH 7.2 and coverslipped in glycerol (pH 8.6). This dye when exposed to green light, produces a red fluorescent Nissl stain (highlighting the neuronal cytoplasm and nucleoli). This dye also stains the glial nuclei but neurons were clearly delineated. This stain permitted total cell counts per ganglion for each region of the intestine. Estimations of NADPH diaphorase positive cells per ganglion and total ganglion cell counts followed the same procedure as that described in section 2.1.1 with the exception that a sample population of 200 ganglia per region from six animals was used in estimations of mean frequency of diaphorase cells.

2.2.2 Chemicals

All of the reagents used in this particular investigation are listed in 2.1.2 with the exception of ethidium bromide which was purchased from Sigma Chemical Co. (Canada).

2.2.3 *Results*- Myenteric Plexus

Laminar preparations of the myenteric plexus taken from the small and large intestine and histochemically treated for NO synthase histochemical activity show all ganglia to contain positively labeled cells (fig. 2.6A-F, and fig. 2.7A and B). Axonal processes could be seen coursing through the primary, secondary and tertiary meshwork fibre bundles (fasciculi) (fig. 2.6A and F), and these could sometimes be followed through up to three ganglia. Most of the labeled cell soma were present within ganglia, but occasionally an extraganglionic cell could be found in the interconnecting fasciculi of the primary and secondary meshworks comprising this plexus (fig. 2.7B).

More than one class of NO labeled neuron was apparent, based on variations in morphological type, size and staining intensity. As in the guinea-pig, background labeling was negligible, yielding an excellent signal to noise ratio. The reaction within neurons, appeared to label the cells' cytoplasm in its entirety including efferent fibres and proximal dendrites and thus afforded sufficient definition of cell shape to allow morphological classification. Of the eight known morphologically classified neurons within the enteric nervous system, as described by Stach (1989), the labeled ganglion cells encompass 2 types; type I, usually found at the poles of the ganglia with radially arranged dendrites, stellate in shape with a centrally located nucleus (fig. 2.6D); type II found primarily at the center of the ganglia, adendritic and oval in appearance (fig. 2.6E). There was little regional variation along the intestinal axis in the proportion of labeled cells in each myenteric ganglion; with the duodenum, jejunum and ileum displaying similar proportions and the colon exhibiting the lowest density of labeled cells. In general, the proportion of NADPH diaphorase labeled cells per ganglion within the different regions did not exceed 25% as presented in Table 2.2.

TABLE 2.2. Proportion of NO Synthesizing Cells Per Ganglion Estimated from Counts of NADPH Diaphorase Positive and Total Cells Per Ganglior. in Various Regions of the Rat Intestine.

	DUODENUM	JEJUNUM	ILEUM	COLON
NO CELLS/GANGLION	5.0 ± 0.2	6.0 ± 0.3	8.0 ± 0.4	5.0 ± 0.3
TOTAL CELLS/GANGLION	23 ± 1	28 ± 1	28 ± 1	42 ± 2
% NO CELLS/GANGLION	22	21	28	12

NOTE: The total number of cells per ganglion represents the mean of cell counts for 100 ganglia per region of tissue examined with the neuronal cytoplasmic stain ethidium bromide. The number of NO synthase-reactive cells per ganglion are presented as the mean ± SEM of counts from 200 ganglia per region from 6 animals. The proportion of NO synthase-reactive cells is presented as percentage of NO cells per total cells per ganglion.

2.2.4 *Results* - Deep Muscular Plexus

Laminar preparations of the myenteric plexus displaying areas where the circular smooth muscle was not completely removed by dissection revealed NO synthase-reactive fibres within the muscle layer (fig. 2.6C). These labeled fibres are characteristic of the nerve fibre ramification within the deep muscular plexus (Gabella 1979).

2.2.5 *Results* - Circular Muscle-Submucosa Interface

In laminar preparations of the colon with the mucosa facing down, a dense network of intensely labeled fibres was found at the interface of the circular muscle and the submucosa (fig. 2.8D). This fibre meshwork found at the circular muscle-submucosa interface first described by Christensen and Rick (1987) is similar to that reported by Krantis (1991a) to contain GABAergic fibers.

2.2.6 *Results* - Submucosa

In contrast to the guinea-pig, the submucosa of the rat consists of two interconnected nerve layers; Henle's nerve plexus, subjacent to the circular muscle layer, and Meissner's nerve layer situated closest to the mucosa.

Figure 2.6. Photomicrographs showing myenteric plexus laminae from the rat small intestine treated for histochemical localization of NO synthase activity. (A) Interconnected ganglia (G) and nerve fibre networks of the jejunal myenteric plexus display a discrete subpopulation of intensely labeled neurons (N). Associated processes (small arrows) can be traced through the primary (Pp), secondary (Ps) and tertiary (Pt) nerve fibre bundles (fasciculi) of the plexus. (B) Duodenum; intensely labeled extraganglionic cells (e) within the primary and secondary fasciculi display NO synthase activity. (C) NO synthase positive nerve fibres can be seen deep within the circular muscle (cm) layer shown here partly separated and folded back revealing the underlying myenteric plexus. (D) Labeled cells with type I morphology in the duodenum (E) Labeled cell with type II morphology in the ileum. (F) NO synthase-reactive nerve fibres (small arrows) are evident in primary, secondary and tertiary fasciculi of this plexus in the ileum. Scale bar = 50 μm.

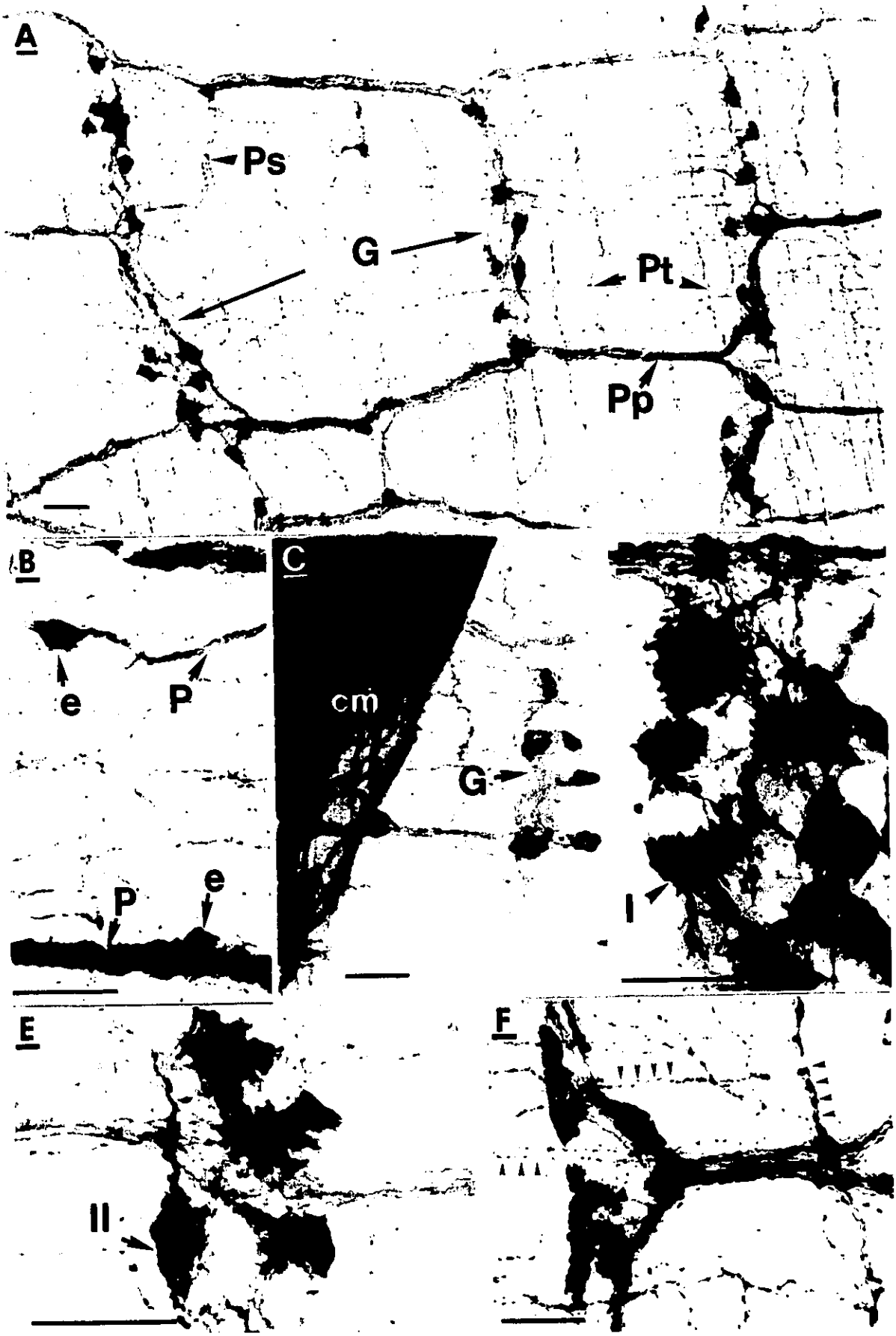
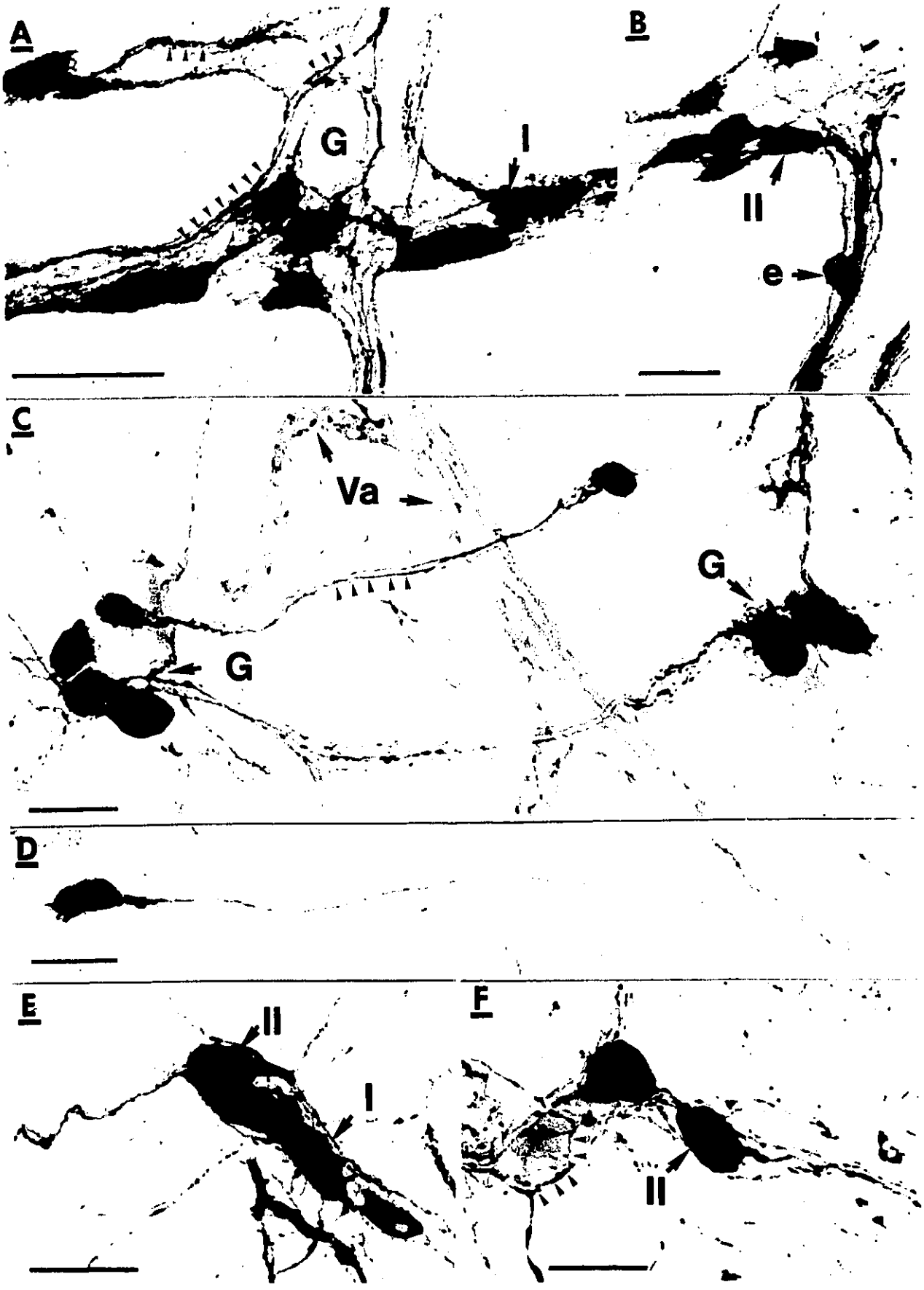

A
Ps
G
Pt
Pp
B
e
P
P
e
C
cm
G
I
E
II
F

Figure 2.7. Photomicrographs showing myenteric and submucosal ganglia from the rat distal colon. (A and B) The myenteric ganglia (G) contain a profusion of labeled fibres (small arrows) and intensely stained cells. NO synthase reactive type I (I) and type II (II) neurons can be identified. (C) In a preparation of the submucosa with Henle's (Schabadasch) plexus uppermost, ganglia (G) containing intensely labeled cells can be seen overlying a submucosal arteriole (Va). Axonal processes (small arrows) can be seen coursing through the ganglia and interconnecting fasciculi. (D). Shows a type II submucosal cell the axon of which could be traced for up to 600 μm. (E and F) High power micrographs showing the distribution of intensely stained varicose fibres and nerve cells in ganglia of Henle's plexus. Type I and II cells are easily identified together with labelled fine varicose fibres (small arrows) that can be seen to ramify around labelled and unlabeled cells. Scale bar = 50


A
G
I
B
II
e
C
Va
G
G
D
E
II
I
F
II

Of the three laminae, NO synthase reactive neural elements were most prevalent within the nerve layer (Henle's plexus) located between the circular smooth muscle layer and the vascular network of the submucosa. Relative cell density varied along the gut axis. In the duodenum (right side of fig. 2.8D), the ganglia of this plexus rarely contained more than one NO synthase positive cell. The proximal to distal portions of the jejunum and ileum displayed an increasing number of NO synthase reactive ganglionic cells, from a rare occurrence in the proximal region to as many as three labeled neurons in the distal segment. Within each ganglion of Henle's plexus of the colon however, (fig. 2.8C) as many as four cells were labeled. In addition, fine varicose fibres were found to display NO synthase activity. For comparison of the different nerve layers, we aligned the micrographs of the colon submucosa adjacent to micrographs of the colon myenteric plexus (fig. 2.7A-C).

Like the labeled myenteric neurons, neurons in Henle's plexus were found to have fibres that project long distances (fig. 2.7D). The longest axonal projection noted was measured at 1.3 mm before becoming obscured from view. The NO synthase-reactive nerve cells observed within Henle's plexus of all the intestinal regions examined, were predominantly of the type II morphology as originally described by Dogiel (1899) and further examined by Stach (Stach 1989) (fig. 2.7E and F). Type I cells were rarely encountered and then only found in the large intestine (fig. 2.7E). Fibre network density varied along the gut axis but did not always correlate with the cell density.

The pattern of labeling of NO positive neural fibres was consistent throughout the rostro/caudal extent of the duodenum. The interconnecting fasciculi contained only a few stained fibres and reactive ganglionic fibres were rare (fig. 2.8D). Conversely, ganglia within the proximal to distal regions of the jejunum and ileum were considerably larger, and contained a profusion of intensely stained fine varicose fibres around clearly unlabeled cells (fig. 2.8A). Compared to the jejunal and ileal segments, NO-related ganglionic fibres of the colon were more diffuse (compare fig. 2.7C, E and F with fig. 2.8A).

Laminar preparations of Meissner's plexus taken from the large intestine displayed a scant distribution of intensely labeled NO synthase-reactive neurons (topographically situated at the mucosal side of the submucosal vascular network) which were increasingly prevalent towards the distal portion of the colon. An organized network of ganglia and interconnecting fibres as described by Gabella (Gabella 1979) in the porcine intestine was observed in the jejunum and ileum only. In fact, this organized network detected with this histochemical stain is identical to that shown in Figure 2.8A.

Figure 2.8. Photomicrographs showing laminae of the submucous plexus from the small intestine. (A) Regional patterns of labeling of Henle's plexus and the underlying vascular network from the ileum are shown. A profusion of very fine intensely labelled varicose fibres occur throughout the large ganglia (G) and interconnecting nerve bundles. (B) Under higher magnification, the intensely labeled puncta of the arterioles (Va) can be easily distinguished from the labeled nerve fibres (arrows) of Henle's plexus, and a large unlabeled venule (Vv) from the ileum. (C) Intensely labeled fine paravascular fibres (small arrows) are seen coursing with an arteriole (Va) in a submucosal preparation from the distal ileum. (D) The intensely labeled fine network of fibres at the circular muscle-submucosa interface is shown in this duodenal preparation. The labeled Henle's ganglia and the vascular plexus can be seen below the stained fibre network (arrows). Scale bar = 50 μm.

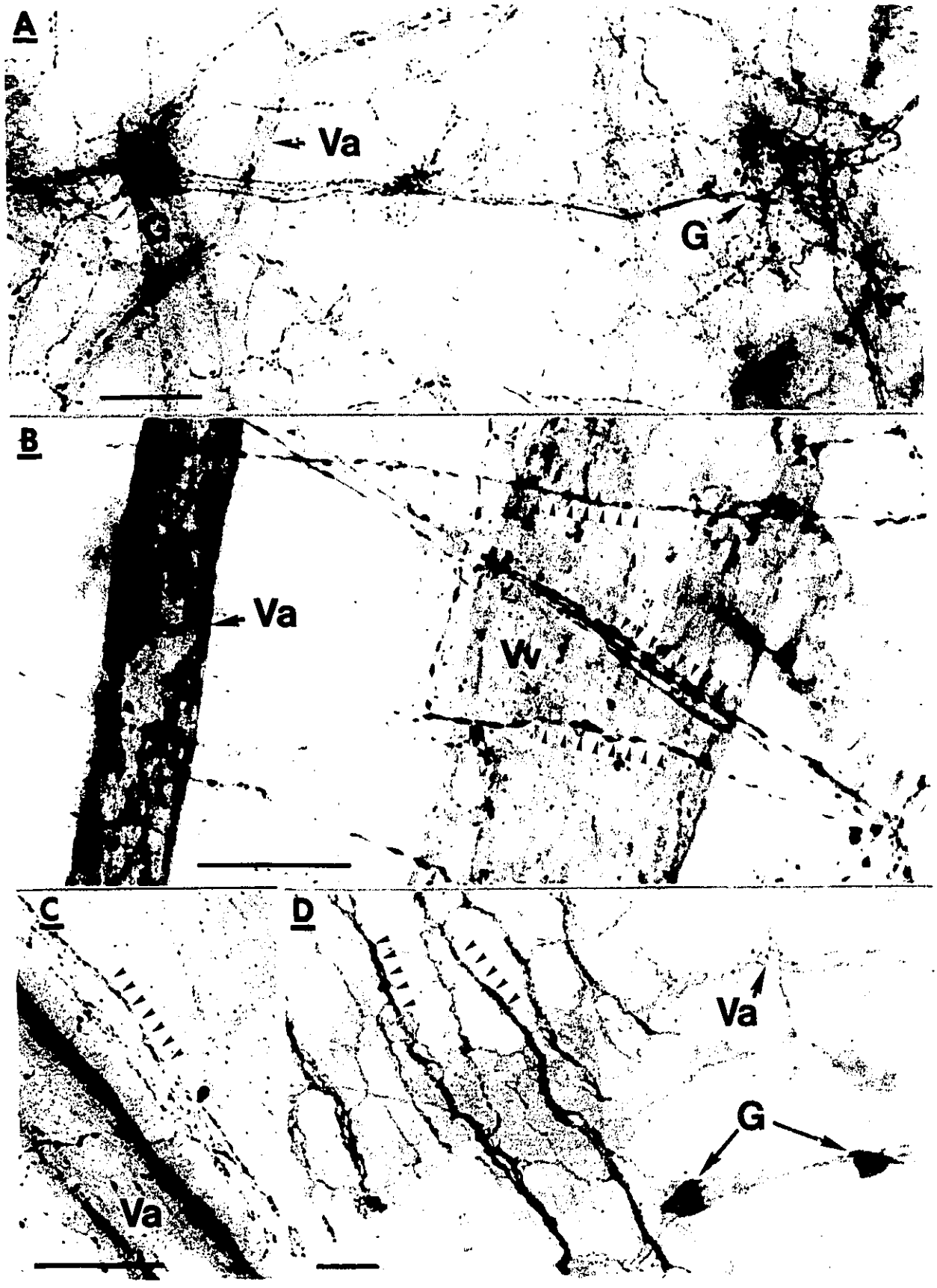
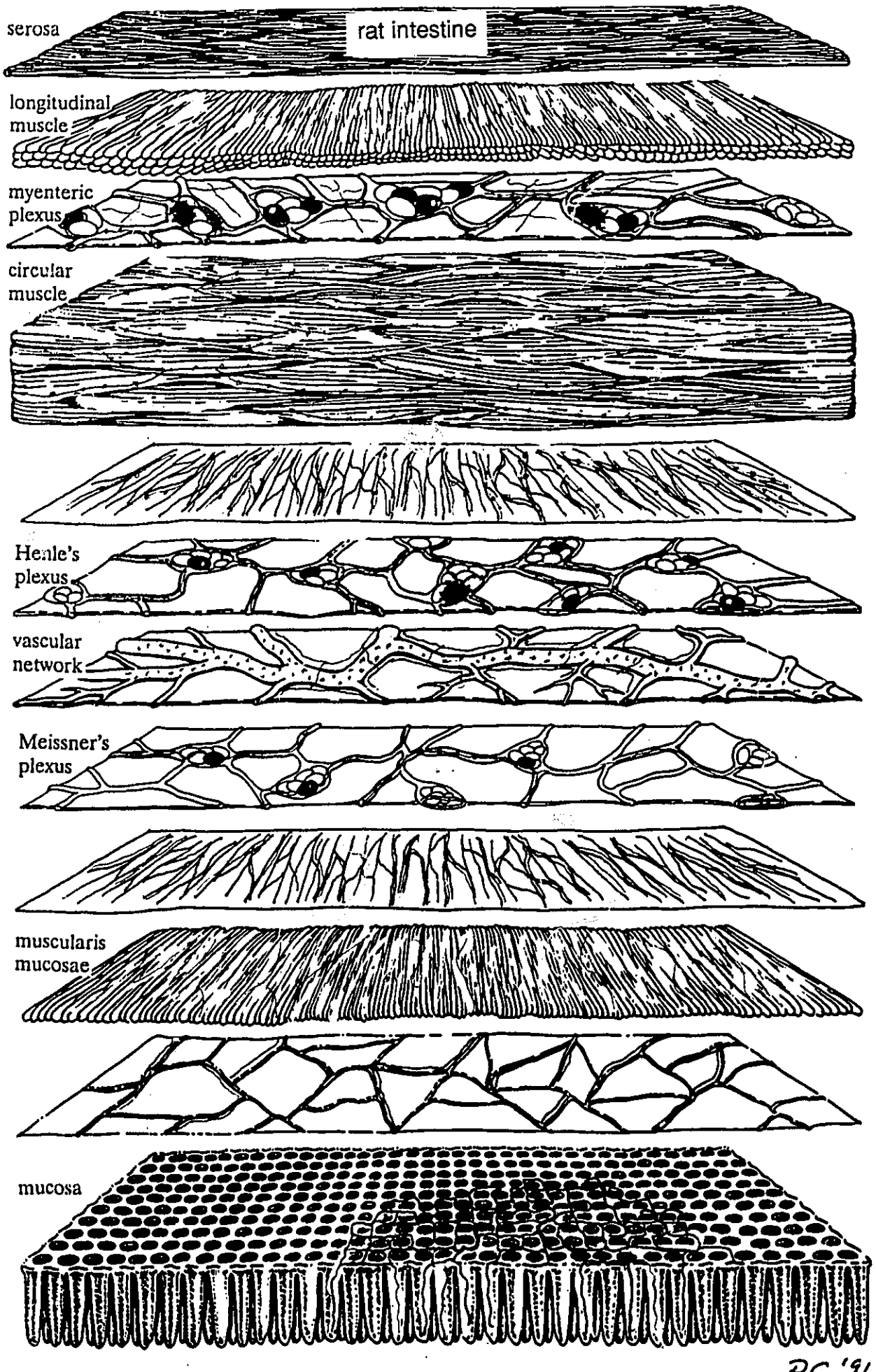

A
Va
G
G
B
Va
W
C
Va
D
Va
G

Figure 2.9. A summary of the NO synthase-related NADPH diaphorase distribution in the rat intestine. Separated component layers of the gut wall are illustrated. Note the presence of Meissner's nerve layer in this species. NADPH diaphorase staining is drawn in blue. Stained myenteric ganglion cells displayed type I and II morphologies. Emergent processes from myenteric ganglia could be seen coursing through the primary, secondary and tertiary fasciculi of this plexus. The circular muscle layer was densely innervated with NADPH diaphorase positive nerve fibres. Meshworks of intensely labeled fibres were visible at the circular muscle submucosa interface, and associated with Henle's and Meissner's nerve layers. Labeled cells were seen in Henle's and Meissner's ganglia in all regions of the intestine. Labeled fibres were also seen ramifying within the muscularis mucosae and forming fine meshworks at the base of the mucosa. Scant fibres were viewed projecting within the mucosal lamina propria. As in the guinea-pig intestine, punctate patches of NADPH diaphorase activity were distributed in a regular pattern along the fine submucosal vessels. Labeled fibres were seen in the paravascular bundles running along side these vessels. Stained fibres ramifying within the muscularis mucosae and underlying mucosa were also observed. Labeled cells with occasional scant discernible processes emerging from the soma were identified at the base of the mucosa subjacent to the muscularis mucosae.


rat intestine
serosa
longitudinal muscle
myenteric plexus
circular muscle
Henle's plexus
vascular network
Meissner's plexus
muscularis mucosae
mucosa
P.S. '91

2.2.7 *Results* - Submucosal Vascular Plexus

Arterioles and venules were found to consistently display a punctate labeling pattern (fig. 2.8B). These patches of NO synthase activity appeared to be uniformly distributed along the entire vessel length but displayed no characteristics of perivascular nerve fibre organization about the vessels. However, densely labeled fibres occurred in paravascular nerve bundles (fig. 2.8C).

Figure 2.9 provides a summary of the observed NO synthase distribution in the rat intestine.

2.2.8 Discussion

The results of this histochemical study confirm the presence of NO synthase-related NADPH diaphorase activity in rat myenteric neurons and their innervation of the circular smooth muscle (Bredt et al. 1990b; Aimi et al. 1993) and expands our understanding of the distribution of NO synthesizing sites within the gut wall. NO synthase reactive neural elements of the myenteric and submucosal nerve layers were identified and characterized. In addition discrete sites of NO synthase activity in the wall of fine arterioles within the submucosa of the rat small and large intestine were detected. With the exception of the neural labeling found in Meissner's plexus in this species, the overall labeling pattern was similar to the findings in the guinea-pig.

The present histochemical technique represents a simple and reliable method for selectively labeling enteric sites of NO synthase activity. The intense blue staining yielded high resolution definition of positive neural elements, thereby allowing the classification of neurons according to established morphological criteria. Within the different ganglionated neural networks, positively stained neural elements were differentially distributed, with the frequency of stained ganglion cells (neurons) being highest in the myenteric plexus. The occurrence of nerve cell labeling was most prevalent in the ileum. In contrast to the myenteric plexus of the guinea-pig intestine treated with this histochemical technique (Nichols et al. 1992), in the rat, only two of the known morphologies according to Stach (1989) of myenteric nerve cells were observed. However, the nerve cell types (type I and II) intensely labeled in rat tissue are a subset of those cells labeled in the guinea-pig intestine (types I, II and VI).

NADPH diaphorase positive neurons exhibiting type I morphology have been recently proposed to be one population of inhibitory motor neurons involved in the aborally-directed inhibitory reflex pathways of the gut (Costa et al. 1991a). Neurons with Dogiel type II morphology account for approximately one-third of all guinea-pig myenteric neurons and are

proposed to have a sensory function (Pompolo et al. 1989; Costa et al. 1991a). The proportion of NO synthase reactive myenteric neurons/ganglion was greatest to be in the ileum and smallest in the colon (Table 2.2). Since 85% of Dogiel type II neurons have been shown to contain calcium-binding proteins (calbindins) (Pompolo et al. 1989) a major proportion of type II neurons have a potentially large capacity to buffer Ca^{2+} and could potentially regulate Ca^{2+}/calmodulin mediated activation of NO synthase. Whether NO plays a role as a transmitter/modulator of enteric sensory neurons in the gastrointestinal tract of the rat remains to be determined. Our findings that putative type II enteric sensory neurons possess the potential to produce NO is supported indirectly by the findings that NADPH diaphorase positive cells have been localized to rat (Aimi et al 1992) lumbar and sacral dorsal root ganglia. In addition, NO presynaptically suppresses noncholinergic slow EPSPs that occur in type II cells by suppressing the ongoing release of a slow excitatory synaptic mediator such as substance P (SP) (Tamura et al 1993).

In the rat small intestine submucosal ganglia displayed far fewer stained cells than observed in the myenteric plexus, while the numbers of stained ganglion cells in the colon submucosa matched those of the myenteric plexus. Although, stained ganglion cells were rare in the submucosa of the small intestine, ganglia and interconnecting fasciculi were richly invested with intensely stained varicose fibres. In the colon, labeled fibres were less profuse. Recently Brown et al (Brown et al. 1992), reported that in the rat gastric mucosa NO may regulate epithelial cell secretion. Since the cell bodies of secretomotor neurons are found in submucous ganglia (Bornstein et al. 1988), these data suggest that NO may be a mediator in intrinsic secretomotor reflex pathways of the gut. The occurrence of a more profuse fibre innervation in regions with the fewest number of positive cells is intriguing. Since the submucosa contains an extensive and complex array of intrinsic and extrinsic nerve fibres (Wilson et al. 1981; Aimi et al. 1991), it is possible that a proportion of the innervation of the submucosa that we found to display NO synthase activity are extrinsic in origin. In addition, a proportion of these fibres may be part of the myenteric modulatory fibres displaying NO synthase activity to be present to a fine network of fibres at the circular smooth muscle/submucosa interface in the rat colon. This network is a consistent feature of the histochemical studies of NO-related enteric innervation of the guinea-pig. According to Christensen and Rick (Christensen et al. 1987) this network is proposed to serve as a link between the myenteric plexus and neuro-vascular networks of the submucosa, and is also believed to be involved in the generation of slow waves in the colon.

Immunohistochemical (Llewellyn-Smith et al. 1992) and NADPH diaphorase histochemical (Nichols et al. 1992) studies of the guinea-pig myenteric plexus, indicates that NO

synthase-positive nerve fibres make synaptic contacts with NO synthase reactive and non-reactive myenteric neurons. The present study reveals NO synthase-reactive ganglionic fibres around labeled and unlabeled cells in Henle's plexus of the rat submucosa. Taken together, these findings suggest that in addition to it's neuroeffector action on the circular smooth muscle of the rodent, NO may regulate the activity of both myenteric and submucous neurons.

NO mediates the action of several vasodilator compounds in both the central and peripheral vasculature (for reviews see Long et al. 1989; Moncada et al. 1991). In addition, evidence supporting the identity of EDRF as NO has been reported for the porcine aorta, where NO was detected in vascular endothelial cells using a newly developed cellular probe (Malinski et al. 1992). In the present study, a consistent pattern of NO synthase localization in the submucosal arterioles was seen in all regions of the rat intestine. This pattern was similar to that found in submucosal blood vessels of the guinea-pig, consisting of a highly organized distribution of discrete patches of NO synthase activity in the wall of the submucosal arterioles. The pattern of labeling was unlike any varicose perivascular, nerve innervation and too sparse to be related to the smooth muscle cells. Instead, the topographical location of these puncta in the vessel wall correlates with the endothelial cell distribution, similar to that observed the cerebral vasculature of Wistar rats (Poeggel et al. 1992) using NADPH histochemistry. Several reports indicate that NO not only plays a local vasodilator role in the gastrointestinal microcirculation (Pique et al. 1989; Walder et al. 1990; Pique et al. 1992), but also behaves as an important factor in the maintenance of vascular integrity in the gastric (MacNaughton et al. 1989; Peskar et al. 1991) and intestinal mucosa (Boughton-Smith et al. 1990; Hutcheson et al. 1990) of the rat. This observation of the localization of intrinsic NO synthase activity in the wall of intestinal microvasculature in the guinea-pig, and now the rat, provides a candidate anatomical locus for the non-neural generation of NO for the control of enteric vasodilation.

In conclusion, the results of these studies show that NO can be synthesized at multiple sites throughout the gut wall of the guinea-pig and rat gastrointestinal tract. In addition to the multiple myenteric nerve types that display intense NO synthase activity, the presence of NO synthase-reactive ganglion cells in the submucosa and fibres within all of the submucosal nerve networks supports the notion that NO, in addition to its apparent myenteric transmitter role (Toda et al. 1990a; Maggi et al. 1991; Shuttleworth et al. 1991; Boeckxstaens et al. 1991a; Boeckxstaens et al. 1991b; Grider et al. 1992; Lefebvre et al. 1992a), plays a role in the control of secretomotor function. Furthermore, the discovery of distinct sites of NO synthase activity in rat and guinea-pig enteric arterioles strongly supports the findings of pharmacological studies by others

(MacNaughton et al. 1989; Pique et al. 1989; Boughton-Smith et al. 1990; Hutcheson et al. 1990; Walder et al. 1990; Peskar et al. 1991; Malinski et al. 1992) which show NO has EDRF-like vasomotor actions in the mammalian gut.

This rich neural and vascular localization of NO synthase activity together with the increasing functional evidence for NO to be involved in the reflex control of gut motility suggests that disruption of the enteric nitrergic system could give rise to a number of gut disorders with abnormal patterns of motility ranging from bacterial and virally-induced vomiting and diarrhea through to apparently idiopathic conditions such as irritable bowel syndrome, delayed gastric emptying, reflux, gastric and duodenal ulceration and pseudo-obstructions such as Hirschsprung's disease.

CHAPTER 3

3.1. The Nitrergic Innervation of the Human Colon

Much of the chemical coding of enteric neurons has been carried out in the guinea-pig intestine. In the human intestine very little is known about the distribution of enteric substances or which substances colocalize in a particular neuron and whether they are similar to the established neurochemical addresses of the guinea-pig intestine. Even less is known about this area in the diseased human gut. One reason for the apparent lack of knowledge is the difficulty in obtaining human tissue, normal or diseased. Another reason may be the level of difficulty in the microscopic dissection of the individual nerve layers of the human gut wall. *The aim of this study was to histochemically assess the nitrergic innervation of the human colon as was done in the guinea-pig and rat intestine and compare the nitrergic innervations across these species.*

Prior to this study NO was identified as a mediator of NANC inhibition of circular smooth muscle in the human esophagus (McKirdy et al 1992) and in both the small (Maggi et al 1991; Stark et al 1993) and large (Burleigh 1992; Boekxstaens et al 1993) intestine. Therefore *we hypothesized that Dogiel type I myenteric ganglionic neurons and associated fiber innervation of the circular smooth muscle of the human colon would display NADPH diaphorase activity.* Since NO mediates vasodilation in the gut, *we also hypothesized that human submucosal vascular sites would also display NADPH diaphorase sites* as was observed in the guinea-pig and rat intestine.

3.1.1. Materials and Methods

All human tissue studied in this investigation was obtained under approval of the Research Ethics Board of the Children's Hospital of Eastern Ontario (CHEO) and according to the Medical Research Council of Canada Guidelines on Research Involving Human Subjects. All processed tissues were sampled for parallel routine pathological investigation in the Department of Pathology at CHEO. The histopathological findings of the pathologist from CHEO were compared with the clinical presentation and progress of the patient. Full-thickness samples were obtained from seven surgically resected specimens of infant (aged 6 months to 1 year) distal colon. All but one surgery was for Hirschsprung's disease. Only normal segments were included in this study. The other specimen of human tissue was obtained from a 1-year-old, otherwise normal, male infant requiring a transverse colostomy at the end of staged treatment for congenital anal stenosis. During elective closure of the colostomy, two segments of normal

human transverse colon were removed. Tissue segments were washed in ice cold 0.1 M sodium phosphate-buffered saline (PBS, pH 7.4) and fixed for 2 hours at 4^0C in 4% paraformaldehyde, and 0.4% picric acid in 0.16 M sodium phosphate buffer (PB, pH 7.0). Segments to be used for isolation of laminar preparations were washed and stored in 0.1M sodium phosphate buffered saline (PBS, pH 7.2) at 4^0C for a minimum of 48 hrs. Separate colon segments were kept at 4^0C in PB (pH 7.2) containing 10% sucrose and 0.1% sodium azide for a minimum of 24 hours prior to sectioning.

Axial and circumferential sections were taken simultaneously at a 14 μm thickness and mounted onto glass slides.

Laminar preparations of myenteric and submucosal nerve layers were isolated from fixed specimens and prepared for staining following the same procedure as described in Chapter 2, section 2.2.1 with the following exception. In a dish filled with PBS containing a sylgard base, the fixed specimen was placed serosa facing up and the serosa was peeled away using fine forceps. The bowel was slit along its longitudinal axis between the plane of the longitudinal and circular muscle layers to ease separation.

Colon sections and laminar preparations, pre-incubated for 15 minutes in PB (pH 8.0) were subjected to 30 and 60 minute incubations respectively at 37^0C in the NO synthase histochemical reaction mixture comprising 1mM NADPH, 0.5mM NBT and 0.3% Triton X-100 in 10 mM PB (pH 8.0). All preparations were washed several times in PB (pH 7.2), allowed to air dry overnight, coverslipped under permount and photographed under brightfield conditions.

The mean frequency of NADPH diaphorase positive cells per ganglion was estimated from 100 ganglia taken from four biopsied specimens of human infant colon. The procedure and the criteria used to carry out this estimation is described has been described in section 2.1.1. The standard Giemsa stain used in section 2.1 and the ethidium bromide stain used in section 2.2 were applied to laminar preparations of the myenteric plexus from the human colon to estimate total cell number per ganglion.

3.1.2. Chemicals

All buffer materials were obtained from BDH (Ontario, Canada) with the exception of ß-NADPH which was purchased from Sigma (Missouri, USA).

3.1.3 *Results*- Myenteric Plexus

Laminar preparations of the myenteric plexus taken from the human infant colon and histochemically treated for NO synthase-related NADPH diaphorase activity showed all ganglia to contain positively labeled cells (fig. 3.1A and B). Axonal processes can be seen coursing through the primary (fig. 3.1B), secondary and tertiary meshwork fibre bundles (see fig. 4.1A; Chapter 4, section 4.4). Preparations of the myenteric plexus where overlying circular muscle has not been completely removed reveals NO synthase reactive fibers (fig. 3.1A). Frequently, NO synthase related extraganglionic cells could be found in the interconnecting fasciculi of the primary meshworks of this plexus (see fig. 4.1A; Chapter 4, section 4.4). The apparent frequency of NO synthase reactive extraganglionic cells in the human colon was greater than that seen in the rodent intestine.

Variations in morphological type and staining intensity of NO labeled neurons were apparent and indicative of more than one class of neuron being associated with this enteric neurotransmitter in the human colon. As in the rodent intestine, the histochemical reaction within labeled neurons, labeled the cells' cytoplasm and associated efferent fibres and proximal dendrites which afforded a suitable definition of cell shape to allow morphological classification. The labeled ganglion cells included type I and type II morphologically classified neurons as described by Stach (1989). The number of labeled cells in each myenteric ganglion was found to be 17 ± 0.4 which is significantly greater than those frequencies estimated in the myenteric plexus of the guinea-pig (9.0 ± 1.0) and rat (5 ± 0.3) colon. Estimation of total cell number per ganglion in the myenteric plexus of the human colon and therefore proportion of NADPH diaphorase positive cells per ganglion could not be determined since the signal to noise ratio observed with the Giemsa and ethidium bromide stains was such that myenteric ganglion cells could not be sufficiently discerned from other mural constituents. In order to attain a good signal to noise ratio laminar preparations have to be relatively clean of circular smooth muscle. This is inherently difficult in the human gut due to relative thickness of the circular muscle layer and difficulty in dissection as compared to the rodent intestine.

3.1.4 *Results* - Deep Muscular Plexus

Axial sections through the plane of the circular muscle revealed a dense innervation of NO synthase-reactive fibres within this muscle layer (fig. 3.1C). This fiber labeling pattern is consistent with the nerve fibre ramification within the deep muscular plexus (Gabella 1979). At

Figure 3.1 . Photomicrographs of laminar preparations (A and B) and axial sections (C and D) taken from the human proximal colon and treated for NADPH diaphorase staining. (A and B) show typical views of the myenteric ganglia (G) and interconnecting fibre bundles. Intensely labeled neurons are plentiful within the ganglia. In addition, the ganglia and nerve bundles contained intensely labeled fibres. In areas where some circular muscle remains, as shown in A, a dense network of intensely labeled fibres could be seen (arrows) . (C) The deep muscular plexus is richly invested with intensely labeled fibres and at high magnification (D) small labeled cells (asterisks) were also evident. Scale bar = 50 μm.


A
G
G
B
Pp
G
C
D

higher magnification small cells reminiscent of ICC cells (see Furness and Costa 1987; p52) of the deep muscular plexus displayed NO synthase-related NADPH diaphorase activity.

3.1.5 *Results* - Submucosa

In contrast to the guinea-pig and rat, the submucosa of the human gut wall consists of three interconnected nerve layers with distinct topographical locations. These include Henle's nerve plexus, subjacent to the circular muscle layer, Meissner's nerve plexus situated closest to the mucosa and an Intermediate plexus situated between these nerve layers but topographically closer to Meissner's plexus (see Chapter 2, fig. 2.1).

For comparison of the different nerve layers, I aligned the micrographs showing laminar preparations of Henle's plexus adjacent to micrographs showing the Intermediate and Meissner's nerve layers (fig. 3.2A to E). A profuse network of intensely stained varicose fibres within individual ganglia and throughout the interconnecting fasciculi was observed in this nerve layer. This meshwork of fibers appeared to be more profuse than in the myenteric plexus and single stained fibres were distinctly varicose in appearance (fig. 3.2A). Efferent projections from stained cells could often be traced up to 1 mm before disappearing from view (fig. 3.2B). Few ganglia contained labeled cells. The maximum number of labeled cells/ganglia was found to be 5. These cells could be identified as type I (fig. 3.2A) and type IV (fig. 3.2C) cell morphologies as described by Stach (1989). Topographically situated below Henle's nerve layer and vascular network a much finer meshwork of fibers and interconnected ganglia could be discerned associated with Meissner's nerve layer (fig. 3.2A). NO synthase reactive neurons were rare in this nerve layer (fig. 3.2A and E, 3.4A-C). No more than 2 cells/ganglion were observed. In certain regions of laminar preparations with Henle's plexus facing up, a finer network of interconnected ganglia could be viewed just beneath Henle's nerve layer but above Meissner's plexus. This is known as the Intermediate plexus (Hoyle et al. 1989). The ganglia of this nerve layer are larger than those of Meissner's plexus but not quite as large as those seen in Henle's nerve layer (compare fig. 3.2D and E). NO synthase positive ganglion cells were most prevalent in the Intermediate nerve layer.

In circumferential sections of the human infant colon, a profusion of NO synthase positive nerve fibers was found in the ganglia of Henle's (fig. 3.3A and B), Meissner's (fig. 3.3C and D) and Intermediate (fig. 3.3C) nerve layer. These stained varicose fibres could be seen to ramify around clearly unlabeled cells within the ganglia (see fig. 3.3A to D). NADPH diaphorase positive varicose nerve fibers could be seen to project from ganglia of Henle's nerve layer and

Figure 3.2. Isolated laminae from the submucosa of the human proximal colon treated for NADPH diaphorase staining. (A) A large ganglion (G) and interconnecting fibre bundles of Henle's plexus (Hp) contrasts with the much smaller ganglia (G) and interconnecting strands of Meissner's plexus (Mp). (B and D) Ganglia (G) of Henle's plexus displaying intensely labeled cells. The efferent process of the stained cell in B could be traced for up to 1 mm within the tissue preparation. The morphology of this cell is reminiscent of 'dendritic' type II cells as defined by Stach. (C) An intensely stained cell typical of Stach type IV (IV) morphology showing polar dendrites and a stout discernible axon hillock alongside a typical type II cell. Panels D and E contrasts the relative size and anatomy of ganglia and interconnections of Henle's plexus (Hp), the Intermediate plexus (Ip) and Meissner's plexus (Mp). Stained ganglion cells were more frequent in Henle's and Intermediate nerve layers.

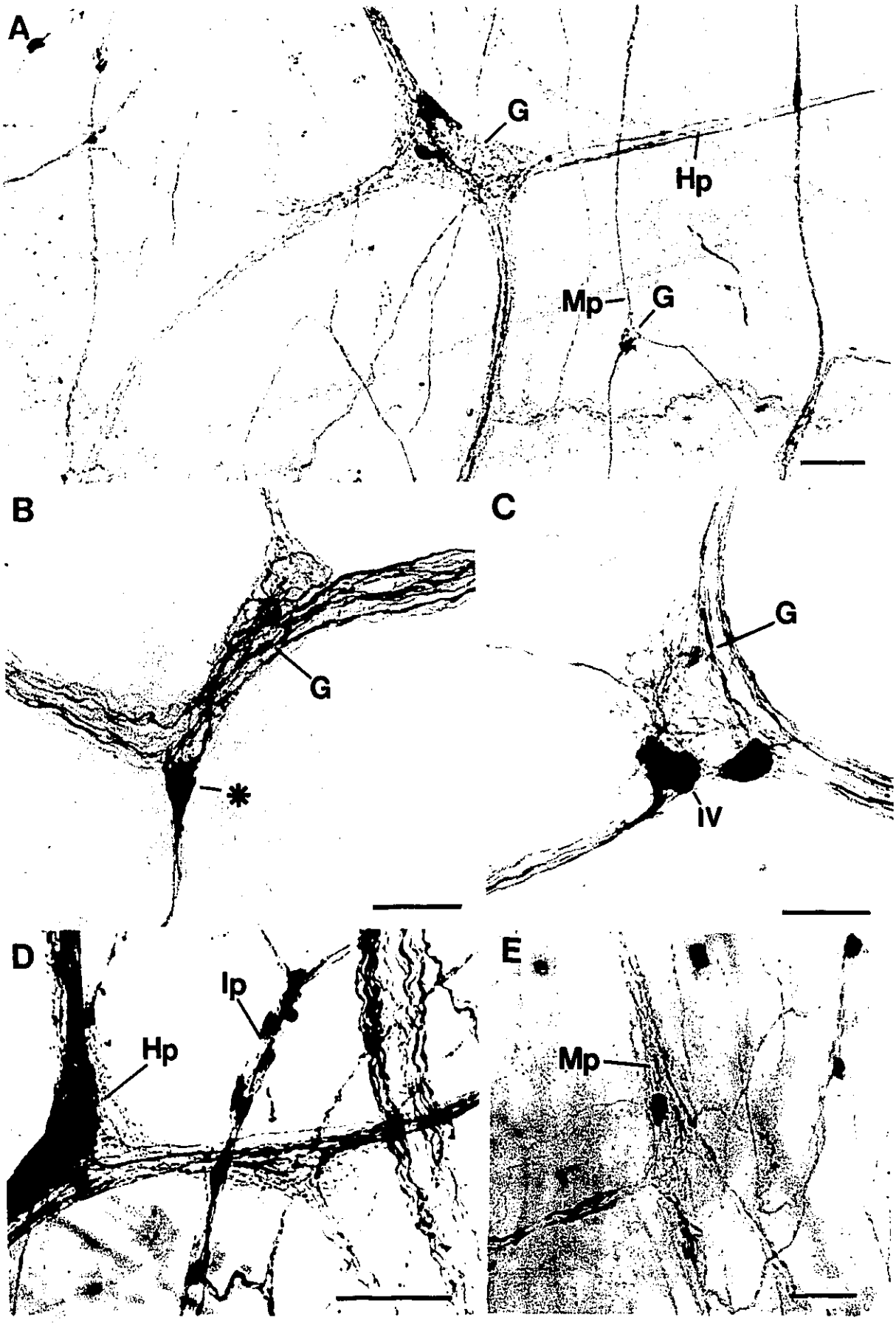

A
G
Hp
Mp
G
B
G
*
C
G
IV
D
Ip
Hp
E
Mp

Figure 3.3. Colour micrographs showing intensely labeled cells and fibres in axial sections of the submucosa from the human proximal colon. Labeling of Henle's plexus (Hp) including nerve bundles and ganglia (G) can be seen in panels A and B. The location of Hp is apparent from the closely juxtaposed circular (cm) muscle layer. (A) Labeled varicose fibres could be seen coursing within the ganglia around clearly unlabeled cells. Some emergent ganglionic fibres could be seen projecting to the overlying circular muscle layer. (B) A labeled cell reminiscent of type II morphology can be seen. (C and D) The extensive NADPH diaphorase labeling of the submucosa includes ganglia (G) and a profusion of fine ganglionic varicose fibres of the Intermediate (Ip) and Meissner's plexus (Mp). Stained paravascular fibres (arrows) can be seen alongside a submucosal blood vessel (bv). Scale bar = 50 μm.

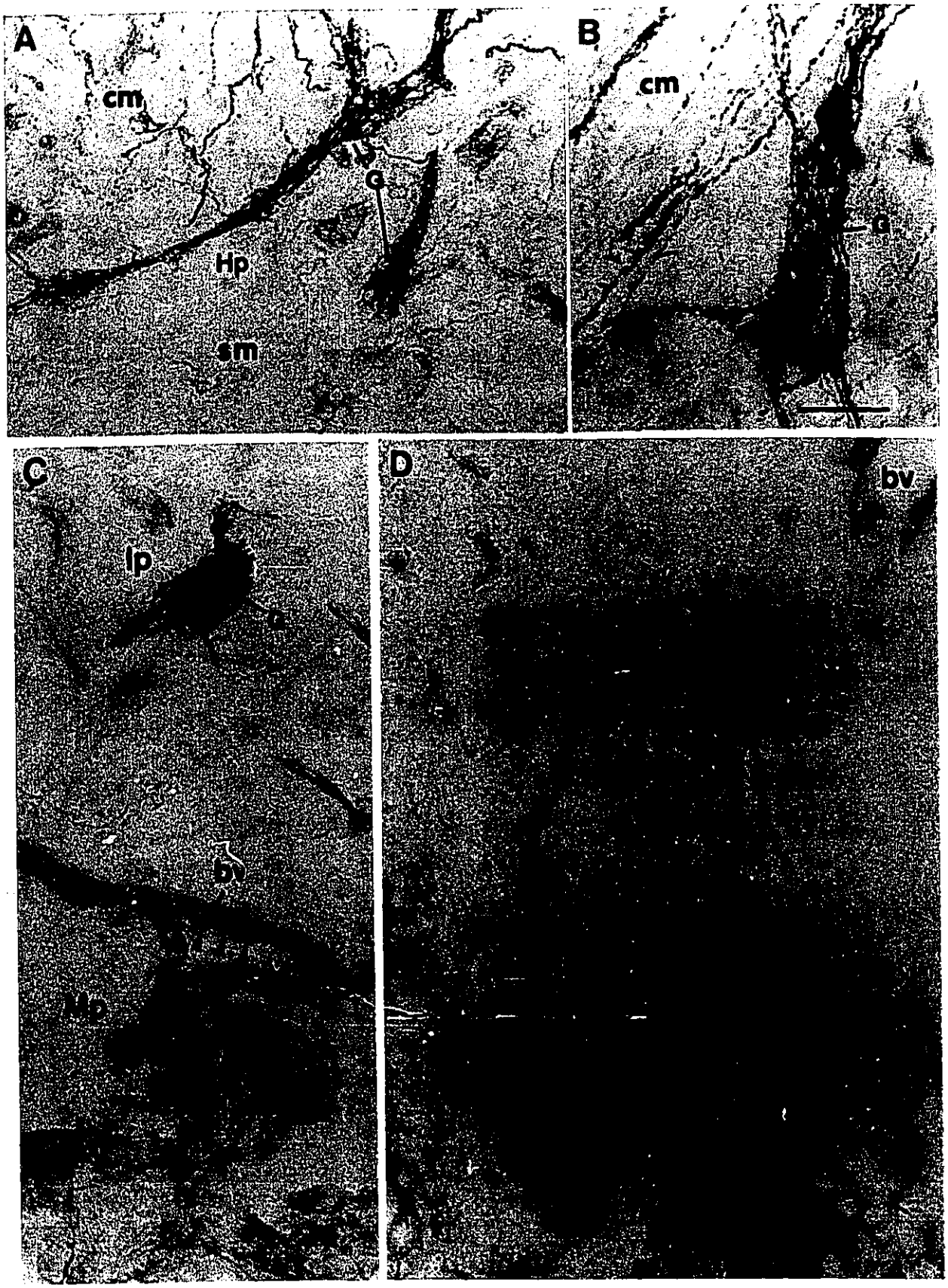

A
cm
c
Hp
sm
B
cm
c
C
lp
bv
Mb
D
bv

Figure 3.4. Colour micrographs of circumferential sections from human colon treated for NADPH diaphorase histochemistry. All panels show the submucosa (sm), muscularis mucosae (mm) and mucosa (m). Within Meissner's plexus (Mp) ganglia displayed intensely labeled cells and fibres (arrows) that appeared also to innervate the muscularis mucosae and mucosa (arrows) as shown in D. The mucosa displayed a distinct fine network of labeled varicose fibres (arrows) subjacent to the muscularis mucosae and in and about the base of the crypts. (C) In addition, a number of small stained nucleated cells, with fine processes (asterisks) were sometimes evident and usually situated at the interface of the muscularis mucosae and mucosa . Scale bar = 50 μm.

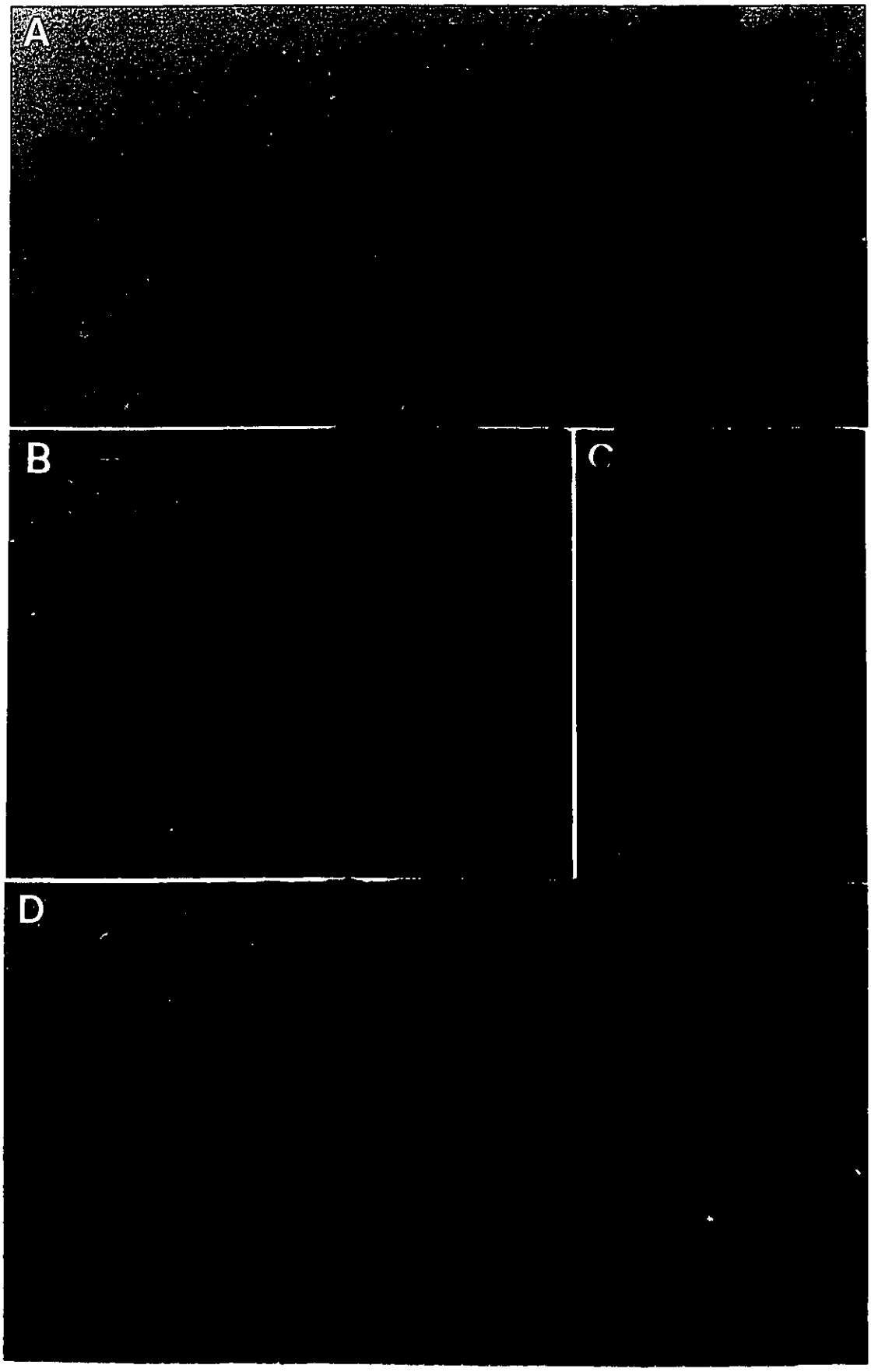
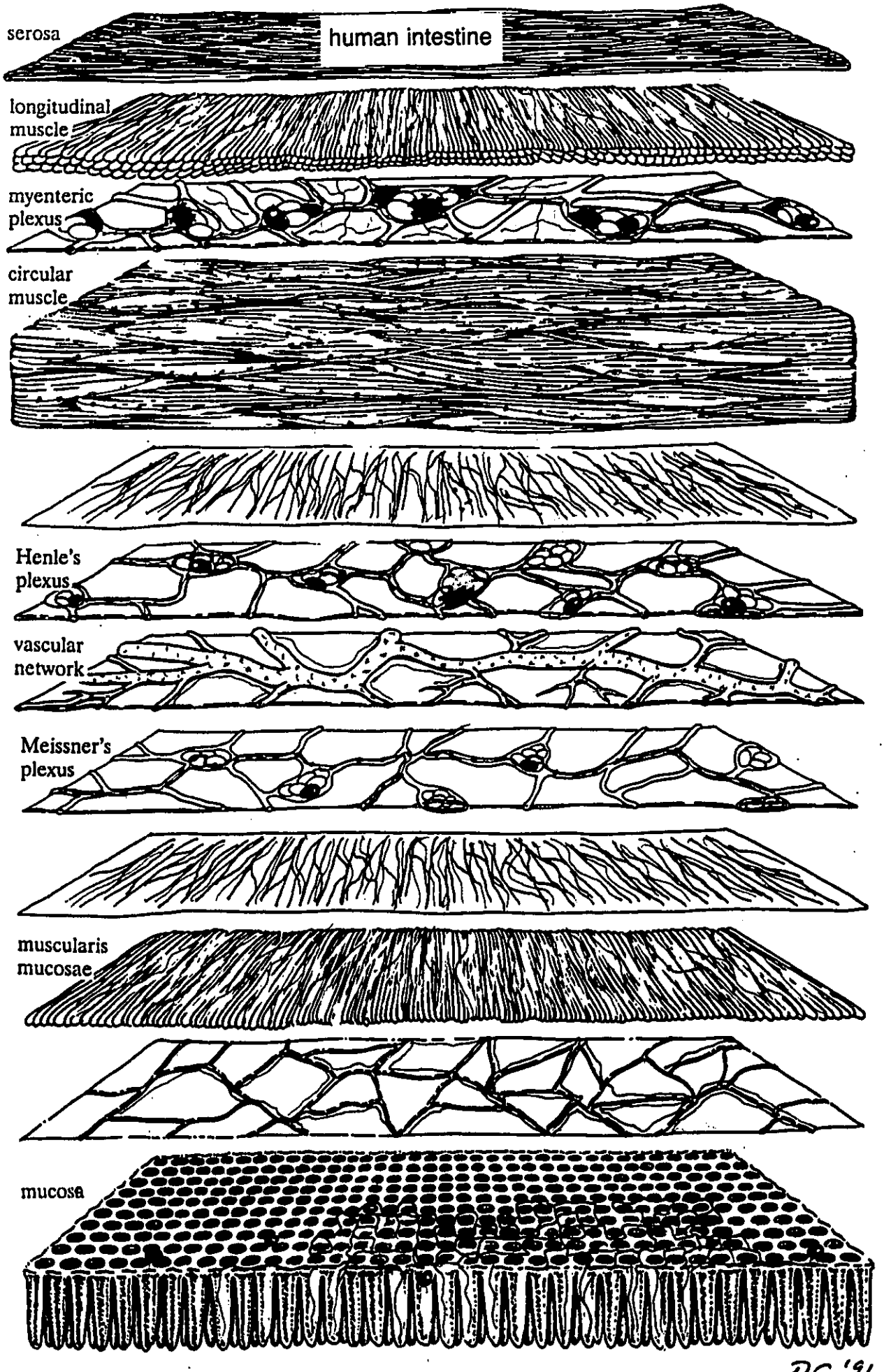

A
B
C
D

Figure 3.5. A summary diagram of the distribution and disposition of NADPH diaphorase labeled elements within individual layers of the human infant colon. Stained myenteric ganglion cells were prevalent. Labeled fibres could be seen coursing within the primary, secondary and tertiary fasciculi of this plexus as well as the associated innervation of the underlying circular muscle layer. Longitudinal muscle fibres also stained. Ganglia of Henle's plexus, Meissner's nerve layer and the Intermediate plexus (not shown in this diagram) displayed a scant distribution of labeled cells and a profusion of stained varicose fibres around clearly unlabeled cells. Stained cells encompassed type I, II and IV Stach morphological types. Fine submucosal vessels a regular pattern of NADPH diaphorase puncta and the paravascular innervation of these vessels contained labeled fibres. A dense meshwork of labeled fibres ramify within the muscularis mucosae and a scant distribution projects within the mucosa. Intensely stained nucleated cells could be seen within the lamina propria of the mucosa. The majority of these cells were situated just beneath the muscularis mucosae, but occasionally they were found further away from this muscle layer.


human intestine
serosa
longitudinal muscle
myenteric plexus
circular muscle
Henle's plexus
vascular network
Meissner's plexus
muscularis mucosae
mucosa
P.S. '91

ramify within the circular muscle layer (fig. 3.3A and B). Finally, scantily distributed fine varicose nerve fibers were evident running alongside (*para*) submucosal arterioles as seen in Figure 3.3C.

3.1.6 *Results* - Muscularis Mucosae and Mucosa

Fine intensely labeled fibers could be seen projecting from Meissner's plexus (fig. 3.4A) through the muscularis mucosae (fig. 3.4B to D) and on into the glandular mucosa (fig. 3.4D). At the base of the glandular mucosa a fine meshwork of labeled fibers could be discerned (fig. 3.4B) as well as discrete nucleated cells within the lamina propria (fig. 3.4C). Some of these cells appeared to possess processes.

Figure 3.5 summarizes the observed NO-related innervation of the infant human colon.

3.1.7 Discussion

The results of this study of the nitrergic innervation of the human infant colon confirm the presence of NO synthase-related NADPH diaphorase activity in myenteric neurons and their innervation of the circular smooth muscle as well as nitrergic neurons and fibers within Henle's plexus as seen by other investigators (Vanderwinden et al. 1992; Timmermans et al. 1993; Timmermans et al. 1994a) Also similar to previous studies; NO synthase reactive neurons of the myenteric and Henle's nerve layers were identified and characterized as having type I and type II nerve cell morphologies based on the classification system of Stach (1989). However, additional findings of this study expands our understanding of the distribution of NO synthesizing sites within the gut wall of the human colon. In addition to these nerve cell morphologies, dendritic type II and type IV cells were also detected in Henle's nerve layer. Unlike previous studies of the nitrergic innervation patterns in the human colon (Vanderwinden et al. 1992; Timmermans et al. 1994a), small cells reminiscent of ICC cells of the deep muscular plexus were found to contain NO synthase activity. Nitrergic neural elements were also found in the Intermediate plexus and Meissner's nerve layer of the submucosa. NO synthase related neurons and fine varicose fibers were observed within ganglia of both nerve plexuses. In addition, labeled fibers from Meissner's nerve layer could be seen projecting to and ramifying within the muscularis mucosae and on into the glandular mucosa. With the exception of the neural labeling in the Intermediate nerve layer, the overall labeling pattern was similar to the findings in the rat intestine. As in the rat and guinea-pig intestine, paravascular nerve fibers were evident alongside

the submucosal blood vessels. However, the nerve cell types (type I and II, and IV) intensely labeled in human tissue as compared to those cells labeled in the guinea-pig (types I, II and VI) and rat (types I and II) intestine indicate that type I and II cells are a consistent morphological type. This morphological diversity of nitrergic neurons has also been observed in the porcine intestine (Timmermans et al. 1994b).

In the guinea-pig (Costa et al. 1991b; Nichols et al 1992; Young et al 1992; McConalogue and Furness 1993), rat (Aimi et al. 1993; Nichols et al. 1993) and human (Timmermans et al. 1993; Timmermans et al. 1994a) intestine most of the NO synthase reactive nerve cells have been morphologically classified as type I neurons. The stained type I neurons and the dense NO synthase reactive fiber pattern in the circular smooth muscle layer as seen in this study provides strong morphological support for a mediator role of NO in NANC inhibition of human intestinal smooth muscle (Boeckxstaens et al. 1993; O'Kelly et al. 1993; Stark et al. 1993). Applied NO elicits membrane hyperpolarization and inhibits mechanical activity of the muscle in isolated preparation of the human jejunum (Stark et al. 1993). Electrical stimulation of NANC nerves in isolated preparations of human small (Stark et al. 1993) and large (Burleigh 1992) intestine is attenuated by hemoglobin (a scavenger of NO). Nitrergic neurons resemble heterogeneous morphologies including those of Stach type IV, of unknown function. This morphological diversity and extensive distribution and disposition of NO synthase neural elements suggests a functional diversity of nitrergic neurons in the human colon.

The finding of NO synthase activity in cells anatomically and morphologically reminiscent of ICC cells of the deep muscular plexus in this study supports previous studies in the canine colon. NO or an NO-related substance is released by isolated ICC cells and ICC cells of the canine intestine have been shown to display NO synthase immunoreactivity (Berezin et al. 1994). A current report indicates that NO release from ICC cells can lead to an increase in intracellular Ca^{2+} levels and therefore NO synthase activity in nearby ICC cells of the network, hence further stimulating NO release. This positive feedback mechanism on the ICC cells can perpetuate as a result of continued NO release and is proposed to amplify signals to the smooth muscle. In this way ICC cells are proposed to behave as intermediates in enteric inhibitory neurotransmission with NO as the mediator (Publicover et al. 1993). As in the guinea-pig and rat intestine, NO synthase activity was not observed in smooth muscle fibers of the human colon. These findings do not support the view that NO is produced and released from gastrointestinal smooth muscle cells (Grider et al. 1992; Berezin et al. 1994).

Stained ganglion cells were observed in Henle's Meissner's and the Intermediate nerve layers of the submucosa. This is not consistent with previous investigations of the nitrergic innervation patterns in the human colon which reported a lack of NO synthase positive nerve cell soma in Meissner's nerve layer and the Intermediate plexus (Timmermans et al. 1993; Timmermans et al. 1994a). Since the cell bodies of secretomotor neurons are found in submucous ganglia (Bornstein et al. 1988), these data support a role for NO as a mediator in intrinsic secretomotor reflex pathways of the gut. Indeed, recent reports indicate that in the rat colon NO donors stimulate intestinal secretion *in vitro* (Wilson et al. 1993) and this appears to be nerve mediated since the prosecretory response to NO is prevented by tetrodotoxin. Ganglia and interconnecting fasciculi of the submucosal nerve layers were found to be richly invested with intensely stained varicose fibres. A proportion of these fibres may be part of the myenteric modulatory fibres displaying NO synthase activity to be present to a fine network of fibres at the circular smooth muscle/submucosa interface in the colon. This network is a consistent feature of the histochemical studies of NO-related enteric innervation of the guinea-pig and rat large intestine and is proposed to serve as a link between the myenteric plexus and neuro-vascular networks of the submucosa, involved in the generation of slow waves (Christensen et al. 1987). Moreover, the submucosa contains an extensive and complex array of intrinsic and extrinsic nerve fibres (Wilson et al. 1981); therefore it is possible that a proportion of the nitrergic innervation of the submucosa and mucosa in the human infant colon that were detected in this and other (Timmermans et al. 1994a) studies are extrinsic in origin. In fact the mucosal nitrergic fibers observed in this study may be sensory afferents since NADPH diaphorase positive neurons have been detected in sensory ganglia (Aimi et al 1991).

Ultrastructural analysis of the guinea-pig myenteric plexus, indicates that NO synthase-positive nerve fibres make synaptic contacts with NO synthase reactive and non-reactive myenteric neurons (Llewellyn-Smith et al. 1992). The present study reveals NO synthase-reactive ganglionic fibres around labeled and unlabeled cells in all nerve layers of the human colon submucosa. Taken together, these findings suggest that in addition to it's neuroeffector action on the circular smooth muscle, NO may regulate the activity of both myenteric and submucous neurons in the human colon.

The extensive distribution of NO synthase related fibers within the nerve and muscle layers, together with the morphological variety of labeled neurons as seen in this study suggests there must be distinct subpopulations of nitrergic neurons within the myenteric and submucosal nerve layers of the human colon. In the rodent, nitrergic neurons and fibres occur

throughout the gut wall within all regions of the intestine. Moreover, the pattern of distribution of this innervation shows regional specificity. It is tempting to speculate that the human gut is also richly innervated by nitrergic elements, where like in the rodent, it may have important functions.

3.2. Identification of Enteric Nitrergic Neural Elements in the Human Colon: Co-distribution with the GABAergic Innervation

GABA is a putative transmitter of enteric interneurons in a variety of mammals (Krantis et al. 1981; Jessen et al. 1983; Ong et al. 1983; Tanaka 1985; Krantis et al. 1986; Roberts et al. 1993). It is not known whether these neurons use other transmitter substances, a common characteristic of enteric neurons. Even less is known about the presence and distribution of the enteric GABAergic system in the human, although GABA and its synthesizing enzyme glutamic acid decarboxylase (GAD) have been measured biochemically in the muscularis externa of the large intestine (Krantis et al. 1981; Jessen et al. 1983; Miki et al. 1983; Ong et al. 1983; Tanaka 1985; Krantis et al. 1986; Roberts et al. 1993).

According to pharmacological studies, a primary target for neurally released GABA in the mammalian gut is the cholinergic excitatory motor nerves, and the intrinsic NANC inhibitory neurons (Krantis et al. 1981; Kaplita et al. 1982; Maggi et al. 1991; Tsai et al. 1993). There is compelling evidence for NO to be released by NANC inhibitory neurons. The findings described in sections 2.1, 2.2 and 3.1 as well as other studies (referenced in these sections) indicate that NO synthase-related neurons and nerve fibres are distributed throughout the layers of the rodent and human gut wall. *We hypothesized that in addition to its proposed inhibitory motor neuron localization, NO may also be synthesized for use as a cellular messenger by other functional populations of enteric neurons, possibly including interneurons.* As enteric neural GABA is exclusive to interneurons, colocalizaton with NADPH diaphorase activity was examined.

Preliminary findings of this study were presented at the 1994 AGA Meeting. The final report was published in 1994 (Nichols et al. 1994).

3.2.1. Materials and Methods

Specimens used for the study described in section 3.1 were parallel processed for this investigation. Preparation of the cryosectioned tissues used in this study is described in section 3.1.1.

Slide mounted tissue sections were reacted for immunofluorescent demonstration of GABA-T. Sections were incubated in rabbit anti-GABA-T diluted (1:400) (personal gift from J.-Y. Wu) in PBS (pH 7.2) containing 0.3% Triton X-100 for 18 hours at 4^0C. Following a 15 minute wash in PBS, tissue sections were incubated in fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit (1:20) (Amersham, Ontario, Canada) diluted in the triton-PBS buffer for 40 minutes at 37^0C, washed, rinsed and coverslipped. Sections were then photographed using a Zeiss axioplan microscope and a 488 nm filter prior to histochemical demonstration of NO synthase-related activity. The histochemical reaction precipitate significantly quenches the GABA-T immunofluorescence.

Sections treated for GABA-T immunofluorescence and photographed were then reacted for histochemical identification of NO synthase-related NADPH diaphorase activity as previously described (section 3.1.1).

3.2.2. Chemicals

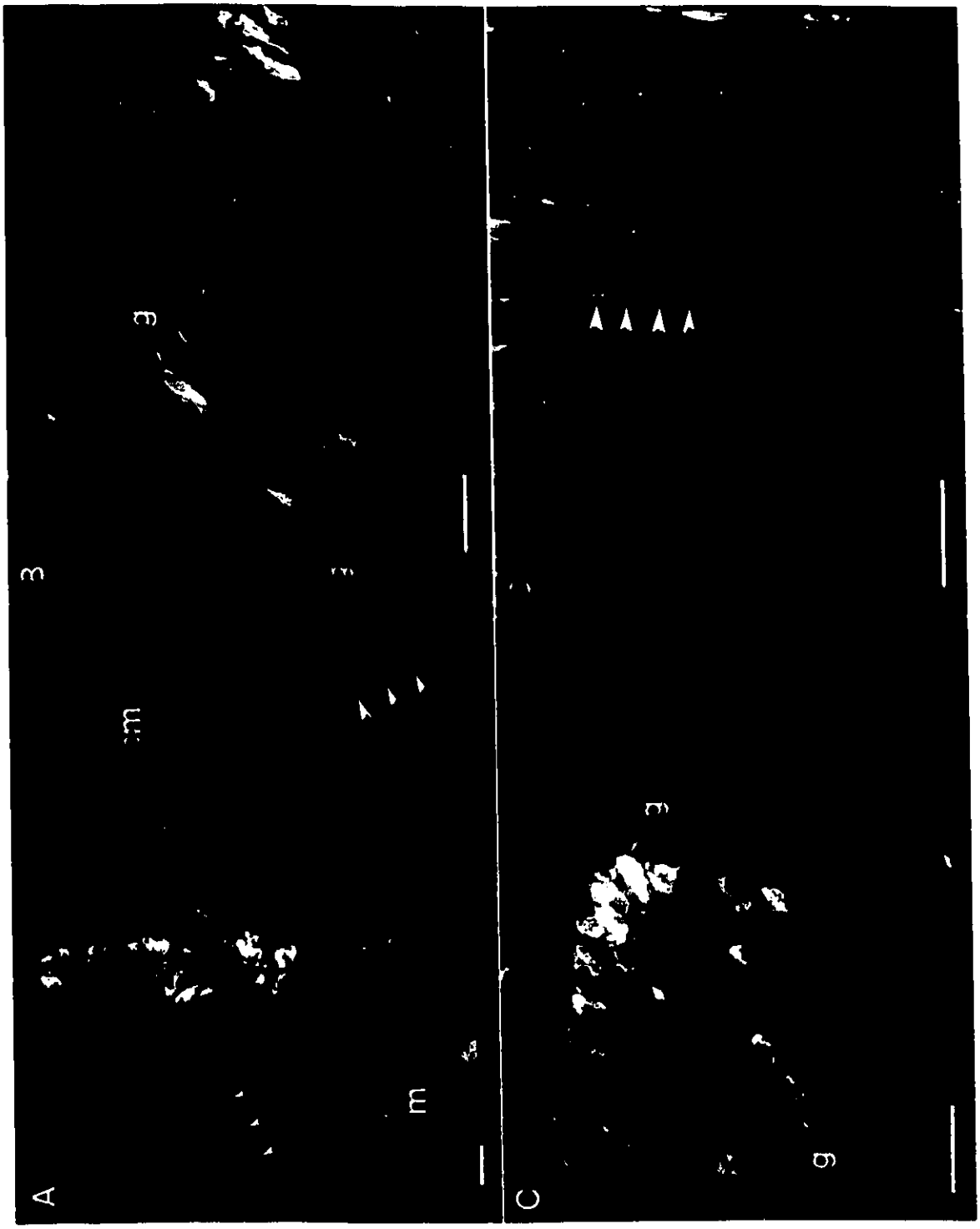
As in section 3.1.2.

3.2.3. *Results* - GABA-T Immunohistochemistry

In 12 μm fixed frozen sections of the human colon treated for GABA-T immunoreactivity (figs. 3.6 and 3.7), intense labeling was found in fine nerve fibre bundles of the longitudinal and circular muscle layers (fig. 3.6A and D) and in nerve cells and fibres of the myenteric (fig. 3.6A-C) and submucosal (fig. 3.7A-C) nerve layers. All ganglia contained GABA-T-immunoreactive (IR) cells with labeling confined to the cytoplasm. Cell nuclei were not labeled.

GABA-T-IR cells in the myenteric plexus were of moderate size with either elongated or small and round soma (figs. 3.6A-C). The elongated cells often had a single narrow process (fig. 3.6B and C). On occasion, very large round cell soma could also be seen (fig. 3.6C) displaying multiple processes. In any particular ganglion an average of 4.0 ± 1.0 GABA-T-IR cells were estimated, with no particular pattern or polarity of localization. Within the muscularis, GABA-T-IR nerve fibers were observed in both the longitudinal (fig. 3.6A) and circular (fig. 3.6A and D) smooth muscle, but there appeared to be a much more dense innervation in the circular muscle layer (fig. 3.6D). Within the submucosa, GABA-T-IR cells and fibers were noted in the ganglia of Henle's plexus, Meissner's plexus and a third separate group of ganglia located nearby the vascular network between Henle's and Meissner's plexuses (fig. 3.7A-C).

Figure 3.6. Axial sections of the myenteric nerve layer and circular smooth muscle layers of the human colon. (A) Intensely GABA-T immunoreactive nerve cells are present in a myenteric ganglion (G). Within the circular muscle (cm) intensely labeled fibre bundles (large arrowheads) are distributed throughout the muscle layer. In contrast, the longitudinal muscle layer (lm) is sparsely innervated (small arrowheads). (B andC) Higher magnification micrographs of myenteric ganglia showing intensely GABA-T immunoreactive cells (g). Labeled cell processes can be seen and the distinctvie elongate soma are evident. (D) Labeling in the circular muscle layer is extensively localized to nerve bundles. Scale bar = 100 μm.


A
m
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g
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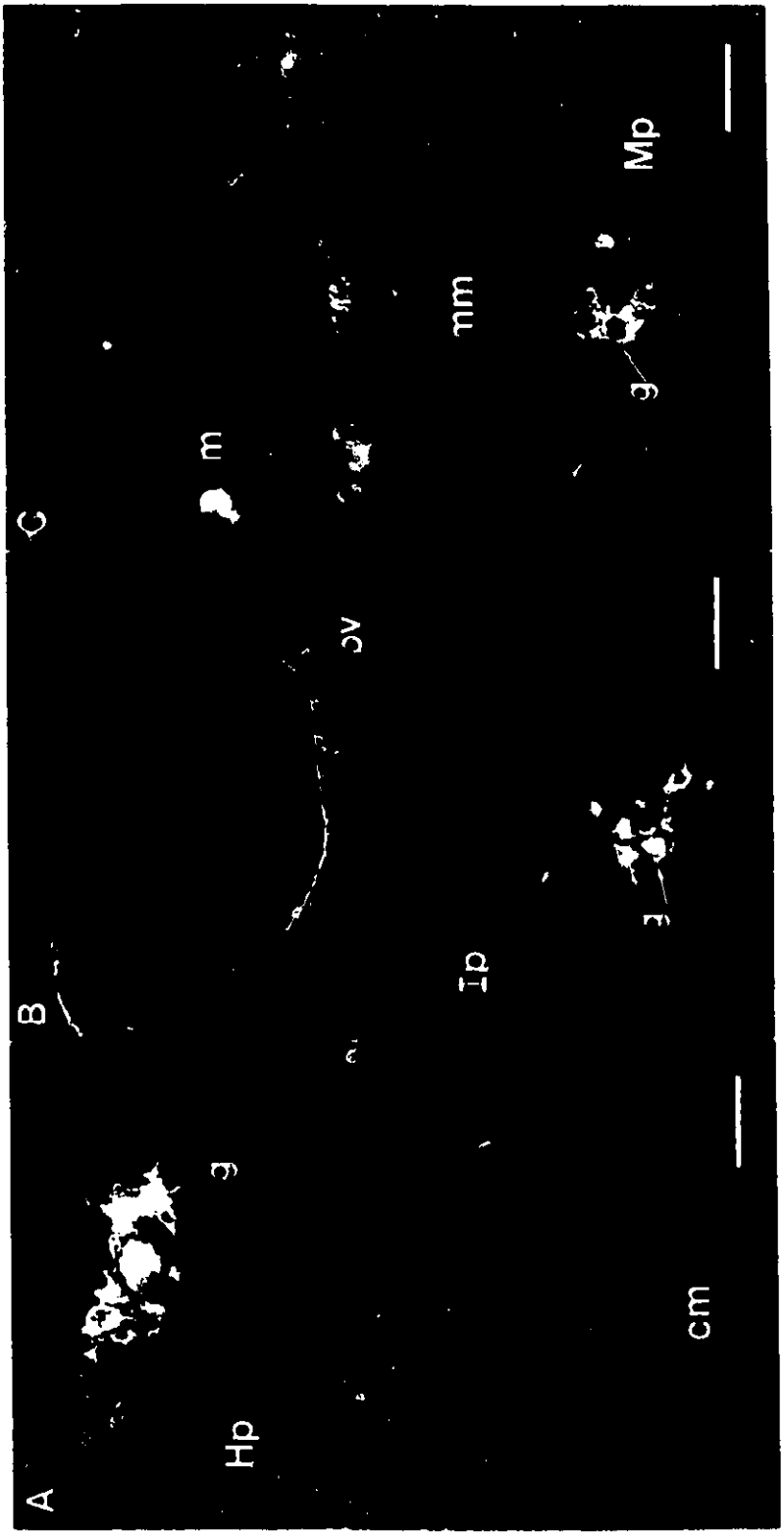
g
g

Figure 3.7. Micrographs of the human colon submucosa showing the Henle's plexus (Hp), Intermediate plexus (Ip) and Meissner's plexus (Mp). (A) Typical ganglion of Henle's plexus containing intensely GABA-T immunoreactive cells can be seen lying close to the circular muscle (cm). The labeled cells included both large (g) and small soma. (B) Ganglia located nearby the vascular plexus (bv) can be easily seen. These ganglia appear to belong to the Intermediate plexus (Ip) and contain intensely labeled nerve cells (g). (C) Labeled nerve cells (g) are also present in ganglia of Meissner's plexus (Mp) adjacent to the muscularis mucosae (mm) and mucosa (m). In the mucosa some faint but non-specific labeling is evident. Scale bar = 50 μm.


A
B
C
Hp
g
m
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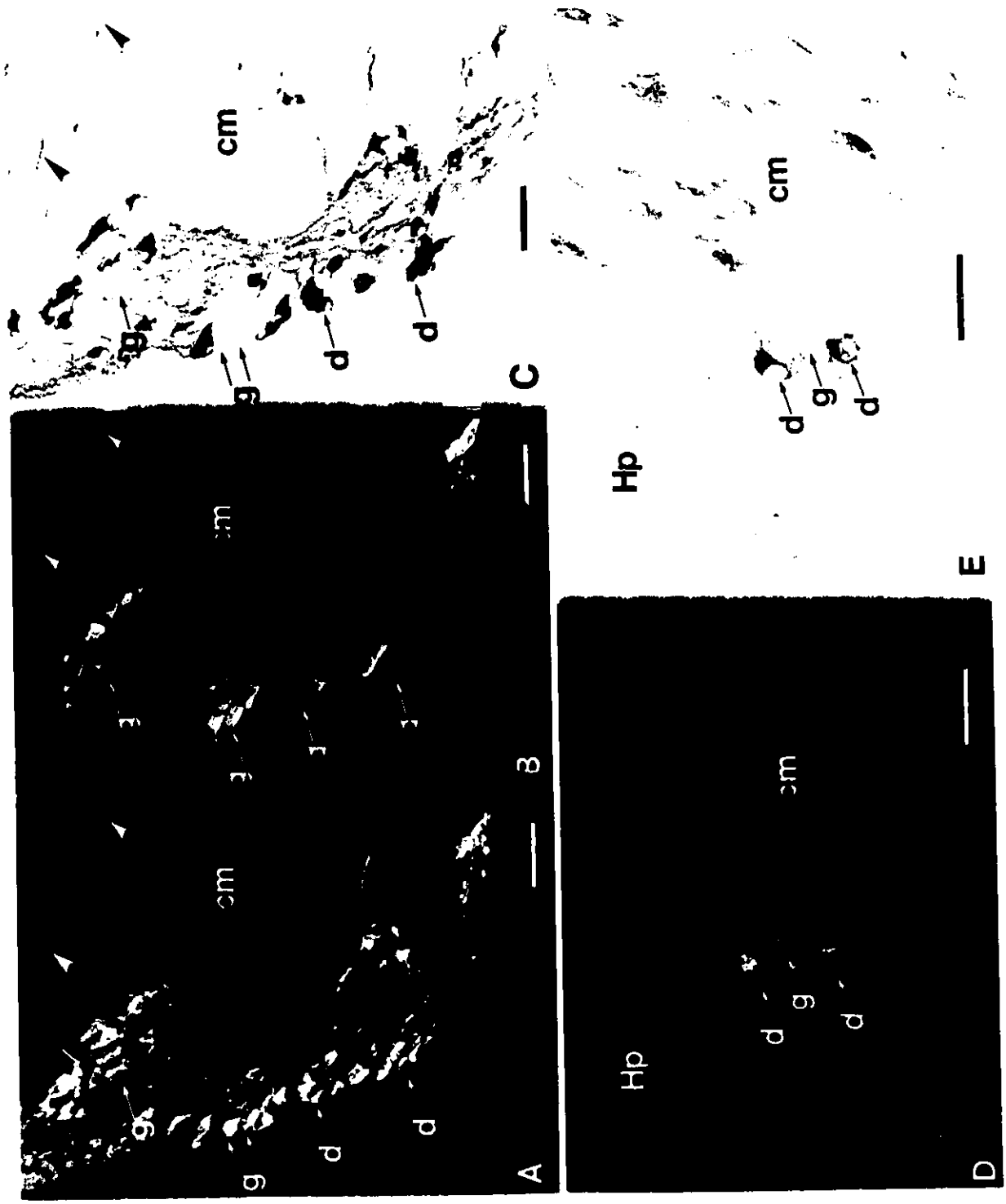
ip
nm
g
Mp
cm

Figure 3.8. Micrographs (A-C) showing a myenteric ganglion and smooth muscle treated for GABA-T immunohistochemistry and NO synthase-related NADPH diaphorase histochemistry. (A) Intensely GABA-T immunoreactive (IR) cells (g,d) and nerve fibres (large arrowheads) can be seen. Following NO synthase histochemical treatment this same tissue section showed a reduction in immunoreactivity. (B) Previously GABA-T-IR cells (d) could no longer be seen. (C) When viewed by light microscopy these same cells (d) were intensely stained for NO synthase-related NADPH diaphorase activity. Within this micrograph, the sites of cells displaying GABA-T immunoreactivity (g) were free of any NO synthase-related activity. (D and E) Representative micrographs of GABA-T immunoreactivity (D) and NO synthase-related activity (E) within a Henle's plexus (Hp) ganglion and the circular muscle (cm) layer. The cells demonstrating colocalization are labeled d. Scale bar = 100 μm.


cm
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This third group of ganglia likely represent the Intermediate plexus as described by Hoyle and Burnstock. It appeared that of these three, the Intermediate plexus showed a comparatively greater number of GABA-T-IR cells (cf. fig. 3.7A-C). Ganglionic nerve cells in each of the submucosal nerve layers were observed to be generally small and ovoid in appearance with centrally or eccentrically located nuclei (fig. 3.7A-C). An occasional ganglion of Henle's plexus possessed a large stout cell as seen in fig. 3.7A.

3.2.4. *Results* - NO Synthase Histochemistry and Colocalization

Selected tissue sections treated for GABA-T-immunoreactivity were decoverslipped and processed for visualization of NO synthase-related NADPH diaphorase activity. In these tissues, a subpopulation of myenteric neurons and innervation of the muscle layers, as well as neurons within a subpopulation of ganglia of the submucosa displayed intense NO synthase-related activity (fig. 3.8C and E). The largest proportion of stained nerve cells were apparent in the myenteric and Henle's plexi. These cells were either elongated or round, and labeling was present to the cytoplasm only (fig. 3.8C and E). Within the myenteric plexus 3.0 (± 0.1) cells per ganglion contained NO synthase-related activity. Of the 4.0 (±0.1) GABA-T-IR cells within a given ganglion, 1.0 (± 0.2) contained NO synthase-related activity (fig. 3.8A-C). Likewise, of the 3.0 (± 0.1) NO synthase-related cells within an individual ganglion, 1.0 (± 0.2) displayed GABA-T-immunoreactivity (fig. 3.8A-C). Similar colocalization of these two neurochemical markers was seen in Henle's plexus (fig. 3.8D and E).

3.2.5. Discussion

In the present study, GABA-T-IR neurons and nerve processes were found to be distributed throughout the muscularis externa and submucosal nerve networks of the human colon. This distribution of labeling correlates with that seen in the muscularis externa of the rat and guinea-pig intestine by high affinity GABA autoradiography and immunohistochemistry for GABA or its synthesizing enzyme glutamate decarboxylase (GAD) (North 1982; Jessen et al. 1983; Gilon et al. 1987; Hills et al. 1987; Oomori et al. 1992). However, unlike the rodent GABA-T immunoreactivity, nerve fibers in human gut were seen to extend to deep within the longitudinal muscle as seen with numerous other transmitter systems (Gabella 1979; Wattchow et al. 1988).

The enteric GABAergic system has been characterized as similar to that of the CNS (Erdo et al. 1986), with presynaptic high affinity uptake and enzymatic degradation being the

principle route of deactivation of neurally released GABA. In the brain, glial cells are associated with GABA synapses, and are involved in the removal of extraneuronal GABA via a high affinity transport system. In the glia, GABA is converted and shunted to the presynapse for resynthesis. GABA-T is the primary catabolic enzyme for the transmitter GABA in the brain and periphery. Although glia have been reported to display GABA-T activity (van Gelder 1965), such activity is low compared to that in GABAergic neurons (Nagai et al. 1983). In the present study, we found the immunoreactive ganglion cells to be intensely labeled, indicative of high levels of GABA-T, and hence enteric GABAergic neurons. These data are in accord with previous studies on GABA-T distribution in rodent gut using a GABA-T histochemical protocol (Krantis et al. 1986). The possibility that non-GABAergic cells including GABAceptive cells or glia possess sufficient levels of GABA-T to have contributed to the observed pattern of labeling is unlikely. Studies in the brain indicate that GABAceptive neurons do not localize GABA-T, at least in amounts detectable by histochemical techniques (Nagai et al. 1983). Furthermore, enteric glia, although more numerous than nerve cells, are small and do not possess any significant capacity for the high affinity uptake of GABA (Krantis et al. 1986; Gilon et al. 1987). It is now known that not all GABAergic neurons express significant levels of GABA-T in the cell soma. However, the evidence is strong that all neurons with GABA-T activity are in fact GABAergic (Nagai et al. 1983; Staines et al. 1984).

The discovery of GABA-T-IR nerve cells and fibres within established ganglionated nerve networks of the submucosa in this study of the human colon, supports the proposal for GABA to be a transmitter of submucosal neurons (Krantis et al. 1991a; Krantis et al. 1991b). Moreover, this identification of GABA-T immunoreactivity in subpopulations of neurons within myenteric ganglia provides additional evidence for the existence of a myenteric GABAergic system. Within the submucosa, both the Henle's and Meissner's nerve networks contained GABA-T-IR ganglion cells. A separate group of ganglia located more centrally within the submucosa appeared to be larger and contained more GABA-T-IR cells than those present in either Henle's or Meissner's networks. Their location is indicative of an Intermediate plexus as described by Hoyle and Burnstock (1989). These results strongly suggest that GABAergic cells are distributed within the three submucosal ganglionated nerve networks of the human colon.

Establishing the cellular morphology of the GABA-T-IR cells was difficult, since fine processes were not easily seen. However, the general shape of the GABA-T-IR cell somata could be discerned, which were either elongated or else small and round. All positive cells displayed labeling throughout their cytoplasm, but not the nuclei. This is consistent with the

findings of studies in the rat intestine (Krantis et al. 1986) and indicative of the mitochondrial localization of GABA-T (Nagai et al. 1983).

These findings also revealed that in the enteric system, GABA-T appears to be a good marker for GABAergic neurons since the distribution of GABA-T labeling correlates with that of other GABAergic markers e.g., GABA itself, GAD or high affinity GABA uptake (van Gelder 1965; Chan-Palay et al. 1979; Tanaka 1985; Krantis et al. 1986; Gilon et al. 1987; Hills et al. 1987; Furness et al. 1989; Krantis et al. 1989; Hope et al. 1991; Krantis et al. 1991a; Krantis et al. 1991b). The advantage of using GABA-T over GAD as a marker for GABAergic neurons is that the former is localized primarily in the cell body whereas the latter is concentrated in the nerve terminals (McLaughlin et al. 1975; Chan-Palay et al. 1979). Hence it is easier to demonstrate colocalization of GABA-T with other transmitter markers, e.g., NO than GAD and NO.

In the same gut tissue and within the same anatomical locations as GABA-T-IR neural elements distinct networks of NO synthase-related neurons and fibres were also identified. In contrast to the rodent (Aimi et al. 1991; Bredt et al. 1991a; Nichols et al. 1992; Young et al. 1992; Nichols et al. 1993; Nichols et al. 1994) the NO synthase-related cell labeling was rare while the fibre labeling was prevalent within Meissner's nerve layer of the submucosa. The extent of NO related innervation and the morphology of NO neurons observed was comparable to that seen in other histochemical studies of NO synthase-related NADPH diaphorase localization in the human colon (Timmermans et al. 1993; Timmermans et al 1994a; Vanderwinden et al 1993). NO synthase-related nerve cells and fibres appeared to occur in greater number than the GABA-T-IR elements. The presence of GABA-T-IR cells and fibres with NO synthase-related activity was an unexpected finding and raises important questions about the functional status of NO in the human gut wall and potentially other mammalian species. The cells colocalizing GABA-T immunoreactivity and NO synthase-related NADPH diaphorase activity represent only a portion of either the NO synthase-related NADPH diaphorase positive or the GABAergic innervations of the colon, suggesting some specialized function for these neurochemically defined cells. Indeed, the ganglia of the Meissner's and the Intermediate plexi contain more GABAergic cells than any cells displaying NO synthase-related NADPH diaphorase activity. These data suggest that GABA and NO may be mediators in intrinsic secretomotor reflex pathways of the gut, since the cell bodies of secretomotor neurons are found in submucous ganglia (Bornstein et al. 1988).

GABA actions in the rodent gut are well known and consistently show that GABA has no direct effects on the enteric smooth muscle (Erdo et al. 1986). Rather, GABA acts on receptors on ganglionic neurons, and is thus proposed to be a transmitter of enteric interneurons,

including those innervating the excitatory and inhibitory motor neurons. An obvious proposal as a result of these findings is that GABA-T-IR cells and cells colocalizing GABA-T/NO synthase-related NADPH diaphorase activity in the gut wall are these interneurons. Interestingly, GABA and NO synthase-related activity have been previously shown to colocalize within interneurons of the spinal cord (Valtschanoff et al. 1972). While it is possible that the cells displaying GABA-T and NO synthase-related activity may be motor neurons, there is no functional evidence to support this.

The notion that NO may be released by gut interneurons has not been widely considered. In contrast to GABA, NO exerts powerful and direct relaxant actions on gut smooth muscle and as such is generally considered to by synthesized and released by NANC inhibitory motor neurons (for review see Sanders et al. 1992)). The distribution of NO related cells in the myenteric plexus together with the rich investment of fibers in the underlying muscularis supports a neurotransmitter role of NO in motor neurons. The number and distribution of NO synthase-related neurons in the gut, in addition to the observation of dense NO synthase-related varicose fibers within ganglia strongly suggest that NO may be a transmitter of enteric interneurons (Aimi et al. 1991; Bredt et al. 1991a; Nichols et al. 1992; Young et al. 1992; Nichols et al. 1993; Nichols et al. 1994). Elegant proof of this has been provided by Young et al. (1992), showing that NO stimulates cGMP production within both myenteric and submucous ganglion cells. On this basis, it is highly unlikely that NO functions only as a transmitter/messenger of NANC inhibitory motor neurons. Investigations in this laboratory (Glasgow et al. 1993) indicate that treatment of anesthetized rats with an inhibitor of NO synthesis although reducing all gastric relaxant activity only inhibits certain components of the relaxant activity in the duodenum. The remaining duodenal relaxations were augmented. It has been proposed that there is more than one mediator of enteric NANC inhibition in the gut (Burnstock 1972; Furness et al. 1980; Manzini et al. 1986; Daniel et al. 1989; Bult 1990; Knudsen et al. 1991; Burleigh 1992; Kow et al. 1992; Sanders et al. 1992). For the rat, at least two different NANC systems occur in the small intestine, with ATP being the major NANC transmitter in the duodenum (Manzini et al. 1986). The augmented duodenal relaxations, could be explained if NO released from enteric neurons was modulating non-NO inhibitory motor neurons, perhaps those releasing ATP. This notion is strengthened by the results of the present study where, GABA-T positive cells and therefore by definition enteric interneurons, display NO synthase-related activity.

3.3. Identification of Enteric Nitrergic Elements in the Human Colon: Co-distribution with Neuropeptide Y Innervation

The results of section 3.2 provided for the first time, anatomical evidence for the potential for NO synthesizing capacity in human enteric interneurons. These findings also display the potential in using neurochemical identification of functionally different types of enteric neurons.

NO and NPY have a wide variety of similar biological actions in the gastrointestinal system, including modulation of food intake, blood flow, motility and secretion (McCulloch et al. 1987; Pique et al. 1989; Taylor 1989; Sheikh 1991; Kubes 1992; Kubes et al. 1992). The findings described in section 3.1 indicate that NO synthase-related neurons and nerve fibers are found throughout the submucosa of the human colon. As enteric neural NPY and NO synthase are found in secretomotor neurons of the rodent intestine (Costa et al. 1991a), *we hypothesized that NO may also be synthesized for use as a cellular messenger by secretomotor neurons in the human intestine*. To test this hypothesis colocalizaton with NADPH diaphorase activity was examined.

A preliminary account of the findings in this section were presented to the meeting of the Canadian Physiological Society (1993). A detailed report of these findings were published in 1994 (Nichols et al. 1994).

3.3.1. Materials and Methods

The specimens of human infant colon used in this study were processed in parallel and prepared for sectioning as described in section 3.1.1.

For immunohistochemical demonstration of NPY, sections were subjected to an 18-hour incubation at 4^0C in primary rabbit anti-NPY antisera (1:400) in PBS (pH 7.2) containing 0.3% Triton X-100, followed by a 15-minute wash in PBS (pH 7.2) and a 40-minute incubation at 37^0C in fluorescein isothiocyanate-conjugated donkey anti-rabbit antisera (1:20) diluted in the same buffer as the primary antibody. In the control preparations, the primary antiserum was preadsorbed with NPY. Sectioned preparations were then coverslipped using 90% glycerol in PBS containing 0.1 mmol/L phenylaminediamine. Using a Zeiss Axioplan microscope, preparations were photographed under fluorescence (488 nm filter) before histochemical demonstration of NO synthase activity because the reaction precipitate tends to quench the immunoreactivity. The position within the section was recorded using the microscope stage x- and y-axis coordinates.

Subsequent to NPY immunofluorescence histochemistry and photography, sections were treated for histochemical identification of NO synthase-related NADPH diaphorase activity as described previously (section 4.1.1). Briefly, colon sections were incubated in 1mmol/L NADPH, 0.5mmol/L nitroblue tetrazolium, and 0.3% Triton X-100 in 10 mmol/L PB (pH 8.0) for 30 minutes. Omission of NADPH served as an appropriated control. After several washes in PB (pH 7.2) to remove excess precipitate, the sections were photographed under bright-field conditions at the recorded coordinates.

3.3.2. Chemicals

Rabbit anti-NPY antibody was purchased from Peninsula Laboratories. Fluorescein isothiocyanate-conjugated donkey anti-rabbit antisera was obtained from Amersham (Ontario, Canada). All buffer materials were purchased from BDH (Ontario, Canada). Nitroblue tetrazolium and ß-NADPH were obtained from Sigma (St. Louis, MO).

3.3.3. *Results* - NPY Immunohistochemistry

Preadsorption of antiserum with NPY resulted in the absence of any positive immunoreactivity. NPY-immunoreactive (IR) nerve cells and fibers were present throughout the nerve and muscle layers of the colon, respectively (figs. 3.9, 3.10. 3.11). The NPY-positive fiber innervation of the circular muscle was extensive with a particular concentration of single fibers (fig. 3.9C) and fiber bundles (fig. 3.10A). Both nonvaricose (fig. 3.9C) and varicose (fig. 3.10A) single NPY-IR fibers were observed within the circular muscle. The NPY-IR innervation of the longitudinal muscle layer (not shown) was sparse, and, for the most part, these fibers appeared to be nonvaricose.

NPY-IR fibers (primarily varicose) were present in ganglia throughout the myenteric plexus. Ganglia showing NPY-IR cells were rare. We observed a total of 17 NPY-IR cells counted in 130 myenteric ganglia (Table 3.1). The positive myenteric ganglia contained up to four reactive cells, as shown in Figure 3.9A. NPY-IR cells were either elongated with stout polar dendritic processes and centrally located nuclei, ovoid, or adendritic with eccentrically located nuclei (fig. 3.9A).

Within the submucosa, three ganglionated nerve layers, as described by Hoyle and Burnstock, were apparent: (1) a nerve layer topographically situated within the submucosa adjacent to the circular muscle layer, the so-called Henle's or Schabadasch plexus; (2) another distinct ganglionated network lying close to the muscularis mucosae, the Meissner's plexus; and

(3) a third layer more centrally located but closer to Meissner's plexus in the vicinity of the fine submucosal blood vessels, the intermediate plexus. Within these nerve networks, a small proportion of the ganglia contained nerve cells intensely immunoreactive for NPY. Of 223 submucosal ganglia observed, a total of 91 NPY-IR cells were estimated (Table 3.1). These submucosal cells were mostly round or oval, absent of dendritic processes, and had unlabeled, eccentrically located nuclei which are morphologically typical of a Dogiel or Stach type II nerve cell (figs. 3.10C and 3.11A). Within Henle's plexus only, there were large NPY-IR cells with radially arranged dendritic processes around the cell soma, typical of a Dogiel or Stach type I morphological description (fig. 3.10A). In this plexus, ganglia displayed NPY-IR varicose fibers (fig. 3.10A). In the Intermediate and Meissner's plexi, most labeled cells could be identified as either ovoid or elongated with eccentrically located nuclei (figs. 3.10A and 3.11A). Varicose NPY-IR fibers were found within the ganglia of each of these submucosal nerve layers and were more prevalent in Meissner's plexus.

The muscularis mucosae contained a rich innervation of NPY-IR varicose fibers (fig. 3.11E). Some of these fibers could be seen branching into the mucosa. More prominent within the mucosa was the presence of a large number of NPY-IR endocrine cells at the base of the crypts and in the mucosal epithelium (fig. 3.11E).

3.3.4. *Results* - NO Synthase Histochemistry and Colocalization

Preparations in which NADPH was omitted from the reaction showed no blue formazan precipitate. Virtually all layers of the human colon wall exhibited NO synthase-related elements. A scant distribution of NO synthase-related nerve fibers in the longitudinal smooth muscle layer (not shown) contrasted with the prevalent NO-related fiber innervation of the circular smooth muscle layer (figs. 3.9D and 3.10B). As with the NPY-IR innervation of the circular smooth muscle, both nonvaricose and varicose fibers containing NO synthase-related NADPH diaphorase activity were observed (figs. 3.9D and 3.10B, respectively). No synthase-reactive nerve fiber bundles were also observed (figs. 3.9D). All ganglia of the myenteric plexus displayed NO synthase-related nerve cells (figs. 3.9B) and fibers. A total of 184 NO synthase-related NADPH diaphorase-positive cells were estimated from a sample population of 130 myenteric ganglia (Table 3.1).

Cells displaying NO synthase-related activity were found in ganglia of Henle's plexus, Intermediate nerve layer, and Meissner's plexus. Within Henle's nerve layer, NPY-IR type I cells were commonly seen (fig. 3.10B). In ganglia of the Intermediate and Meissner's

Figure 3.9. Axial sections of the myenteric plexus and circular muscle layers from the human colon. (A) An intensely fluorescent (NPY-immunoreactive) elongated cell (large arrow) is present in the ganglion (G). (B) Bright-field micrograph of A. The NPY-immunoreactive elongated cell of A is also positive for NO synthase-related activity. (C) A rich innervation of NPY-IR fibre bundles (arrowheads) within the circular muscle is evident. (D) Bright-field micrograph of C; codistribution of NO synthase-related elements within the circular muscle (arrowheads). Scale bar = 50 μm.

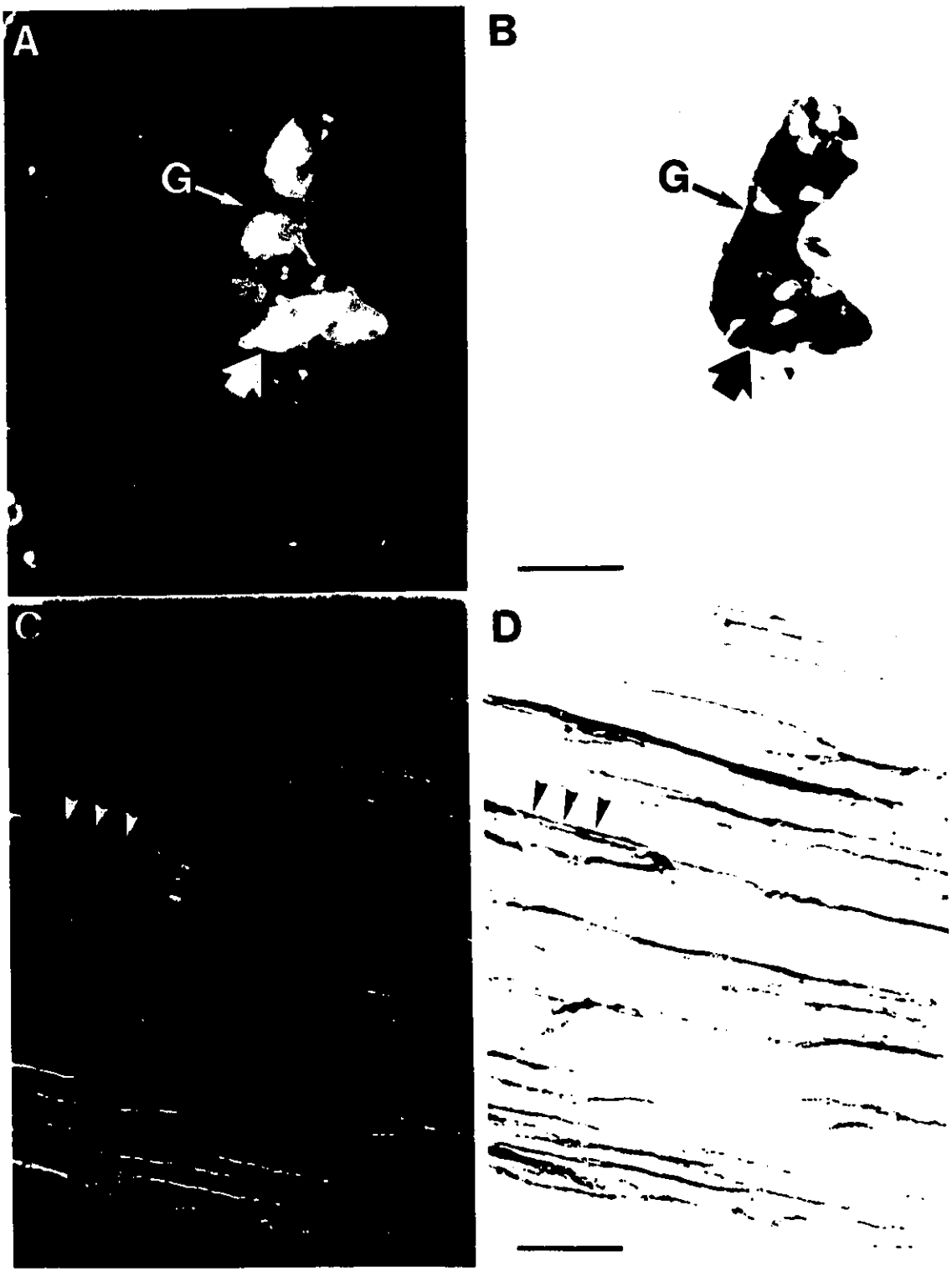
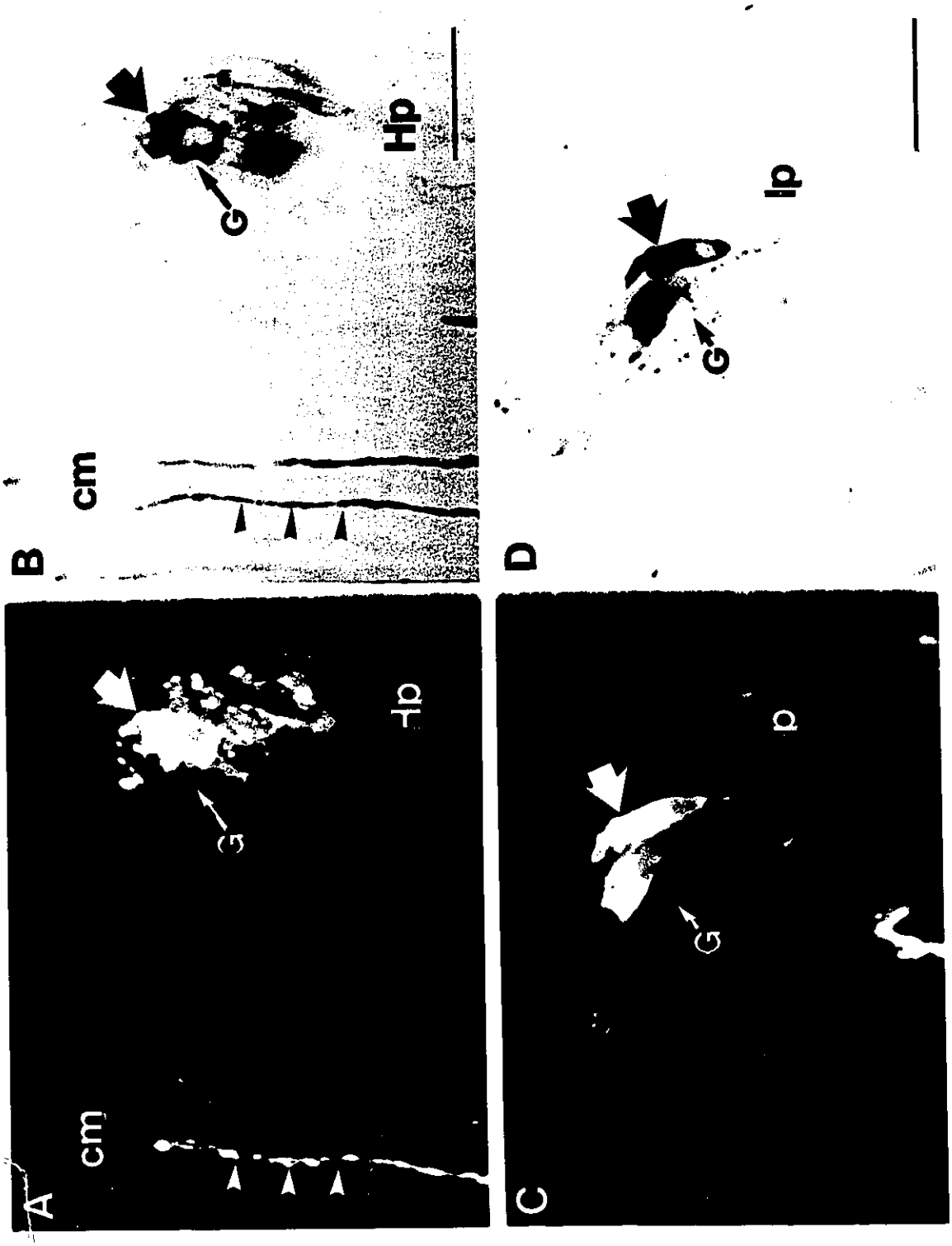

A
G
B
G
C
D

Figure 3.10. Micrographs showing the circular muscle and underlying Henle's nerve plexus. (A) Immunofluorescence micrograph showing NPY-immunoreactive nerve fibres (arrowheads) of the circular muscle (cm). A large, intensely labeled, type I ganglion cell (large arrow) is present in a ganglion of Henle's plexus (Hp). (B) Bright-field micrograph of A showing the nerve fibres (arrowheads) and large ganglion cell (large arrow) with NO synthase-related activity. (C) Immunofluorescence micrograph showing a ganglion containing two NPY-immunoreactive cells. Ip, Intermediate plexus. (D) In the light micrograph presentation of C, the same cells show NO synthase-related activity. One of these cells is distinctly elongated (large arrow). Scale bar = 50 μm.


A
cm
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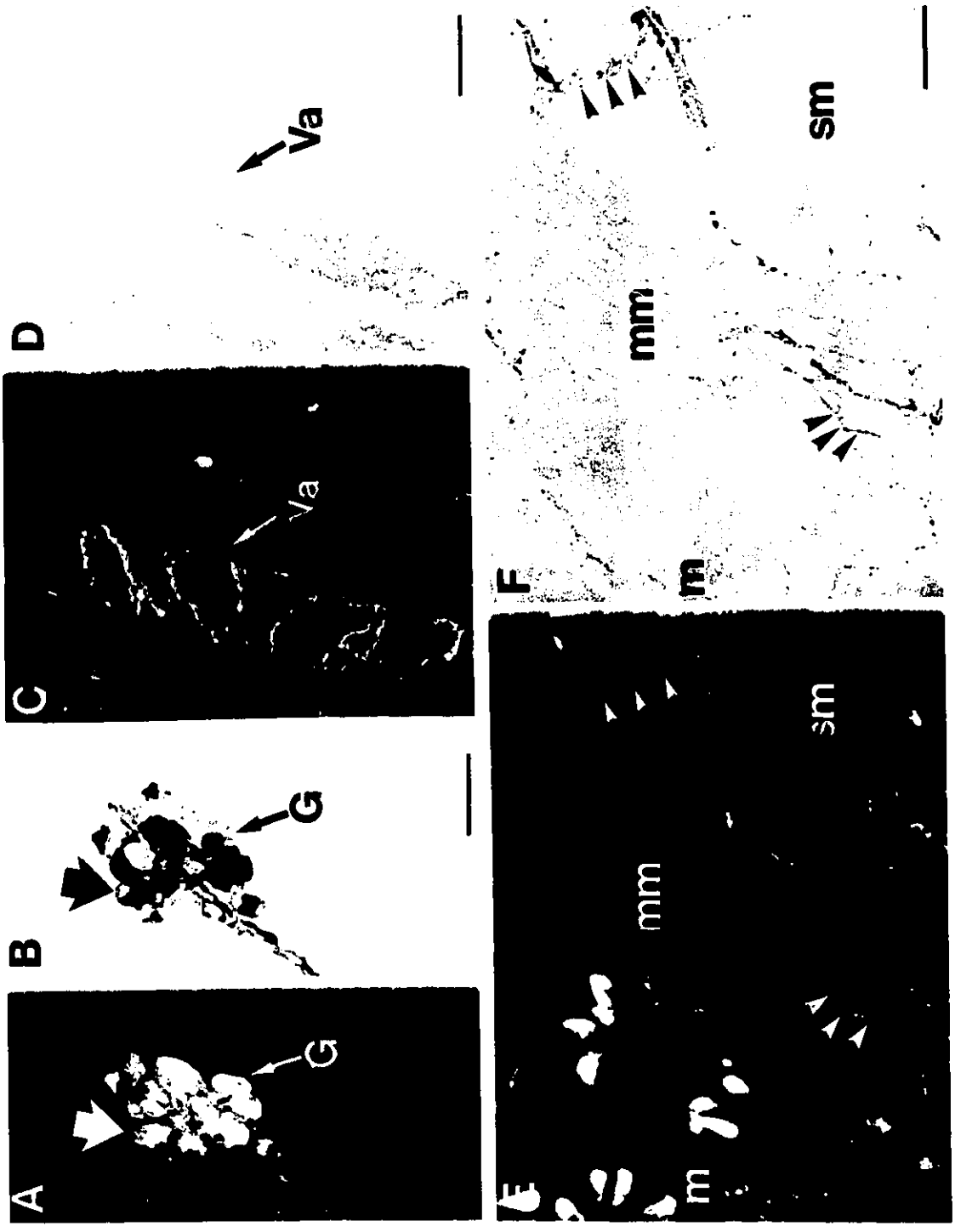
lp

Figure 3.11. Micrographs showing the submucosa, muscularis mucosae, and mucosal layers. (A) Immunofluorescent micrograph showing a ganglion containing many NPY-immunoreactive nerve cells. (B) Bright-field micrograph of A showing the same ganglion containing NO synthase-related cells. One of the nerve cells colocalizing NO synthase-related activity is indicated (large arrow). (C) Immunofluorescent micrograph showing NPY-immunoreactive perivascular innervation of a submucosal arteriole (Va). (D) Bright-field micrograph of A showing an obvious absence of NO synthase-related perivascular fibres. (E) NPY-immunoreactive nerve fibres in the submucosa (sm) and muscularis mucosae (mm) can be seen. In addition, intensely labeled small endocrinelike cells are present in the mucosa (m). (F) Bright-field micrograph presentation of E, showing colocalization of NO synthase-related activity in nerve fibres only. Scale bar = 50 μm.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E
mm
m
sm
F
mm
m
sm

plexi, only a type II morphology could be distinguished as NO synthase positive (figs. 3.10D and 3.11B). Not all submucosal ganglia showed NO synthase-related activity. Of 223 observed submucosal ganglia, 75 NO synthase-related cells were estimated (Table 3.1). Finally, NO synthase positive fiber innervation was evident in the muscularis mucosae (fig. 3.11F).

Myenteric and submucosal ganglia and nerve bundles of the circular smooth muscle and muscularis mucosae contained elements colocalizing NO synthase activity and NPY immunoreactivity. Within ganglia of the myenteric plexus (fig. 3.9A and B), Henle's nerve layer (figs. 3.10A and B), Intermediate nerve layer (figs. 3.10C and D and 3.11A and B), and Meissner's Plexus (not shown), nerve cells colocalized NPY-IR and NO synthase activity. Of 130 observed myenteric ganglia, 9 of 17 NPY-IR cells (53%) also showed NO synthase-related activity (Table 3.1). Within the 223 observed submucosal ganglia, 16 of 91 NPY-IR cells (18%) were also positive for NO synthase-related NADPH diaphorase activity (Table 4.1). Within the circular smooth muscle (figs. 3.9C and D and 3.10A and B) and muscularis mucosae (figs. 3.11E and F), an extensive codistribution of NPY and NO synthase-reactive innervation was observed. In contrast, perivascular innervation and mucosal endocrine cells positive for NPY did not colocalize NO synthase-related activity and NPY immunoreactivity (figs. 3.11C-F). A summary of the anatomic findings is shown in Table 3.2.

Table 3.1. Total Counts of NPY-IR Cells, NADPH Diaphorase-Positive Cells, and Cells Colocalizing NPY and NADPH Diaphorase Activity

	Myenteric plexus			Submucosa		
	NPY	NADPH diaphorase	NPY and NADPH diaphorase	NPY	NADPH diaphorase	NPY and NADPH diaphorase
Total no. of cells	17	184	9	91	75	16
Total no. of ganglia	130	130	130	223	223	223

NOTE. Measurements were obtained from a sample population of 130 ganglia from the myenteric plexus and 223 ganglia from the submucosa in 30 cross sections.

Table 3.2. A Semiquantitave Assessment of Cell and Fiber Distributions Showing NPY immunoreactivity Alone, NO synthase Activity Alone, or Coexistence of NPY immunoreactivity and NO Synthase Activity.

	LM fibers	MP cells/fibers	CM fibers	Hp cells/fibers	Ip cells/fibers	Mp cells/fibers	MM fibers
NPY	+	+/++	+++	+/++	+++/+	++/+++	++
NO synthase	++	+++/+++	+++	++/+	++/+	+/++	++
NPY/NO synthase	+	+/+	+++	+/+	++/+	+/++	++

CM, circular muscle; Hp, Henle's plexus; Ip, Intermediate plexus; LM, longitudinal muscle; MM, muscularis mucosae; Mp, Meissner's plexus; MP, myenteric plexus. +, ++, +++ indicate the amount of labeling observed from least to most.

3.3.5. Discussion

Colocalization of NPY immunoreactivity in neural elements displaying NO synthase-related activity was observed in the muscularis externa, myenteric plexus, submucosa, and muscularis mucosae of infant sigmoid and right transverse colon. NO synthase-related activity was absent in the NPY-positive perivascular innervation and mucosal endocrine cells. The distribution of NPY-positive endocrine cells in the mucosa was qualitatively similar to that previously reported (Kawana et al. 1990). These data indicate a substantial codistribution of NPY-IR and NO synthase-related activity within all nerve layers in the wall of the human infant colon and, in particular, in a proportion of the NPY-IR submucosal neurons, suggesting that NO synthesis potential may also exist in secretomotor neurons. This is not surprising given the extensive overlap of function of these two substances. Both NPY and NO exert inhibitory actions; however, NPY is understood to interact with neurons, whereas NO can relax smooth muscle directly (Sanders et al. 1992). NO, like NPY, may function as an inhibitory transmitter of interneurons (Glasgow et al. 1993). both NPY and NO exert secretomotor actions (McCulloch et al. 1987; Kubes 1992). Within the submucosa, NPY is indicated to be localized to cholinergic secretomotor neurons (Costa et al. 1991a).

Kawana et al. (1990) described NPY-IR ganglion cells within the human colon submucosa and myenteric nerve layers as either round or elongated and as more numerous in the submucosa. Our findings confirm those of Kawana et al.(1990). In addition, we identified two distinct cell morphologies (type I type II) for NPY-IR ganglion cells and further determined that

NPY-positive cells in the submucosa are present in Henle's, Meissner's, and the Intermediate nerve networks. These cells appeared qualitatively more abundant in Henle's plexus. Overall, the type II cells appeared to be more numerous within cells colocalizing NPY-IR and NO synthase activity. Costa et al. (1991) propose that type I cells are motor neurons, whereas type II are sensory in function. It is intriguing that type I neurons were found to colocalize NPY and NO synthase-related activity in Henle's plexus because it has been proposed that this plexus is primarily a motor plexus, providing the interface between the myenteric plexus and the submucosa (Hoyle et al. 1989). It is interesting to note that in a study of the adult human sigmoid colon, a complete lack of NPY-IR cells within the submucosa has been reported (Crowe et al. 1992). This may indicate a developmental change in the neurochemistry of human enteric neurons. Moreover, an extensive codistribution of NPY and NO in the human intestine has not been observed the rodent intestine (Nichols et al. 1993). Instead, in the guinea-pig (Grider et al. 1993) and ferret (Dey et al. 1993) gastrointestinal tract, NO-synthesizing neurons colocalize VIP. Parallel findings have been noted in gallbladder neurons in which VIP, but not NPY, colocalized with NO synthase in guinea-pigs but not humans (Talmage et al. 1993). These findings suggest that functionally equivalent neurons of the rodent intestine may differ chemically in the human intestine.

It is proposed that a large proportion of NPY nerve fibers in the human colon represent extrinsic splanchnic innervation, including the perivascular NPY nerve fibers (Holst et al. 1989). Extrinsic NPY fibers are also reported to be derived from the pelvic plexus (Kawana et al. 1990), including parasympathetic preganglionic and a few sympathetic postganglionic nerve trunks. In their study of the infant colon, Kawana et al. (1990) distinguish two sources of extrinsic NPY fibers: one associated with perivascular nerves and another arising from nerve fasciculi. In the present study, it was not possible to determine which of the nerve fibers in the muscularis externa and the muscularis mucosae originated from outside the gut. However, the lack of any NO-related labeling of perivascular fibers strongly suggests that sympathetic nerves innervating the gut do not synthesize NO. This finding is supported by McConalogue and Furness (1993) where lesion of extrinsic sympathetic fibers to the guinea-pig colon had no effect on intramural NADPH diaphorase positive fiber labeling. Moreover, the lack of any NO innervation of the colonic blood vessels further supports the notion that any vascular actions of NO in the gut are most likely related to its endothelium-derived relaxing factor properties (Moncada et al. 1989). This is consistent with our findings (Nichols et al. 1992; Nichols et al. 1993; Nichols et al. 1994) that show the presence of NO synthase-related activity within discrete intracellular deposits localized in the blood vessel wall of the rodent and human gut.

Chapter 4

4.1 The Nitrergic Innervation in Patients with Hirschsprung's Disease

In 1888, Harold Hirschsprung provided the first pathological report of a disease, which included such characteristics as impaired intestinal motor function, progressive abdominal distension and which appeared to be fatal in two cases (Hirschsprung 1888). Analysis by autopsy revealed grossly distended large distal bowel due to an obvious mechanical obstruction. Hirschsprung named the disease *congenital megacolon* otherwise known as Hirschsprung's disease (HSCR), which is grossly recognized by a markedly dilated colon proximal to a narrowed (stenotic) distal colonic segment.

Histological investigations of the large intestine from patients with HSCR revealed the absence of ganglionic cells in the affected segment of colon (Meier-Ruge 1974) and an orally situated hypoganglionic region spanning the dilated segment (transition zone) which eventually gives rise to a normoganglionic region proximal to the dilatation. In addition, in positions normally occupied by the myenteric ganglia and submucous ganglia, thick bundles of nerve fibers were found that were not present in normal colon (Whitehouse et al. 1948). The apparent absence of ganglionic cells as could be determined by general cell histological stain, brought forth a new term for the disease to indicate that there is a characteristic 'aganglionic' segment.

Segregation analyses reveal that HSCR has an estimated population incidence of 1/5000 live births and a sibling recurrence risk of 4%, with males being 3.5-4.0 times more likely to be affected (Meier-Ruge 1974; Badner et al. 1990). This male/female ratio has been reported to drop with the length of the aganglionic segment and is approximately 1:1 in aganglionosis of the entire colon (Meier-Ruge 1974). Although the rectum and lower sigmoid are the portions of the bowel most involved, there are some cases where the entire large intestine is aganglionic. On this basis HSCR has been anatomically divided into two types. Short - segment HSCR which involves aganglionisis in only the rectum and rectosigmoid colon accounts for approximately 80% of all cases. The long - segment form of the disease involves variable lengths (region) of the proximal bowel.

Interestingly the 'aganglionic' character of the diseased segment, fails to account for all aspects of HSCR including the variability in the clinical presentation, and the lack of correlation between the severity of the symptoms and the length of the 'aganglionic' segment.

These facts suggest, that factors other than the absence of nerves are also involved in the etiology of this disease.

Histochemical and ultrastructural analyses reveal that the circular muscle and muscularis mucosae are ramified more extensively with adrenergic and cholinergic nerves than that of the normal bowel (Garrett et al. 1969) and the density of the cholinergic fiber innervation was found to be associated with the severity of the obstruction apparent with the disease. Although originally described as hypertrophic (Whitehouse et al. 1948), these autonomic fiber bundles were found to be ultrastructurally normal (Howard et al. 1970). The increased intramural cholinergic nerve fiber innervation of the aganglionic region is considered to be extrinsic parasympathetic, and fibres projecting from adjacent ganglionated regions of the colon. There is also a proliferation of extrinsic sympathetic adrenergic nerve fibers in HSCR affected bowel (Hanani et al. 1989).

A number of immuno- and histocytochemical studies using nerve-specific antibodies, antibodies to enteric neuropeptides and NADPH diaphorase staining associated with NO synthase activity, have attempted to further characterize the anatomical and neurochemical nature of the innervation of the aganglionic bowel. These studies reveal a disorganized pattern and apparent reduction of peptidergic nerves containing somatostatin, vasoactive intestinal polypeptide (VIP), enkephalin (ENK), substance P (SP) and calcitonin gene-related peptide (CGRP) (Bishop et al. 1981; Larsson et al. 1983; Tsuto et al. 1985; Hirose et al. 1989; Tsuto et al. 1989). The source of these peptidergic nerve fibers in the aganglionic bowel is unknown but are proposed to originate from anally projecting neurons (i.e. VIP, ENK) proximal to the aganglionic region, or from sensory afferent innervation of the gut (i.e. SP and CGRP) (Hanani et al 1989). A complete loss of NO synthase reactive neurons and fibers in the aganglionic bowel have been reported by independent groups (Vanderwinden et al. 1992; Kobayashi et al. 1994; O'Kelly et al. 1994). In contrast to these findings, neuropeptide Y (NPY) fiber innervation is elevated (Hamada et al. 1987; Larsson et al. 1988). This may be explained by the fact that NPY is colocalized in extrinsic sympathetic noradrenergic nerves found in the aganglionic bowel (Hamada et al. 1987).

The obstruction associated with HSCR is characterized by the absence of normal peristalsis and constriction in the diseased segment. Functional analyses of HSCR using isolated preparations of muscle strips from aganglionic and 'normoganglionic' segments of colon confirm anatomical observations. Electrical stimulation of neurons in normoganglionic segments elicited relaxation followed by contractions and contractions were blocked by atropine (Hanani et al.

1986; Larrson et al. 1987; Okamoto et al. 1987). The same treatment of aganglionic segments revealed only strong contractions in the absence of atropine and relaxations in the presence of atropine (Hanani et al. 1986). Since relaxation responses could be elicited (in the absence of extrinsic sympathetic input which causes relaxation of the gut muscularis), the NANC inhibitory motor nerves were assumed to be functional in the aganglionic bowel. Others have reported a lack of NANC relaxation responses in isolated segments of aganglionic bowel (Kubota et al. 1983). Moreover, the release of acetylcholine is significantly greater in aganglionic bowel compared to ganglionic bowel and elevated levels of acetylcholine have been measured in the colon of Hirschsprung's patients (Ikawa et al. 1980). Thus cholinergic nerves are functional and appear to release abnormally high levels of acetylcholine. Enteric cholinergic nerves exert excitatory actions while the enteric NANC neurons and extrinsic sympathetic nerves provide the inhibitory innervation of the enteric smooth muscle (Furness et al. 1987). An increase in the activity of cholinergic nerves and/or a decrease in the NANC inhibitory activity can result in contraction of the gut muscularis. This is the premise which is proposed for the obstruction which occurs in HSCR. However while the cholinergic and NANC neurons receive somatic inputs, cholinergic neurons also receive inhibitory inputs to their nerve endings which modulates cholinergic activity by inhibiting acetylcholine release. These inhibitory neural inputs include nerves releasing NPY, noradrenaline, ENK, GABA and NO.

Constriction of the aganglionic bowel is also proposed to be due to an increased sensitivity of the intestinal muscles as a result of the absence of normal innervation; termed denervation hypersensitivity. The elevated acetylcholine levels would then evoke stronger contractions in the hypersensitive muscle. A normal response to acetylcholine or its muscarinic analogues in HSCR has been reported by several independent groups (Kamijo et al. 1953; Penninckx et al. 1975; Hanani et al. 1989). Taken together these findings indicate that the dysmotility associated with HSCR is more likely neurogenic rather than myogenic. Clearly, a mechanical obstruction exists in the aganglionic bowel which has been described as 'a state of spastic contraction (Ehrenpreis 1970).

Although the cellular and molecular origins of aganglionosis are unclear, there are several hypotheses regarding the etiology of the disease. It is proposed that HSCR is a developmental defect arising from a failure of neural crest cell migration, colonization or differentiation in situ. Alternatively, a vascular etiology has been proposed involving colonic ischemia leading to ganglion cell death (Earlam et al. 1972).

HSCR was hypothesized to be a congenital disease caused by a failure of neural crest cell migration to the hindgut and/or a failure of crest cells to differentiate into enteric neurons as determined by the enteric microenvironment. Since neuroblasts (neural crest cells) of the vagus nerve migrate to the intestinal wall from the 7th to the 12th week, aganglionosis of the distal colon was hypothesized to result from a late defect of neuroblast migration, colonization or differentiation (Meier-Ruge 1974; Gershon 1989). Considering that enteric neurons are phenotypically diverse and the ENS possesses a unique structure subsequent studies attempted to determine whether the fates of neural crest-derived emigres are i) predetermined (programmed) or ii) their migration, colonization and differentiation are influenced by the microenvironments encountered as they migrate, or as they develop in the wall of the gut.

That the enteric environment may be abnormal in patients with HSCR comes from observations in a mouse model of HSCR, the lethal spotted (*ls/ls*) mutant mouse. *ls/ls* mice display all the classical features of HSCR including congenital megacolon secondary to aganglionosis coli. Examination of the aganglionic region in *ls/ls* mice reveals that the muscularis mucosae is abnormal, much thicker than the more proximal bowel. There is the same number of cells per unit length of tissue as in the controls, but the individual muscle cells of the abnormal tissue are hypertrophic and the spaces between cells are widened (Tennyson et al. 1986). Biological analysis of this mouse model of HSCR has revealed failure of neural crest cell migration, secondary to a defect in cell adhesion or failure of colonization of neural crest cells in the gut due to a failure of differentiation (Webster 1973; Jacobs-Cohen et al. 1987; Gershon 1989). Co-culture explant studies have revealed that crest-derived cells from non-mutant sources are unable to colonize the aganglionic region of the *ls/ls* mouse bowel (Jacobs-Cohen et al. 1987). Likewise, crest-derived cells from *ls/ls* mice are unable to colonize control mouse, chick or quail gut (Rothman et al. 1986). Immunocytochemical analysis of the developing *ls/ls* bowel reveals that the basal laminae components, laminin and type IV collagen, are profuse and disorganized in the aganglionic region (Gershon 1989). These matrix or adhesion materials appear to lie in the path that the migrating crest-derived cells normally take to colonize the distal colon. These components of the basal laminae are proposed to activate receptors on migrating crest-derived cells, and stop migration. Since these receptors are expressed by crest-derived cells after they colonize the gut, it is proposed that it is the overproduction of matrix factors of the enteric (vs migrating) microenvironment which prematurely activates these receptors and prevents colonization and differentiation (ibid).

From these findings, HSCR was considered to have a genetic etiology. Support for this is provided in part by animal models for HSCR, in the mouse, rat and the horse, where in each of these cases, a single-gene mutation is the cause of aganglionic megacolon (Lyon et al. 1989; McCabe et al. 1990). It has been found in the mouse that three independent loci each lead to aganglionosis. These include piebald-lethal (s^l) and lethal-spotting (*ls*) and dom spotting (*Dom*) (Lane 1966). Among these mouse mutations, s^l leads to a megacolon phenotype, exhibits white spotting in coat color, and maps to a region of mouse chromosome 14 identical to human chromosome 13 (Puffenberger et al. 1994). Targeted disruption of the endothelin-B receptor gene (EDNRB) to create a null mutation in mice manifests the recessive phenotype of megacolon and white spotting (Hosoda et al. 1994). In the human this EDNRB gene maps to chromosome 13. Indeed a population of HSCR patients display de novo interstitial deletions of chromosome 13. EDNRB is a proposed candidate gene for HSCR susceptibility. EDNRB encodes for endothelin-A (ET_A) and endothelin-B (ET_B), two endothelin receptor subtypes. ET_A and ET_B have different affinities for the endothelin peptides. While ET_A has a greater affinity for endothelin-1 or endothelin-2 both potent vasoconstrictors, ET_B is a preferential receptor for endothelin-3 (ET-3) which has vasodilatory actions. In the mouse, the interaction of ET_B receptor and ET-3 is essential for normal development of enteric neurons from neural crest cell-derived cells (Baynash et al. 1994) since targeted disruption of the mouse ET-3 gene in *ls /ls* mice also produced the megacolon and coat color spotting phenotype. Taken together with the fact that EDNRB mutation in mice and humans causes megacolon suggests that this ET_B-ET-3 interaction plays an important in the development of enteric ganglia in humans.

Recently, a gene for HSCR has been mapped to chromosome 10 (Angrist et al. 1993; Lyonnet et al. 1993) and is linked to the RET proto-oncogene locus (Romeo et al. 1994). A small population of HSCR patients with RET mutations have been identified (Edery et al. 1994). The RET gene products, the receptor tyrosine kinases are cell-surface molecules that transduce signals for cell growth and differentiation (Schudardt et al. 1994). These receptors are expressed in the developing peripheral nervous system of mice. Targeted disruption of these receptors manifests in total lack of neural crest-derived enteric ganglion neurons in mice (Pachnis et al. 1993). Therefore, expression of the RET gene is essential for development of the enteric nervous system since it forms part of a signaling pathway necessary for growth and differentiation. At the RET or EDNRB locus, mutations are associated with incomplete penetrance and a higher penetrance in males than females.

These observations as well as the existence of dominant HSCR families with no linkage to RET or ENRB suggest that it may be necessary for additional susceptibility genes to be expressed in order to cause HSCR (Hosoda et al. 1994; Puffenberger et al. 1994). One pathogenic model for HSCR suggests that it may be associated with a "random variation in the number of ganglion cells with a detrimental cellular phenotype" such that a "threshold effect might exist on the number of defective cells necessary to induce a clinically detectable aganglionosis". Otherwise, additional cellular processes may be required to precipitate ganglion cell loss in HSCR, in a similar fashion as the "second hit" phenomenon required at tumor suppressor genes in predisposed cells induce tumorogenesis (Hosoda et al. 1994; Puffenberger et al. 1994).

An alternative etiology has been proposed by Earlam (Earlam et al. 1972), where HSCR could occur as a result of an ischemic insult in utero. According to Earlam aganglionosis, stenosis and atresia are three types of gut lesions that can occur following occlusion of enteric blood supply in utero. *In all cases* the bowel is obstructed distally and dilated proximally however the obstruction can be complete (atresia) or incomplete (stenosis) and present in the neonatal period, or later if the obstruction is partial. The ischemic insult could be imposed when the gut undergoes rotational strains early in development. From the fifth to the tenth week of gestation, the bowel undergoes a 270^0 counterclockwise rotation to assume its adult position. The ischemic insult would occur at the site made hypoxic by rotation-induced occlusion of its main mesenteric arterial input. The region which is under excessive rotational strain would eventually unwind and assume a normal rotation as is observed in the adult (Earlam et al. 1972). Permanent intestinal malrotations are known to be an underlying cause of partial or complete obstruction of the duodenum or colon (Walker-Smith et al. 1985). Furthermore, Earlam proposed that this etiologic mechanism, differing only in its severity, could explain the anatomic distribution of these conditions occurring most often in the duodenum (causing aganglionic megaduodenum) and colon (i.e. Hirschsprung's disease). In all cases the bowel situated farthest away from the arterial blood vessels supplying the gut (i.e. supplied by terminal branches of the mesenteric arterial supply) is that which may become aganglionic. These fine arterial branches supplying these regions would be more susceptible to occlusion induced by rotational strain than the main arterial supply to other regions of the gut.

Following experimentally imposed ischemia in chick embryo in utero the muscularis becomes contracted and the bowel stenotic (Earlam et al. 1972). Earlam proposes that atretic bowel (destruction of all cell types) may be due to prolonged ischemic insults, while destruction of ganglion cells without damage to surrounding tissue, would occur following a

temporary ischemic insult. This hypothesis is based on the concept that neural tissue is most sensitive to anoxia. The optimum period of time for temporary ischemia to induce neural damage without affecting other constituent tissue is around 4 hours (Cannon et al. 1913; Hukura et al. 1965; Earlam et al. 1967; Szurszewski et al. 1968; Earlam et al. 1972; Okamoto et al. 1987). Earlams proposal has been disputed by two independent groups who were able to show complete recovery of ischemic induced lesions in dog and rabbit intestine following a temporary occlusion of 1-3 hours (Meier-Ruge 1974). However, Earlam stressed that at a 4 hour ischemic insult is the minimum required to induce aganglionosis.

Earlam (1972) suggested that the anatomical appearance of the HSCR affected bowel is more compatible with the cells having once been present and then destroyed rather than never having migrated to the gut, since the space between the external muscle layers where the myenteric plexus should be, display ghost-like ganglia with neural fibres rather than cells. In a congenital lesion occurring at such an early stage, evidence of these spaces would not be expected.

Additional support for a vascular etiology in HSCR is that there is recurrence of ganglion cell loss in histologically normal bowel following resection of the HSCR affected segment. The recurrence rate is quite high and is thought to be due to interrupted blood supply to the remaining gut during surgery (Ehrenpreis 1965; Nixon 1966).

In summary, HSCR is know regarded as a multifactorial disease state since multiple pathogenic factors have found to be associated with this condition. Regardless of the etiological basis of HSCR, a major factor underlying obstruction in this disease, appears to be an increased cholinergic excitatory transmission acting in conjunction with a functional defect in or reduced capacity of the intrinsic NANC inhibitory transmission in the aganglionic colon. This latter defect would account for the inability of the diseased bowel to relax. While abnormalities of adrenergic, cholinergic and peptidergic neural innervations have been reported, there is no clear explanation for the occurrence of the spastic aganglionic segment of bowel.

Little is known about NO in the pathological mammalian gut, in particular the human. *Since NANC function is disrupted in HSCR and NO is a NANC transmitter we hypothesized that the nitrergic innervation of the diseased bowel in these patients would be disrupted.* The aim of this study was to examine the neuronal NO synthase, in normal and HSCR affected human gut. Utilizing the methodology and experience gained during my analysis of the nitrergic system in the rodent, and in normal human colon, NADPH diaphorase histochemistry was used to examine NO synthase distribution in the ENS in ten patients with HSCR and three

age matched controls. In addition, a subpopulation of these specimens were subjected to biochemical assessment of NO synthase activity.

4.2 Materials and Methods

Resected bowel specimens were obtained from ten infants with Hirschsprung's disease (confirmed histologically by standard Hematoxylin and Eosin staining) who underwent pull-through surgery at the Children's Hospital of Eastern Ontario. Two of the patients were diagnosed with long-segment HSCR while the rest were diagnosed with rectosigmoid aganglionosis (short-segment HSCR). The age of the infants ranged from 6 months to 1 year. In addition, normal control colon specimens were obtained from three age-matched infants who underwent closure of colostomy. All human tissue studied in this investigation was obtained under approval of the Research Ethics Board of the Children's Hospital of Eastern Ontario (CHEO) and according to the Medical Research Council of Canada Guidelines on Research Involving Human Subjects. All processed tissues were sampled for parallel routine pathological investigation in the Department of Pathology at CHEO. The histopathological findings of the pathologist from CHEO were compared with the clinical presentation and progress of the patient.

Tissue was processed for cryostat sections, laminar preparations and biochemical assessment of NO synthase activity (conversion of ^{14}C-L-arginine to ^{14}C-L-citrulline). For tissue sections, segments were taken from the normally ganglionated ('proximal') colon as well as from the apparent transition zone and the aganglionic ('distal') segment. For whole mounts and biochemical analysis segments were taken from the proximal and distal portions of the specimen. Specimens to be sectioned or dissected were subjected to the same fixation procedure as that described in section 3.1.1. Specimens to be used in the biochemical analysis were immediately snap frozen in liquid nitrogen and stored at -80^{0}C until the assay was performed.

The preparation of cryostat sections and laminar preparations used in this study is identical to that used in section 3.1.1. Tissue sections were cut simultaneously in the axial and circumferential planes of both normal and diseased segments. This arrangement of sections allowed the detection of subtle differences in the morphology and intensity of NO synthase-related diaphorase staining in the normal vs diseased tissue.

NADPH diaphorase staining of laminar preparations and sectioned tissue followed the same methodologies as described in Chapter 3 section 3.1.

4.3 Chemicals

As in section 3.1.

4.4 *Results* - NO Synthase Histochemistry: Normal vs 'Aganglionic' Colon

Figure 1 compares laminar preparations taken from the myenteric and Henle's plexus of normal (normoganglionic regions of HSCR affected gut or control gut) and 'aganglionic' bowel. As described in section 3.1.3, the myenteric plexus of control specimens revealed positively labeled cells with neurites coursing through the primary, secondary and tertiary meshworks of this plexus (fig. 4.1A). All ganglia contained NO synthase-related NADPH diaphorase positive cells. In addition, extraganglionic cells were observed in the primary nerve fiber bundles. These cells always displayed a classical Dogiel type II morphology. The myenteric plexus of the distal bowel from 8 out of 10 patients displayed a profusion of very large NO synthase positive nerve fiber bundles coursing through this nerve layer (fig. 4.1B). This feature was most obvious in laminar preparations. The submucous plexus from normal and aganglionic segments of colon are shown in Figure 4.1 (C and D). In Henle's plexus of control specimens ganglia contained a rich innervation of NO synthase positive varicose nerve fibers coursing about clearly unlabeled cells (fig. 4.1C). Not all Henle's ganglia contained positively labeled nerve cells however a maximum of 5 NO synthase positive cells could be seen in any one ganglion. Henle's plexus of the aganglionic bowel displayed a similar pattern of innervation as was observed in the myenteric plexus (fig. 4.1D). No ganglia were observed. Instead large dense nerve fiber bundles containing NO synthase reactive fibers were evident with multiple fiber projections and secondary branchings. This dense NO synthase positive fiber bundle ramification of the myenteric and submucous plexuses was not evident in sectioned tissues.

In 2 out of 10 patients the histologically diagnosed aganglionic bowel displayed a different innervation pattern from the patient population described above. Figure 4.2 displays axial (longitudinal axis of the gut) sections from the normoganglionic and so called 'aganglionic' segments of these patients is presented in Figure 4.2. In the control specimen, ganglia contain a subpopulation of intensely labeled NO synthase reactive cells (fig. 4.2A). The underlying circular muscle displays a dense innervation of NO synthase reactive fibers. However, in the so called 'aganglionic' region as depicted in Figure 4.2B, NO synthase positive cells are evident. Compared to the control gut, there is a sparsity not only in the number of cells present in any one ganglion but also in the NO synthase positive fiber innervation of the underlying circular muscle layer. At higher magnification, the NO synthase reactive fiber innervation of the circular muscle

Figure 4.1. Photomicrographs showing the myenteric and submucosal layers in laminar preparations of the normoganglionic (A and B) and diseased (B and D) bowel. (A) Intensely stained cells are present in a myenteric ganglion (G), and within the interconnecting fibre bundle (Pp). The latter cells displayed classic extraganglionic type II (eII) morphology. Intensely labeled fibres could be traced within the ganglion, coursing around clearly unlabeled cells and within the secondary (Ps) and tertiary (Pt) meshworks of the myenteric plexus. (B) This pattern is not evident in the distal 'aganglionic' colon. Ganglia are absent, and instead, an extensive innervation by large fibre bundles containing intensely labeled nerve fibres can be seen. (C) Intensely labeled neurons are present in a ganglion of Henle's plexus (Hp). Intensely labeled varicose fibres form a rich network within the ganglion and on into the interconnecting fibre bundles. Overlying the submucosa, are intensely labeled fibres (arrows) of the circular muscle. (D) Within the submucosa of the distal colon ganglia were not evident. However, very large nerve bundles with a rich investment of intensely labeled fibres were always present in the diseased segment. Scale bar = 50 μm.

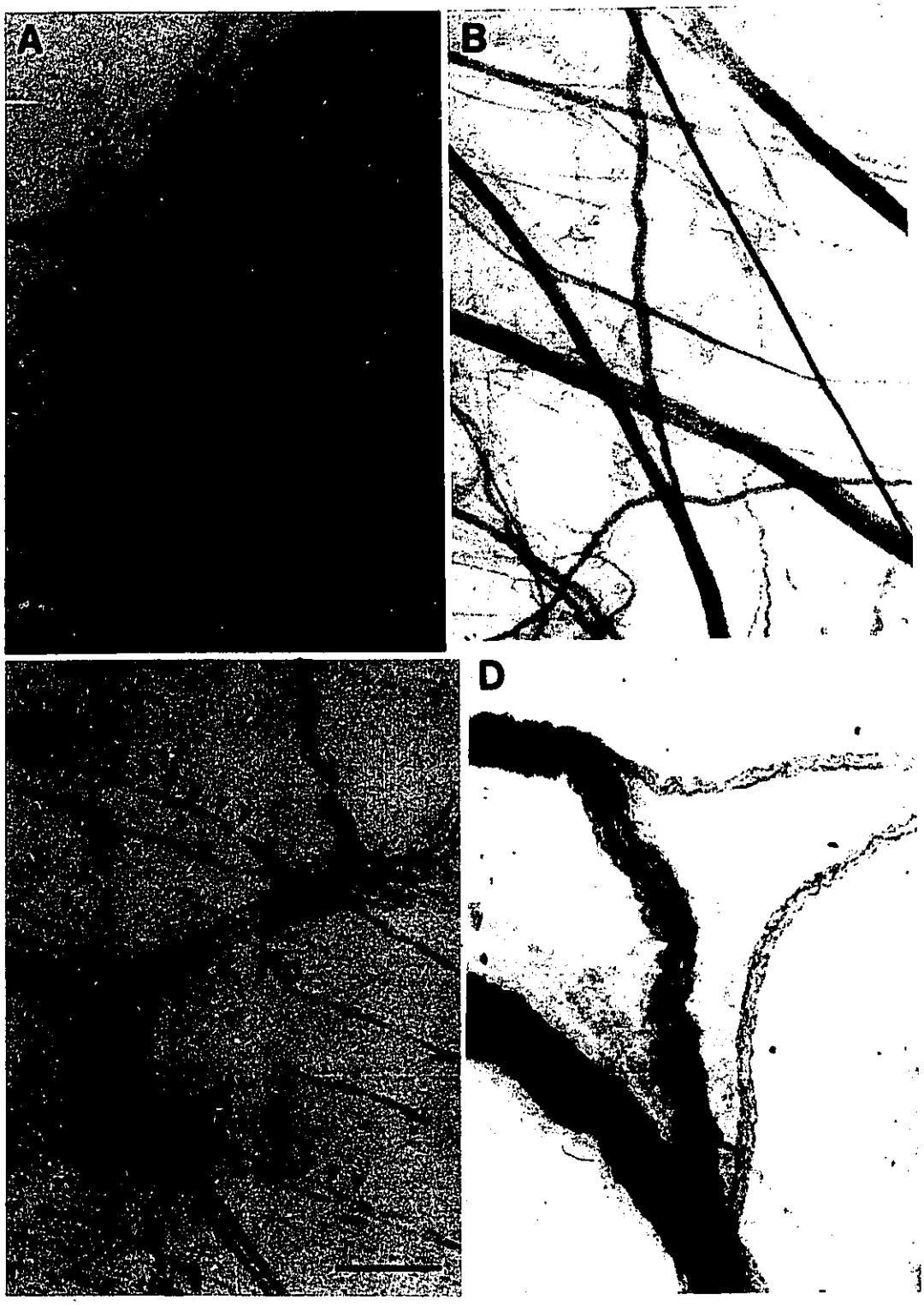

A
B
D

Figure 4.2. Micrographs showing circumferential sections of the human colon treated for NADPH diaphorase histochemistry. Panels A and C represent typical sections through the proximal normoganglionic colon. Panels B and D show comparative sections taken from the distal 'aganglionic' colon. A and B are comparative sections through the deep muscular plexus and C and D show comparative labeling patterns of the submucosa (sm), muscularis mucosae (mm) and mucosa (m). Note the distinct stout intensely labeled circular muscle fibre bundles (asterisks) in the diseased segment (B). The muscularis mucosae (mm) also displayed increased innervation with large caliber NADPH diaphorase positive fibres (arrows). Within the submucosa large fibres are also evident (arrows) and these can be distinguished from the labeled blood vessels (bv). Scale bar = 50 μm.

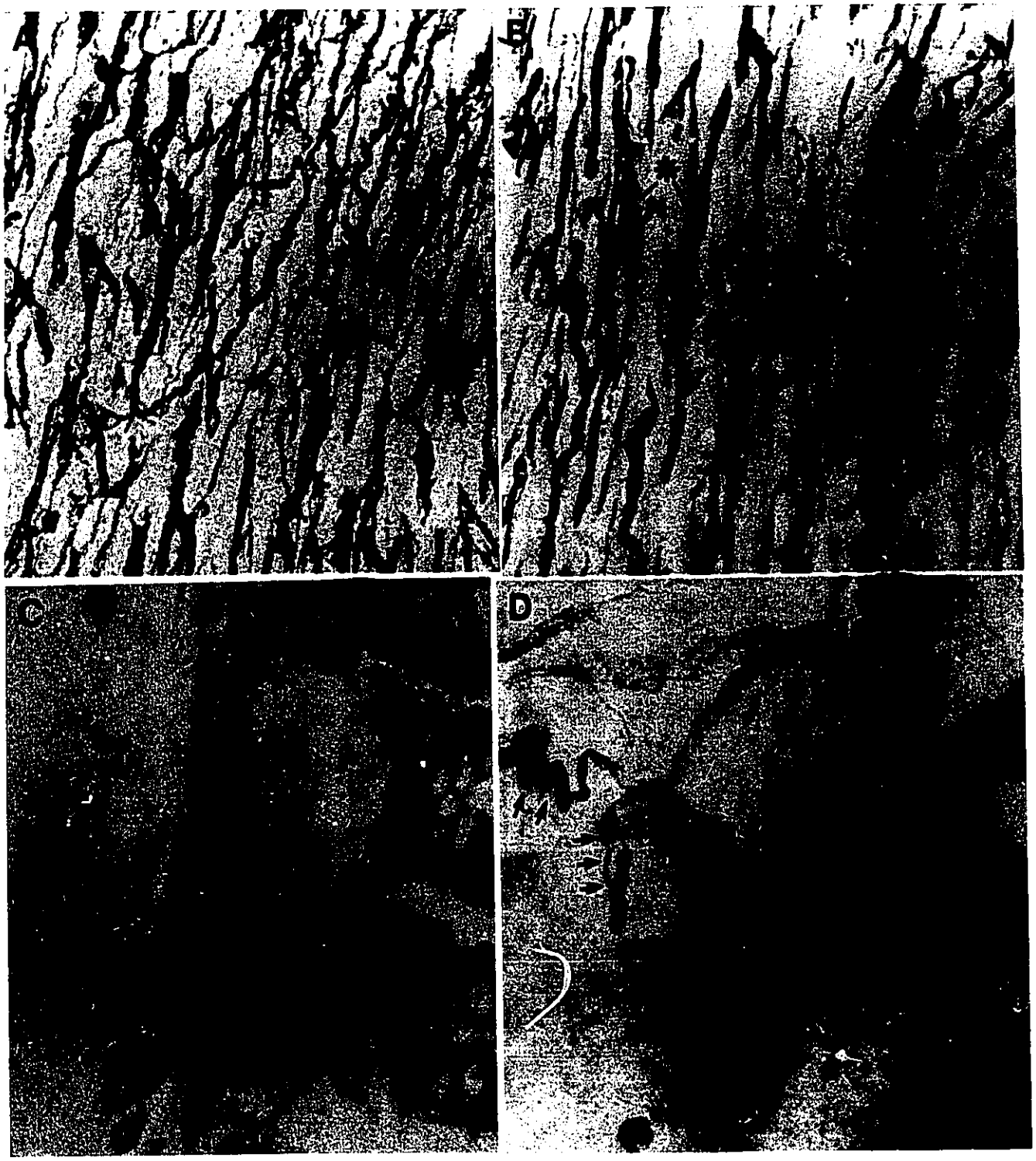
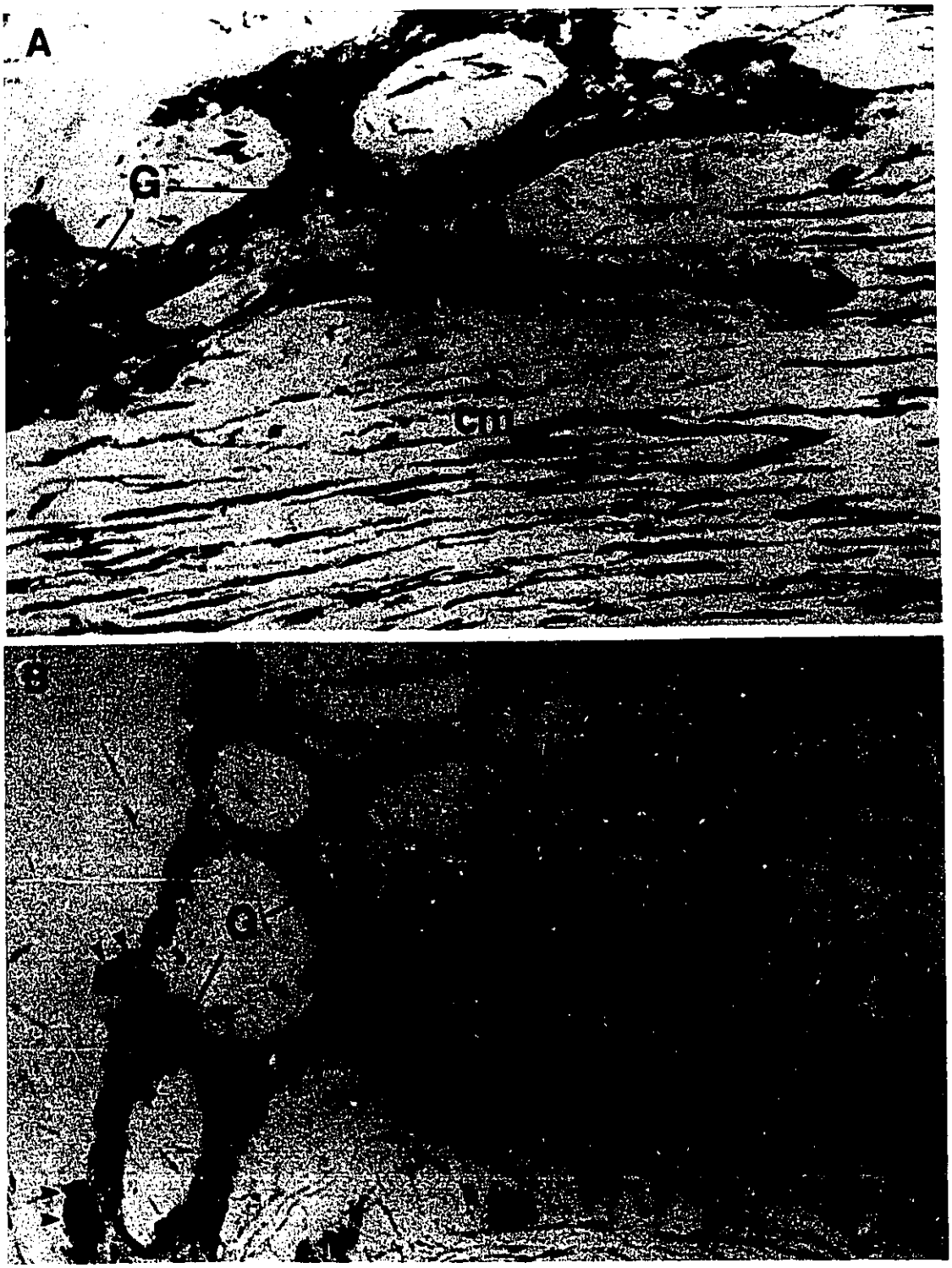

A
B
C
D

Figure 4.3. Axial sections (14 μm) cut from the colon treated for NADPH diaphorase histochemistry.

(A) Normoganglionic bowel: Myenteric ganglia (G) containing intensely labeled cells can be seen lying between the longitudinal (lm) and circular (cm) muscle layers. Scale bar = 50 μm.

(B) Distal 'aganglionic' bowel: Axial section taken from the distal so called 'aganglionic' region of the same patient. Note the loss of cells in the ganglia (G) and the presence of intensely labeled fibres in the muscle layers. Within the ganglia a small number of intensely labeled cells (small arrow heads). Scale bar = 50 μm.


A
G
Cm
B

layer of the control bowel is profuse (fig. 4.3A) compared to the more diffuse ramification of fibers in the distal constricted bowel (fig. 4.3B). The fiber innervation of the diseased segment appeared much thicker and in general, disarrayed. Within the muscularis mucosae of the diseased bowel, this feature of the NO synthase positive fiber innervation was more prominent (fig. 4.3C vs D). In addition positively labeled ganglionic fibers of the submucosa from diseased bowel displayed a dramatic tortuosity (fig. 4.3D).

4.5 *Results* - NO Synthase Activity: Normal vs 'Aganglionic' Colon

Total NO synthase (T-NOS) activity was significantly reduced in the distal constricted ('aganglionic') bowel as compared to the normoganglionic regions of these patients (n=3) (0.32 ± 0.06 and 0.64 ± 0.09 nmol NO/min/g tissue; $p<0.001$) (fig. 4.4A). This decrease in T-NOS activity could be attributed to a 3-fold decrease in constitutive NO synthase (C-NOS) activity (0.2 ± 0.04 and 0.57 ± 0.09; $p<0.001$) (fig. 4.4B). There was no significant difference in inducible NO synthase (I-NOS) activity between regions (fig. 4.4B).

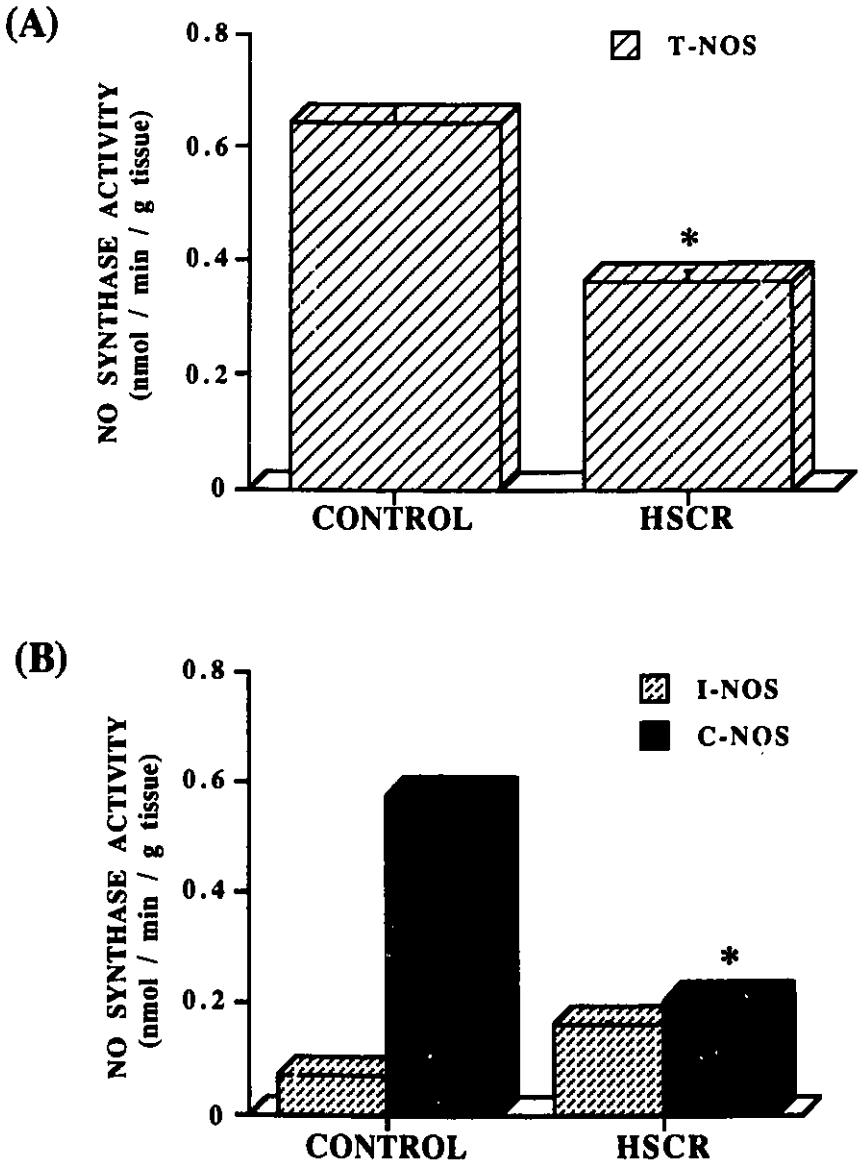


FIGURE 4.4: Total (A) or inducible and constitutive (B) NO synthase activity in HSCR affected vs proximal (control) colon. Asterisks (*) denote statistical significance ($p<0.001$). Values are expressed as means ± SEM.

4.6. Discussion

For the majority of patient tissues examined in this study axial and circumferential sections displayed classical signs of HSCR, that being a complete absence of neurons in the myenteric and submucous plexuses and associated innervation of the muscularis in the constricted segment as determined by the standard H and E stain, and NO synthase-related NADPH diaphorase staining. Several other groups assessing NO synthase innervation in sectioned tissue specimens from patients with HSCR have reported identical findings in the so called aganglionic bowel (O'Kelly et al. 1993; Vanderwinden et al. 1993; Kobayashi et al. 1994). However, unlike these studies, we identified large dense nerve fiber bundles containing NO synthase-related NADPH diaphorase positive fibers in both the myenteric and submucosal nerve layers in laminar preparations taken from the aganglionic segments of these patients. These nerve fiber bundles are reminiscent of extrinsic autonomic innervations of the gut. This is consistent with the finding that a large population of NADPH diaphorase positive neurons and fibers is found in the autonomic ganglia of the rat (Aimi et al. 1991). However, lesion of extrinsic sensory and sympathetic fibers to the guinea-pig colon had no effect on the intramural NADPH diaphorase positive fiber innervation (McConalogue et al. 1993). The apparent lack of fine NO synthase related nerve fibers in both nerve layers of the aganglionic bowel, suggests a disruption in the descending NANC innervation from more proximal hypoganglionic or normoganglionic regions.

The second and most significant finding of this study is that in the so-called 'aganglionic' region of bowel from 2 patients, there is a survival of at least one type of enteric ganglion cell, the NO synthesizing myenteric neuron. In addition the circular muscle and muscularis mucosae of the so called 'aganglionic' bowel from these 2 patients displayed a profuse innervation of NO synthase related nerve fibers. In general these NO synthase reactive fibers were more tortuous in appearance compared to control counterparts. These findings suggest that although enteric ganglionic neurons have been lost, a subpopulation of NO synthase positive myenteric neurons survive. In confirmation of this, other investigators in this laboratory have examined some of the known peptidergic innervations within these same affected segments and noted a complete absence of the expected nerve fibres. This novel and surprising finding of the selective survival of a subpopulation of myenteric neurons, suggests there may be a neurotoxicological basis rather than developmental origin for this disease in a subpopulation of the patients with HSCR.

Selective survival of NO synthesizing neurons occurs in CNS lesions such as Huntington's chorea (Ferrante et al. 1985) *In the striatum of these patients, there is a significant*

reduction in the density of NADPH diaphorase positive nerve cells compared to controls but a select subpopulation of these cells are spared, much like the findings of this study. These data may indicate that a similar phenomenon in the gut may be occurring in a subpopulation of patients with HSCR at least with respect to myenteric nitrergic neurons. Indeed, the possibility for a multifactorial pathogenesis for HSCR is supported by the multiple developmental defects (i.e. multigenic anomalies) that lead to the ganglion cell loss in the large intestine. Nonetheless, a normal migration of crest-derived cells must occur in this group, since nerve cells are present. The innervation anomalies may result from a local disruption in the microenvironment, which has to be less severe or which occurs at a later time point than the one responsible for agenesis.

In this study, HSCR affected segments of colon display a significantly reduced level of constitutive NO synthase activity as compared to age-matched controls. This reduction in the production of NO could be explained either by a loss of stimulatory input to the NO synthesizing neurons and/or a reduction in neuron number and/or a change in the regulation of the production of NO synthase. Three patients were biochemically assessed for NO synthase activity, of which one patient exhibited selective sparing of NO synthase positive myenteric neurons. Interestingly, NO synthase activity was not significantly different between our two defined patient groups.

Whether NO is involved in the death of surrounding neurons and/or the survival of cells producing it in the colon of HSCR patients is unknown. This phenomenon is proposed to occur in certain neurodegenerative disorders of the CNS. It has been shown that central neurons isolated in tissue culture undergo lesion damage via the excitotoxic actions of glutamate analogues (Choi 1988; Dawson et al. 1991b; Dawson et al. 1991c) and this damage is mediated by the actions of NO (Garthwaite 1991; Dawson et al. 1991b; Dawson et al. 1993). The nitrergic neurons themselves are protected from the toxic effects of NO due to some special characteristic(s) which may include high levels of superoxide dismutase or that the Ca^{2+} levels required to activate NO production are inhibitory to guanylate cyclase (a site of NO action) (Koh et al. 1986; Dawson et al. 1993). Thus, the neurons that are activated to produce NO may be resistant to its toxic actions such that under pathological conditions which lead to the death of other neurons, these cells would survive. Therefore, it would be expected that NO synthesizing neurons would selectively survive neurodegenerative disorders such as is observed in Huntington's disease (HD), where a subpopulation of nitrergic neurons are selectively spared in the striatum of patients suffering from this condition; the etiology of which includes a primary or secondary pathological mechanism involving NO (Coyle et al. 1993). However, unlike HD which has an adult onset,

neuronal damage in HSCR occurs either in utero, or in the early postnatal period, and involves immature neurons. It has been proposed that immature neurons are more vulnerable to neuronal degeneration and that NO is involved in the process (Wu et al. 1995). If we assume that NO is a contributing factor to a neurodegenerative-type lesion in Hirschsprung's disease in some patients, then both ischemia and neurotoxicity are possible candidate mechanisms since these mechanisms are responsible for degenerative disorders of the brain including stroke, and ischemia-reperfusion (see Coyle et al. 1993).

Neurotoxic and ischemic lesions in the CNS have also been shown to be associated with programmed cell death (Le et al. 1995). Programmed cell death (PCD) which is a critical process in embryonic development, especially in shaping the nervous system (Raff et al. 1993). Morphologically PCD differs from necrosis; in PCD there is no evidence of inflammation whereas in necrosis the loss of membrane and mitochondrial integrity triggers infiltration of inflammatory cells. It is interesting to note that in the so called 'aganglionic' region of patients with HSCR, there is no evidence of inflammation. PCD is part of the cell cycle along with cell growth and proliferation and is a naturally occurring event induced by environmental stimuli such as trophic factors or other extracellular stimuli particularly in the developing nervous system. PCD is controlled by local environmental signals; and unlike necrosis, PCD can be induced or repressed by the loss or addition of defined activation signals (Raff et al. 1993). Other triggers of PCD are factors or events (eg. ischemia) that lead to a sustained elevation in intracellular Ca^{2+} levels (Hockenbury et al. 1993; Richter 1993).

Since PCD shares a molecular pathway with the normal cell cycle, it is sensitive to the same environmental stimuli and mechanisms that regulate cell growth and proliferation (Berges et al. 1993). A cell will divide or die depending on the overriding effect of regulatory proteins or summation of signal inputs. A regulatory protein which suppresses PCD is the *bcl-2* gene product Bcl-2. Bcl-2 is an integral membrane protein localized in the inner mitochondrial membrane (Hockenbury et al. 1993), nuclear envelope and endoplasmic reticulum (ibid) which functions to prevent pro-oxidant induced PCD. But cell damage or the onset of a developmental program can derepress the death program and commit the cell to die. For example, neurons require the presence of growth factors to survive but neurons overexpressing bcl-2 do not. Therefore in the developing nervous system, a trophic-dependent tissue, PCD is readily inducible in response to changes in trophic factors or other extracellular stimuli. It might be possible therefore, that the environmental factors involved in the permanent loss of neurons during the

development of the ENS in HSCR are not only inhibiting colonization and differentiation in situ but also activating PCD.

In the case of the mouse model of HSCR overproduction of extracellular matrix factors (i.e. laminin and collagen type IV) (Hockenbury et al. 1993) is believed to prevent colonization and differentiation whereas leading to aganglionosis perhaps low levels of these factors promote migration, colonization and differentiation but overproduction triggers PCD. However, in PCD, "the cell is induced to commit suicide by specific signals in an otherwise normal microenvironment" (Raff et al. 1993). These signals act by suppressing an intrinsic cell suicide program that is constitutively expressed in cells and operates by default when a cell is deprived of such signals. One such signaling pathway may involve NO. NO is an inhibitor of cell growth and proliferation (Marsden et al. 1991; Sarkar et al. 1995). Inhibition of cell growth and differentiation can induce the cell to enter the death cycle or undergo PCD (Berges et al. 1993) Therefore, deregulation (or reduction) of NO production could prevent its inhibitory action on cell growth and proliferation and trigger the target cell to undergo PCD. NO producing neurons may have higher concentrations of a survival factor such as Bcl-2 and therefore are rescued from PCD. Interestingly, NO has been shown to inhibit the production of collagen (an extracellular matrix component) (Kolpakov et al. 1995). Therefore deregulation of NO production might contribute to the proposed defect in the production of collagen in HSCR associated with the inability of neural crest-derived cells to colonize the distal bowel and differentiate. If we reconsider Earlams proposal that ischemia during development of the ENS is the trigger for ganglion cell loss (Earlam et al. 1972) then the possibility exists that the nerve cell loss is due to ischemia induced PCD (Raff et al. 1993) and NO could be a candidate mediator. Indeed, genetic evidence for a role of pro-oxidant (ischemia) induced nerve cell death is provided by familial amyotrophic lateral sclerosis (Hockenbury et al. 1993). Whether this is a trigger for some forms of HSCR deserves consideration.

The findings of this study may provide insight at least in part, to mechanisms underlying the functional obstruction in HSCR. NO is a major inhibitory NANC neurotransmitter of the gut, and is also produced by a subpopulation of enteric motor neurons and interneurons. The reduced nitrergic innervation would account for the disruption of NANC function in this disease. While Hanani and colleagues (1989) have shown that NANC inhibitory neurons are functional in isolated preparations of diseased bowel, others (Kubota et al. 1983) have shown that NANC relaxations are non-functional in the aganglionic bowel. The existence of at least two patient populations as defined by the findings of this study may explain the conflicting functional

evidence. Nonetheless functional studies clearly show that there is a defect in, or reduced capacity of the NANC inhibitory neuroeffector transmission in the aganglionic colon. A reduction in the number of NANC NO producing neurons and/or a loss or withdrawal of stimulatory inputs to these cells might contribute to this observed defect in HSCR.

Chapter 5

5.1. Characterization of the Nitrergic System in the Rodent and Human Vasculature

In the gastrointestinal tract, NO is considered to play an important role in the control of blood flow (Pabst 1987; Pique et al. 1989; Walder et al. 1990; Pique et al. 1992). In order to document the sites of NO synthase activity in the enteric vasculature, enzyme activity was localized in relation to mural constituents in fixed frozen sections of the rat and human gut wall using a CD31-type antibody raised against endothelial cell membrane glycoproteins. The findings described in the following pages provide the first histochemical evidence for NO synthesis in both endothelial and smooth muscle cells of rat and human submucosal arterioles and venules. Aspects of these findings were presented at the AGA Meeting, 1993 (Nichols et al. 1993). A complete report was subsequently published (Nichols et al. 1994).

5.1.1. Materials and Methods

Segments (2-4cm in length) of ileum and colon, rapidly removed from freshly decapitated male Sprague Dawley rats (350-450g; n=4) were placed immediately in pre-chilled 0.1M sodium phosphate buffered saline (pH 7.2; PBS) to clean the tissue of surface blood and debris. For laminar preparations, the segments were opened along the mesenteric border, pinned (mucosa facing up) and exposed to a 2hr fixation at 4^0C in a modified Zamboni's fixative consisting of 4% paraformaldehyde, 0.2% picric acid mixture in 0.16M sodium phosphate buffer (PB; pH 6.9). Fixative was cleared with several rinses in cold PBS. Human tissue was obtained from a 1 year old otherwise normal male infant requiring a transverse colostomy at end staged treatment of congenital anal stenosis. During elective closure of the colostomy two (2-4 cm length) segments of normal human transverse colon were removed and prepared as described above.

Fixed gut segments which were stored for at least 24 hrs at 4^0C in 0.1M PB (pH 7.2) containing 10% sucrose. Cryostat sections were cut at 10 μm thickness and mounted onto electrostatic glass slides. Sections were taken in both tangential (parallel to the layers of the gut wall) and coronal (perpendicular to the gut axis and through the lumen) orientations. Slides containing prepared sections, if not immediately exposed to histochemical treatment for NO synthase activity, were stored at -80^0C.

Laminar preparations from the submucosa were prepared by fine dissection of fixed segments of the small and large intestine as described in section 2.1. The serosa,

longitudinal smooth muscle layer and myenteric plexus were peeled back from a superficial incision made along one end of the tissue segment. The remaining portion of the tissue segment wall consisted of some remaining circular smooth muscle, the submucosa, muscularis mucosae and mucosa. Once the muscularis mucosae and mucosa were peeled away from the remaining portion of the segment wall remnants of circular smooth muscle were removed. In this orientation, the plexus submucous externus or Henle's plexus was exposed revealing the underlying vascular plexus.

The histochemical reaction for NO synthase in both laminar preparations and frozen sections was greatly facilitated by incubation overnight at 4^0C in 0.1M PBS pH 7.2 containing 3% Triton X-100 and 10% sucrose prior to the histochemical reaction.

Following a 15 minute incubation at room temperature in 10 mM PB (pH 8.0), the fixed and pretreated tissue preparations were incubated in a reaction medium consisting of 1mM NADPH, 0.5mM nitroblue tetrazolium (NBT), and 0.3% Triton X-100 in 10mM PB, (pH 8.0) for 30 min. (frozen sections) or 1hr (laminar preparations) at 37^0C. The stained tissue sections and laminar preparations were then rinsed several times in 0.1M PBS (pH 7.2). Laminar preparations were air dried and coverslipped in permount.

Tissue sections reacted for NO synthase histochemistry were subsequently treated for immunohistochemical demonstration of endothelial cells using a monoclonal mouse anti-human endothelial cell (membrane glycoprotein, CD31) antibody JD70. Tissue sections were incubated overnight at 4^0C in the presence of JD70 (1:40), washed in 0.3% Triton-X-100 in PBS (pH 7.2) and subjected to a 40 minute incubation at 37^0C with a FITC-conjugated sheep-anti-mouse antibody (1:20 Sigma). Double labeled sections were given a final wash with 10mM PBS (pH 7.2), and coverslipped using a mounting medium consisting of 0.1mM phenylaminediamine and 90% glycerol in PBS. Visualization of FITC was carried out using a Zeiss Axioplan microscope with a 488 nm filter.

5.4.2. Chemicals

Nitroblue tetrazolium salt and the FITC-conjugated sheep-anti-mouse antibody was obtained from Sigma. The CD31-type monoclonal antibody (JD70) was purchased from DAKO. All other chemicals used in this investigation were from BDH.

5.1.3. *Results* - Submucosal Vasculature viewed in Laminar Preparations

Submucosal laminar preparations from the human colon and rat small (see fig. 5.1A and B) and large intestine, treated for NO synthase histochemistry, displayed a network of intensely labeled neural fibres. In addition, fine arterioles and venules were stained with distinctive patterns of labeling. Both large and small venules displayed small, weakly-positive patches of reactivity in combination with a reaction which appeared to outline the cellular boundaries of each endothelial cell. In contrast, arterioles displayed only concise, intense patches of NO synthase activity. Each endothelial cell appeared to display a single patch. There was no observable NO synthase activity contained within the perivascular nerve fiber innervation of these vessels and these anatomical data suggest that the NO synthase enzymatically reactive sites as seen here are intrinsic to the vessel wall.

EDNO appeared the same across a wide range of vessel sizes. The largest stained arteriole was measured at approximately ~34 μm in diameter and the smallest observable vessel caliber where NO synthase activity could be seen was ~5 μm across. The largest caliber stained venule seen was measured at 150 μm across. Qualitatively the intensity of puncta observed in the venules irrespective of caliber size was less than that seen in arterioles. Venular endothelial labeling also included a faint staining, discernible from the background, about the borders of each cell (fig. 5.1A).

5.1.4. *Results* - Submucosal Vasculature viewed in Tissue Cross-Section

To determine if in fact the NO synthase visualized histochemically was restricted to the endothelial layer of enteric vessels studied in laminar preparations, double stained tissue sections (for NO synthase and an endothelial cell immunofluorescence marker) were studied. NO synthase activity was found to be associated with both endothelial and smooth muscle cells of the intimal and medial layers within the wall of all submucosal vessels (figs. 5.2A-D). Double staining allowed a distinction between the endothelium and the smooth muscle layers of both arteriole and venules (fig. 5.2A and B).

Each endothelial and smooth muscle cell appeared to have one patch of NO synthase activity associated with it (fig. 5.2C and D). In addition these NO synthase patches appeared to predominate at the luminal aspect of the endothelial cells (large arrows in fig. 5.2D). In rat, scantily distributed fine varicose nerve fibers were evident running alongside (*para*) the adventitial layer of the arterioles as seen in the example of Figure 5.2D.

Figure 5.1. Photomicrographs of NO synthase histochemistry in laminar preparations of Henle's plexus and the underlying vascular network from rat ileum. A: The arteriole (Va) and venule (Vv) display uniformly distributed intensely labelled patches of NO synthase -related NADPH diaphorase activity. B: High magnification of the arteriole reveals the longitudinal orientation of these patches. This vascular labelling was distinct from the overlying intensely labelled nerve fibres (arrowheads in A and B) of Henle's plexus. Scale bar = 20μm.

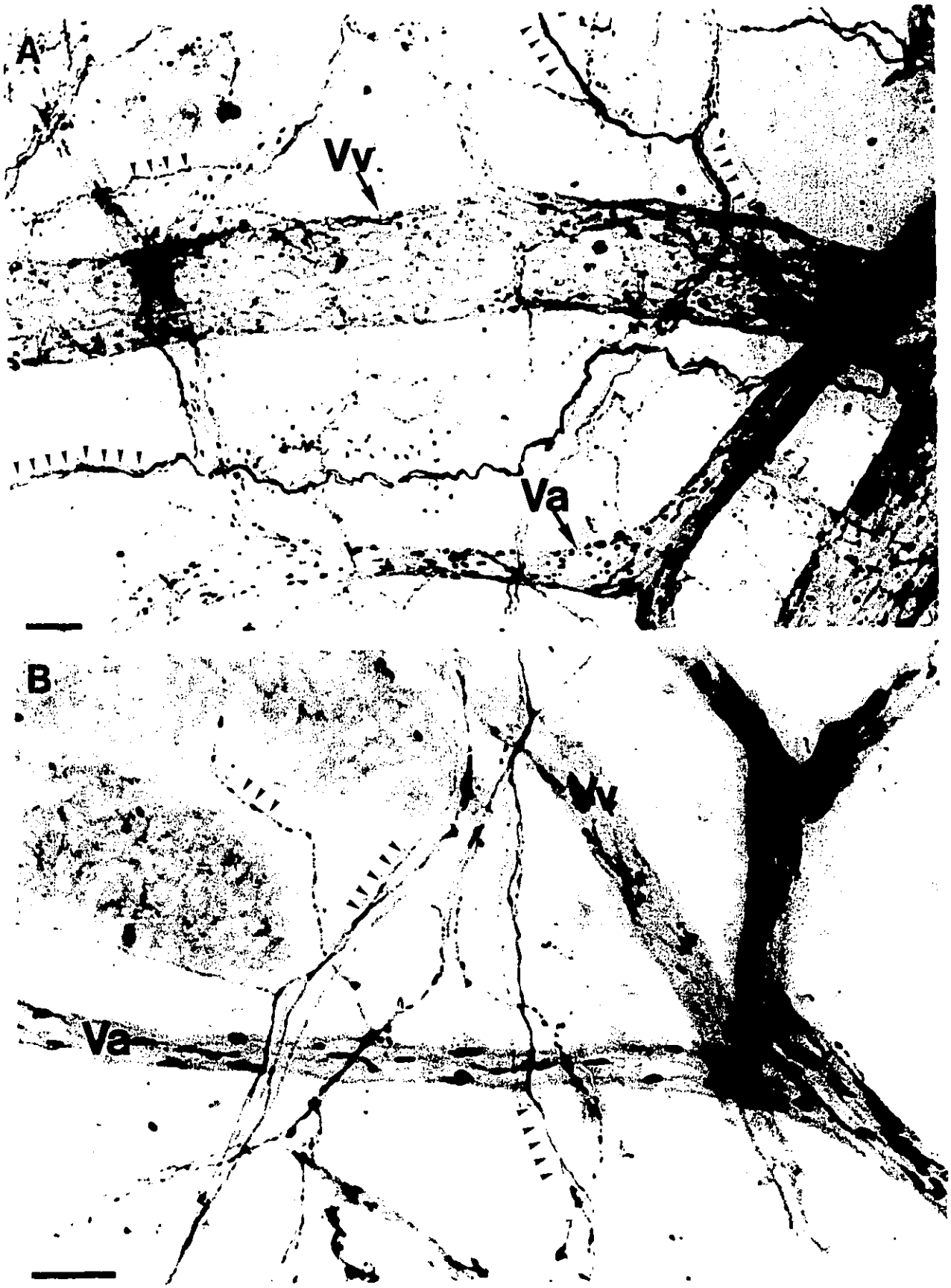
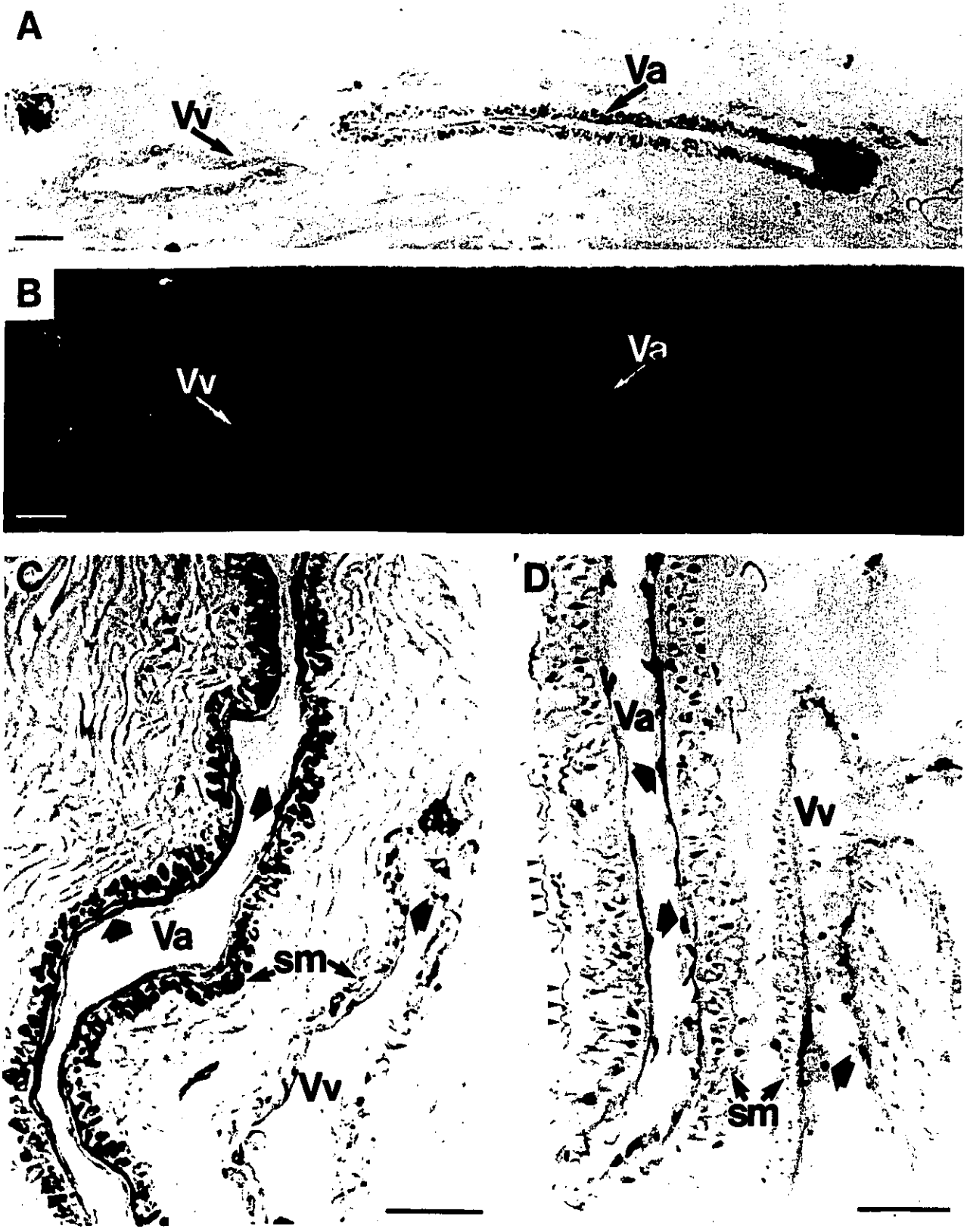

A
Vv
Va
B
Vv
Va

Figure 5.2. Photomicrographs of tangential sections through human (A, B and C) and rat (D) colon submucosa. A & B: Light (A) and fluorescent (B) micrographs of the same section. In both the arteriole (Va) and venule (Vv) CD31 immunoreactivity as seen in B, distinguishes the endothelium. C&D: Higher magnification of human (C) as compared to rat (D) arteriole and venule reveals NO synthase reactive patches to be localized to endothelial (large arrowheads) and smooth muscle (sm) cells. In the rat and not human, fine varicose nerve fibres (small arrowheads) can be seen situated close to the adventitial layer of the arteriole. Scale bar = 20μm.


A
Va
Vv
B
Va
Vv
C
Va
sm
Vv
D
Va
Vv
sm

There was a differential staining intensity in the vessel wall between species. In the submucosal vessels of the human intestine, NO synthase activity was more evident than that seen in the rat (fig. 5.2C and D). In both species, staining was more distinctive in arterioles than in venules (fig. 5.2C and D).

5.1.5. Discussion

The results of this study show that both endothelial and smooth muscle cells of the rat and human submucosal arterioles posses the potential for NO synthesis. Furthermore, this NO synthesis potential displays a compartmentalized distribution with the activity restricted to a small discrete patch within individual endothelial and smooth muscle cells of arterioles and boundaries between cells of venules. This contrasts with the diffuse cytoplasmic distribution of NO synthase in neuronal cells. The localization of NO synthase activity within the enteric vascular endothelium of human and rat arterioles and venules confirms previous molecular and biochemical studies in other peripheral vessels (Palmer et al. 1988; Busse et al. 1990; Radmonski et al. 1990; Busse et al. 1992; Forstermann et al. 1992; Janssens et al. 1992; Michel et al. 1992; Pollock et al. 1992; Boje et al. 1993), including reports of NO synthase activity in arterial smooth muscle (Wood et al. 1990; Schini et al. 1991). Activity was found within vascular smooth muscle of both venules and arterioles. This particular localization of NO synthase activity was unexpected since only the inducible isoform of NO synthase has been reported in vascular smooth muscle cells (Schini et al. 1991; Busse et al. 1992; Charpie et al. 1993), and tissues used in this study were not subject to treatments expected to trigger induction. This observation is consistent with the view that the inducible form of NO synthase (mNOS) is present under normal physiological conditions (Kobzik et al. 1993). These anatomic data strongly support the proposed vasodilator role of NO in the mammalian gastrointestinal tract (Pique et al. 1989; Walder et al. 1990; Pique et al. 1992) and together with my previous findings (Nichols et al. 1992; Nichols et al. 1993; Nichols et al. 1994) support the role for NO in enteric vasomotor regulation .

EDNO effects a relaxant response and prevents platelet aggregation and adhesion to the endothelium by increasing cyclic GMP levels through its allosteric action on soluble guanylyl cyclase in both the underlying vascular smooth muscle and nearby blood platelets. These actions of EDNO preclude it as an intercellular messenger molecule that effects increased blood flow and reduced platelet aggregate formation and resulting thrombosis (Moncada et al.

1991). NO has also been shown to modulate microvascular permeability in the gastrointestinal tract (Kubes 1992). Vascular smooth muscle-derived NO behaves as an autocrine factor that plays a role in modulation of vasomotor tone (Charpie et al. 1993). NO also functions as a modulator of vascular smooth muscle cell protein synthesis and production of extracellular matrix components (Kolpakov et al. 1995).

There is both basal and stimulated release of NO from the endothelium of both artery and vein. Stimulated NO release is effected by certain agonists (ACH, ATP or bradykinin) or by physical stimuli such as fluid shear stress (Pohl et al. 1986; Busse et al. 1991; Ignarro et al. 1991; Lamontage et al. 1992; Busse et al. 1993) or low arterial PO_2 (Pohl et al. 1989). These forms of NO release, occur through different mechanisms and are proposed to regulate differing vascular functions (Busse et al. 1991).

Reports indicate that arteries and veins differ in their basal and stimulated production of EDNO. Vallance et al (Vallance et al. 1989) report that inhibition of EDRF synthesis in vivo increases arterial but not venous tone in humans. In venules, several vasodilatory substances act independently of the endothelium (Shepherd et al. 1975). DeMay and Vanhoutte (DeMay et al. 1981) and Furchgott et al. (Furchgott et al. 1981) have reported a significantly greater response of veins versus arteries to the endothelium-independent inhibitory effect of adenosine. Similarly, isoproterenol induced relaxations of canine veins do not require the presence of functional endothelial cells (Furchgott et al. 1981). Taken together these observations point to a diminished importance of the endothelium to NO physiology in veins as opposed to arteries or perhaps differential sensitivity of the smooth muscle (DeMay et al. 1982). The striking difference in intensity and distribution of NO synthase activity illustrated by the findings in this report provides an anatomic basis for these functional differences.

The restricted distribution of NO synthase activity within the endothelial cells suggests that EDNO is synthesized by a Ca^{2+}- and calmodulin-dependent dioxygenase (Forstermann et al. 1991; Michel et al. 1992) located in the membrane fraction of endothelial cells. This isoform of NO synthase accounts for 95% of the NO synthase activity found in endothelial cells (Forstermann et al. 1992). In addition to this particulate enzyme, there is a cytosolic Ca^{2+}- and calmodulin-dependent enzyme that represents <5% of the endothelial NO synthase activity (Forstermann et al. 1992). The endothelium of peripheral arterioles also expresses a constitutive Ca^{2+}-independent form (both cytosolic and membrane bound) and an endotoxin- or cytokine-induced (inducible) Ca^{2+}-independent form of NO synthase (Gross et al. 1991; Lamas et al. 1991; Radomski et al 1990). An endothelial cDNA encoding a human vein NO synthase isoform

has also been recently cloned. This enzyme is constitutively expressed and displays NO synthase histochemical activity (Janssens et al. 1992). These differentially distributed endothelial cell NO synthases have been shown to be structurally similar and may represent a posttranslational or cotranslational modification of the same protein (Michel et al. 1992; Michel et al. 1993). Whether the NO synthases found in the endothelial and smooth muscle cells of gut microvessels in this study represent the same or different isoforms of NO synthase is yet to be determined.

In conclusion, it would appear that NO synthase is compartmentalized within the wall of venules and arterioles. The intracellular compartment in which NO is synthesized may be a factor that dictates its biological activity. Current ultrastructural studies have confirmed and extended these findings indicating that NO synthase is associated with the Golgi apparatus within the endothelial cells (O'Brien et al. 1995). Molecular analysis reveals that the Golgi compartmentalization is necessary for the enzyme to respond to intracellular signals and produce NO (Sessa et al. 1995). Endothelial NO synthase represents a novel Golgi-associated protein in the mammalian system. Similar ultrastructural and molecular analyses of NO synthase in vascular smooth muscle cells have not been done. The subcellular punctate patches of NO synthase activity, as seen in this study, to be localized to the most luminal aspect of the endothelial cells, may be the most opportune site for a readily available 'pool' of NO. The smooth muscle cell patch may then represent a reserve pool of NO synthase within the vessel wall which may be required when the tissue is under a local stress.

5.2. Distribution of NO synthase Activity in Postcapillary Venules of the Human Infant Colon

The use of endothlelial cell immunohistochemistry in axial and circumferential sections of human infant colon in section 5.4 also revealed specialized postcapillary venules known as high-endothelial venules (HEV) within Peyer's patch follicles, the lymphoid tissue of the gut. These vessels are easily identified by their characteristic cobblestone appearance in tissue cross-sections, and their cuboidal endothelium (Granger et al. 1984). The HEV represent arteriovenous communications linking the arterial and venous vascularization of the Peyer's patch follicle. Together an anastomotic meshwork is formed on the lateral and serosal surfaces of the follicle (perifollicular region) but not the surface facing the intestinal mucosa. This meshwork extends in regions between Peyer's patches, known as the interfollicular region with T lymphocytes, and to the lamina propria of the mucosa (Granger et al. 1984). This study describes

the distribution and disposition of NO synthase-related NADPH diaphorase activity in the HEV of the human infant colon.

5.2.1. Materials and Methods

See section 5.1.1 for preparation of tissue sections and staining procedures.

5.2.2. Chemicals

See section 5.1.2.

5.2.3. *Results*

Figure 5.3 depicts HEV on the lateral surface of a Peyer's patch in a tangential (axial) section through the human colon stained for NO synthase-related NADPH diaphorase activity (fig. 5.3A) and the endothelial cell immunohistochemical marker, JD70 (fig. 5.3B). Staining of the HEV are evidenced by the classical cobblestone appearance of the endothelial cells (fig 5.3B). Vessels in the perifollicular regions identified with CD31 immunoreactivity were either circular in shape or irregular with end processes (fig. 5.3B and D). Double-staining (for NO synthase and CD31 immunofluorescence) allowed a distinction of endothelial cells from surrounding constituents of the Peyer's patch and extralymphoid regions. The endothelial cells appeared as cuboidal or cylindrical cells measuring approximately 10mm in height (fig. 5.3B). There was a strong reaction for NO synthase-related activity in each endothelial cell of the HEV within the Peyer's patch (fig. 5.3A). Sections histochemically stained in the absence of NADPH displayed no staining of these vessels.

An HEV at higher magnification (fig. 5.3C and D) revealed that these intensely stained patches of NO synthase activity could have different staining patterns in each endothelial cell of the same venule. Associated with each endothelial cell was a patch of NO synthase activity which occurred at either the luminal or adluminal aspects of these cells or at the juncture between cells. (fig. 5.3C). In general, HEV in cross section appeared as an endothelial sheet or as an irregular aggregation of rather large (approximately 10-15 μm in diameter) endothelial cells with the semblance of a lumen. The luminal diameters of the observed HEV ranged from 10 to 30 μm.

HEV in the extralymphoid regions including the interfollicular (T lymphocyte region) area and mucosal lamina propria also stained at an equal intensity (fig. 5.4). Staining of the HEV also provided the opportunity to discern the anatomical localization of these venules in the human intestine. In the interfollicular regions, HEV were seen subdjacent to the circular

Figure 5.3. Photomicrographs of tangential sections through human colon submucosa. A and B are light (A) and fluorescent (B) micrographs of the same section showing the perifollicular region of a Peyer's patch (Pf) which is distinct from the interfollicular region (If). CD31 immunoreactivity, as seen in B, distinguishes the endothelial cells of the HEV (large arrows) . Each endothelial cell (small arrow) possesses NO synthase reactive patches. (C and D) Higher magnification of an HEV reveals NO synthase reactive patches to be localized to the luminal aspect of the endothelial cells (small arrow). Patches of NO synthase activity, as seen in C, can also be seen at the adluminal aspect of the HEV as well as at the juncture between endothelial cells. Scale bars = 30 μm.

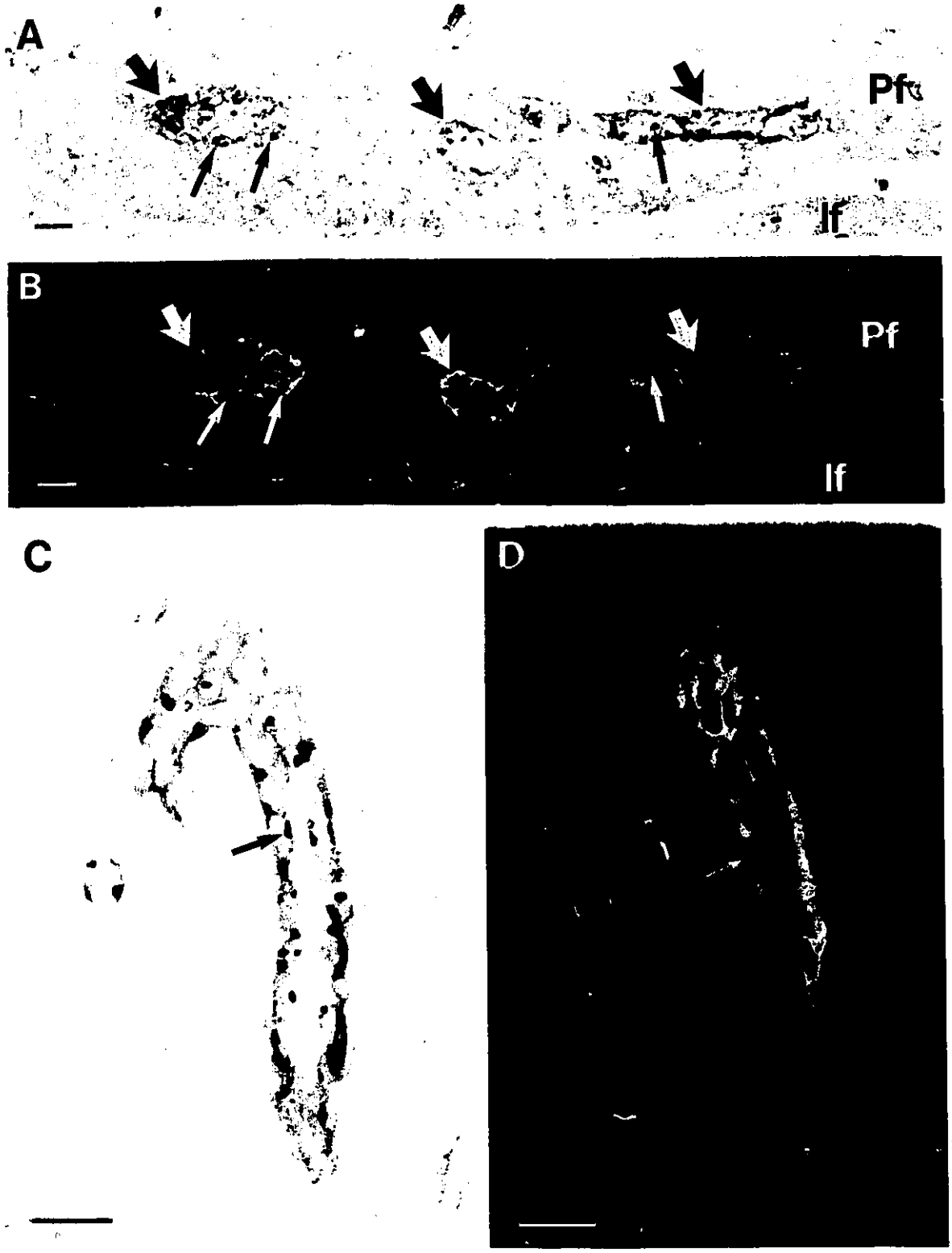

A
Pf
If
B
Pf
If
C
D

Figure 5.4. Photomicrographs of cross sections through human colon depicting the anatomical localization of the HEV. (A) Portion of an HEV in the interfollicular region of the submucosa (sm) close to the circular smooth muscle (cm). (B) An HEV (asterisk) in the interfollicular region adjacent to the muscularis mucosae (mm). NO synthase reactive patches are smaller than stained nerve cells (small arrows) and larger than stained nerve fibre (small triangles) varicosities seen in the muscularis mucosae. (C) Extralymphoid HEV (asterisks) within the glandular mucosa (m). Compare nerve fibre (small triangles) varicosities with NO synthase stained patches of the intramucosal HEV endothelial cells. Scale bars = 50 μm.

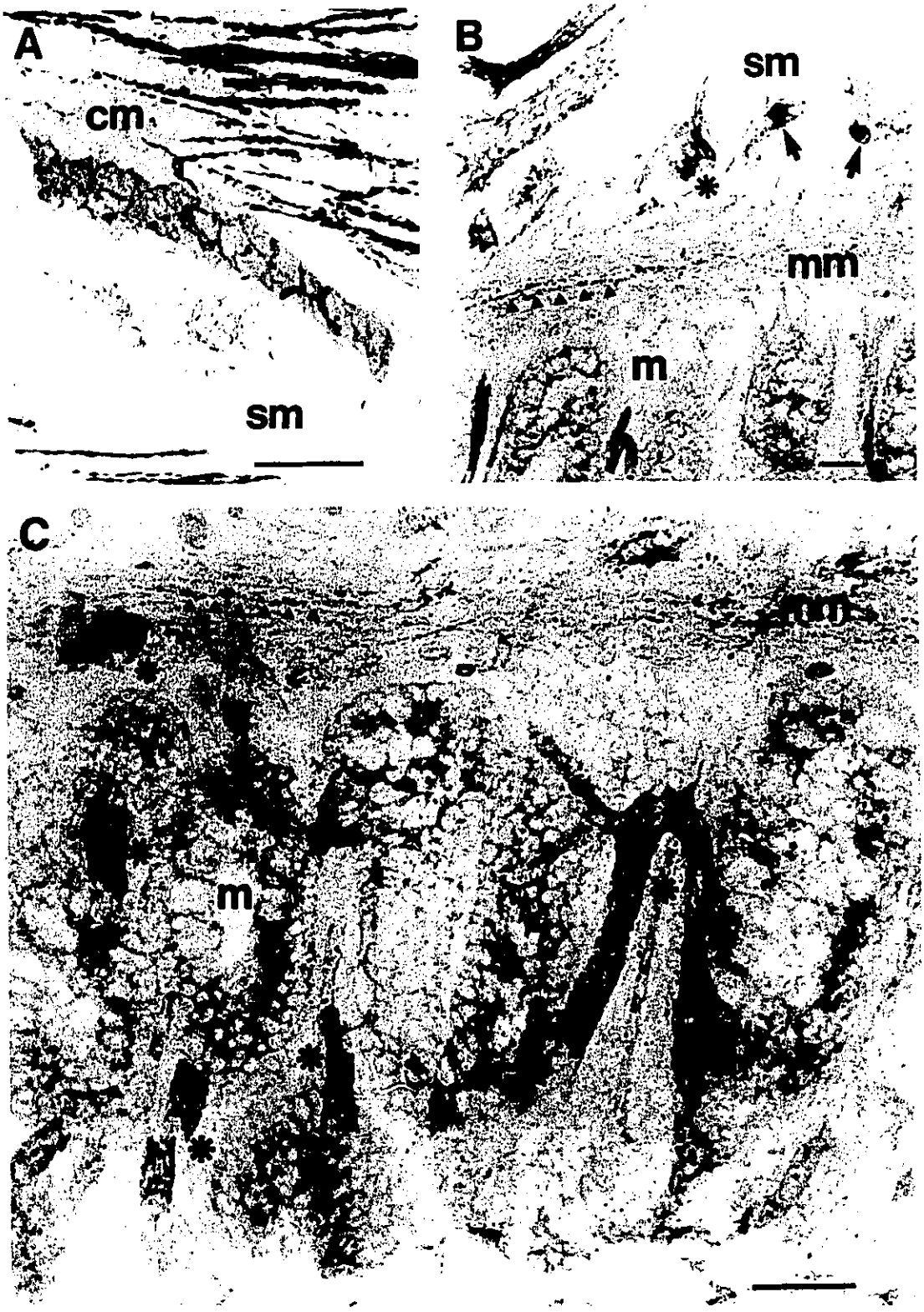
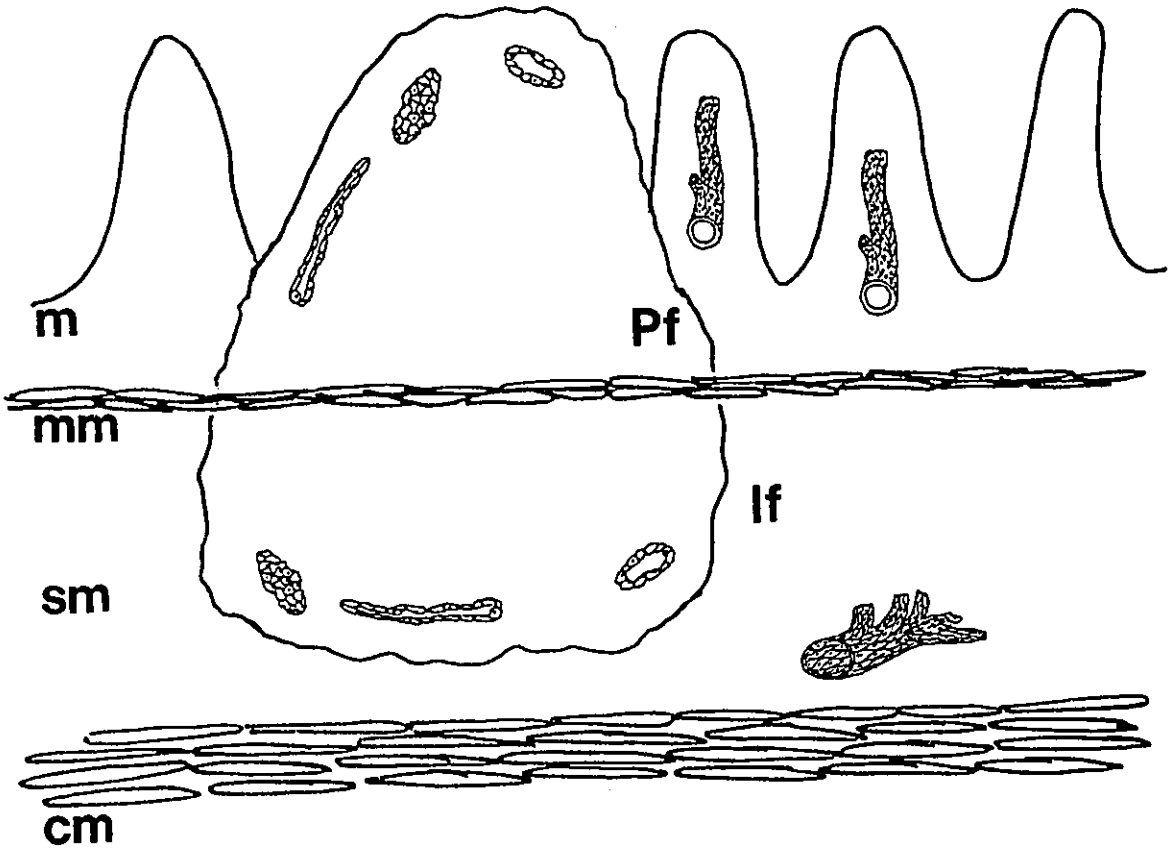

A
cm
sm
B
sm
mm
m
C
mm
m

Figure 5.5. A diagram showing the distribution of HEV in the human large intestine and the mural constituents in cross section including the mucosa (m), muscularis mucosae (mm), submucosa (sm) and circular smooth muscle (cm). There is a dense vascularization in the perifollicular (Pf) and interfollicular (If) regions. The interfollicular HEV give rise to mucosal (m) HEV which surround the Peyer's patch.


m
Pf
mm
lf
sm
cm

smooth muscle (fig. 5.4A), muscularis mucosae (fig. 5.4B) and within the glandular mucosa of the colon (fig. 5.4C). In these regions the HEV appeared as a sheet of endothelial cells possibly due to the orientation of the venules within the plane of the section and are distinct from the arterioles and venules irrigating these areas as seen in the previous section. The patches of NO synthase activity associated with each endothelial cell were smaller than the submucosal nerve cells (measuring approximately 25 mm) (fig. 5.4B) and larger than nerve fiber varicosities (fig. 5.4C).

A diagrammatic representation of the wall of the human large intestine showing the distribution and localization of the HEV identified in this study is presented in Figure 5.5.

5.2.4. Discussion

Staining of the HEV in this study provided a general anatomic picture of the distribution of these venules in the human intestine as summarized in the diagram of figure 5.5. Intensely stained HEV were localized to perifollicular and interfollicular regions as well as intramucosal areas. This distribution parallels the organization of lymphatic microvessels as shown using in vivo intravital microscopy (Nagata et al. 1994). This makes sense, since lymphocytes in the circulation migrate across the HEV to be stored or transported within lymphatic vessels.

The results of this study show that endothelial cells of gut-associated HEV in the human colon possess the potential for NO synthesis. Each cell displayed at least one discrete patch of NO synthase activity. However these NO synthase reactive patches displayed various subcellular distributions; luminal, adluminal and at the juncture between cells. This differential NO synthase staining pattern may indicate that individual endothelial cells might have been in different functional states. The occurence of patches of NO synthase activity is in accord with my previous studies of the mammalian intestine displaying enzymatic activity in endothelial cells of other microvessels (Nichols et al. 1992; Nichols et al. 1993; Nichols et al. 1994). This staining pattern is also consistent with the endothelial NO synthase isoform being a membrane-associated protein (Pollock et al. 1993; Loesch et al. 1994). Electron microscopic observations have revealed that endothelial NO synthase, identified histochemically or immunohistochemically, exists in perinuclear granules. These granules are associated with membranous structures believed to be the endoplasmic reticulum and cytoplasmic vesicles (Tomimoto et al. 1994). In addition to providing information on the cellular localization of this enzyme, this electron

microscopic data further confirmed the validity of NADPH diaphorase histochemistry as a marker of endothelial NO synthase.

High endothelial venules (HEV) are specialized postcapillary venules found in lymphoid organs such as Peyer's patches of the gut wall. HEV have long been regarded as blood vessels characteristic of lymphoid tissue. Very little is known about endothelial cells of HEV beyond the fact that these endothelial cells differ from those of other blood vessels in morphology and their height (Ropke et al. 1972; Radmonski et al. 1990). In addition, they have surface staining for immunoglobulin G which has not been found in endothelial cells of other blood vessels (Yamaguchi et al. 1983). The HEV are located between arteriovenous communications and venous sphincters and provide sites of migration of small lymphocytes from the blood vascular system into lymphoid organs (such as the spleen or Peyer's patch) (Yamaguchi et al. 1983). To gain access to the lymph node, circulating lymphocytes must first interact (adhesion) with the columnar-shaped high endothelial cells of the HEV and then pass (emigration) between them. From the lymph node parenchyma, the lymphocytes pass into the efferent lymphatics and recirculate back into the blood stream via the thoracic duct and the left subclavian vein (Pabst 1987). This migration of lymphocytes referred to as lymphocyte recirculation, permits antigen-specific lymphocytes to 'home to' and come into contact with their appropriate antigen in the peripheral lymphoid tissues. There is also a targeted migration of lymphocytes to specific lymphoid organs. For example lymphocytes 'homed' to the gut are selectively transported across *gut specific* HEV into Peyer's patches (Salmi et al. 1991).

Lymphocyte migration and recirculation is determined by events at the luminal endothelial cell-lymphocyte interface. The traffic of lymphocyte subsets is directed by binding to HEV in lymphoid tissues and in sites of inflammation. Regulation of these events is critical since migration is a selective event in that only lymphocytes are removed from the circulation (Doe 1989; Salmi et al. 1991). Adhesion and emigration pathways used by lymphocytes are also used in other physiologic settings, for example, by neutrophils interacting with acutely inflamed endothelium. Intravital microscopic studies, as well as in vitro models of neutrophil interactions with endothelial cells, demonstrate that this process appears to be different to that used by recirculating lymphocytes (Butcher 1991).

In general however, the movement of leukocytes (i.e. lymphocytes and neutrophils) are dependent on several factors. Cell migration patterns depend not only on the type of cell, but also on its stage of activation. In addition, vascular endothelium varies throughout the body, which influences cell migration. For example, the HEV found in (secondary) lymphoid

tissues are different from HEV or even non-specialized postcapillary venules of non-lymphoid tissues. Moreover, the small vessel endothelium among non-lymphoid tissues varies considerably between different tissue types. However, in all such cases the molecules present on endothelium are locally regulated under inflammatory conditions. The types of cells which migrate across different endothelial beds are affected by all of these determinants. Generally, the migration of leukocytes (eg. lymphocytes, neutrophils) depends on the interacting cells' surface charge, the hemodynamic shear force in the vascular bed and the expression of complementary sets of adhesion molecules on the leukocytes and the endothelium. Once cells have left the vasculature, they use different sets of adhesion molecules to maneuver through the tissues (see Roitt et al. 1993).

Regulation of neutrophil-endothelial interactions is not well understood, however there is now evidence for NO, produced by endothelial cells in the gut, to play a key role. Inhibition of NO production with analogues of the synthetic precursor L-arginine (N^G-monomethyl-L-arginine or L-NMMA and N^G-nitro-L-arginine methyl ester or L-NAME) which inhibit the constitutive and inducible forms of NO synthase activates neutrophil adhesion to and emigration across the endothelium of feline gut-associated mesenteric (non-specialized, non-lymphoid) postcapillary venules. This effect is reversed by L-arginine (Kubes et al. 1991; Kubes 1992). The NO donor S-nitroso-*N*-acetylpenicillamine (SNAP) is a potent inhibitor of neutrophil-endothelial interaction in vitro (Ma et al. 1993). In addition, NO has been shown to decrease PAF-stimulated neutrophil adhesion to postcapillary venules (Gaboury et al. 1992). Together these findings indicate that NO exerts a negative modulatory influence on neutrophil-endothelial interactions. There is also indirect evidence that NO may be involved in neutrophil emigration following recognition and binding to the endothelium. Inhibition of NO production causes an increase in protein and fluid leakage out of the microvasculature within the feline mesentery. The mechanism(s) by which NO inhibits neutrophil adhesion and emigration to the endothelium is still unclear. It is proposed that the drop in NO production may trigger the activation of other inflammatory cells which then may cause release of substances that effect an increased microvascular permeability (Kubes et al. 1992). Another proposed mechanism by which NO regulates neutrophil-endothelial interaction is via the endothelial-derived P-selectin, a member of the adhesion glycoprotein superfamily. Exogenous NO is proposed to prevent neutrophil adherence to the venular endothelium, at least in part, through a decreased expression of P-selectin (Gauthier et al. 1994). The source of NO production for these actions remains to be

determined however, endothelium-derived NO is the proposed endogenous modulator of neutrophil adhesion and emigration (Kubes et al. 1992).

Whether NO plays a role in lymphocyte-HEV interaction or emigration of blood borne lymphocytes into the Peyer's patch parenchyma or regulation of lymphocyte homing to Peyer's patches is as yet undetermined. There is some indirect evidence to support such a notion. Pertussis toxin has been shown to have an inhibitory action on lymphocyte-HEV adhesion events and is dependent on a G protein activating ADP-ribosyltransferase pathway (Bargatze et al. 1993). NO also activates ADP-ribosyltransferase activity (Brune et al. 1989) which suggests the possibility that NO may have a role in this process. In vitro studies demonstrate that the kinetics of (neutrophil-endothelial) adhesion events in inflamed endothelium seem too slow (occurring over minutes) to explain the very rapid interactions of circulating lymphocytes with HEV in vivo, which occur within seconds (Bjerknes et al. 1986). NO-sensitive G protein-ADP-ribosyltransferase signaling pathways are well qualified for the very rapid alterations in cellular behavior required for lymphocytes to adhere to HEV (Bargatze et al. 1993).

In conclusion, our results demonstrate that human intestinal HEV have NO synthesis potential. Direct evidence for NO to exert an effect on lymphocyte-high endothelial interactions in Peyer's patches or other lymphoid tissue awaits further study. However, in light of the role of NO in neutrophil-endothelial interactions, the importance of ADP-ribosyltransferase activity involved in lymphocyte binding to HEV, the fact that NO activates ADP-ribosyltransferase activity and these anatomical findings, the possibility that NO affects lymphocyte-HEV interactions deserves attention.

Chapter 6

6.1 The Enteric Nitrergic System and the Pathogenesis of Experimental Duodenal Ulcer

Duodenal ulcer disease has a complex etiology with multiple pathogenic factors including increased secretion of gastric acid (see Szabo 1984) and altered bicarbonate secretion (Isenberg et al. 1987). These were the earliest recognized contributing factors to the human disease state and led to the original therapies utilizing agents with anti-secretory or acid neutralizing actions. The rate of ulcer healing was significantly increased with these treatments, but a high frequency of side effects and ulcer recurrence was also reported (Salena et al. 1987).

The human disease state is distinguished by the development of ulcers on the anterior and/or posterior aspect of the duodenal bulb, immediately distal to the pyloric sphincter (Brooks 1985). In the acute form of the disease, focal lesions only pervade the mucosal surface of the duodenum (Poulsen et al. 1977; Brooks 1985), whereas in the chronic state, lesions may further penetrate through the layers of the intestinal wall. The clinical severity appears to be associated with the number (1 or 2) and size of the ulcers (see Brooks 1985). As aggressive factors (eg. acid) within the lumen of the duodenum, overcome the mucosal defense mechanisms (eg. alkaline secretions) a breach in the duodenal mucosa may occur. Local disturbances in acid secretion and neutralization are not in of themselves only inadequate for ulcer formation (Kirkegaard et al. 1980), but they are not always associated with the disease and fail to explain the specific localization (focal nature) of the lesions (Jacobson et al. 1975). These findings are indicative of the multifactorial nature of this disease (Szabo 1984).

Using the most effective animal model of the disease, the cysteamine-HCl treated Sprague-Dawley rat, I sought to investigate some alternate explanations for the cause of duodenal ulceration. Much information about this disease has been obtained from the Sprague-Dawley rat gastrointestinal tract which is used widely as a model for gastrointestinal disease including duodenal ulceration. The three major pathogenic factors in duodenal ulcer formation as determined from multiple studies of the CSH experimental model are proposed to be secretion, motility and mucosal damage and defense (Szabo 1984). While the stability of these contributing factors has an anatomical and vascular basis, disruption to any one of these functions depends on the neuroendocrine status of the individual.

Cysteamine-HCl (CSH) administered to rats by oral or systemic routes reliably results in the formation of one solitary or two opposing duodenal ulcers, within 2 cm of the

pyloric sphincter (Szabo 1978; Krantis et al. 1989). Ulcers occur within 24 hours on the anterior and/or posterior wall of the duodenum and may penetrate to the liver or pancreas. Compared to other models, CSH induced duodenal ulceration most closely imitates the morphology and pathology of the human disease condition (Robert et al. 1974; Szabo 1978; Krantis et al. 1989). CSH-treated rats exhibit increased gastric acid output (Szabo, 1977; Ishii et al. 1976; Kirkegaard et al. 1980), and reduced alkaline secretions (Kirkegaard et al. 1981; Adler et al. 1983; Isenberg et al. 1987). Not surprisingly therefore, CSH induced ulcers respond to therapeutic strategies involving antacids, and antisecretory agents (Robert et al. 1974; Szabo 1984). However in man, these strategies are problematic (see above).

Factors other than acid secretion (Table 3.1) play a significant role in the development of duodenal ulceration but the manner and extent to which they may interact to initiate lesion formation is unclear. These factors include changes in patterns of gastroduodenal motility and blood flow as well as neuroendocrine status. Stress, in its many forms, can lead to any one or all of these changes. Ultimately, a combination of these pathogenic alterations creates an imbalance between aggressive and defensive factors.

TABLE 6.1. Some factors which may contribute to duodenal mucosal injury

Increased gastric acid secretion
Decreased bicarbonate secretion
Altered patterns of duodenal mucosal blood flow/ischemia-reperfusion
Altered gastroduodenal motility patterns
Increased free radical generation
Increased levels of Helicobacter pylori in the duodenum
Stress

The presence of acid in the duodenum is a requirement for ulcer formation to occur, but duodenal ulcer patients may exhibit increases, decreases and no change in gastric acid secretion compared to normal levels (Jacobson et al. 1975). In (acid) hypersecretory syndromes such as Zollinger-Ellison, multiple randomly localized lesions are formed over the proximal to distal duodenum rather than the focal lesions observed in patients with duodenal ulcer disease (see Szabo 1984). Interestingly, rats subjected to pentagastrin administration in order to stimulate and attain similar levels of gastric acid secretion as that seen in the CSH model, do not produce ulcers

(Kirkegaard et al. 1980). Therefore, in the CSH rat model of duodenal ulcer and in the human disease state, the presence of acid must only be a contributing factor to ulcerogenesis and does not account for the specific localization of duodenal ulcers. Moreover, the level of acid secretion must be considered in the context of the impairment of acid neutralization in the duodenum. Secretion of bicarbonate for bulk neutralization of acid in the duodenum comes from multiple sources including the duodenum (from submucosal Brunner's glands and mucosal epithelial cells), the pancreas, and the liver (Flemström 1994). In patients with duodenal ulcer bicarbonate secretion from the duodenal mucosa (Isenberg et al 1987) and the pH at the surface of this epithelium (Danesh et al 1987; Quigley et al 1987) were found to be significantly lower. These findings were proposed to be due to a defect in the duodenal bicarbonate secretory processes, or of neural pathways mediating reflex control of bicarbonate secretion. In the CSH treated rat, the pancreatic secretions are elevated while production and secretion of duodenal bicarbonate is reduced (Szabo 1984). Moreover, CSH, at a dose proposed not to affect the bicarbonate secretory processes, inhibited the ability of the duodenal mucosa to detect luminal acid (Adler et al. 1983; Briden et al. 1985; Ohe et al 1988). Taken together the findings of these human and animal studies suggest that there may be a defective mediation of stimuli of the secretion, increased sympathetic inhibition of the secretion or a combination of these mechanisms. Irrespective of the mechanism, this diminished function may result in a decrease in acid disposal in the proximal duodenal segment, and in turn increase the acid load in this region.

An additional and often overlooked factor necessary for efficient neutralization of acid in the duodenum is gastroduodenal motor activity. Regional motility patterns guide the delivery of alkaline secretions to the duodenal bulb from more distal sites thereby controlling the availability of alkaline secretions in the proximal duodenum for neutralization of the acidic chyme. Similarly, the rate of delivery, or emptying of acidic chyme from the stomach (gastric emptying) is also a determinant of the size of the acid load present in the duodenal bulb. Moreover, gastric motility also regulates the mixing of food with acidic gastric secretions, thereby diluting secreted protons before delivery to the duodenum (Merseau et al. 1984). However, patients with severe duodenal ulcer exhibit delayed gastric emptying (Monto et al. 1976). In the CSH induced duodenal ulcer model, gastric emptying and duodenal motility are also disrupted and these alterations occur in advance of changes in acid and bicarbonate secretions (Poulsen et al. 1982; Tanaka et al. 1989). Non-propagating motor activity patterns have been reported to be disrupted according to in vitro motility recordings taken from rat duodenal segments within 15 to 30 minutes following treatment with CSH. The investigators reported changes in motor activity, particularly

an increase in phase III responses of the migrating myoelectric complex (MMC) (see Szabo et al 1984). These alterations in MMC's were accompanied by increased transit time in the proximal duodenum and a decreased transit time in the distal duodenum (where pancreatic secretions enter). Thus motility disruptions could reduce effective transport of alkaline secretion from the distal duodenum to the duodenal bulb. In support of these findings, Monto et al (1976) observed a reduced frequency of orally directed contractions in patients with duodenal ulcer as compared to control subjects. Current reports by investigators in this laboratory using intraluminal strain guages to monitor in vivo motility patterns in conscious unrestrained rats following CSH administration, reveal a reduced frequency of contractile activity but a significant increase in the amplitude of contractions (associated with the force of contraction). In addition there was an increased frequency of relaxant responses but a reduced amplitude of relaxations (A. McKay and A. Krantis, unpublished observations).

CSH induced disruptions in gastroduodenal motility have been proposed to account at least in part for the specific localization of lesion damage to anterior and posterior sites of the proximal duodenum in Mersereau and Hinchey's (1984) 'channel hypothesis'. In CSH treated rats but not control animals, they observed duodenal channeling of gastric effluent to the sites of eventual lesion formation. This channeling phenomenon is produced by pyloric constriction. Therefore the focal lesions observed in duodenal ulcer patients may be a result of altered local gastroduodenal motility patterns.

In contrast to Mersereau and Hinchey's 'channel hypothesis', the anatomy of the human duodenal vascular supply has also been proposed to explain the specific localization of duodenal ulcers. Examination of the human proximal duodenum by intravital microscopy revealed the existence of end arteries within 2 cm of the pylorus; mesenteric arteries that do not connect with the submucous vascular plexus by anastomoses, but directly supply regions of the mucosa (Piasecki 1971). Interestingly, a study of canine duodenum showed that none of the mucosal arteries of this region were extramural (mesenteric) in origin (Piasecki 1975) and unlike man, this species does not exhibit chronic duodenal ulceration. These data are intriguing and provide indirect evidence for end arteries to play a role in ulcerogenesis. An increased force of smooth muscle contraction in the proximal duodenum could give rise to constriction of these end arteries and focal ischemia due to the lack of collateral blood flow to these sites (fig. 6.1). Support for the occurrence of these events comes from several studies. As discussed, both *in vitro* and *in vivo* investigations of the effects of CSH on gastroduodenal motility report observed increases in contractile motor activity in the gastroduodenum. Interestingly, large increases in

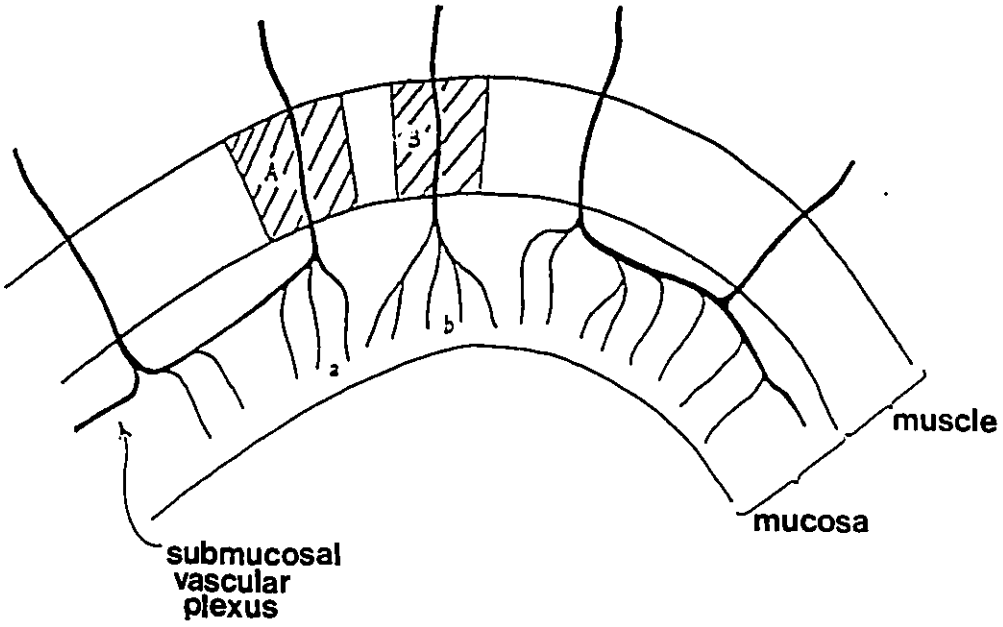


Figure 6.1. A schematic illustration of the anatomy of duodenal end arteries which may predispose a mucosal region to lesion formation. 'a' depicts the anatomy of vascularized mucosa showing anastomotic connections between arteries of the vascular plexus. Contraction of the smooth muscle about A would restrict blood flow but would not render the dependent mucosa ischemic, since supplemental flow is provided by anastomotic connections. 'b' depicts the mucosal region which is completely dependent on the blood supply from the duodenal end artery (B) since it receives no supplemental blood supply. Therefore, restriction of flow through B (eg. due to contraction of the muscularis) could impose ischemic damage and potentially lead to ulceration in this region of the mucosa.

tonic contraction or basal tone of a gut segment decreases mean mesenteric blood flow (Chou 1989) which can lead to a temporary ischemic insult. Inadequate blood flow can lead to disruption of gut barrier function seen as 'leaks' in the gut mucosa and this can occur in as little as 30 minutes of imposed ischemia. Maximal leakiness or permeability of the mucosa can be reached within 12 to 24 hrs after injury (see Landow et al. 1994).

The defense and maintenance of mucosal integrity are dependent on local blood flow (Miller 1988). Since the mucosal microcirculation provides a 'sink' for proton back diffusion, changes in local blood flow directly affects the duodenal acid load. In addition, mucosal blood flow is associated with duodenal bicarbonate secretion (Szabo et al. 1980; Flemström 1994). Therefore, bulk neutralization of acid may be jeopardized under conditions of compromised blood flow (i.e. ischemia). The findings obtained from two studies where different techniques of blood flow measurements were used in CSH induced duodenal ulcer, together, provide a picture of the transmural changes in local blood flow patterns in experimental ulcer. Within 15 to 30 minutes after CSH administration (the same time frame for changes in motility patterns), there is a decrease in the perfusion of the mucosal surface (villi tips) (hydrogen clearance technique, Yabana et al. 1989) followed by a transient increase in blood flow to the total thickness of the duodenum (microsphere technique, Szabo et al. 1980). These observations suggest that local hypoxia/ischemia (reduced flow) to the duodenal villi is followed by a reactive hyperemia in the extramucosal regions of the duodenum (Szabo 1984). Ischemia-reperfusion in the duodenal mucosa in the face of unbuffered acid may induce lesion formation. An ischemic insult could lead to tissue damage upon tissue reperfusion due to the formation of oxygen-derived free radicals and membrane lipid peroxidation which would disrupt mucosal integrity. Indeed the reintroduction of oxygen to ischemic tissue has been demonstrated to result in the explosive generation of oxygen-derived free radicals (Flaherty et al. 1988). In this regard, ligation of individual gastric mucosal arteries to produce focal mucosal ischemia induces gastric mucosal ulcers (Piasecki et al. 1989). Unfortunately, similar studies have not been carried out in the duodenum to confirm whether experimentally produced ischemia leads to the formation of duodenal ulcers. However, ultrastructural assessment of duodenal ulcer formation indicates that the earliest recognizable gross lesion occurs at the villi tips as soon as 30 minutes following the administration of CSH and is reminiscent of early ischemic (necrotic) damage (Brooks 1985; Pfeiffer et al. 1987; Tanaka et al. 1989). Focal necrosis occurs at the villus tips within 5 hours of CSH administration, followed by successive erosion within 12 hours to full ulcer formation visible from 18 to 24 hours (Poulsen et al. 1977). These final hours of duodenal ulcer formation

are accompanied by inflammatory cell infiltration. Scanning microscopy (Giampolo et al. 1980) reveals that as early as 2 hours following CSH administration, there is opening of intercellular junctions and microvillar disorganization in the absorptive cells, with subepithelial bleb and edema formation and degeneration of the lamina propria. From 8 to 12 hours post CSH, there is an observed necrosis and desquamation of absorptive cells exposing the lamina propria to luminal fluids. Focal necrotic damage and atrophy is observed to progress to formation of an 'ulcer crater' from 12 to 24 hours following CSH. Ulcers consistently occur 2 to 5 mm from the pylorus with ulcers formed on the anterior wall of the duodenum sometimes followed by a second ulcer located opposite on the posterior wall (Poulsen et al. 1977; Giampolo et al. 1980).

Support for the role of free radicals in duodenal ulceration comes from multiple studies which show that administration of oxygen-derived removing agents to duodenal ulcer rats (Salim 1990a; Salim 1990b; Salim 1990c; Krantis et al. 1993), significantly reduces lesion formation and stimulates healing of both acute and chronic duodenal ulceration. In addition, these compounds administered to duodenal ulcer patients (Salim 1990c) significantly reduced ulcer recurrence. The mechanism(s) leading to formation of these free radicals in duodenal ulcer disease has yet to be determined.

Helicobacter pylori (a non invasive gut pathogen) appears to play an important pathogenic role in the development of duodenal ulceration in man. Eradication of H. *pylori* with bismuth and antibiotics combined with the H_2 receptor blocker cimetidine to reduce acid causes rapid healing of ulcers and a lower rate of recurrent ulceration compared to cimetidine alone (Hentschel et al. 1993). The contribution to the ulcerogenic mechanism related to H. *pylori* is unclear. The presence of H. pylori is not sufficient for pathogenesis of the disease. Moreover, in intact *ex vivo* rat stomach (Saita et al. 1992) and rat mucosal cells *in vitro* (Micots et al. 1993) exposure to H. *pylori* alone is insufficient to cause injury. *However, following an ischemic insult H. pylori accelerates ulceration* (Saita et al. 1992). Thus the disruption to mucosal integrity which occurs during the latter stages of duodenal ulceration as discussed, would expose the mucosal lamina propria to the bacterium within the lumen of the duodenum and accelerate the ulcer process. In addition analysis of duodenal biopsies reveal that H. *pylori* is rarely found in the human duodenum and this is believed to be due to the presence of bile creating a non-hospitable environment (Kozol et al. 1994). Instead the bacterium is washed down from the stomach and once there triggers an inflammatory response which in certain individuals can indirectly cause tissue injury by enabling exposure of the mucosa to luminal aggressive factors, and consequent duodenal ulcer formation. This is supported by the finding that gastritis and duodenitis often

accompany duodenal ulcer disease. Interestingly, H. pylori is not found in Sprague-Dawley rats and hence is not a factor in the CSH induced duodenal ulceration. Indeed, this is the one factor that appears to set the two ulcerative conditions apart.

A consensus recommendation was that H. *pylori* should be eradicated in all patients with duodenal ulcer (see Freston 1994). The present most effective therapeutic modality for long-term management of duodenal ulcers is a triple therapy using bismuth (to treat for the presence of H. *pylori*), amoxicillin or tetracycline and metronidazole (Hentschel et al. 1993). This treatment regimen eradicates H. *pylori* in 80-90% of patients and the recurrence rate is low. The chief problem with this therapy is the low compliance. In addition, the gastrointestinal side effects, particularly from bismuth, have been reported to be very unpleasant (Hentschel et al. 1993) and occurs in at least 30% of patients treated by this therapy. The need to eradicate H. *pylori* not withstanding; recent clinical trials indicate that administration of cimetidene with free radical scavengers significantly reduces the recurrence of ulceration (Salim 1990c) implicating the role of free radicals in the pathogenesis of duodenal ulcer and the possibility for alternative effective treatment modalities.

Researchers in this laboratory are using the rat CSH duodenal ulcer model to derive basic information about the involvement of enteric GABAergic neurons and their target sites in the pathogenesis of this disease. Since pharmacologic and physiologic studies show that NANC inhibitory enteric neurons including a subpopulation releasing NO are targeted by intrinsic enteric GABAergic neurons (Booeckxstaens et al 1990), the aim of this study was to extend the investigation of the enteric nitrergic system by examining the effects of nitrergic compounds on the development of experimental duodenal ulcer.

Enteric transmitter substances regulate gastroduodenal motility and blood flow. Enteric neurotransmitters which have been shown to be affected by the development of duodenal ulceration include GABA, somatostatin, serotonin, and dopamine. GABA is a transmitter of enteric interneurons in a variety of mammals where it is proposed to play a role in the modulation of acid secretion and motility. CSH-induced duodenal ulceration is associated with alterations in levels of GABA (Krantis and McKay 1990) and the peripheral GABAergic system can modulate ulcer formation (A. McKay and A. Krantis, unpublished observations).

Both pharmacological and anatomical evidence indicates that GABA is present to interneurons throughout the rodent gut wall (Krantis et al. 1981; Jessen et al. 1983; Kerr et al. 1986; Davenger et al. 1987; Hills et al. 1987; Krantis et al. 1987; Roberts et al. 1988; Krantis et al. 1991a; Krantis et al. 1991b). The enteric actions of GABA are mediated via i) bicuculline-

sensitive, Cl^--dependent post-synaptic $GABA_A$ receptors on excitatory cholinergic motor neurons and NANC inhibitory motor neurons, and through ii) bicuculline-insensitive, baclofen-sensitive $GABA_B$ receptors. Whereas $GABA_A$ receptors mediate stimulation of motor activity, the $GABA_B$ sites mediate inhibition of the cholinergic motor neurons (Krantis et al. 1980; Krantis et al. 1981; Giotti et al. 1983; Ong et al. 1983; Maggi et al. 1984; Kerr et al. 1986; Krantis et al. 1987; Krantis et al. 1993; Roberts, 1993). The administration of GABA and aminooxyacetic acid (an inhibitor of GABA degradation) systemically exacerbates CSH induced ulcer formation while bicuculline (a $GABA_A$ receptor antagonist) reverses this effect (Krantis et al. 1989). It appears that systemic (presumably enteric) $GABA_A$ receptors are involved in this aggravation of duodenal ulcer formation since systemically administered GABA is unable to cross the blood-brain-barrier (Rapoport 1976; Krantis 1983). In the rat stomach and duodenum, CSH stimulates NANC inhibitory neurons in vitro (interfering with neurogenic responses) and this action is not due to $GABA_A$ receptor- or electrical stimulation of these neurons (Krantis 1987; Krantis et al. 1989). Oral or subcutaneous administration of baclofen ($GABA_B$ receptor agonist), significantly reduces CSH induced ulcer formation (A. McKay and A. Krantis unpublished observations). GABA is colocalized with gastrin in antral mucosal G cells (Davenger et al. 1994). In addition, GABA stimulates gastrin release via $GABA_A$-receptor sites (Harty et al. 1983) as well as stimulating the neural cholinergic modulation of gastrin and somatostatin release (Harty et al. 1991). For ease of discussion, Krantis and colleagues (1995) describe GABAergic actions mediated by $GABA_A$ receptors as A-GABAergic and $GABA_B$ related actions as B-GABAergic. In addition to its proposed role in control of motility and acid secretion, there is now preliminary evidence for a B-GABAergic reduction in mucosal blood flow (Knight et al. 1994). Taken together, these studies provide direct evidence that peripheral GABAergic receptor sites may represent new target sites for duodenal ulcer therapy. When taken in the context of GABAergic neurons targeting NO neurons, the need to determine the involvement of GABA/NO in duodenal ulcerogenesis becomes obvious.

In addition to nitrergic neurons being targeted by A-GABAergics, NO may also be a transmitter of gut interneurons, since it colocalizes with GABA (Nichols et al. 1994) and NO modulates ATP-NANC inhibitory neurons in the rat duodenum (Glasgow et al. 1993). In the canine small intestine NO appears to exert a tonic inhibitory effect on intestinal myoelectric activity by reducing the frequency of the MMC pacesetter and by suppressing the postprandial activity (Maczka et al 1993). In addition NO stimulates gastric emptying presumably by reducing pyloric sphincter tone (Calignano et al 1993). *In vivo* studies in the rat indicate that NO also appears to

stimulate gastric acid secretion by altering gastric blood flow and gastrin release (Pique et al 1992; Bilski et al. 1994). While NO does not have a direct effect on the parietal cell, it mediates the vasodilation associated with the acid secretory process (Pique et al 1992) potentially allowing a maximal rate of secretion to be attained. This indirect action of NO on acid secretion from the parietal cell is proposed to be due either to an increased supply of a secretory stimulant to the parietal cell via the circulation or to support of the metabolic demands of secretion (Pique et al 1992). In addition NO inhibits neural modulation of acid secretion from these cells (Barrachina et al. 1994). NO inhibits duodenal bicarbonate via a depressant action on neurally mediated secretion (Hallgren et al 1993; Takeuchi et al 1993). However NO has also been shown to play a stimulatory role in gastroduodenal alkaline secretion in response to acid (Bilski et al. 1994). NO stimulates pancreatic secretion and this prosecretory effect is proposed to be due to its vasodilatory actions and consequent increased supply of hormone to the secretory cells and/or by mediating neurally stimulated pancreatic exocrine secretion (Konturek et al. 1993).

NO plays an integral part in the maintenance of mucosal integrity (MacNaughton et al. 1989). In addition, NO is synthesized in enteric blood vessels (Nichols et al. 1994) and plays a critical role in the local regulation of blood flow in the gut (Pique et al. 1989; Walder et al. 1990; Pique et al. 1992). Local hyperemic responses are regulated by NO. Following brachial artery occlusion treatment with the NO synthase inhibitor L-NMMA did not affect peak blood flow but significantly decreased the duration of hyperemia or flow debt repayment (reperfusion) (Tagawa et al 1994). Thus, EDNO synthesis contributes to the postischemic hyperemic response. Moreover, NO mediates early postischemic vasodilation (Greenberg et al. 1995).

Based on these motor and neuroendocrine actions of NO *we hypothesized that the nitrergic system may be involved in the pathogenesis of duodenal ulceration.* If enteric nitrergic sites are involved in ulcerogenesis, this raises the prospect not only for new avenues for the study of the duodenal ulcer process, but also potentially important therapeutic target sites for this disease. In order to address this question, *the aim of this study was directed towards determining the extent of involvement of nitrergic mechanisms in experimental duodenal ulceration by examining the effects of CSH on NO innervation and production as well as assessing the effects of various systemically administered nitrergic compounds on cysteamine-HCl induced duodenal ulceration.* Since the B-GABAergic system has an ameliorating effect on duodenal ulceration, *this study also set out to examine whether modulation of the GABAergic and nitrergic systems would be advantageous.*

6.2. Materials and Methods

Male Sprague-Dawley rats (Charles River, Quebec) weighing 200-350g were housed as described in section 2.1.1 and allowed a week to adapt. Experimental procedure carried in this study complies with the set guidelines of the Canadian Council on Animal Care.

6.2.1 Induction of Duodenal Ulceration

Duodenal ulcers (DU) were induced following the procedure described previously (Krantis et al. 1993). The rats were fasted for 24 hr with full access to water. Subsequently, cysteamine-HCl (CSH) was administered in two intragastric bolus doses of 2.8mg/kg, 2 hr apart. Animals were then given water containing 0.1% CSH for 24 hr. Sham controls were treated in the same manner but 0.9% saline was administered in place of CSH. Individual groups of animals were treated with various drugs prior to the administration of CSH. All drugs were administered subcutaneously. The treatment groups included:

(1) L-arginine 150 mg/kg;

(2) N^G-nitro-L-arginine methyl ester (L-NAME) at 37.5mg/kg;

(3) N^G-nitro-L-arginine (L-NNA) at 37.5mg/kg;

(4) N^G-iminoethyl L-ornithine (L-NIO) at 50 mg/kg all administered 1 hr prior to CSH administration.

These dosages of NO synthase substrate and inhibitors are based on previous in vivo studies (Rees et al 1990; Lefebvre et al 1992b). Lastly,

(5) baclofen 400 µg/kg given 2 hr prior to CSH administration and L-NAME 37.5 mg/kg given 1 hr prior to CSH administration.

All animals were euthanised 24 hrs following the second injection of CSH. The rats were then laporotomized and the gastroduodenum was excised and treated to allow: (1) examination of the the mucosal surfaces for lesion formation; (2) NADPH diaphorase histochemical analysis in circumferential sections (see section 6.2.2); (3) assessment of NO synthase mRNA using *in situ* hybridization in circumferential sections of duodenum (see section 6.2.3) or (4) immediately frozen in liquid nitrogen for subsequent biochemical analysis of NO synthase activity (see section 6.2.4). In the first group the incidence, profile and severity of duodenal ulceration was assessed with the various drug treatments after the method of Krantis and Nicholson (1989). The 'incidence' of duodenal ulceration was calculated by the presence or absence of duodenal lesions. A 'profile' of ulceration was obtained from the number of ulcers observed macroscopically on the mucosal surface of the duodenum. The severity of the mucosal

lesions were determined using macroscopic measurement of the extent of each lesion (the product of the length and breadth) to derive the area in mm^2 of the lesion. This was considered to be a determinant of the 'intensity of ulceration'. Evaluations of intensity were done under randomized, blinded conditions.

6.2.2 NO Synthase Histochemistry

Proximal segments of duodenum (1 cm) were removed from control and CSH treated rats, washed in PBS (pH 7.4) and immersed in modified Zamboni's fixative for 2 hrs at 4^0C (see section 2.1.1). Specimens were washed in PBS (pH 7.4) to clear the tissue of fixative and incubated for at least 24 hrs in PB (pH 7.4) containing 10% sucrose for cryoprotection. Cryoprotected specimens were frozen on dry ice with cold CO_2, sectioned at -20^0C to achieve thickness of 14 µm, and mounted on glass slides. Slide mounted sections were incubated in the NADPH diaphorase medium (see section 2.1.1) for 30 min. at 37^0C, rinsed in PB (pH 7.4) and subsequently incubated in standard eosin stain for 30 seconds at room temperature (RT).

6.2.3 *In Situ* Hybridization

Identical lengths of duodenal segments taken from control and CSH treated rats were washed in sterile ice cold PBS (pH 7.4) and immersed in 4% paraformaldehyde in 0.1M sodium phosphate buffer (pH 7.4) for 48 hrs at 4^0C. All subsequent procedures were carried out under sterile procedures to prevent the degradative action of RNAases on the tissue mRNA. Fixative was cleared in three changes of sterile PBS (pH 7.4) and incubated overnight in sterile 10% sucrose in PB (pH 7.4) for cryoprotection. Cryoprotected specimens were frozen on dry ice, sectioned at -23^0C using a blade that had been immersed in 5% H_2O_2 in sterile water for at least 1 hr prior to use to inactivate RNAases. Sections were taken in the circumferential axis of the duodenum at a 20 µm thickness, mounted on 3-aminopropyltriethoxysilane treated glass slides and stored at -20^0C until the *in situ* hybridization (ISH) procedure was carried out.

A 39mer oligonucleotide (3'-TTG GGC ATG CTG AGG GCC ATT ACC CAG ACC TGT GAC TCT-5') complimentary to the mRNA coding for amino acids [151-163] of rat brain NO synthase [EC 1.14.13.39] (Bredt et al. 1991b) was kindly provided by Dr. J. Fryer, Department of Anatomy and Neurobiology, University of Ottawa.

Tailing of the NO synthase mRNA oligonucleotide probe was carried out using terminal deoxynucleotidyltransferase for incorporating digoxigenin (DIG) labeled-dUTP nucleotide to the 3"-end. To make 50 µl (final volume) of labeling mixture, constituents were

added in the following order. To redistilled water, 1M sodium cacodylate buffer with 2mM dithiothrietol and 300mM Tris-HCL (pH 7.2) was added and mixed with $CoCl_2$ for a final concentration of 2mM. Subsequently, 1μg (corresponding to approximately 200 pmol) of the probe, 5nmol of digoxigenin-11-dUTP in sodium cacodylate buffer and 30 units of terminal deoxynucleotidyl transferase in 1.2 μl of sodium cacodylate buffer were added. The reaction mixture was incubated at 37^0C for 5 hrs.

Tissue sections, from untreated and CSH treated rats, 20 μm in thickness were air-dried at room temperature (RT) for 15 min., and immersed in standard Bouin's fixative at RT for 30 min. to quench the high levels of endogenous intestinal alkaline phosphatase activity. Fixative was cleared in two 10 min. changes of 70% ethanol and washed in redistilled water. Proteolytic treatment was carried out with proteinase K, 5μg/ml in 0.1M Tris-HCL, pH 8.0 at 37^0C for 20 min. to permeabolize the fixation associated cellular protein matrix and to enhance the signal. Sections were then hybridized with 500 ng/ml of DIG-labeled probe in hybridization buffer consisting of 50% formamide, 6X SSC (1XSSC = 0.15M sodium chloride, and 0.015M sodium citrate, pH 7.4) 50mm Tris-HCL and 2.2% sodium dodecyl sulphate (SDS) in Denhardts solution. Hybridization was allowed to proceed overnight at RT in a chamber humidified with 50% formamide in 1X SSC. Posthybridization washes included 2X SSC (2 changes over 20 min.) and 0.5X SSC (2 changes over 40 min.) at RT. Detection of hybridized DIG-labeled probe was achieved by high affinity anti-DIG antibodies conjugated to alkaline phosphatase (AP). A 2 hr. incubation at RT with sheep anti-DIG antibody conjugated to AP diluted 1:500 in 0.025M Tris-HCL (pH 7.5) containing 0.3% Triton-X-100 was followed by two 10 min. washes in 0.1M Tris-HCL pH 7.5. Detection of AP activity was performed by development in bromochloroindoyl phosphate - nitroblue tetrazolium (BCIP-NBT) containing 1mM levamisole for 5 hrs. to inhibit endogenous AP activity. In order to examine the specificity of the hybridization three controls were employed. These include a) omission of the probe to eliminate the possibility of positive chemography (due to high levels of endogenous AP activity in the gut), b) pretreatment of sections with RNAase to prevent binding of probe to RNA in the tissue, and c) prehybridizations with excess unlabeled oligonucleotide (using 10X the concentration of labeled probe and incubating for 30 min. at RT) before application of labeled probe to eliminate or significantly reduce signal by competitive binding of unlabeled probe to NO synthase mRNA.

A colorimetric standard curve was prepared by allowing the color development to proceed for six different time points from 2 to 72 hrs in sections of duodenum from control rats.

The curve was used to semiquantitatively correlate color intensity of staining with absolute levels of NO synthase mRNA as seen in control vs CSH treated rats.

6.2.4 Biochemical Measurement of NO Synthase Activity

NO synthase activity was assessed using the ^{14}C-L-arginine to ^{14}C-L-citrulline conversion assay (Salter et al 1991). Briefly, frozen segments of duodenum were thawed on ice and placed in ice cold Krebs buffer (pH 7.4) consisting of 115mM NaCl, 8mM KCl, 2mM KH_2PO_4, 25mM $NaHCO_3$, 1.3mM $CaCl_2$ and 2.4mM $MgCl_2$. Tissues weighing 50-100mg were placed in 250 µl of a homogenization buffer (pH 7.4) at 4^{0}C consisting of 10mM HEPES, 320mM sucrose, 1mM dithiothrietol, 0.1mM ethylenediaminetetraacetic acid (EDTA), 10µg/ml soybean trypsin inhibitor, 10µg/ml leupeptin and 2µg/ml aprotinin. Using a Polytron homogenizer (Brinkmann Instruments, Rexdale, Ontario), tissues were homogenized for 20 sec at high speed and centrifuged at 14000 x g for 10 min at 4^{0}C. Subsequently, two separate 20 µl aliquots of the supernatant were added to:

1) 50 µl of incubation buffer at 37^{0}C consisting of 975µM NADPH, 190µM $CaCl_2$, 948µM $MgCl_2$, 38mM KH_2PO_4, 6mM valine, 58µM L-arginine and 0.047 µCi ^{14}C-L-arginine;

2) incubation buffer also containing 977 µM Ca^{2+} chelator ethylene glycol-bis (ß-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA) to differentiate between the Ca^{2+}-dependent constitutive and the Ca^{2+}-independent inducible enzyme isoform. An internal control sample [to assess background (BG) activity] consisted of 50 µl of incubation buffer at 37^{0}C with ^{14}C-L-arginine and 20 µl of filtered deionized water (ddH_2O). All mixtures were vortexed and placed in a metabolic shaking incubator for 10 min. at 37^{0}C. Separation of ^{14}C-L-arginine from ^{14}C-L-citrulline was achieved using ion exchange chromatography. Resin (Na- form, 100-200 mesh, Bio-Rad) was mixed with concentrated NaOH and rinsed several times (~20 times) with ddH_2O to attain a pH of 8.0. Samples were loaded onto Dowex 50W-X8 ion exchange columns (1 ml volume) and eluted with 2ml ddH_2O. 1ml aliquots of eluent were mixed with 14ml of liquid scintillation fluid, vortexed and placed in a Beckman LS-9000 scintillation counter for radioactive counts. NO synthase activity is expressed as nmol/min/g tissue. The activity of the different isoforms of NO synthase were then calculated Those samples which did not contain EGTA (a calcium chelator) in the reaction mixture, reflected total NO synthase activity, while those containing EGTA reflected NO synthase activity of the inducible isoform. The activity of constitutive NO synthase was calculated from the difference between total and inducible NO

synthase activities. The following is a sample calculation of the amount of ^{4}C-L-arginine converted to ^{14}C-L-citrulline or NO synthase activity. [(2 x sample CPM) - (2 x BG CPM)] x dilution factor x [^{14}C activity in incubation buffer / total CPM] x [mol cold arginine in incubation buffer / mols of hot arginine in incubation buffer] / tissue weight x 1000 mg / incubation time = nmol NO / min./ g tissue.

6.3. Drugs and Chemicals

All drugs were directly dissolved in 0.9% saline. Cysteamine (2-aminoethanethiol HCl) was obtained from Aldrich (Milwaukee, Wisconsin). Baclofen (Lioresal) was a gift from Ciba-Geigy. L-NIO was purchased from Transduction Laboratories (Kentucky) L-arginine, L-NAME, EDTA, ß-aminoethyl ether EGTA, NADPH, soybean trypsin inhibitor, leupeptin, aprotinin and valine were purchased from Sigma (St. Louis, Missouri). NaCl, $CaCl_2$. KCl, MgCl, NaOH, $NaHCO_3$, KH_2PO_4, HEPES, and dithiothreitol were obtained from BDH (Montreal, Quebec). ^{14}C-L-arginine and ^{14}C-L-citrulline were obtained from NEN Dupont (Mississauga, Ontario). Dowex 50W-X8 resin (Na-form, 100-200 mesh) and the chromatography columns were purchased from Bio-Rad Laboratories (Richmond, California).

6.4 Statistical Analysis

For comparisons of NO synthase activity in the individual treatment groups, group means were compared using a one-way analysis of variance (ANOVA). A value of $P<0.05$ was considered to be statistically significant. When significance was found, the least significant difference test was used for multiple comparisons. The relationship between the incidence or profile of CSH induced duodenal ulceration with pretreatment with NO synthase inhibitors was assessed by chi-square (Fisher's exact test of proportions) test. For comparison of the area of CSH induced lesions with NO synthase treatment, a Student's t-test for unpaired observations was used. Data for ulcer incidence and profile are expressed as percentages. All other quantitative data (NO synthase activity and lesion area) are expressed as the mean ± SEM (standard error of the mean) of the number of independent determinations.

6.5 RESULTS

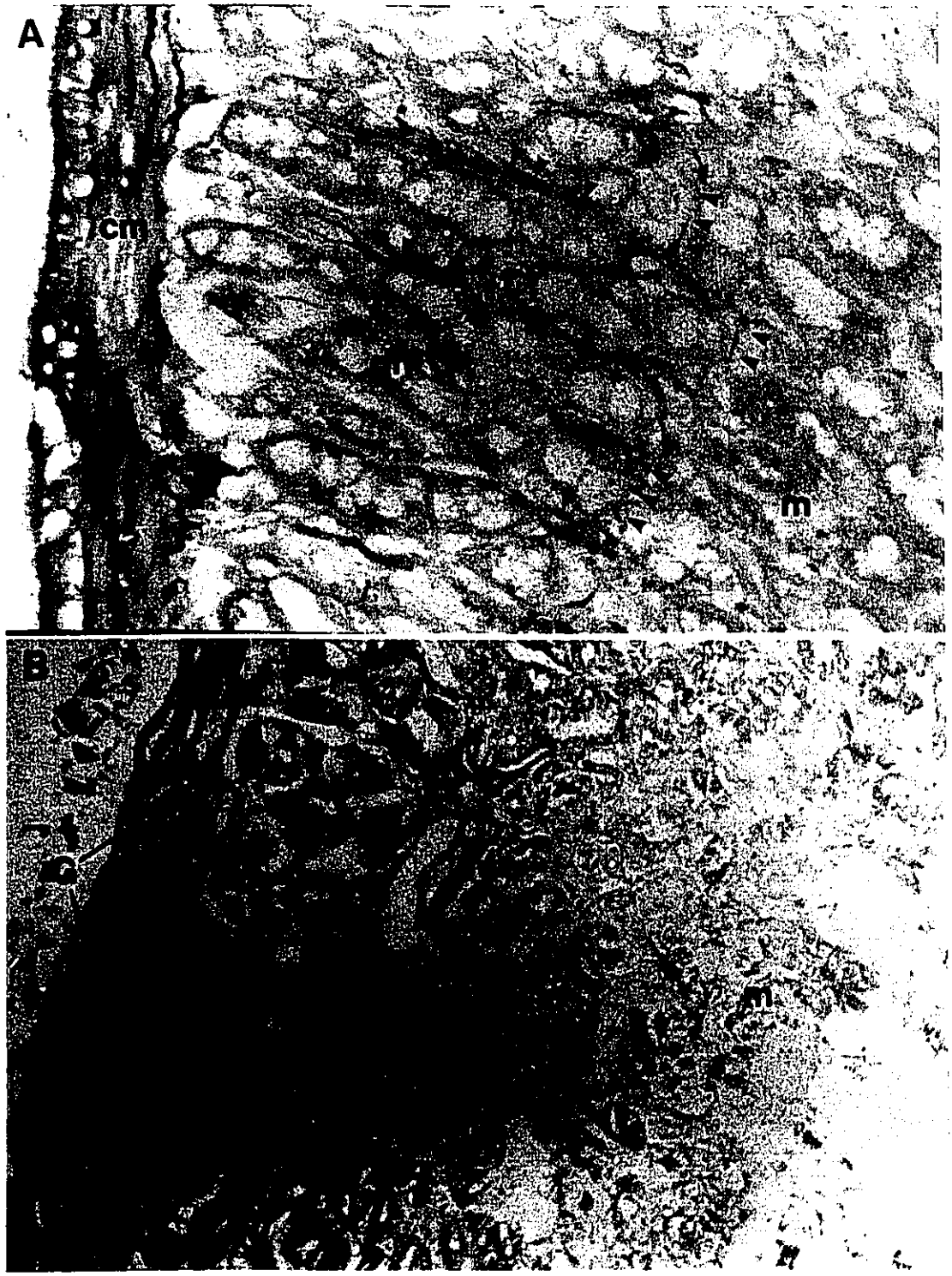
If the nitrergic system plays a role in ulcerogenesis then it might be expected that duodenal ulceration is associated with alterations in nitrergic innervation patterns and/or changes in NO production.

The administration of the alkyl compound cysteamine-HCl (CSH) to rats is known to induce duodenal ulceration at 24 hrs (Szabo 1978; Krantis et al. 1989). To determine the potential effects of duodenal ulceration on nitrergic innervation patterns and on NO synthase activity, this experimental model of duodenal ulcer was employed. Two doses of CSH (28mg/kg) were administered to rats (total n=52) and 24 hrs later duodenal segments were removed for histochemical identification of NO synthase-related NADPH diaphorase reactivity and determination of NO synthase activity. Administration of CSH in this manner resulted in adequate levels of ulceration without any mortality.

6.5.1 CSH Induced Changes of Nitrergic Innervation Patterns in the Duodenum

Figure 6.2 displays the typical nitrergic innervation patterns observed in circumferential sections of duodenum from control (n=4) and CSH treated (n=4) rats. In addition to the NO synthase related neurons observed in the myenteric plexus and associated fiber innervation of the circular muscle, there was a profusion of labeled fibers ramifying within the Brunner's glandular submucosa (fig. 6.2A). In addition labeled fibres could be seen ramifying within the muscularis mucosae and within the mucosal laminar propria (fig. 6.2A). In the anterior region of the duodenum where lesion damage was observed, the labeled nerve fibers of the circular muscle displayed a more diffuse and disorganized innervation pattern with similar staining intensity to that of control animals (fig. 6.2B). In addition the glandular submucosa appeared to be significantly retracted, with obvious enlargement of microvessels. NO synthase positive fiber innervation of the Brunner's glandular submucosa appeared scant and greatly reduced. Lesion damage was evident with a significant loss of integrity in the villi of the mucosa and appearance of inflammatory infiltrate at the site of the damage. As a result the labeled fibre innervation of the muscularis mucosae and mucosa as seen in the duodenum of the control animal were also lost.

Figure 6.2. Circumferential sections (14 μm) of the proximal duodenum taken from a control rat (A) and a rat pretreated with cysteamine-HCl (B). Tissue sections were subsequently stained for NO synthase-related NADPH diaphorase histochemistry and counterstained with eosin to visualize transmural constituents. In control rats, there was intense NADPH diaphorase staining of the myenteric ganglia and circular muscle (cm) as well as a network of fibres coursing within the Brunner's glandular submucosa (gm) and ramifying along the base of the villi (arrows). In the cysteamine-HCl treated rat (B) the labeling within the circular muscle layer (cm) was of a similar intensity to control tissue, however the pattern of innervation was altered. Labeled fibre innervation of the circular muscle appeared disrupted and the glandular submucosa was considerably retracted with profoundly reduced diaphorase staining. Lesion formation and necrotic damage is apparent in the mucosa (m) with typical loss of integrity and inflammatory cell infiltrate.


A
cm
m
B
m

6.5.2 CSH Induced Changes in NO Synthase Activity

CSH caused a significant increase in total NO synthase (T-NOS) activity as compared to the saline (control) group from 0.55 ± 0.06 to 1.39 ± 0.09 nmol NO/min/g tissue ($p<0.001$) (fig. 6.3A). This increase in T-NOS activity was due to a 3-fold increase in constitutive NO synthase (C-NOS) activity from 0.29 ± 0.04 to 0.99 ± 0.09 ($p<0.001$) (fig. 6.3B). There was no significant difference in inducible NO synthase (I-NOS) activity in CSH vs the saline treatment group (fig. 6.3B).

To determine if the CSH effected increase in constitutive NO synthase activity could be attenuated separate groups of animals were pretreated with L-nitro arginine methyl ester (L-NAME) (37.5 mg/kg; n=38), nitro-L-nitro arginine (L-NNA) (37.5mg/kg; n=39), or iminoethyl ornithine (L-NIO) (50 mg/kg) 1 hr prior to CSH administration. All inhibit the activity of both the constitutive and inducible NO synthase isoforms but preferentially target constitutive NO synthase (Pollock et al. 1991). Moreover, L-NNA is a more potent inhibitor of neuronal NO synthase activity than L-NAME (Buisson et al. 1992; Buisson et al. 1993; Klatt et al. 1994). L-NIO is proposed to be 10 times more potent in inhibiting endothelial NO synthase than L-NAME (Rees et al. 1990).

Both L-NAME and L-NNA attenuated the CSH induced increase in T-NOS activity (1.39 ± 0.09 to 0.67 ± 0.07 nmol NO/min./g tissue; $p<0.001$) and (1.39 ± 0.09 to 0.41 ± 0.03 nmol NO/min./g tissue; $p<0.001$) respectively (fig. 6.3A). These attenuating effects of L-NAME and L-NNA were due to a significant decrease in C-NOS activity by 69% (0.99 ± 0.09 to 0.31 ± 0.04 nmol NO/min./g tissue; $p<0.001$) and 83% (0.99 ± 0.09 to 0.17 ± 0.01 nmol NO/min./g tissue; $p<0.001$) respectively (fig. 6.3B). L-NIO however did not attenuate the CSH induced increase in T-NOS activity (fig. 6.3A), nor did it significantly reduce the CSH induced rise in C-NOS activity (fig. 6.3B).

L-NAME and L-NNA had no significant effect on I-NOS activity (0.39 ± 0.05 to 0.36 ± 0.05 nmol NO/min./g tissue; $p>0.05$) and (0.39 ± 0.05 to 0.24 ± 0.05 nmol NO/min./g tissue; $p>0.05$) respectively (fig. 6.3B). However, L-NIO pretreatment displayed a 3-fold increase in I-NOS activity compared to the saline and CSH treated groups. (0.26 ± 0.03 and 0.39 ± 0.05 to 0.86 ± 0.06; $p<0.001$) (fig. 6.3B). Rats pretreated with L-NAME or L-NNA displayed no significant difference in T-NOS, I-NOS or C-NOS activities as compared to the saline group (fig. 6.3A and B).

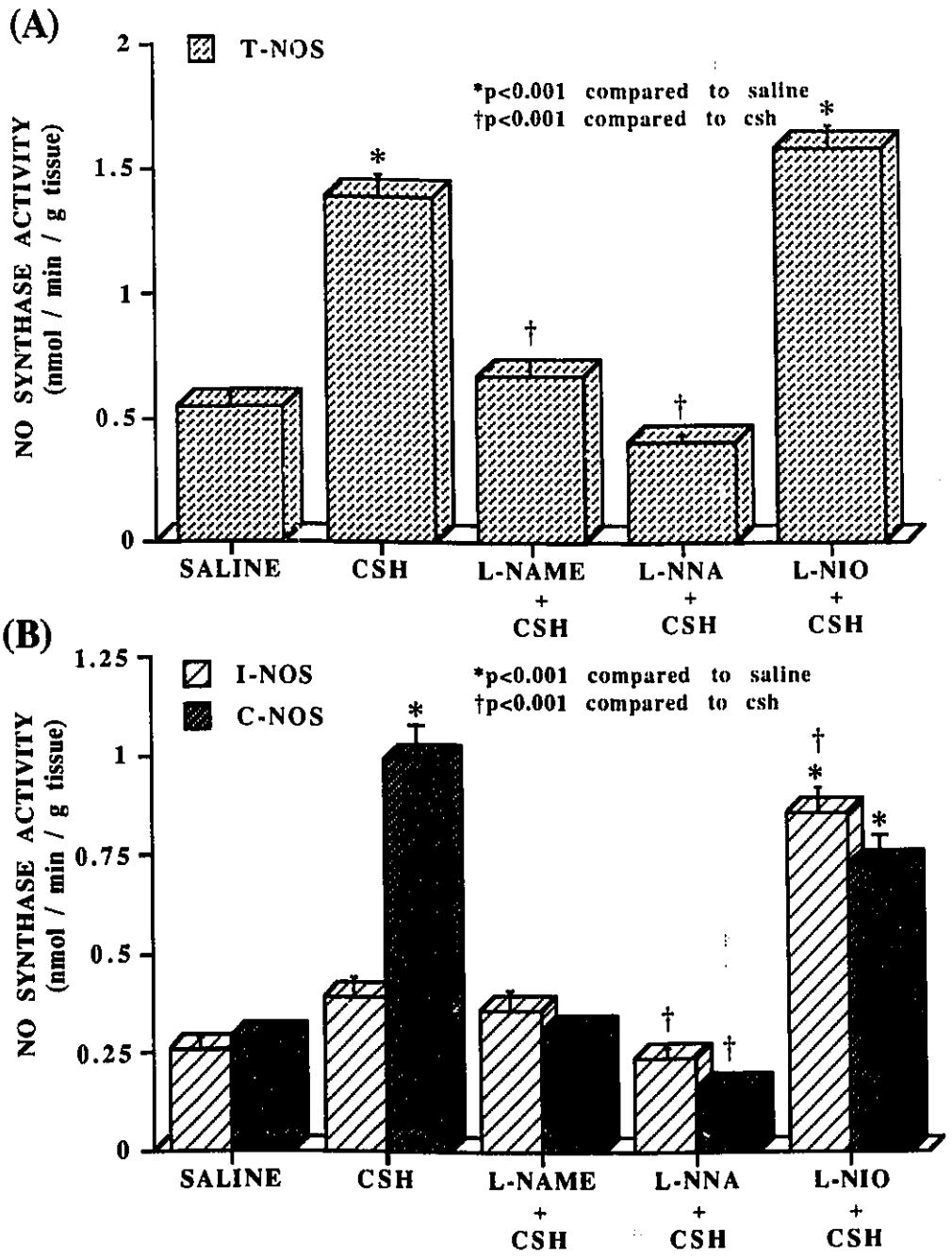


FIGURE 6.3. NO synthase activity 24 hr after saline or CSH treatment. Graph A represents total NO snthase activity and B shows a breakdown into inducible and constitutive activities following treatment. The treatments represented in the abscissa include saline, CSH alone or 1 hr pretreatment with L-nitro arginine methyl ester (L-NAME), nitro-L- nitro arginine (L-NNA) or iminoethyl -L-ornithine (L-NIO) with CSH. Values obtained from 18-52 rats are expressed as means ± SEM. Asterisks denote statistical significance (P<0.001) when compared to saline (*) or CSH (†) group.

6.5.3 Summary

The findings discussed in section 6.5.1 show that CSH treatment causes a disruption in the nitrergic fiber innervation of the muscularis and a reduction in the density of NO synthase reactive fibers coursing in and around the Brunner's glandular submucosa and mucosa of the duodenum The intensity of staining between the treatment groups appeared similar. Since NO normally inhibits neuronal regulation of duodenal bicarbonate secretions (Hallgren et al 1993; Takeuchi et al 1993) and has been shown to play a stimulatory role in gastroduodenal alkaline secretion in response to acid (Bilski et al. 1994) the reduced nitrergic innervation in the duodenum of CSH treated rats may lead to a disruption in the neural regulation of the bicarbonate secretory process.

The results presented in section 6.5.2 indicate that CSH induced duodenal ulceration is associated with a 3-fold increase in the activity of the Ca^{2+}-dependent isoform of NO synthase, while Ca^{2+}-independent NO synthase activity does not appear to be affected. This is consistent with the notion that elevated constitutive NO synthase activity is involved in CSH induced ulceration. This elevated enzyme activity could be from neuronal, endothelial, epithelial or even smooth muscle sources. The NO synthase inhibitors L-NAME, and L-NNA attenuated this CSH induced increase in Ca^{2+}-dependent NO synthase activity. However, L-NIO which is proposed to preferentially inhibit the endothelial (vs neuronal) NO synthase activity, did not attenuate CSH's effect on Ca^{2+}-dependent NO synthase activity. This suggests that the elevation in Ca^{2+}-dependent NO synthase activity associated with CSH induced ulceration may derive more substantially from sources other than the endothelium.

The CSH induced increase in Ca^{2+}-dependent NO synthase activity could be due to either upregulation of the synthesis or activity of the enzyme or both. If CSH asserts its regulatory effect on only a small subpopulation of the nitrergic elements producing NO synthase, *in situ* hybridization (ISH) represents the most appropriate method to detect an associated change in message level. *On this basis, an ISH procedure was established for the detection of NO synthase mRNA in sections of duodenum from normal and CSH treated rats in order to observe any potential changes in NO synthase mRNA levels. In addition, I sought to determine if CSH induced ulceration could be modulated using NO synthase substrate or inhibitors and evaluate the effectiveness of different NO synthase inhibitors as anti-duodenal ulcer drugs.*

6.5.4 *In Situ* Hybridization

ISH was employed to visually assess whether experimental ulcer induces changes in the expression of NO synthase mRNA. Figure 6.4A and B displays probe labeled sections of duodenum from control (6.4A, n=3) and CSH treated (6.4B, n=3) rats which were processed using the ISH protocol developed in this study. Ganglionic cells of the myenteric plexus and submucosa expressed NO synthase mRNA in the duodenum of control and CSH treated animals. When the colorimetric curve was used to semiquantitatively correlate color intensity of staining in labeled elements with absolute levels of NO synthase mRNA as seen in control vs CSH treated rats, there appeared to be no observable difference in staining intensity. By inference, this suggests that there is no visible difference in the levels of NO synthase mRNA in CSH treated and control rats. Figure 6.4C shows a control section of duodenum that was pretreated with RNase to demonstrate that the signal results from binding of the probe to RNA in the tissue. Staining was absent in control tissue sections in both control and CSH treated rats.

6.5.5 Effect of NO Synthase Activity Manipulation on the Incidence of CSH Induced Duodenal Ulceration

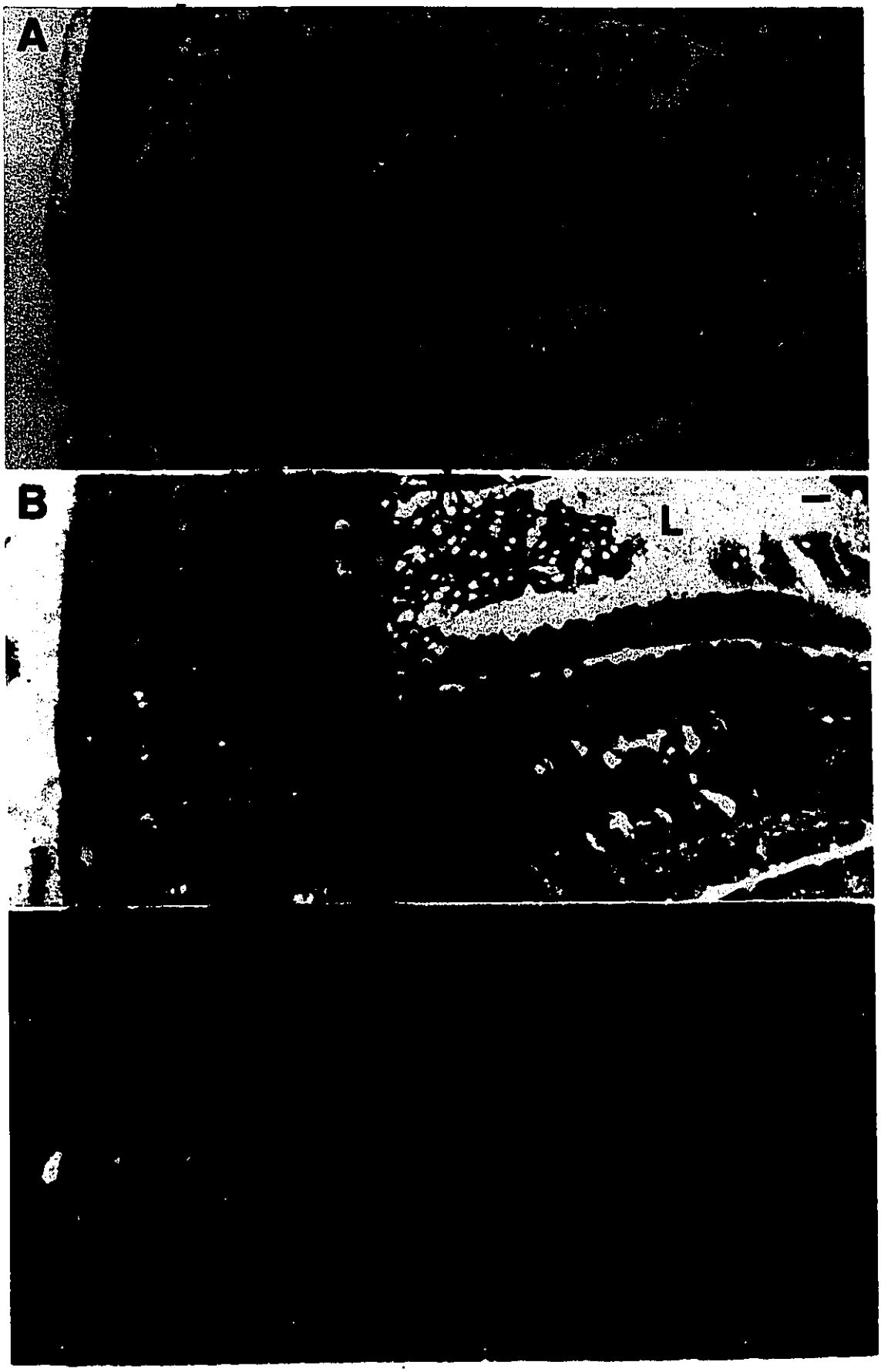
The overall effects of NO synthase substrate and inhibitors on the incidence, profile and severity of CSH induced ulceration are shown in Figures 6.5, 6.6 and 6.7 respectively. Rats (total n=34) administered two doses of CSH (28 mg/kg) showed macroscopic signs of duodenal ulceration at 24 hrs. Ulcers were seen on the anterior and/or posterior aspects of the duodenal wall, usually situated within 2 cm distal to the pyloric sphincter. As in the experiments of section 6.5.2, treatment with CSH resulted in no mortality of animals. As can be seen in the histograms of Figure 6.5, A through D, incidence of duodenal ulceration ranged from 58 to 83% across the three CSH groups (n=10-24). A range of 8 to 33% displayed mucosal irritation, evidenced by blanching and/or slight reddening of the luminal wall. 8 to 20% of all CSH treated rats showed no signs of mucosal disruption and were considered normal.

The incidence of ulceration in animals receiving L-ARG (150mg/kg) 1 hr prior to CSH treatment or CSH treatment alone was nearly identical (fig. 6.5A).

Pretreatment with L-NAME (37.5mg/kg) significantly reduced the ulcer incidence (fig. 6.5B). Duodenal ulceration was reduced by 75% while those displaying a normal or irritated mucosa were increased by 33% and 44% respectively (fig. 6.5B).

L-NNA at a dose of 37.5 mg/kg also reduced the effects of CSH treatment. L-NNA pretreatment caused nearly identical shifts to L-NAME in the incidence of CSH induced

Figure 6.4. In situ hybridization light micrographs of circumferential sections (14 μm) of rat duodenum following hybridization with the NO synthase probe (A and B) and probe plus RNase pretreatment (control, C). Tissue sections in A and C were taken from a control rat while the section displayed in B was obtained from a CSH treated rat. Nerve cell staining of the myenteric (Mg) and submucosal (Sg) ganglia is evident in both the control (A) and CSH treated (B) rat. (C) No staining of ganglion cells was evident in the RNase pretreated sections. L indicates the site of lesion formation. Scale bar in B = 100 μm.


A
B
L

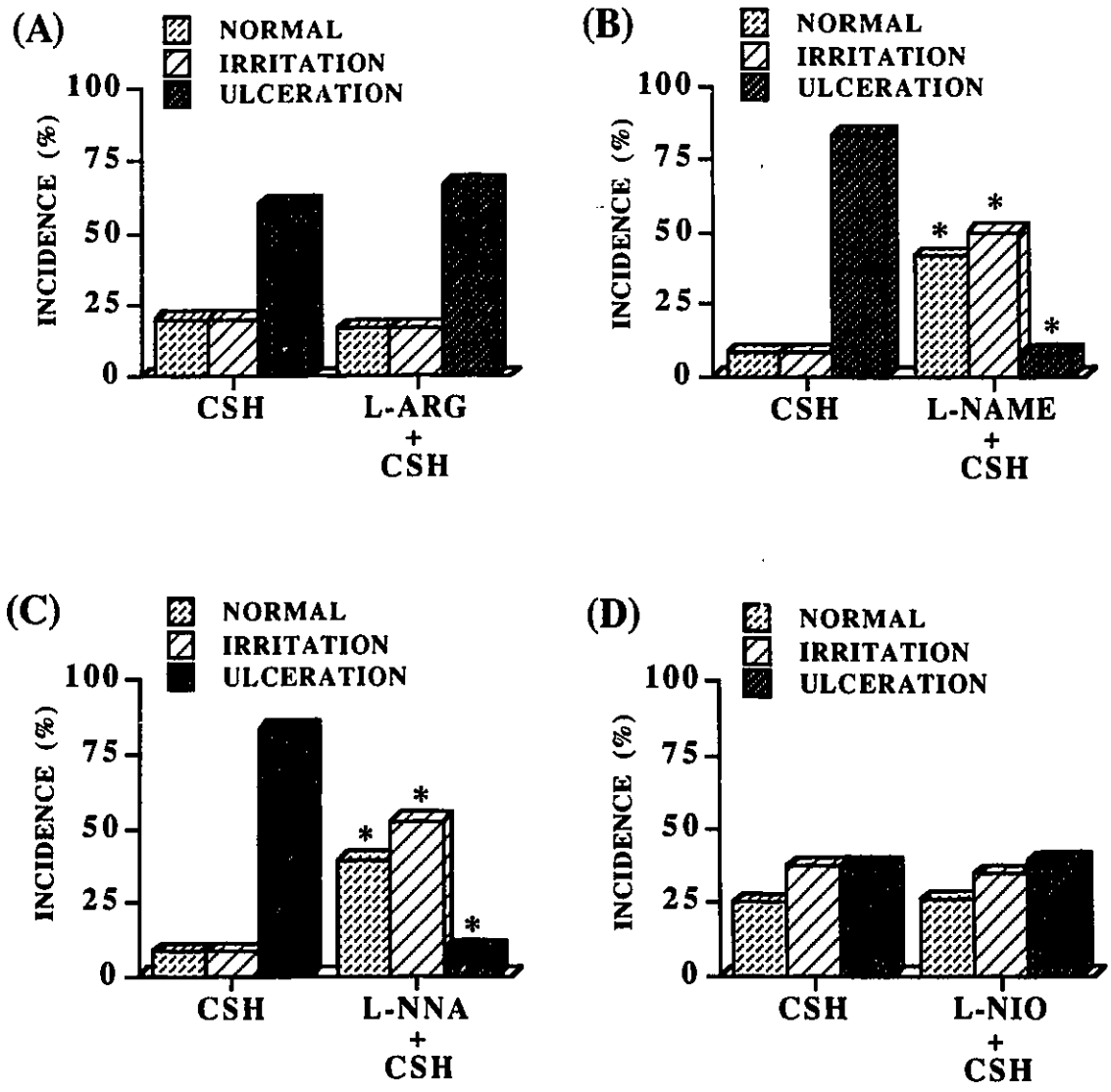


FIGURE 6.5. The effects of nitrergic compounds on CSH induced duodenal ulcer incidence. The treatment groups are represented in the abscissa. Each graph represents the incidence of duodenal ulceration 24 hr post CSH administration, with 1 or more lesions per animal considered a single incident. The different treatments are include (A) L-arginine (L-ARG), (B) L-nitro arginine methyl ester (L-NAME), (C) nitro-L-nitro arginine (L-NNA) or (D) iminoethyl -L-ornithine (L-NIO). All drugs were applied as a single bolus 1 hr before CSH. Asterisks (*) denote statistical significance ($p<0.0001$) when compared to each control (CSH) group. Values are expressed as percentages.

ulceration. A 75% decrease in ulceration was observed in the face of an increase in the incidence of animals with a normal or irritated mucosa of 31% and 44% respectively (fig. 6.5C).

L-NIO pretreatment did not produce a significant change in ulcer incidence (fig. 6.5D).

6.5.6 Effect of NO Synthase Substrate and Inhibitors on the Profile of Ulceration

The profile of CSH induced duodenal ulceration as presented in Figure 6.6 shows that within the CSH treatment groups (fig 6.6A-D), 17 to 42% had no ulcers, whereas 8 to 33% exhibited solitary and 30 to 50% displayed opposing (two) ulcers . This profile of ulceration was significantly increased by 20% in animals pretreated with L-ARG, where 6 of 12 rats displayed opposing ulcers compared to only 3 of 10 CSH treated rats (fig. 6.6A).

L-NAME also shifted the ulcer profile (fig. 6.6B), causing a 45% reduction in the number of animals with opposing ulcers (6 of 12 animals vs 1 of 18 animals), an increase of 75% in the number of animals with no ulcers (2 of 12 vs 15 of 18 animals) and an increase of 25% in the number of animals (4 of 12 vs 2 of 18 animals) with solitary ulcers.

Pretreatment with L-NNA gave virtually identical results to L-NAME, with a 42% decrease in animals with opposing ulcers (from 6 to 1 out of every 12 animals) along with a 75% rise in the number of animals with no ulcers (from 2 to 10 out of every 12 animals) and a 24% increase in animals with solitary ulcers (1 to 4 out of every 12 animals) (fig. 6.6C).

While L-NIO did not alter ulcer incidence (see section 6.5.4), pretreatment with this inhibitor resulted in a significant shift in ulcer profile compared to control. This shift encompassed a 12% decline in the number of animals with opposing ulcers (from 7 to 4 of 24 animals) to a 14% increase (from 2 to 5 of 24 animals) in the number of animals with solitary ulcers (fig. 6.6D). The number of animals with no ulcers was the same in both groups (15 of 24 animals).

6.5.7 Effect of NO Synthase Substrate and Inhibitors on the Severity of Ulceration

Figure 6.7 shows the area of the lesions (index of the severity of ulceration) formed due to CSH treatment. The average lesion area from the CSH treated groups was 4.5 ± 1.47 mm^2. For ease of graphical presentation, the lesion area for groups pre-exposed to NO synthase substrate or inhibitors are presented in Figure 6.7 as a percentage of their own group.

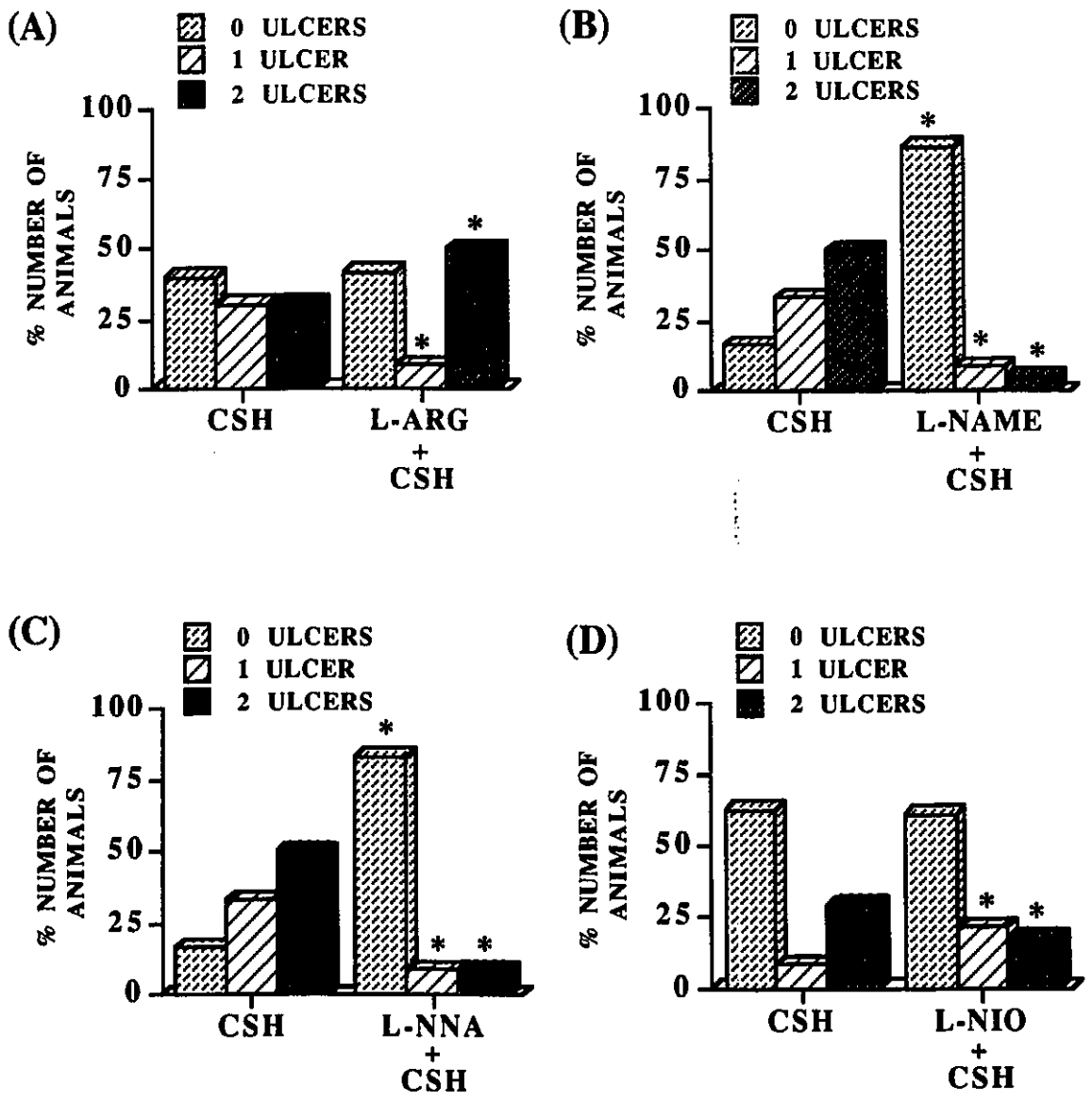


FIGURE 6.6. The effects of nitrergic compounds on the profile of CSH induced duodenal ulceration. The treatment groups are represented in the abscissa. Each graph represents the profile of duodenal ulceration, with 0, 1, or 2 ulcers. The different treatment groups include: (A) L-arginine (L-ARG), (B) L-nitro-arginine methyl ester (L-NAME), (C) nitro-L-nitro arginine (L-NNA) or (D) iminoethyl-L-ornithine (L-NIO). All drugs were applied as a single bolus 1 hr before CSH. Asterisks (*) denote statistical significance ($P<0.0001$) when compared to each control (CSH) group. Values are given as percentages.

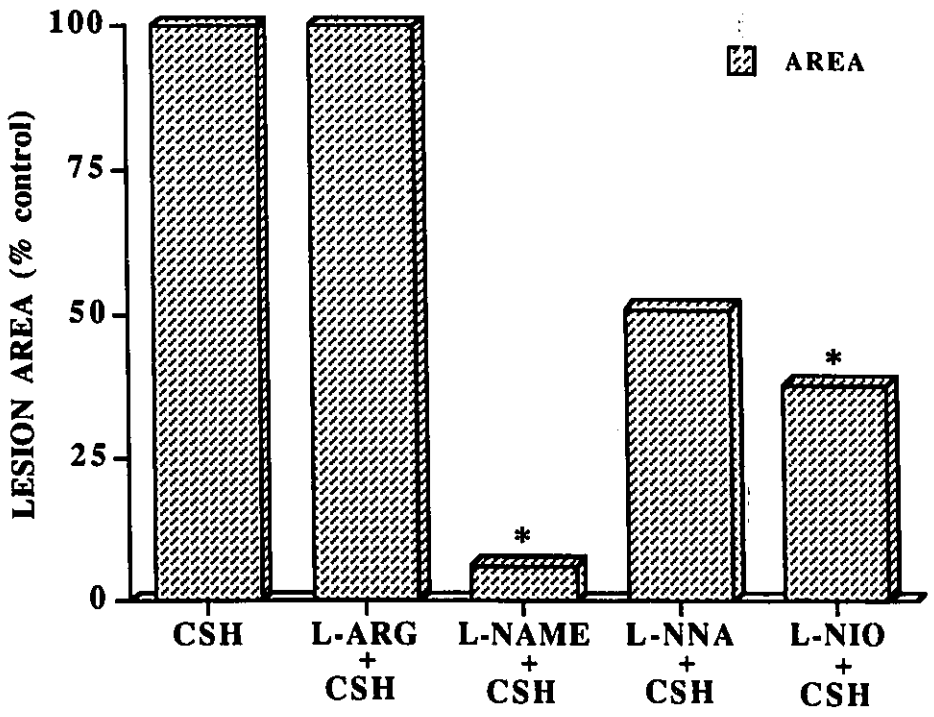


FIGURE 6.7. The effects of nitrergic compounds on the severity of CSH induced ulcers. The various treatment groups are represented in the abscissa and the severity of ulceration is represented in the ordinate as lesion area. Drugs were given as a single bolus dose 1 hr before CSH. Asterisks (*) denote statistical significance ($p<0.0001$) from comparisons of the mean lesion area of the treatment group to the control (CSH induced) lesion area. Values are expressed as percentage of control.

Statistical comparison of the actual mean lesion area of the control vs treatment groups shows no significant change in the lesion area of CSH induced ulcers in animals pretreated with L-ARG as compared to the CSH control (1.81 ± 0.56 to 1.89 ± 0.5 mm^2; $p>0.05$). L-NAME significantly reduced the severity of CSH induced ulceration decreasing lesion area by 94% (3.91 ± 0.82 to 0.25 ± 0.01 mm^2; $p<0.001$). L-NNA also decreased the size of the lesion by 49% (3.91 ± 0.82 to 2 ± 1 mm^2; $p>0.05$) but this was not statistically significant. L-NIO significantly decreased the size of the ulcers by 62% (3.63 ± 0.6 to 1.39 ± 0.22 mm^2; $p<0.001$).

6.5.8 Summary

The semiquantitative findings described in section 6.5.4, suggest that CSH treatment does not lead to upregulation of the production of NO synthase in the rat duodenum. Although these data are substantive, more direct quantitative measurements of NO synthase

mRNA levels would be useful. Modification of this ISH protocol to radioactively or fluorescently label the probe and determination of density of staining with an image analysis system would achieve this. ISH represents the best method for visualization of the source of NO synthase.

The results of sections 6.5.5 to 6.5.7 support the proposal that modulation of Ca^{2+}-dependent NO synthase activity alters the incidence, profile and severity of CSH induced ulceration. Administration of NO synthase substrate and inhibitors 1 hr before CSH affected the parameters of CSH induced duodenal ulceration in different ways. The results of these findings are summarized in table 6.1.

L-ARG, a NO synthase substrate, aggravated CSH induced ulceration with a significant shift in ulcer profile, while all other parameters were unchanged. The NO synthase inhibitor L-NAME significantly reduced all of the measured parameters. Treatment with L-NNA in the same manner significantly reduced the incidence and profile but not the severity of ulceration. Finally, L-NIO significantly reduced the profile and severity of CSH induced ulcers.

Based on these results, it would appear that the constitutive isoform of NO synthase may be a potential target for the development of anti-ulcer drugs, which used singularly or in combination may present an alternative or else an adjunct to current therapies.

Previous studies carried out by our group have shown that CSH induced ulceration can be modulated through peripheral $GABA_A$- and $GABA_B$- receptor sites. $GABA_A$ receptor stimulation aggravates CSH induced ulceration whilst treatment with the $GABA_B$-receptor agonist protected against CSH induced ulceration (Krantis et al. 1989; Krantis et al. 1990). Thus the enteric B-GABAergic system like the nitrergic system represents a target site for drug therapy for the treatment of duodenal ulcer disease. Furthermore targeting these systems may be synergistically advantageous. This notion was examined and expanded upon herein by also evaluating the effects of combined treatment with baclofen and L-NAME.

6.5.9 Effects of Baclofen and L-NAME on Duodenal Ulceration

Animals pretreated with either L-NAME or baclofen (BAC) demonstrated significant reductions in the incidence, profile, and lesion area of CSH-induced duodenal ulceration.

As can be seen in the histogram of Figure 6.8B, the incidence of duodenal ulceration in the CSH group (n=12) was about 63% while 25% displayed mucosal irritation, evidenced by blanching and/or slight reddening of the luminal wall and 12% of all CSH treated rats showed no signs of mucosal disruption and were considered normal. The profile of

ulceration due to CSH treatment (fig. 6.8B) displayed 25% and 38% of the animals with solitary and opposing ulcers respectively, as well as 37% which exhibited no ulcers. The average area of the CSH induced lesions was 3.68 ± 0.98 mm^2 (fig. 6.9). As in section 6.5.2 and 6.5.4, treatment with CSH resulted in no mortality of animals.

BAC (40 μg/kg; n=8) pretreatment caused a 38% reduction in CSH induced duodenal ulceration and a 38% increase in animals exhibiting mucosal irritation (fig. 6.8A). BAC also shifted the profile of CSH induced ulceration with a 25% and 13% decrease in the number of animals with solitary or opposing ulcers respectively, as well as a 31% rise in the number of animals with no ulcers (fig. 6.8B). As shown in Figure 6.9, BAC significantly reduced the size of CSH induced lesions by 74% (5.3 ± 0.8 to 1.38 ± 0.6 mm^2; $p>0.05$).

Rats (n=6) pretreated with L-NAME (37.5 mg/kg) alone 1 hr before CSH, displayed a shift in the incidence of CSH induced ulceration with a 46% decrease in ulceration as well as a 4% and 42% increase in animals displaying a normal or irritated duodenal mucosa respectively (fig. 6.8A). L-NAME also reduced the number of animals with solitary or opposing ulcers by 8% and 38% respectively while causing a 46% increase in the number of animals with no ulcers (fig. 6.8B). L-NAME caused a more significant reduction in the severity of CSH induced ulceration than BAC decreasing lesion area by 73% (3.68 ± 0.98 to 1 mm^2;$p<0.001$) (fig. 6.9).

CSH-treated rats (n=8) that were pretreated with a combination of BAC (120 min. before CSH administration) and L-NAME (60 min. prior to CSH administration), were completely free of ulcers (Fig. 6.8A and B). Microscopic appearance of the duodenal wall from these rats was normal (fig. 6.9).

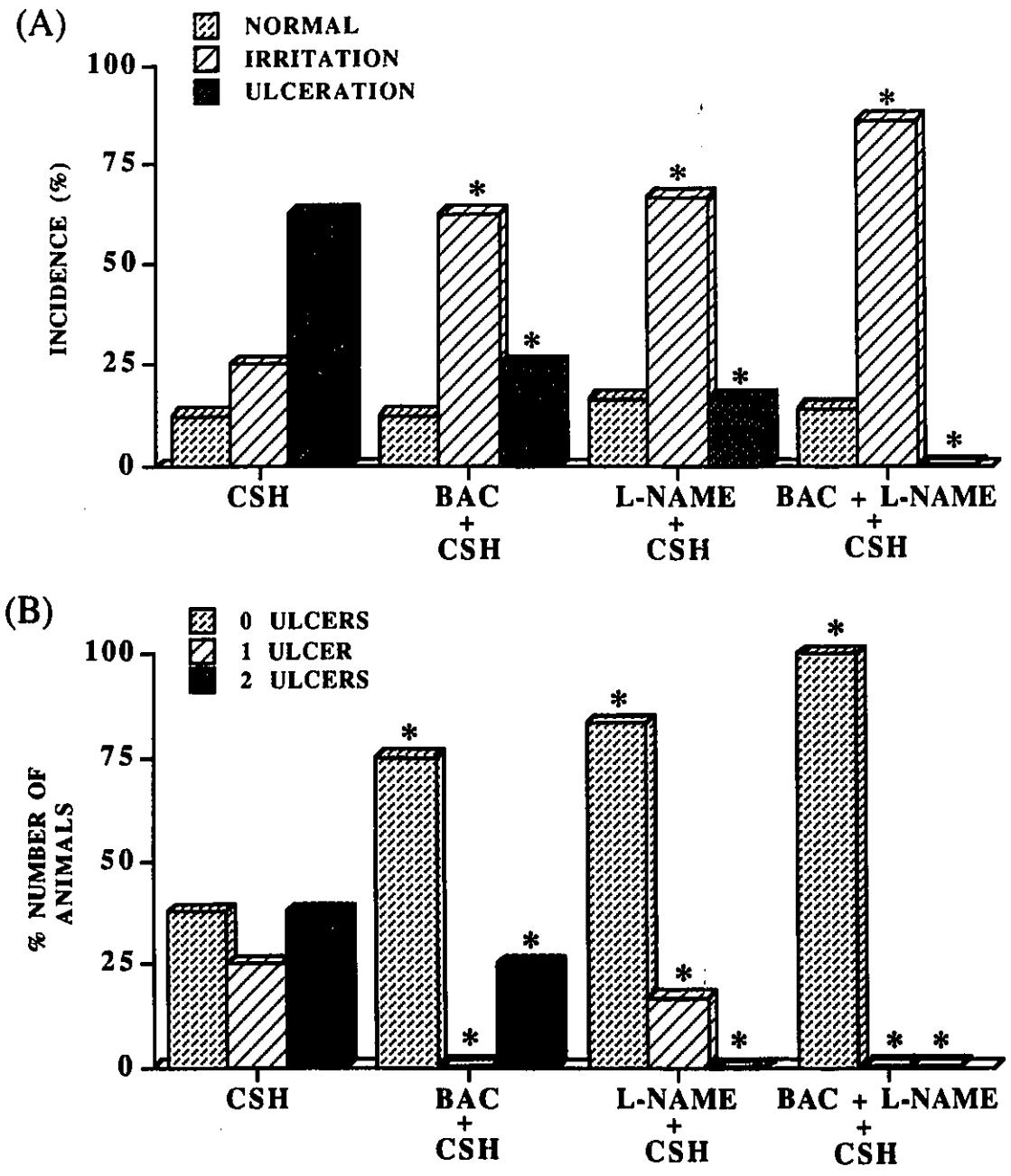


FIGURE 6.8. The effects of separate and combined treatment with baclofen (BAC) and L-NAME on the incidence (A) and profile (B) of CSH induced duodenal ulceration. The various treatments are given in the abscissa. Separately, BAC or L-NAME was applied as a single bolus 1 hr before CSH. Both drugs were administered as a single bolus 2 hrs before (BAC) and 1 hr before (L-NAME) CSH. Asterisks (*) denote statistical significance ($p<0.001$) as compared to the CSH (control) group. Values are expressed as percentages.

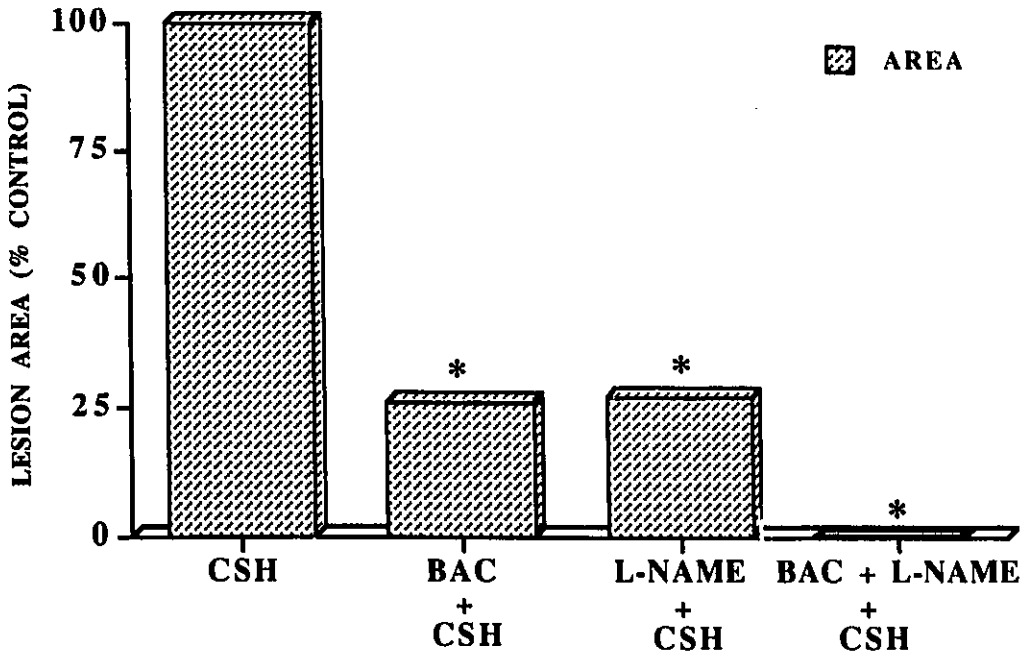


FIGURE 6.9. The effects of separate and combined treatment with baclofen (BAC) and L-NAME on CSH induced lesion area. The treatment groups are represented in the abscissa. Separately BAC and L-NAME were administered 1 hr prior to CSH. Combined treatment involved administration of BAC 2 hr followed by L-NAME 1 hr before CSH. Asterisks (*) denote statistical significance ($p<0.001$) from comparisons of mean lesion area of each treatment group to that of each CSH (control) group. However, values are expressed as percentage of control.

6.5.10 Summary

The results of this study reveal that single bolus pretreatment with BAC or L-NAME significantly improve CSH induced duodenal ulceration. When these pretreatments were combined, CSH induced ulceration was eliminated. This effect was the most potent anti ulcer effect ever seen in this laboratory using this experimental model of duodenal ulcer disease. Whether these treatments accelerate the healing of ulcers requires urgent examination. Preliminary data obtained by Dr. Krantis and coworkers (unpublished observations) suggests that baclofen given orally, does indeed accelerate healing of CSH induced ulceration.

6.6. Discussion

The results of these studies suggest that NO generated by the Ca^{2+}-dependent constitutive form of NO synthase plays an important role in the development of experimental duodenal ulceration, and that inhibiting constitutive NO production confers protection from duodenal ulcer formation. In our experimental duodenal ulcer model, constitutive NO synthase activity was significantly increased. Acute administration of L-NAME or L-NNA prior to CSH administration, attenuated the CSH induced increase in constitutive NO synthase activity and resulted in a significant amelioration of the CSH induced lesion. By contrast, administration of L-NIO in the same manner, did not attenuate the observed increase in activity of the constitutive isoform. Since L-NIO is proposed to preferentially target the endothelial NO synthase isoform, one might speculate that CSH may have a greater effect on the neuronal isoform of this enzyme.

Experimental duodenal ulceration is associated with, among other pathogenic factors, alterations in motility and blood flow patterns. *However these pathogenic changes represent the earliest observable signs leading to the focal necrotic damage of the duodenal mucosa.* Interestingly, they occur within the same time frame post CSH treatment and deserve consideration. The mechanisms behind these CSH induced changes is unclear, but the findings of this study would suggest that NO may be a mediator. The questions then are, how might CSH be causing elevation of constitutive NO synthase activity and how would increased NO production contribute to the CSH induced changes in motility or blood flow?

The increased activity in constitutive NO synthase may be due either to a change in the activity of the enzyme or altered synthesis of the enzyme or both. Although only semiquantitative, assessment of NO synthase mRNA in this study using ISH, indicated that CSH treatment probably does not upregulate enzyme synthesis. More definitive evidence for this could be derived by isolation of mRNA and Northern blot analysis using the probe obtained for this study.

Interestingly, CSH has been shown to not only stimulate a subpopulation of NANC inhibitory motor neurons, but to inhibit the stimulation of these neurons by GABA or nicotinic agonists (Krantis 1987; Krantis et al. 1989). These actions of CSH were not due to neural inhibition of the NANC neurons or to the inability of the enteric muscle to relax (Krantis 1987). Nonetheless, stimulation of nitrergic NANC inhibitory neurons could potentially cause the elevation in constitutive NO synthase activity as seen in this study. CSH is a potent nucleophilic agent which can bind to carbon centers of membrane (and other) proteins (Szabo 1978).

Therefore CSH could inhibit transmitter-evoked activation of NANC neurons via interaction with receptor sites which are distinct from the transmitter receptor sites

Whether the actions of CSH in this model of duodenal ulceration are exerted via specific intracellular signaling events involving changes in NO production, is unknown, but the findings of this study suggest such a notion. However, the potential for NO to exert toxic actions is evidenced by its ability to target multiple aspects of cellular function. NO produced in nanomolar concentrations can inhibit cellular respiration by competing with cytochrome oxidase for oxygen (Brown et al. 1994). NO is also proposed to exert toxic actions via its stimulatory effect on ADP ribosylase and resultant post-tranlational modification of intracellular proteins (Wallis et al. 1993). This deregulation of ADP ribosylase can lead to inhibition of glycolosis by inhibiting glyceraldehyde-3-phosphate dehydrogenase (Zhang et al 1994). NO also leads to inhibition of the rate-limiting enzyme for DNA replication, ribonucleotide reductase, and to DNA breakdown (Stamler 1994).

Another means by which CSH could trigger constitutive NO synthase activity is through its effects on blood flow. Rats treated with CSH display morphological signs as well as blood flow changes reminiscent of focal ischemia in the duodenum (Brooks 1985; Tanaka et al. 1989). Under physiological conditions the mucosa is protected by NO formed by the constitutive NO synthase (Miller 1988). However, tissue injury ensues when high levels of constitutively formed NO occur. Ischemia and reperfusion events, are often associated with overproduction of NO (Matheis et al 1992, Patel et al 1993; Caldwell et al 1994) as well as leading to generation of reactive oxygen species such as superoxide (O_2^-) (Flaherty et al 1988). Since NO contains an unpaired electron (i.e. is paramagnetic) it reacts rapidly with O_2^- to form peroxynitrite ($ONOO^-$) in high yield which may induce membrane lipid peroxidation (Radi et al 1991). Under conditions of low pH $ONOO^-$ may decompose to nitrogen dioxide (NO_2) and the highly toxic hydroxyl radical (OH^-) (Beckman et al 1990). This 'dual' role of constitutively derived NO in mucosal protection vs mucosal injury has been under intense investigation. Local intra-arterial infusion of low doses of the NO-donor, S-nitroso-N-acetylpenicillamine (SNAP) attenuates mucosal injury induced by local intra-arterial infusion of endothelin-1 (a potent vasoconstrictor released by endothelial cells). Interestingly, higher doses of SNAP cause necrotic damage (Lopez-Belmonte et al 1993). It was proposed from these findings that the mucosal injury occurs as a result of a combination of NO and superoxide to form the cytotoxic $^{\bullet}ONOO^-$. Therefore, the endothelin-imposed vasoconstriction and associated oxidative stress (in the face of significantly elevated levels of NO) exacerbated the hyperemic response precipitating lesion formation. Whether CSH has an effect on

endothelin release has, to my knowledge, not been assessed. Nonetheless, these findings support the notion that CSH induced elevation in NO associated with an ischemic insult could contibute to the precipitation of lesion formation.

There are two lines of evidence in distinguishing oxidative stress as a causal event in ulcer formation: (i) anatomical evidence for oxidative (necrotic) damage and (ii) free radical scavengers or inhibitors of processes generating oxygen radicals (i.e. antioxidants) reduce lesion damage. In the rat CSH experimental model of duodenal ulcer as well as in human trials, administration of free radical-removing agents along with inhibitors of acid secretion not only reduce experimental ulcer formation (Salim 1990a; Salim 1990b; Krantis et al. 1993) but reduce the rate of recurrence of ulcers in duodenal ulcer patients (Salim 1990c).

The role of NO in neuronal toxicity in the CNS has been recently discovered (Dawson et al. 1991b) and NO synthase inhibitors have been shown to be neuroprotective (Dawson et al. 1993). Moreover, NO appears to mediate hypoxic-ischemic neuronal injury, and this damage occurs via neural processes, independent of vascular NO effects. It can be hypothesized that net neuronal stimulation precipitated from a hypoxic/ischemic insult, as is proposed to occur in excitotoxic lesion in the CNS, could lead to duodenal lesion formation. A local ischemic insult could result from contraction of smooth muscle about duodenal mesenteric end arteries or via vasoconstriction of these arteries. The focal nature of the lesion sites within the duodenal bulb could be explained by the anatomy and location of these blind ended (no anastomotic connections) arteries. Reperfusion following an ischemic insult at already poorly perfused sites of the duodenum would raise cytosolic calcium levels and increase local constitutive NO production in neurons and/or endothelial cells. Endothelium-derived NO has been shown to be a local mediator of early postischemic vasodilation (Greenberg et al. 1995), which is a protective adaptation that ensures prompt restoration of blood flow when flow is interrupted abruptly and ischemia is induced (Greenway 1984). Direct transmural electrical stimulation of the rat small bowel duodenal end arteries evokes a pronounced and persistent vasodilation that is NANC in nature (Biber et al. 1973; Remak et al. 1990). Therefore oxidative species could be formed and react with the available NO to form $^{\bullet}ONOO^{-}$.

Ischemia induced increase in NO production might also affect local motility patterns. Interestingly, examination of the damage imposed due to experimentally-induced ischemia in the dog, indicates the muscle can become irreversibly damaged and contracted possibly due to the elevated cytosolic calcium levels (Earlam et al. 1967). This is consistent with the *in vivo* motility responses to CSH where a decreased frequency of contractions with a

corresponding increase in the amplitude of contractile activity was observed (A. McKay, A. Krantis, unpublished observations). However, in addition to these responses an increased frequency of relaxations and a reduced amplitude in relaxant activity was also seen. Moreover, several studies of experimentally induced ischemia in the gut, indicate that ischemia leads to motility disturbances (Hukura et al. 1965; Earlam et al. 1967; Szurszewski et al. 1968) and although these changes are in part neurogenic, the mechanism(s) behind these disturbances are not discussed. Nonetheless, an ischemia induced state of contraction of the muscularis could lead to constriction of the end arteries again leading to a hypoxic/ischemic insult. One might envisage that this cycle of events might be perpetuated and reach a threshold which initiates or precipitates lesion formation. The extent or severity of the damage might be contingent upon the length of time the stress is present, and/or the extent of the imposed ischemia.

NO may contribute to CSH induced ulcerogenesis by its involvement with other pathogenic factors associated with duodenal ulcer formation or via other pathogenic mechanisms. Since the NO stimulates acid secretion (Pique et al. 1989; Barrachina et al. 1994; Bilski et al. 1994), this enhanced secretory activity contributing to CSH-induced duodenal ulceration could be due in part to an increase in NO synthase activity, hence NO production. Our findings that inhibitors of NO synthase display anti-ulcer actions further support such a notion. Moreover NO has been shown to inhibit duodenal bicarbonate secretion (Hallgren et al 1993; Takeuchi et al 1993) or stimulate pancreatic bicarbonate secretion (Konturek et al. 1993). However, this stimulatory action of NO on pancreatic secretion may not be beneficial since back diffusion of bicarbonate is compromised during ulcerogenesis due to disrupted duodenal motility patterns (Monto et al 1976). The predominating effect of NO on bicarbonate secretion in ulcerogenesis, therefore, may be to inhibit duodenal bicarbonate secretion. Taken together the actions of NO on acid and bicarbonate secretory processes could lead to an increase in the acid load within the duodenum.

Whether the source of the elevated NO production associated with CSH induced ulceration is neuronal, endothelial or epithelial is difficult to ascertain without more specific inhibitors of the neuronal or endothelial constitutive isoforms. *In vitro* and *in vivo* studies led to the proposal that L-NIO preferentially inhibits the activity of the endothelial isoform of NO synthase (Rees et al. 1990) but inducible NO synthase was not assessed in these studies. In my study, L-NIO did not attenuate the CSH induced increase in constitutive NO synthase activity but rather led to a significant increase of the inducible NO synthase activity compared to the control and CSH groups. This was unexpected and indicates that the effects of NO synthase inhibitors

may depend on the conditions of study which may confer a lack of specificity. It might be speculated that L-NIO used under the conditions used in this study results in the activation of the inducible isoform. If we consider however, that L-NIO does preferentially inhibit the endothelial NO synthase isoform, then these findings indicate that the elevated NO synthase activity associated with ulceration in this animal model may from sources other than the endothelium (i.e. neuronal, epithelial), but modulation of the endothelial isoform can affect particular parameters of ulceration.

At present, no one NO synthase inhibitor is specific for a particular isoform. However, the use of NO synthase inhibitors in this study provided some insight into the contribution of NO to the different parameters of CSH induced ulceration. Since L-NAME has anti-muscarinic properties (see Griffith et al. 1995), the beneficial effects of L-NAME to ameliorate CSH induced ulcer formation may in part be due to this action since gastroduodenal contraction amplitude is significantly increased in the rat CSH model of duodenal ulcer (unpublished observations). L-NNA does not interact with muscarinic receptors and was used in this study to attempt to determine whether this action of L-NAME contributed to its anti-ulcer properties. The actions of L-NAME and L-NNA on the incidence and profile of CSH induced ulceration were not significantly different from one another suggesting that the anti-muscarinic effect of L-NAME may not contribute to the reduction of these parameters of ulcer formation. Interestingly, L-NAME significantly reduced the area of CSH induced lesions whereas L-NNA had no significant effect on this parameter. Therefore the anti-muscarinic actions of L-NAME and perhaps the hypercontractile state of the duodenum associated with CSH induced ulcer formation, may contribute most specifically to the severity of ulceration. Equally as interesting is the finding that, L-NIO, which is proposed to be a much more potent inhibitor of endothelial NO synthase than L-NAME, significantly reduced the profile and severity of ulceration. Conversely, L-NNA in low doses is proposed to inhibit neuronal NO synthase with little effect on the endothelial isozyme (Buisson et al. 1992; Buisson et al. 1993). L-NNA significantly reduced all parameters except the severity of CSH induced ulceration. Taken together the size (area) of the ulcers may depend in large part on the actions of endothelial-derived NO, but this isozyme may also contribute in a less significant way to the number (profile) of ulcers formed.

The results of this study also shows the powerful therapeutic potential of combining the $GABA_B$ agonist baclofen and constitutive NO synthase inhibitors for the treatment of duodenal ulceration. $GABA_B$ agonists are already used clinically for spastic disorders and therefore could be easily prescribed for a new indication. The NO synthase inhibitors however,

have well known side effects in animals; lethargy and reduced respiratory rate due to the pulmonary hypotension elicited by these drugs as well as increasing blood pressure (Rees et al 1990). The magnitude and type of side effects in humans, of course, may differ substantially from the observations made in animals. Clearly, the development of site specific NO synthase inhibitors deserves attention.

The mechanism(s) by which baclofen and L-NAME completely prevent duodenal ulceration is unclear. Baclofen dose-dependently decreases duodenal mucosal flow when administered systemically (Knight et al. 1994) which may attenuate the post-ischemic vasodilatory actions of NO in vivo. Baclofen prejunctionally inhibits acetylcholine release from cholinergic excitatory motor neurons (Krantis et al. 1980; Krantis et al. 1981; Giotti et al. 1983; Ong et al. 1983; Maggi et al. 1984; Kerr et al. 1986; Krantis et al. 1987; Krantis et al. 1993; Roberts, 1993). In this way, baclofen treatment might be equivalent to a drug having anti-muscarinic properties, which may be a factor in its anti-ulcerogenic effect. Interestingly, reduction in neuronal ischemic injury in the brain and spinal cord is associated with elevated levels of GABA, and the $GABA_A$ receptor agonist muscimol appears to prevent this injury (Gonzales et al 1992; Matsumoto et al 1991; Madden 1994). Baclofen does not exhibit these beneficial effects in cerebral ischemia (Rosenbaum et al 1990). As discussed, ischemia is proposed to be a contributing factor in CSH induced duodenal ulceration. Stimulation of A-GABAergic sites aggravates CSH induced ulceration while stimulation of the B-GABAergic system represses the CSH evoked duodenal mucosal injury. Taken together these phenomena are intriguing considering the opposite nature of $GABA_A$ mediated events in the gut compared to those in the CNS .

At this point the question to consider is how NO synthase inhibitors and baclofen act together to prevent CSH induced duodenal ulceration? To do so these agents would have to be acting, at least in part, via different mechanisms. Baclofen has been shown to have a vasoconstrictor action on duodenal blood vessels (Knight et al. 1994). Hence both NO synthase inhibitors and baclofen can lead to vasoconstriction and prevent hyperemic-induced damage as well as inhibit cholinergic motor neurons and reduce contractile force on enteric smooth muscle (which would reduce constriction of end arteries). However, two mechanisms set these agents apart. NO synthase inhibitors, such as L-NAME, reduce acid secretion and stimulate bicarbonate secretion while baclofen, or $GABA_B$ receptors, do not appear to play a role in these critical events. These separate actions of NO synthase inhibitors could account for the anti-ulcer effect of the baclofen and L-NAME treatment on CSH induced duodenal ulceration.

It is important to note that the rats used in this experimental model of duodenal ulcer do not possess *H. pylori* and therefore do not require this bacterium to develop ulceration. Perhaps in humans this bacterium predisposes an individual to ulcer recurrence rather than playing a direct role in ulcerogenesis.

The findings of this study collectively show that NO derived from the Ca^{2+}-dependent isoform of NO synthase plays a role in CSH induced ulcerogenesis. This represents one of the few instances in gut pathology where the Ca^{2+}-dependent rather than the Ca^{2+}-independent NO synthase has been shown to contribute to the disease state. NO should be included in the repertoire of pathogenic factors for duodenal ulcer disease not as a defensive factor via its effects on blood flow, but rather as an aggressive factor. The lack of specificity and reported systemic side effects of NO synthase inhibitors indicate that alternatives to pharmacologic or antagonist inhibition of NO deserve further investigation. It must be considered however that NO is critical in aspects of brain, cardiovascular and many other organ systems function, suggesting the need for site-specific pharmacologic intervention, to prevent undesirable side effects.

CHAPTER 7

Studies embodied in this thesis compare and contrast the nitrergic innervation and vascular patterns of NO synthase localization in the guinea-pig, rat and human intestine. Intestinal segments of all the species examined revealed intense NO synthase-related NADPH diaphorase staining of morphological type I myenteric ganglion cells and associated nerve fiber innervation of the muscularis. This subfeature of the nitrergic labeling has also been observed by several groups in the guinea-pig (Costa et al. 1991c; Llewellyn-Smith et al. 1992; Young et al. 1992; McConalogue et al. 1993), rat (Schmidt et al. 1992; Aimi et al. 1993) and human (Timmermans et al. 1993; Vanderwinden et al. 1993; Kobayashi et al. 1994; O'Kelly et al. 1994; Timmermans et al. 1994a) intestine. This pattern of innervation is consistent with NO being a transmitter of NANC motor neurons (Sanders et al. 1992; Stark et al. 1992; Brookes 1993). In addition to characterizing type I cell labeling, I also found that cells exhibiting type II morphology (in all species examined), and neurons reminiscent of type IV (human) and type VI (guinea-pig) morphology, also display NO synthase activity. Type II neurons originally described by Dogiel are proposed to be enteric sensory neurons. Their cell soma lie within the myenteric and submucous plexuses and they project to the villi of the mucosa as well as to other myenteric or submucous ganglia (Furness et al. 1990). These are the only neurons of the gut which exhibit this 'dual' projection indicative of the structure of a sensory neuron. In addition they are never found to project to the muscle layers (typical of type I neurons) and lack fast, excitatory synaptic input which indicates that they are not motor neurons or interneurons (Hendriks et al. 1990). Interestingly, the majority of Dogiel type II neurons contain calcium-binding proteins (calbindins) (Song et al. 1994) and therefore have a potentially large capacity to buffer Ca^{2+}. Therefore they can easily regulate Ca^{2+}/calmodulin mediated activation of NO synthase. Whether type II neurons and hence NO plays a role in mediating sensory information in the rodent or human gut remains to be determined. The functional significance of type IV and type VI neurons is unknown.

My results show rich nitrergic nerve fibre innervation of the myenteric ganglia and the interconnecting primary, secondary and tertiary meshworks of this plexus in all three species. Several laboratories have observed this pattern of innervation in the guinea-pig (Llewellyn-Smith et al. 1992; Young et al. 1992) and rat (Aimi et al. 1993) intestine. However, the observed nitrergic fibre innervation of the tertiary plexus in the human colon has not been noted by other groups (Timmermans et al 1993; Vanderwindin et al 1993; Kobayashi et al 1994; O'Kelly et al 1994; Timmermans et al 1994a). More intriguing is the suggestion by Llewellyn-Smith and coworkers (1983; 1993) that the human intestine does not possess a tertiary plexus. The findings

of this study clearly show that a tertiary meshwork does indeed exist in the myenteric plexus of the human colon (see fig. 4.1A; Chapter 4, section 4.4).

NO synthase-related NADPH diaphorase activity was never found in intestinal smooth muscle cells of the rodent or human intestine. This contradicts the view that NO can also be produced and released from gastrointestinal smooth muscle cells (Grider et al. 1992; Berezin et al. 1994). However, the possibility exists that there may be a smooth muscle NO synthase structurally and chemically distinct from the neural and endothelial form of the enzyme.

A consistent feature of the gut wall observed in this study was the distribution of nitrergic ganglionic cells and profuse innervation of fine varicose nitrergic nerve fibers in the submucosa of the small (guinea-pig and rat) and large (guinea-pig, rat and human) intestine. Although the structural organization of the myenteric plexus is relatively consistent among these species, the anatomy of the neural innervation of the submucosa is variable. Unlike the rat and human intestine, the submucosa of the guinea-pig intestine does not possess a Meissner's plexus and unlike the human intestine, the guinea-pig and rat submucosa does not possess an Intermediate plexus. This large ratio of labeled fibres to neurons has been observed by other laboratories investigating the guinea-pig large intestine (Young et al. 1992; McConalogue et al. 1993), but labeled neurons were never seen in the small intestine. However, my findings show that all regions of the guinea-pig small and large intestine possess nitrergic submucosal ganglionic neurons. Consistent with the findings of this study, others have also observed this pattern of labeling in Henle's plexus of the small and large intestine of the rat (Aimi et al. 1993) . However, this study provides additional information regarding the existence of a profuse nitrergic fibre innervation and scant neuronal staining within Meissner's nerve layer and associated innervation of the muscularis mucosae and mucosa in the rat intestine. The findings of this study in the human large intestine, confirm the presence of a high density of labeled fibres and low frequency of nitrergic neurons in both Henle's and Meissner's nerve layers (Takaki et al. 1985; Timmermans et al. 1993; Timmermans et al. 1994a), and provide new evidence for nitrergic neurons and fibres within the Intermediate nerve layer, as well as nitrergic innervation of the muscularis mucosae and mucosa Within the mucosa, intensely stained cells were also observed.

NO is produced by vascular endothelial cells and regulates blood flow in the gut. My examination of the vascular networks of the submucosa and mucosa of all three species reveal that fine submucosal vessels display a characteristic punctate staining pattern when viewed in laminar preparations. Moreover, fine paravascular nitrergic fibers could often be seen coursing alongside these vessels. When this localization of NO synthase activity was analyzed in more

detail in axial sections of the rodent and human intestine, the punctate patches of NAPDH diaphorase activity were found to be associated with both endothelial (one punctate patch per cell) and smooth muscle cells of submucosal arterioles and venules. In addition, endothelial cells of specialized postcapillary venules in human intestinal lymph nodes; the high-endothelial venules (HEV), also displayed these punctate patches of NADPH diaphorase activity. Recent ultrastructural studies using NO synthase immunohistochemical and histochemical analysis, have confirmed and extended these findings indicating that this punctate patch of NO synthase activity is associated with the Golgi apparatus within the endothelial cells (O'Brien et al. 1995).

Taken together with the findings of other groups, the results of my studies indicate that the nitrergic innervation in the rodent and human intestine is more extensive than previously thought. The diversity of cell types in the myenteric and submucous plexuses and abundance of fibre types within the submucosal layer displaying NO synthesis potential supports the notion that NO may potentially play a role in a diversity of gut functions. Pharmacological investigations confirm these anatomical findings. In addition to its established role as an inhibitory transmitter of a subpopulation of NANC motor neurons NO must now be considered a transmitter of interneurons. Moreover, the extensive and rich nitrergic innervation of the submucosa and mucosa as well as the vascular localization of NO synthase activity strongly suggests that NO plays an important role in secretomotor and vasomotor functions in the gut.

The potential for NO to be a transmitter of interneurons is strongly supported by my discovery that a subpopulation of GABAergic interneurons in the myenteric and submucosal nerve layers in the human colon also display NO synthase-related NADPH diaphorase activity. This codistribution also extended to the innervation of the muscularis. In the myenteric plexus, NO synthase reactivity was found in almost 40% of GABA-T immunoreactive myenteric neurons. These findings provide the first anatomical evidence in support of the proposal that NO is a transmitter of enteric interneurons in the human colon.

The possibility that NO is localized to NPY submucosal neurons and hence 'secretomotor', was evaluated by looking for parallel distributions of nitrergic neurons and NPY neurons in the submucosa of the human colon. NO synthase and NPY coexist in submucosal secretomotor neurons of the rodent intestine and both play a role in gut secretomotor function. NO and NPY are also similarly involved in other biological actions in the mammalian intestine including blood flow and motility. It is not surprising then, that an extensive codistribution pattern of NO synthase reactive and NPY immunoreactive innervations was found. Subpopulations of neurons containing both NADPH diaphorase activity and NPY are present in

the myenteric and submucosal ganglia. In addition nerve fibres in the deep muscular plexus of the circular muscle layer and within the muscularis mucosa displayed NADPH diaphorase activity and NPY immunoreactivity. Approximately 53% of NPY immunoreactive ganglion cells in the myenteric plexus contained NADPH diaphorase activity whereas, only 18% of NPY positive submucosal ganglion cells also displayed NADPH diaphorase reactivity. As expected from other studies of the rodent intestine in this thesis, NADPH diaphorase activity was not found colocalized to the NPY immunoreactive perivascular innervation nor did mucosal endocrine cells positive for NPY stain positive for NADPH diaphorase. These findings support the notion that NO is a transmitter of secretomotor neurons in the human intestine.

Since the enteric GABAergic system plays a role in the pathogenesis of cysteamine-HCL (CSH)-induced duodenal ulceration and CSH interacts with enteric NANC inhibitory neurons targeted by enteric GABAergic neurons, I sought to test the hypothesis that CSH induced ulcer formation is mediated, at least in part, by NO. Findings from this study indicate that experimental duodenal ulcer is associated with a disorganized pattern of nitrergic innervation in the muscularis, a disruption and diminution of labeled fibres about the Brunner's glands in the submucosa and a significant increase in constitutive NO production. This CSH induced increase in constitutive NO synthase activity could be attenuated by NO synthase inhibitors. In CSH treated rats, lesion damage was significantly reduced by pretreatment with NO synthase inhibitors whilst pretreatment with substrate to NO synthase, L-arginine, was found to exacerbate lesion formation by increasing the profile (number) of ulcers; other parameters of ulceration remained unchanged. Clearly, NO plays a role in ulcerogenesis.

Neuronally-derived NO is involved in NANC relaxations of the intestinal muscularis, and prejunctional inhibition of cholinergic contractions, while endothelial-derived NO controls local blood flow. Hence NO plays a role in motility and vasomotor function in the gut. In addition, NO is involved in mediating early post-ischemia reperfusion events. Moreover, neurotoxic and ischemic lesions in the CNS are mediated, at least in part, by NO. In the gut, disruptions to local blood flow and motility patterns are the earliest recognizable functional disturbances associated with experimental duodenal ulcer. Moreover, these functional changes occur within similar time frames post CSH treatment. Experimental ischemia has been shown to lead to a disruption of motility patterns and consistently leads to contraction of the muscularis (Hukura et al. 1965; Earlam et al. 1967; Szurszewski et al. 1968). The observed increase in contractile activity in experimentally induced duodenal ulcer as seen in both in vitro (Monto et al. 1976) and in vivo (A. Krantis, personal communication) studies could lead to an ischemic insult.

Contractions of the smooth muscle about the duodenal end arteries which provide no collateral blood supply render the duodenum susceptible to ischemic insult (Biber et al. 1973; Remak et al. 1990). These end arteries irrigate two focal sites of the anterior and posterior duodenal wall 1.5 cm from the pylorus where the focal lesions are reportedly developed in both the animal model and human condition. Assessment of the effect of different inhibitors (proposed to preferentially inhibit the neuronal or endothelial constitutive isoform of NO synthase) on the incidence, profile and severity of CSH induced ulcer formation provided some insight into the potential contribution of these different isoforms as well as permitting speculation of the potential contribution of neurotoxic or ischemic mechanisms to ulcer formation. L-NAME, L-NNA and L-NIO were the inhibitors employed in this study.

While L-NIO is proposed to preferentially inhibit endothelial NO synthase, L-NNA at low doses is a more potent inhibitor of neuronal NO synthase and L-NAME also has anti-muscarinic actions. L-NAME and L-NNA but not L-NIO attenuated the CSH induced increase in constitutive NO synthase activity. L-NIO significantly reduced the profile and severity of ulcers, L-NNA significantly reduced the incidence and profile of the lesions and L-NAME significantly reduced all measured parameters of CSH induced ulceration. These findings suggest that: (i) CSH induced ulceration is associated with increased constitutive NO production; (ii) a large part of this elevated NO is probably neuronally-derived; (iii) both neuronally-derived and endothelial-derived NO contribute to the profile of ulceration (iv) neuronally-derived NO preferentially contributes to the incidence (occurence) of ulceration; (v) endothelial-derived NO preferentially contributes to severity (size) of the ulcers such that, an ulcer may be formed but is exacerbated by EDNO. This may be due to its role in mediating reperfusion events associated with ischemia; (vi) anti-muscarinic actions may play a role in the incidence of ulceration, the severity of ulceration or both. NO synthase inhibitors, particularly L-NAME, are effective anti-ulcer drugs in experimental duodenal ulceration.

These findings and the knowledge that modulation of enteric $GABA_B$-receptor sites also improves experimentally induced ulceration provided the impetus to investigate the 'combined' actions of NO synthase inhibition with L-NAME and $GABA_B$ stimulation with baclofen, on CSH induced ulceration. The combination of these two drugs completely prevented ulcer formation. This effect may be due to combined and/or separate actions of these compounds. Both of these compounds reduce blood flow in the gut. However, baclofen prejunctionally inhibits acetylcholine release to the muscle and therefore has an 'anti-muscarinic'-type action, while L-NAME modulates acid and bicarbonate secretion.

If more specific inhibitors for neuronal vs endothelial isoforms of constitutive NO synthase can be developed, the source of elevated NO synthase activity associated with cysteamine-HCl induced duodenal ulceration could be elucidated. The notion that the neuronal isoform contributes largely to duodenal ulcerogenesis is supported by studies showing that mutant mice which lack neuronal NO synthase have significantly smaller lesions following cerebral artery occlusion than control animals (Huang et al 1994). In addition future studies should examine the effects of baclofen and/or NO synthase inhibitors in experimentally evoked ischemia (specifically partial occlusion of end arteries) in the duodenum. This would allow some insight into the ability of the GABAergic or nitrergic systems to modulate ischemic-type damage in the gut as they do in the brain. From the point of view of clinical relevancy, it is necessary that we now examine the role of NO synthase inhibitors and baclofen in duodenal ulcer healing.

Hirschsprung's disease (HSCR) represents another intestinal disorder with an underlying impairment in motor function involving net neuronal excitation and contraction of the muscularis which gives way to a functional obstruction in the large bowel. Proposed etiological mechanisms include ischemia and/or abnormal neuronal signaling during or subsequent to development of the ENS in the large bowel and/or genetic susceptibility. The diseased, constricted large bowel of patients with HSCR has been identified by the absence of ganglion cells, a recessive phenotype associated with this disease, referred to as aganglionosis. The length of the aganglionic segment is variable and does not correlate with the variation in severity of the clinical symptoms. Two patient populations were defined in this study, those that exhibited a lack of ganglion cells in 'aganglionic' bowel, and those that exhibited a selective 'survival' of NADPH diaphorase myenteric ganglion cells in the diseased segment. These anatomical findings may explain the conflicting functional evidence for relaxation responses in the large bowel of patients diagnosed with HSCR. Some groups report a reduced NANC mediated relaxation response (Hanani et al. 1989) while others report absence of these responses in the diseased large bowel (Kubota et al. 1983). Moreover, even in the patients of this study that exhibit the 'classical' phenotypic feature of aganglionosis, the myenteric and submucous plexuses in these patients displayed a dense, tortuous innervation of NADPH diaphorase positive fibre bundles, reminiscent of autonomic innervation of the bowel. This feature has hitherto not been reported in previous examinations of diseased bowel using NO synthase histochemistry or immunohistochemistry (Vanderwinden et al. 1993; Kobayashi et al. 1994; O'Kelly et al. 1994). Since NADPH diaphorase positive neurons have been localized to autonomic ganglia of the rat (Aimi et al. 1991), and lesion of extrinsic sympathetic and sensory innervation to the guinea-pig bowel does not

affect the density of submucosal NADPH diaphorase innervation (McConalogue et al. 1993), these stained fibre bundles could be from parasympathetic sources.

The curious feature of the localized lesions seen in the bowel of patients with HSCR is the ganglion cell destruction without damage to surrounding tissue. This characteristic and our findings that in a subpopulation of patients with HSCR, there is a reduction of NADPH diaphorase activity but survival of a select subpopulation of NADPH diaphorase positive cells is reminiscent of the findings in the striatum of patients with Huntington's disease (HD). Neurodegenerative disorders in the brain such as HD are proposed to be due to two mechanisms, neurotoxicity and ischemia, not mutually exclusive events. Moreover, evidence is accumulating that these lesions may arise as a result of programmed cell death due to aberrant local environmental signaling events associated with neurotoxicity or ischemia (Hockenbury et al. 1993; Richter 1993; Le et al. 1995). Finally, there is evidence that NO may be a mediator of these events (Dawson et al. 1993; Le et al. 1995; Wu et al. 1995). Clearly, future investigations should consider the type of lesion occurring in HSCR.

The findings of the anatomical and pathological assessments of the nitrergic system in this thesis, not only provide new and relevant information about the integration of nitrergic innervation in the ENS, but indicate that a pathological defect associated with a change in constitutively produced NO, may contribute to the development of certain gut disorders like experimental duodenal ulcer and HSCR. Moreover, the underlying etiological factors associated with these disease states include ischemia and changes in motility secondary to changes in neuronal signaling, both of which may involve NO. Future investigations of experimental duodenal ulcer and HSCR should consider the type of lesions that are produced i.e. necrosis vs PCD. The notion that lesions formed in duodenal ulcer disease may be due to necrosis and those of HSCR may be due to PCD with ischemia and neurotoxicity as potential underlying mechanisms deserves attention. It is intriguing that the underlying primary or secondary pathological factors contributing to the formation of a localized lesion in both conditions include ischemia and/or disruptions in motility patterns secondary to the net excitatory response of neuronal stimulation and/or repression.

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