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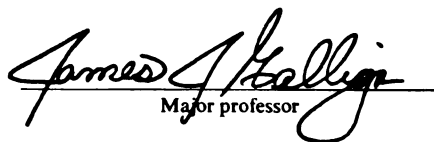
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of Guinea Pig Ileum

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Anne Marie R. Yunker

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**ANATOMICAL AND FUNCTIONAL ALTERATIONS IN ENTERIC NEURONS
FOLLOWING EXTRINSIC DENERVATION OF GUINEA PIG ILEUM**

Volume I

By

Anne Marie R. Yunker

A DISSERTATION

**Submitted to
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ABSTRACT

ANATOMICAL AND FUNCTIONAL ALTERATIONS IN ENTERIC NEURONS FOLLOWING EXTRINSIC DENERVATION OF GUINEA PIG ILEUM

By

Anne Marie R. Yunker

Digestion is controlled by extrinsic nerves originating outside the gastrointestinal tract and by enteric nerves found within the gut. Extrinsic nerves innervate enteric neurons and submucosal arterioles, and regulate gastrointestinal function. However, normal digestion quickly returns after destruction of extrinsic nerves, suggesting loss of extrinsic innervation induces alterations in enteric neurons.

Surgical denervation of segments of guinea pig ileum destroyed extrinsic nerves and induced c-Fos-immunoreactivity (ir) in submucosal and myenteric neurons one week after denervation. New substance P (SP)- and vasoactive intestinal polypeptide (VIP)-ir nerves were associated with submucosal arterioles beginning four weeks after denervation. Systemic administration of 6-hydroxy-dopamine eliminated sympathetic nerves and induced new VIP-ir but not SP-ir nerves, whereas capsaicin treatment eliminated extrinsic sensory nerves and induced new SP-ir but not VIP-ir innervation of arterioles. These data indicate loss of extrinsic sympathetic and sensory nerves is followed by the appearance

of VIP- and SP-ir paravascular nerves, respectively. Immature axons contain nonphosphorylated (np) heavy molecular weight neurofilament protein (NF-H)-ir, and mature axons contain phosphorylated (p) NF-H-ir. Normally, enteric somata contained npNF-H-ir and axons contained pNF-H-ir. However denervation-induced SP- and VIP-ir axons did not contain pNF-H-ir, suggesting denervation induces axonal sprouting. Retrograde tracer (fluoro-gold) was injected into arterioles to determine if axonal sprouts originated from submucosal neurons. Greater arteriolar innervation would increase the number of uptake sites, resulting in more labelled submucosal neurons. Denervation did not alter the number of fluoro-gold cells, implying new SP-ir and VIP-ir nerves did not originate from submucosal ganglia.

Loss of extrinsic sensory innervation increased nitric oxide (NO) synthase (NOS)-ir in myenteric neurons and potentiated neurogenic longitudinal muscle relaxations. These relaxations were attenuated by NOS antagonists, suggesting denervation also increases inhibitory innervation of smooth muscle. Finally, NO modulates SP neurotransmission and non-adrenergic, non-cholinergic contraction amplitudes were attenuated after denervation. Therefore, the data in this dissertation demonstrate extrinsic denervation induces functional and anatomical adaptations that allow enteric neurons to control gastrointestinal function in the absence of extrinsic nerves.

ACKNOWLEDGEMENTS

Where the telescope ends, the microscope begins.
Which of the two has the grander view?

Victor Hugo, *Les Misérables*
1862

I have been blessed to have known a number of outstanding people, but three men have made an indelible mark on my life and this dissertation. I am honored and privileged to have completed my dissertation in the laboratory of James Galligan. Jim has provided an ideal environment for research by allowing his graduate students freedom to explore their own interests, and encouraging excellence in the classroom and the laboratory. His enthusiasm for research and his dedication to teaching are surpassed only by his wealth of knowledge. Without Jim's impetus, I never would have learned about an enzyme called NADPH diaphorase, nor would I have learned critical thinking.

For the past eight years, all triumphs and failures have been shared with Michael Thompson. Michael has had faith and strength when I had none, and has always expressed interest and encouragement in the work necessary to complete this dissertation. He has taught me about the rewards that come from dedication and perseverance in work and relationships, and about the strength in inner peace. Michael has brought light, love, and joy into my life.

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LIST OF ABBREVIATIONS

ACh	acetylcholine
AP-1	activator protein 1
ATP	adenosine triphosphate
ANS	autonomic nervous system
CGRP	calcitonin gene-related peptide
Ca²⁺	calcium
C	celsius
CNS	central nervous system
ChAt	choline acetyltransferase
CMMPP	circular muscle-myenteric plexus preparation
EC₅₀	concentration of drug producing half-maximal effect
IC₅₀	concentration of drug producing half-maximal inhibition
DTAF	dichlorotriazinyl amino fluorescein
DMPP	1,1 dimethyl-4-phenyl-piperazinium iodide
DRG	dorsal root ganglia
EDTA	[ethylenedinitrilo]tetraacetatic acid
EGTA	ethylene glycol-bis (β-aminoethyl ether)
ENS	enteric nervous system
EJP	excitatory junction potential

EPSP	excitatory post-synaptic potential
fEPSP	fast excitatory post-synaptic potential
Fg	fluoro-gold
FITC	fluorescein isothiocyanate
GABA	gamma amino butyric acid
GI	gastrointestinal
GA	glyoxylic acid
g	gram
GAP-43	growth-associated protein-43
GC	guanylate cyclase
HEPES	[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
Hz	hertz
h	hour
6-OH-DA	6-hydroxy-dopamine
IEG	immediate early gene
Ig	immunoglobulin
ir	immunoreactivity
Cy3	indocarbocyanine
iNOS	inducible nitric oxide synthase
IJP	inhibitory junction potential
L_i	initial length
KDa	kilodalton
LDCD	large dense-cored vesicles

L _o	length for development of optimum tension
LMMPP	longitudinal muscle-myenteric plexus preparation
L	lumbar
min	minute
mM	millimolar
mV	millivolt
MEM	modified essential medium
DMMA	N ^G -monomethyl-D-arginine
LMMA	N ^G -monomethyl-L-arginine
M	molar
mACh	muscarinic receptor
M _x	muscarinic receptor subtype
nm	nanometer
NADH	β-nicotinamide adenine dinucleotide, reduced
NADPH	β-nicotinamide adenine dinucleotide phosphate, reduced
nACh	nicotinic receptor
NF	neurofilament
NF-H	neurofilament protein, heavy molecular weight
NF-L	neurofilament protein, light molecular weight
NF-M	neurofilament protein, medium molecular weight
nNOS	neuronal nitric oxide synthase
NPY	neuropeptide Y
NO	nitric oxide

NOS	nitric oxide synthase
DNAME	nitro ^G -D-arginine methyl ester
LNA	nitro ^G -L-arginine
LNAME	nitro ^G -L-arginine methyl ester
NANC	non-adrenergic, non-cholinergic
npNF	nonphosphorylated neurofilament protein
NE	norepinephrine
PNS	peripheral nervous system
PBS	phosphate buffered saline
pNF	phosphorylated neurofilament protein
pnpNF	phosphorylated and nonphosphorylated neurofilament
K ⁺	potassium
psi	pounds per square inch
sec	second
SBS	short bowel syndrome
sEPSP	slow excitatory post-synaptic potential
SSV	small synaptic vesicles
SNP	sodium nitroprusside
SEM	standard error of the mean
SMI	Sternberger Monoclonal Incorporated
sc	subcutaneous
SP	substance P
SPME	substance P methyl ester

NK _x	tachykinin receptor
T	thoracic
TRITC	tetramethyl rhodamine isothiocyanate (rhodamine)
TTX	tetrodotoxin
TRSC	texas red sulfonyl chloride
TH	tyrosine hydroxylase
UV	ultraviolet
u	units
VIP	vasoactive intestinal polypeptide

INTRODUCTION

1. HISTORICAL OVERVIEW

The Cell Theory and the Neuron Doctrine

The era of modern biology began in the 1830s when anatomists used now-primitive staining techniques to examine structures in nonvertebrate and vertebrate tissues. These scientists included Jacob Schleiden and Theodore Schwann who made a series of pioneering observations that led to the development of the Cell Theory. This treatise advanced the idea that living tissue is not an amorphous mass as previously thought, but rather comprised of small structural units called "cells". The cells would somehow communicate with each other in such a manner as to allow coordinated physiological activity of the entire tissue. Although the Cell Theory did not provide any structural or functional evidence that actually explained the mechanisms of communication between cells, it resulted in an expanded interest in cellular anatomy, culminating in an explosion of histological staining of peripheral and central tissues. Unfortunately, many tissues were impossible to study as most histological stains did not discriminate between heterogeneous populations of cells. In 1873 Camillo Golgi introduced a silver impregnation method that allowed the great neuroanatomist Ramòn y Cajal to stain neurons in peripheral tissues such as the small intestine (Cajal, 1895), gallbladder, and pancreas (Cajal and Sala, 1891), as well as neurons in the central nervous system (CNS). Golgi's silver stain was superior

to all other stains at that time as it labelled approximately 1-7% of all neurons, thus allowing observation of single cells. Furthermore, the entire structure of the neuron sequestered the silver stain, allowing the anatomy of the neuron to be visualized. Results from use of the silver stain led Cajal to propose the Neuron Doctrine, which stated that neurons are the basic signaling unit of the nervous system, and each neuron contains several processes that may be involved in processing and subsequent transmittal of information (Principle of Dynamic Polarization). Cajal's Neuron Doctrine provided the first modern neuroscientists a structural and functional working model for their explorations of the nervous system, and Golgi's silver stain provided a tool to visualize nervous tissues that is still in use today.

Early Anatomical Observations of the Gastrointestinal Tract

Prior to Cajal's use of Golgi's silver stain, a handful of anatomists used difficult and unreliable staining techniques to examine peripheral and central tissues. Although the presence of peripheral ganglia had been known by the second century (Galen, ca. A.D. 129-199), written records do not contain a description of ganglia found within the gastrointestinal (GI) tract until the middle of the 19th century. In 1840, R. Remak described microscopic ganglionic cells in the walls of the pharynx and stomach. This observation was followed by reports from George Meissner (1857), Leopold Auerbach (1862), and Thomas Billroth (1858) which provided the first detailed descriptions of ganglionated plexuses found within the walls of the GI tract.

Classification of the Enteric Nervous System

In 1869, the word "enteric" first appeared (Webster's Ninth New Collegiate Dictionary, 1988) and was defined as "of or relating to the intestines", or in more general

usage, "referring to the alimentary tract". Today, the enteric nervous system (ENS) is defined as the network of neurons and support cells found within the walls of the GI tract, pancreas, and gallbladder. The ENS was originally classified by John Langley (1921) as a division of the autonomic nervous system (ANS), as he wrote

"...we should expect the cells of Auerbach's and Meissner's plexuses to be on the course of the bulbar and sacral nerves, but as there was no clear proof of their central connexion, and as their obvious histological characters differed from those of any other peripheral nerve cell, I placed them in a class by themselves as the enteric nervous system."

Although most authors for the past 60 years have erroneously followed Catherine Hill's (1927) conclusion that many ganglionic cells in the ENS represent parasympathetic postganglionic neurons, Langley cited anatomical and functional evidence to support his classification of the ENS as distinct from parasympathetic nerves.

First, he noted that enteric ganglia were not on the course of either cranial (i.e., bulbar or vagal) or sacral nerves, and most enteric ganglia did not appear to receive parasympathetic preganglionic innervation. Although parasympathetic preganglionic nerves in the vagus synapse on some cells in the small intestine, fewer than 20,000 vagal afferent and efferent nerve fibers pass through the guinea pig diaphragm (Irwin, 1931) to innervate an estimated 10^7 neurons in the ENS. This efferent to enteric neurons ratio of 1:5000 would be the greatest divergence ratio in the nervous system, assuming all enteric neurons represented parasympathetic postganglionic neurons. The parasympathetic preganglionic nerve to postganglionic neurons divergence ratio is approximately one for most autonomic ganglia (see Furness and Costa, 1987), implying that most enteric neurons are not postganglionic cells and do not receive parasympathetic innervation.

Langley also observed that the histological characteristics of enteric neurons were

not similar to other peripheral neurons. The best study of the morphology of enteric neurons was performed by Alexander Dogiel (1899) who used methylene blue to illustrate the remarkable structure and diversity of enteric neurons. As compared to the simple ovoid and circular cytoarchitecture of parasympathetic ganglionic cells, enteric neurons possessed numerous branching filamentous and lamellar dendrites that are more similar to neurons in the CNS than to neurons in the ANS. The recent development of immunohistochemical techniques has also supported Langley's intuitive conclusion. Enteric neurons contain a variety of peptides and proteins that are not found in parasympathetic postganglionic cells, or are found co-expressed in different combinations than those proteins found in parasympathetic neurons. Furthermore, each enteric ganglion appears to have a random selection of cell morphologies and cell markers, unlike other autonomic ganglia, where morphologically and functionally similar cells are organized into discrete clusters or groups of cells. Finally, the ultrastructure of enteric ganglia differs from that of other autonomic ganglia, as enteric ganglia lack internal capillaries and connective tissue found in other autonomic ganglia (Cook and Burnstock, 1976). Although other autonomic ganglia contain collagen, fibroblasts, macrophages, and mast cells, enteric ganglia contain only tightly packed neurons and glia that are not encompassed by satellite cell sheaths found in other autonomic ganglia (reviewed in Llewellyn-Smith *et al.*, 1983). Therefore, based on morphological and histological properties, enteric ganglia do not resemble autonomic ganglia, but rather resemble the organizational ultrastructure of the CNS.

In addition to anatomical observations, Langley knew of the work of William Bayliss and Ernest Starling (1899; 1900a; 1900b), that demonstrated the existence of

polarized reflexes in small and large intestine isolated from extrinsic innervation. Such reflexes were not observed in any other peripheral tissue that had been examined, allowing Langley to argue that complex reflex pathways such as the peristaltic reflex occurred independently of parasympathetic and sympathetic nerves. Furthermore, the ability to maintain reflex activity when isolated from CNS innervation implies that all neural elements responsible for the reflex, including primary afferent (sensory) neurons, interneurons, and motor neurons must reside within the ENS. This indicated that unlike other autonomic ganglia, enteric neurons do not function simply as relays in centrifugal pathways. In fact, Langley and other physiologists such as Walter Cannon (1906) observed that digestion was not compromised proceeding surgical destruction of extrinsic nerves, further suggesting that neurons in the ENS exhibit a high degree of autonomy and are responsible for the physiological processes of digestion.

Although Langley's description of the ANS may now be considered an oversimplification (Furness and Costa, 1987), embryological data have further supported classification of the ENS as a separate division of the ANS. The ontogeny of enteric neurons has been studied since the mid-1950s (Yntema and Hammond, 1954), resulting in the conclusion that similar to parasympathetic neurons, enteric neurons are derived from neuroblasts from vagal and sacral regions of the neural crest (Le Douarin and Teillet, 1973; Pomeranz and Gershon, 1990; Gershon *et al.*, 1993). Unlike parasympathetic neurons, enteric neuroblasts migrate from the crest to the immature gut along the vagus nerve (reviewed in Gershon *et al.*, 1993) and differentiate into enteric neurons. In contrast to parasympathetic and enteric neurons, the precursor cells of sympathetic nerves originate from the trunk region of the neural crest (Le Douarin, 1982).

Furthermore, the presence or absence of local hormones and growth factors results in further differentiation of sympathetic and parasympathetic neurons, whereas enteric neurons are thought to differentiate in the absence of exogenous molecules (Carnahan *et al.*, 1991). Taken together, these development studies suggest that enteric neurons mature differentially than do other neurons of the ANS.

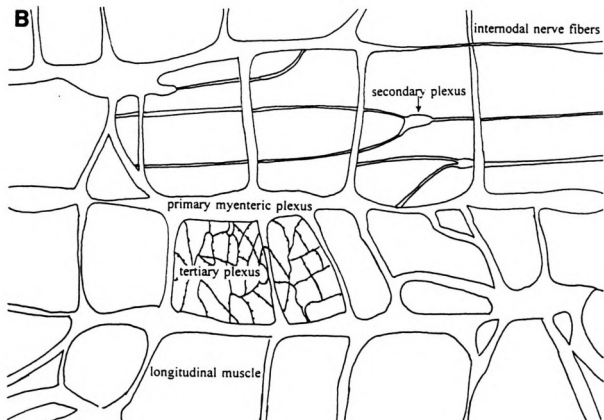
2. INTRINSIC INNERVATION OF THE GASTROINTESTINAL TRACT

Arrangement of Enteric Plexuses

The ENS is comprised of two interconnecting networks, or plexuses, of nerves. The submucosal plexus (Meissner's plexus) is a network of small ganglia and connecting nerve fibers found in connective tissue of the submucosal layer located between mucosal and circular muscle layers (Figure 1a). Submucosal ganglia contain approximately 8 cells/ganglion (Young *et al.*, 1993), and these neurons project to mucosa and arterioles found within the submucosal layer of the GI tract to regulate secretion, absorption, and blood flow. In contrast, the myenteric plexus (Auerbach's plexus) is composed of three plexuses located between longitudinal and circular muscle layers of the GI tract (Figure 1b). The primary plexus contains interganglionic (internodal) nerve fibers, as well as myenteric ganglia. Myenteric ganglia contain approximately 105 cells/ganglion (Young *et al.*, 1993a). The secondary plexus is comprised of nerve fibers originating from primary myenteric ganglia and nerve fibers, and secondary plexus nerves innervate circular muscle (Auerbach, 1864; Brookes *et al.*, 1992). Finally, the tertiary plexus is formed by meandering bundles of nerve fibers located in spaces between the primary plexus and provides functional innervation to longitudinal muscle (reviewed in Furness

Figure 1. Anatomy of the enteric nervous system. A. Camera lucida drawing of transverse section of guinea pig ileum stained with hematoxylin and eosin. Nuclei of epithelial, endocrine, nervous and muscle cells are shown. Magnification is 160 X. Calibration bar for A is 5 μm . B. Camera lucida drawing of whole mount preparation of guinea pig ileal myenteric plexus-longitudinal muscle. The myenteric plexus adheres to longitudinal muscle and can not be physically separated. Magnification is 40 X. Calibration bar for B is 100 μm .

Figure 1.

A**B**

and Costa, 1987; Llewellyn-Smith *et al.*, 1993).

The muscle layers of the GI tract receive additional innervation other than the nerve fibers originating from ganglionated layers. The circular muscle receives innervation from circular muscle plexus and deep muscular plexus, in addition to innervation from the secondary plexus of the myenteric plexus. The circular muscle plexus is orientated parallel to circular muscle fibers, and receives innervation from primary and secondary plexuses of the myenteric plexus. In contrast, the deep muscular plexus is orientated parallel to circular muscle and separates a thin layer of muscle cells from circular muscle (Gabella, 1974), thus suggesting that circular muscle is asymmetrically innervated by the ENS. Although a longitudinal muscle plexus is found in some species including humans, an analogous plexus is not found in rodents. However, nerve fibers of the tertiary plexus can be localized in small troughs on the surface of longitudinal muscle (Llewellyn-Smith, 1986), indicating that nerve fibers of the tertiary plexus form neuroeffector junctions with longitudinal muscle.

Cytoarchitecture of Enteric Neurons

The most elegant and intuitive study of the morphology of enteric neurons was performed in the late 1800s by Alexander Dogiel. Dogiel used methylene blue to stain enteric neurons in large and small intestines of rats, guinea pigs, rabbits, cats, and humans (Dogiel 1899), and concluded that there are three separate classes of neurons in the myenteric plexus. Dogiel type I cells were described as flattened cells with stellate or angular morphologies that measured 10-40 μm in the long axis, and 10-20 μm in the short axis (Figure 2). These cells have 2-22 broad and flat dendrites that frequently terminate in multiple short tufts. The axons from Dogiel type I cells arise from cell

somata or dendrites, and are occasionally observed to bifurcate and/or contain spines. Dogiel was able to trace these axons for short distances, and concluded that although they projected to other ganglia, they eventually appeared to enter the circular muscle. Dogiel type II cells are 20-50 μm in the long axis, and 10-20 μm in the short axis, and have angular, spindle, or star-shaped smooth cell somata. Type II cells have long and filamentous processes that originate from 3-10 thick dendrites, and single axons which arise from either pronounced axon hillock-like structures or dendrites. Dogiel traced these axons to other ganglia within the plane of the myenteric plexus. Finally, Type III cells are approximately the same size and possess the same morphology as Dogiel type I cells. However, type III cells have 2-10 dendrites that are less broad than type I dendrites and taper into branched dendrites that frequently terminate in filamentous distal dendrites as they project away from the cell soma. Similar to axons from type II cells, axons of type III axons originated from cell somata or dendrites and project to interganglionic nerve fibers. As Dogiel type III cells are similar to type I cells, many researchers confused these two cell morphologies. For example, Catherine Hill (1927) used violet staining to observe neurons in submucosal ganglia, and concluded that type III cells were actually a variant of type I cells. However, Dogiel clearly stated that type III cells were unique and different than type I cells, and are found predominantly in the submucosal plexus (reviewed in Furness and Costa, 1987).

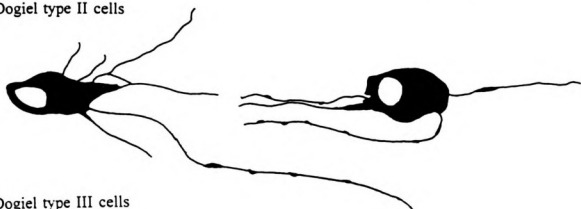
Although Dogiel's description accounts for the majority of cells in the ENS, many cells can not be categorized by his classification scheme. In one classification scheme advocated more recently (Costa *et al.*, 1986a) 44% of all myenteric neurons visualized by immunocytochemistry coupled to indirect fluorescence could not be placed in Dogiel's

Figure 2. Cytoarchitecture of enteric neurons. Camera lucida drawings of three morphologies described by Dogiel (1899). Dogiel type I and II cells are found in myenteric ganglia, whereas type III cells are localized in submucosal ganglia. Type I neurons were stained by NADPH diaphorase histochemistry, type II neurons were stained with antiserum against nonphosphorylated heavy neurofilament protein, and type III neurons were stained with antiserum against choline acetyltransferase. Neurons are magnified by 320 X. Calibration bar is 9 μ m.

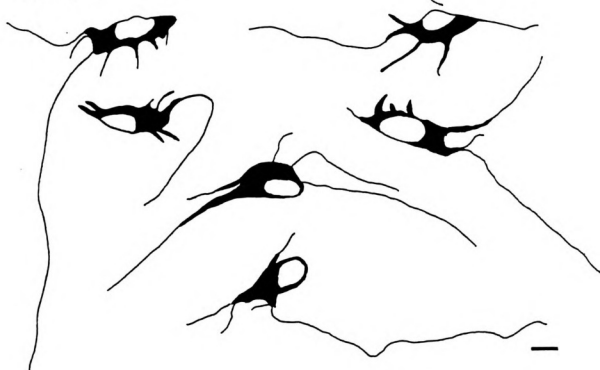
Figure 2.
Dogiel type I cells



Dogiel type II cells



Dogiel type III cells



scheme. However, over 90% of neurons could be classified in Dogiel's scheme when myenteric neurons were randomly filled with Lucifer Yellow, implying that the discrepancy in labelling could result from differences in methodology. Although Dogiel's classification scheme accounts for many neurons in the ENS, at least some enteric neurons do not fit any of his categorizes, suggesting a more detailed scheme is needed.

Dogiel speculated that a functional significance underlaid the morphological differences in enteric neurons. For example, as Dogiel type I cells appeared to project to circular muscle, he postulated that they were motor neurons. Although Dogiel was frequently unable to trace projections of type II cells, he concluded that they were likely to be intrinsic sensory neurons, as these cells appeared to send multiple long axon-like dendrites towards the mucosal surface. Furthermore, axons of Dogiel type II cells projected to myenteric neurons in other ganglia, allowing sensory information to be transmitted to other myenteric neurons. However, until the invention of electrophysiological techniques, the idea that physiological functions were correlated to cell morphology remained untested and highly debated (see Hill, 1927; Gabella, 1976).

Ultrastructure of Enteric Neurons

Dogiel's observations were limited by the light microscopes in existence at the end of the nineteenth century. Electron microscopy studies have yielded additional information on the structure and organization of enteric neurons, as eight classes of myenteric neurons have been identified based on microanatomical differences (Cook and Burnstock, 1976). Two classes of enteric neurons have axo-somatic synapses and perikarya that are 8-13 μm in diameter. However, (i) small cells near the ganglionic surface contain occasional large dense-cored vesicles and are surrounded by enteric glial

cells, whereas (ii) small cells in the interior of the ganglia have neither dense-cored vesicles or contacts with glial cells. A variation of (iii) small cells with axo-dendritic synapses and average perikaryon diameter of 14-18 μm are only observed in the stomach. Four classes of medium-sized myenteric neurons are observed, with diameters of 20-25 μm . Elliptical shaped neurons (iv) measuring 20 μm in the long axis, and 10 μm in the short axis contain numerous ribosomes and rough endoplasmic reticulum, resulting in an electron-dense appearance. These cells are found in the interior of the ganglia and have axo-somatic synapses. A second population of medium-sized neurons (v) also have axo-somatic synapses and appeared electron-dense, as they contain numerous and distinctive mitochondria. The final two classes of medium-sized myenteric neurons have broad dendrites (vi) or thin dendrites (vii), and are located at the periphery and the interior of ganglia, respectively. Finally, two populations of neurons have large perikarya of 30-35 μm , although some neurons in this class are found outside of myenteric ganglia. Large neurons within the ganglia (viii) have small and faint mitochondria and few synaptic contacts. Submucosal ganglionic neurons do not differ from each other in their ultrastructural organization, as they possess broad and thin dendrites, axo-somatic and axo-dendritic synapses, and perikarya that measure 12-30 μm (Wilson *et al.*, 1981).

Neurochemistry of Enteric Neurons

Pharmacological studies in the early 20th century by researchers such as Langley, Dixon, Dale (1913 and 1914), Loewi (1926), and Cannon (1921) convincingly illustrated that parasympathetic nerves released acetylcholine (ACh) and sympathetic nerves released norepinephrine (NE). However, identity of many neurotransmitters in the ENS remained unresolved, for biochemical protocols, and histochemical techniques such as

immunohistochemistry, were not available prior to the early 1980s.

Norepinephrine was the first neurotransmitter to be identified in peripheral and central tissues by histological methods. Eränkö (1952) developed a protocol for the anatomical localization of catecholamines using hot formaldehyde vapor. This technique proved to be difficult as the water content had to be controlled carefully, ultimately leading to development and use of glyoxylic acid (GA) to reveal neuronal stores of catecholamines (Furness and Costa, 1975). These histological techniques were advantageous as preparations of peripheral tissues could be incubated in hot formaldehyde vapor or glyoxylic acid, and then examined using fluorescent microscopy. As NE fluoresced bright blue, innervation could be easily visualized. However, histological techniques did not allow localization of any other catecholamines, such as dopamine and epinephrine, or any other neurotransmitters, such as ACh.

Beginning in the 1970s, immunological techniques were used to raise antibodies or antiserum against putative enzymes and neurotransmitters. Thomas Hökfelt *et al.* (1975) were the first group to apply such techniques to the ENS for they described somatostatin-immunoreactive (ir) neurons in myenteric ganglia. This observation suggested that peptides may also be used by enteric neurons as neurotransmitters or as modulators of neurotransmission. Immunohistochemical studies have found enteric neurons contain a plethora of possible neurotransmitters including angiotensin (reviewed in Costa *et al.*, 1987), bombesin (reviewed in Costa *et al.*, 1991), calcitonin gene-related peptide (CGRP) (Furness *et al.*, 1985), cholecystokinin 1-8 (Schultzberg *et al.*, 1980) dynorphin 1-8 and 1-17 (Costa *et al.*, 1985a), met- and leu-enkephalin (Furness *et al.*, 1983), histamine, galanin (reviewed in Furness *et al.*, 1988), gastrin-releasing peptide 1-27

(Costa *et al.*, 1984), neuropeptide Y (NPY) (Furness *et al.*, 1983b; Furness *et al.*, 1985; Keast *et al.*, 1984b), neurotensin, peptide histidine isoleucine amine (reviewed in Furness *et al.*, 1982), pituitary adenylate cyclase activating peptide (Sundler *et al.*, 1992), somatostatin (Hökfelt *et al.*, 1975; Costa *et al.*, 1980; Costa and Furness, 1984; Keast *et al.*, 1984a), substance K, substance P (SP) (Costa *et al.*, 1981; Keast *et al.*, 1984b), and/or vasoactive intestinal peptide (VIP) (Furness and Costa, 1979; Costa and Furness, 1983; reviewed in Furness and Costa, 1982a; Keast *et al.*, 1984b). Antiserum or antibodies have also been raised against enzymes involved in the synthesis of neurotransmitters. For example, enteric neurons have been shown to contain choline acetyltransferase (ChAt)-ir, the enzyme responsible for production of ACh; nitric oxide synthase (NOS)-ir, the rate-limiting enzyme for synthesis of nitric oxide (NO); and/or tyrosine hydroxylase (TH), the rate-limiting enzyme for synthesis of catecholamines. Some enteric neurons have also been shown to contain serotonin-ir (Costa *et al.*, 1982; Griffith and Burnstock, 1983; Furness and Costa, 1982), as well as uptake of gamma amino butyric acid (GABA) (Krantis and Kerr, 1981). Therefore, although the functions controlled by the ENS appear simple compared to those mediated by the CNS, each enteric neuron may release multiple neurotransmitters and neuromodulators that in turn, may influence arterioles, GI smooth muscle, and other enteric neurons.

Criteria for Identification of Neurotransmitters in the ENS

The ENS contains a wide variety of putative peptidergic and aminergic neurotransmitters as described in the preceding section. A number of these molecules have met the criteria for identification as neurotransmitters. Although some authors claim as many as six criteria must be met for a molecule to be a neurotransmitter (Paton, 1958;

Werman, 1966; Orrego, 1979), known exceptions exist for every condition except two: The molecule must be released from neurons upon nerve stimulation, and exogenous application of the molecule to effector tissues must mimic the effects of nerve stimulation. Although these criteria appear simplistic, even today, many neurochemicals found in enteric neurons can not be conclusively identified as "neurotransmitter" or as "neuromodulator". Many experimental paradigms may allow substances to reach sites that are not accessible in physiological conditions (reviewed in Furness and Costa, 1987). However, a number of molecules have been positively identified as neurotransmitters in the ENS, including ACh, NE, NO (or a NO-like molecule), opiate peptides, serotonin, SP, and VIP.

Chemical Coding of Enteric Neurons

Although the preceding discussion of neurochemistry of enteric neurons suggests that these neurons are very complex, a surprising pattern of chemical coding has emerged from early immunohistochemical studies by Hökfelt *et al.* (Schultzberg *et al.* (1980)). These studies used immunohistochemical and histological techniques to localize two substances simultaneously in the same cell. Hökfelt's group were the first to report that some submucosal neurons in rat and guinea pig contained somatostatin and cholecystokinin, but other submucosal neurons did not contain either neuropeptide. This observation led John Furness, Marcello Costa, and co-workers to further investigate the possibility that enteric neurons possessed a chemical code, in which neurons could be classified according to their neuropeptide and amine content. The resulting studies performed over the next ten years indicated that distribution of different peptide-containing neurons is distinct, and that each peptide or amine may serve as a marker for

a different functional set of neurons. For example, an amazingly simple pattern was observed in submucosal ganglia of guinea pig, as all submucosal neurons could be placed in one of four categories: 45.3% of submucosal neurons contained dynorphin-, galanin-, and VIP-immunoreactivity (ir); 28.6% of neurons contained ChAT-, cholecystokinin-, CGRP-, NPY-, and somatostatin-ir; 10.8% of neurons contained ChAT- and SP-ir; and 14.4% of cells contained ChAT-ir only (Furness *et al.*, 1984; Furness *et al.*, 1985; reviewed in Furness and Costa, 1987; reviewed in Costa *et al.*, 1991).

Immunohistochemical and functional studies have revealed that chemical content of submucosal neurons is correlated to functions of neurons. For example, in guinea pig ileum, dynorphin-, galanin-, and VIP-ir neurons are thought to mediate non-cholinergic secretomotor activity and may mediate vasodilation in some arterioles (reviewed in Surprenant, 1994; but see Galligan *et al.*, 1990). Cholinergic secretomotor activity is regulated by neurons containing ChAT-, cholecystokinin-, CGRP-, NPY-, and somatostatin-ir, whereas cholinergic vasodilatory nerves contain ChAT- and calcitonin-ir (Brookes *et al.*, 1992). Finally, ChAT- and SP-ir cells have been proposed to be intrinsic sensory neurons (reviewed in Furness *et al.*, 1984; Furness *et al.*, 1985; Furness and Costa, 1987; Song *et al.*, 1992).

Subclasses of myenteric neurons may also be categorized by morphology, immunohistochemical content, targets of innervation, and proposed functions (reviewed in Costa *et al.*, 1986a; Furness and Costa, 1987). Myenteric neurons may provide excitatory or inhibitory innervation to circular or longitudinal muscle (motor neurons), receive and transmit sensory innervation (intrinsic sensory neurons), and function as interneurons in reflex or effector pathways. Immunohistochemistry and anatomical tracing

with DiI, a carbocyanine dye, have identified five myenteric projections to circular muscle: (i) large Dogiel type I cells contain dynorphin-, gastrin-releasing peptide-, neurofilament (NF) protein-, and VIP-ir; (ii) small Dogiel type I cells contain dynorphin-, enkephalin, NPY-, and VIP-ir; (iii) small neurons contain ChAt-ir; (iv) small neurons contain ChAt- and SP-ir; and (v) small Dogiel I cells contain ChAt-, enkephalin, NF-, and SP-ir (Brookes *et al.*, 1991b). In contrast, similar experiments have not provided a clear classification scheme for innervation to longitudinal muscle; however, most neurons appear to contain ChAt-ir, and 47% of these cells contain SP-ir (Brookes *et al.*, 1992). There is little information regarding intrinsic sensory neurons, although most authors agree that enteric sensory neurons exist within myenteric ganglia. As described previously, Dogiel (1899) hypothesized that filamentous type II cells function as sensory nerves. The majority of cells with this morphology contain calbindin-, somatostatin-, and SP-ir (Costa *et al.*, 1986b; Pompolo and Furness, 1988; Furness *et al.*, 1990). Therefore, the direct implication of the theory of chemical coding is simply an extension of Dogiel's work; namely, that neurons with similar morphologies and chemical content have similar projections and mediate the same physiological functions.

3. SOME FUNCTIONS OF INTRINSIC INNERVATION

Viscerofugal Neurons

As described previously, Langley (1921), Bayliss and Starling (1899, 1900), Cannon (1906), and Alvarez (1948) were among the first physiologists to observe that enteric reflexes such as peristalsis and the vasodilator reflex could occur independently of extrinsic innervation. However, removal of prevertebral ganglia abolishes reflexes that

occur over long lengths of the digestive tract (Kuntz and Van Buskirk, 1941), suggesting segments of the GI tract may be interconnected by projections between the ENS and prevertebral ganglia. Functional and histological studies support this hypothesis, as reflex activity is observed when transected regions of the gut are connected only by mesentery and prevertebral (celiac) ganglia (Kuntz and Saccomanno, 1944). Furthermore, nerve fibers are found in decentralized celiac ganglia (Kuntz, 1938), implying prevertebral ganglia receive innervation from the viscera. Retrograde labelling of segments of the GI tract with Fast Blue has proven that some enteric neurons project to celiac, superior mesenteric, or inferior mesenteric ganglia (Messenger and Furness 1992; Messenger and Furness, 1993). Viscerofugal enteric neurons are found only in myenteric ganglia, with increasing density from the stomach to large intestine. It is imperative to note that very few neurons in the ileum project to prevertebral ganglia, as injection of inferior mesenteric ganglia does not label myenteric neurons, and injection of superior mesenteric ganglia labels 0.025% of myenteric neurons, whereas injection of celiac ganglia labels 0.1% of myenteric neurons.

Intrinsic Sensory Neurons

Approximately 75% of ileal viscerofugal neurons have cytoarchitectural properties consistent with Dogiel type II neurons, as they appear to send filamentous dendrites circumventrally towards the mucosal surface, and their axons project within myenteric ganglia (Messenger and Furness, 1992; Messenger and Furness, 1993). Such observations of bipolar or pseudounipolar enteric cells resulted in the hypothesis that these neurons represent intrinsic sensory neurons (Dogiel, 1899). Mucosal distortion and stretching of the GI tract can produce polarized reflexes such as peristalsis (Yuan *et al.*, 1991).

Furthermore, the presence of such reflexes in extrinsically-denervated ileum supports the conclusion that some enteric neurons function as sensory cells (Bayliss and Starling, 1899; Bayliss and Starling, 1900; Trendelenburg, 1917). Although most researchers agree that enteric sensory neurons exist, no one has been able to conclusively prove their location, cytoarchitecture, chemical composition, or electrophysiological properties. As described previously, silver impregnation or methylene blue staining reveals pseudo-unipolar or bipolar neurons in submucosa and myenteric plexuses; these neurons are proposed to be sensory cells (Dogiel, 1899; Lawrentjaw, 1931). Kirschgessner and Gershon (1988a) found 20-50% of all submucosal neurons and 2% of myenteric neurons in rat small intestine express N-acetyl-lactosamine carbohydrate differentiation antigens. As these antigens are commonly found in primary sensory neurons, their localization in submucosal neurons may indicate enteric sensory neurons. *In vitro* functional studies has supported the conclusion that submucosal ganglia contain sensory neurons, as mucosal stimulation induces immediate early gene (IEG) expression in submucosal and myenteric neurons, suggesting stimulus-invoked nuclear activity (Kirschgessner and Gershon, 1988a; Hunt *et al.*, 1987). Interestingly, the ganglionic nicotinic receptor (nACh) antagonist hexamethonium prevented induction of IEG in myenteric neurons, suggesting sensory information is received and transmitted by submucosal neurons. Finally, similar to neurons in dorsal root ganglia (DRG), a subset of submucosal neurons do not appear to receive synaptic input (Surprenant, 1984), further implying intrinsic sensory cells are found in submucosal ganglia. However, immunohistochemical studies have found calcium binding proteins typical of sensory neurons in brain nuclei (Rogers, 1989; Winsky *et al.*, 1989), sensory ganglia (Kuramoto *et al.*, 1990; Rogers, 1989), and in Dogiel type II

myenteric neurons (Furness *et al.*, 1990; Brookes *et al.*, 1991b). These data provide at least preliminary evidence that intrinsic neurons may be located in myenteric ganglia, as well as submucosal ganglia.

Cholinergic Vasodilatory Neurons

Nerve stimulation of extrinsic nerves produces vasodilation in some segments of the GI tract (Dale, 1913; Kewenter, 1965). However, the first evidence for enteric regulation of blood flow was provided by Biber and co-workers (1971). Stimulation of intramural nerves (Biber *et al.*, 1973) or mucosa of the small intestine (Biber *et al.*, 1973) increased blood flow by 30-150%, even after extrinsic nerves had been destroyed, indicating that enteric neurons mediate nerve-evoked vasodilation. Although vasodilation was blocked when tetrodotoxin (TTX) or local anesthetics were applied to the mucosa, vasodilation remained after application of α - or β -adrenoreceptor antagonists or chlorisondamine, a nACh receptor antagonist. Unfortunately, the identity of the vasodilator substance and the origin of vasodilator neurons were not identified, as it was almost impossible to study responses of arterioles with outside wall diameter of less than 100 μm .

Although parasympathetic nerves release ACh to relax peripheral arterioles, neither pharmacological nor anatomical studies of enteric cholinergic innervation could be performed on submucosal arterioles. Until 1993, immunohistochemical demonstration of cholinergic innervation was almost impossible, as virtually all antibodies raised against ChAt did not recognize autonomic cholinergic nerve fibers. Furthermore, although some nerve fibers can be followed for long distances after immunohistochemistry (described in Furness and Costa, 1987), nerves associated with submucosal arterioles have multiple

collaterals that cross-over and run with arterioles in multiple planes, resulting in difficulty following a single nerve fiber from its origin to its target (Bornstein *et al.*, 1987; Galligan *et al.*, 1990). Anatomical tracing of innervation to submucosal arterioles also proved to be near impossible, as retrograde dyes are taken up by sympathetic and primary afferent nerves found in close association with blood vessels, thus limiting availability of dye for uptake by vasodilator neurons. Furthermore, most anatomical tracers used in the CNS are of limited use in the ENS, due to distances between *en passant* innervation and submucosal arterioles or GI smooth muscle.

The development of the Diamtrak on-line arteriole video-monitoring system (Neild, 1989) has allowed small diameter resistance vessels to be studied, and resulting data have demonstrated that submucosal neurons provide cholinergic vasodilation in normal small intestine. Electrical stimulation of submucosal ganglia or interganglionic nerve fibers results in frequency-dependent, TTX-sensitive vasodilation of precontracted arterioles (Neild *et al.*, 1990; Galligan *et al.*, 1990; Andriantsitohaina and Surprenant, 1992). This vasodilation is mediated by submucosal neurons, as surgical destruction of underlying myenteric plexus and extrinsic innervation does not alter the amplitude or duration of vasodilation; however, vasodilation is eliminated by destruction of interganglionic submucosal nerve strands (Neild *et al.*, 1990). Vasodilation is potentiated by acetylcholinesterase inhibitors, and is totally inhibited by muscarinic ACh (mACh) antagonists. Construction of antagonist concentration-response curves reveals that ACh acts through M₃ receptors (Neild *et al.*, 1991) thought to be located on endothelial cells (Andriantsitohaina and Surprenant, 1992). Activation of these receptors leads to relaxation of arterioles *via* release of NO (Andriantsitohaina and Surprenant, 1992;

Surprenant, 1994).

Immunohistochemical studies have identified cholinergic neurons in submucosal ganglia. As described previously, ChAT-ir is found in approximately 54% of submucosal neurons (Furness and Costa, 1983a; Furness *et al.*, 1984; reviewed in Furness and Costa, 1987; Schemann *et al.*, 1993), and approximately 20% of these cells contain calretinin and have projections consistent with vasodilatory nerves (Pompolo and Furness, 1988). Therefore, anatomical and functional data prove the presence of enteric cholinergic vasodilatory neurons within submucosal ganglia.

Non-Cholinergic Vasodilatory Neurons

Although immunohistochemical techniques have identified non-cholinergic vasodilatory substances including VIP and CGRP in submucosal neurons, and early experiments suggested the presence of non-cholinergic vasodilation (Biber *et al.*, 1971; Biber *et al.*, 1973), muscarinic antagonists inhibit neurogenic vasodilations. Exogenous application of VIP or SP, but not CGRP, dilates submucosal arterioles (Galligan *et al.*, 1990). However, the actions of VIP are mediated by VIP-evoked depolarizations of cholinergic vasomotor neurons (Mihara *et al.*, 1985; Surprenant, 1985) as mACh receptor antagonists such as pirenzepine inhibit responses to VIP, and cholinesterase inhibitors such as eserine potentiate vasodilation response (Galligan *et al.*, 1990). Muscarinic receptor antagonists do not alter dilation in response to SP, indicating tachykinin (NK) receptors are present on smooth muscle cells of submucosal arterioles.

Vasoconstrictor Neurons

At this time, evidence suggests that enteric neurons do not mediate vasoconstriction of arterioles, as electrical stimulation of submucosal ganglia does not

elicit vasoconstriction of submucosal arterioles in normal or extrinsically-denervated ileum (Galligan *et al.*, 1990; reviewed in Surprenant, 1994).

Cholinergic Motor Neurons

Anatomical and functional data have proven that ACh is produced and released by enteric excitatory motor neurons. As described previously, many myenteric neurons contain ChAT-ir (Furness *et al.*, 1983a; Furness *et al.*, 1984; reviewed in Furness and Costa, 1987; Schemann *et al.*, 1993), and cholinergic neurons innervate longitudinal and circular muscle layers as revealed by anatomical tracing techniques (Brookes *et al.*, 1991b; Brookes *et al.*, 1992). Acetylcholine production may be assayed by measuring outflow of [³H]ACh from [³H]choline-labelled tissues. These experiments demonstrate that myenteric ganglia are the major site of ACh synthesis, as longitudinal muscle-myenteric plexus preparations (LMMPPs) produce approximately 10 times that of longitudinal muscle alone (Szerb, 1975). Furthermore, this technique has been used to verify release of ACh from myenteric nerves (Dale and Feldberg, 1934), and nerve-evoked release of [³H]ACh (Szerb 1975, 1976) is inhibited by TTX and botulinus toxin, proving enteric neurons release ACh.

The actions of ACh on circular and longitudinal muscle, and the contribution of ACh in the peristaltic reflex have been well-characterized. Distension of small intestine elicits an orally-directed excitatory reflex that contracts circular muscle (Bayliss and Starling, 1899). Early experiments demonstrated that TTX and mACh receptor antagonists such as hyoscine (scopolamine) inhibit peristalsis (reviewed in Kosterlitz and Lees, 1964), and that ACh release is associated with peristalsis (Ikeda *et al.*, 1958). However, it was impossible to determine the location of cholinergic neurons in the reflex.

In 1990 Tonini and Costa invented a partitioned organ bath which allowed drugs to be applied specifically to the site of contraction (oral segment), site of stimulation (aboral segment), or to intermediate segments of isolated guinea pig ileum. Distension of the aboral end elicited reflex contractions of circular muscle in the oral segment, and application of mACh receptor antagonists applied to oral or aboral segments inhibited 95% of reflex contractions (Tonini and Costa, 1990). These data suggest that cholinergic interneurons at the site of distension, and motor neurons in the oral segment mediate the excitatory phase of peristalsis.

This hypothesis is strengthened by *in vitro* experiments utilizing nerve-muscle preparations, as low-frequency transmural stimulation contracts LMMPPs or circular muscle myenteric plexus preparations (CMMPPs). Muscarinic receptor antagonists such as piperazine, a M_1 receptor antagonist, inhibit contractile activity (Tonini and Costa, 1990), indicating ACh acts at mACh receptors on GI smooth muscle to produce contractions. Furthermore, contractions are enhanced by anti-cholinesterases (reviewed in Kosterlitz and Lees, 1964). Finally, electrophysiological experiments demonstrate nerve stimulation induces transient depolarizations in smooth muscle cells from circular muscle. These depolarizations, or "excitatory junction potentials" (EJPs) are sensitive to mACh antagonists (Bennett and Burnstock, 1968), providing additional evidence that enteric cholinergic neurons account for the majority of the excitatory reflex of peristalsis.

Non-Adrenergic Non-Cholinergic Excitatory Motor Neurons

Although hyoscine increases the threshold value necessary to trigger peristalsis, decreases the number of complete peristalses, and decreases the amount of fluid ejected, hyoscine and other muscarinic antagonists are unable to totally prevent peristaltic activity

(Tonini *et al.*, 1981). These observations support Kosterlitz and Watt's (1963) earlier observations of a non-cholinergic excitatory component of graded reflex contractions of the GI tract. Fifty years earlier, Von Euler and Gaddum (1931) illustrated that extracts made from intestinal tissue contained a peptide that contracted intestinal smooth muscle (Bury and Mashford, 1977), thus implying that an excitatory peptide may also be involved in mediation of peristalsis.

Anatomical and functional studies have suggested SP is an excitatory neurotransmitter in the GI tract. Substance P-like immunoreactivity is localized in axons (Nilsson *et al.*, 1975; reviewed in Furness and Costa, 1987), and neurons of submucosal and myenteric plexuses (Pearse and Polak, 1975; reviewed in Furness and Costa, 1987). Extrinsic denervations of loops of ileum does not alter SP content of LMMPPs as measured using a bioassay, further suggesting that most SP is produced by enteric neurons (Franco *et al.*, 1979b). Measurements of SP confirmed that myenteric ganglia, not longitudinal muscle, are the source of the majority of SP within the GI tract (Franco *et al.*, 1979b). Bioassays (Franco *et al.*, 1979a, 1979b), radioimmunoassays (Baron *et al.*, 1983; Donnerer *et al.*, 1984; Holzer, 1984), and radioimmunoassays coupled to gel exclusion chromatography (Holzer, 1984), have all proven that transmural stimulation of the GI tract or LMMPPs releases SP from myenteric neurons. Furthermore, SP release is frequency-dependent, inhibited by TTX and removal of calcium (Ca^{2+}) ions, mimicked by potassium (K^{+})-induced depolarization, and results in contraction of ileal smooth muscle (Franco *et al.*, 1979a, 1979b). Taken together, these data indicate that electrical stimulation of enteric nerves releases SP. Increased ileal intraluminal pressure also releases SP concomitant to initiation of peristalsis (Donnerer *et al.*, 1984), further

suggesting that SP mediates the non-cholinergic component of peristalsis.

Studies of nerve-muscle preparations have proven that SP is a neurotransmitter in the GI tract. In the presence of atropine or hyoscine, trains of electrical stimulation result in neurogenic contractions of LMMPPs (Ambache and Freeman, 1968; Costa and Furness, 1972), and contractions are mimicked by application of exogenous SP (Bury and Mashford, 1977; reviewed in Furness and Costa, 1987). Nerve- and SP-evoked contractions are antagonized by prior exposure to SP (Franco *et al.*, 1979a, 1979b), suggesting desensitization of SP receptors occur. Development of peptide and non-peptide antagonists to SP receptors (also referred to as "tachykinin receptors" (NK receptors)) has provided further evidence for SP as a neurotransmitter to longitudinal and circular muscle. For example, D-Arg¹, D-Pro², D-Trp^{7,9}-SP, Leu¹¹-substance P, and D-Pro², D-Trp^{7,9}, Leu¹¹-substance P attenuate neurogenic hyoscine-insensitive contractions of longitudinal and circular muscle, but do not alter hyoscine-sensitive responses (Gintzler and Hyde, 1983; Costa *et al.*, 1985b). Similarly, the novel non-peptide antagonist 2(CP96345-1) inhibits non-cholinergic contractions as well as binding of [³H] SP at NK₁ receptors (Snider *et al.*, 1991). As NK₁ receptors are found in high density on GI smooth muscle (reviewed in Gerard *et al.*, 1993), and application of SP produces EJPs in smooth muscle of the intestine (Fujisawa and Ito, 1982), these data provide strong evidence that SP acts on receptors on smooth muscle to produce contractions. Neurokinin receptor antagonists also inhibit hyoscine-resistant excitatory reflex activity in the intestine, but do not alter hyoscine-sensitive contractile activity (Costa *et al.*, 1985c), further strengthening the conclusion that SP is an important neurotransmitter in peristalsis.

Non-Adrenergic Non-Cholinergic Inhibitory Motor Neurons

Transmural stimulation relaxes precontracted LMMPPs in the presence of mACh antagonists (reviewed in Furness and Costa, 1987). These relaxations are not mediated by sympathetic nerves, as they are observed following destruction of sympathetic nerves (Furness, 1969) or after addition of direct or indirect-acting sympatholytic drugs (Burnstock *et al.*, 1966; Campbell, 1966). Electrophysiological recordings of smooth muscle cells reveal that relaxation is not due to inhibition of release of excitatory enteric neurotransmitters, as transmural stimulation results in transient hyperpolarization of smooth muscle cells (Burnstock *et al.*, 1963; Burnstock *et al.*, 1966; Bennett and Burnstock, 1968). Hyperpolarizations, or "inhibitory junction potentials" (IJPs) are also recorded after extrinsic denervation or addition of sympatholytic drugs (Burnstock *et al.*, 1966), proving that enteric neurons release an inhibitory neurotransmitter. This conclusion is supported by *in vitro* and *in vivo* observations, as relaxation of the lower esophageal sphincter after swallowing, relaxation of the stomach during eating, and relaxation of intestine aboral to a bolus during peristalsis can occur after loss of extrinsic innervation (Bayliss and Starling, 1899, 1900b; reviewed in Furness and Costa, 1987; reviewed in Sanders and Ward, 1992).

Until recently, the identity of enteric inhibitory neurotransmitters was not known. Early research focused on adenosine 5'-triphosphate (ATP) or a related purine compound (Burnstock *et al.*, 1970; Burnstock 1972; Burnstock 1975), as stimulation of myenteric ganglia releases ATP, adenosine diphosphate, and adenosine monophosphate. Furthermore, application of exogenous ATP relaxes numerous precontracted smooth muscle preparations from a number of species (Burnstock *et al.*, 1970). Although these data

suggest purines mediate non-adrenergic, non-cholinergic (NANC) relaxations, three pieces of evidence prove that another molecule must be involved. First, many nerves, including extrinsic nerves, release ATP during neurotransmission, suggesting most nerves may not specifically release ATP as a neurotransmitter. Secondly, histological techniques have not localized ATP stores in enteric neurons with Dogiel type I morphology (reviewed in Furness and Costa, 1987). However, the most damaging evidence against ATP comes from the observation that analogues of ATP do not relax a number of GI smooth muscle preparations (Costa *et al.*, 1986b), suggesting that ATP is not an inhibitory neurotransmitter in all tissues.

In the 1980s, vasoactive intestinal polypeptide (VIP) was hypothesized to mediate relaxation of GI smooth muscle. Unlike ATP, suitable immunohistochemical techniques allowed for visualization of VIP, and antiserum raised against VIP recognized a subpopulation of myenteric neurons and varicose nerve fibers associated with circular and longitudinal muscle layers (Costa *et al.*, 1980). Transmural nerve stimulation releases VIP (Fahrenkrug *et al.*, 1978), and application of exogenous VIP relaxes some smooth muscle preparations. However, some GI smooth muscle, such as ileal longitudinal muscle of guinea pig does not respond to VIP (Costa *et al.*, 1986b), leading to the conclusion that VIP is not an inhibitory neurotransmitter for all GI smooth muscle.

In 1990, David Bredt and co-workers demonstrated the presence of nitric oxide synthase (NOS) in a population of myenteric neurons found in small intestine of rat. As NOS is the rate-limiting enzyme that de-imidates L-arginine to equimolar concentrations of L-citrulline and nitric oxide (NO) (Palmer *et al.*, 1988; Palmer and Moncada, 1989; reviewed in Moncada *et al.*, 1991; Sanders and Ward, 1992) (Figure 4), this observation

suggested that NO is synthesized and released from myenteric neurons. Numerous anatomical studies have localized NOS-ir in myenteric neurons in all species and GI segments examined to date, including guinea pig small intestine (Costa *et al.*, 1991; Costa *et al.*, 1992; Young *et al.*, 1992) and colon (McConalogue and Furness, 1993), and rat antrum, duodenum, ileum, caecum, proximal colon and distal colon (Belai *et al.*, 1992). Nitric oxide synthase-ir is found in large and small Dogiel I cells located at the periphery of myenteric ganglia, and in small, oval neurons in the interior of myenteric ganglia, suggesting enteric motor neurons and interneurons contain NOS-ir. Immunoreactivity is not found in non-neural cells, except for rare mucosal endocrine cells (Costa *et al.*, 1992), but numerous NOS-ir varicose nerve fibers are found in myenteric and submucosal ganglia, internodal strands, secondary and tertiary plexuses, and circular and deep muscular plexuses associated with circular muscle (Costa *et al.*, 1992). Although retrograde tracing experiments have not been performed, extrinsic denervation of loops of ileum does not eliminate NOS-ir nerves, proving they originate from enteric neurons (Costa *et al.*, 1992; McConalogue and Furness, 1993). Removal of the myenteric plexus (myectomy) eliminates NOS-ir nerves in submucosal ganglia and circular muscle, indicating that NOS-ir nerves originate from myenteric ganglia. Furthermore, myectomies result in accumulation of NOS-ir in nerves oral to the lesion, implying NOS-ir nerves innervate aboral neurons or muscle (Costa *et al.*, 1992; McConalogue and Furness, 1993). These projections are consistent with data from analysis of peristalsis, as descending relaxation proceeds distension of the lumen (Bayliss and Starling, 1899; Bayliss and Starling, 1900; Costa and Furness, 1976; Furness and Costa, 1987).

Extensive functional studies have supported the hypothesis that NO is synthesized

and released from myenteric neurons. Nitric oxide is released from enteric nerves following transmural stimulation (Bult *et al.*, 1990; Wiklund *et al.*, 1993b; Shuttleworth *et al.*, 1994), or application of nicotinic agonists (Shuttleworth *et al.*, 1994). Furthermore, in the presence of TTX, application of exogenous NO or drugs that liberate NO-like molecules relax esophageal sphincter (Shikano *et al.*, 1988), taenia coli (Shikano *et al.*, 1988), duodenum longitudinal muscle (Toda *et al.*, 1990), ileum longitudinal muscle (Toda *et al.*, 1990; Osthaus and Galligan, 1991), and circular muscle (Boeckxstaens *et al.*, 1990). These relaxations mimic nerve-evoked NANC relaxations produced by transmural nerve stimulation of the GI tract (Shuttleworth *et al.*, 1991; Osthaus and Galligan, 1992; reviewed in Sanders and Ward, 1992), implying that the identity of the unknown inhibitory NANC neurotransmitter is NO or a NO-like molecule.

Biochemical experiments have shown that guanidine nitrogen atoms can be substituted with a methyl or nitrile group, forming N^G-monomethyl-L-arginine (LMMA) (Ress *et al.*, 1989) or N^G-nitro-L-arginine (LNA), respectively. Some L-arginine analogs including LNA inhibit NO production by permanently binding to, and inhibiting NOS (Furfine *et al.*, 1993); alternatively, most substituted L-arginine analogs are competitive antagonists (Figure 4), thus inhibiting binding of L-arginine to NOS (Palmer and Moncada, 1989; Mayer, 1989; reviewed in Moncada, 1991; reviewed in Sanders and Ward, 1992). However, NOS antagonists such as LMMA do not affect arginine-utilizing enzymes such as arginase or arginine decarboxylase, indicating specificity for NOS (Granger *et al.*, 1990). *In vitro* pharmacological experiments have found that application of substituted L-arginine analogs attenuate transmural nerve stimulation-induced neurogenic relaxations of lower esophageal sphincter (Tottrup *et al.*, 1986), stomach

(Boeckxstaens *et al.*, 1990), small intestine (Toda *et al.*, 1990; Osthaus and Galligan, 1991), and colon (Shuttleworth *et al.*, 1991; Humphreys *et al.*, 1991). However, these drugs do not alter muscle responsiveness to mACh receptor agonists such as bethanechol, indicating that NOS antagonists do not affect contractile properties of GI smooth muscle. Oxyhemoglobin binds extracellular NO, independent of NOS; as expected, hemoglobin attenuates NANC neurogenic relaxations in tissues taken from the entire GI tract (reviewed in Sanders and Ward, 1992). Finally, removal of extracellular Ca^{2+} eliminates enzyme activity (Bredt and Snyder, 1990b), as well as muscle relaxation (reviewed in Furness and Costa, 1987). Taken together, these data indicate NO or NOS antagonists inhibit nerve-evoked relaxations, indicating that NO is released from enteric nerves.

As discussed previously, distension of the small intestine stimulates peristalsis, resulting in relaxation of aboral ileum (Bayliss and Starling, 1899; Costa and Furness, 1976). In rat proximal colon, neurogenic relaxations are not affected by desensitization to ATP or VIP, but are inhibited by LNA (Hata *et al.*, 1990), indicating NO is important in peristalsis. Furthermore, NOS antagonists alter threshold volume of initiation, rate of propulsion of solids, and results in dyscoordination of peristalsis, (Costa *et al.*, 1991). Taken together, these data provide physiological evidence that NO mediates at least part of the descending inhibitory reflex during peristalsis.

Electrophysiological studies have verified that NO, or a similar molecule, accounts for at least part of the transient hyperpolarization of smooth muscle cells associated with relaxation of GI smooth muscle. In canine duodenum, application of exogenous NO or the NO-donor sodium nitroprusside (SNP) hyperpolarizes resting membrane potentials in

a concentration-dependent manner (Stark *et al.*, 1993). Exogenous NO is unlikely to act on myenteric neurons, as SNP-induced IJPs are not attenuated by TTX or low Ca^{2+} -high Mg^{2+} buffer. Furthermore, L-, but not D-, arginine analogs, attenuate nerve-evoked, but not SNP-induced, IJPs. These data indicate that NOS antagonists do not act on smooth muscle cells. As predicted, oxyhemoglobin and removal of Ca^{2+} attenuated nerve- and SNP-evoked IJPs (Stark *et al.*, 1993). Therefore, electrophysiological studies agree with *in vitro* functional data, and prove that NO, or a NO-like molecule, is an important intrinsic inhibitory neurotransmitter. However, although these data demonstrate that NO mediates NANC relaxations, it should be noted that characteristics of agonist- and electrical field stimulation-induced IJPs are distinct, as hyperpolarization and repolarization of neurogenic IJPs occur in half the time as compared to agonist-evoked IJPs. Furthermore, although oxyhemoglobin and L-arginine analogs attenuate neurogenic NANC IJPs and relaxations of smooth muscle, they do not totally inhibit either event. Biochemical studies have proven L-arginine analogs inhibit NO formation. Therefore, it is probable that other molecules, including ATP and VIP, are important in mediation of NANC neurotransmission.

Cholinergic Interneurons

Anatomical tracing from GI smooth muscle reveals that most motor neurons to circular and longitudinal muscle contain ACh. However, myenteric interneurons also contain ACh. Although anatomical studies have been difficult due to limitations with antiserum raised against ChAt, both Brookes *et al.* (1992) and Schemann *et al.* (1993) identified a population of small ChAt-ir neurons. These neurons were not labelled by retrograde dye applied to circular or longitudinal muscle, further suggesting that some

non-motor myenteric neurons contained ChAt-ir.

Studies of reflex activity of myenteric neurons have provided convincing evidence that ACh is used in communication between enteric neurons. Luminal distension evokes ascending contractile activity and descending relaxations (Bayliss and Starling, 1899; Bayliss and Starling, 1900a; Costa and Furness, 1976). This reflex activity involves nACh and mACh receptors, as hyoscine (scopolamine) and hexamethonium inhibit contractions oral to the site of stimulus (Costa and Furness, 1976). However, until invention of partitioned organ baths, it was impossible to determine the anatomical location and relative contribution of cholinergic interneurons in the peristaltic reflex. As described previously, Tonini and Costa (1990) found that reflex contractions of circular muscle were antagonized by hexamethonium or hyoscine applied near the site of distension. Therefore, these data provide *in vitro* evidence of cholinergic interneurons near the site of ileal distension, as well as cholinergic neurotransmission between enteric neurons.

Electrophysiological recordings from myenteric neurons confirm that nACh and mACh receptors are localized on enteric neurons. Focal stimulation of interganglionic nerves results in a number of synaptic potentials including transient (20 msec) depolarizations of neuronal membranes called "fast excitatory post-synaptic potentials" (fEPSPs). Many fEPSPs are mediated by nACh receptors, as nACh antagonists such as tubocurarine and hexamethonium block nerve-evoked fEPSPs (Nishi and North, 1973a). Exogenous application of ACh from electrodes placed close to cell somata mimic fEPSPs (Nishi and North, 1973a; North *et al.*, 1980), and these responses are also antagonized by nACh antagonists (North *et al.*, 1980). Inhibition of cholinesterase by physostigmine

prevents metabolism of ACh and prolongs the amplitude and duration of nerve-evoked fEPSPs (North and Nishi, 1974). Taken together, these functional and electrophysiological studies demonstrate enteric neurons release ACh to activate nACh receptors on enteric neurons.

Muscarinic receptor antagonists such as hyoscine also inhibit reflex contractile activity (Costa and Furness, 1976; Tonini and Costa, 1990). Electrical stimulation of interganglionic nerve strands in the myenteric plexus produces depolarizations of myenteric neurons called "slow excitatory post-synaptic potentials" (sEPSPs). Many sEPSPs are attenuated or abolished by muscarinic antagonists (North and Tokimasa, 1982; North *et al.*, 1985), and mACh agonists can mimic sEPSPs in some cells (North *et al.*, 1985), providing further evidence for cholinergic interneurons in the ENS.

Non-Adrenergic Non-Cholinergic Interneurons

Many synaptic events in the ENS are inhibited by TTX and removal of Ca^{2+} (Wood and Mayer, 1979), but are not affected by cholinergic antagonists such as hexamethonium, atropine, curare, or physostigmine. These synaptic events are not affected by direct- or indirect-acting sympatholytic drugs, suggesting enteric interneurons may use neuropeptides or amines in neurotransmission (reviewed in Furness and Costa, 1987). Recent experiments have demonstrated that some enteric neurons use ATP to mediate fEPSPs, as application of ATP mimics fEPSPs. Furthermore, suramin, a purinergic receptor antagonist, attenuates nerve- and ATP-evoked depolarizations in the presence of hexamethonium (Galligan and Bertrand, 1994). Taken together, these data indicate that ATP may be an enteric neurotransmitter, although further studies are needed to determine the origin of purinergic innervation.

Electrophysiological studies have also suggested that SP and serotonin are used in communication between enteric neurons. As described previously, SP-ir is found in myenteric neurons and in basket-like innervation of some myenteric neurons (Nilsson *et al.*, 1980; reviewed in Furness and Costa, 1987), and physiological stimuli releases SP as measured by radioimmunoassay (Holzer, 1984) and high performance liquid chromatography (Murphy *et al.*, 1982). Binding studies (Drapeau *et al.*, 1990) and functional studies (Fox and Daniel, 1986; Kilbinger *et al.*, 1986) have suggested NK receptors are located on some enteric neurons. Application of SP for short periods of time to enteric neurons mimics neurogenic sEPSPs recorded in myenteric neurons (Katayama *et al.*, 1979; Johnson *et al.*, 1980; Johnson *et al.*, 1981). Although antagonists for neuronal NK receptors have not been developed, chymotrypsin hydrolyzes SP, and decreases the amplitude and duration of neurogenic and agonist-induced sEPSPs (Johnson *et al.*, 1981). Therefore, these data provide compelling evidence that SP is used as a neurotransmitter by non-motor neurons. Similar anatomical and functional evidence also suggests that serotonin can mediate some sEPSPs (Katayama and North, 1978; Wood and Mayer, 1979; reviewed in Furness and Costa, 1987).

4. EXTRINSIC INNERVATION OF THE GASTROINTESTINAL TRACT

Extrinsic Paravascular and Perivascular Innervation

As nerves of the ENS are referred to as "intrinsic", all nerves originating from sources located outside of the wall of the gut are identified as "extrinsic". Extrinsic sympathetic, parasympathetic, and sensory (primary afferent) nerves originate from sources located outside of the GI tract and enter the GI tract by utilizing the tract provided

by submucosal arterioles (Figure 3). Extrinsic paravascular innervation is orientated parallel to arterioles, and lies along the blood vessels within and outside of the adventitia. This paravascular innervation may then remain associated with the arterioles to regulate micro-circulation, or project away from arterioles to innervate mucosal crypts, enteric ganglia, and smooth muscle. Perivascular extrinsic innervation form a continuous network of anastomosing nerve fibers that comprise the perivascular plexus found in adventitia and superficial plane of submucosal arterioles. These nerves remain associated with the vasculature to regulate microcirculation.

Anatomy of Efferent Sympathetic Innervation

The axons of cholinergic sympathetic preganglionic cell bodies in thoracic (T) and lumbar (L) intermediolateral gray matter of the spinal cord enter the splanchnic nerves, and terminate on noradrenergic postganglionic prevertebral celiac, inferior mesenteric, superior mesenteric, and pelvic ganglia (Langley, 1921; Furness and Costa, 1974a; reviewed by Szurszewski and King, 1989). Although variations exist among species, the small intestine is mainly innervated by mesenteric ganglia which receive preganglionic axons originating from T₉ to T₁₀ spinal roots (reviewed by Ronan and Gonella, 1981). The majority of postganglionic sympathetic neurons found in celiac-mesenteric ganglia project *via* splanchnic nerves to the stomach and small intestine as perivascular and paravascular nerves, as interruption of mesenteric nerves or removal of prevertebral ganglia eliminates sympathetic innervation (Furness and Costa, 1971; Furness and Costa, 1978). Although most sympathetic innervation to the small intestine originates from prevertebral ganglia, some sympathetic innervation originates from cervical chain ganglia and projects within vagal nerves to the upper small intestine (Lundberg *et al.*, 1978;

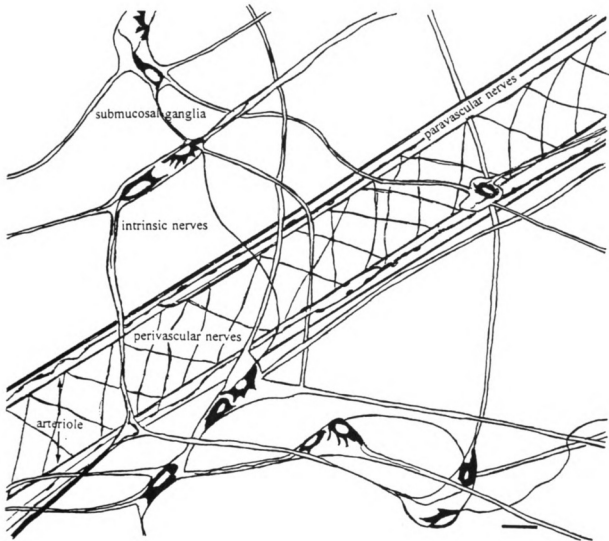


Figure 3. Extrinsic and intrinsic innervation of submucosal arterioles. Drawing of whole mount preparation of submucosal plexus of guinea pig ileum. Arterioles receive paravascular and perivascular innervation from extrinsic sympathetic and sensory nerves, as well as nerves from intrinsic vasodilatory neurons. Calibration bar is approximately 20 μm .

Muryobayashi *et al.*, 1968; Ahlman, 1978).

Early histological studies used hot formaldehyde vapors or glyoxylic acid (GA) to induce fluorescence of neuronal stores of catecholamines, resulting in staining that could be visualized using fluorescence microscopy. More recently, antibodies against TH, the rate-limiting enzyme for the production of catecholamines, or dopamine β -hydroxylase, the enzyme that catalyzes formation of NE from dopamine (Furness *et al.*, 1979) have been extensively used in a number of peripheral tissues, including the GI tract, to identify sympathetic nerves. Histological and immunohistochemical studies of the gut have revealed a dense network of noradrenergic innervation around submucosal arterioles and a well-developed peri-neuronal innervation in submucosal and myenteric ganglia (Furness and Costa, 1975). Interestingly, sympathetic innervation to submucosal arterioles also contains NPY-ir, although sympathetic nerves to enteric ganglia do not contain detectable NPY-ir (Ekblad *et al.*, 1984). The accompanying veins receive little sympathetic innervation. Although a sparse network of sympathetic nerves is found in the mucosa and circular muscle, little innervation is found with the longitudinal muscle layer of the small intestine. Noradrenergic cell bodies are not observed in submucosal or myenteric ganglia in all regions of the GI tract except the colon (reviewed in Furness and Costa, 1974; Furness and Costa, 1987).

Anatomy of Efferent Parasympathetic Innervation

Cholinergic parasympathetic preganglionic cell bodies in the rostral portion of dorsal motor nucleus and nucleus ambiguus of the medulla oblongata synapse on cholinergic cells in enteric ganglia as revealed by retrograde and anterograde tracing (Satomi *et al.*, 1978; Zhang *et al.*, 1991). However, the nuclear ambiguus provides only

a minor innervation below the esophagus (Kalia and Sullivan, 1982); therefore, most anatomical tracing has focused on the dorsal motor nucleus. Interestingly, fewer than 20,000 vagal fibers (afferent and efferent) pass through the guinea pig diaphragm (Irwin, 1931), suggesting that most enteric neurons and effector tissues do not receive parasympathetic innervation. This conclusion has been supported by anatomical tracing techniques, for injection of anterograde tracer in the dorsal motor nucleus of the vagus results in labelled nerve fibers in only 30% of myenteric ganglia of the small intestine. These results imply that vagal nerves may exert effects by innervating a small number of command neurons in enteric ganglia (Wood, 1987; Kirchgeßner and Gershon, 1989). Vagal efferent nerves are not found in adventitia, muscle layers, submucosal plexus, or mucosa (Kirchgeßner and Gershon, 1989; Berthoud *et al.*, 1990). Finally, very few nerve fibers in the small intestine degenerate after vagotomy (Honjin, 1951; Stovichek and Nikulin, 1970), further demonstrating the paucity of innervation by parasympathetic nerves. Although other organs of the GI tract receive parasympathetic preganglionic innervation originating from the retrofacial nucleus, nucleus retroambiguus, and/or lateral intermediate gray matter and Onuf's nucleus of sacral spinal cord segments S₁₋₅ (reviewed by Gillis *et al.*, 1989), anatomical tracing studies have not revealed such projections to the small intestine.

Although histological markers are not specific for acetylcholine, acetylcholinesterase has been used in some central tissues as a potential marker for cholinergic cells. However, as cholinergic and non-cholinergic peripheral nerves are associated with acetylcholinesterase, the stain is not useful in localization of cholinergic somata or axons (Silver, 1974). Antibodies have been recently raised against choline

acetyltransferase (ChAt), the enzyme responsible for synthesis of ACh (Eckenstein *et al.*, 1981; Kimura *et al.*, 1980). Unfortunately, cholinergic innervation to the ENS has been difficult to study using immunohistochemistry. Although nerve fibers containing ChAt-ir have been observed to be associated with submucosal and myenteric ganglia (Furness *et al.*, 1983a; Schemann *et al.*, 1993), few parasympathetic nerve fibers degenerate after vagotomy (Stovichek and Nikulin, 1970), implying that the ChAt-ir nerve fibers associated with the ganglia are predominantly from the ENS. Furthermore, although extrinsic nerves are thought to enter the wall of the GI tract using arterioles as tracts, ChAt-ir is not seen in nerve fibers associated with the arterioles (Furness *et al.*, 1983). Taken together, these results suggest that there is little vagal innervation to the small intestine, and most cholinergic innervation is from the ENS.

Anatomy of Afferent Vagal Innervation

The GI tract receives extrinsic sensory innervation originating from autonomic and somatic nervous systems. Pseudo-unipolar sensory neurons found in nodose (inferior vagal) ganglia send their central processes to the nucleus tractus solitarius of the medulla, and their peripheral process to the GI tract *via* the vagus nerve, as revealed by anatomical tracing. For example, application of the retrograde dye fluoro-gold to the stomach or the pancreas reveals sensory vagal nerve terminals in nodose ganglia (Dockray *et al.*, 1989). Unfortunately, such studies have not been performed on the small intestine. However, similar results are expected, as histological studies have revealed that 70-90% of vagal nerve fibers at the diaphragmic level are unmyelinated afferent nerves and only 10-20% of these nerves serve as efferent innervation (Irwin, 1931; Agostini *et al.*, 1957, Hoffman and Schnitzlein, 1961; Grundy and Scratcherd, 1989).

Sympathetic and spinal ganglionic afferent innervation contains specific neurochemical markers thus allowing anatomical localization of these nerves. Unfortunately, immunohistochemical methods have not revealed any neuropeptides that are found exclusively in parasympathetic afferent nerves. Vagal afferent nerve fibers do not contain ChAt-ir, but in some regions of the gut such as the stomach, vagal nerves may contain detectable levels of CGRP-, SP-, and/or VIP-ir (Pederson *et al.*, 1981; Raybould *et al.*, 1989). It should be noted that these neuropeptides are also found in spinal ganglionic afferent innervation. However, bilateral vagotomy does not alter CGRP-, SP-, or VIP-ir in the small or large intestine (Gibbins *et al.*, 1985; Goehler *et al.*, 1988; Su *et al.*, 1987; Sternini, 1991), suggesting a paucity of vagal afferent innervation to the intestine.

Anatomy of Innervation from Dorsal Root Ganglia

Surgical denervation combined with anatomical tracing techniques have indicated that the majority of extrinsic sensory innervation to the GI tract originates from spinal sensory ganglia (reviewed by Sternini, 1993). Pseudo-unipolar sensory neurons located in dorsal root ganglia (DRG) send their central unmyelinated processes to laminae I and V of the dorsal horn, and their peripheral processes into splanchnic nerves (Grundy and Scratcherd, 1989). It should be noted that these primary sensory nerves give off numerous collateral branches at prevertebral ganglia (Lee *et al.*, 1985), that may modulate the electrical properties of sympathetic postganglionic neurons (Aldskogius *et al.*, 1986; Mathews *et al.*, 1982; reviewed in Elvin *et al.*, 1993).

Spinal afferent nerves enter the wall of the gut as perivascular nerve fibers as revealed by immunohistochemistry (Papka *et al.*, 1984; Gibbins *et al.*, 1985; Uddman *et*

al., 1986). Neurons in the DRG contain a variety of neuropeptides, including CGRP, cholecystokinin, dynorphin, enkaphalins, galanin, somatostatin, SP, and VIP (reviewed by Dockray *et al.*, 1991). However, projections to the GI tract contain only two of these peptides, as SP-ir and CGRP-ir are always found co-localized in nerve fibers of the DRG (Gibbins *et al.*, 1985). Furthermore, application of the retrograde dye DiI to the small intestine results in labelling of SP-ir/CGRP-ir cells, but almost no other cells in the dorsal root ganglia located primarily at T₆-L₁ (Vickers and Costa, unpublished data, in Costa *et al.*, 1991). As discussed previously, these SP/CGRP-ir nerve fibers form a perivascular plexus, and branch to innervate submucosal and myenteric ganglia. Nerve fibers from the DRG are also found in the mucosa, but not associated with circular or longitudinal muscle layers (Gibbins *et al.*, 1985; Costa *et al.*, 1985; reviewed in Sternini, 1991).

5. FUNCTIONS OF EXTRINSIC INNERVATION

Anatomical evidence has shown that the GI tract receives sympathetic, parasympathetic, and primary afferent innervation originating from sources located outside of the gut. Although the connecting pathways are well-understood, the function of many of these nerves remains a controversy even today. However, almost all investigators now accept that sympathetic nerves provide inhibitory innervation, whereas parasympathetic nerves may provide excitatory and inhibitory innervation to the GI tract.

Regulation of Motility by Sympathetic Nerves

One of the first pharmacological experiments to examine the effects of sympathetic nerve stimulation on the GI tract was performed by Langley and Dickinson (1889), as they discovered that application of nicotine to celiac or mesenteric ganglia prevented

motility of the stomach. Direct application of nicotine to splanchnic nerves did not alter motility, thus indicating that nACh receptors were located on postganglionic neurons. Stimulation of thoracic pre-ganglionic nerve fibers to superior mesenteric ganglia produced identical results, as motility of the small intestine was inhibited (Ludwig, 1852; Pflüger, 1857; Lister, 1858; Basch, 1873; Bayliss and Starling, 1899). Furthermore, stimulation of mesenteric nerves to a segment of intestine generated a substance that relaxed an unstimulated piece of intestine, thus providing the first demonstration of an intestinal neurotransmitter. Finkleman (1930) found that inhibition of motility of isolated rabbit duodenum was mimicked by application of exogenous epinephrine, and both agonist- and nerve-mediated inhibition of motility were attenuated by adrenergic antagonists or desensitization of the muscle. However, it took an additional twenty years for Mann and West (1951) to deduce that sympathetic nerves released predominantly NE, and not epinephrine.

Similar *in vivo* experiments suggested that NE is capable of inhibition of contractions mediated by parasympathetic innervation, as vagal stimulation produces contractions of the ileum that are greatly reduced by sympathetic nerve stimulation. This inhibition of contractile activity is not due to direct sympathetic inhibition of vagal activity, as similar findings were made following chronic vagotomies (Jansson and Lisander, 1969). However, the site of action of NE was not known, although the mechanism of action of NE did not appear to be at longitudinal muscle, as muscle responsiveness to ACh was not altered by stimulation of sympathetic nerves (Kernter, 1965). This conclusion was supported by histological studies, as sympathetic nerves were not observed in the muscle layers of the gut of small mammals (Norberg, 1964; Gabella and Costa, 1967; Costa and

Gabella, 1971).

The controversy regarding the site and mechanism of action of sympathetic innervation was answered using histology, *in vitro* measurements of outflow of ACh, and electrophysiological techniques. Histological demonstration of formaldehyde vapor-induced fluorescence of sympathetic nerve fibers proved that the terminal axons of postganglionic sympathetic nerves were found within the GI tract (Norberg, 1964). Furthermore, the pattern of fluorescent nerves within myenteric ganglia suggested that sympathetic inhibition of motility occurred at enteric neurons, as most myenteric neurons has *en passant* innervation by noradrenergic nerve fibers (reviewed in Llewellyn-Smith *et al.*, 1983). These anatomical data supported the hypothesis of Kernter (1965), for he suggested that NE acted at enteric neurons and not on circular or longitudinal muscles. Functional evidence for this hypothesis was generated using electrophysiological studies. Neurotransmission by inhibitory motor neurons normally results in hyperpolarization of muscle cells, as described previously. However, stimulation of sympathetic nerves did not produce IJPs, thus providing conclusive evidence that GI smooth muscle does not receive direct innervation from sympathetic nerves. Furthermore, this study demonstrated that NE did not stimulate myenteric inhibitory motor neurons, as release of inhibitory neurotransmitters would produce IJPs. Measurements of ACh outflow from perfused segments of ileum supported Kernter's hypothesis, as stimulation of sympathetic nerves reduced ACh release by approximately 45% (Vizi and Knoll, 1971). Similar results were obtained in tissues in which vagal innervation had been destroyed. For example, following extrinsic denervation of loops of ileum, application of exogenous NE significantly reduced intrinsic release of ACh *in vitro* (Lee, 1970; Furness and Burnstock,

1975). Taken together, these data demonstrate that NE inhibits enteric excitatory neurons. The final support for sympathetic inhibition of enteric cholinergic neurons is obtained from electrophysiological studies. Application of exogenous NE or stimulation of mesenteric nerve fibers at did not affect membrane properties of myenteric neurons (Hirst and McKirdy, 1974; Nishi and North, 1973b). However, stimulation of sympathetic innervation resulted in pre-synaptic inhibition of cholinergic neurotransmission (Hirst and McKirdy, 1974), that was shown to be mediated by α_2 -adrenergic receptors (Drew, 1978; Frigo *et al.*, 1984). Therefore, sympathetic nerves inhibit excitatory myenteric neurons, resulting in decreased motility of GI smooth muscle.

Regulation of Blood Flow by Sympathetic Nerves

Sympathetic innervation provides extrinsic regulation of blood flow through the microvasculature of the GI tract. Normally, mesenteric and splanchnic vessels contain approximately 25-30% of the total blood volume of the body (Lacroix, 1960), and intestinal mucosa has the highest vascular perfusion of any peripheral tissue in the body. Perfusion of the mucosa allows for secretory and absorptive processes to occur. These processes are partially mediated by extrinsic sympathetic nerves that mediate vasoconstriction of resistance vessels of the GI tract. Dale (1913) reported that stimulation of sympathetic nerves constricted arterioles, and α_1 adrenergic receptor antagonists and indirect sympatholytic drugs attenuated or inhibited neurogenic vasoconstrictions. However, in a number of species, stimulation of extrinsic nerves results in EJPs in smooth muscle cells and corresponding vasoconstrictions which are insensitive to α_1 adrenergic receptor antagonists and catecholamine-depletion by reserpine (Evans and Surprenant, 1992). However, vasoconstriction is dependent on extrinsic innervation, as

destruction of sympathetic nerves eliminates vasoconstrictions. In addition to NE, sympathetic nerves also contain and release ATP or a related molecule, as activation of P_{2x} purinoreceptors constricts submucosal arterioles (Evans and Suprenant, 1992). Although sympathetic postganglionic cell bodies within celiac ganglia contain NPY, a potent vasoconstrictor (Edvisson *et al.*, 1984; reviewed in Furness and Costa, 1987), a physiological role for NPY in control of mesenteric blood flow has not been found.

Regulation of Motility by Efferent Vagal Nerves

Although nerve stimulation of the sympathetic nervous system produces clear inhibition of motility of the GI tract, and transection of splanchnic nerves results in a marked increase in intestinal motility (Bayliss and Starling, 1899) conflicting data has resulted from *in vivo* studies of parasympathetic innervation. For example, Bayliss and Starling (1899) and van Harn (1963) observed increased ileal motor activity following vagal stimulation in many animals. However, these authors also observed that identical stimulation of vagal nerves could stimulate and inhibit motility, or only inhibit intestinal motility. Such results led Bayliss and Starling to conclude vagal nerves contain both excitatory and inhibitory efferent innervation. However, vagal excitatory and inhibitory innervation appeared to exhibit unique stimulation requirements and latency of activation, as excitatory responses always occurred within two seconds of nerve stimulation, whereas inhibitory events developed twenty to thirty seconds after nerve stimulation. An alternative explanation of the observed mixed motor activity following vagal stimulation was provided by Kewenter (1965). He noted the differences in time of onset of excitatory and inhibitory effects, and hypothesized that inhibition of motility following vagal stimulation resulted from reflex activation of sympathetic innervation. Activation of vagal

nerves would decrease systemic blood pressure, and activate baroreceptor reflexes, thus increasing sympathetic nerve activity. Increased sympathetic outflow would then inhibit motility by activating α_2 adrenoreceptors as discussed previously. Furthermore, increased sympathetic nerve outflow stimulates the medullary region of adrenal glands, resulting in increased concentrations of circulating catecholamines. Therefore, circulating NE would then act to inhibit motility at non-innervated adrenergic receptors on smooth muscle. *In vivo* stimulation of distal processes of vagal innervation in adrenalectomized animals failed to produce inhibition of motor activity (Kewenter, 1965), thus supporting this hypothesis. Finally, intravenous administration of atropine, a mACh receptor antagonist prior or during vagal stimulation in normal animals resulted in only increased motor activity of the intestine (Kewenter, 1965), further suggesting that parasympathetic efferent innervation provided only excitatory innervation to the GI tract.

Kewenter's conclusions generated a great deal of controversy over the identity of parasympathetic neurotransmitters, as well as the physiological functions of vagus nerves. Closer examination of the early *in vivo* work revealed that use of anesthetization, site of vagal nerve stimulation, and stimulus parameters for nerve stimulation could all alter responses to vagal nerve stimulation. For example, single-shock stimulation of vagal nerve-endings did not alter intestinal motility (reviewed in Ronan and Gonella, 1981). However, vagal stimulation with trains of pulses induced contractions and enhanced amplitude and duration of pre-existing contractions of circular and longitudinal muscle (Gonella, 1964; Ronan and Gonella, 1981). Furthermore, these effects were antagonized by atropine (Fukuda, 1968), suggesting vagal stimulation released ACh. Interestingly, unlike Kewenter's (1965) study, other investigators demonstrated that addition of atropine

(Fukuda, 1968) or high frequency vagal stimulation (reviewed by Ronan and Gonella, 1981) relaxed GI smooth muscle. Although there are many interpretations of these data, vagal parasympathetic postganglionic cholinergic nerves form excitatory synapses on enteric excitatory neurons (Campbell 1966, 1970; Martinson, 1965). High frequency vagal stimulation may release adenosine (Burnstock, 1972; Burnstock, 1975), NO (Tottrup *et al.*, 1981), serotonin (Wood, 1981), or VIP (reviewed in Furness and Costa, 1987) from myenteric neurons, resulting in relaxation of circular and longitudinal muscle.

If vagal efferents innervate GI tissue, then transection of vagal nerves would be expected to decrease activity of GI smooth muscle. However, Kewenter (1965) observed only minor changes in intestinal basal tone and motility after vagal section, suggesting that parasympathetic nerves are normally not active. An alternative hypothesis has resulted from recent development of anatomical tracing techniques. Use of the anterograde tracer DiI has illustrated that cholinergic parasympathetic pre-ganglionic parasympathetic nerves from dorsal motor nuclei provide peri-neuronal innervation to adrenergic post-ganglionic neurons in the celiac and superior mesenteric ganglia (Berthoud and Powley, 1993). Although the function is not known, it has been hypothesized that this innervation allows vagal influence on GI motility without requiring actual vagal innervation within the GI tract. Therefore, if this hypothesis is correct, sectioning of the vagus during a period of sympathetic nerve quiescence would not alter the basic pattern of motility, as this motor activity would be predominantly controlled by the intrinsic rhythm of GI smooth muscle and enteric neurons. However, during sympathetic nerve activity, stimulation of parasympathetic nerves would increase activity of the ileum. This potential explanation would also explain the seeming negative data of such investigators as Kewenter, Bayliss

and Starling (1901), and Alvarez (1929), in that section of the parasympathetic nerves did not alter GI motility of the intestine.

Therefore, although few parasympathetic nerves are found within the small intestine, vagal nerves may influence activity of enteric and postganglionic sympathetic neurons. However, the physiological effects of parasympathetic nerve activity may be mediated by sympathetic tone to the GI tract. Investigators such as Bayliss and Starling (1901) and Alvarez (1929) found it difficult to study vagally-mediated motor responses in proximal and distal small intestine in the presence of sympathetic innervation. *In vivo* studies by Kewenter (1965) found elevated basal sympathetic nerve activity in GI tissues unresponsive to vagal nerve stimulation. Greater frequency stimulation of sympathetic nerves was necessary to inhibit motility of GI smooth muscle in tissues sensitive to vagal nerve stimulation, as compared to tissues which were unresponsive to vagal nerve stimulation (Kewenter, 1965). Intravenous injections of guanethidine and ergotamine confirmed these observations, as regions of the GI tract which were sensitive to parasympathetic nerve stimulation were not affected by indirect-acting sympatholytic drugs. Finally, application of exogenous guanethidine potentiated vagally-mediated motor responses. Taken together, these data suggest that sympathetic innervation provides physiological antagonism of parasympathetic innervation of the GI tract. Therefore, vagal tone may not be observed under normal conditions due to strong tonic sympathetic inhibition to some regions of small intestine.

Regulation of Blood Flow by Efferent Vagal Nerves

Although Sir Henry Dale (1913) demonstrated that autonomic nerves to the GI tract release a vasodilatory substance, until recently it was impossible to study innervation

of submucosal arterioles, as changes in arteriolar diameter could not be monitored. In other peripheral tissues, stimulation of parasympathetic nerves relaxed resistance vessels with intact endothelial cells (Furchgott, 1980). *In vitro* and *in vivo* application of exogenous ACh also decreased blood pressure, implying ACh mediates parasympathetic-induced vasodilation of arteries (Furchgott, 1981). However, vagal stimulation near the diaphragm did not alter jejunal blood flow in animals with intact sympathetic nerves and adrenal glands (Kewenter, 1965). Similar results were observed in the ileum, suggesting parasympathetic nerves do not regulate intestinal blood flow (Kewenter, 1965).

Other investigators observed high frequency vagal stimulation constricted submucosal arterioles, suggesting this experimental protocol also stimulated sympathetic nerves. Intravenous injection of guanethidine attenuated vasoconstriction and uncovered excitatory motor activity, suggesting parasympathetic vasodilation was masked by sympathetic vasoconstriction (reviewed in Chou, 1989). These results are difficult to interpret, as vagal nerves contain efferent and afferent nerve fibers. Therefore, stimulation of vagal nerves may antidromically activate afferent nerves, resulting in local release of vasodilatory substances. Alternatively, parasympathetic nerves may release ACh and dilate submucosal arterioles. Proceeding destruction of sympathetic nerves, vagal stimulation relaxes arterioles in stomach (Martison, 1965; Thieffin *et al.*, 1990) and colon (Hultén *et al.*, 1969a, 1969b). Furthermore, these vasodilations are partially antagonized by atropine, suggesting vasodilation is mediated by nerve-evoked ACh. Unfortunately, these experiments did not determine if cholinergic vasodilations were mediated by parasympathetic or enteric neurons.

Sensory Reception and Effector Functions by Afferent Nerves

According to the classical scheme of autonomic pharmacology, parasympathetic nerves mediate cholinergic responses and sympathetic nerves are responsible for adrenergic effects. Similar to the peripheral nervous system (PNS), smooth muscle and enteric neurons also receive innervation from extrinsic sensory nerves originating in DRG. Although specialized somatic and visceral sensory receptors are common, extrinsic afferent innervation to the GI tract do not exhibit such morphological specializations, resulting in difficulty in localization of extrinsic sensory nerve endings. Furthermore, unlike other peripheral sensory nerves, extrinsic sensory nerves to the viscera do not respond to specific modalities. For example, electrophysiological studies of conduction of impulses in vagal afferent nerves have recorded activity after exposure to mechanical or chemical stimuli (reviewed in Grundy and Scratcherd, 1989). Current attempts at categorization of modalities of these nerves have failed; however, extrinsic sensory nerves are simplistically divided into chemoreceptors and mechanoreceptors. Chemoreceptors are sensitive to protons, glucose, amino acids, and other simple molecules, whereas mechanoreceptors may respond to deflection of mucosal crypts, or movements of GI smooth muscle (Grundy and Scratcherd, 1989). Therefore, similar to other peripheral tissues, the GI tract receives efferent sympathetic and parasympathetic innervation and afferent sensory innervation. However, at this time, relatively little information exists about visceral sensory nerves as compared to peripheral sensory nerves.

Traditionally, sensory nerves have been viewed as a receptive and afferent system which activates effector systems. Studies of formation of the wheal and flare after inflammation or irritation of the skin (reviewed in Holzer, 1988) led Langley and

Anderson (1894) to propose that the receptive-afferent-efferent pathway of sensory nerves was mediated by a reflex that occurred entirely within a single neuron. The axon reflex hypothesized that the peripheral process of sensory nerves bifurcates, allowing one branch to send afferent information to the CNS. Nerve impulses would travel antidromically to the second branch which innervates the effector organ, such as an arteriole. The resulting antidromic activation of the local efferent branch would then mediate vasodilation (Lewis and Marvin, 1927).

This scheme has been challenged by Peter Holzer (1988), as he observed that antidromic activation of the peripheral stump of transected dorsal roots resulted in vasodilation in arterioles in the GI tract and peripheral tissues, and motor responses of the ileum, although the afferent pathway had not been activated (Holzer, 1991). His studies suggest that sensory nerves transmit effector information by a mechanism separate from that of the receptive-afferent-efferent pathway of Langley and Anderson. Although it is not known if a single neuron can mediate both afferent and efferent information, sensory nerves may have an effector role in regulation of blood flow, trophic and immunological processes, and activity of autonomic ganglia and visceral smooth muscle (reviewed in Holzer, 1988). Therefore, irrespective of the mechanism for mediation of effector functions, sensory nerves can not be simply viewed as afferent nerve fibers.

The potential local efferent function of sensory innervation from nodose and DRG would have been almost impossible to study without the discovery of capsaicin. Since the 1800s, physicians have known that intake of capsicol, a gelatine capsule containing oil extract of the red pepper *Capsicum annum*, would improve digestion and peristalsis (Högyes, 1878). Hot chili peppers of the genus *Capsicum* contain capsaicin (8-methyl-N-

vanillyl-6-noneamide), a neurotoxin which defunctionalizes a large group of sensory neurons (Jancsó, 1967), thus making it a valuable tool to study sensory innervation. Subcutaneous administration (nM- μ M) or perineuronal application (μ g/kg range) of small concentrations of capsaicin to neonatal rodents permanently destroys most primary afferent sensory neurons. Capsaicin appears to be especially toxic to small, dark-bodied type B neurons found in sensory ganglia (Szolcsányi *et al.*, 1974; reviewed in Holzer, 1991). These neurons have been shown to give rise to either unmyelinated or thinly myelinated "C" nerve fibers (Jancsó *et al.*, 1977; Scadding, 1980) that comprise approximately 70% of all visceral afferent innervation (Buck and Burks, 1986). Although adult animals are less sensitive to capsaicin, systemic administration or perineuronal application of capsaicin to any part of sensory nerves produces an acute excitatory phase. This event is characterized by a tetrodotoxin (TTX)-insensitive depolarization of peripheral sensory nerve endings, massive release of neuropeptides and neuromodulators, and depletion of neurotransmitters, ultimately resulting in desensitization. However, cumulative doses of capsaicin or systemic administration of mg/kg concentrations of capsaicin produces long-lasting or permanent loss of sensory nerves in adult rodents, including guinea pigs as assayed by immunocytochemistry of small intestine and DRG and radioimmunoassay of the GI tract and DRG (Gibbins *et al.*, 1985; Sternini *et al.*, 1987; reviewed by Holzer, 1988; Holzer, 1991; Buck and Burks, 1986). This loss is specific for extrinsic sensory neurons and enteric sensory nerves are not effected (Gamse *et al.*, 1980; Gasparovic *et al.*, 1964; Jessell *et al.*, 1978; Holzer, 1988; Buck and Burks, 1986; Furness *et al.*, 1982).

Sensory Reception by Afferent Vagal Nerves

Traditionally, studies of parasympathetic innervation of the GI tract have viewed vagus nerves as containing efferent nerve fibers. However, as discussed previously, histological studies of vagal nerves at the diaphragmic level have revealed that 80-90% of vagal nerves are unmyelinated primary afferent nerves (Agostini, 1957). Although identification of stimuli and modalities of extrinsic nerves have been difficult to elucidate, afferent vagal nerves in the vagus appear to respond to stimuli originating from muscle and mucosa. For example, the frequency of nerve impulses recorded from vagal nerves increases after distension of the stomach, suggesting that vagal sensory nerve fibers either innervate or have specialized receptors for mechanical stimuli (Grundy and Scratcherd, 1989). Similar studies also reveal increased frequency of nerve impulses recorded from vagal nerves following exposure of vagal nerves to protons (hydrogen) or ethanol (reviewed in Holzer, 1991), implying that vagal nerves also transmit sensory information. However, it is not known if a single vagal nerve fiber can respond to both mechanical and chemical stimuli.

Regulation of Motility by Afferent Vagal Nerves

As discussed previously, stimulation of vagal nerves produces excitation, inhibition, or excitation and inhibition of GI smooth muscle. Therefore, Barthó and Szolcsányi (1978) used capsaicin to selectively stimulate extrinsic sensory nerves to the ileum. Application of small concentrations of capsaicin to segments of isolated ileum results in non-cholinergic contractions of the ileum (Toh *et al.*, 1955; Barthó and Szolcsányi, 1978), associated with release of SP as measured at the vascular bed (Donnerer *et al.*, 1984). Taken together, these data suggest that extrinsic sensory afferent

nerves release excitatory neurotransmitters on GI smooth muscle. Electrical stimulation of vagal nerves in the stomach release CGRP, SP, and VIP as assayed by radioimmunoassay (Pedeson *et al.*, 1981; Raybould *et al.*, 1991). However, sensory vagal nerves are unlikely to be involved in control of motility of the ileum, as bilateral subdiaphragmal vagotomy does not prevent capsaicin-induced non-cholinergic contractions in rodents (Barthó and Szolcsányi, 1978). These contractions are unlikely to result from an action of capsaicin on enteric neurons, as contractions are abolished by destruction of extrinsic innervation to the ileum prior to administration of capsaicin (Barthó and Szolcsányi, 1978). Final evidence for a lack of vagal afferent regulation of motility comes from stimulation of extrinsic nerves associated with mesenteric arterioles in guanethidine-treated preparations. Low frequency (10 Hz) nerve stimulation produces contractions that are attenuated by nACh antagonists such as hexamethonium or mecamlamine, but not by capsaicin, implying stimulation of non-sensory extrinsic nerves releases ACh. This observation further strengthens the argument against an efferent role of vagal sensory nerves in regulation of motility of the ileum (Szolcsányi and Barthó, 1978). It should be noted however that vagal sensory nerves may be involved in reflex control of GI motility in the duodenum and stomach, as perfusion of an isotonic mixture of protein, fat, and carbohydrates decreases gastric tone by a non-adrenergic, non-muscarinic pathway (De Ponti and Malagelada, 1987; Azpiroz and Malagelada, 1986; Raybould *et al.*, 1991).

Regulation of Blood Flow by Afferent Vagal Nerves

Proceeding destruction of sympathetic nerves or prejunctional inhibition of release of NE, vagal stimulation produces vasodilation of submucosal arterioles (reviewed in Jodal

and Lundgren, 1989). This vasodilation is only partially attenuated by atropine, suggesting release of a non-cholinergic vasodilatory substance (Martison, 1965; Hultén *et al.*, 1969a; Hultén *et al.*, 1969b). A similar non-cholinergic vasodilation is observed following *in vivo* or *in vitro* administration of low concentrations of capsaicin in a number of species (Rózsa, 1986; Thieffn *et al.*, 1990; Hottenstein *et al.*, 1991). As low doses of capsaicin stimulate sensory nerves, these data suggest that stimulation of vagal sensory nerves mediate non-cholinergic vasodilation. Interestingly, vagal stimulation in capsaicin-treated animals produced vasodilations that were almost completely antagonized by atropine, implying that vagal sensory nerves mediate part of vagal neurogenic relaxation (Raybould, 1991; Thieffn *et al.*, 1990).

Sensory Reception by Innervation from Dorsal Root Ganglia

There are similarities between sensory nerves from nodose and DRG. For example, little is known regarding transmission of sensory information to DRG. Electrophysiological studies suggest that splanchnic nerves from DRG innervate or contain specialized chemoreceptors or mechanoreceptors in mesenteric ganglia (Grundy and Scratcherd, 1989). These nerves also transmit visceral pain (reviewed in Mayer and Raybould, 1990). Interestingly, vagal and spinal ganglionic sensory nerves may preferentially innervate and transmit chemical and mechanical information from myenteric ganglia or smooth muscle and mucosa, respectively, suggesting the source of sensory information preferentially activates vagal or dorsal root afferent nerve fibers.

Regulation of Motility by Innervation from Dorsal Root Ganglia

There is a paucity of information on the effect of stimulation of DRG cells on motility of the GI tract (Gillis, 1989). Nerve stimulation of dorsal roots at T₉ elevates intragastric pressure, although it is unclear if elevation results from activation of efferent sympathetic nerves by afferent nerves, or by antidromic activation of spinal ganglionic nerves (Semba and Mizonhsi, 1978). The most information about regulation of motility by spinal ganglionic primary afferent nerves has resulted from use of capsaicin on isolated segments of ileum. As described previously, administration of capsaicin improves peristalsis *in vivo* (Högyes, 1878; Barthó and Szolcsányi, 1978), and application of small concentrations of capsaicin to isolated segments of ileum *in vitro* causes contractions (Szolcsányi and Barthó, 1978; Barthó and Szolcsányi, 1978; Barthó *et al.*, 1982). These contractions result from stimulation of extrinsic DRG afferent nerves, as they are inhibited by TTX and extrinsic denervation of the ileum, but by not bilateral vagotomy (Barthó and Szolcsányi, 1978). Capsaicin-induced contractions do not involve neurotransmission through ganglionic nicotinic receptors, as hexamethonium, DMPP, and nicotine do not reduce the effects of capsaicin (Barthó and Szolcsányi, 1978; Barthó *et al.*, 1982). However, muscarinic antagonists such as hyoscine or atropine attenuate approximately 75% of the contractions (Barthó *et al.*, 1982), and substance P (D-Pro²-D-Trp^{7,9}), a NK receptor antagonist, abolishes atropine-resistant capsaicin-induced contractions. This tachykinin receptor antagonist also reduces the amplitude of capsaicin-induced cholinergic contractions (Barthó *et al.*, 1982). Taken together, these data indicate that sensory nerves from DRG release SP which acts directly on smooth muscle, and indirectly on cholinergic (Holzer, 1981) and SP-containing enteric neurons (Holzer, 1994). Identical results are

obtained after electrical stimulation of extrinsic peri-arterial nerves (Szolcsányi and Barthó, 1978; Barthó *et al.*, 1982), further suggesting that innervation from DRG serves an efferent function in regulation of motility.

Although functional studies provide compelling evidence for a role of capsaicin-sensitive DRG neurons in control of motility, immunohistochemical studies do not reveal primary afferent nerves in the longitudinal or circular muscle layers of the GI tract. However, occasional varicose nerve fibers containing SP-ir and CGRP-ir are observed as single varicose axons in internodal strands, or in myenteric ganglia (Gibbins *et al.*, 1985). If nerves from the DRG release excitatory neurotransmitters such as SP or CGRP on enteric neurons then EPSPs should result. Takaki and Nokayama (1988) found that as predicted, mesenteric nerve stimulation evoked slow EPSPs and increased excitability in approximately 30% of myenteric neurons examined. These membrane depolarizations were mimicked by application of SP or CGRP (Palmer *et al.*, 1986), thus further supporting Holzer's hypothesis of efferent functions of sensory nerves.

Regulation of Blood Flow by Innervation from Dorsal Root Ganglia

Nerve fibers from DRG are also involved in regulation of submucosal blood flow. As discussed previously, nerve fibers from the dorsal root ganglia are closely associated with submucosal arterioles, suggesting that sensory nerves may have a role in regulation of blood flow. In the presence of guanethidine, stimulation of proximal processes of splanchnic nerves dilates submucosal arterioles (Lembeck and Holzer, 1979). Systemic or peri-neuronal application of capsaicin at DRG prevents this vasodilation, providing further evidence that sensory nerves from spinal ganglia mediate vasodilation. Substance P, or a similar molecule, appears to be necessary for endothelium-dependent vasodilation

(Gamse *et al.*, 1980; Lembeck and Holzer, 1979), while CGRP may mediate endothelium-independent vasodilation in some tissues of the GI tract (reviewed in Holzer, 1991; Sternini, 1991). However, the mechanism of peptide-induced relaxation may vary in different resistance vessels.

6. AUTONOMY OF THE ENTERIC NERVOUS SYSTEM

As described previously, peristalsis *in vivo* (Bayliss and Starling, 1899; 1900a; 1900b) or *in vitro* (Trendelenberg, 1917) does not require extrinsic innervation, as stereotypical oral contractions and aboral relaxations around a bolus of food occurred even after extrinsic innervation had been destroyed. Langley (1921) used these findings to argue that reflexes occurred in the absence of extrinsic innervation because the complete reflex arc, including sensory neurons, interneurons, and motor neurons was found within the wall of the GI tract. However, many reflexes such as receptive relaxation of the stomach and inhibition of bile by colon distension require extrinsic innervation and are inhibited by loss of extrinsic nerves (Shapiro and Woodward, 1959; reviewed in Furness and Costa, 1987). Furthermore, extrinsic innervation has a profound effect on GI function, as previously discussed. Sympathetic nerves directly innervate submucosal arterioles and some myenteric neurons, resulting in vasoconstriction (Dale, 1913; Evans and Surprenant, 1992) and inhibition of excitatory enteric neurons and motility (Vizi and Knoll, 1971; Lee, 1970; Furness and Burnstock, 1975; Hirst and McKirdy, 1974), respectively. Parasympathetic innervation does not appear to have major actions in the small intestine (Kewenter, 1965). Extrinsic sensory innervation from DRG directly innervates submucosal arterioles (Lembeck and Holzer, 1979) and myenteric neurons

(Holzer, 1981; Holzer, 1994; Gibbins *et al.*, 1985; Takaki and Nokayama, 1988) and mediates reflex vasodilation and increased contractile activity. Therefore, these data provide convincing evidence that extrinsic nerves have important roles in regulation of GI functions. However, surgical destruction of extrinsic nerves results in only a brief period of digestive dysfunction, frequently categorized by diarrhea (Alvarez, 1948). Normal digestion then returns, although extrinsic innervation has not been re-established within the GI tract. These observations suggest that loss of extrinsic innervation induces anatomical and functional adaptations of enteric neurons, resulting in functional autonomy of the ENS. Such alterations may allow enteric neurons to function in the absence of modulatory extrinsic innervation, or even to control processes previously mediated by extrinsic innervation.

Anatomical Evidence of Denervation-Induced Alterations of Enteric Nerves

Surgical denervation procedures were developed originally to determine the contribution of extrinsic innervation to GI functions. In the past twenty years, denervation procedures have been used to establish the origin of nerves within the GI tract (Furness and Costa, 1978; Costa *et al.*, 1981; Furness and Costa, 1982; Costa and Furness, 1983; Furness *et al.*, 1983b; Costa *et al.*, 1984; Furness and Costa, 1987; Costa *et al.*, 1992). However, Galligan *et al.* (1988) observed that surgical denervation of loops of guinea pig ileum or severing celiac ganglionic axons alters innervation of submucosal arterioles. As described previously, extrinsic perivascular and paravascular sympathetic and SP- and CGRP-ir sensory nerves are associated with arterioles. One week after surgical denervation, these nerves were not detected by immunohistochemistry, although faint SP-ir nerves were found associated with arterioles. These nerves were different from

extrinsic sensory nerves, as they were thinner than extrinsic sensory nerves, had fewer and smaller varicosities, did not contain CGRP-ir, and were insensitive to systemic administration of capsaicin. Galligan and co-workers also described formation of a perivascular plexus that contained VIP-ir, although VIP-ir is only rarely associated with submucosal arterioles in normal ileum. Taken together, these data suggest that loss of extrinsic innervation resulted in new SP- and VIP-ir innervation of submucosal arterioles. After extrinsic denervation, neither population of extrinsic nerve fibers survive, yet the denervated small intestine does not become ischemic, suggesting the ENS has responded to loss of extrinsic innervation to regulate vasoconstriction and vasodilation.

Functional Evidence of Denervation-Induced Alterations of Enteric Nerves

The Diamtrak online video-monitoring system (Neild, 1989) was used to examine neurogenic regulation of blood flow in submucosal arterioles of surgically-denervated preparations. Loss of extrinsic innervation had profound effects on enteric vasomotor neurons, as vasodilation of small arterioles (measuring less than 40 μm) was 160% greater than normal preparations, and vasodilation of larger arterioles (measuring greater than 40 μm) was 75% larger than normal vasodilation (Galligan *et al.*, 1990). Although stimulation of 67% of submucosal ganglia in control and normal preparations dilated arterioles (Neild *et al.*, 1989; Galligan *et al.*, 1990), stimulation of 97% of submucosal ganglia dilated arterioles in surgically-denervated preparations (Galligan *et al.*, 1990). These results suggest loss of extrinsic innervation increased vasodilator substances in almost all submucosal ganglia. Interestingly, although mACh receptor antagonists inhibited all neurogenic vasodilations in normal preparations, these drugs attenuated only a portion of neurogenic vasodilation after denervation (Galligan *et al.*, 1990). The

tachykinin receptor antagonists spantide, D-Arg¹, D-Pro², D-Trp^{7,9}, Leu¹¹-SP, and D-Arg¹, D-Phe⁵, D-Trp^{7,9}, Leu¹¹-SP inhibited the non-cholinergic component of vasodilation (Galligan *et al.*, 1990). Extrinsic denervation did not alter arteriolar responsiveness to muscarine, suggesting differences in neurogenic vasodilation were not due to differences in receptors on smooth muscle or endothelium. Taken together, these data illustrate that new SP-ir nerves observed after extrinsic denervation of loops of ileum have functional significance in neurogenic vasodilation of submucosal arterioles. As enteric SP-ir paravascular nerves and non-cholinergic vasodilations are not observed in preparations from unoperated animals, extrinsic denervation appears to induce anatomical and functional alterations in enteric neurons.

Statement of Purpose

The studies by Galligan and co-workers support the hypothesis that loss of extrinsic innervation induces compensatory changes in enteric neurons to allow continuation of digestive processes. The first goal of this dissertation was to determine the source of new SP- and VIP-ir nerves associated with submucosal arterioles by combining anatomical tracing with the retrograde dye fluoro-gold and immunohistochemistry. Galligan and coworkers (1988) demonstrated new SP-ir innervation was not sensitive to capsaicin, suggesting these nerves originated from enteric sources. However, the source of this innervation was not identified. The second goal was to determine the mechanism for the appearance of new SP- and VIP-ir nerves. Anatomical studies have shown axonal sprouting, phenotypic alterations in existing nerve fibers, and up-regulation of existing neurochemicals can occur following axotomy of peripheral nerves or decentralization of autonomic nerves. Immunohistochemical studies for neuropeptides and

neurofilaments were used to discriminate between these possibilities. Surgical denervation of the intestine destroys extrinsic sympathetic, parasympathetic, and sensory nerves. Therefore, the third study used neurotoxins to selectively eliminate sympathetic or sensory nerves to determine the identity of extrinsic innervation responsible for altered innervation of submucosal arterioles. Fos is an early immediate gene protein encoded by *c-fos*, and is an index of increased or increasing nuclear activity. Therefore, localization of Fos-ir was used to determine if specific subpopulations of enteric neurons responded to loss of extrinsic nerves. As NADPH diaphorase and NOS-ir are found in perivascular innervation in some species excluding guinea pig, the fifth goal of this dissertation was to use NADPH diaphorase staining to determine if extrinsic denervation induced staining in enteric neurons. Such changes would be indicative of induction of NOS, and a possible role of nitric oxide originating from enteric neurons. Therefore, the first portion of this dissertation will further characterize alterations in innervation of submucosal arterioles and elucidate the source and underlying mechanism for the new innervation.

As described below, neuronal NADPH diaphorase and NOS are the same enzyme, thus providing an easy and convenient histological stain for nitrinergic nerves. In preliminary studies of possible nitrinergic innervation of submucosal arterioles in guinea pig ileum, a significant increase of myenteric neurons containing NADPH diaphorase-staining was observed after surgical denervation of loops of ileum. Nitric oxide mediates neurogenic relaxation of GI smooth muscle, and is produced by the rate-limiting enzyme NOS. Therefore, the preliminary histological experiments indicated alterations in myenteric ganglia that could potentially lead to altered control of motility. This hypothesis was tested using anatomical techniques and functional assays. The sixth goal

of this dissertation was to characterize NADPH diaphorase staining following loss of extrinsic innervation, and to determine the identity of extrinsic innervation responsible for the observed changes in staining. Similar studies were performed using antiserum raised against NOS. The seventh study used *in vitro* nerve-muscle preparations to determine if increased NADPH diaphorase and NOS-ir in myenteric nerves resulted in altered functional innervation of longitudinal muscle. A biochemical assay for indirect measurement of NO was also used to further characterize denervation-induced alterations in NOS. These studies indicated calcium-independent production of NO could occur after extrinsic denervation, and immunohistochemical localization of an inducible form of NOS was performed. Finally, *in vitro* longitudinal muscle-myenteric plexus preparations revealed neurogenic contractions proceeding relaxations were reduced in preparations made from denervated loops of ileum. As previous work had suggested NO mediates release of excitatory enteric neurotransmitters, circular muscle-myenteric plexus preparations were used to determine if such a mechanism could occur in guinea pig ileum. Taken together, the studies on innervation of submucosal arterioles and longitudinal muscle have strengthened the hypothesis that loss of extrinsic innervation results in alterations in enteric nerves, and have provided the first evidence of alterations in inhibitory innervation of the GI tract.

The remainder of the introductory section of the dissertation provides background information on specific approaches used to achieve the goals outlined in the preceding section, as well as a rationale for the chosen model utilized in the studies.

7. NEUROFILAMENT PROTEINS

The neuronal cytoskeleton is composed of structural proteins that may be important in maintenance of axoplasmic space (Shaw, 1986; Shaw, 1991), or in regulation of cell morphology, axon caliber (Hoffman *et al.*, 1984), intracellular Ca^{2+} (Lefebvre and Mushynski, 1987, 1988) or axonal transport (Hoffman *et al.*, 1985). These structural proteins include the intermediate filament proteins, which in turn, are comprised of proteins including the neurofilament protein triplet. The neurofilament triplet is the major intermediate filament protein in neurons, and is made of heteropolymers of three separate gene products (Csoznek *et al.*, 1980) with SDS-PAGE estimated molecular weights of 68 KDa (light molecular weight neurofilament, NF-L), 150 KDa (medium molecular weight neurofilament, NF-M) and 200 kDa (heavy molecular weight neurofilament protein, NF-H) (Hoffman and Lasek, 1975; Liem *et al.*, 1978). Although the subunits of the neurofilament triplet have homologous primary and secondary structures (reviewed in Shaw, 1991), antibodies can discriminate between the subunits in immunoblots (Shaw and Weber, 1981; Lee *et al.*, 1982) and in immunohistological experiments (Trojanowski *et al.*, 1986). Interestingly, such studies have demonstrated that although all neurons were thought to contain neurofilaments, some neurons do not contain neurofilament triplet proteins (Shaw and Weber, 1981; Shaw *et al.*, 1981a, 1981b; Sharp *et al.*, 1982; Debus *et al.*, 1982; Trojanowski *et al.*, 1985; Vickers and Costa, 1992), or lack one or more of the subunits (Goldstein *et al.*, 1983; Liem *et al.*, 1978; Trojanowski *et al.*, 1985).

Microheterogeneity of Neurofilament Proteins

Studies with antiserum raised against NF-L, NF-M, or NF-H reveal that neurofilament subunits are unequally distributed within individual neurons. Microheterogeneity in the distribution of neurofilament proteins was first described in DRG neurons *in vitro*. Antisera raised against NF-L or NF-M recognized DRG somata but were unable to recognize axons (Sharp *et al.*, 1982; Debus *et al.*, 1982). However, axons contained NF-H-ir, suggesting an unequal distribution of NF proteins within individual neurons. Similar results are obtained in the brain, as NF-L- and NF-M-ir are found in somata and dendrites of pyramidal cells in the cerebellum (Debus *et al.*, 1982; Trojanowski *et al.*, 1985) and cortex (Shaw *et al.*, 1981; Dahl, 1983), although NF-H-ir is localized only in axons. Furthermore, heavy weight NF protein-ir is not found in cell bodies and dendrites of hippocampus, brain stem (Shaw *et al.*, 1981), and spinal motor neurons (Dahl, 1983). This pattern of NF-ir is hypothesized to have functional significance, as NF-L and NF-M are thought to represent integral components of neuronal cytostructure, whereas NF-H may only be incorporated into axons to serve a yet-defined physiological role (reviewed in Trojanowski *et al.*, 1985; 1986). However, other immunohistochemical studies detected NF-H-ir in cerebellar Purkinje cell somata and dendrites (Goldstein *et al.*, 1983), resulting in much controversy over the true distribution of the components of NF-triplet.

Phosphorylation of Neurofilament Proteins

In 1983, Goldstein, Sternberger and Sternberger advanced the field of neurofilament research and explained the discrepancy for conflicting reports of distribution of NF-H. Goldstein and co-workers raised and characterized a series of monoclonal

antibodies raised against rat hypothalamus, and described a number of antibodies that specifically recognized NF-M, NF-H, or NF-M and NF-H as shown by Western analysis. Although the series of antibodies should have recognized similar structures in brain slices, they found the antibodies could be classified into four groups based on their pattern of immunoreactivity, as NF-H antibodies recognized (i) synapses, (ii) neurofibers, (iii) perikarya and dendrites, or (iv) neurofibers and perikarya (Sternberger and Sternberger, 1983). After investigating the apparent discrepancy in NF-H immunoreactivity, they concluded that most heterogeneity in NF-H-ir was not pre-translational, but resulted from post-translational modifications of NF epitopes. Phosphatase treatment of brain sections or gels destroyed immunoreactivity of antibodies that preferentially recognized neurofibers, but did not alter antibodies that recognized perikaryon. Conversely, treatment with the proteolytic enzyme trypsin abolished immunoreactivity of antibodies that preferentially recognized perikaryon, but did not alter immunoreactivity of antibodies that recognized axons (Sternberger and Sternberger, 1983). As phosphatase removes phosphate groups, these data indicated axons, but not perikarya, contained phosphorylated heavy molecular weight neurofilament protein (pNF-H-ir). However, perikarya in the CNS contained non-phosphorylated or lightly-phosphorylated heavy molecular weight neurofilament protein (npNF-H-ir), as phosphatase treatment did not alter the epitopes of perikaryon-specific antibodies. Taken together, these data indicate that a single neuron can contain heterogenous distribution of phosphorylated and nonphosphorylated NF-H proteins.

As discussed previously, some central and peripheral neurons do not appear to contain NF proteins. However, perikarya, dendrites, and axons differ in their composition

of neurofilament triplet protein subunits, as well as in the phosphorylation of subunit proteins. Biochemical studies have shown the presence of multiple phosphorylated sequences of NF-M and NF-H (Goldstein *et al.*, 1983; Nixon *et al.*, 1989; Sternberger and Sternberger, 1983). The carboxyl-terminal tail domain of NF-M and NF-H contain as many as 54 repeats of hexapeptide sequences containing KSP (lysine, serine, proline) motifs (Julien and Mushynski, 1982). Several protein kinases including cAMP dependent protein kinase, multifunctional Ca^{2+} /calmodulin dependent protein kinase, and protein kinase C phosphorylate serine in KSP sequences *in vivo* and *in vitro* (Leterrier *et al.*, 1981; Tanaka *et al.*, 1984; Vallano *et al.*, 1985; Sihag *et al.* 1988; reviewed in Shaw, 1991) resulting in highly phosphorylated NF-M, and especially NF-H proteins. Furthermore, most antibodies raised against NF-M and NF-H prior to 1983 recognized phosphorylated epitopes of NF proteins. As a result, NF-M- and NF-H-ir was found only in structures which contained phosphorylated epitopes, such as axons. However, at least some somata contain npNF-M- and npNF-H-ir, as development of antibodies against npNF-M and npNF-H reveal the presence of npNF-M- and npNF-H-ir in central (Sternberger and Sternberger, 1983) and peripheral (Carden *et al.*, 1985) perikarya and dendrites.

The physiological ramifications of phosphorylation of NF-proteins is not known at this time. Highly-phosphorylated carboxyl terminals of NF-H protein are sterically hindered and project radially into axoplasmic space. As discussed previously, phosphorylated KSP sequences are more abundant in axons compared to dendrites. Furthermore, axons are more stable to proteases (Goldstein *et al.*, 1988; Sternberger and Sternberger, 1983), suggesting phosphorylation may protect the carboxyl terminals from

degradatory cellular processes. Alternatively, phosphorylation of neurofilament proteins may actually induce charge repulsions that increase spacing between 10 nm intermediate filament proteins, resulting in filling of axoplasmic space (Shaw, 1986; Shaw, 1991), or in control of axon caliber (Hoffman *et al.*, 1984; Hoffman *et al.*, 1985). Greater axoplasmic space or axon caliber would then allow for greater conduction velocity (reviewed in Hoffman *et al.*, 1984; Hoffman *et al.*, 1985). Although phosphorylation of NF-M and NF-H does not effect the ability of the subunits to assemble into 10 nm filaments (Georges *et al.*, 1986), the addition of pNF-H may stabilize bundles of axons. Covalent or weak interactions between phosphate groups on neighboring axons may increase axonal stability and fascicular strength by allowing interactions between 10 nm filaments. However, until site-directed mutagenesis is used to destroy KSP sequences, the function of phosphorylation of NF-H will not be known.

Neurofilament Proteins are Developmentally-Regulated

Neurofilament triplet proteins show a common developmental pattern in rats (Nasal *et al.*, 1986), amphibians (Weasel *et al.*, 1989), and other vertebrates (reviewed in Shaw, 1991). During embryogenesis, abundant NF-L-like proteins is found in perikarya, dendrites, and axons. The NF-L protein associates with weakly phosphorylated NF-M (Nasal *et al.*, 1986) in all structures of the cell. However, it is hypothesized that incorporation of weakly phosphorylated NF-H may provide a signal to the cell, resulting in maturation of the cytoskeleton, and transformation of the developing neuron into a differentiated adult neuron. Although the exact temporal sequence is unknown, protein kinases such as cAMP-dependent protein kinase and protein kinase C phosphorylate serine residues found in KSP sequences, and transform weakly phosphorylated NF-H to an adult pattern

comprised of a maximum of forty-three phosphorylation sites on the carboxyl terminal (Lee *et al.*, 1991; Schilling and Pilgrim, 1990).

Studies of axonal sprouting of sciatic nerves reveal that axonal injury by crushing or axotomy may result in recapitulation to immature, lightly phosphorylated NF-H. For example, after sciatic nerve lesion, enzyme-linked immunoabsorbant assays (ELISAs) and immunohistochemical studies show a significant decrease in the immunoreactivity of SMI 31 (Schilling and Pilgrim, 1990). As described previously, SMI 31 selectively recognizes a phosphorylated epitope on extensively phosphorylated NF-H, thus indicating that critical phosphates have been lost or removed. Furthermore, SMI 32, a monoclonal antibody raised against a nonphosphorylated epitope of NF-H significantly increases upon nerve injury. The increase in nonphosphorylated epitopes may result from loss of phosphorylation of previously-mature axons. Alternatively, increased npNF-H-ir may occur by axonal sprouting or branching of new nerves, thus revealing new nonphosphorylated epitopes that have not had the opportunity to become phosphorylated. This theory of axonal sprouting or branching is supported, as the increase in SMI 32 following injury appears to occur at the tips of axons where axonal growth would progress (Pestronk *et al.*, 1990). Interestingly, the weakly phosphorylated NF-H is the predominant form of NF-H during normal embryogenesis and growth of axons.

Neurofilaments in Autonomic Nerves

Many studies have characterized neurofilament protein-like immunoreactivity (NF-ir) in the CNS, but few studies have examined neurofilament localization in the autonomic nervous system (ANS). As described previously, the first studies of microheterogeneity of NF proteins were done on cultures of dorsal root ganglia. Although sensory ganglia

are not part of the ANS, a subpopulation of small DRG cells contain NF-M-ir *in situ* (Vitadello *et al.*, 1987). Neurofilament-M-ir is not observed in large cells. Neurofilament triplet-ir is observed also in parasympathetic and sympathetic ganglia. The best characterization of neurofilament triplet-ir is in celiac ganglion of guinea pig, as 20% of postganglionic sympathetic neurons contain NF-L-, NF-M-, and npNF-H-ir (Vickers *et al.*, 1990). Most of the NF-positive neurons do not contain NPY-, but do contain somatostatin-ir (Vickers *et al.*, 1990), suggesting neurofilament proteins may be expressed in some cells to serve a certain purpose or function that is not needed in other cells.

Some enteric neurons contain NF-ir, although the distribution of immunoreactivity is dependent on the target antigen and the antibody used. For example, previous reports have found that 15% of myenteric neurons, and no submucosal neurons, contain NF-L-, NF-M- and NF-H-ir in the guinea pig ileum (Costa *et al.*, 1989). NF-proteins were found in (i) dynorphin-, gastrin releasing peptide-, and VIP-ir in anally-projecting Dogiel type I cells and (ii) dynorphin-, enkephalin-, and SP-ir small orally-projecting neurons (Costa *et al.*, 1989). However, in retrograde tracing experiments from GI smooth muscle, Brookes *et al.* (1991b; 1992) concluded NF-triplet proteins co-localized with VIP- but not SP-ir in Dogiel type I neurons projecting to circular muscle. Interestingly, they did not observe NF-ir neurons to project to the longitudinal muscle (Brookes *et al.*, 1992). Other researchers have reported a larger distribution of neurofilament subunits in myenteric neurons, as antiserum raised against 50,000 molecular weight degradation product reacts with all three peptides in the NF triplet, and recognizes approximately 10-50% of myenteric neurons (Björklund *et al.*, 1984). Similar to other studies, monoclonal antibodies raised against npNF-M and npNF-H recognize some myenteric neurons,

whereas pNF-H-ir is observed in axons in internodal strands and the primary plexus (Eaker *et al.*, 1990). Unfortunately, these studies did not quantify the number or determine the identity of NF-ir myenteric neurons. Finally, although Costa *et al.* (1989) did not observe NF-protein in submucosal neurons, NF-M has been found in SP- or CGRP-ir submucosal neurons in rat ileum (Buchan *et al.*, 1991). Therefore, these studies suggest that at least some enteric neurons contain neurofilament proteins.

8. FOS PROTEIN

The *fos* oncogene was discovered in a virally-induced murine osteosarcoma (Finkel *et al.*, 1966), and a cellular homologue (*c-fos*) has been observed in developing mammalian cells. The *fos* oncogene encodes Fos, a 62-65 kDa nuclear phosphoprotein that has been implicated in normal cell growth (Kovary and Bravo, 1991; Lau and Nathans, 1985; Lau and Nathans, 1987), differentiation (Curran and Morgan, 1985; Krujer *et al.*, 1985; and Milbrandt, 1986), and development (Alcantara and Greenough, 1993; Gubits *et al.*, 1988), as mitogens and differentiation-specific antigens induce rapid and transient expression of Fos. *In situ* hybridization and immunocytochemistry has revealed that Fos can be rapidly and transiently induced in pheochromocytoma 12 cells, post-mitotic neurons in the brain and spinal cord, and dorsal root ganglia. Stimuli known to induce Fos in select populations of neurons include pharmacological agents such as pentylenetetrazol (reviewed in Morgan and Curran, 1988), seizures (Dragunow and Robertson, 1987), light (Aronin *et al.*, 1990), sound (Brown *et al.*, 1988; Ehret and Fischer, 1991), ischemia (Wessel *et al.*, 1991), hormones and growth factors (Milbrandt, 1986; Bartel *et al.*, 1989; Krujer *et al.*, 1985), transection or stimulation of peripheral

nerves (Leah *et al.*, 1991; Chi *et al.*, 1993a; Chi *et al.*, 1993b), stimulation of visceral afferent nerves (Hunt, 1987; Birder *et al.*, 1991), and serum, phorbol esters, neurotransmitters, and other agents that increase intracellular Ca^{2+} and cAMP (Greenberg *et al.*, 1985; 1986). Electron microscopic studies have demonstrated that although neuronal excitation induces c-Fos within neuronal nuclei, glial, ependymal, and endothelial cells do not express Fos protein (Mugnaini *et al.*, 1989). Although the physiological role of Fos is not clear, expression of Fos appears to be correlated with increased nuclear activity, and Fos may act as a messenger between short-lasting extracellular signals and long-term nuclear events in neurons (reviewed in Morgan and Curran, 1989). Therefore, increases in Fos may be involved in long-term processing of information generated upon short-term stimulus.

As many types of stimuli can induce Fos, this has led to the hypothesis that neuronal activity and Fos-ir are related. Fos has been used in place of 2-deoxyglucose in mapping of polysynaptic pathways in the CNS (Schoppman and Stryker, 1981; Bullitt, 1990). For example, stimulation of cerebral cortex results in increased electrical activity, increased uptake of 2-deoxyglucose, and induction of Fos-ir in overlapping populations of motor and sensory cerebellar neurons (Morgan and Curran, 1989). Fos immunohistochemistry and *in situ* hybridization are useful also as markers for physiological activity (Hunt, 1987). For example, noxious stimuli result in expression of *c-fos* protein in spinal cord and thalamic nuclei previously identified by electrophysiological and lesion experiments to be involved in transduction of noxious information to higher brain centers (Bullitt, 1990). Therefore, Fos-ir has been shown to be a valid marker for neurons with increased, or increasing, nuclear activity.

Regulation of Fos

The *fos* gene and corresponding protein are closely regulated in normal neurons, as expression of the gene can have profound effects on cell functioning. The normal c-*fos* gene product transforms fibroblasts *in vitro* (Verma, 1986), resulting in abnormal cell division. In the process of studying uncontrolled mitosis, *cis* and *trans* regulatory elements and proteins have been discovered, leading to a better understanding of control of expression of Fos protein. Furthermore, as Fos is expressed in neurons associated with high amounts of plasticity and trans-synaptic activity, these regulators may be crucial in cellular learning, regulation of neuronal phenotype, and other processes necessary for development and regeneration.

The cellular *fos* gene can be regulated by multiple second messengers, including Ca^{2+} , cAMP, diacylglycerol, and protein kinase C. Calcium (through nifedipine-sensitive channels or from intracellular stores) and cAMP activate cAMP-dependent protein kinase (protein kinase A). Protein kinase A phosphorylates serine 133 of cAMP responsive element binding protein (CREB) (reviewed in Sheng and Greenberg, 1990). Phosphorylated CREB then binds to the cAMP response element (CRE) located at positions -57 to -63 upstream of the mRNA cap (reviewed in Verma and Sassone-Corsi, 1987). Although nonphosphorylated CREB represses transcriptional activity of intermediate early genes (IEGs) (Lamph *et al.*, 1990), deletion of CRE also significantly reduces transcription of *fos*, indicating cAMP induces Fos protein through CREB. Calcium can act also *via* an unidentified Ca^{2+} /calmodulin-regulated kinase, ultimately resulting in transcription of *fos*. This biochemical analysis agrees with *in vitro* data, as influx of Ca^{2+} through voltage-dependent L-type Ca^{2+} channels leads to formation of Ca^{2+} -

calmodulin complex, and induction of *fos* mRNA and Fos protein (Morgan and Curran, 1986; Greenberg, 1985; Greenberg, 1986).

Although regulation of Fos occurs at the level of DNA, *fos* mRNA and Fos protein can also be directly controlled. For example, second messengers can activate protein kinases, resulting in phosphorylation of serine and threonine residues of Fos (reviewed in Verma and Sassone-Corsi, 1987). Depending on the induction stimulus and the site of phosphorylation, addition of phosphate groups may increase or decrease the stability of the molecule (Morgan and Curran, 1988). Finally, translation of *fos* mRNA requires removal of a 67 base pair adenine-thymidine-rich region from the 3' noncoding sequence. This region is transcribed into destabilizing motifs, thus promoting degradation of mRNA by 3' exonucleases (reviewed by Verma and Sassone-Corsi, 1987). Therefore, Fos can be regulated by extracellular signals at multiple targets.

Functions of Fos

Upon stimulation by second messengers such as cAMP, protein kinase C (PKC), or elevation of intracellular Ca^{2+} , *fos* mRNA is transcribed and translocated into neuronal cytoplasm within minutes. Cellular machinery manufactures Fos protein, which moves back into the nucleus (reviewed in Verma and Sassone-Corsi, 1987; Morgan and Curran, 1988). Fos protein interacts with Jun, the family of proteins encoded by *jun* IEGs, via leucine zippers, resulting in formation of a heterodimeric transcription factor complex (Halazonetis *et al.*, 1988). The Fos-Jun heterodimer binds with high affinity to DNA protein-binding elements with consensus sequence -TGACTCA-, also known as activator protein-1 (AP-1) binding sites. Binding of AP-1 has profound effects on transcription, as activation of AP-1 allows Fos to activate or repress transcription, depending on the

identity of Jun protein in the heterodimeric complex. Therefore, an extracellular signal induces Fos protein, which influences transcription of late response genes through an AP-1 binding site. Late response proteins include Ca^{2+} binding proteins (Marsiakowski and Shooter, 1988), vesicle-associated proteins (Stein *et al.*, 1988), and neuronal-specific intermediate proteins such as peripherin and light- and medium-weight neurofilament proteins (Lindenbaum *et al.*, 1988). Late response genes also include a number of neurotransmitters and neuromodulators such as preproenkephalin, prodynorphin, neuropeptide Y, and cholecystokinin. The presence of AP-1 binding sites on these genes allows short-lived stimuli to influence long-term changes in structural proteins and neurotransmitters. Finally, Fos may not directly regulate some late response genes, but may activate synthesis of transcription-factor proteins which, in turn, regulate secondary target genes (Morgan and Curran, 1989).

9. NITRIC OXIDE SYNTHASE

NADPH Diaphorase

Some of the first attempts to study morphology of central and peripheral neurons used endogenous enzymes such as cholinesterases and dehydrogenases to catalyze reactions to stain subpopulations of neurons. Dehydrogenase histochemistry utilizes endogenous dehydrogenases to produce reduced cofactors. Diaphorases, a family of enzymes found in neurons and non-neuronal tissue, transfer electrons from the reduced co-factors to tetrazolium salt. The reduction of tetrazolium precipitates colored formazan crystals, allowing visualization of stained cells with conventional light microscopy (Friede, 1961; Scherer-Singler *et al.*, 1983). Ultrastructural analysis of diaphorase-stained CNS

cells reveals that formazan crystals associate with endoplasmic reticulum (Wolf *et al.*, 1992) and other organelle membranes (Hope and Vincent, 1989), although this relationship is not observed in enteric neurons (Llewellyn-Smith *et al.*, 1992). In contrast, dehydrogenase staining in non-neuronal cells produces formazan staining that is only associated with mitochondria (Walker, 1963).

Anatomical and biochemical techniques have identified two distinct diaphorase enzymes. Nonspecific diaphorases, such as NAD(P)H:quinone oxidoreductase (DT-diaphorase) are localized in heterologous populations of cells, including hepatocytes, parachymal and mammary cells, mucosal crypts (Walker, 1963), and soluble brain fractions (reviewed in Hope and Vincent, 1989). Nonspecific diaphorases can use NADH or NADPH to form formazan; however, neuronal NADPH diaphorase requires β -NADPH. Although the function of neuronal NADPH diaphorase activity is unknown, non-specific diaphorases catalyze 2-electron reductions of quinones (Ma *et al.*, 1992). NADPH diaphorase staining was first described in subpopulations of neurons in neocortex, striatum, midbrain, and cerebellum of normal tissue (Thomas and Pearse, 1961). Interestingly, although neuropathologies such as ischemia, Huntington's disease, Alzheimer's diseases, and excitatory amino acid-toxicity eliminate large populations of neurons, NADPH diaphorase stained cells are spared (Thomas and Pearse, 1964; Ferrante *et al.*, 1985; Koh *et al.*, 1986), resulting in a great deal of interest in the identity and role of NADPH diaphorase.

Biochemical studies of NADPH diaphorase activity revealed that the reaction is enzymatically-catalyzed, as it is abolished by pre-treatment with proteases, heat, acid- and alkaline denaturation (Hope and Vincent, 1989). Unfortunately, more detailed

characterization of NADPH diaphorase was impossible as a specific assay was not known. However, in 1990 Bredt and Snyder utilized a 2',5'-ADP-linked agarose column to purify NOS from crude cerebellar supernatant preparations. As ADP-linked columns are useful in purification of NADPH-requiring enzymes, Hope and co-workers (1991) used a similar protocol to isolate NADPH diaphorase. They found NADPH diaphorase co-purified with NOS in a single peak. Furthermore, both NOS and NADPH diaphorase activity were immunoprecipitated from crude supernatants with NADPH diaphorase-specific antiserum, but not with non-immune rabbit serum, indicating that neuronal NADPH diaphorase is an isoform of NOS. Immunohistochemical studies supported this conclusion, as NADPH diaphorase staining and NOS-ir were co-localized in neurons in the corpus striatum, cerebral cortex, retina, myenteric plexus, and cells of the adrenal medulla (Dawson *et al.*, 1991). Furthermore, although human kidney cells do not stain for NADPH diaphorase, following transfection with NOS cDNA, NADPH diaphorase staining is observed (Dawson *et al.*, 1991), providing further evidence that NOS and neuronal NADPH diaphorase are the same enzyme.

Discovery of Nitric Oxide Synthase

Since the discovery of ACh, some researchers observed exogenous ACh contracted resistance vessels, while other researchers reported cholinergic vasodilation (reviewed in Moncada *et al.*, 1991). Furchgott and Zawadzki (1980) observed that intact endothelial cells were necessary for relaxation, as endothelial cells released a labile and humoral molecule that relaxed precontracted aortic strips (reviewed in Moncada *et al.*, 1991). Numerous groups incorrectly identified the molecule as arachidonic acid lipooxygenase (Singer and Peach, 1983), cytochrome P-450 enzymes (Pinto *et al.*, 1985), and a molecule

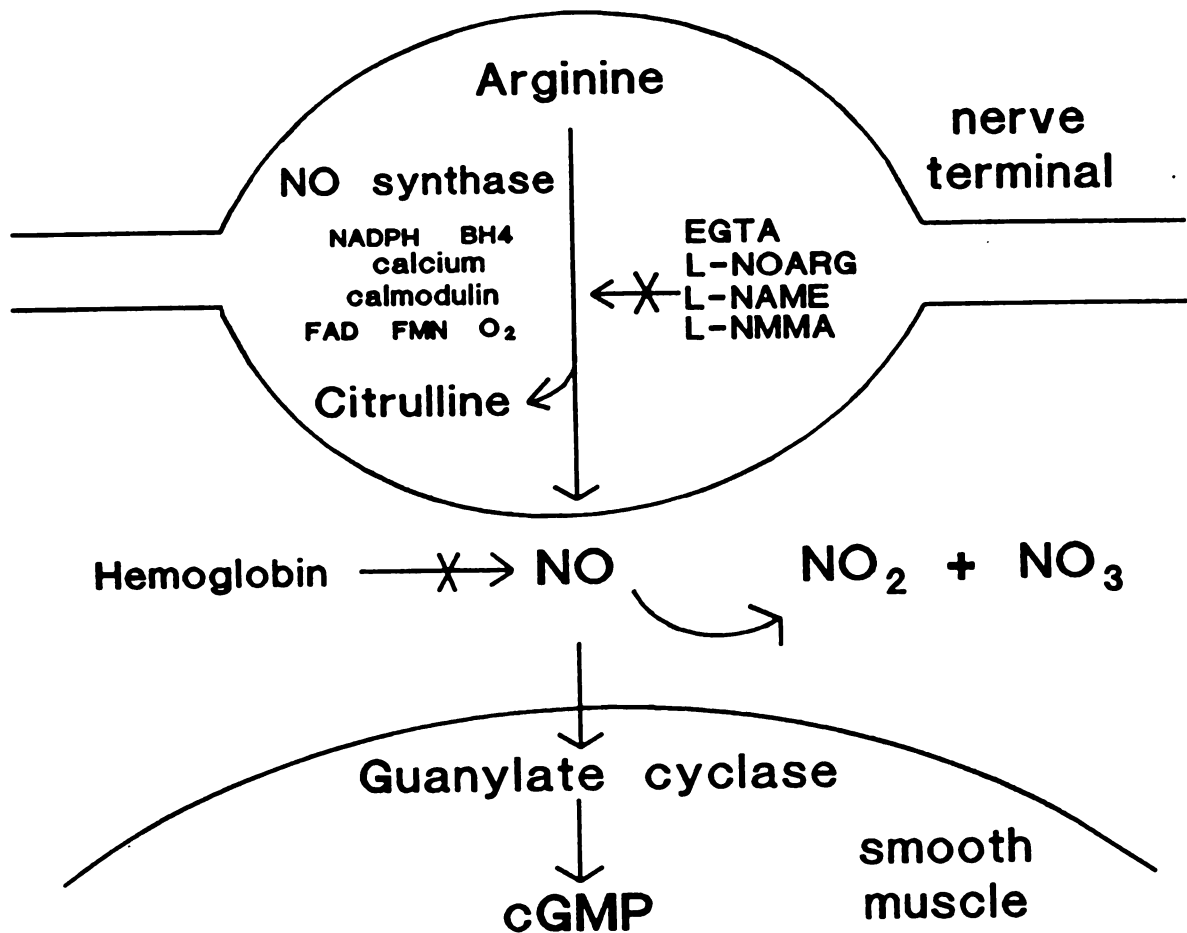


Figure 4. Nitric oxide is produced from L-arginine by the rate-limiting enzyme nitric oxide synthase. Nitric oxide synthase is found in neurons and produces equimolar concentrations of NO and citrulline. Type I isoform of NOS requires NADPH, BH₄, Ca²⁺, and calmodulin, and can be antagonized by chelation of extracellular Ca²⁺, antagonism of NOS by substituted L-arginine analogs, and binding of extracellular NO by hemoglobin. Nitric oxide is known to activate guanylate cyclase in smooth muscle, resulting in elevated concentrations of cGMP. Diagram kindly provided by Dr. J.J. Galligan.

with a carbonyl center (Griffith *et al.*, 1984). However, in 1986 Furchgott suggested that endothelium derived relaxing factor was nitric oxide (NO, NO[•]), and Palmer and coworkers (1987) provided Furchgott's hypothesis to be correct. Two years later, Palmer's group (1989) and Bredt and Synder (1989) independently showed that NO formation was accompanied by stoichiometric conversion of arginine to citrulline and NO. Bredt and Synder (1990b) purified NOS from rat cerebellum, and identified it as a 150 kDa monomer protein that required Ca²⁺, calmodulin, and NADPH. Purification of NOS allowed production of antiserum against NOS, NOS-ir to be observed in central and autonomic neurons (Bredt *et al.*, 1990), and the sequence of neuronal NOS to be determined. The sequence of cerebellar neuronal NOS has 58% homology with NADPH-cytochrome P-450 reductase (Bredt *et al.*, 1991b), thus making it the first example of a soluble cytochrome P-450 in eukaryotes (White and Marletta, 1992). The cDNA sequence of type I isoform found in brain contains consensus binding sites for calmodulin, NADPH, 6(R)tetrahydro-L-biopterin (BH₄), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and heme, as well as 12 phosphorylation sites (Bredt *et al.*, 1991a). Finally, a number of protein kinases including cAMP-dependent protein kinase, protein kinase C, and Ca²⁺/calmodulin-dependent protein kinase phosphorylate NOS, resulting in decreased enzyme activity *in vitro* (Bredt *et al.*, 1991a).

As neuroscientists identified NOS in neuronal cells, cardiovascular pharmacologists identified type III isoform of NOS in endothelial cells (reviewed in Moncada *et al.*, 1991). Interestingly, the cDNA clone of endothelial NOS is only 60% identical with rat brain NOS isoform (Lamas *et al.*, 1992; Marsden *et al.*, 1992). Type I and III isoforms of NOS are similar as they are constitutively expressed, activated by Ca²⁺ (EC₅₀ 350 nM)

(Schmidt *et al.*, 1992), and not dependent upon new protein synthesis. However, induction of type II NOS in macrophages, neutrophils, kupffer cells, hepatocytes (Evans *et al.*, 1992) and vascular smooth muscle is dependent on new RNA and protein synthesis, and is controlled by transcriptional factors. Although type I and III NOS are regulated by intracellular Ca^{2+} concentrations and phosphorylation by protein kinase A, inducible NOS (iNOS) cDNA does not have binding sites for Ca^{2+} or calmodulin (Stuehr *et al.*, 1991; Mitchell *et al.*, 1992); however, calmodulin does associate with the enzyme after translation. Furthermore, induction of type II isoform of NOS results in long-lasting release of NO, compared to transient production of NO by type I and III isoforms. However, all purified NOSs are flavoproteins containing one FAD and one FMN per subunit, in addition to one BH_4 (Hevel and Marletta, 1992) and one ferroprotoporphyrin complex (White and Marletta, 1992; Klatt *et al.*, 1992).

Putative Functions of Nitric Oxide Synthase

As discussed previously, NOS catalyzes a 5-electron oxidation of one of two chemically equivalent guanidino nitrogens of L-arginine to L-citrulline and NO in the presence of molecular oxygen and NADPH. However, accumulating evidence strongly suggests that NOS has a number of possible physiological functions. In the absence of L-arginine and BH_4 , type I NOS oxidizes NADPH in a calmodulin-dependent uptake of molecular oxygen and oxidation of NADPH, resulting in production of hydrogen peroxide (H_2O_2) (Mayer *et al.*, 1992). Interestingly, this mechanism resemble the production of H_2O_2 by neutrophils. NOS binds and reduces cytochrome C two to three fold faster than oxidation of arginine. This mechanism does not involve superoxide radical formation but is due to a direct Ca^{2+} /calmodulin-dependent interaction between NOS and cytochrome C

(Klatt *et al.*, 1992). Finally, NOS can associate with cytochrome P₄₅₀ and induce-Ca²⁺ independent hydroxylation of N-ethylmorphine, suggesting NOS can participate in cellular electron transfer processes (Hevel and Marletta, 1992). Although the functions of such reactions are not known, they may be involved in generation of pathophysiological states (Schmidt *et al.*, 1992).

Putative Actions of Nitric Oxide

Nitric oxide binds to the heme moiety of soluble guanylate cyclase (Rapoport and Murad, 1983), resulting in conformational change and activation of the enzyme (Craven and DeRubertis, 1978). Guanylate cyclase (GC, GTP pyrophosphate-lyase (cyclizing)) can then convert 3', 5' guanosine triphosphate to guanosine 3', 5'-cyclic monophosphate (cGMP) (Rapoport and Murad, 1983). Elevated intracellular cGMP levels may then have a number of actions within neuronal and muscle cells. For example, cGMP-dependent mechanisms can mediate relaxation of smooth muscle (reviewed in Ward and Sanders, 1992). Alternatively, cGMP can regulate various protein kinases such as cGMP-dependent protein kinase (protein G kinase) (Gerzer and Hamlet, 1988), nucleoside 3', 5'-monophosphate phosphodiesterases, and ion channels (Schmidt *et al.*, 1992; Ward and Sanders, 1992). For example, cGMP has been shown to directly act to open cation channels in photoreceptor cells, or to close Na⁺ channels in kidney cells (reviewed in Garthwaite, 1991). A second mechanism of action of NO may be *via* endogenous ADP-ribosyltransferases found in neural and non-neuronal tissues. Nitric oxide can stimulate endogenous ADP-ribosylation of a protein in platelets (Bruce and Lapetina, 1989), and NO donors such as SNP enhance ADP-ribosylation of a neuronal 37 kDa protein identified as glyceraldehyde-3-phosphate dehydrogenase (Zheng and Synder, 1992; Zheng

and Synder, 1993). Elevated cGMP may also accelerate the rate of mono-ADP ribosylation of proteins such as glyceraldehyde-3-phosphate dehydrogenase, resulting in decreased target activity (Zheng and Synder, 1992).

Nitric oxide may mediate physiological processes by altering voltage-gated ion channels. Sodium nitroprusside and NO increase the open probability of Ca^{2+} -activated K^{+} channels in canine colonic muscle (Ward and Sanders, 1992). Furthermore, release of NO from nerves (Ramagopal and Leighton, 1989) or interstitial cells (Publicover *et al.*, 1993), or application of exogenous NO (Publicover *et al.*, 1993) decreases intracellular Ca^{2+} concentrations in smooth muscle cells, suggesting NO may affect ion channels.

Anatomical Evidence of Nitric Oxide Synthase in Enteric Nerves

Although non-specific dehydrogenase histochemistry has been used to determine the total number of neurons in the GI tract (Gabella, 1967), the first report of NADPH diaphorase by Gershon and Kirchgessner (1986) found staining in all submucosal neurons and one-third of myenteric neurons in guinea pig small intestine. However, Young *et al.* (1992) observed NADPH diaphorase staining in approximately one-tenth of myenteric neurons and no submucosal neurons in guinea pig ileum. Furthermore, similar to the findings of Dawson and co-workers (1991), Young *et al.* reported that NADPH diaphorase and NOS-ir were always localized in the same populations of myenteric neurons. Intensely NADPH diaphorase stained neurons also contained bright NOS-ir, whereas weak NADPH diaphorase staining was associated with faint NOS-ir, although this pattern has not been observed by all investigators (Belai *et al.*, 1992). However, addition of L-arginine or N^{G} -nitro-L-arginine does not alter NADPH diaphorase staining, suggesting formation of NO and reduction of tetrazolium salt occur at different sites on the enzyme

(Young *et al.*, 1992).

Until recently, no other laboratories had access to NOS antiserum, requiring most studies on putative nitrinergic innervation to be performed with only NADPH diaphorase staining. NADPH diaphorase histochemistry staining is found in primary, secondary, and tertiary plexuses of myenteric plexus from the esophagus to proximal portion of the colon (Belai *et al.*, 1992; Aimi *et al.*, 1993; McConalogue and Furness, 1993; Nichols *et al.*, 1993), including gallbladder (Talmage and Mawe, 1993) and pancreas (Shimosegawa *et al.*, 1993). Interestingly, some studies have reported that most NADPH diaphorase stained cells in colchicine-treated tissue also contain VIP-ir (Costa *et al.*, 1992; Aimi *et al.*, 1993; Shimosegawa *et al.*, 1993; Talmage and Mawe, 1993), suggesting the presence of multiple NANC inhibitory neurotransmitters in the same neurons. Immunohistochemical experiments with antiserum raised against NOS have confirmed a close correlation of NOS- and VIP-ir, but not NOS- and SP-ir, or NOS-ir and cholinesterase staining, in myenteric neurons of colchicine-treated tissue (Costa *et al.*, 1992). Therefore, these anatomical data indicate that VIP and NOS are contained in a population of myenteric neurons that do not contain SP or ACh.

Anatomical Evidence of cGMP Formation in the GI Tract

As described previously, *in vitro* pharmacological experiments have demonstrated that NO, or a NO-like molecule, mediates NANC inhibitory neurotransmission in the GI tract. Anatomical and functional studies have strongly suggested that NO can relax GI smooth muscle through activation of GC. Although antibodies have found cGMP-like-ir in glial cells in the brain and periphery (De Vente and Steinbusch, 1992; Aoki *et al.*, 1993), basal cGMP-ir is not observed in enteric neurons and smooth muscle cells.

However, electrical field stimulation or addition of exogenous NO increases cGMP-ir in NADPH diaphorase negative myenteric neurons and smooth muscle cells in small intestine and colon (Shuttleworth *et al.*, 1993; Young *et al.*, 1993b). Furthermore, exogenous NO (Du and Conklin *et al.*, 1992) and electrical field stimulation (Torphy *et al.*, 1986) also increases cGMP levels in lower esophageal smooth muscle as measured by radioimmunoassay. Stimulus-induced cGMP-ir is prevented by NLA, a NOS antagonist, and methylene blue, a soluble GC antagonist (Shuttleworth *et al.*, 1993). Taken together, these data indicate that nerve stimulation increases NO production and release, resulting in activation of guanylate cyclase and formation of cGMP.

Characterization of NOS in Enteric Nerves

Few studies have examined biochemical properties of NOS in the GI tract, although Kostka *et al.* (1993) studied production of L-citrulline and cGMP as measures of NOS activity in purified synaptosomes made from canine deep muscular plexus. Similar to cerebellar NOS, enteric NOS requires NADPH and exogenous calmodulin for production of citrulline and cGMP. As predicted, increased concentrations of L-, but not D-arginine potentiate production of citrulline and cGMP, indicating stereo-specificity of a substrate-dependent enzyme. However, unlike the findings of Bredt and Synder (1990), addition of exogenous Ca^{2+} is not necessary for cGMP or citrulline production, indicating necessary Ca^{2+} must be supplied by an endogenous source. Finally, 0.1 mM EGTA does not totally inhibit citrulline or cGMP production, suggesting NO is formed by Ca^{2+} -dependent and Ca^{2+} -independent forms of NOS (Kostka *et al.*, 1993).

Functional Evidence of Nitric Oxide Release by Enteric Nerves

As described previously, *in vitro* nerve-muscle preparations made from all segments of the GI tract demonstrate frequency-dependent neurogenic relaxations that are attenuated by substituted L-arginine analogs (reviewed in Ward and Sanders, 1992). Similar results are observed after application of DMPP, a nACh receptor agonist, indicating cholinergic receptors may be found on inhibitory motor neurons. Measurements of metabolites of NO support the conclusion that electrical and pharmacological stimulation releases NO, as NO_2^- and NO_3^- concentrations increase after electrical or pharmacological stimulation of pieces of ileum (Shuttleworth *et al.*, 1994; Wiklund *et al.*, 1993).

Reflex studies and *in vivo* motility studies also demonstrate that enteric nerves release NO. Receptive relaxation of the stomach allows intake of a large volume without a similar magnitude increase in gastric pressure. Receptive relaxation in response to pressure or DMPP is antagonized by NOS antagonists, suggesting that NO is important in relaxation (Desai *et al.*, 1991). Similar results are observed in the ileum, as NOS antagonists decreases compliance of the intestinal wall at all distension rates, indicating that accommodation of the small intestine is also mediated by NO (Waterman *et al.*, 1994). NOS antagonists also change the threshold volume of initiation of peristalsis, decrease the rate of propulsion of solids in the ileum (Costa *et al.*, 1991) and stomach (Orihata and Sarma *et al.*, 1994), and produces dyscoordination of peristalsis, thus providing yet further evidence of NO release *in vivo*.

10. MECHANISMS OF NEURONAL PLASTICITY

Plasticity in Neuronal Phenotype

During development, many progenitor neural crest cells transiently express catecholamines although these sympathetic markers are lost before maturation (reviewed in Gershon *et al.*, 1993). Similarly, embryonic and neonatal ganglia express a wide variety of neuropeptides not found in adult ganglia (reviewed in Rao and Landis, 1992), whereas aging ganglia express neurotransmitters and neuromodulators not found in younger ganglia (reviewed in Hendry, 1992). Although neuroscience tends to view expression of neurotransmitters as a static event, cellular processes such as changes in ion flux, and binding of neurotransmitters, neuromodulators, cytokines, and growth factors activate second and third messengers. These intracellular messengers activate ribonucleases, protein kinases, and regulatory elements on DNA to temporally control the amount of neurotransmitter produced. However, decentralization and axotomy studies of autonomic ganglia have revealed that phenotypic expression of neurotransmitters is constantly controlled, and can be changed under some circumstances. For example, adult cholinergic neurons of the iris normally do not express the sympathetic markers TH or NPY. However, following sympathectomy, TH and NPY are expressed in previously-cholinergic neurons (Björklund *et al.*, 1985), suggesting a switch from cholinergic to noradrenergic function. Similar results were obtained from studies of eccrine sweat glands concentrated in footpads. Pharmacological and physiological studies have proven that neurogenic sweating is mediated by mACh receptors (Landis and Keefe, 1983; Stevens and Landis, 1987), and catecholamines are absent from sweat gland innervation of adult rats. However, transplantation or cross-innervation of sweat glands results in

expression of sympathetic markers (reviewed in Rao and Landis, 1992), illustrating a switch in neuronal phenotype. Sympathetic neurons can also be induced to function as cholinergic neurons. Although pure cultures of superior cervical ganglia (SCG) neurons contain sympathetic markers and synthesize catecholamines, conditioned medium induces expression of ChAt, elevated production of ACh, decreased sympathetic markers, and decreased synthesis of catecholamines (Walicke *et al.*, 1977).

Regulation of Neuronal Phenotype

There is a general paucity of information concerning the regulation of cholinergic and adrenergic neurotransmitter selection in developing neurons and even less is known regarding regulation of NANC neurotransmitters. However, it is clear that multiple extracellular signals govern the transmitter status of neurons at any given time. As described previously, neonatal SCG neurons can either secrete catecholamines and form adrenergic synapses, or release ACh, and form cholinergic synapses. In 1977, Walicke *et al.* illustrated that elevated K^+ , veratridine, or electrical stimulation depolarized SCG neurons and favored development of sympathetic characteristics. However, in the absence of electrical activity, cholinergic properties were expressed. These observations suggest trans-synaptic activity is an important regulator of neuronal phenotype and function. Furthermore, activation of preganglionic nerves induces mRNA (Black *et al.*, 1986), protein (Meuller *et al.*, 1969), and increases activity of TH (Meuller *et al.*, 1969; Zigmond *et al.*, 1989). Conversely, decentralization depresses expression of TH (Meuller *et al.*, 1969; Zigmond *et al.*, 1989), indicating regulation of transcription and translation of TH. Pre-synaptic activity also may be important in regulation of neuropeptides such as SP and VIP. Although most adult rat SCG neurons do not contain SP, *in vivo* and *in vitro*

decentralization of rat SCG, application of TTX, or addition of nACh receptor antagonists increases synthesis of preprotachykinin mRNA and SP in adult SCG neurons (Kessler and Black, 1982; Kessler *et al.*, 1981; Kessler *et al.*, 1983; Black *et al.*, 1986). As predicted, veratridine-induced depolarization attenuates the intracellular concentration of SP, although alterations in cellular content do not result from release of SP (Black *et al.*, 1986). Vasoactive intestinal polypeptide expression also may be regulated by pre-synaptic activity, as SCG neurons normally contain little VIP-ir, and VIP-ir increases after decentralization. However, Sun *et al.* (1992) observed depolarization-induced elevations in VIP- and SP-ir in SCG explants and primary cultures, leading the authors to conclude that sympathetic nerve activity actually increases SP and VIP levels. Unfortunately, this conclusion is not supported *in vivo*, for very little SP and VIP is found in SCG of normal animals.

Axotomy or crushing of autonomic or somatic nerves alters mRNA and protein levels for a number of peptides. For example, unilateral axotomy of the sciatic nerve induces NPY, VIP, and galanin mRNA in DRG neurons, and results in a greater number of NPY-, VIP-, or galanin-ir DRG neurons (Noguchi *et al.*, 1993). Although mRNA for CGRP and somatostatin is expressed in normal and control cells, axotomy decreases both RNA and protein levels of CGRP and somatostatin, indicating that multiple regulatory mechanisms are involved in control of neuropeptide expression. Changes in neuropeptide levels are also observed proceeding section of postganglionic SCG axons, as mRNA and protein for VIP, but not SP, significantly increases after axotomy (Rao *et al.*, 1992). These results suggest that the target of innervation secretes regulatory molecules which are taken up and retrogradely transported by presynaptic nerves. These regulatory

molecules would influence neurotransmitter expression (reviewed in Hendry, 1992), similar to the mechanisms by which nerve growth factor (NGF) regulates neuronal survival (Hendry *et al.*, 1974). Although few regulatory molecules have been identified, cytokines such as ciliary neurotrophic factor (CNTF) and cholinergic differentiation factor (CDF), (also known as leukemia inhibitory factor, LIF) stimulate expression of VIP in SCG neurons *in vitro* (Nawa and Patterson, 1990). In this proposed model, an inhibitory molecule would originate from the target of innervation and repress VIP expression in normal neurons. Axonal crushing or axotomy would prevent retrograde transport of inhibitory molecules, resulting in up-regulation of neuropeptide expression. Finally, SP levels may be regulated by NGF, as ciliary ganglionectomy or decentralization of SCG increases NGF (reviewed in Hendry, 1992), exogenous NGF increases SP concentration and TH activity in the iris (Kessler *et al.*, 1983; Kessler, 1985) and SCG (reviewed in Rao and Landis, 1992), and antibodies against NGF inhibit induction of SP and TH. The cellular mechanisms for such regulation are unknown at this time.

In development, neurons and myoblasts may develop independently of each other. However, neurons can influence target tissue, and target tissue has profound effects on neuronal development such as in formation of correct synapses and junctions (reviewed in Hendry, 1992). However, it is feasible that although neurons and myoblasts produce growth factors such as insulin-like growth factor, fibroblast growth factor, CNTF, and CDF, these molecules are not retrogradely transported, but act extracellularly *via* a membrane-bound receptor to generate a second messenger. Therefore, the ability of these molecules depends on the presence of the molecule itself and a receptor on the neuron of interest. These two criteria agree well with antidotal data, as studies have illustrated the

importance of the type of neuron (Iacovitti *et al.*, 1982; Walicke *et al.*, 1977) (the presence of a receptor), as well as the importance of the target of innervation (reviewed in Landis, 1990) (the source of cytokines and/or growth factors). Although a clear example has not been found, basic fibroblast growth factor (bFGF) is found in adrenal medullary chromaffin granules (Westermann *et al.*, 1990), and bFGF impregnated gel foams can prevent death of preganglionic adrenal neurons following destruction of the adrenal medulla (Blottner *et al.*, 1989). At this time, it is unknown if bFGF is retrogradely transported, or acts *via* a second messenger, or if bFGF can regulate neurotransmitters, in addition to controlling cell fate. However, CNTF regulates survival of developing and adult neurons, and regulates transcription of VIP, somatostatin, and CGRP (Symes *et al.*, 1993), providing preliminary evidence that regulation by target-derived molecules may occur.

Axonal Sprouting and Regeneration of Adult Neurons

Anatomical studies have provided two general explanations for plasticity in autonomic and peripheral nerves. As described above, denervation can induce alterations in neuronal phenotype, manifested as an upregulation of existing neurotransmitters, or a switch in phenotypic expression. The second mechanism for plasticity involves formation of axonal sprouts, complete regeneration of previously-damaged nerves, and formation of correct synapse or junctions. It has been well documented that following lesion of peripheral nerves, or autonomic preganglionic or postganglionic nerves, the lesioned nerves form regenerative axonal sprouts that will often reinnervate the denervated target (Guth, 1956). For example, 2 to 4 months after lesion of preganglionic cervical sympathetic nerves, stimulation of T₁ results in vasoconstriction of arterioles in the ear,

whereas stimulation of T₄ dilates the pupils (Langley 1897). These data indicate that not only did preganglionic nerves regrow after axotomy, but reformed the correct neuro-effector junctions. Furthermore, there are numerous examples of axonal sprouting and regeneration of nerves expressing a new phenotype as compared to innervation prior to surgery. For example, after chemical sympathectomy, the cholinergic innervation to the vas deferens increases, presumably to replace the noradrenergic innervation (Evans *et al.*, 1979). Alternatively, the source of innervation may not be altered, but levels of neurotransmitters or neuromodulators are affected by axotomy. Following unilateral superior cervical ganglionectomy in neonatal rats, the remaining SCG maintains the ipsilateral innervation, and assumes control of the contralateral iris, tarsal muscle, orbital muscle and choroidal vasculature (reviewed in Smith *et al.*, 1991). The new innervation from the contralateral SCG increases muscle size and contractile strength, although innervation still originates from SCG neurons. Anatomical studies reveal that although 21% of SCG neurons contained NPY under normal or sham conditions, following ganglionectomy, 85% of SCG neurons express NPY, indicating either an upregulation of NPY, or expression of NPY in many neurons not previously synthesizing NPY.

Regulation of Axonal Sprouting and Regeneration

The mechanisms for axonal sprouting, regeneration, and synapse formation are poorly understood at this time. A multitude of molecules have been identified in developing and regenerating nerves that may be important, and a discussion of each is beyond the scope of this review. However, Cajal (1928) demonstrated that regenerating axons preferentially grow towards deafferented stumps of transected nerve, as regrowth is greatly decreased upon removal of the distal nerve stump. These observations suggest

that the distal nerve, myelin, or support cells releases soluble chemoattractant molecules that induces sprouting and guides regrowing axons along the correct path. The molecules currently thought to be involved in sprouting of peripheral axons, include NGF (Hendry, 1992; Kuchel *et al.*, 1992; Van der Zee *et al.*, 1992), insulin (Ekström *et al.*, 1993; Edbladh *et al.*, 1994), laminin, fibronectin (Wang *et al.*, 1992), insulin-like growth factor II (Glazner *et al.*, 1993; Edbladh, 1994), ciliary neurotrophic factor (Sendtner *et al.*, 1992), amyloid precursor protein (Alvarez *et al.*, 1992), and map-associated kinases (Hendry, 1992). However, it should be noted that it is improbable that a single molecule can initiate and maintain axonal growth, and select and maintain the correct synapses.

11. RATIONALE FOR USE OF GUINEA PIG ILEUM

The neurons of the small intestine regulate a number of physiological functions, including blood flow and motility. The submucosal arterioles and longitudinal muscle were chosen as models for these studies for four reasons. First, both neuroeffector targets receive intrinsic and extrinsic innervation important in regulation of digestive functions. Second, other investigators have characterized the physiological functions and anatomy of extrinsic and intrinsic innervation to the arterioles and longitudinal muscle in normal small intestine. As a result of this characterization, specific anatomical markers can be chosen to selectively label extrinsic sympathetic, extrinsic primary afferent, intrinsic excitatory, or intrinsic inhibitory innervation. Finally, functional assays for inhibitory innervation exist to allow correlation of alterations in regulation of function.

METHODS

1. ANIMALS

Albino male guinea pigs (250-400 g) were obtained from the Michigan Department of Public Health (Lansing, MI) or Charles River Breeding Laboratories (Indianapolis, Indiana) and maintained under conditions of controlled temperature and lighting with Guinea Pig chow from Harlan Teklad or Purina and water provided *ad libitum*.

2. EXTRINSIC DENERVATION

Guinea pigs were anesthetized with 50 mg/kg ketamine and 5 mg/kg xylazine administered intramuscularly. A loop of ileum with attached mesenteric arcade was exposed through an abdominal incision, and extrinsic perivascular and paravascular nerves associated with the mesenteric blood vessels innervating the loop of ileum were crushed with watchmaker forceps (Furness and Costa, 1978). The arterioles were bathed with a solution of 1% lidocaine to prevent vasospasm. The loop of intestine was identified by a piece of 5-0 silk suture left encircling the mother arteriole. The ileum was returned to the abdominal cavity, and the incision was closed using 4-0 surgical silk. Sham surgeries were identical to the denervation surgeries, except that the nerves were not crushed. Animals were given sc injections of Combiotic^R, an aqueous suspension of penicillin and dihydrostreptomycin (Webster, Syracuse, NY), and placed under an incandescent lamp to maintain body temperature until the animal was returned to its home cage.

As surgical denervation destroyed sympathetic, parasympathetic and sensory nerves, neurotoxins were used to selectively destroy a single population of nerves. Guinea pigs were administered subcutaneous injections of 250 mg/kg of 6-hydroxy-dopamine (6-OH-DA) (Sigma) on days 1, 3, 5, and 7 to selectively destroy sympathetic innervation to the GI tract (Furness and Costa, 1974a), as 6-OH-DA is accumulated by catecholaminergic neurons resulting in a restrictive degenerative action of the compound (Tranzer and Thoenen, 1967). Control animals received subcutaneous injections of 0.1% ascorbic acid. Small unmyelinated primary afferent nerves were eliminated by daily subcutaneous injections of increasing concentrations of capsaicin (0.5 mg/kg to 50 mg/kg) (Papka *et al.*, 1984; Galligan *et al.*, 1988; Holzer, 1991). Control animals received sc injections of 80% PBS, 10% Tween 80, and 10% ethanol.

3. PREPARATION OF ILEUM FOR IMMUNOHISTOCHEMISTRY

Guinea pigs were anesthetized *via* halothane inhalation, stunned, and exsanguinated. A segment of ileum was removed 10-20 proximal to the ileocecal junction and placed in oxygenated (95% O₂, 5% CO₂) Krebs' solution containing ((mM) NaCl, 117; KCl, 4.7; CaCl₂, 2.5; MgCl₂, 1.2; NaHCO₃, 25; NaH₂PO₄, 1.2; and glucose, 11). The segment of ileum was then transferred into a petri dish lined with silicone elastomer (Sylgard^R, Dow Corning, Midland, MI), the mesenteric attachment was removed, and the intestine was opened along the mesenteric border. The tissue was gently stretched into a rectangular sheet, pinned onto a piece of balsa wood, and fixed according to one of three protocols. Most preparations were fixed overnight in Zamboni's solution (4% paraformaldehyde and 15% picric acid) maintained at 4° C (Zamboni *et al.*, 1967;

Stefanini *et al.*, 1967 Costa *et al.*, 1980), cleared in dimethyl sulfoxide to remove fixative from the tissue, and rehydrated in 0.01 M phosphate buffered saline (PBS), pH 7.1. Some ileal sheets were fixed for 3 h at room temperature in 4% paraformaldehyde, and washed in PBS. Finally, some preparations were fixed in 2% paraformaldehyde containing 0.2% picric acid for 4 h at room temperature, and washed in PBS to remove residual fixative (Schemann *et al.*, 1993). Following fixation, tissues were stored at 4°C in PBS with 0.1% sodium azide as a preservative.

As some neuropeptides such as substance P are difficult to localize in enteric somata, some pieces of ileum were incubated for 8 h at room temperature in Eagle's Modified Essential Medium (MEM) containing 100 μ M colchicine to block microtubule elongation, resulting in enhanced neuropeptide levels of somata (Furness *et al.*, 1983).

Prior to any immunohistochemical procedure, the fixed tissue was washed at least twice in PBS. The mucosa was removed by scraping with a scalpel, or by peeling strips away from the submucosal plexus with a pair of watchmaker forceps. The submucosal plexus and circular muscle were separated from the LMMP using blunt dissection (Costa *et al.*, 1980), resulting in a submucosal layer or submucosal layer and attached circular muscle, and a myenteric plexus and longitudinal muscle (LMMP) preparation. Representative samples of the separated laminae measuring approximately 0.5 cm² were placed in PBS in individual wells of Falcon^R 24 well tissue culture plates (Becton Dickinson, Lincoln Park, NJ) lined with silicone elastomer.

4. IMMUNOHISTOCHEMISTRY

Tissues were incubated for 48 h at room temperature in antiserum and/or antibodies raised against various neuropeptides, enzymes, or other cellular proteins. The

preparations were incubated occasionally in a single antiserum; however, the majority of tissues were incubated in two antibodies raised in different species of animals to allow simultaneous co-localization of two different antigens *via* indirect immunofluorescence (Sternberger, 1974; Costa *et al.*, 1987). The primary antisera, sources, dilutions, and references for characterization are summarized in Table 1. The tissues were washed three times in PBS, and incubated for 2 h at room temperature in the appropriate secondary antisera conjugated to a fluorescent molecule.

Bound substance P antiserum was localized using 1:80 goat anti-rat IgG conjugated to tetramethyl rhodamine isothiocyanate (Chemicon, Temecula, CA), Texas red sulfonyl chloride (TRSC, Texas red) (Jackson Immunologicals, West Grove, PA), or fluorescein isothiocyanate (FITC) (Sigma, St. Louis, MO). Bound antiserum raised against phosphorylated heavy neurofilament protein (pNF-H; SMI 31), or nonphosphorylated heavy neurofilament protein (nPNF-H; SMI 32) were visualized by 1:200 goat anti-mouse IgG and IgM conjugated to rhodamine (Chemicon), 1:160 goat anti-mouse IgG conjugated to dichlorotriazinyl amino fluorescein (DTAF) (Jackson), or 1:800 goat anti-mouse IgG conjugated to indocarbocyanine (Cy3) (Jackson). Bound antiserum raised against an epitope accessible in phosphorylated and non-phosphorylated heavy neurofilament protein (SMI 33) was localized by 1:200 goat anti-mouse IgG and IgM conjugated to rhodamine (Chemicon), or 1:1000 goat anti-mouse IgM conjugated to Cy3 (Jackson). Antibodies or antiserum raised against CGRP, cFOS, constitutive NOS, inducible NOS, NPY, or VIP were visualized using 1:80 goat anti-rabbit IgG conjugated to FITC (Sigma), DTAF (Chemicon), or rhodamine (Chemicon), or 1:800 goat anti-rabbit IgG conjugated to Cy3. The tissues were rinsed three times in PBS to remove unbound secondary antiserum,

Table 1. Antisera used to study denervation-induced alterations in the enteric nervous system of guinea pig ileum.

Antigen Code	Target Antigen	Host	Dilution	Source	Reference
ChAt P3	ChAt	rabbit	1:200	Biometra	Schemann <i>et al.</i> , 1993
CGRP AB1971	CGRP 28-37	rabbit	1:800	Amersham	Gibbins <i>et al.</i> , 1985
cFOS OA-11-823	cFOS 1-16	sheep	not determined	Cambridge Research	not available
cFOS AB-1	cFOS 128-152	mouse	not determined	Oncogene Sciences	not available
cFOS SC-52	cFOS 3-16	rabbit	1:1000	Santa Cruz	not available
cNOS	cerebellar NOS	rabbit	1:1000	B. Mayer	Klimaschewski <i>et al.</i> , 1992
iNOS	inducible NOS	rabbit	1:500	M. Marletta	not available
NPY RPN1702	NPY 1-35	rabbit	1:400	Amersham	Furness <i>et al.</i> , 1985
npNF-H SMI 32	hypothalamus	mouse	1:500	Sternberger	Sternberger <i>et al.</i> , 1983
pnpNF-H SMI 33	hypothalamus	mouse	1:1000	Sternberger	Sternberger <i>et al.</i> , 1983
pNF-H SMI 31	hypothalamus	mouse	1:1000	Sternberger	Sternberger <i>et al.</i> , 1983
SP NCL34HL	SP 1-11	rat	1:200	Serotec	Cuello <i>et al.</i> , 1979
VIP 7913	VIP 1-28	rabbit	1:400	J. Walsh	Furness <i>et al.</i> , 1981

mounted on microscope slides (Corning, Corning, NY) with bicarbonate-buffered glycerol (pH 8.6) (Costa *et al.*, 1980), and coverslipped (Corning). The tissues were visualized with an inverted Nikon diaphot microscope equipped with an epi-fluorescent collector lens at magnification of 400x. The barrier cube G1B (580 nm dichroic mirror, 546 nm excitation filter, 590 nm barrier filter) was used to visualize TRITC (fluorochrome excitation 557 nm, fluorochrome emission 576 nm), TRSC (fluorochrome excitation 596 nm, fluorochrome emission 620 nm), and Cy3 (fluorochrome excitation 550 nm, fluorochrome emission 570 nm). Barrier cube B-2E (510 nm dichroic mirror, 450-490 nm excitation filter, 530 nm barrier filter) was used to visualize FITC (fluorochrome excitation 492 nm, fluorochrome emission 520 nm) and DTAF (fluorochrome excitation 492 nm, fluorochrome emission 520 nm). Kodak Technical Pan film (Rochester, NY), rated at 100 ASA was used for black and white photography, and Kodak Ektachrome elite slide film rated at 400 ASA was used for color photography.

Peptide neutralization was used to verify that primary antiserum recognized the intended antigen. Antiserum against NPY, CGRP, neurofilaments (SMI 31, SMI 32, or SMI 33), or fos were incubated with 10-fold excess (by weight) of porcine sequence NPY (Sigma), rat sequence CGRP (Sigma), neurofilament purified from bovine spinal cord (Sigma), or human sequence fos peptide (amino acid residues 3-16) (Santa Cruz Biotechnology, Santa Cruz, CA). Previous studies have shown that immunoreactivity for SP, VIP (Galligan *et al.*, 1988), iNOS (M. Marletta, personal communication), and cNOS (Klimaschewski *et al.*, 1992), is eliminated by incubation of the antibody with the intended antigen. To verify that the secondary antisera did not recognize guinea pig immunoglobulin, tissues were incubated in diluted secondary antiserum for 2 h at room

temperature. Finally, tissues were incubated in primary antiserum and incorrect secondary antiserum to verify that cross reactions did not occur.

As SMI 31, SMI 32, and SMI 33 recognize phosphorylated, nonphosphorylated, and phosphorylation-independent epitopes in immunoblots and tissues (Sternberger and Sternberger, 1983; Watson *et al.*, 1989), control experiments were performed to verify their specificity in guinea pig ileum. Fixed submucosal tissues were incubated for 18 h at 22°C in 400 µg/mL *E. coli* alkaline phosphatase (Sigma) in 0.1 M Tris HCl, pH 8.0, with 0.01 M phenylmethylsulfonyl fluoride (PMSF) to dephosphorylate neurofilament protein epitopes. Alternatively, tissues were incubated for 10 min at 37°C in 400 µg/mL trypsin (Gibco, Grand Island, NY) in 0.05 M Tris HCl, pH 7.6, 0.3 M NaCl and 0.02 M CaCl₂, or in trypsin and phosphatase treatments sequentially. Control tissues were incubated in the buffers only. Tissues were then washed with PBS, and processed as described previously.

As SMI 31, 32, and 33 are sensitive to the phosphorylation status of the neurofilament proteins, phosphate-free buffer (0.05 m Tris buffer with 1.5% sodium chloride, pH 7.6) was also tested to determine if the presence of phosphate in the diluent buffer could alter the patterns of immunoreactivity in the submucosal plexus (Trojanowshi *et al.*, 1985; Trojanowski *et al.*, 1986).

5. HISTOCHEMISTRY

Some tissues were counterstained with bisbenzimidazole to stain nuclear material according to the protocol of Swanson and Sawchenko (1982). Tissues were incubated for 5 min at 22°C in 1 µg/ml of bisbenzimidazole (Hoechst dye 33528) (Sigma) in PBS, pH 7.2,

washed with PBS, and mounted in buffered glycerol for fluorescence microscopy.

Hematoxylin and eosin staining using standard procedures was performed on normal, denervated, and control ileum fixed in 4% paraformaldehyde by the Michigan State Histotechnology Center.

NADPH diaphorase histochemistry was used as a putative anatomical marker for cNOS (Brent *et al.*, 1991). Tissues were incubated in 0.2 mM nitro blue tetrazolium (p-nitrotetrazolium blue, 2, 2'-Di-p-nitrophenyl-5'-5'-diphenyl-[3-3'-dimethoxy-4,4'-diphenylene]-ditetrazolium chloride) (Sigma) and 1 mM NADPH (Sigma) in 0.1 M Tris-HCl with 1% Triton X-100, pH 8.0 for 30 min at 37°C (Scherer-Singler *et al.*, 1983). The tissues were washed in 0.01 M PBS, coverslipped with buffered glycerol, and visualized using differential interference contrast optics at 100, 200, and 400x magnification.

NADH diaphorase histochemistry was used as a neuronal stain (Gabella, 1967). Tissues were processed as for NADPH diaphorase staining, except NADH was substituted as the electron donor.

6. VERIFICATION OF EXTRINSIC DENERVATION

At the appropriate time (one, four, seven, ten, sixteen, or twenty-four weeks after denervation), the small intestine was removed as described previously. The denervated loop was identified by the loop of silk suture, and a small piece of denervated and control tissue from the same animal was placed in a 2% solution of glyoxylic acid (Sigma) at room temperature (Costa and Furness, 1974b). The mucosa was scrapped away, and the submucosal and myenteric plexuses were teased apart with two pairs of watchmaker

forceps. The tissues were stretched flat on a glass coverslip, incubated for 5 min at 100°C, and coverslipped with mineral oil. Glyoxylic acid reacts with tryptamine in catecholamines in an acid-catalyzed step to form the weakly fluorescent 1,2,3,4-tetrahydro- β -carboline-1-carboxylic acid *via* a Schiff's base. This compound can under antioxidative decarboxylation to 3,4-dihydro- β -carboline; alternatively, it can undergo a second intramolecular acid-catalyzed reaction with GA to yield 2-carboxymethyl-3,4-dihydro- β -carbolinium salt (Björklund *et al.*, 1972). These end-products give off blue fluorescence (fluorochrome excitation 405 nm, fluorochrome emission 450 nm) when exposed to UV light (cube V-2A, dichroic mirror 430 nm, excitation filter 400 nm, barrier filter 450 nm) (Furness and Costa, 1974). Denervation after one and four weeks was considered acceptable if there was a 90% reduction in catecholamine fluorescence in the submucosal plexus. As extrinsic nerves eventually return to the denervated segment, control segments from the same animal are used to determine if the putatively-denervated segment contained fewer sympathetic nerves.

As described previously, sensory nerves from the dorsal root ganglia contain SP-ir and CGRP-ir in the same nerves. Therefore, immunohistochemistry for SP and CGRP was used to determine if the denervation procedure was successful.

7. INJECTION OF RETROGRADE TRACER

Fluoro-gold (Fg) (Fluorochrome Inc., Englewood, CO) was injected into submucosal arterioles to identify the origin of intrinsic nerves associated with arterioles in normal and denervated ileum. The ileum was removed, and a 2 cm length of ileum was placed in a petri dish lined with silicone elastomer. The ileum was opened along the

mesenteric border, stretched into a rectangle, and the mucosa was removed with fine forceps. A small piece (5 cm²) of submucosa was removed using fine scissors and any adhering circular muscle was removed with fine forceps. The submucosa was stretched and attached with small stainless steel pins to a small organ chamber (2 ml volume) lined with silicone elastomer. The mounted submucosal preparation was transferred to the stage of an inverted Nikon Diaphot microscope, and arterioles were visualized by differential interference contrast optics at a final magnification of 400x. The preparations were continuously superfused with oxygenated Krebs' solution heated to 36°C. Glass micropipettes were broken back, beveled to a tip diameter of 8-15 µm, and filled with a solution of 4% fluoro-gold dissolved in distilled water. The micropipette was positioned with a micromanipulator under visual guidance on the surface of an arteriole, and then advanced into the arteriolar wall. Pulses of nitrogen (10 psi, 0.3 Hz, 300 msec duration, 5 min) were used to eject fluoro-gold into one arteriole per preparation. Following injection of fluoro-gold, the micropipettes were left in place for an additional 10 minutes to minimize leakage of tracer from the injection site. The organ chamber with attached submucosal preparation was incubated for 24 hours in Eagle's MEM supplemented with 10,000 U/ml penicillin and 10 µg/ml penicillin to allow retrograde transport to occur. After 24 h, the tissues were incubated in fresh MEM supplemented with 100 µM colchicine for an additional 24 h, and then fixed in Zamboni's solution as described previously. Some tissues were then processed for immunohistochemical localization of SP- and/or VIP-ir, and coverslipped with buffered glycerol. Fluoro-gold fluorescence (fluorochrome excitation 323 nm, fluorochrome emission 408 nm) was viewed with UV light (cube V-2A).

8. IN VITRO RELAXATIONS OF LONGITUDINAL MUSCLE

Longitudinal muscle-myenteric (LMMP) plexus preparations were made from segments of ileum using a modification of the procedure described by Kosterlitz *et al.* (1964). The mesentery was removed for segments of ileum, and LMMP preparations were made by threading a 5 cm piece of ileum onto a glass rod. The LMMP was teased away from the submucosal and mucosal layers using a cotton swab. The resulting LMMP was divided in half, mounted in plexiglass holders equipped with platinum foil electrodes, and placed in 10 or 20 mL baths containing 1 μ M scopolamine and 3 μ M guanethidine in warmed (36°C) and continuously oxygenated (95% O₂ and 5% CO₂) Krebs' solution. The free end of each segment was attached with a thread to an isometric strain gauge force transducer (FT03C, Grass Instrument, Quincy, MA) and each tissue was stretched to its optimum length (L_o) for development of active tension. The muscle was contracted with 3 μ M histamine and nerve-mediated NANC relaxations were induced by transmural electrical stimuli provided by a Grass S48 stimulator (60 V, 0.5 msec pulse duration, 1000 msec train duration, 0.25 trains per second, variable stimulation (1, 2, 5, 10, 20, or 50 Hz)) and a constant current isolator (Burnstock *et al.*, 1966). Responses were recorded using a Grass 7D polygraph. Data were expressed as a percent of 20 Hz relaxation or contraction.

9. LENGTH-TENSION RELATIONSHIPS OF LONGITUDINAL MUSCLE

As denervation-induced alterations in neurogenic relaxations could result from alterations in muscle properties, passive and active tension was measured in normal, extrinsically denervated, and control animals. The strips of muscle were clamped in

plexiglass and metal holders, and mounted in plexiglass holders as described previously. The length of the muscle was altered through a micrometer ratchet system (Percy *et al.*, 1990). The initial length (L_i) was defined as the absolute length of the muscle where there is no tension in the muscle. The tissue was stretched 0.5 mm, and the responses were allowed to equilibrate. The amplitude of this response at steady state represented passive tension (P). Active tension was elicited by addition of 3 μ M histamine. The peak amplitude of the agonist-induced response was measured, and was named active tension (A). This value was summed with P to produce total tension (T). The tissues were washed 3 times, 5 minutes each, and the procedure was repeated until A did not increase over 3 consecutive trials. Data were normalized by dividing the length of each tissue by its L_o , whereas tension was normalized by dividing active or passive tension by the maximum active tension for each tissue. The length of the muscle was adjusted to the optimal length for the development of active tension (L_o) prior to nerve stimulation.

10. IN VITRO CONTRACTIONS OF CIRCULAR MUSCLE

Whole ileal preparations were prepared by dividing the ileum into transverse rings approximately 0.5 cm wide. Alternatively, a 2 cm piece of ileum was opened along the mesenteric attachment, the mucosa was removed using fine forceps and a dissecting microscope, and the remaining tissue was cut into strips perpendicular to the long axis of the mesenteric border. All preparations were mounted in the plane of the circular muscle. The tissues were attached to plexiglass holders and isometric force transducers as described previously for the longitudinal muscle. The tissues were allowed to equilibrate for 90 minutes, and they were stretched until 1 g of resting tension was maintained.

Frequency-response curves were generated using parameters previously described for stimulation of longitudinal muscle.

Transmural stimulation produced a monophasic contraction that was altered by many agonists and antagonists. All concentration-response curves were constructed using cumulative concentrations of agonist or antagonist. However, non-cumulative concentrations of substance P methyl ester (SPME) and bethanechol were used, as rapid desensitization occurred.

11. NITRIC OXIDE SYNTHASE ASSAY

NOS activity was measured according to the method of Mitchell *et al.* (1992), based on the method of Bredt and Synder (1989). Longitudinal muscle-myenteric plexus preparations were made as described previously. The tissue was homogenized on ice in buffer (pH 7.5) containing (mM): Tris-HCl, 50; EDTA, 0.1; EGTA, 0.1; 2-mercaptoethanol, 12; leupeptin, 0.002; pepstatin, 0.001; and phenylmethylsulfonyl fluoride, 1. Nitric oxide synthase activity was measured in tissue homogenates as the formation of L-[¹⁴C]citrulline from L-[¹⁴C]arginine (Bredt and Synder, 1990). In most experiments, tissue homogenates (50 μ l) were incubated with 3 μ M [¹⁴C]arginine (331.2 mCi/mmol) (Dupont New England Nuclear, Boston, MA) and exogenous cofactors or antagonists in a reaction volume made up to 100 μ l with HEPES (pH 7.5). Homogenates were incubated for 25 min at 37°C, and the reaction was stopped by addition of 1 ml of 20 mM HEPES (pH 5.5) containing 2 mM EGTA and 2 mM EDTA. After mixing, 1 ml samples were applied onto chromatographic columns (Dowex AG 50W-X8 resin, 1.1 g resin/column, Na⁺ form, 200-400 mesh) (Biorad, Rockville Center, NY) and [¹⁴C]citrulline

was eluted with 2 x 1 ml aliquots of water. After addition of scintillation fluid, radioactivity was measured using a Beckman scintillation counter. Although preliminary experiments demonstrated that columns bound $98 \pm 1\%$ (3 preparations) of [^{14}C]arginine, background counts resulting from trace [^{14}C]arginine were measured by applying tissue-free homogenates to 3 control columns/treatment. The background counts were averaged, and subtracted from counts of samples containing tissue. [^{14}C]Citrulline production was expressed as pmole/mg protein, and protein concentrations were measured as described by Bradford (1976), using bovine serum albumin (Sigma) for standards, and protein assay dye reagent concentrate from Biorad. Finally, formation of [^{14}C]citrulline was confirmed by thin layer chromatography (Iyengar *et al.*, 1987).

12. DRUGS

All drugs were purchased from Sigma Chemical Co, except CP-96,345-1 (dihydrochloride salt of [(2S,3S)-*cis*-2-(diphenylmethyl)-*N*-[(2-methoxyphenyl)-methyl]-1-azabicyclo[2,2,2]octan-3-amine], which was the gift of Pfizer (Groton, CN), and 8-Bromo-cGMP, which was purchased from Research Biochemicals (Natick, MA). Papaverine and tetrodotoxin (TTX) were dissolved in dilute acetic acid, N^{G} -nitro-L-arginine (LNA) was dissolved in dilute hydrochloric acid, and all other drugs were dissolved in deionized water. Oxyhemoglobin and superoxide dismutase were maintained on ice after reconstitution, and sodium nitroprusside (SNP) was made fresh daily immediately prior to use.

13. STATISTICAL ANALYSIS OF DATA

Data were expressed as mean $\pm$ standard error of the mean. In anatomical studies, the ratio of cells/ganglia between two groups were compared using the non-parametric Mann-Whitney or Wilcoxon rank-sum (Mann-Whitney-Wilcoxon) test, whereas comparisons between three or more unpaired groups were made using the Kruskal Wallis Test, followed by the Student Newman Keuls Test for *a posteriori* comparisons. In functional studies, comparisons between two groups were made using a 2-tailed student's T test after verification of equivalent variance using the F statistic, whereas multiple comparisons were made using analysis of variance (ANOVA), proceeded by the Least Significant Differences test (Steel and Torrie, 1960). The effect of each drug was expressed as the percent increase or decrease as compared to 20 Hz stimulation, and the concentration of the drug producing half-maximal (EC_{50}) or half-maximal inhibition (IC_{50}) was determined for each tissue, and the average values were expressed as the geographic mean (Fleming *et al.*, 1972). The 95% confidence limits were calculated using the S.E.M. of the average EC_{50} and Student's *t* value for *n* - 1 degrees of freedom (Kenakin, 1987). Similar statistical tests were performed on data generated by measurement of citrulline production, except the Duncan Multiple Range Test was utilized proceeding two-way ANOVA with replicates, and Dunnett's Test was used to compare several means to a single control. Differences among groups were considered significant if the probability of type I error was less than 5%.

RESULTS

1. EXTRINSIC DENERVATION ALTERS VASCULAR INNERVATION

Innervation of Submucosal Arterioles in Normal Ileum

Four populations of nerve fibers were associated with submucosal arterioles in normal guinea pig ileum. Glyoxylic acid (GA)-induced fluorescence of neuronal stores of catecholamines and immunohistochemistry for TH (data not shown) and NPY (data not shown) revealed a dense paravascular and perivascular plexus surrounding all submucosal arterioles (Figure 5A). Varicose and non-varicose catecholaminergic nerve fibers covered the surfaces of submucosal and myenteric ganglia, and some sympathetic nerve fibers formed pericellular baskets around submucosal and myenteric neurons. Submucosal and myenteric ganglia did not contain catecholaminergic neurons, as GA-induced fluorescent or TH-ir neurons were not observed. Immunohistochemistry for SP- and CGRP-ir revealed that paravascular varicose nerve fibers always contained SP- and CGRP-ir in the same nerves (Figures 7A and 7B). Substance P-/CGRP-ir nerve fibers were brightly fluorescent with large varicosities, and these nerves had a larger caliber than other nerve fibers. Substance P- without CGRP-ir, and CGRP- without SP-ir was never observed in nerve fibers associated with submucosal arterioles, although some submucosal neurons contained SP- or CGRP-ir. Thin SP-ir nerve fibers with small varicosities were found within submucosal ganglia, although they were not observed to associate with arterioles.

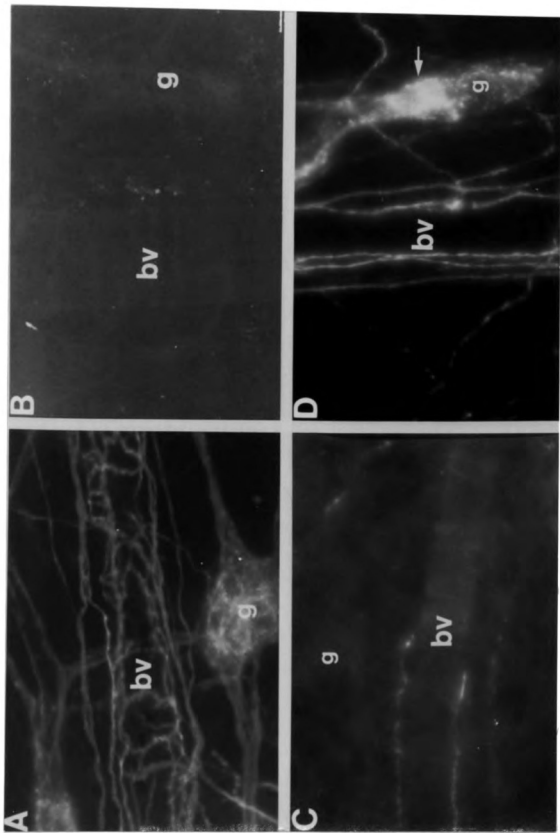
Antiserum raised against VIP recognized occasional thin paravascular nerve fibers with small varicosities (Figure 8A). Antiserum raised against ChAt identified faint, thin, non-varicose nerve fibers closely associated with adventitial surfaces of arterioles. Unlike other vascular nerves, ChAt-ir nerve fibers did not form a paravascular or perivascular plexus, but appeared to enter and leave the plane of the tissue, resulting in short segments of immunoreactive nerve fibers (data not shown). Finally, antiserum against NOS and dehydrogenase staining for NADPH diaphorase activity did not label perivascular or paravascular innervation in normal ileum (Figures 25A and 31A) indicating nitrinergic nerve fibers were not found with submucosal arterioles in normal preparations. However, thin, varicose NOS-ir nerve fibers formed pericellular baskets around some submucosal ganglionic neurons (Figure 31). Taken together, these results indicate that submucosal arterioles are innervated by nerves containing SP-/CGRP-ir, NPY-/TH-ir, or VIP-ir. Arterioles also receive cholinergic innervation as demonstrated by the presence of ChAt-ir nerve fibers. However, normal paravascular and perivascular innervation do not contain SP- without CGRP-ir, CGRP- without SP-ir, or NOS-ir.

Sympathetic Nerves Are Destroyed by Extrinsic Denervation

As described previously, glyoxylic acid reacts with neuronal stores of catecholamines, resulting in blue fluorescent sympathetic nerve fibers in submucosal (Figure 5A) and myenteric plexus (Figure 6A) preparations when viewed with fluorescent optics and an UV barrier filter. One and four weeks after surgical denervation, very little catecholamine fluorescence could be detected in submucosal (Figure 5B) or myenteric plexus (Figure 6B) preparations from 16/18 animals as compared to shams (2 animals) or controls (18 animals). Control segments taken oral or aboral to denervated segments

Figure 5. Glyoxylic acid-induced fluorescence of neuronal stores of norepinephrine in sympathetic nerve fibers in submucosal plexus preparations from normal and denervated ileum. A. Norepinephrine containing nerve fibers in normal intestine. B. Four weeks after extrinsic denervation, glyoxylic acid-induced fluorescent nerve fibers are not detected. C. Norepinephrine containing nerve fibers return to the intestine 10 weeks after extrinsic denervation. D. Sympathetic nerve fibers have not reformed the perivascular plexus 26 weeks after denervation. g, submucosa ganglion; bv, arteriole; arrow, neuron. Bar in B is approximately 30 μ m.

Figure 5.



contained a distribution and density of GA-fluorescent nerve fibers that appeared identical to segments taken from normal animals. Furthermore, 1 and 4 weeks after loss of extrinsic innervation, occasional bright yellow serotonin-containing enterochromaffin cells were identified, indicating that the absence of GA-induced fluorescence resulted from loss of extrinsic nerves, and not unintentional inhibition of the chemical reactions necessary for fluorescence of catecholamines (see methods). Finally, one week after denervation, single paravascular sympathetic nerves were associated with large ($< 40\ \mu\text{m}$) arterioles, but these nerves were very short, and were likely to be the distal portion of degenerating axons.

Approximately 10 weeks after denervation (10 animals), GA-induced fluorescent nerve fibers were not associated with many submucosal arterioles or submucosal ganglia, and GA-induced fluorescent nerve fibers were not found within interganglionic nerve strands. However, some small arterioles ($< 40\ \mu\text{m}$) had one or two fluorescent paravascular nerve fibers closely opposed to vascular walls (Figure 6C). Faint meandering paravascular nerve fibers were occasionally observed with some arterioles. The non-meandering paravascular nerves frequently ended with bright, half-mooned or crescent-shaped protrusions that were consistent with growth cones in the ANS. Perivascular innervation was not associated with most submucosal arterioles, although sympathetic nerve fibers were observed to cross over large arterioles ($> 50\ \mu\text{m}$) with multiple pairs of paravascular nerve fibers. In addition to their close association with arterioles, some single, thin, non-varicose nerve fibers projected away from arterioles towards non-fluorescent ganglia.

Ten weeks after surgical extrinsic denervation, punctate GA-induced fluorescence

of catecholamines was observed over the surface of myenteric ganglia (Figure 6C), and sympathetic nerve fibers were found within interganglionic nerve strands of the primary plexus. Similar to re-innervation of submucosal arterioles, some sympathetic nerve fibers within primary interganglionic nerve bundles ended in bright, crescent-shaped structures, which were frequently associated with a helical-like twisting of nerve endings. Sympathetic nerve fibers were never associated with longitudinal muscle.

Twenty-six weeks after extrinsic denervation (5 animals), GA-induced fluorescent nerve fibers were found with every submucosal arteriole (Figure 5D). Multiple paravascular sympathetic nerve fibers were both closely- and loosely- associated with every arteriole, and some nerve fibers contained bright varicosities and heterogeneous fluorescence. However, very few perivascular nerve fibers were observed with small arterioles ($< 50\ \mu\text{m}$) (Figure 5D) indicating that the density of sympathetic innervation was not re-established. Occasional GA-induced fluorescent nerve fibers were not associated with arterioles or ganglia, but disappeared in and out of the plane of focus (Figure 5D). As these nerve fibers were not observed in normal or control preparations, they may represent aberrant sympathetic projections. Finally, bright sympathetic nerve fibers were located in interganglionic nerve strands, and punctate staining was found in all submucosal ganglia. Interestingly, in every preparation examined, occasional submucosal ganglia contained bright catecholaminergic neurons with eccentric, non-fluorescent nuclei (Figure 5D). Although the significance of these catecholaminergic neurons is not known, enteric neurons transiently express catecholamines during development from sympathoadrenal precursor cells (Carnahan *et al.*, 1991; but see Gershon *et al.*, 1993). Therefore, phenotypic expression of sympathetic markers by some

enteric neurons after surgical denervation may mimic prenatal neurotransmitter expression (recapitulation to ontogeny), suggesting the presence of extrinsic nerves are crucial in the regulation of enteric phenotype.

Similar to submucosal preparations, all myenteric ganglia contained bright, punctate GA-induced fluorescence, although catecholaminergic neurons were not observed. Sympathetic nerves were found within interganglionic nerve tracts 26 weeks after extrinsic denervation (Figure 6D). Finally, although the number of sympathetic nerve fibers increased 26 weeks after denervation, control and normal preparations contained more fluorescence, suggesting the density of sympathetic innervation to myenteric ganglia had not been re-established by 26 weeks. Alternatively, the absence of catecholamine fluorescence may indicate decreased catecholamine production although extrinsic nerves have reinnervated ileal segments.

Extrinsic Sensory Nerves are Destroyed by Extrinsic Denervation

As described previously, innervation of the small intestine from DRG can be visualized by colocalization of SP- and CGRP-ir. One and four weeks after surgical denervation, SP-/CGRP-ir nerve fibers were not observed in submucosal preparations (Figures 7C and 7D), although bright SP-/CGRP-ir was found in thick, varicose paravascular nerve fibers in normal (Figures 7A and 7B), control (data not shown), and sham-denervated ileum (data not shown). Myenteric plexus preparations did not contain extrinsic SP-/CGRP-ir nerve fibers one week after loss of extrinsic nerves (data not shown), although SP- or CGRP-ir myenteric neuron cell bodies could be observed. As normal and control myenteric plexus preparations also contained few SP-/CGRP-ir nerve fibers (data not shown), submucosal plexus preparations were used to monitor

Figure 6. Glyoxylic acid-induced fluorescence of neuronal stores of norepinephrine in sympathetic nerve fibers in myenteric plexus preparations from normal and denervated ileum. A. Norepinephrine containing nerve fibers in normal intestine. B. Four weeks after extrinsic denervation, glyoxylic-acid induced fluorescence is not observed. C. Fluorescent sympathetic nerves return to the intestine 10 weeks after extrinsic denervation. D. The brightness and density of sympathetic innervation has not returned to normal by 26 weeks after extrinsic denervation. g, myenteric ganglion. Bar in D is approximately 40 μm .

Figure 6.

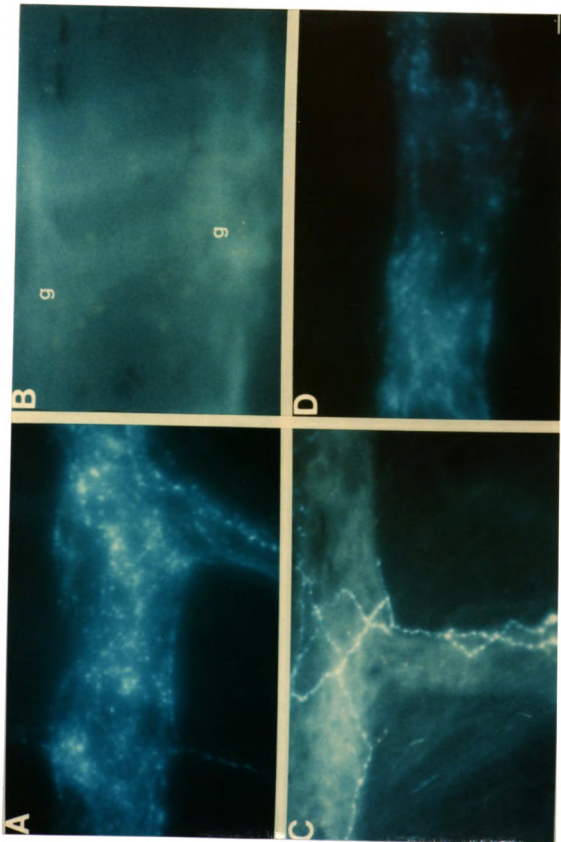
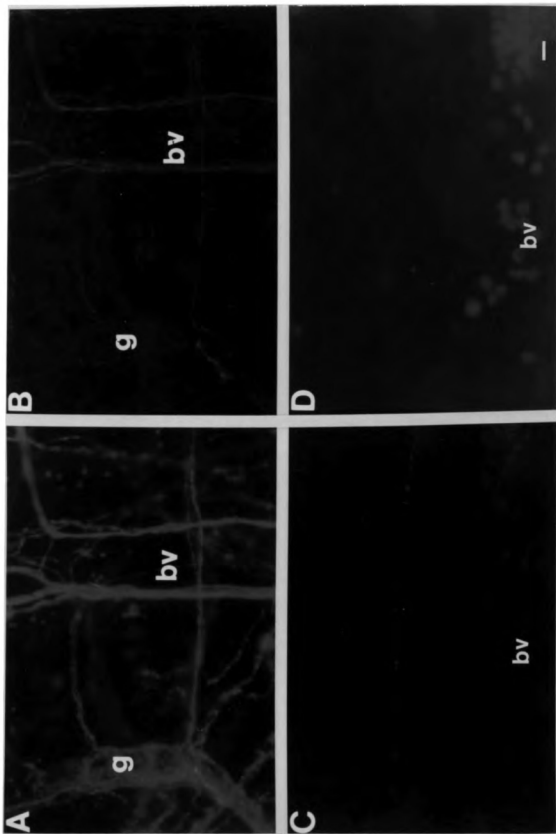


Figure 7. Co-localization of substance P and calcitonin gene related peptide-immunoreactivity in extrinsic primary afferent nerves in submucosal plexus preparations from normal and denervated ileum. A. SP-ir in perivascular and paravascular nerve fibers in normal intestine. B. Co-localization of CGRP-ir with SP-ir in nerve fibers associated with arterioles. C. SP-ir nerve fiber associated with an arteriole 4 week after denervation. D. The nerve fiber in C does not contain CGRP-ir. bv, arteriole. Bar in D is approximately 10 μ m. Note the fluorophore in A and D is Texas Red, whereas FITC-conjugated secondary antibodies were used in B and C.

Figure 7.



reinnervation of enteric neurons by extrinsic sensory nerves. Substance P-/CGRP-ir nerve fibers were not observed 7 weeks after surgical extrinsic denervation. Single, varicose SP-/CGRP-ir nerve fibers were rarely associated with submucosal arterioles 10 weeks after denervation, although occasional paravascular SP-/CGRP-ir nerves reappeared 24 weeks after loss of extrinsic nerves (Figures 22C and 22D). Brightly fluorescent SP-/CGRP-ir nerve fibers were loosely-associated with arteriolar walls, and few perivascular SP-/CGRP-ir nerve fibers transversed the surface of arterioles. Taken together, these data indicate that extrinsic sensory nerves reinnervate the ileum, and begin to return approximately 10 weeks after surgical disruption of extrinsic innervation. However, similar to reinnervation by sympathetic nerves, the density of extrinsic sensory nerves appears to remain decreased 26 weeks after denervation, indicating 26 weeks is not sufficient time for extrinsic nerve fibers to regrow into the intestinal wall. Alternatively, surgical extrinsic denervation may permanently reduce the density of extrinsic innervation to ileal segments.

Extrinsic Denervation Increases Vasoactive Intestinal Polypeptide-Immunoreactivity Innervation of Submucosal Arterioles

As described previously, few single, short, smooth or varicose VIP-ir nerve fibers are associated with submucosal arterioles in normal ileum (Figure 8A). A similar pattern and density of VIP-ir innervation associated with arterioles was observed in control (Table 2) and sham-operated animals (data not shown), as few perivascular nerve fibers contained VIP-ir, although VIP-ir was observed in some submucosal neurons and in nerve fibers in interganglionic nerve bundles which crossed over the surface of arterioles.

One week after surgical extrinsic denervation, more varicose and smooth VIP-ir

nerve fibers were associated with arterioles as compared to control tissues taken oral or aboral to denervated segments of ileum (Table 2). Furthermore, occasional pairs of paravascular VIP-ir nerve fibers were located on either side of some arterioles (Figure 8B); this pattern was never observed in arterioles from control preparations. Finally, after surgical extrinsic denervation, some VIP-ir nerves could be followed for distances over 1 mm, although VIP-ir nerve fibers in control preparations appeared and disappeared within 300 μm .

Four weeks after loss of extrinsic innervation, VIP-ir nerve fibers associated with most arterioles (Figure 8C), and a sparse VIP-ir perivascular nerve plexus formed around most arterioles by 7 weeks after surgery (Figure 8D). Although immunohistochemical methods did not allow quantitative measures of VIP-ir, VIP-ir vascular innervation appeared brighter, and fluorescence was more homogenous as compared to controls. Paravascular and perivascular VIP-ir nerve fibers were found with almost every arteriole 10 weeks after denervation, and a VIP-ir plexus was associated with arterioles 16 and 24 weeks after surgical denervation. These data support and expand the time course of denervation-induced alterations in VIP-ir vascular innervation previously reported (Galligan *et al.*, 1988), and indicate that enteric nerves exhibit neuronal plasticity by increasing the density of VIP-ir nerve fibers associated with submucosal arterioles following loss of extrinsic innervation.

Extrinsic Denervation Results in New Substance P-Immunoreactive Innervation of Submucosal Arterioles

Loss of extrinsic innervation also resulted in marked alterations in the density and distribution of SP-ir vascular innervation. As described previously, bright SP-ir is found

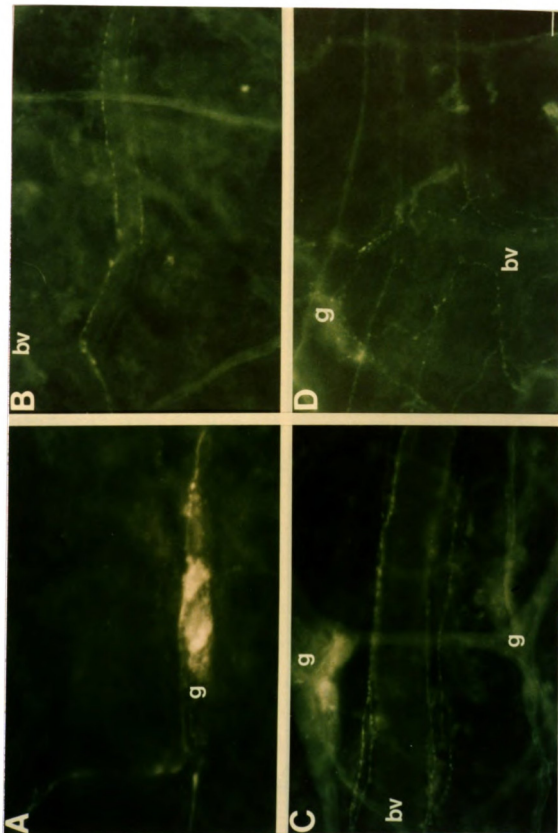
Table 2. Survey of the number of arterioles with nerve fibers containing vasoactive intestinal polypeptide-immunoreactivity in normal, control, and denervated ileum.

Weeks After Dener- vation	Arteriolar Size (μm)						
	0-10	11-20	21-30	31-40	41-50	51-60	61-70
normal	0/7	1/11	3/14	2/16	0/5	0/4	0/3
week1 control	0/7	1/12	2/17	1/7	1/10	1/7	0/7
week1 denerv.	4/10	8/10	18/20	7/11	2/7	2/5	0/4
week4 control	0/8	3/8	1/9	1/8	2/4	1/3	0/7
week4 denerv.	4/8	12/13	14/16	7/7	7/8	0/4	0/8
week10 control	0/5	1/9	1/13	0/11	1/5	0/6	0/3
week10 denerv.	7/7	10/10	11/12	9/10	3/7	1/5	6/10
week24 control	1/8	1/7	0/17	1/14	0/6	0/3	2/10
week24 denerv.	8/9	13/15	11/12	4/12	1/5	1/4	1/5

Description of the fraction of submucosal arterioles receiving VIP-ir nerves/total submucosal arterioles examined. A single VIP-ir paravascular nerve was associated with controls. See text for description of innervation of arterioles. Data are taken from a minimum of 3 preparations for each time point.

Figure 8. Vasoactive intestinal polypeptide-immunoreactivity in intrinsic nerve fibers in submucosal plexus preparations from normal and denervated ileum. A. VIP-ir in submucosal plexus from normal ileum. B. Paravascular VIP-ir nerve fibers associate with an arteriole 1 week after denervation. C. Perivascular and paravascular VIP-ir nerves associate with an arteriole 7 weeks after extrinsic denervation. D. VIP-ir nerves form a perivascular vascular plexus 10 weeks after denervation. bv, arteriole; g, submucosal ganglion. Bar in D is approximately 40 μ m.

Figure 8.



co-localized with CGRP-ir in extrinsic, capsaicin-sensitive varicose paravascular nerve fibers (Figures 7A and 7B). Although SP-ir nerve fibers that surrounded submucosal ganglionic neurons did not contain detectable CGRP-ir, SP-ir nerve fibers without detectable CGRP-ir were not found to associate with submucosal arterioles in normal preparations.

Extrinsic SP-/CGRP-ir nerve fibers were not observed 1 and 4 weeks after extrinsic denervation. However, four weeks after surgical extrinsic denervation, faint SP-ir axons were associated with arterioles (Figures 7C and 7D). These axons were unlikely to be extrinsic primary afferents, as they did not contain CGRP-ir, had small varicosities, were not sensitive to systemic capsaicin administration, and appeared to have a smaller caliber than extrinsic sensory nerve fibers. Substance P-ir vascular nerve fibers were not found in control or sham-denervated submucosal preparations, indicating the appearance of these axons resulted from loss of extrinsic innervation and not surgical trauma. Four weeks after denervation, paravascular SP-ir nerve fibers were only associated with small arterioles (Table 3), and SP-ir nerve fibers never contained VIP-ir (data not shown). The number of arterioles with SP-/but not CGRP-ir nerve fibers and the density of new SP-ir nerve fibers/arteriole increased 7, 10, and 16 weeks after loss of extrinsic innervation. Furthermore, SP-ir paravascular nerve fibers were associated with greater lengths of arterioles, and some arterioles had multiple non-varicose SP-ir nerve fibers associated on either side of the vessel (see Figures 21A and 21B) 7 and 10 weeks after denervation. Although SP-ir nerve fibers were closely associated with submucosal arterioles 4 and 7 weeks after denervation, SP-ir nerve fibers were located 15-35 μm away from arterioles 16 weeks after denervation. Finally, a loose paravascular SP-ir plexus surrounded

Table 3. Survey of the number of arterioles with nerve fibers containing substance P-, but not calcitonin gene-related peptide-immunoreactivity in normal, control, and denervated ileum.

Weeks After Dener- vation	Arteriolar Size (μm)						
	0-10	11-20	21-30	31-40	41-50	51-60	61-70
normal	0/10	0/10	0/9	0/7	0/6	0/3	0/3
week1 control	0/9	0/10	0/13	0/5	0/5	0/4	0/7
week1 denerv.	5/8	4/7	8/10	6/8	0/4	0/5	0/4
week4 control	0/8	0/9	0/11	0/7	0/4	0/4	0/7
week4 denerv.	5/9	9/12	15/18	9/10	1/5	0/5	0/8
week10 control	0/5	0/7	0/12	0/6	0/4	0/6	0/3
week10 denerv.	6/8	13/13	17/17	9/11	2/6	1/4	6/10
week24 control	0/10	0/11	0/7	0/8	0/3	0/2	2/10
week24 denerv.	7/10	10/12	9/12	7/11	0/5	0/4	1/5

Description of the fraction of submucosal arterioles receiving nerves containing SP-, but not CGRP-ir nerves/total submucosal arterioles examined. See text for description of innervation of arterioles. Data are taken from a minimum of 3 preparations for each time point.

arterioles by 16 weeks after denervation. Although some bright, thick, varicose nerves contained SP/CGRP-ir, thin SP-ir nerves without CGRP-ir could be detected at this late time point (see Figures 21A and 21B).

Specificity of Primary and Secondary Antiserum

As immunohistochemistry was used to localize extrinsic and enteric nerves, it was imperative to determine the specificity of antisera raised against ChAt, CGRP, cFos, NOS, NPY, npNF-H, pnpNF-H, pNF-H, SP, and VIP. All of the secondary antisera recognized the intended IgG epitope of primary antisera, as fluorescence was not observed in tissues incubated in secondary antiserum without primary antiserum. Furthermore, secondary antisera did not bind to incorrect primary antisera except goat anti-mouse IgG and M conjugated to rhodamine and goat anti-mouse IgG conjugated to Cy3. Both antisera recognized bound antiserum raised against SP and were not used in co-localization studies. Goat anti-rabbit IgG conjugated to Cy3 bound some of the mouse and rat antiserum. Therefore, this secondary antiserum was not used for co-localization studies. All of the primary antisera were able to recognize the target peptide as peptide neutralization for CGRP, cFos, NPY, npNF-H, pnpNF-H, pNF-H, SP, and VIP prevented immunoreactivity.

Specificity and Effectiveness of Chemical Denervations

As described in the preceding pages, surgical denervation of loops of guinea pig ileum eliminates all extrinsic innervation. Therefore 6-OH-DA and capsaicin were used to destroy extrinsic sympathetic and sensory nerves, respectively. Glyoxylic acid-induced fluorescence of neuronal stores of catecholamines was used to verify effectiveness of 6-OH-DA treatment, as this histological technique could be performed in minutes utilizing

fresh tissue. As soon as one week after systemic administration of 6-OH-DA, almost no catecholamine fluorescence could be detected in submucosal or myenteric plexus preparations. However, segments taken from vehicle-treated controls contained a distribution of GA-fluorescent nerve fibers identical to segments taken from normal animals. Treatment with 6-OH-DA did not alter the density or appearance of SP-/CGRP-ir nerve fibers, or the localization of SP-, CGRP-or NPY-ir in enteric nerve cell bodies or axons, indicating that 6-OH-DA was a specific neurotoxin for sympathetic nerve fibers. Chemical denervation by capsaicin did not alter localization of SP- or CGRP-ir in enteric nerve cell bodies or nerve fibers, or the density and appearance of GA-induced fluorescent nerve fibers. However, capsaicin administration destroyed extrinsic sensory nerve fibers as visualized by immunohistochemical detection of SP-/CGRP-ir nerve fibers. Taken together, these data agree with previously published data illustrating the specificity of 6-OH-DA and capsaicin.

Specificity of Surgical Extrinsic Denervations

Hematoxylin and eosin staining was used to determine if surgical manipulations of loops of ileum or direct loss of extrinsic nerves damaged mucosa or altered physical properties of GI smooth muscle. Abrupt cessation of blood flow to the gut results in rapid gangrene, ultimately producing peritonitis (reviewed in Gallavan *et al.*, 1989). Because of its high resting demand for nutrients and oxygen, the mucosa is exquisitely sensitive to oxygen deprivation (Gallavan *et al.*, 1989), unlike slowly-responding smooth muscle. During oxygen deprivation, mucosal cells are unable to exclude luminal contents such as bacteria and proteolytic enzymes, resulting in sloughing and disruption of mucosal crypts, and ulceration (Gallavan *et al.*, 1989). Breakdown of the mucosal barrier allows

fluids and blood to enter the lumen, where microorganisms produce distension of the bowel. Therefore, the mucosa was examined in cross-sections of ileum to determine if mucosa damage occurred after surgical extrinsic denervation.

Submucosal arterioles were crushed for only 3 sec/crush during surgical denervations, after which time blood flow did not appear to be interrupted. This observation was supported by morphological observations of denervated loops of ileum. One week after surgery, loops of denervated ileum were pink and oxygenated, and did not appear different in size or color than segments of ileum oral or aboral to the denervation. Hematoxylin and eosin staining was used to stain denervated and control tissues, thus allowing cellular structures to be observed. Coded slides of surgically-denervated or control transverse sections were undistinguishable from each other, as mucosal crypts did not appear to be altered by loss of extrinsic nerves. Finally, a board-certified pathologist did not observe mucosal damage manifested as sloughing or ulceration in surgically-denervated or control tissues. Infiltration by macrophages and neutrophils also was not observed. Taken together, these results suggest that surgical extrinsic denervation does not interrupt blood flow to denervated segments.

During disease and changes in diet, hyperplasia and/or hypertrophy can occur, resulting in increased thickness of GI smooth muscle. Hyperplasia (increased muscle cell number) and hypertrophy (increased cell volume not associated with mitosis) occur after stenosis or transection of the GI tract (reviewed in Gabella, 1989). To determine if surgical manipulations or loss of extrinsic innervation altered nuclear events in muscle cells resulting in hyperplasia or hypertrophy of smooth muscle cells, the wet weight of LMMPs was examined in control and denervated preparations. Following preparation

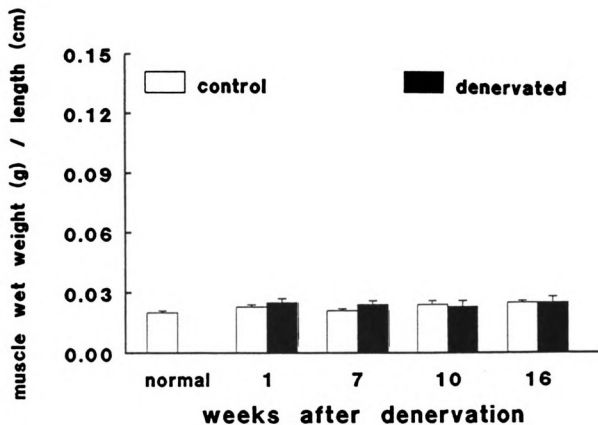
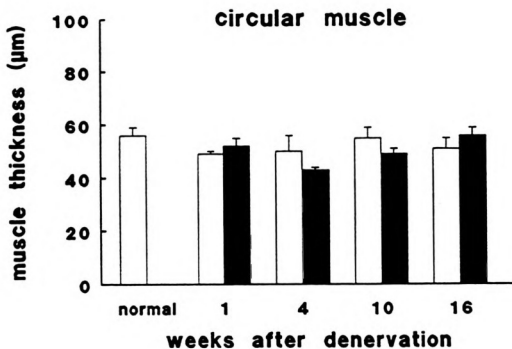
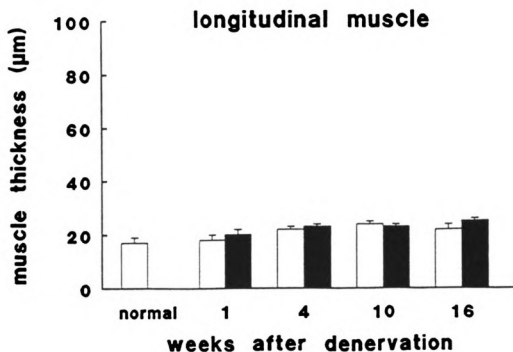


Figure 9. Weight of longitudinal muscle myenteric plexus preparations from normal, control, and denervated ileum. LMMPPs were prepared as described in methods, weighted in a known volume of water, and the length of each preparation was recorded. Data are expressed as means of muscle weight/length of preparation $\pm$ SEM for 3-4 preparations from 3-4 animals for each time.

Figure 10. Thickness of circular and longitudinal muscle in transverse sections from normal, control, and denervated ileum. Transverse sections of ileum were prepared as described in methods, stained for hematoxylin and eosin, and muscle thicknesses were measured using an eye-piece micrometer at 400x magnification. A. Thickness of circular muscle in normal, control, and denervated preparations. B. Thickness of longitudinal muscle. *Open bars* represent values from control preparations and *solid bars* represent values from denervated preparations. Data are expressed as means of muscle thicknesses $\pm$ SEM for 6-9 sections from 2-3 animals for each time.

Figure 10.

A**B**

of LMMPPs, LMMPPs were weighed in a known volume of water, and the length of each preparation was recorded. These data were normalized for the length of each tissue, and the values compared for control and denervated segments. An increase in this ratio would be expected if hypertrophy or hyperplasia occurred. As shown in Figure 9, loss of extrinsic nerve fibers did not alter the weight of LMMPPs, suggesting that extrinsic denervation did not induce hyperplasia or hypertrophy of longitudinal muscle cells.

Potential changes in circular and longitudinal muscle were investigated further by examining hematoxylin and eosin stained transverse sections of control and surgically-denervated ileum. The borders that separate longitudinal and circular muscle from other laminae of the ileum are discrete and easily visualized (see Figure 1A), and an eye-piece micrometer was used to measure the thickness of circular and longitudinal muscle in normal, control, and denervated ileum. As shown in Figures 10A and 10B, extrinsic denervation did not alter the thickness of circular or longitudinal muscle as compared to sections taken from animal-matched control segments. These data support the conclusion that neither muscle division, nor expansion of existing muscle cells, occurred after surgical extrinsic denervation.

2. NEW SUBSTANCE P-IMMUNOREACTIVE NERVES DO NOT ORIGINATE FROM SUBMUCOSAL GANGLIA

Introduction and Experimental Rationale

There are three possible mechanisms to account for the presence of new SP- and VIP-ir nerve fibers associated with submucosal arterioles after loss of extrinsic innervation. First, increased SP- and VIP-ir nerves may not represent an increase in the total number of intrinsic nerve fibers, but may result from increased content of SP or VIP in nerves previously associated with arterioles. As discussed previously, the concentrations of protachykinin mRNA and SP increases after decentralization of SCG neurons (Kessler *et al.*, 1981). These data suggest that loss of trans-synaptic activity upregulates the expression of SP. Although increased SP and VIP is possible, this explanation does not account for all of the alterations observed in vascular innervation after extrinsic denervation. Upregulation of the concentrations of neuropeptides occurs quickly after perturbation of nerves (Rao and Landis, 1992), and neuropeptide levels do not continue to increase over time after decentralization (Rao and Landis, 1992). As the number of VIP- and SP-ir nerve fibers increased over time, an alternative mechanism appeared plausible. Alterations in innervation associated with arterioles may result from changes in phenotypic expression of enteric neurons, such as *de novo* synthesis of neuropeptides previously not expressed. Following long-term sympathectomy, dopamine β -hydroxylase-ir appears in cholinergic nerves of rat cerebral arteries (Mione *et al.*, 1991), although this protein is normally only expressed by sympathetic nerves. These results imply that loss of sympathetic innervation induces translation and/or transcription of dopamine- β -hydroxylase. Therefore, surgical extrinsic denervation may result in new

patterns of expression of neuropeptides not previously observed in existing vascular nerves. Finally, new SP- and VIP-ir nerves may result from axonal sprouting of enteric nerves. Axonal sprouting is induced in response to nerve injury such as transection and crushing (see introduction). For example, autonomic preganglionic nerve fibers form new axons after partial denervation of SCG (Murray and Thompson, 1957). Similarly, sensory nerve fibers sprout after destruction of sympathetic nerve fibers in sweat gland of rats (Yodlowski *et al.*, 1984), and peripheral nerve injury triggers central (Woolf *et al.*, 1992) and peripheral (Pestronk and Crachman, 1988) sprouting of myelinated afferent nerve fibers. Therefore, appearance of new innervation associated with submucosal arterioles may result from sprouting of existing axons, increased concentrations of previously-existing (but not detectable) neurotransmitters, or *de novo* synthesis of new neuropeptides in nerves previously associated with submucosal arterioles.

As described in the previous section, enteric neurons respond to loss of extrinsic innervation by increasing the density of SP- and VIP-ir nerve fibers associated with submucosal arterioles. The presence of these nerve fibers is not altered by systemic capsaicin administration (Galligan *et al.*, 1988), further indicating that the nerves originate from submucosal or myenteric ganglia. However, previous experiments did not determine the mechanism by which new innervation resulted, or the source of these new nerves. These questions were addressed by combining immunohistochemistry with retrograde tracing of enteric nerves innervating submucosal arterioles in normal, control, and surgically-denervated preparations of submucosal plexus. Fluoro-gold (Fg) is a useful retrograde neurotracer in the CNS, as it is not taken up by fibers of passage. Furthermore, Fg fluorescence can be detected in fresh and fixed tissues using fluorescence

microscopy. Therefore, it was hypothesized that if Fg was injected into submucosal arterioles, then nerve fibers innervating arterioles would retrogradely transport Fg, resulting in labelling of enteric neurons. If loss of extrinsic innervation stimulates axonal sprouting from submucosal neurons, then the number of Fg-labelled neurons/ganglion would increase as compared to control preparations. Alternatively, if loss of extrinsic innervation upregulates or stimulates *de novo* synthesis of SP and/or VIP in nerve fibers previously associated with arterioles, then the numbers of Fg uptake sites and Fg-labelled submucosal neurons/ganglion would be similar in control and denervated tissues. However, as previous work has demonstrated four classes of submucosal neurons (Furness and Costa, 1987), increased Fg-labelled neurons/ganglion could result from axonal sprouting of non-SP or non-VIP neurons. Therefore, co-localization of Fg and SP- or VIP-ir would determine if increased Fg-labelling in submucosal neurons resulted from axonal sprouting of VIP- or SP-ir neurons.

If axonal sprouting does not occur, then new nerve fibers associated with arterioles may result from increased concentrations of SP and VIP in enteric nerves previously associated with submucosal arterioles. Alterations in the number of Fg-labelled neurons containing SP- or VIP-ir would indicate increased synthesis of new neurotransmitters in nerves previously associated with arterioles. Unfortunately, these experiments would not discriminate between elevated synthesis of previously existing neurotransmitters from *de novo* synthesis and altered neuronal phenotype. Furthermore, this experimental design assumes increased concentrations of SP or VIP in nerve fibers near arterioles would also substantially increase neuropeptide concentrations in the cell somata, allowing detection using immunohistochemistry.

The Retrograde Tracer Fluoro-gold Labels Submucosal Neurons in Normal Preparations

Proceeding successful cannulation of 30-45 μm resistance vessels, pressure injections of fluoro-gold filled arterioles with yellow fluorescent dye with very little leakage outside the arteriole. Micropipettes were not withdrawn immediately after injection, as a greater amount of Fg remained within arterioles thus greatly reducing the spread of fluorescence over sites of injection (Figure 11A). Twenty-four or forty-eight hours after injection, occasional nucleated satellite cells were labelled with Fg (Figure 11B). These cells were found near injected arterioles or surrounding nearby ganglia and did not have a morphology consistent with submucosal neurons. Although immunohistochemical studies were not performed to identify these cells, their morphology was consistent with Schwann cells and enteric glia. Enteric glial cells are frequently associated with submucosal ganglia and have long processes which radiate from centrally-located perikarya to terminate in small swellings (Gershon and Rothman, 1991). Schwann cells are found near blood vessels and are distinguished by centrally-located perikarya and symmetrical flat or thin cytoplasm projections.

Fluoro-gold labelling was also observed in submucosal ganglia (Figure 11C). In normal preparations, 0.19 ± 0.02 neurons/submucosal ganglion (67 neurons, 386 ganglia, 3 animals, 4 preparations) contained Fg. These neurons had bright homogeneously-stained cytoplasm, broad and flat dendrites consistent with Dogiel type III neurons, and dark, less-stained eccentrically-placed nuclei (Figure 11C). Occasional axons could be traced for short distances ($< 50 \mu\text{m}$). It was likely that neuronal labelling with Fg resulted from specific retrograde transport by enteric nerves and not uptake of Fg by cell bodies, as preliminary experiments revealed at least 24 hours was required for neurons to contain Fg.

Figure 11. Fluoro-gold labelling of neurons in submucosal plexus preparations from normal and denervated ileum. A. Whole mount preparation showing Fg-staining over arteriolar site of injection. B. Occasional small, non-ganglionic cells contained Fig. C. Fg-labelled submucosal ganglionic neuron in normal ileum. D. Fg-labelled submucosal ganglionic neuron in ileum denervated 6 weeks prior to experiment. bv, arteriole; g, submucosal ganglion; arrow, labelled cell or neuron. Bar in D is approximately 30 μm .

Figure 11.

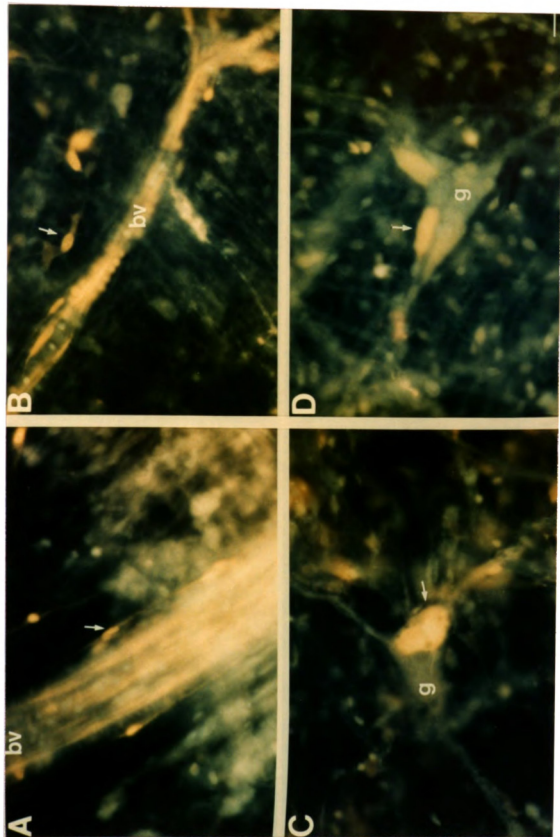


Table 4. Percentage of Fluoro-gold-labelled neurons containing substance P- or vasoactive intestinal polypeptide-ir in submucosal ganglia in normal, control, and denervated ileum.

Treatment (Weeks after Denervation)	SP-ir/Fg-labelled cells (SP cells, Fg cells)	VIP-ir/Fg-labelled cells (VIP cells, Fg cells)
normal	6 ± 6% (1,18)	0 ± 0% (0,23)
control, <i>week 6</i>	5 ± 3% (2,21)	4 ± 4% (1,38)
denervated, <i>week 6</i>	6 ± 4% (1,13)	13 ± 8% (2,33)
control, <i>week 8</i>	0 ± 0% (0,6)	0 ± 0% (0,20)
denervated, <i>week 8</i>	7 ± 7% (1,23)	8 ± 8% (1,9)

Data were tested for significance ($p < 0.05$) with the Wilcoxon rank-sum test for nonparametric data.

Furthermore, injections directly above arterioles failed to labelling of submucosal neurons, indicating Fg-labelled neurons did not occur by uptake of Fg located outside of arterioles. Co-localization studies with antiserum raised against SP or VIP revealed that 6 ± 6% of Fg-filled neurons contained SP-ir, whereas 0 ± 0% of Fg-filled neurons contained VIP-ir in normal submucosal preparations (Table 4). As 56% of submucosal neurons contain either VIP- or SP-ir (reviewed in Furness and Costa, 1987; see introduction), these data indicate that Fg positive submucosal neurons must either contain ChAt, or ChAt/cholecystokinin/CGRP NPY/ and somatostatin (see introduction).

Extrinsic Denervation Does Not Result In Substance P-Immunoreactive Axonal Sprouts from Submucosal Neurons

Four weeks after surgical denervation, 0.18 ± 0.02 submucosal neurons/ganglion (34 neurons, 198 ganglia, 3 preparations, 2 animals) were labelled with Fg. Similar to control and normal preparations, these neurons were located at the periphery of ganglia, contained homogeneously-stained cytoplasm, and had eccentrically-placed nuclei. Their morphology was consistent with Dogiel type III neurons. Furthermore, loss of extrinsic innervation did not alter the number of Fg-labelled neurons as compared to animal-matched controls (0.19 ± 0.02 submucosal neurons/ganglion, 35 neurons, 180 ganglia, 3 preparations, 2 animals) (Figure 12), indicating axonal sprouting of submucosal neurons did not occur. Similar results were obtained 6 (Figure 11D) and 8 weeks after surgical extrinsic denervation, as 0.22 ± 0.04 (76 neurons, 341 ganglia, 6 preparations, 3 animals) and 0.14 ± 0.02 (34 neurons, 244 ganglia, 5 preparations, 3 animals) Fg-labelled submucosal neurons/ganglion were observed 6 and 8 weeks after loss of extrinsic nerves. These results were not different from animal-matched controls 6 (0.2 ± 0.06 Fg neurons/ganglion, 41 neurons, 204 ganglia, 4 preparations, 2 animals) and 8 (0.19 ± 0.02 Fg neurons/ganglion; 30 neurons, 163 ganglia, 4 preparations, 2 animals) weeks after loss of extrinsic innervation, indicating axonal sprouting from submucosal nerves did not occur at later times after surgical denervation.

Some preparations were processed for immunohistochemical detection of SP- and/or VIP-ir. Six and weeks after surgical denervation, $6 \pm 4\%$ and $13 \pm 8\%$ of Fg neurons contained SP- and VIP-ir, respectively, and similar results were observed 8 weeks after loss of extrinsic innervation (Table 4). Although $6 \pm 2\%$ of Fg neurons contained SP in control animals, $0 \pm 0\%$ of Fg neurons contained VIP-ir, indicating VIP may

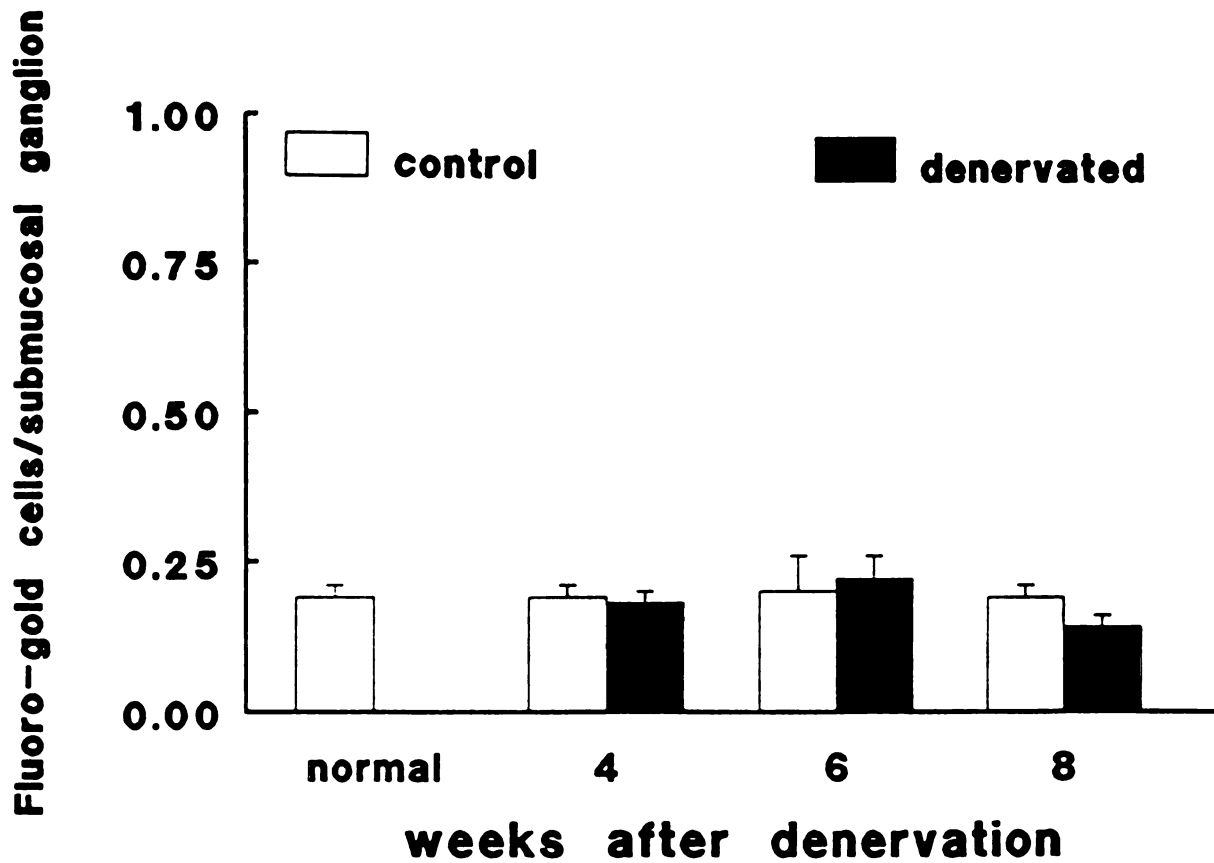


Figure 12. Number of Fluoro-gold labelled neurons in submucosal ganglion from normal, control, and denervated ileum. Retrograde transport was allowed for 48 hours in all preparations. Data are means of Fg-cells/submucosal ganglion $\pm$ SEM for 3-6 preparations for each time examined.

increase in a population of submucosal neurons following extrinsic denervation, although extrinsic denervation does not induce axonal sprouting of submucosal neurons. However, SP- and VIP-ir were examined in less than 30 Fg-labelled neurons for each time point, suggesting additional experiments are necessary to determine if there is a statistically-significant increase in VIP-ir Fg-labelled neurons after denervation.

3. LOCALIZATION OF PHOSPHORYLATED AND NONPHOSPHORYLATED HEAVY NEUROFILAMENT PROTEIN IN ENTERIC NERVES

Introduction

In order to use phosphorylation-sensitive antibodies raised against NF-H, it was necessary to characterize their binding in preparations made from normal guinea pig ileum. Therefore, antibodies raised against phosphorylated, nonphosphorylated, and phosphorylation-independent epitopes of NF-H were used to determine if discrete subpopulations of enteric neurons contained NF-H, and if the distribution of phosphorylated NF-H was homogeneous in extrinsic and intrinsic nerves. As described previously, SMI 31, SMI 32, and SMI 33 are monoclonal antibodies raised against neurofilament proteins from rat hypothalamus (Sternberger and Sternberger, 1983). Immunological and immunohistochemical studies have shown that SMI 31 recognizes an extensively phosphorylated epitope on NF-H (pNF-H), and to a lesser extent, on NF-M, SMI 32 reacts with a nonphosphorylated epitope on NF-H (npNF-H), and SMI 33 recognizes a nonphosphorylated epitope that is accessible in both phosphorylated and nonphosphorylated NF-H (pnpNF-H) (Sternberger and Sternberger, 1983). Although this series of antibodies can recognize NF-M, approximately 90% of total reactivity is with the 200 Kda neurofilament subunit (Pestronk *et al*, 1990; Yee *et al.*, 1988; Watson *et al.*, 1989), suggesting the nomenclature of "NF-H" is appropriate.

Specificity of Sternberger Monoclonal Antibodies

Staining with SMI 31, SMI 32, and SMI 33 was abolished following pre-incubation of each antiserum with neurofilaments isolated from bovine spinal cord, indicating that each antibody recognized neurofilament proteins (data not shown). Trypsin

and phosphatase pretreatment of submucosal preparations was used to verify that each antibody was specific for pNF (SMI 31), npNF (SMI 32), or pnpNF (SMI 33). Pretreatment of submucosal preparations with alkaline phosphatase dephosphorylates phosphorylated NF-H-ir, and resulted in decreased SMI 31 localization, but not binding of SMI 32 or SMI 33, indicating that binding of SMI 31 requires the presence of phosphate groups on the target antigen. Pretreatment with trypsin did not alter SMI 33 binding, but abolished staining by SMI 32. As trypsin is a serine protease that cleaves the carboxyl terminal end of arginine and lysine residues unless such sites are protected by post-translational modifications, such as phosphorylation (Carden *et al.*, 1985; Sternberger and Sternberger, 1983; Nixon *et al.*, 1982), these results strongly suggest that SMI 32 recognizes a nonphosphorylated epitope. Trypsin did not alter staining by SMI 31, presumably due to the presence of phosphorate groups on NF-H. Sequential trypsin and alkaline phosphatase treatment destroyed pNF-H- and npNF-H-ir. Additional staining was not revealed with SMI 32, suggesting that potential epitopes are not masked by phosphorylation. Finally, the pattern of staining for SMI 33 was not altered by phosphatase or trypsin, suggesting SMI 33 recognizes a nonphosphorylated epitope that is accessible in phosphorylated and nonphosphorylated neurofilaments (Sternberger and Sternberger, 1983; Watson *et al.*, 1989).

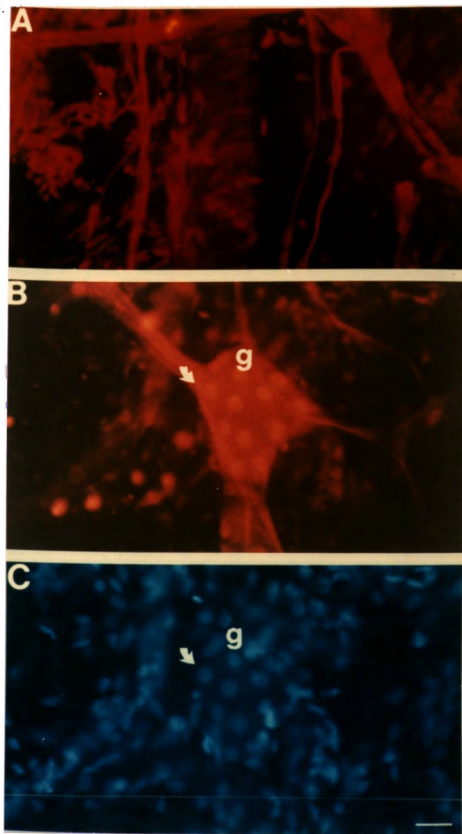
The patterns of immunoreactivity for pNF-H-, npNF-H-, and pnpNF-H-ir in the submucosal plexus were similar irrespective of the buffer used to dilute the antibodies. This observation suggests that binding of SMI 31, 32, or 33 is not altered by the presence of phosphate in the diluent buffer.

Phosphorylated NF-H-ir Is Found in Submucosal Nuclei and Nerve Fibers

Incubation with SMI 31, SMI 32, and SMI 33 gave different staining patterns in the submucosal and myenteric plexuses. NF-H is the most phosphorylated component of the neurofilament triplet, as it contains approximately 22 moles of phosphate/mole of polypeptide (Julien and Mushynski, 1982). As a result, most antiserum raised against NF-H recognizes a phosphorylated epitope (Carden, 1985). Similar to other studies in the CNS (Sternberger and Sternberger, 1983) and ENS (Eaker *et al.*, 1990), little pNF-ir was observed in neuronal cytoplasm, as dendrites and somata of submucosal neurons did not contain NF-H-ir. However, strong pNF-H-ir was localized in nerve fibers associated with submucosal arterioles (Figure 13A), interganglionic nerve strands (Figure 13B), and occasionally in single axons transversing through the submucosal plexus (Figure 13B). However, distinct elliptical and round-shaped bodies were stained in submucosal preparations (Figure 13B), with the brightest staining within submucosal ganglia. There were 7.1 ± 0.5 (7,355 organelles, 1,116 ganglia, 13 preparations, 4 animals) bodies/submucosal ganglion that measured $19 \pm 2 \mu\text{m}$ in the long axis and $9.0 \pm 0.3 \mu\text{m}$ in the short axis. This immunoreactivity was co-localized with the nuclear stain bisbenzimidazole (Figures 13B and 13C), suggesting that SMI 31 also recognizes an epitope found in ganglionic nuclei. The pattern of immunoreactivity was not uniform in the nuclei, as 4-6 circular areas of less than $1 \mu\text{m}$ were devoid of staining in each nucleus. Furthermore, there was frequently a dark ring devoid of any immunoreactivity surrounding the nucleus, and the remaining cytoplasm of the cell contained very faint filamentous staining which appeared continuous with the interganglionic strands. The co-localization of SMI 31 and bisbenzamide suggest that enteric nuclei contain a protein

Figure 13. Phosphorylated heavy molecular weight neurofilament protein-immunoreactivity in nuclei and nerve fibers in submucosal plexus preparations in normal ileum. A. pNF-H-ir in nerve fibers associated with submucosal arterioles. B. SMI 31-ir in organelles found within a submucosal ganglion. C. Co-localization with Hoechst dye 33528 (bisBenzimide, bB) within a submucosal ganglion identifies organelles as nuclei. bv, arteriole; arrow in A indicates perivascular nerve fiber, arrow in B indicates stained SMI 31-ir nuclei. Calibration bar in C is 30 μ m.

Figure 13.



with sequence homology to neurofilament proteins. There is some homology between intermediate filament proteins and lamins, a family of structural proteins found within the nuclear envelope (McKeon, 1986). However, nuclear staining has not been reported in other tissues using antiserum raised against pNF-H, suggesting that enteric nuclei contain unique phosphorylated proteins that may protect the nuclear organelles against mechanical forces that develop during motility (see Sharkey *et al.*, 1990). Alternatively, SMI 31 may recognize phosphorylated KSP or other similar sequences such as those found in histone H1 or some other nuclear protein (Wood, 1982).

As extrinsic sympathetic, parasympathetic, and sensory nerve fibers are associated with submucosal arterioles, immunohistochemical studies were used to determine the identity of pNF-H-ir nerve fibers in the vascular plexus. Previous work described in the preceding pages has demonstrated that each population of nerves contains unique neuropeptides, as sympathetic nerves contain NPY-ir (Furness *et al.*, 1985), and extrinsic sensory nerves contain CGRP- and SP-ir (Gibbins *et al.*, 1985). Enteric nerve fibers associated with submucosal arterioles contain VIP-ir but not NPY-, SP-, or CGRP-ir (reviewed in Furness and Costa, 1987). Phosphorylated NF-H-ir was found co-localized with NPY- (data not shown), CGRP- (Figures 15C and 15D), and VIP-ir (Figures 15E and 15F) in nerve fibers associated with arterioles. This pattern of co-localization suggests that pNF-H-ir is found in extrinsic and intrinsic nerves associated with submucosal arterioles.

The identity of pNF-H-ir nerves were verified using surgical and chemical extrinsic denervations. Surgical extrinsic denervation of loops of ileum destroys extrinsic innervation, as previously described. Loss of extrinsic innervation abolished pNF-H-ir

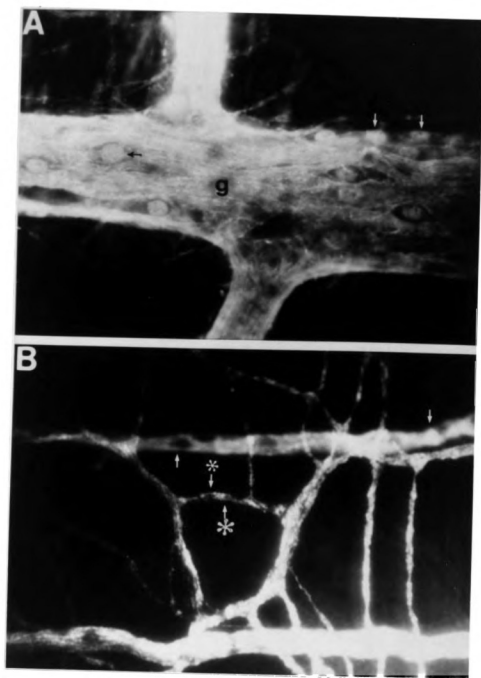
nerves associated with submucosal arterioles, but not pNF-H-ir in interganglionic nerve strands, one week after surgical denervation (Figure 16A). Phosphorylated NF-H-ir nerve fibers were not altered in tissues taken from unoperated segments of ileum from the same animals (Figure 16B). Phosphorylated NF-H-/NPY-ir, pNF-H/CGRP-ir, or pNF-H-/VIP-ir nerve fibers were rarely observed after extrinsic denervation, and sham surgeries did not affect immunoreactivity or localization of any antigen (data not shown). As predicted, administration of capsaicin, but not the vehicle, eliminated pNF-H-/CGRP-ir nerve fibers associated with arterioles (Figures 16C and 16D) one week after treatment; capsaicin administration did not affect pNF-H-/NPY-ir or pNF-H-/VIP-ir nerves. Finally, systemic 6-OH-DA treatment destroyed pNF-H-/NPY-ir nerves associated with arterioles (Figures 16E and 16F), but did not alter nerves containing pNF-H-/CGRP-ir or pNF-H-/VIP-ir. Systemic vehicle administration did not affect pNF-H-ir or neuropeptide-ir (data not shown). Taken together, these data indicate that extrinsic sympathetic and sensory nerves contain pNF-H-ir.

Phosphorylated NF-H-ir Is Found in Myenteric Nuclei and Nerve Fibers

Bright pNF-H-ir was localized in interganglionic nerve bundles of primary and secondary plexuses, and faint pNF-H-ir was observed in the tertiary plexus (Figure 17A and 17B). However, pNF-H-ir was not observed in nerve varicosities. SMI 31-ir was co-localized with bisbenzamide in cell organelles (data not shown), suggesting SMI 31 recognizes an epitope found in all enteric nuclei. Bright SMI 31-ir was localized in myenteric ganglia (108 ± 16 nuclei/ganglion; 13,066 nuclei, 100 myenteric ganglia, 9 preparations, 3 animals). Nuclear staining by SMI 31 was not uniform, as small circular or ovoid areas were devoid of immunoreactivity, similar to the pattern of SMI 31-ir

Figure 14. Phosphorylated heavy molecular weight neurofilament protein-immunoreactivity in nuclei and nerve fibers in myenteric plexus preparations in normal ileum. A. pNF-H-ir nerve fibers of primary and secondary plexuses. B. Faint pNF-H-ir in tertiary plexus. g, myenteric ganglion; arrow indicates secondary plexus; arrow with asterisk indicates tertiary plexus; small arrow indicates nuclei immunoreactive for SMI 31. Calibration bar in D is 30 μ m.

Figure 14.



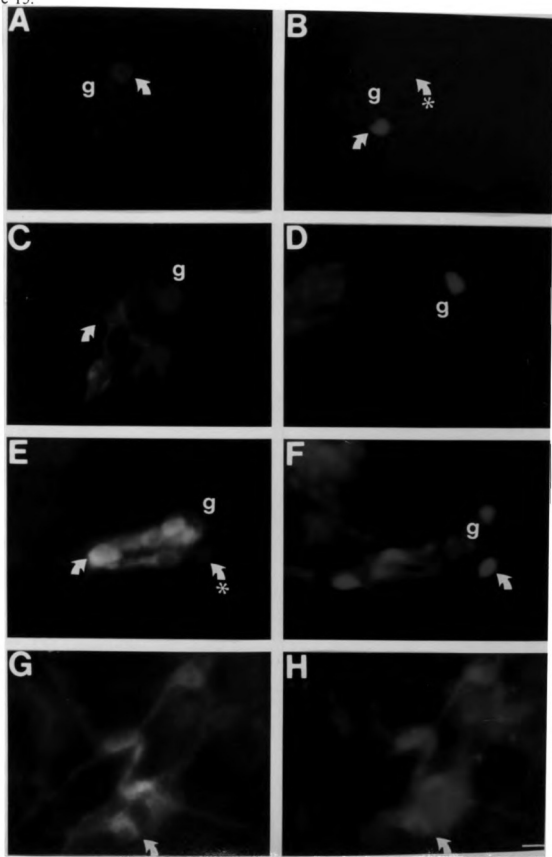
in submucosal ganglionic nuclei. Faint SMI 31-ir was also observed in non-ganglionic nuclei. Neither surgical or chemical denervation affected SMI 31-ir in nerve fibers in the myenteric plexus (data not shown), further suggesting pNF-H-ir is found in extrinsic and intrinsic innervation of the GI tract.

Nonphosphorylated NF-H-ir Is Found in Substance P-Immunoreactive Submucosal Neurons

SMI 32 gave a different pattern of immunoreactivity as compared to staining with SMI 31. Submucosal interganglionic nerve strands, nerve fibers associated with submucosal arterioles, and ganglionic nuclei did not contain detectable npNF-H-ir. However, 1.3 ± 0.1 cells/ganglion (3,358 cells, 2,690 submucosal ganglia, 43 preparations, 4 animals) contained npNF-H-ir. These neurons were frequently located near interganglionic nerve bundles at the periphery of ganglia (Figure 15). The np-NF-H-ir neurons had eccentrically-placed dark nuclei devoid of immunoreactivity and uniformly stained cytoplasm. Although dendrites were not immunoreactive, occasionally, proximal processes of axons were visible. The np-NF-H-ir cells were classified by three morphologies consistent with Dogiel type III morphology. Approximately 46% of all np-NF-H-ir cells were 38 ± 2 μm in the long axis and 14 ± 1 μm in the narrow axis. These cells had axons that were usually found within the ganglia. Approximately 38% of all SMI 32-ir cells were pyramidal or square-shaped and measured 27 ± 1 μm in the long axis and 20 ± 1 μm in the short axis. The axons from these cells frequently were associated with interganglionic nerve fibers. These axons did not enter the body of the ganglia. Finally, the remaining 16% of the npNF-H-ir submucosal neurons did not fit into either category and were 23 ± 3 μm in the long axis and 13 ± 2 μm in the short axis.

Figure 15. Nonphosphorylated heavy molecular weight neurofilament protein-immunoreactivity in submucosal nerve cell bodies in normal ileum. A. CGRP-ir in a single ganglionic neuron. B. npNF-H-ir is localized in a cell without CGRP-ir. C. NPY-ir in a submucosal ganglion. D. Corresponding co-localization of npNF-H-ir. The arrow represents a npNF-H-ir neuron that does not contain NPY-ir. E. Intense VIP-ir in submucosal ganglion; the arrow indicates a VIP-ir neuron. F. Corresponding co-localization of npNF-H-ir. Notice arrow marks npNF-H-ir neuron; this neuron does not contain VIP-ir. G. SP-ir in ganglionic neurons and a beaded varicosity. Note that one ganglion does not contain SP-ir neurons. H. npNF-H-ir is found in the same population of SP-ir cells. g, submucosal ganglion. Bar in H is approximately 30 μ m.

Figure 15.



The axons from these cells frequently were not visible. Submucosal tissues were incubated with SMI 32 and antiserum raised against neuropeptides to determine the subclass(es) of neurons containing np-NF-H-ir (Table 5). Nonphosphorylated-NF-H-ir was not observed to be co-localized with CGRP (11 preparations) (Figures 15A and 15B), NPY (10 preparations) (Figure 15C and 15D), or VIP (16 preparations) (Figure 15E and 15F). However, in colchicine-treated tissues, $92 \pm 2\%$ of all np-NF-H-ir neurons also contained SP-ir (Figures 15G and 15H) while $94 \pm 2\%$ of all SP-ir neurons contained np-NF-H-ir. Conversely, almost all npNF-H-ir neurons contained SP-ir. Surgical- or chemical-extrinsic denervation did not alter np-NF-H-ir in submucosal neurons (data not shown).

Nonphosphorylated NF-H-ir Is Found in Motor and Sensory Myenteric Neurons

Interganglionic nerve fibers and nerve fibers in the secondary and tertiary plexuses of the myenteric plexus did not contain npNF-H-ir. However, npNF-H-ir was observed in 32 ± 3.0 myenteric neurons/ganglion (10,522 cells, 350 ganglia, 26 preparations, 6 animals) (Figure 16). These cells were located at the peripheral border as well as in the interior of the ganglia. Rarely, homogeneously-stained, large extra-ganglionic neurons contained npNF-H-ir. Nonphosphorylated NF-H-ir was observed in dendrites and axons of many neurons. In some neurons, 4-15 broad and flat dendrites could be observed, with occasional axons emerging from either a dendrite-like process or the cell soma, consistent with Dogiel type I morphology. Some npNF-H-ir neurons had smooth somata with stained axon hillocks, and contained 4-8 filamentous dendrites. These neurons represent Dogiel type II neurons. Nonphosphorylated NF-H-ir axons exhibited a number of projections, as they appeared to exit the ganglia without entering internodal nerve bundles,

projected out of the ganglia within the internodal nerve bundles, or projected within the body of the ganglia. However, npNF-H-ir was most frequently observed only in proximal portion of axons over distances up to 50 μm . Bifurcations in axons were observed rarely. Nonphosphorylated-NF-H-ir myenteric cells could not be categorized by shape. They had an average size of $34 \pm 1 \mu\text{m}$ in the long axis, and $20 \pm 1 \mu\text{m}$ in the narrow axis, with the largest npNF-H-ir cell measuring $55 \times 28 \mu\text{m}$ (long and narrow axis), and the smallest cell measuring $20 \times 10 \mu\text{m}$ (long and narrow axis, respectively). Myenteric neurons exhibited diversity in the pattern of npNF-H-ir in the cytoplasm and in the intensity of staining. The majority of small npNF-H-ir myenteric cells contained fluorescence throughout the soma. However, approximately 25% of all npNF-H-ir cells contained filamentous np-NF-H-ir throughout the cytoplasm, and were frequently the largest npNF-H-ir cells in the ganglion.

Tissues were incubated with SMI 32 and antiserum against neuropeptides to identify the subclasses of myenteric neurons that contain np-NF-H-ir (Table 6). Nonphosphorylated NF-H-ir myenteric neurons did not contain CGRP- (12 preparations) or NPY-ir (12 preparations). Although the myenteric plexus contains a dense plexus of SP-ir nerve fibers originating from myenteric neurons, this study was unable to identify individual SP-ir myenteric neurons, even after incubation in colchicine containing medium as bright SP-ir axons covered SP-ir myenteric nerve cell bodies. Although many myenteric neurons contain SP-ir in myenteric ganglia, immunohistochemical methods can only detect faint levels of SP. Part of the difficulty in detection of SP results from bright extrinsic and intrinsic varicose nerve fibers that project over the surface of myenteric ganglia, masking SP-ir in myenteric neurons. Rare SP-ir neurons were found to contain

npNF-H-ir, indicating that some SP neurons may contain npNF-H-ir (data not shown), similar to the chemical coding in submucosal ganglia. Co-localization studies have also determined that some npNF-H-ir neurons also contained either VIP-ir (26 preparations) (Figures 18C and 18D) or NOS-ir (Figures 18E and 18F) (10 preparations), although some VIP-ir and NOS-ir cells did not contain detectable amounts of np-NF-H-ir. Surgical- or chemical- denervation did not alter np-NF-H-ir in myenteric neurons (Figures 18A and 18B).

Phosphorylation-Independent NF-H-ir Is Found in Submucosal Perikarya and Nerve Fibers

Unlike SMI 31 or SMI 32, SMI 33 recognizes a nonphosphorylated epitope on NF-H that is accessible on phosphorylated and nonphosphorylated neurofilament protein. In submucosal preparations, pnpNF-H-ir was found in interganglionic nerve fibers, nerve fibers associated with the arterioles, and in 2.1 ± 0.1 submucosal neurons/ganglion (1,080 cells, 538 ganglia, 10 preparations) (Figures 17A and 17B). The immunoreactivity was homogenous in the cytosol, and was not observed in the nucleus. SMI 33 appeared to recognize the same population of submucosal cells identified by SMI 32, as pNF-H- and pnpNF-H-ir cells were located at the periphery of the ganglion, shared similar morphologies, and did not have visible dendrites. As SMI 32 and SMI 33 differ in their heavy chain, co-localization studies could be performed using secondary antiserum raised against IgG to visualize npNF-H-ir or IgM to visualize pnpNF-H-ir. Such studies revealed that npNF-H-ir and pnpNF-H-ir were co-localized in almost all cells examined (data not shown).

Table 5. Number of peptide- and nonphosphorylated NF-H-ir cells in submucosal ganglion of guinea pig ileum.

Peptide	Peptide-ir cells/ganglion (cells;ganglia)	np-NF-H-ir cells/ganglion (cells;ganglia)	Co-localization of neuropeptide- and np-NF-H-ir (% np-NF-H-ir cells)
CGRP	1.6 ± 0.1 (1,637;1,190)	1.4 ± 0.1 (850;608)	0%
NOS	0.003 ± 0.002 (4;957)	1.4 ± 0.1 (1,260;900)	0%
NPY	1.6 ± 0.1 (1,322;879)	1.3 ± 0.1 (912;671)	0%
SP	1.2 ± 0.1 (746;661)	1.3 ± 0.1 (817;661)	94 ± 2%
VIP	3.5 ± 0.2 (4,308;1,478)	1.3 ± 0.1 (1,574;1,046)	0%

Table 6. Number of peptide- and nonphosphorylated NF-H-ir cells in myenteric ganglion of guinea pig ileum.

Peptide	Peptide-ir cells/ganglion (cells;ganglia)	np-NF-H-ir cells/ganglion (cells;ganglia)	Co-localization of neuropeptide- and np-NF-H-ir (% np-NF-H-ir cells)
CGRP	2.0 ± 0.6 (186;135)	31 ± 3.0 (2,604;84)	0%
58-			
NOS	10 ± 1.7 (1,256;144)	30 ± 3.0 (1,574;144)	29 ± 5%
NPY	1.1 ± 0.3 (216;156)	35 ± 3.4 (1,784;50)	0%
SP	not counted		not calculated
VIP	3.0 ± 0.3 (740;244)	31 ± 1.8 (4,652;413)	10 ± 3%

Figure 16. Nonphosphorylated heavy molecular weight neurofilament protein-immunoreactivity in myenteric nerve cell bodies in normal and denervated ileum. A. npNF-H-ir is found in many neurons in whole mount preparations of myenteric plexus from normal ileum. B. npNF-H-ir is not altered by loss of extrinsic nerves one week prior to experiment. C. VIP-ir in a single myenteric neuron. D. Nonphosphorylated NF-H-ir is localized in a neuron with VIP-ir (arrow), as well as neurons without VIP-ir. E. NOS-ir myenteric neurons. F. Nonphosphorylated NF-H-ir is localized in neurons with NOS-ir (arrow), and in neurons that do not contain NOS-ir. Bar in G is approximately 30 μ m.

Figure 16.

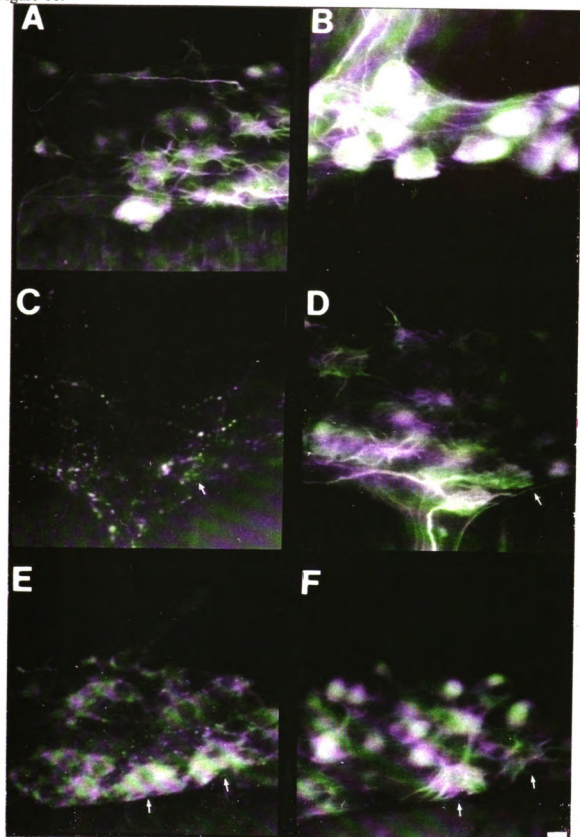
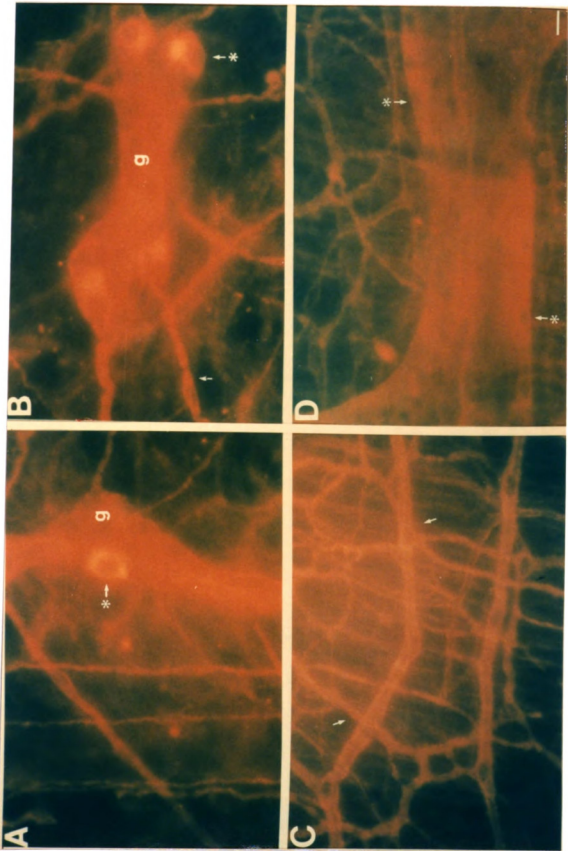


Figure 17. Heavy molecular weight neurofilament protein-immunoreactivity in whole mount submucosal and myenteric plexus preparations from normal ileum. A. SMI 33-ir paravascular innervation associated with submucosal arterioles. B. SMI 33-ir submucosal neurons and interganglionic nerve fibers. C. SMI 33-ir interganglionic nerves and nerve fibers of secondary and tertiary plexus. D. SMI 33-ir is localized in many, but not all, myenteric neurons. arrow, SMI 33-ir nerve; arrow and asterisk indicate SMI 33-ir neuron; g, ganglion. Bar in D is approximately 30 μ m.

Figure 17.



Phosphorylation Independent NF-H-ir Is Found in Myenteric Perikarya and Nerve Fibers

In the myenteric plexus, pnpNF-H-ir was localized in interganglionic nerve strands of the primary plexus, as well as in secondary and tertiary plexuses (Figure 17C), and nerve fibers closely associated with circular muscle. Interestingly, other investigators (Brookes *et al.*, 1992) have not observed NF-H-ir in the tertiary muscle plexus, demonstrating variations in localization of neurofilaments possibly due to differences in antibodies. Although pnpNF-H-ir appeared homogenous throughout the nerve fibers, strong pnpNF-H-ir was localized at the periphery of the primary plexus and the nerve strands of the secondary plexus. Within the myenteric ganglia, 36 ± 4 neurons/ganglion (1,990 cells, 130 ganglia, 12 preparations) contained pnpNF-H-ir. Similar to npNF-H-ir myenteric cells, these neurons were located throughout the ganglion, and processes could be observed in some cells. Although the majority of pnpNF-H-ir cells contained homogenous immunoreactivity, approximately 25% of all cells contained filamentous pnpNF-H-ir (Figure 17D).

4. NEW VASCULAR INNERVATION RESULTS FROM AXONAL SPROUTING

Introduction and Experimental Rationale

As discussed in the introduction, numerous studies have revealed that the neurofilament triplet proteins are closely-regulated during development, as incorporation of NF-H and phosphorylation of NF-M and NF-H coincides with maturation of axons (reviewed in Shaw, 1991). Furthermore, during regeneration of peripheral nerves, the pattern of incorporation and phosphorylation of NF-M and NF-H is very similar to the pattern expressed during development (Schilling and Pilgrim, 1990). Specifically, few KSP sequences in NF-H are phosphorylated in growing and regenerating peripheral axons (reviewed in Shaw, 1991; Schilling and Pilgrim, 1990; Pestronk *et al.*, 1990). Similar results are observed in developing and regenerating DRG neurons, as immature axons do not contain NF-H-ir, although NF-H-ir is found in adult axons (Shaw *et al.*, 1981; reviewed in Shaw, 1991). Therefore, these results suggest incorporation and phosphorylation of NF-H is involved in maturation of axons (see Shaw, 1986).

In the previous section, trypsin and phosphatase were used to demonstrate the selectivity of SMI 31 and SMI 32 for phosphorylated and nonphosphorylated NF-H, respectively. Furthermore, characterization of SMI 31 and 32 revealed microheterogeneity in the distribution of pNF-H and npNF-H, as SMI 31-ir was only observed in axons (and nuclei), whereas SMI 32-ir was only found in neuron cell bodies. These studies indicate that SMI 31 and SMI 32 may be used to determine if surgical extrinsic denervation induces axonal sprouting of enteric SP or VIP nerve fibers, resulting in new vascular innervation. As previously discussed, retrograde tracing studies with Fg indicated that extrinsic denervation did not stimulate axonal sprouting of submucosal neurons.

Therefore, if axonal sprouting occurs, then the nerve fibers must originate from neurons in myenteric ganglia. Although it was impossible to trace SP- or VIP-ir myenteric nerves to submucosal arterioles in transverse sections of ileum, it was hypothesized that loss of extrinsic innervation induces axonal sprouting of myenteric nerves, resulting in increased density of nerves associated with submucosal arterioles. This hypothesis was tested using whole mount submucosal preparations and immunohistochemistry for neurofilament proteins. If loss of extrinsic innervation induced axonal sprouting of myenteric nerve fibers containing SP or VIP, then pNF-H-ir should not be observed in SP- or VIP-ir nerve fibers, NF-H is not phosphorylated in growing and regenerating axons (see proceeding section). However, if the SP- and VIP-ir nerves associated with submucosal arterioles result from either upregulation or *de novo* synthesis of SP or VIP, then these pre-existing mature nerve fibers should contain pNF-H-ir. Therefore, immunohistochemical methods for co-localization of SP or VIP and SMI 31 or SMI 32 were used to determine if new SP- or VIP-ir nerve fibers originated from myenteric ganglia.

Substance P-Immunoreactive Vascular Nerves Are New Axons

As described previously, multiple pNF-H-ir nerve fibers were associated with arterioles in normal ileum, and the majority of these axons were destroyed by extrinsic innervation. This result was not unexpected, as extrinsic sensory and sympathetic nerve fibers and intrinsic VIP-ir nerve fibers contained pNF-H. However, although pNF-H-ir was observed in enteric nerve fibers in interganglionic nerve strands, few pNF-H-ir nerve fibers were associated with arterioles after surgical extrinsic denervation, suggesting that few intrinsic nerve fibers are normally not associated with arterioles. This observation argues against the hypothesis that surgical extrinsic denervation increases the concentration

of SP and VIP in intrinsic axons previously associated with arterioles, as intrinsic axons were not associated with submucosal arterioles. Therefore, the hypothesis of denervation-induced axonal sprouting was further investigated by examining co-localization of NF-H- and SP- or VIP-ir nerves in surgically-denervated preparations.

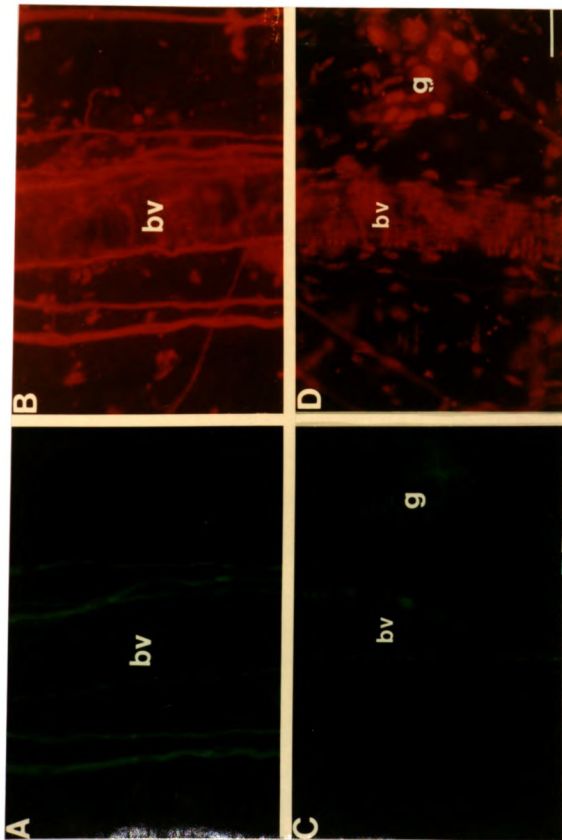
In normal and control tissues, SP- (Figures 18A and 18B) and CGRP-ir (data not shown) nerve fibers associated with submucosal arterioles always contained strong pNF-H-ir. As previously described, these nerve fibers are sensitive to capsaicin and represent extrinsic sensory nerves. Four weeks after surgical extrinsic denervation, faint, thin, non-varicose SP-ir nerve fibers did not contain pNF-H-ir (Figures 18C and 18D). However, pNF-H-ir was observed in submucosal nuclei and interganglionic nerve strands of the same preparations, suggesting that the lack of pNF-H-ir in nerve fibers associated with arterioles did not result from experimental error, but actual represented loss of pNF-H-ir.

Ten weeks after surgical extrinsic denervation, SP- and pNF-H-ir were co-localized in some paravascular nerve fibers loosely associated with arterioles (data not shown). However, most SP-ir nerve fibers did not contain pNF-H-ir, as nerve fibers closely associated with arterioles contained SP-ir, but not pNF-H-ir. Finally, 24-26 weeks after surgical denervation, some SP-ir nerves contained pNF-H-ir (data not shown). However, extrinsic sensory nerve fibers contain SP-ir. As these nerve fibers begin to return 10 weeks after extrinsic denervation, it was difficult to determine if SP-/pNF-H-ir nerve fibers associated with submucosal arterioles originated from intrinsic or extrinsic sources. Therefore, co-localization of pNF-H-ir (SMI 31) and CGRP were examined to attempt to identify the source of SP-ir nerves.

Proceeding surgical extrinsic denervation, SP-ir vascular nerve fibers did not

Figure 18. Co-localization of substance P- and phosphorylated heavy molecular weight neurofilament protein-immunoreactivity in nerve fibers in submucosal plexus preparations from control and denervated ileum. A. SP-ir perivascular nerve fibers are associated with a submucosal arteriole in normal intestine. B. pNF-H-ir is co-localized with SP in normal vascular innervation. C. Ten weeks after extrinsic denervation, multiple SP-ir nerves are associated with arterioles. D. Co-localization of pNF-H-ir reveals some SP-ir nerves are pNF-H-ir. bv, arteriole; g, ganglion. Bar in D is approximately 50 μ m.

Figure 18.



not contain CGRP-ir, although extrinsic sensory nerve fibers contain SP-/CGRP-ir. Twenty-four weeks after extrinsic denervation, many CGRP-ir nerve fibers did not contain pNF-H-ir (data not shown). The pattern of localization of CGRP- and pNF-H-ir indicate that regenerating sensory nerve fibers do not contain pNF-H. However, occasional nerve fibers co-localized CGRP- and pNF-H-ir, suggesting that extrinsic sensory nerve fibers do not contain pNF-H-ir during regeneration, but incorporate and phosphorylate NF-H after stabilization or maturation. Therefore, after surgical extrinsic denervation, submucosal preparations contain mature (CGRP-/pNF-H-ir) and regrowing (CGRP-/but not pNF-H-ir) nerve fibers, thus accounting for observed differences in co-localization of CGRP and pNF-H. Similarly SP-/pNF-H-ir nerves may represent mature extrinsic sensory nerve fibers, such as those associated with submucosal arterioles. Alternatively, SP-/pNF-H-ir nerves may indicate mature intrinsic nerve fibers in submucosal ganglia and interganglionic nerve strands.

New Substance P-Immunoreactive Nerves Lack NF-H

Co-localization studies with antiserum against NF-H and SP indicated that many SP-ir nerve fibers associated with submucosal arterioles do not contain pNF-H-ir after extrinsic surgical denervation. However, these studies were inconclusive, as they were unable to determine if the absence of pNF-H-ir resulted from disruption of a phosphorylated epitope necessary for antigenicity, or if SP-ir nerve fibers did not contain heavy molecular weight neurofilament protein. To distinguish between these possibilities, SMI 32 was used. Previous characterization of SMI 32 revealed that the antibody recognizes a nonphosphorylated epitope on NF-H protein. If the absence of pNF-H-ir resulted from dephosphorylation after surgical extrinsic denervation, then SMI 32 should

recognize nerve fibers containing SP. However, if the absence of pNF-H-ir resulted from loss of the NF-H subunit, then SP-ir nerve fibers should not contain npNF-H-ir.

In preparations made from normal and control tissues, SMI 32 binding was observed in SP-ir nerve cell bodies in submucosal ganglia, but not in paravascular or perivascular innervation associated with arterioles (Figure 15). One and four weeks after extrinsic denervation, somata of some submucosal neurons contained npNF-H-ir; few npNF-H-ir nerve fibers were observed in preparations from denervated segments of ileum (Figures 19A and 19B). However, occasional fiber-like structures were observed, and these structures were not associated with submucosal arterioles or ganglia (Figure 19C). These fibers could be followed for 300-500 μm , and contained occasional oval swellings, consistent with retracting or dying axons. Therefore, the only npNF-H-ir nerve fibers were observed early after denervation, and were likely to represent dying extrinsic nerves. Similar results were obtained with a monoclonal antibody raised against pnpNF-H-ir (SMI 33), as nerve fibers were not observed after extrinsic denervation. However, the absence of npNF-H-ir and pnpNF-H-ir nerve fibers strongly suggests that the absence of pNF-H-ir (SMI 31) resulted from the absence of NF-H in the neurofilament triplet assembly, and not dephosphorylation of KSP sequences.

Vasoactive Intestinal Polypeptide-Immunoreactive Vascular Nerves are New Axons

Co-localization studies were performed on tissues from extrinsically-denervated segments of ileum to determine if denervation-induced alterations of VIP-ir nerve fibers associated with submucosal arterioles resulted from axonal sprouting of myenteric ganglia. As described previously, VIP-ir and pNF-H-ir were co-localized in sparse, thin nerves associated with submucosal arterioles in normal and control preparations. However, four

Figure 19. Nonphosphorylated heavy molecular weight neurofilament-protein immunoreactivity in submucosal plexus preparations from denervated ileum. A. SP-ir is found in paravascular nerves one week after extrinsic denervation. B. npNF-H-ir is not observed in vascular innervation. C. SMI 32-ir nerve fiber-like structures are localized 7 weeks after extrinsic denervation. Arrows in C indicate nerve fiber-like structure. g, ganglion. Calibration bar in C is approximately 40 μm .

Figure 19.

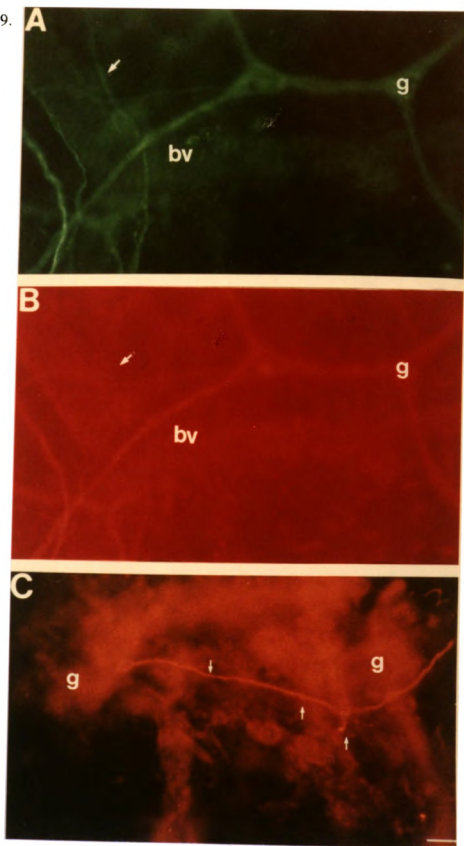
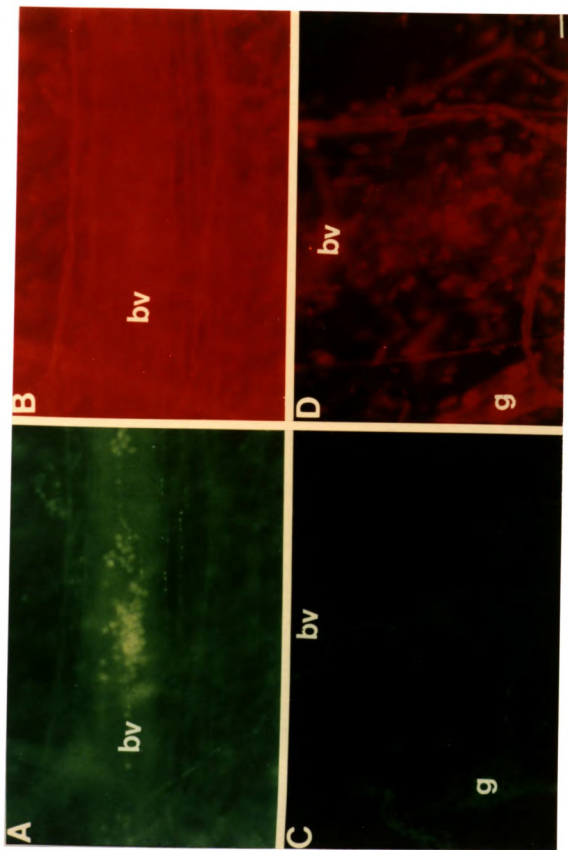


Figure 20. Co-localization of vasoactive intestinal polypeptide- and phosphorylated heavy molecular weight neurofilament protein-immunoreactivity in nerve fibers in submucosal plexus preparations from denervated ileum. A. VIP-ir paravascular nerve fibers in submucosal preparations denervated 4 weeks prior to experiment. B. pNF-H-ir is not observed to be co-localized with VIP-ir. C. VIP-ir nerve fibers associated with an arteriole denervated sixteen weeks previously. D. pNF-H-ir is observed in some VIP-ir nerve fibers. bv, arteriole, Bar in D is approximately 50 μ m.

Figure 20.



weeks after extrinsic denervation, the density of VIP-ir nerve fibers increased, and pNF-H-ir was not observed in most VIP-ir nerve fibers (Figures 20A and 20B). However, rare VIP-ir nerve fibers contained pNF-H-ir, and were likely to represent VIP-ir nerve fibers observed in normal ileum. Ten weeks after loss of extrinsic nerve fibers, most VIP-ir paravascular and perivascular nerve fibers did not contain detectable pNF-H-ir. Sixteen (Figures 20C and 20D) and twenty-four weeks after denervation, some VIP-ir nerve fibers did not contain pNF-H-ir, similar to the results observed with SP-ir vascular nerves. Finally, although VIP-ir paravascular nerve fibers in contact with arterioles did not contain pNF-H-ir, some nerve fibers that were found 30-50 μm from the arterioles contained pNF-H-ir and VIP-ir (Figures 20C and 20D). Therefore, these data suggest that extrinsic denervation induces axonal sprouting in myenteric neurons, culminating in new SP- and VIP-ir nerve fibers associated with submucosal arterioles.

New Vasoactive Intestinal Polypeptide Nerves Lack NF-H

As pNF-H-ir was not observed in many VIP-ir nerve fibers, SMI 32 and SMI 33 were used to determine if the absence of pNF-H-ir resulted from an absence of NF-H protein or from dephosphorylation of KSP sequences. Four, ten, and sixteen weeks after surgical extrinsic denervation, npNF-H- and pnpNF-H-ir were not observed in nerve fibers in submucosal plexus preparations (data not shown), indicating as with SP-ir nerve fibers, the lack of pNF-H-ir resulted from the absence of NF-H, and not from changes in the phosphorylation status of NF-H.

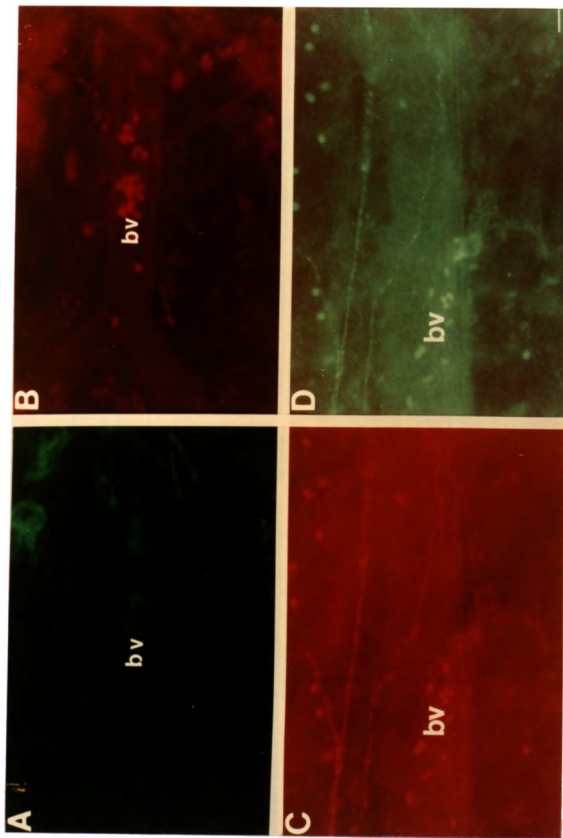
New Substance P-Immunoreactive Vascular Nerves Result from Loss of Extrinsic Sensory Innervation

Surgical extrinsic denervation alters innervation of submucosal arterioles and regulation of neurogenic vasodilation (Galligan *et al.*, 1990), and axonal sprouting of myenteric neurons appears to be one mechanism by which new SP- or VIP-ir vascular nerves appear. However, the stimulus for induction of alterations in innervation of GI microvasculature are unknown. To begin to address this question, animals were treated with 6-OH-DA or capsaicin to selectively eliminate a single population of extrinsic nerves. Substance P- and VIP-ir nerve fibers were then examined to determine if loss of a single population of nerve fibers was sufficient stimulus to alter innervation associated with submucosal arterioles.

Following neurotoxin- and vehicle-treatment, animals were allowed to recover 1 or 4 weeks for 6-OH-DA, or 1 or 9 weeks for capsaicin. One week after 6-OH-DA treatment, glyoxylic acid-induced fluorescent nerves were not observed, although occasional patches of catecholamine fluorescence was observed. As nerve fibers were not clearly visible, these areas of catecholamines may have been degenerating sympathetic nerve fibers. Immunohistochemistry for SP and CGRP revealed SP-ir submucosal ganglionic neurons and SP-/CGRP-ir nerve fibers associated with arterioles similar to those observed in preparations from vehicle-treated animals. Substance P-ir nerve fibers without CGRP-ir were not observed 1 or 4 weeks after sympathectomy (data not shown), suggesting that the appearance of SP-ir nerve fibers after surgical extrinsic denervation may not have resulted from loss of sympathetic nerve fibers. Later time points in 6-OH-DA treated animals could not be examined, as administration of 6-OH-DA, but not the vehicle, resulted in skin lesions at the site of injection, requiring animals

Figure 21. Co-localization of substance P- and calcitonin gene-related peptide-immunoreactivity in submucosal plexus preparations from ileum taken from animals treated with capsaicin or from surgically-denervated segments. A. SP-ir nerve fibers associated with an arteriole 9 weeks after capsaicin-treatment. B. Nerve fibers in C do not contain CGRP-ir. are associated with submucosal arterioles one week after systemic capsaicin treatment. C. SP-ir nerve fibers are found associated with arterioles 26 weeks after surgical extrinsic denervation. D. CGRP co-localizes with SP-ir in C, suggesting that primary afferent innervation has returned to the denervated segment, although the number of primary afferent nerve fibers is reduced compared to controls. bv, arteriole; g, ganglion. Bar in D is approximately 40 μ m. Note that the fluorophore in A and D are FITC, whereas Texas Red conjugated secondary antibodies were used in B and C.

Figure 21.



to be used at early times after denervation.

One week after capsaicin treatment, SP-/CGRP-ir or SP-/without CGRP-ir nerve fibers were not observed to be associated with submucosal arterioles (data not shown). However, by 9 weeks after administration of capsaicin, faint, thin, non-varicose SP-ir nerves without CGRP-ir were associated with arterioles similar to SP-ir nerve fibers observed after surgical extrinsic denervation (Figures 21A and 21B). These results suggest that loss of extrinsic sensory nerve fibers, but not sympathetic nerve fibers, is responsible for initiating cellular events that result in new SP-ir vascular innervation. Finally, these results further support the conclusion that increased SP-ir nerve fibers associated with arterioles results from loss of extrinsic innervation, and not from direct or indirect alterations due to surgical trauma.

New Vasoactive Intestinal Polypeptide-Immunoreactive Vascular Nerves Result From Loss of Sympathetic Innervation

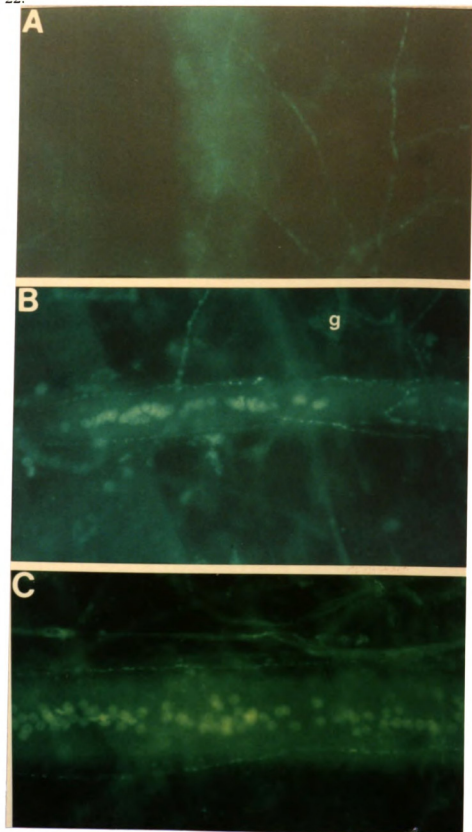
One week after treatment of animals with capsaicin, strong VIP-ir was observed in submucosal and myenteric ganglia, as bright neurons and nerve fibers were easily located. However, multiple VIP-ir nerve fibers were not observed to associate with submucosal arterioles (Figure 22A). Four weeks after sympathectomy, bright VIP-ir nerve fibers were associated with some arterioles, and multiple VIP-ir paravascular nerve fibers were observed (Figure 22B). Few VIP-ir nerve fibers were found to be associated with submucosal arterioles in vehicle-treated animals (Figure 22C), suggesting that loss of sympathetic nerves during extrinsic denervation induced alterations in VIP-ir innervation of submucosal arterioles.

One week after capsaicin treatment, VIP-ir was observed in submucosal neurons,

and occasional thin, short smooth or varicose nerve fibers associated with submucosal arterioles. The pattern and distribution of VIP-ir was similar to that in preparations made from normal or vehicle-treated animals. Finally, nine weeks after capsaicin administration, submucosal neurons and occasional paravascular nerve fibers contained VIP-ir (data not shown). However, there was neither qualitative nor quantitative evidence to suggest that loss of primary afferent nerves increased VIP-ir in submucosal plexus preparations. These observations indicate that loss of sympathetic, but not sensory nerves, is responsible for increased VIP-ir vascular innervation following loss of extrinsic innervation.

Figure 22. Vasoactive intestinal polypeptide-immunoreactivity in submucosal plexus preparations from ileum taken from animals treated with 6-hydroxy-dopamine. A. Few VIP-ir nerves are associated with submucosal arterioles 1 week after 6-OH-DA treatment. B. Multiple VIP-ir paravascular nerve fibers are associated with submucosal arterioles 4 weeks after 6-OH-DA treatment. C. VIP-ir in vehicle-treated controls appears similar to VIP-ir in normal preparations. bv, arteriole; g, submucosal ganglion. Bar in C is approximately 30 μ m.

Figure 22.



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**ANATOMICAL AND FUNCTIONAL ALTERATIONS IN ENTERIC NEURONS
FOLLOWING EXTRINSIC DENERVATION OF GUINEA PIG ILEUM**

Volume II

By

Anne Marie R. Yunker

A DISSERTATION

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for the degree of**

DOCTOR OF PHILOSOPHY

**Department of Pharmacology and Toxicology
and Neuroscience Program**

1995

5. EXTRINSIC DENERVATION INCREASES NITRIC OXIDE SYNTHASE-IMMUNOREACTIVITY IN MYENTERIC NEURONS

Introduction and Experimental Rationale

Neurogenic relaxation of arterioles is mediated at least in part by endothelium derived relaxing factor (EDRF). Although some controversy exists as to the chemical identity of EDRF, the molecule is closely related to NO. As loss of extrinsic innervation results in profound anatomical and functional changes in regulation of neurogenic vasodilation, it was hypothesized that surgical extrinsic denervation would alter NOS in nerve fibers associated with submucosal arterioles. As antiserum against NOS was not available, NADPH diaphorase histochemistry was used as a measure of NOS-ir in submucosal plexus preparations in normal, control, and surgically-denervated preparations.

Extrinsic Denervation Does Not Alter NADPH Diaphorase Staining in the Submucosal Plexus

Few structures contained NADPH diaphorase staining in preparations made from normal submucosal plexus, for only 0.002 ± 0.002 neurons/submucosal ganglion (3 neurons, 1254 submucosal ganglia) contained formazan precipitate, and nerve fibers were not observed to contain NADPH diaphorase staining (Figure 23A). Dark staining was observed within occasional mucosal crypts that had not been dissected away from the submucosal plexus, indicating that epithelial cells contained NADPH diaphorase activity. Finally, dark and punctate staining was found within arterioles, as endothelial cells contained NADPH diaphorase respectively. Capacitance vessels were not stained.

One week after surgical elimination of extrinsic nerves, NADPH diaphorase staining was observed in endothelial cells and mucosal crypts, but not in interganglionic

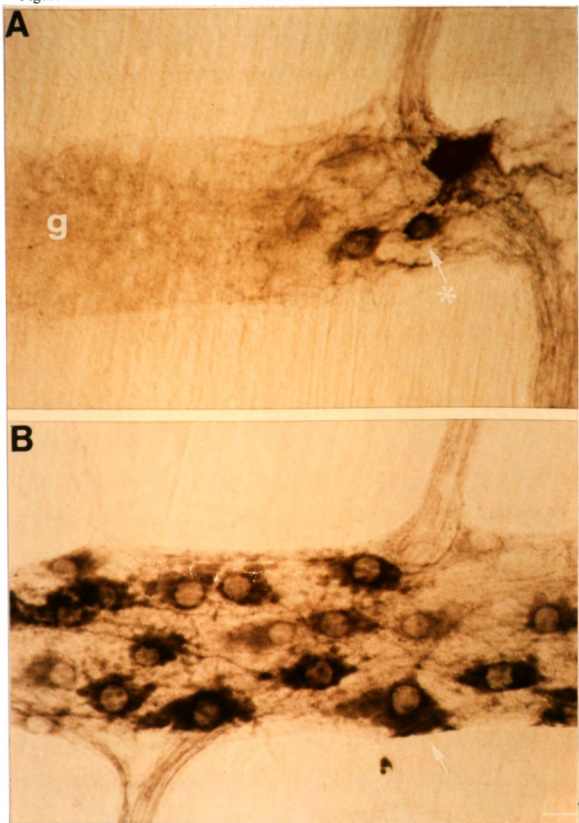
nerves, or in nerve fibers associated with arterioles (data not shown). The number of NADPH diaphorase stained submucosal neurons was not altered, as 0.002 ± 0.001 NADPH diaphorase positive neurons/submucosal ganglion (2 neurons, 871 submucosal ganglia) were observed in control tissues, whereas 0.001 ± 0.001 neurons /submucosal ganglion in denervated preparations contained NADPH diaphorase (1 neuron, 963 submucosal ganglia). Similar results were obtained 4, 8, and 16 weeks after loss of extrinsic innervation (data not shown), indicating that loss of extrinsic nerves does not increase NADPH diaphorase staining in submucosal nerves.

Extrinsic Denervation Increases Myenteric NADPH Diaphorase Staining

Although extrinsic denervation did not alter NADPH diaphorase staining in the submucosal plexus, myenteric plexus-longitudinal muscle preparations were processed simultaneously with submucosal plexus preparations to verify that NADPH diaphorase staining occurred. Subpopulations of myenteric neurons in normal guinea ileum are known to contain NADPH diaphorase (Gershon and Kirchgessner, 1986; Young *et al.*, 1992), thereby allowing a positive control for NADPH diaphorase staining. In normal myenteric plexus preparations, 9.6 ± 1.2 neurons/ganglia (2942 cells, 314 ganglia) stained for NADPH diaphorase. These cells were classified into two morphologies (Figure 23): $16 \pm 3\%$ of stained neurons were 35-45 μm in the long axis, with lamellar dendrites and a single axon. These cells were darkly stained, frequently located at the periphery of ganglia, and were classified as Dogiel type I cells. The remainder of the neurons were 10-29 μm in the long axis, lightly stained, and located in the interior of ganglia. The processes of these cells were frequently not visible, but some lamellar dendrites were observed.

Figure 23. NADPH diaphorase staining in whole mount myenteric plexus preparations from normal and denervated ileum. A. Myenteric ganglion from normal ileum processed for NADPH diaphorase histochemistry. Large, darkly stained cells (arrow), and small, more lightly stained cells are present. B. One week after extrinsic denervation, the number of cells stained for NADPH diaphorase increased. Large, darkly stained neurons (arrow) and small neurons are present. Calibration bar in B is 45 μ m.

Figure 23.



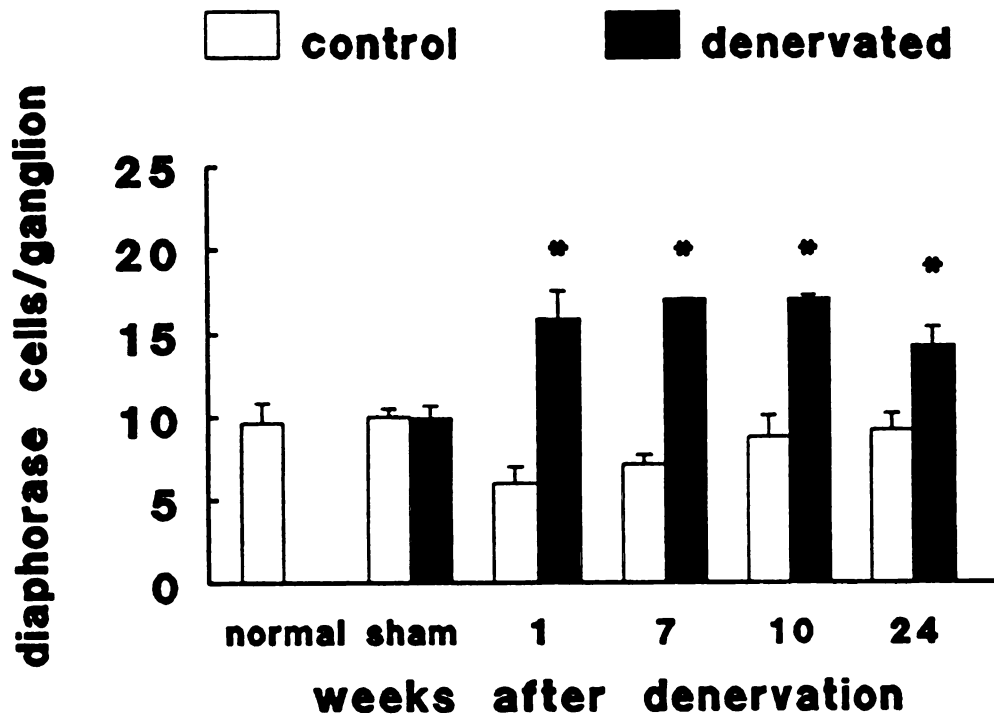
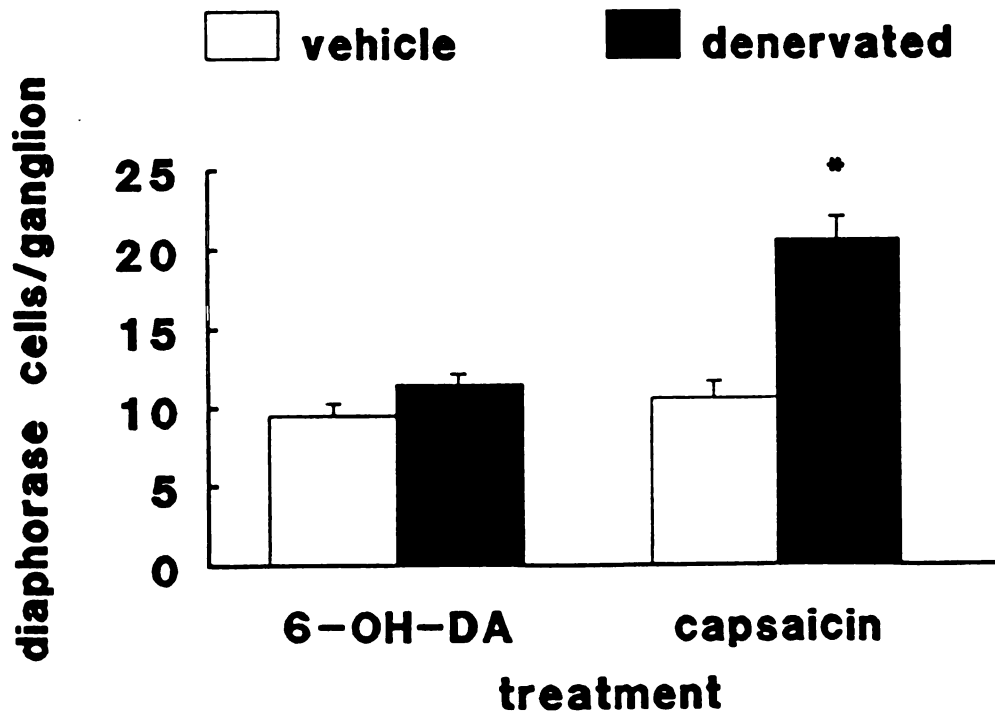
These two classes of NADPH diaphorase-stained neurons may represent motor neurons and interneurons, respectively (Young *et al.*, 1992; see introduction). Finally, nerve bundles in primary and secondary plexuses contained dark NADPH diaphorase staining, and little staining was observed in the tertiary plexus. Tissues fixed in Zamboni's solution did not exhibit staining.

One week after extrinsic denervation the number of NADPH diaphorase stained cells in myenteric ganglia was increased as compared to animal matched controls (Figure 23). An average of 15.9 ± 1.7 stained neurons/myenteric ganglion (3351 neurons, 216 ganglia) were in the denervated region, whereas control segments from the same animals contained 6.0 ± 1.0 neurons/ganglion (1597 neurons, 298 ganglia) (Figure 24). The proportion of large, darkly-stained neurons to that of lightly stained neurons was not altered by denervation ($16:83 \pm 2\%$) as compared to controls ($18:81 \pm 2\%$), although denervated preparations were more darkly stained. The number of NADPH diaphorase stained myenteric neurons/ganglion from sham-operated animals (9.9 ± 0.7 , 831 neurons, 87 ganglia, 4 preparations, 2 animals) was not different from animal-matched controls (10.0 ± 0.5 , 1772 neurons, 177 ganglia) (Figure 24), indicating that surgical trauma and handling of the ileum did not increase NADPH diaphorase.

The number of NADPH-diaphorase stained neurons/myenteric ganglion remained elevated at 7 and 10 weeks after denervation (Figure 24). Although numerous extrinsic nerves were observed in all ganglia 24 weeks after denervation, the number of NADPH diaphorase stained neurons remained elevated by $55 \pm 1\%$. This was the smallest increase in NADPH diaphorase stained neurons observed at any time, possibly as a result of re-establishment of interactions between extrinsic and intrinsic nerves.

Figure 24. The number of NADPH diaphorase stained neurons/ganglion in myenteric plexus preparations from normal, sham, control, and denervated ileum. A. Number of NADPH diaphorase positive neurons/myenteric ganglion in preparations obtained from normal, sham, or extrinsically-denervated ileum. Controls were from areas of ileum oral or aboral to the denervated loop. B. Number of NADPH diaphorase positive neurons obtained from animals treated with 6-hydroxy-dopamine or capsaicin to destroy sympathetic or primary afferent innervation, respectively. Controls were from age-matched vehicle-treated animals. Data are mean of diaphorase positive neurons/ganglia $\pm$ SEM from at least 3 preparations per animal.

Figure 24

A**B**

NADPH Diaphorase Staining is Not Non-Specific Diaphorase Activity

Dicumarol is an oral anti-coagulant that prevents electron transfer from NAD(P)H to FAD, and binds with high affinity to non-specific, but not NADPH-dependent, diaphorases. Pre-treatment of submucosal and myenteric plexus preparations with 0.1 mM dicumarol for 1 hr at room temperature did not inhibit staining of myenteric neurons, but reduced staining of mucosal crypts. However, if β -NADH was substituted for β -NADPH, non-specific diaphorase staining occurred, as 8 ± 0.05 submucosal neurons/ganglion (6399 neurons, 807 submucosal ganglion) (Figure 25B) and 59 ± 5 myenteric neurons/ganglion (4414 neurons, 83 myenteric ganglion) contained NADH diaphorase staining (Figure 25D). Unlike the results from NADPH diaphorase staining, these neurons were lightly stained, and neither axons nor dendrites were ever visible. Furthermore, NADH histochemistry stained most neurons in submucosal ganglia, and approximately 60% of myenteric neurons, unlike NADPH diaphorase staining. NADH histochemistry was sensitive to dicumarol, as neurons did not stain for NADH diaphorase after the preparations were incubated in dicumarol (data not shown). Finally, NADPH staining was not observed if NADPH was excluded from the reaction mixture, and NADH staining was not observed if NADH was excluded from the reaction mixture. Taken together, these results indicate that NADPH diaphorase staining does not result from non-specific diaphorase activity, but is consistent with reports of neuronal NADPH diaphorase activity.

Increased Number of NADPH Diaphorase Positive Neurons Is Not Associated With Cell Death or Mitosis

NADH diaphorase staining has been used previously as an estimate of the total number of neurons within the GI tract (Gabella, 1976). An increase in the number of

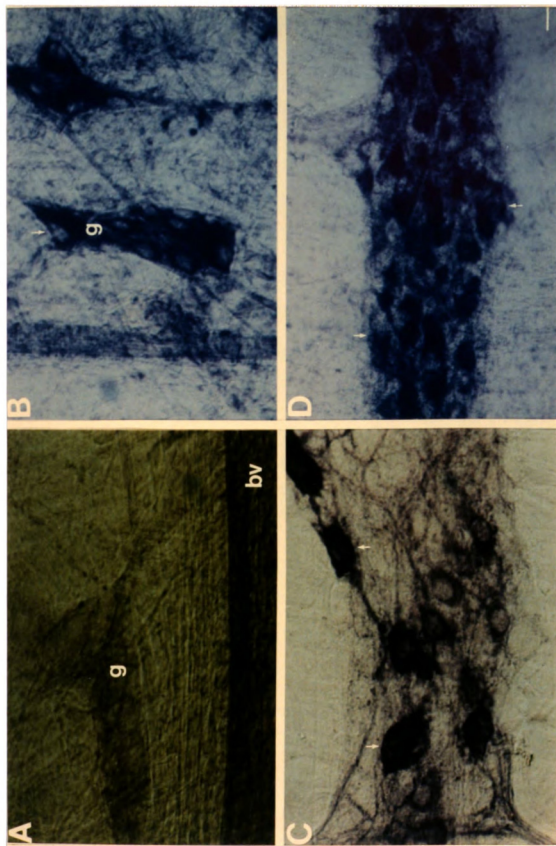
NADPH diaphorase positive myenteric neurons may result from one of two mechanisms. First, loss of extrinsic innervation may increase the total number of myenteric neurons/ganglia, and new neurons contain NADPH diaphorase staining. Therefore, increased NADPH diaphorase staining would not indicate altered phenotypic expression of mature neurons, but rather birth of new neurons. Although CNS neurons can not divide after maturation, electron micrographs reveal progenitor-like cells within myenteric ganglia (Burnstock and Cooke, 1976), that have been proposed to be premitotic cells. Alternatively, increased NADPH diaphorase staining in myenteric ganglia may represent increased catalytic activity, increased production of existing enzyme, or *de novo* synthesis of neuron specific diaphorase, all resulting in altered phenotypic expression of some enteric neurons.

As shown in Figure 26, loss of extrinsic nerves neither increased nor decreased the number of NADH diaphorase-stained neurons in submucosal (Figure 26A) or myenteric (Figure 26B) ganglia. These results indicate that loss of extrinsic innervation does not stimulate division and development of progenitor cells. Secondly, these data indicate that programmed cell death does not occur after elevated NADPH diaphorase staining. Following ventral root avulsion, Wu *et al.* (1992; 1993) observed increased NADPH diaphorase staining prior to loss of motor neurons, suggesting that expression of NADPH diaphorase may play an undefined role in apoptosis. Finally, these data indicate that unlike NADPH diaphorase staining, NADH staining is not effected by denervation, further supporting the conclusion that NADPH diaphorase histochemistry does not result from non-specific diaphorase activity.

Although NADH diaphorase histochemistry has been used as an estimate of cell

Figure 25. NADPH diaphorase and NADH diaphorase staining in submucosal and myenteric plexus whole mount preparations from normal ileum. A. Submucosal preparation stained by NADPH diaphorase histochemistry. B. Submucosal preparation processed for NADH diaphorase histochemistry. C. NADPH diaphorase histochemistry in a myenteric ganglion. D. Myenteric preparation processed for NADH diaphorase histochemistry. bv, arteriole; g, ganglion; arrows indicate positively-stained neurons. Bar in D is approximately 40 μ m.

Figure 25.



numbers, the recent development of antiserum against nerve cell bodies (Young *et al.*, 1993a) has estimated that NADH diaphorase-positive myenteric neurons represent only 60-80% of myenteric neurons. These results indicate a better method of labelling myenteric neurons is needed. Preliminary experiments with methylene blue gave sporadic staining in preparations of myenteric plexus and was not used. However, as SMI 31 and bisbenzamide co-localized in enteric nuclei, SMI 31 was used to visualize ganglionic nuclei in control and denervated preparations. To prevent inclusion of enteric glia in cell counts, only nuclei with visible nucleoli were counted, as glial nuclei lack these structures (Gershon and Rothman, 1991). Similar to results obtained with NADH diaphorase staining, loss of extrinsic innervation did not affect the number of submucosal (Figure 27A) or myenteric (Figure 27B) nuclei counted anytime after denervation. Taken together, these data indicate that extrinsic denervation does not induce mitosis of immature neurons, and does not result in programmed cell death. However, loss of extrinsic innervation induces alterations in some myenteric neurons, resulting in expression of a new phenotype.

Increased NADPH Diaphorase Staining Results from Loss of Sensory Innervation

As described previously, treatment with 6-OH-DA decreased catecholamine fluorescence in submucosal and myenteric ganglia; SP/CGRP-ir was not affected. Treatment with 6-OH-DA did not change the number of NADPH diaphorase positive neurons in myenteric ganglia (11.4 ± 0.7 cells/ganglion, 3630 cells, 326 ganglia) compared to vehicle-treated controls (9.4 ± 0.8 cells/ganglion, 4312 cells, 568 ganglia) (Figure 24B). Capsaicin-treatment eliminated SP/CGRP-ir nerve fibers, and resulted in increased NADPH diaphorase staining. NADPH diaphorase staining was darker, and the

Figure 26. The number of NADH diaphorase stained neurons/ganglion in myenteric and submucosal plexus preparations from normal, control, sham, or denervated ileum. A. Number of NADH diaphorase positive cells/ganglion in submucosal plexus preparations obtained from normal, sham, or extrinsically-denervated ileum. Controls were taken from an area of ileum oral or aboral to the denervated loop. B. Number of NADH diaphorase-positive cells/ganglion in myenteric plexus preparations obtained from normal, sham, or extrinsically-denervated ileum. Data are means $\pm$ SEM from at least 4 preparations per animal.

Figure 26.

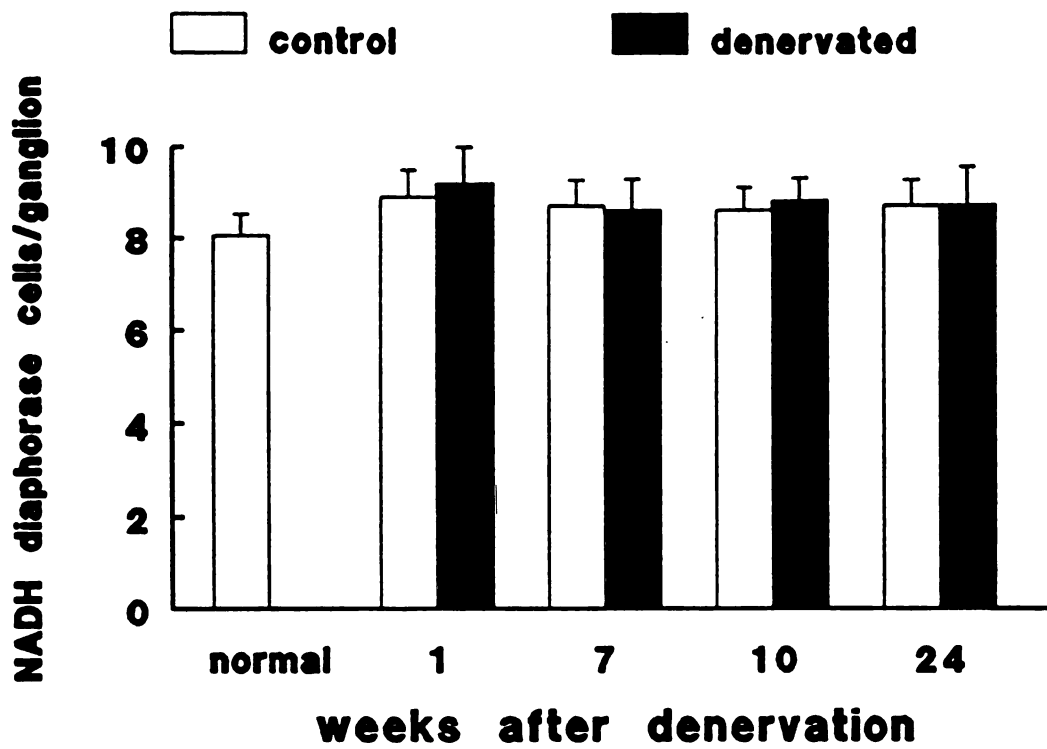
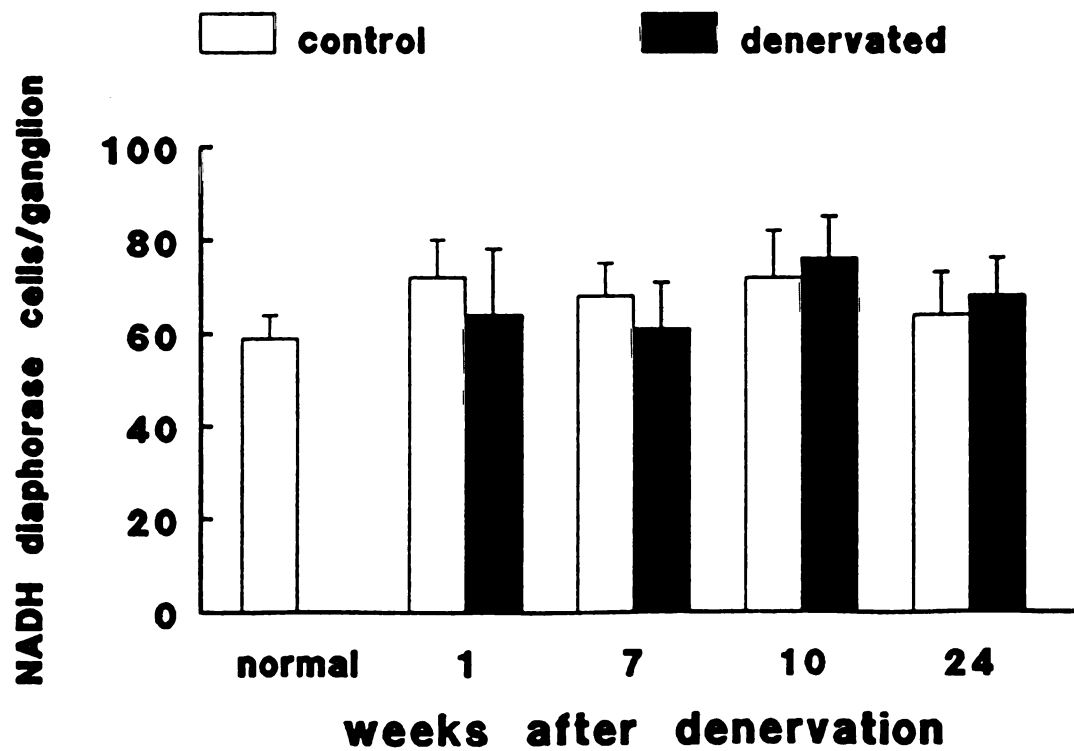
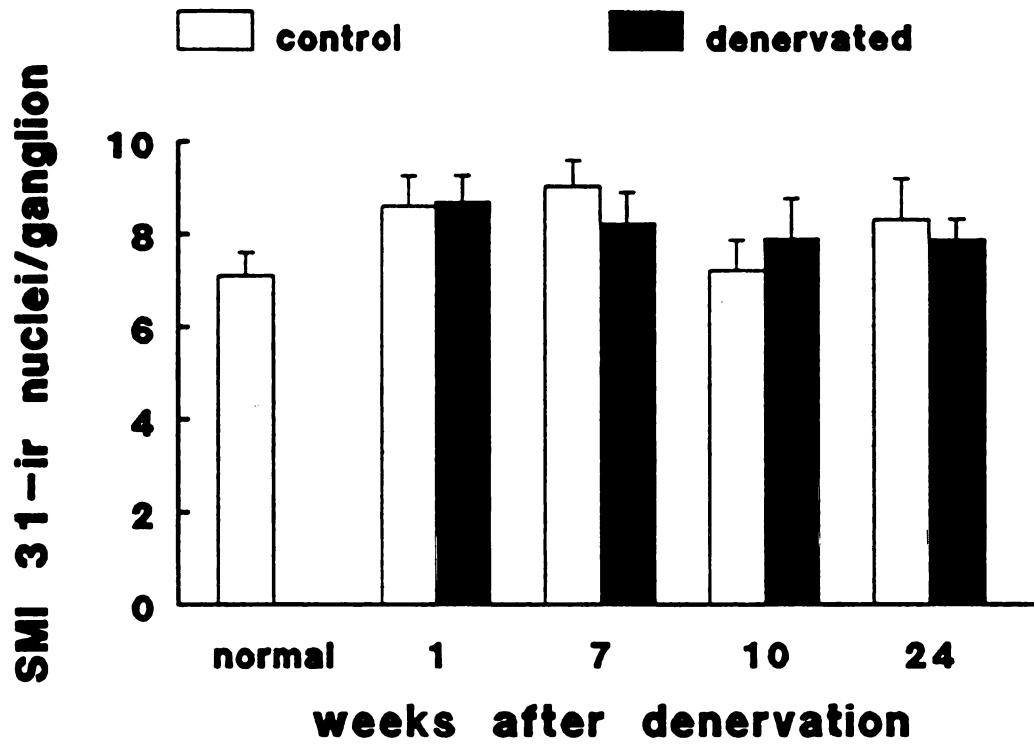
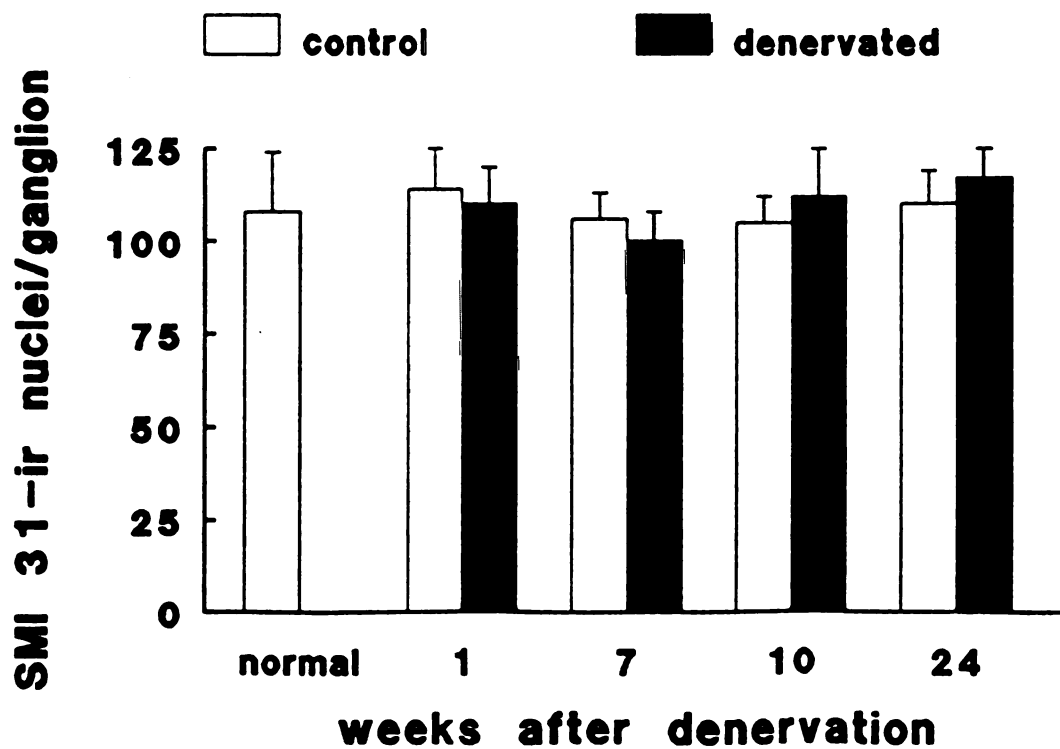
A**B**

Figure 27. The number of SMI 31-immunoreactive nuclei/ganglion in myenteric and submucosal plexus preparations from normal, sham, control, and denervated ileum. A. Number of SMI 31-ir nuclei/ganglion in submucosal plexus preparations obtained from normal, sham, or extrinsically-denervated ileum. Controls were taken from an area of ileum oral or aboral to the denervated loop. B. Number of SMI 31-ir nuclei/ganglion in myenteric plexus preparations obtained from normal, sham, or extrinsically-denervated ileum. Data are means $\pm$ SEM for at least 3 preparations per animal.

Figure 27.

A**B**

number of NADPH-diaphorase positive neurons (20.5 ± 1.4 cells/ganglion, 5150 neurons, 286 ganglia) increased by $94 \pm 2\%$ as compared to vehicle-treated controls (10.6 ± 1.08 neurons/ganglion, 4299 neurons, 423 ganglia).

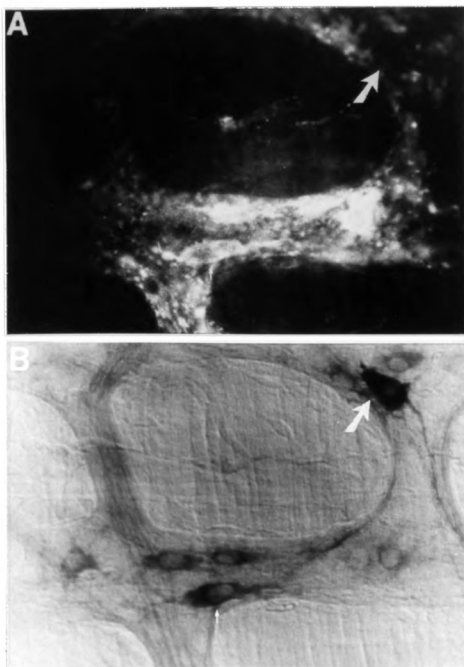
Nitric Oxide Synthase-Immunoreactivity and NADPH Diaphorase Staining are Co-Localized in Myenteric Neurons

Polyclonal antiserum raised against porcine cerebellar NOS was used to verify that denervation-induced increase in NADPH diaphorase staining represented denervation-induced alterations in NOS in myenteric ganglia. In preparations from normal ileum, NOS-ir was not observed in interganglionic, paravascular, and perivascular nerves (Figure 34A), and only 0.003 ± 0.002 neurons/submucosal ganglia contained NOS-ir (4 neurons, 957 submucosal ganglia, 10 preparations, 3 animals). However, similar to results from NADPH diaphorase staining, 8.7 ± 0.6 neurons/myenteric ganglion were immunoreactive for NOS (4002 neurons, 456 ganglia, 26 preparations, 7 animals) (Figure 30). Nitric oxide synthase-ir was located in two populations of myenteric neurons (Figures 28 and 29), as $17 \pm 2\%$ of neurons were large ($>30 \mu\text{m}$), brightly-fluorescent Dogiel type I neurons located at the periphery of myenteric ganglia. The majority of NOS-ir neurons were small ($< 30 \mu\text{m}$), less-brightly stained, and found within the interior of ganglia. Dendrites and axons of smaller neurons were not visible, and their morphology was difficult to resolve. However, they appeared to have small, smooth somata, and may represent interneurons. Nerve bundles in primary and secondary plexuses of myenteric plexus, and deep muscular plexus of the circular muscle contained bright NOS-ir, although little staining was observed in the tertiary plexus.

Co-localization studies with NADPH diaphorase were performed to determined the

Figure 28. Co-localization of nitric oxide synthase-immunoreactivity and NADPH diaphorase staining in myenteric ganglion from normal ileum. A. Whole mount preparation of myenteric plexus processed for NOS-ir. Large, darkly stained cells (arrow) and small, more lightly stained neurons are present. B. NADPH diaphorase staining is found in many NOS-ir neurons (arrows). Bar in B is 40 μ m.

Figure 28.



proportion of neurons containing NADPH diaphorase and NOS-ir. Tissues were fixed in paraformaldehyde, and processed for NOS-ir. The number of neurons/ganglion was recorded, the tissues were washed in PBS, and processed for NADPH diaphorase histochemistry. As preliminary studies revealed that dark NADPH diaphorase staining concealed immunofluorescence, preparations were incubated in nitroblue tetrazolium and NADPH for only 15 min. The decreased incubation time allowed most NADPH diaphorase cells to be visualized, as 8.4 ± 0.56 NADPH diaphorase positive cells/myenteric ganglion (1,768 neurons, 212 myenteric ganglia, 8 preparations, 3 animals) were observed. In 13 preparations (3 animals), 80-100% of NOS-ir neurons/preparation contained NADPH diaphorase staining (Figure 28), with an average of $88 \pm 2\%$ of NOS-ir neurons containing NADPH diaphorase staining. In the same preparations, 70-100% of NADPH diaphorase stained neurons/preparation contained NOS-ir, as $85 \pm 4\%$ of NADPH diaphorase stained neurons contained NOS-ir. Although some darkly stained NADPH diaphorase neurons contained bright NOS-ir, occasional bright NOS-ir neurons did not contain detectable NADPH diaphorase. Taken together, these results indicate that there is a strong relationship between NOS-ir and NADPH diaphorase staining in enteric neurons.

Extrinsic Denervation Increases the Number of Nitric Oxide Synthase-Immunoreactive Myenteric Neurons

One week after surgical extrinsic denervation, control segments contained 8.7 ± 0.5 NOS-ir neurons/myenteric ganglia (2,868 NOS-ir neurons, 342 myenteric ganglia, 12 preparations, 4 animals). However, the number of NOS-ir neurons in denervated segments significantly increased, as 13 ± 0.5 NOS-ir neurons/myenteric ganglion (4,184

Figure 29. Nitric oxide synthase-immunoreactivity in myenteric plexus preparations from control and denervated ileum. A. NOS-ir in myenteric ganglion taken from control segment. B. Four weeks after extrinsic denervation, increased neurons contain NOS-ir. Large, darkly stained neurons (arrow) and small, lightly stained neurons are present. Calibration bar in B is approximately 40 μm .

Figure 29.

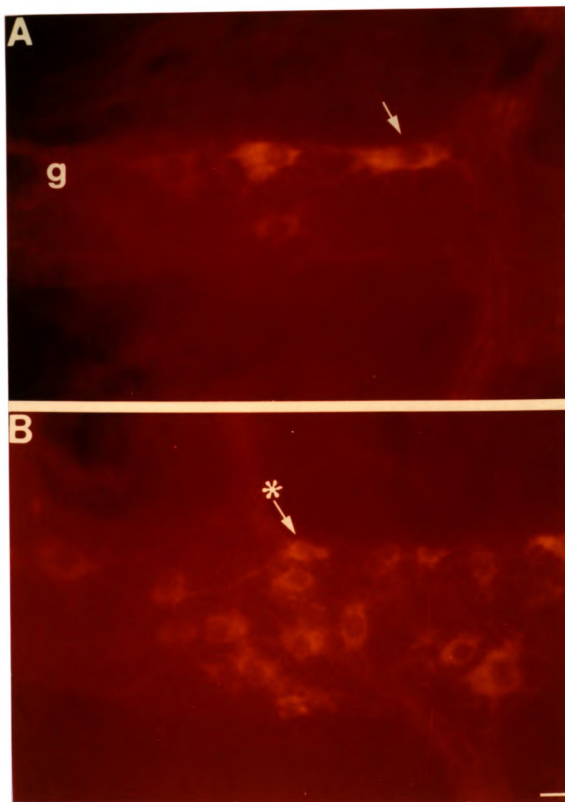
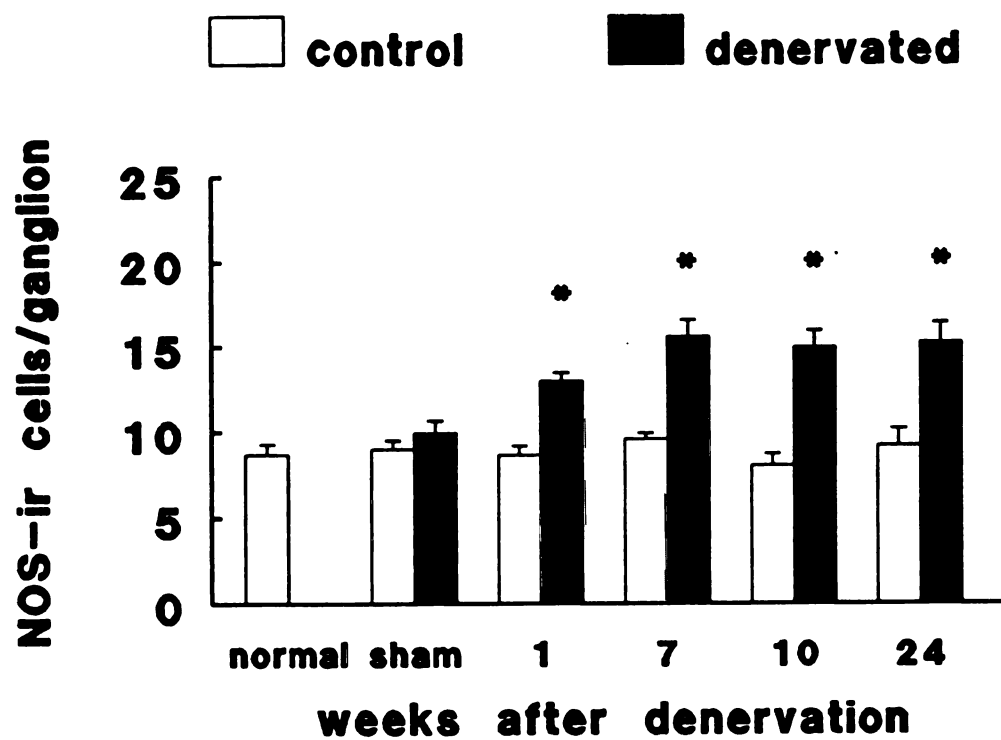
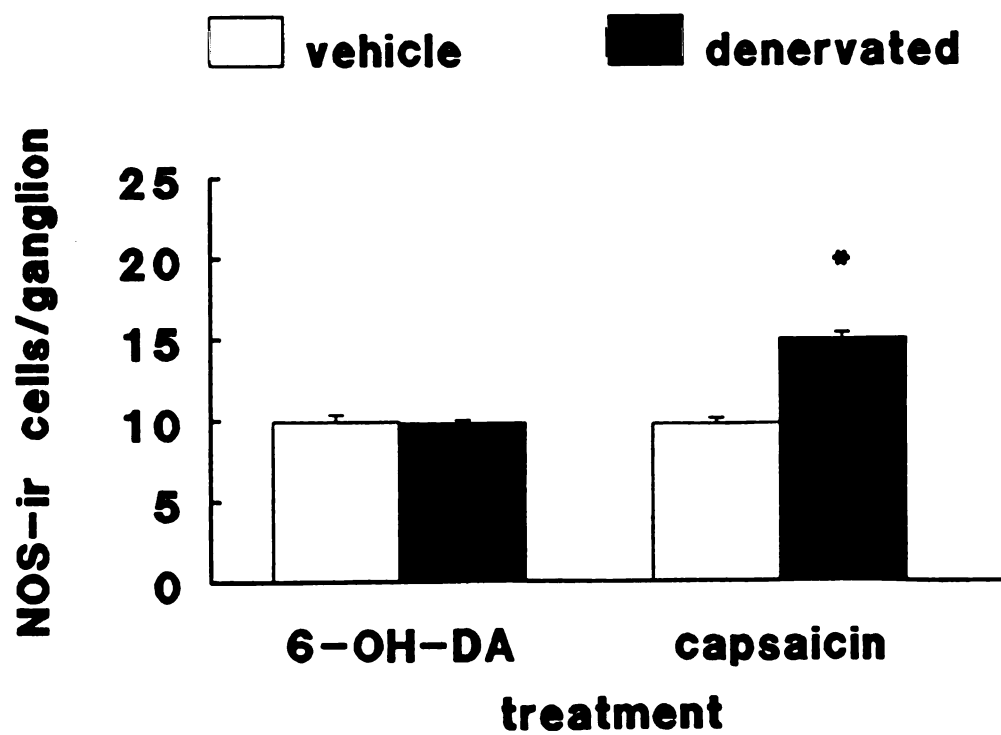


Figure 30. The number of nitric oxide synthase-immunoreactive neurons/ganglion from myenteric plexus preparations from normal, control, sham, and denervated ileum. A. Number of NOS-ir neurons/ganglion in myenteric plexus preparations obtained from normal, sham, or extrinsically-denervated ileum. Controls were from an area of ileum oral or aboral to the denervated loop. B. The number of NOS neurons/myenteric ganglion obtained from animals treated with 6-OH-DA or capsaicin to destroy sympathetic or primary afferent innervation, respectively. Controls were from age-matched vehicle-treated animals. Data are means $\pm$ SEM from at least 3 preparations per animal.

Figure 30.

A**B**

NOS-ir neurons, 328 myenteric ganglia, 12 preparations, 4 animals) were observed 30). The ratio of large, brightly-fluorescent:small neurons was not altered by ($18:82 \pm 3\%$) (Figure 29). The number of NOS-ir myenteric neurons/ganglion sham-denervated segments of ileum (8.1 ± 0.5 NOS-ir neurons/myenteric ganglion) not different from animal-matched controls (7.9 ± 0.4 NOS-ir ganglion), indicating that increased NOS-ir in myenteric ganglia was not the result of surgical stress. Finally, similar to results obtained utilizing NADPH diaphorase histochemistry, the number of NOS-ir neurons remained elevated 4, 7, 10, 16, and 24 weeks after loss of extrinsic nerves (Figure 29), further supporting the hypothesis that NOS is increased by loss of extrinsic nerves.

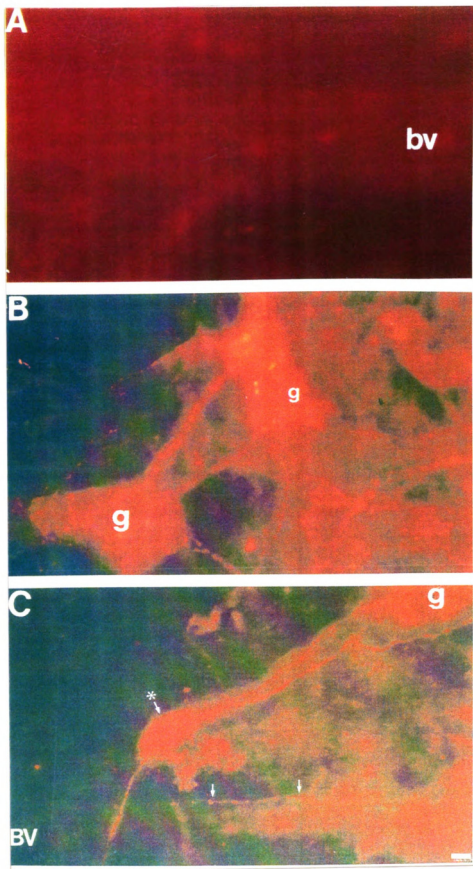
As NOS-ir in nerve fibers in interganglionic nerve strands and secondary nerve fibers is very bright in normal tissues, it was impossible to use immunohistochemical methods to determine if extrinsic denervation increased the amount of protein in these nerves. However, NOS-ir was not observed in the tertiary plexus in normal, control, or denervated tissues at any time (Figure 29).

Increased Nitric Oxide Synthase Results from Loss of Sensory Innervation

Systemic administration of 6-OH-DA did not alter the number of NOS-ir neurons in myenteric ganglia (9.8 ± 0.2 NOS-ir neurons/ganglion, 2,976 neurons, 304 ganglia, 3 animals) compared to tissues taken from vehicle-treated controls (9.9 ± 0.4 NOS-ir neurons/ganglion, 4,656 neurons, 456 ganglia, 3 animals) one week after destruction of sympathetic nerve terminals (Figure 30). However, systemic administration of capsaicin significantly increased the number of NOS-ir neurons/myenteric ganglion as compared to vehicle-treated controls (Figure 30), indicating that loss of primary afferent nerves is responsible for increased NOS observed in myenteric ganglia after extrinsic denervation.

Figure 31. Nitric oxide synthase-immunoreactivity in submucosal plexus preparations from normal and denervated ileum. A. Little NOS-ir is observed in submucosal ganglia or in vascular innervation. B. Four weeks after extrinsic denervation, occasional NOS-ir nerves are associated with arterioles (arrow). C. Four weeks after extrinsic denervation, occasional NOS-ir submucosal neurons are observed in ganglia (arrow with asterisk). bv, arteriole; g, ganglion. Bar in C is approximately 30 μ m.

Figure 31.



Loss of Extrinsic Innervation Does Not Increase Nitric Oxide Synthase in Submucosal Neurons

As described previously, only rare submucosal neurons contain NADPH diaphorase staining or NOS-ir in normal tissues. However, NOS-ir was examined in submucosal neurons to verify that loss of extrinsic innervation did not induce NOS-ir in submucosal neurons. One week after loss of extrinsic nerves, 0.02 ± 0.01 submucosal neurons/ganglion contained NOS-ir (30 neurons, 2106 ganglia, 14 preparations, 5 animals), whereas control preparations contained 0.01 ± 0.0 submucosal neurons/ganglion (6 neurons, 1,078 ganglia, 8 preparations, 3 animals) (Figure 31). Significant differences were not observed at any time after extrinsic denervation, indicating that loss of extrinsic nerves did not increase NOS in submucosal neurons (Figure 31C). However, as soon as four weeks after extrinsic denervation, occasional thin, non-varicose NOS-ir nerve fibers were closely associated with submucosal arterioles (Figure 31B). Although NOS-ir nerves were not observed with arterioles measuring over 60 μm , NOS-ir nerves were seen with arterioles as small as 10 μm (Table 7). Single NOS-ir nerves were usually associated with arterioles; however, 36% of 30-50 μm arterioles with NOS-ir innervation had a pair parallel NOS-ir nerve fibers associated on either side of the arteriole. The majority of arterioles from control and sham preparations did not have NOS-ir nerve fibers (Figure 31), indicating the appearance of NOS-ir nerve fibers resulted from loss of extrinsic nerves. Seven and ten weeks after extrinsic denervation, paravascular nerves closely-associated with arterioles, and some meandering nerve fibers were observed. Similar to earlier times, NOS-ir was not observed in control submucosal preparations. Finally, rare NOS-ir nerves were associated with submucosal arterioles 24 weeks after extrinsic denervation.

Table 7. Survey of the number of arterioles with NOS-ir nerves in normal, control, and surgically-denervated ileum.

Weeks After Dener- vation	Arteriolar Size (μm)						
	0-10	11-20	21-30	31-40	41-50	51-60	61-70
normal	0/14	0/14	0/15	0/12	0/8	0/4	0/7
week1 control	0/9	0/12	1/11	1/10	0/7	0/6	0/7
week1 denerv.	0/10	0/10	0/17	0/11	1/8	0/6	0/4
week4 control	0/9	0/16	1/17	1/8	0/7	0/6	0/7
week4 denerv.	1/7	0/10	5/17	7/11 [*]	7/10 [*]	0/6	0/8
week7 control	0/8	0/20	0/18	0/14	1/13	0/6	0/3
week7 denerv.	2/10	9/19 [*]	3/13 [*]	2/13	3/7	0/5	6/10 [*]
week24 control	0/6	0/16	1/20	1/17	0/14	0/5	2/10
week24 denerv.	0/8	6/16	9/19 [*]	4/12	3/12	2/7	1/5

Description of the fraction of submucosal arterioles receiving NOS-ir nerves/total submucosal arterioles examined. A single NOS-ir paravascular nerve was associated with arterioles, unless marked by *, to denote two nerves associated with a single arteriole. Data are taken from a minimum of 4 preparations for each time point.

Extrinsic Denervation Does Not Increase Vasoactive Intestinal Polypeptide in Myenteric Neurons

As described previously, immunohistochemical studies have demonstrated NOS-ir, NADPH diaphorase, and VIP-ir are co-localized in the same population of myenteric neurons (see introduction). Furthermore, explant and denervation studies of autonomic and dorsal root ganglia have demonstrated that decentralization or axotomy of ganglionic neurons increases VIP levels by 100-240 fold (reviewed in Rao and Landis, 1992). Therefore, VIP-ir was examined in submucosal and myenteric ganglia to determine if VIP was altered by loss of extrinsic nerves.

In normal preparations, bright VIP-ir was observed in varicose nerve fibers within ganglia and tertiary plexuses, and 3.0 ± 0.3 neurons/myenteric ganglion (740 neurons, 244 myenteric ganglia, 14 preparations, 4 animals) contained VIP-ir (Figure 33A). Vasoactive intestinal polypeptide-ir was observed in the cytoplasm of single neurons located in the interior of the ganglia (Figure 32A). These neurons had single axons that projected within the ganglia for short distances, and lamellar or flat dendrites characteristic of Dogiel I or III neurons. Most VIP-ir neurons were associated with other VIP-ir neurons that were in clusters of 2-6 neurons/ganglia. One week after extrinsic denervation, the myenteric plexus contained 3.0 ± 0.5 VIP-ir neurons/ganglion (751 neurons, 376 ganglia, 21 preparations, 5 animals), which was not different from control preparations taken oral or aboral to the denervated segments (2.2 ± 0.3 VIP-ir neurons/ganglion (751 neurons, 376 ganglia, 21 preparations, 5 animals) (Figure 33A). Similar results were at all time points examined (Figure 32A). Finally, extrinsic denervation did not alter the number of bunches, or the number of neurons/bunch (data not shown).

Figure 32. Vasoactive intestinal polypeptide-immunoreactivity in myenteric plexus preparations from normal and extrinsically-denervated ileum. A. VIP-ir in myenteric ganglion taken from control segment of ileum. B. Four weeks after extrinsic denervation, VIP-ir is not altered in myenteric ganglia. Calibration bar in B is approximately 40 μm .

Figure 32.

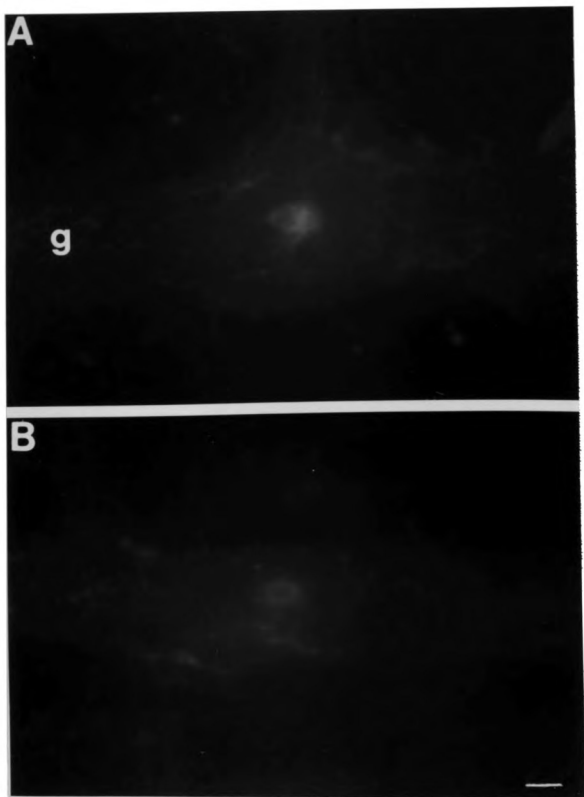
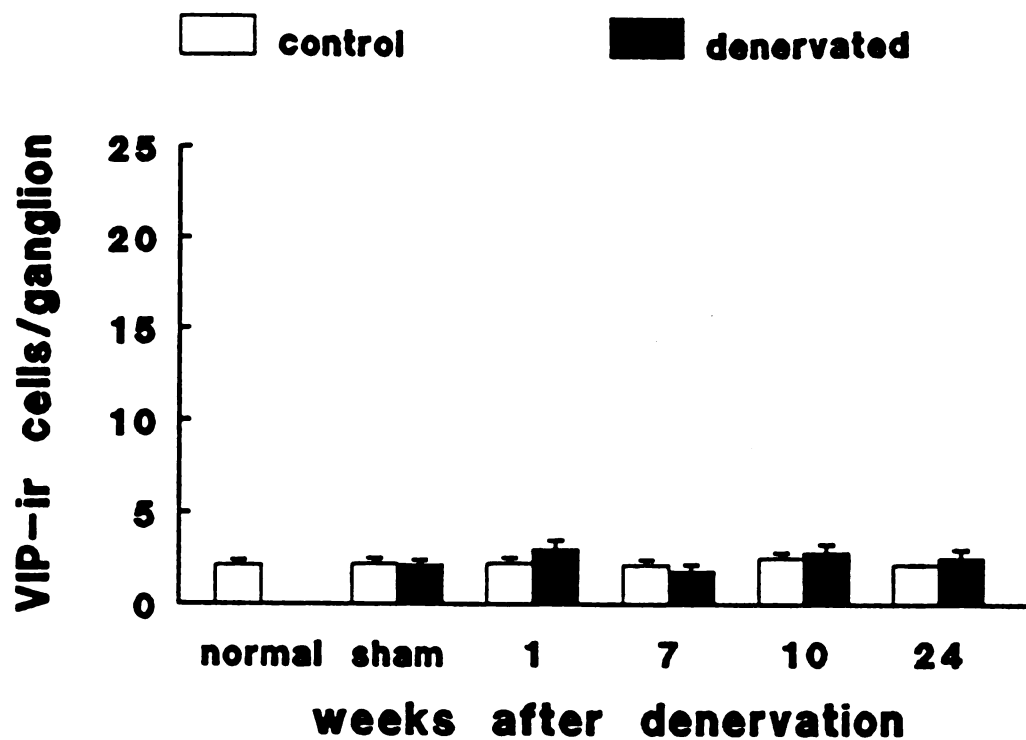
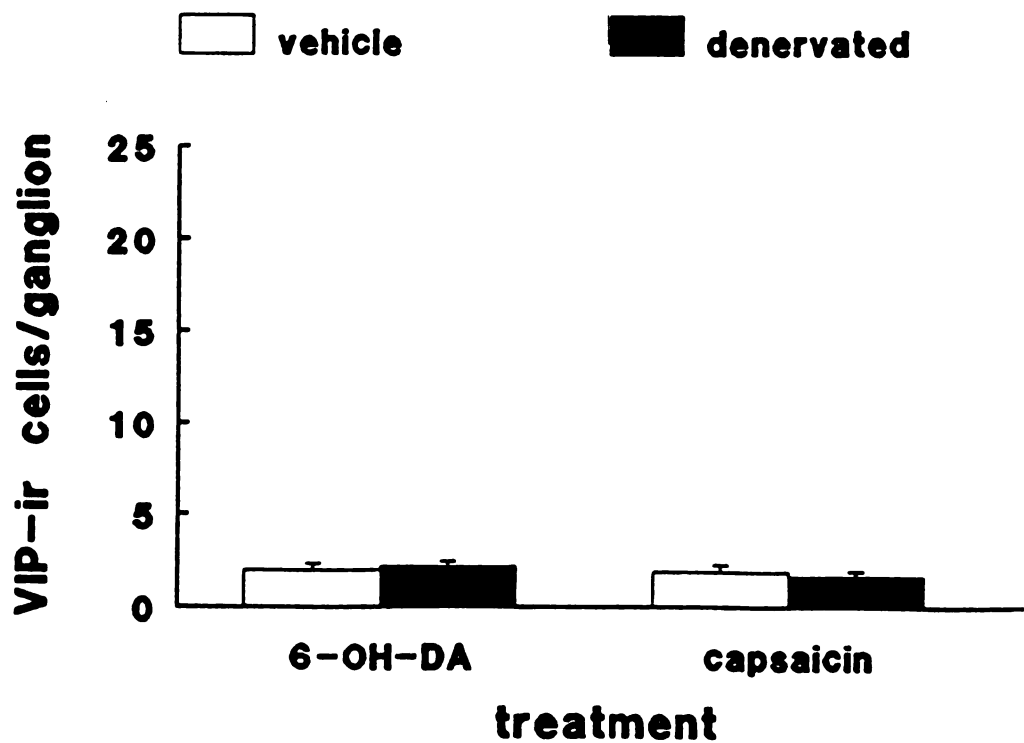


Figure 33. The number of vasoactive intestinal polypeptide-immunoreactive neurons/ganglion in myenteric plexus preparations from normal, sham, and denervated ileum. A. Number of VIP-ir neurons/ganglion in myenteric plexus preparations from denervated and control loops. B. The number of VIP-ir neurons/myenteric ganglia obtained after animals were treated with 6-hydroxy-dopamine or capsaicin to destroy sympathetic or primary afferent innervation, respectively. Controls were taken from age-matched vehicle-treated animals. Data are means $\pm$ SEM from at least 3 preparations per animal.

Figure 33.

A**B**

Extrinsic Denervation Does Not Increase Vasoactive Intestinal Polypeptide in Submucosal Neurons

Strong, cytoplasmic VIP-ir was observed in 3.5 ± 0.2 submucosal neurons/ganglia (4,308 neurons, 1,478 ganglia, 25 preparations, 8 animals) with Dogiel III morphology. One week after loss of extrinsic nerves, the number of VIP-ir neurons/ganglia was not altered, as 4.0 ± 0.6 VIP-ir cells/ganglia (3,222 neurons, 805 ganglia, 28 preparations, 5 animals) were observed in denervated tissues, whereas control preparations contained 3.0 ± 0.6 VIP-ir cells/ganglia (4,197 neurons, 1,396 ganglia, 31 preparations, 5 animals). Furthermore, extrinsic denervation did not alter the number of VIP-ir submucosal neurons/ganglia at all time points examined (data not shown), indicating that loss of extrinsic nerves does not increase VIP in submucosal ganglia.

6. NEUROGENIC RELAXATIONS ARE POTENTIATED FOLLOWING EXTRINSIC DENERVATION

Introduction and Experimental Rationale

As described in the introduction, transmural stimulation of precontracted longitudinal muscle-myenteric plexus preparations (LMMPPs) results in a neurogenic relaxation of smooth muscle followed immediately by a rebound or off contraction. Previous work has shown conclusively that NO mediates a large proportion of neurogenic relaxations of GI smooth muscle in all species thus far examined (see introduction). LMMPPs from guinea pig ileum have been extensively used to demonstrate that myenteric neurons release NO, and that NO has similar pharmacological properties as the neurogenic NANC inhibitory neurotransmitter. Therefore, relaxations of LMMPPs from control and extrinsically-denervated segments of ileum were examined in order to determine if increased quantity of NOS-ir myenteric neurons had a functional consequence on GI motility. Specifically, it was hypothesized that the increased number of NOS-ir myenteric neurons would increase production and release of NO in denervated preparations, resulting in larger neurogenic relaxations of LMMPPs as compared to control preparations. Furthermore, as the pharmacology of neurotransmission to ileal smooth muscle has been well-characterized, functional alterations in other neurotransmitters may be detected in this model after loss of extrinsic nerves.

Extrinsic Denervation Does Not Alter Passive or Active Tensile Properties of Longitudinal Muscle

Histamine (0.3-30 μ M) contracted LMMPPs from normal, control, and denervated segments of ileum. After a rapid contraction, a small desensitization occurred, and

sustained active tension was maintained until histamine was removed by washing. As pre-contracted tissues were necessary to study neurogenic relaxations, the first study determined if loss of extrinsic innervation altered the histamine concentration-response curve. Four (2 animals, 12 preparations) and seven weeks (2 animals, 11 preparations) after extrinsic denervation, the amplitude of histamine-evoked contractions of LMMPPs was not different than contraction amplitudes from LMMPPs made from control segments oral or aboral to the denervated segment (Figure 34A). These data indicate that loss of extrinsic nerves does not alter histamine receptors or the contractile apparatus of longitudinal muscle.

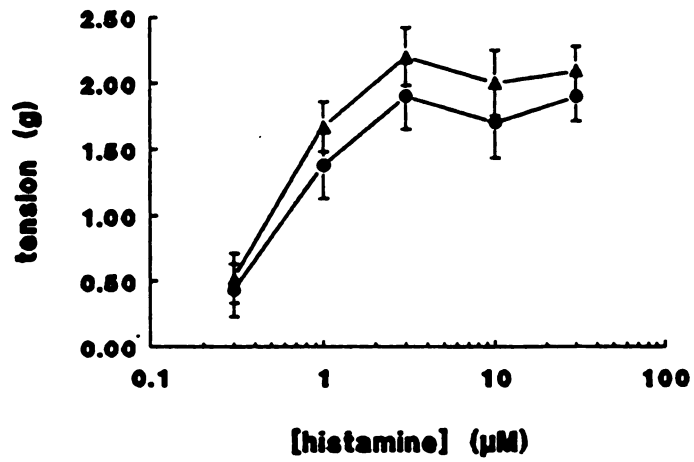
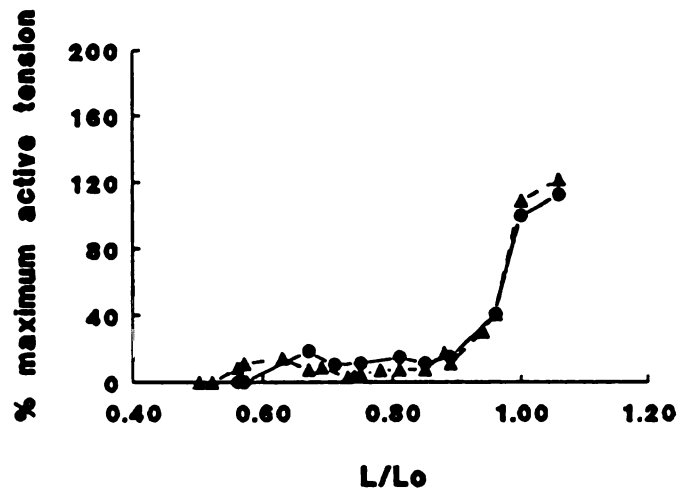
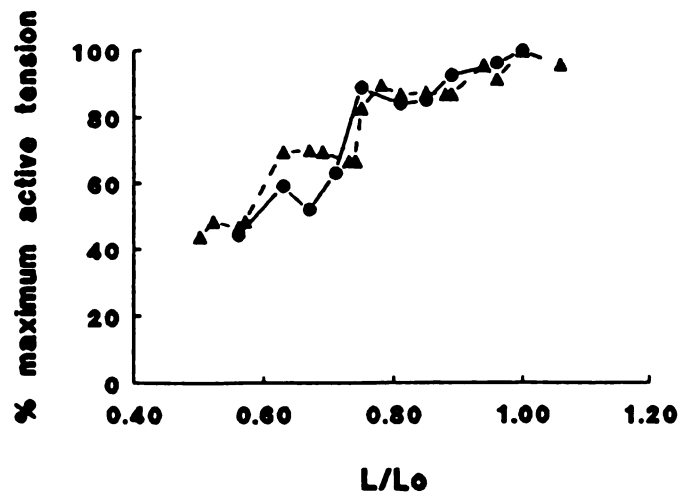
Generation of active and passive tension by longitudinal muscle was further examined in LMMPPs made from denervated and control loops of ileum. Seven weeks after extrinsic denervation, the relationship between the length of longitudinal muscle and the development of passive (Figure 37B) and active (Figure 37C) were not affected by loss of extrinsic nerves.

Extrinsic Denervation Increases Neurogenic Relaxations of Longitudinal Muscle-Myenteric Plexus Preparations

In the presence of histamine (3 μM), transmural stimulation (20 Hz) of pre-contracted LMMPPs from normal ileum resulted in TTX-sensitive (300 nM) neurogenic relaxations of longitudinal muscle. These relaxations were attenuated in a concentration-dependent manner by oxyhemoglobin and L-, but not D-arginine analogs (data not shown), similar to the findings of Osthaus and Galligan (1992). These drugs were unlikely to directly affect smooth muscle, as papeverine (10 μM)-induced relaxations were not altered by 100 μM LNA or 10 μM hemoglobin (data not shown). Finally, SNP

Figure 34. Concentration-response curves for the effects of histamine on NANC relaxations of longitudinal muscle-myenteric plexus preparations from control and denervated ileum, and length-tension relationships for longitudinal muscle from control and denervated ileum. A. Concentration-response curves for the effects of histamine on LMMPPs from control and surgically-denervated loops of ileum. Controls were taken oral or aboral to the denervated segment. B. Passive tension relationships for control and surgically-denervated LMMPPs. Passive tension was generated by stretching the preparations by 0.5 mm increments and allowing the preparations to reach steady-state tension as described in the text. Data were pooled from 6 preparations from 2 animals, and were normalized for maximum active tension. C. Active tension relationships for control and surgically-denervated ileum. Active tension was generated by application of histamine (3 μ M), and the maximum agonist-induced contractile event was measured as described in the text. Data were pooled from 6 preparations from 2 animals and were normalized for maximum active tension. Preparations were denervated 4 or 7 weeks prior to experiment.

Figure 34.

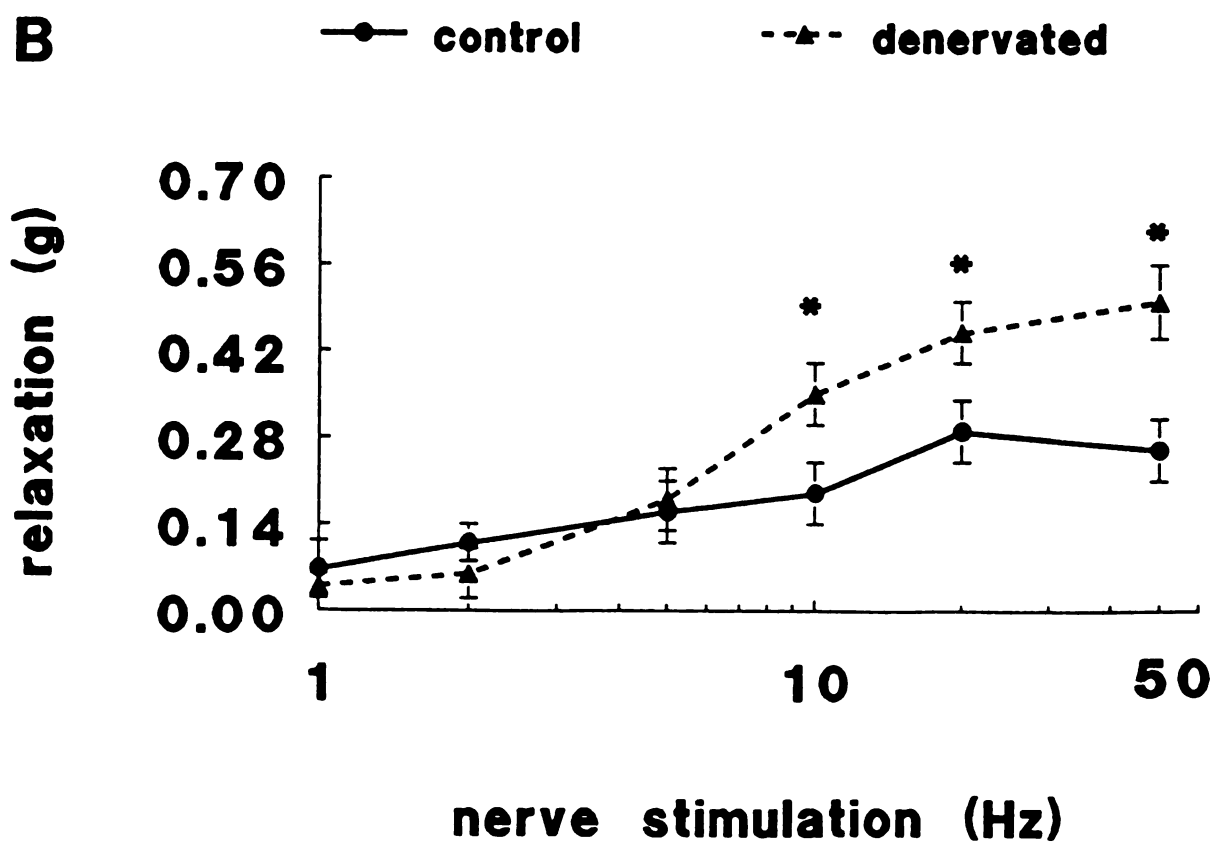
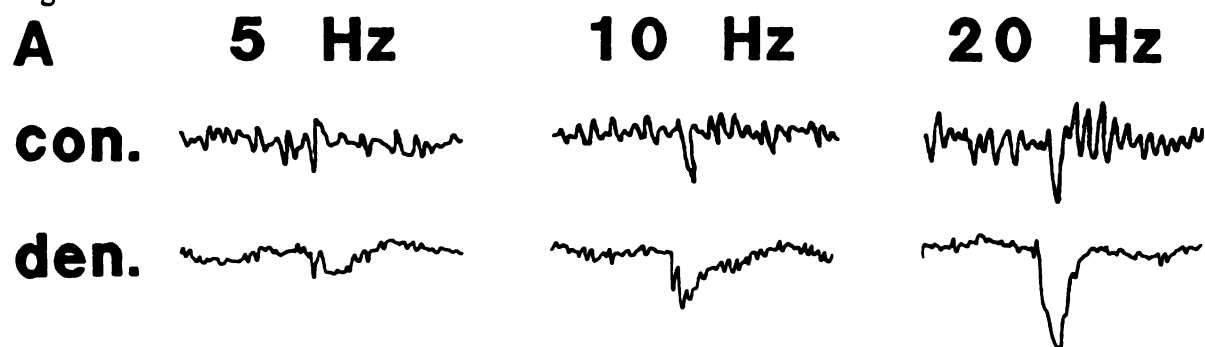
A**B****C**

decomposes to NO and this exogenous NO-donor mimicked neurogenic relaxations (Figure 37), in agreement with other studies (Osthaus and Galligan, 1991).

Four and seven weeks after surgical extrinsic denervation, transmural stimulation (20 Hz) of histamine (3 μ M)-contracted LMMPPs from surgically-denervated ileum produced relaxations with larger amplitudes and duration of relaxation as compared to control preparations from the same animals (Figure 35A). All relaxations were inhibited by TTX (300 nM), indicating the relaxations resulted from stimulation of enteric nerves. Frequency-response curves for extrinsically-denervated and control LMMPPs were examined to determine if the frequency of nerve stimulation affected the amplitude of relaxation of LMMPPs, and if this relationship was altered by loss of extrinsic nerves. As shown in Figure 35B, the relaxation amplitude of control and denervated preparations was dependent on the frequency of nerve stimulation, as very small relaxations were observed at low frequency nerve stimulation, and the amplitude of relaxations increased as the frequency of nerve stimulation increased. Furthermore, increasing the frequency of nerve stimulation potentiated the amplitude of neurogenic relaxations in normal (data not shown), control, and surgically-denervated preparations (Figure 35B). Although the amplitude of relaxation of LMMPPs from control and surgically-denervated segments of ileum was similar when 1, 2, and 5 Hz train stimuli was used, significantly larger relaxations were observed in denervated preparations at 10, 20, and 50 Hz nerve stimulation (Figure 35). For example, the amplitude of nerve-evoked relaxations of LMMPPs from denervated segments of ileum was 13% larger than controls when nerves were stimulated at 5 Hz; however, the relaxation amplitude of LMMPPs prepared from denervated segments of ileum was 93% larger when intrinsic nerves were stimulated at

Figure 35. Neurogenic NANC relaxations of precontracted longitudinal muscle-myenteric plexus preparations from normal and denervated ileum. A. Nerve-mediated relaxations of longitudinal muscle-myenteric plexus preparations from an unoperated (con., control) and surgically-denervated (den.) loop of ileum. Relaxations were induced by trains of electrical stimulation, as described in the text. The horizontal axis is 0.1 min, and the vertical axis is 0.5 g. B. Electrical stimulation of LMMPPs produces a frequency-dependent increase in the amplitude of relaxation in control and denervated tissues. Differences were not observed between normal and control LMMPPs (data not shown). Ileal preparations were denervated 4-7 weeks prior to the experiment and data are means of amplitudes of nerve-evoked relaxations of pre-contracted ileum from 8-14 preparations. * represents $p < 0.05$ as compared to animal-matched controls.

Figure 35.



50 Hz.

Extrinsic Denervation Increases Nitrinergic Neurogenic Relaxations of Longitudinal Muscle Myenteric Plexus Preparations

Frequency-response curves were generated in the presence and absence of LNA (300 μ M) to determine if the denervation-induced potentiation of neurogenic relaxations resulted from increased production of NO. In LMMP preparations from normal animals (17 preparations, 4 animals), LNA (300 μ M) significantly attenuated relaxations generated at 10-50 Hz nerve stimulation (data not shown). However, differences were not observed at 1-5 Hz, indicating the small amplitude relaxations observed at these frequencies were not mediated by NO. Similar results were obtained in LMMP preparations from control and denervated LMMP preparations (Figure 36), as neurogenic relaxations produced by 10-50 Hz nerve stimulation were inhibited by LNA (300 μ M). These data indicate that denervation-induced potentiation of neurogenic relaxations resulted from increased NO, as relaxations were attenuated by a known, previously-characterized NOS antagonist. Furthermore, LNA did not attenuate relaxations of control or denervated preparations at frequencies below 5 Hz, suggesting that low frequency relaxations are not mediated by NO, but by an unidentified inhibitory neurotransmitter. Finally, the frequency-dependence of LNA offers an explanation for the lack of potentiation of low frequency relaxations after extrinsic denervation. As described previously, loss of extrinsic nerves increases NOS-ir and NADPH diaphorase staining in myenteric neurons, and potentiates neurogenic relaxations at 10-50 Hz stimulation. However, extrinsic denervation may not potentiate low frequency relaxations, as loss of extrinsic nerves does not alter the unidentified neurotransmitter(s) released at 1-5 Hz.

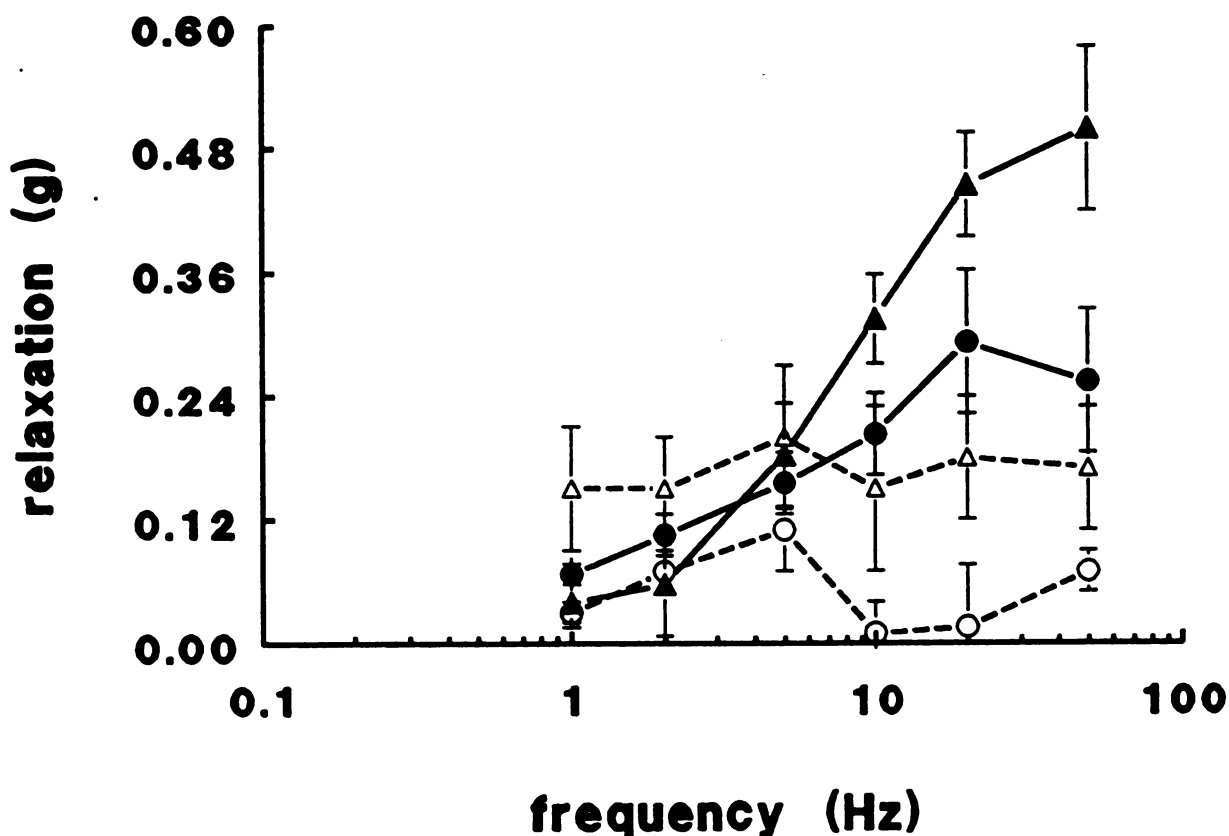


Figure 36. Nitro-L-arginine attenuates neurogenic NANC relaxations of LMMPPs from control and denervated ileum. Amplitudes of neurogenic relaxations of LMMPPs from control (closed circles) and denervated (closed triangles) segments of ileum are dependent on the frequency of nerve stimulation. LNA (300 μ M) significantly attenuated neurogenic relaxations generated at 10, 20, and 50 Hz stimulation in control (open circles) and denervated (open triangles) preparations. LNA was added to tissues 30 min prior to experiments. Data are means of amplitude of relaxations of precontracted tissues $\pm$ SEM for 12 preparations from 2 animals denervated 4 weeks prior to experiment. Although not indicated, nerve-evoked relaxations (10-50 Hz) are significantly larger in denervated preparations, and LNA significantly attenuated relaxations in control and denervated preparations at 10-50 Hz transmural stimulation ($p < 0.05$).

Denervation-Induced Potentiation of Neurogenic Relaxations Does Not Result From Alterations in Longitudinal Muscle

Proceeding extrinsic denervation, larger neurogenic relaxations could result from increased release of inhibitory neurotransmitter, such as NO. However, loss of extrinsic nerves may induce alterations in longitudinal muscle, resulting in increased sensitivity to inhibitory neurotransmitters. Concentration-response curves for sodium nitroprusside (SNP) were examined in LMMPPs from control and denervated regions to determine if extrinsic denervation potentiated the responsiveness of longitudinal muscle to exogenous NO. As shown in Figure 37, extrinsic denervation did not alter SNP-induced (1-30 μ M) relaxations of precontracted LMMPPs, as the amplitude of relaxations were not different in denervated or control preparations. These data indicate that denervation-induced potentiation of relaxations does not result from changes in sensitivity of longitudinal muscle, but from increased inhibitory neurotransmitter release.

Loss of Primary Afferent Innervation Increases Nitrinergic Neurogenic Relaxations of Longitudinal Muscle Myenteric Plexus Preparations

As surgical extrinsic denervation destroys extrinsic sympathetic and sensory nerves, 6-OH-DA and capsaicin were used to eliminate sympathetic and sensory nerves respectively. The amplitude of neurogenic relaxations was not altered one and four weeks after administration of 6-OH-DA, as the frequency-response relationship from preparations made from ileum of 6-OH-DA-treated animals (8 preparations, 2 animals) were not different from frequency-response relationships from vehicle-treated animals (6 preparations, 2 animals) (Figure 38A). However, seven weeks after administration of capsaicin (14 preparations, 3 animals) significantly larger neurogenic relaxations were observed at 20 and 50 Hz nerve stimulation as compared to preparations from

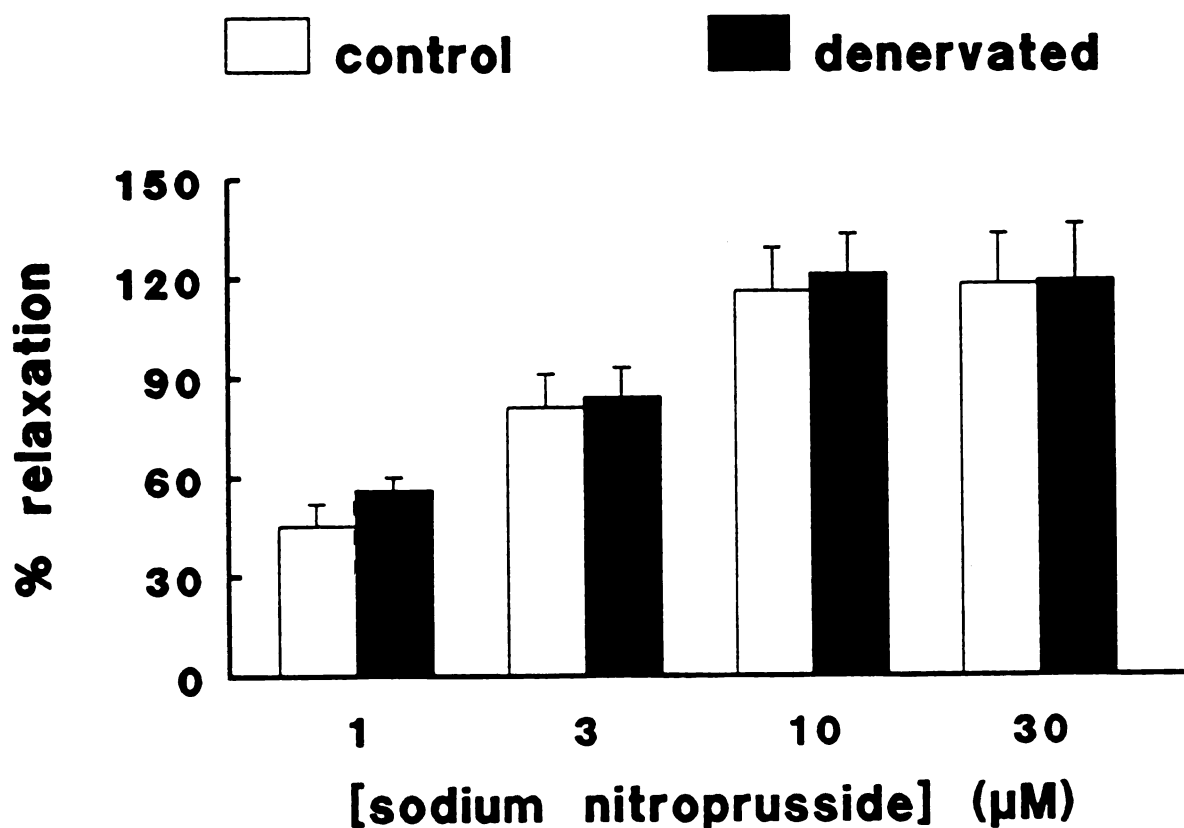


Figure 37. Sodium nitroprusside mimics neurogenic relaxations in longitudinal muscle-myenteric plexus preparations from control and denervated ileum. Non-cumulative concentrations of SNP were applied to pre-contracted LMMPPs, and the data are expressed as means of % loss of histamine-induced tone $\pm$ SEM. Animals were denervated 4 and 7 weeks prior to the experiment.

vehicle-treated animals (9 preparations, 2 animals) (Figure 38B). These relaxations were attenuated by LNA in a concentration-dependent manner (1-300 μM) (data not shown), similar to results obtained from surgically-denervated ileum. Taken together, these results indicate that loss of extrinsic sensory nerves, but not sympathetic nerves, accounts for increased potentiation of neurogenic relaxations.

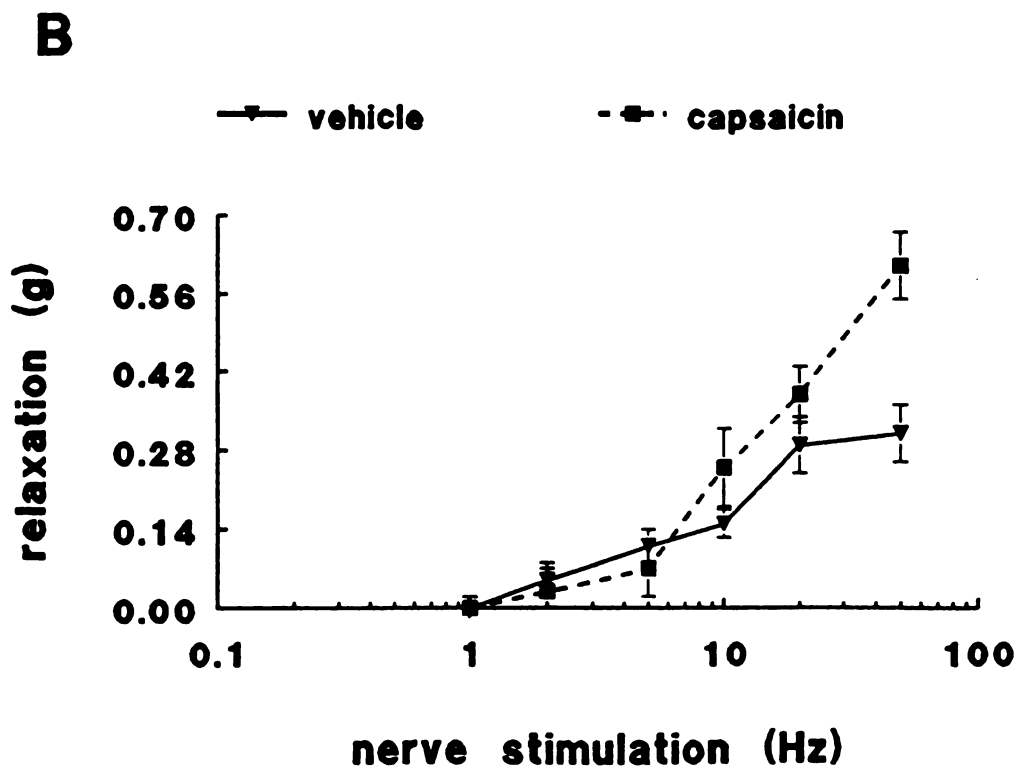
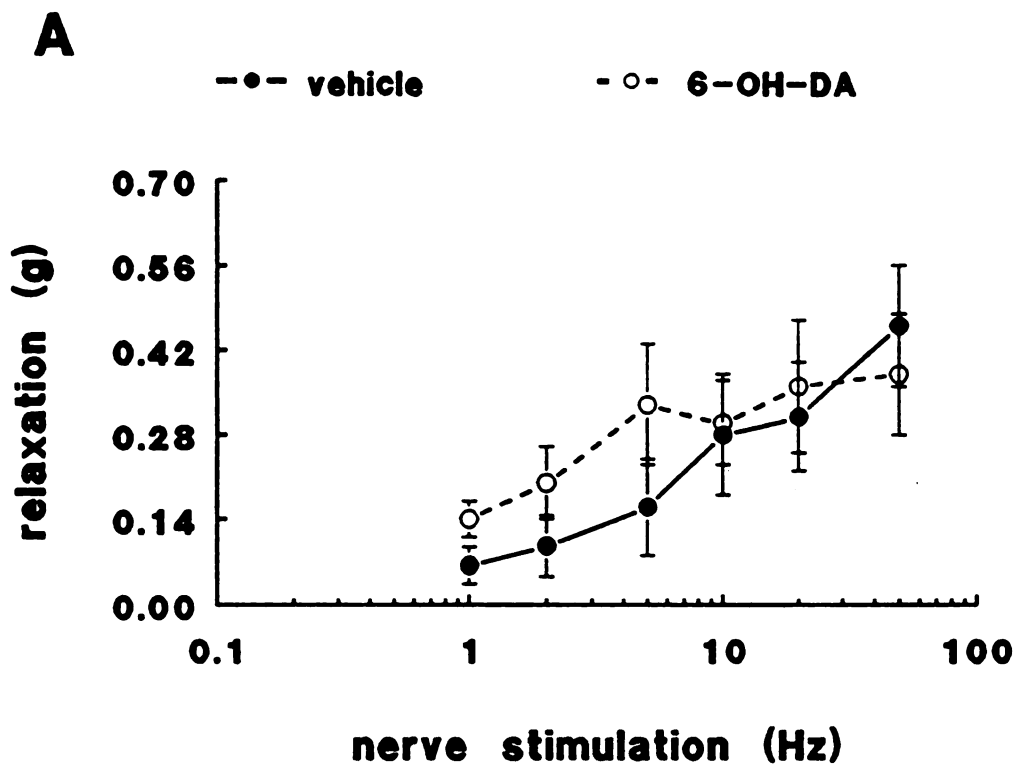
Extrinsic Denervation Decreases Rebound Contractions of Longitudinal Muscle Myenteric Plexus Preparations

Following neurogenic relaxations of longitudinal muscle, a monophasic contractile event is observed in normal tissues (Osthaus and Galligan, 1992). These contractile events, referred to as rebound or off contractions, are mediated by SP. In normal tissues, SP, or substance P methyl ester (SPME), a NK_1 receptor agonist, produced concentration-dependent (1-300 nM) contractions in the presence or absence of TTX (300 nM) (data not shown). Furthermore, CP-96,345-1, a non-peptide NK_1 receptor antagonist, antagonized SP contractions in a concentration-dependent manner (Figure 39), resulting in parallel shifts in SP concentration-response curves (Figure 39). Schild analysis of these data revealed a slope not significantly different from 1 (Figure 39B), allowing estimation of the concentration of CP-96,345-1 necessary to produce a 2-fold shift in the SP concentration response curve (pA_2 ; $\text{pA}_2=8.23$). Schild analysis also allowed estimation of the antagonist dissociation constant (K_b ; $K_b = 5.8 \text{ nM}$). This constant is similar to dissociation constants for NK_1 receptors in guinea pig ileum (see Gerard *et al.*, 1993), indicating CP-96,345-1 is a valuable antagonist in the study of tachykinin receptors.

Four and seven weeks after extrinsic denervation, the amplitude of rebound contractions were significantly attenuated as compared to control preparations (Figures

Figure 38. Frequency dependence of neurogenic NANC relaxations of longitudinal muscle-myenteric plexus preparations from animals treated with 6-OH-DA or capsaicin. A. Electrical stimulation of LMMPPs produced a frequency-dependent increase in the amplitude of relaxations in vehicle- and 6-OH-DA-treated preparations. B. Electrical stimulation of LMMPPs produced a frequency-dependent increase in the amplitude of relaxations in vehicle- and capsaicin-treated preparations. * represents $p < 0.05$ as compared to vehicle-treated controls.

Figure 38.



38A and 38B). Frequency-response curves were generated to determine if denervation-induced attenuation of nerve-evoked NANC contractions was frequency dependent. As shown in Figures 41A and 41B, increased frequency of nerve stimulation potentiated the amplitudes of rebound contractions in all tissues examined. Furthermore, loss of extrinsic innervation attenuated the amplitudes of rebound contraction as compared to responses of control preparations at all frequencies examined. These data indicate that denervation-induced alterations in rebound contractions are not dependent on the frequency of nerve stimulation.

CP-96,345-1 (1-300 nM) inhibited rebound contractions in control and denervated tissue in a concentration-dependent manner (Figure 41A), indicating that loss of extrinsic nerves did not alter the identity of neurotransmitter used to mediate rebound contractions. Furthermore, CP-96,345-1 did not alter the bethanechol concentration-response curves of longitudinal muscle (Figure 40B). These data indicate that CP-96,345-1 does not inhibit neurogenic activity by non-selective alterations in contractibility of longitudinal muscle, but rather inhibits SP neurotransmission by an action at NK₁ receptors on longitudinal muscle.

Use of the NK₁ receptor antagonist did not determine if the denervation-induced loss of extrinsic innervation decreased release of excitatory neurotransmitters, or decreased contractibility of longitudinal muscle. However, as described previously, surgical denervation did not alter the amplitude of histamine contractions, indicating loss of extrinsic nerves did not affect contractile properties of longitudinal muscle. Finally, a similar attenuation of rebound contractions was observed in preparations made from animals treated with capsaicin (Figure 43A), but not 6-OH-DA (Figure 43B).

Figure 39. CP-96,345-1 antagonized substance P-induced contractions of LMMPPs from normal ileum. A. Concentration response relationships for SP in the absence and presence of indicated concentrations of CP-96,345-1. Data are mean $\pm$ SEM of a minimum of eight observations per point. B. Schild analysis of data in A yielded a slope of 1.07 ± 0.11 (not significantly different from 1.0) and a pA_2 value of 8.23. Data kindly provided by Dr. J.J. Galligan from experiments performed by C. Martin.

Figure 39

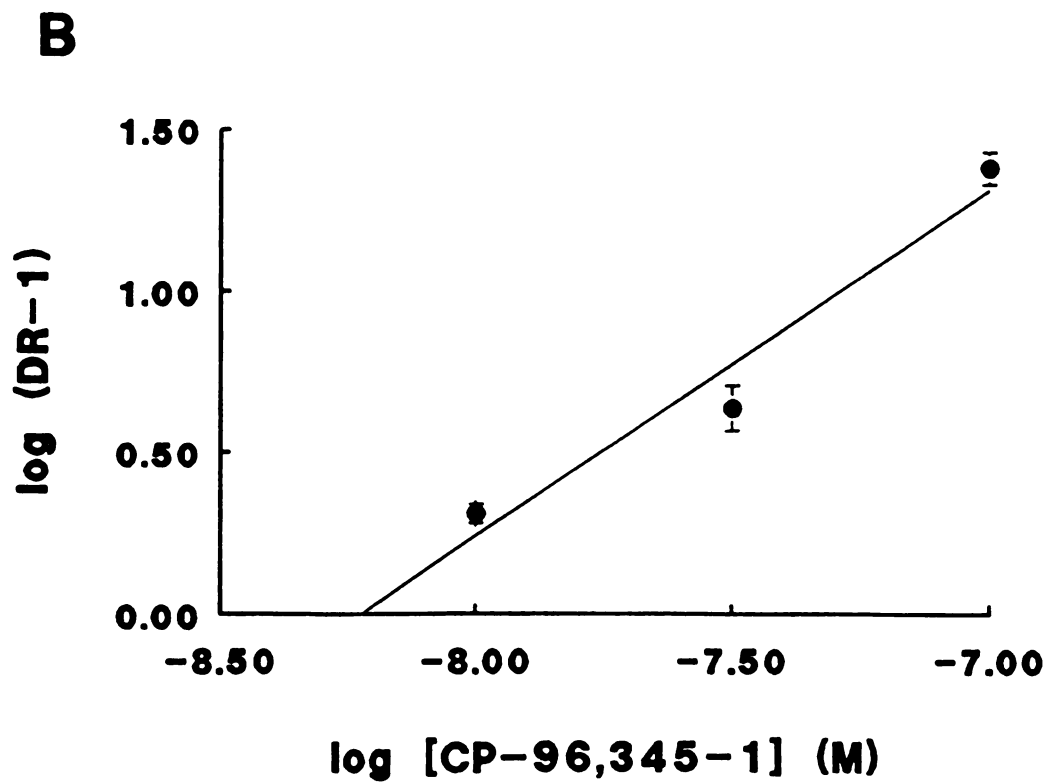
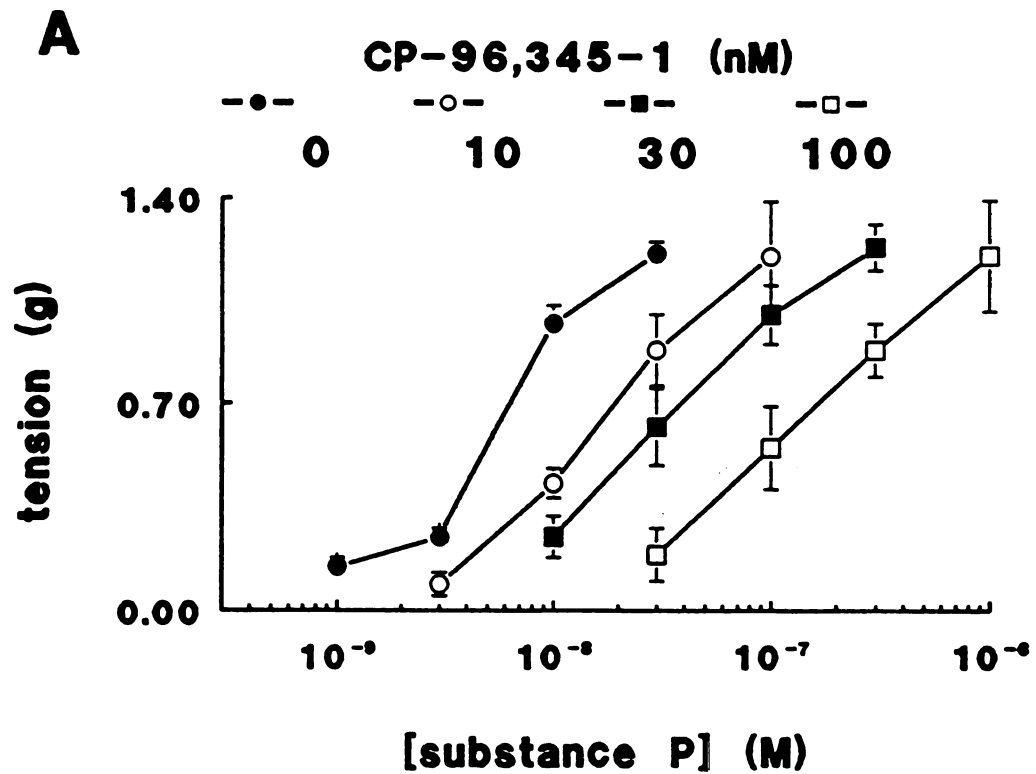


Figure 40. Concentration-response curves for CP-96,345-1-mediated inhibition of nerve-evoked, non-cholinergic contractions of normal, control, and denervated LMMPPs. A. CP-96,345-1 inhibits nerve-evoked non-cholinergic contractions of LMMPPs in a concentration-dependent manner. B. CP-96,345-1 did not affect contractions produced by bethanechol in normal ileum. Data are expressed as mean of contraction amplitude (g), from a minimum of nine preparations.

Figure 40.

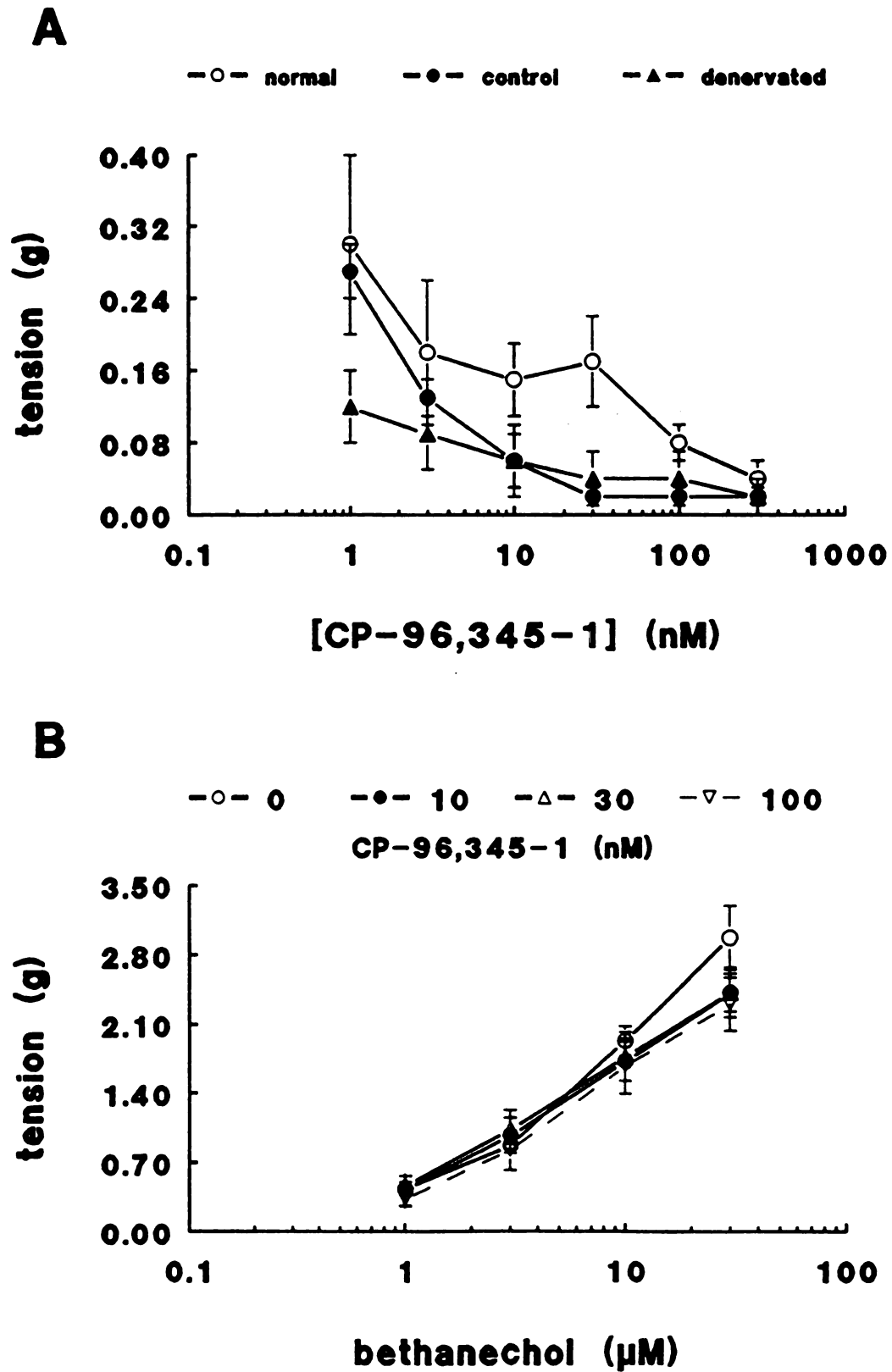


Figure 41. Neurogenic NANC contractions of longitudinal muscle-myenteric plexus preparations from control and denervated ileum. A. Nerve-mediated contractions of longitudinal muscle-myenteric plexus preparations from an unoperated and surgically-denervated loop of ileum. Contractions were induced by trains of electrical stimulation, as described in the text. The horizontal axis is 0.1 min, and the vertical axis is 0.5 g. B. Electrical stimulation of LMMPPs produces a frequency-dependent increase in the amplitude of contraction in control and denervated tissues. Differences were not observed between normal and control LMMPPs. Ileal preparations were denervated 4-7 weeks prior to the experiment. * represents $p < 0.05$ as compared to animal-matched controls.

Figure 41.

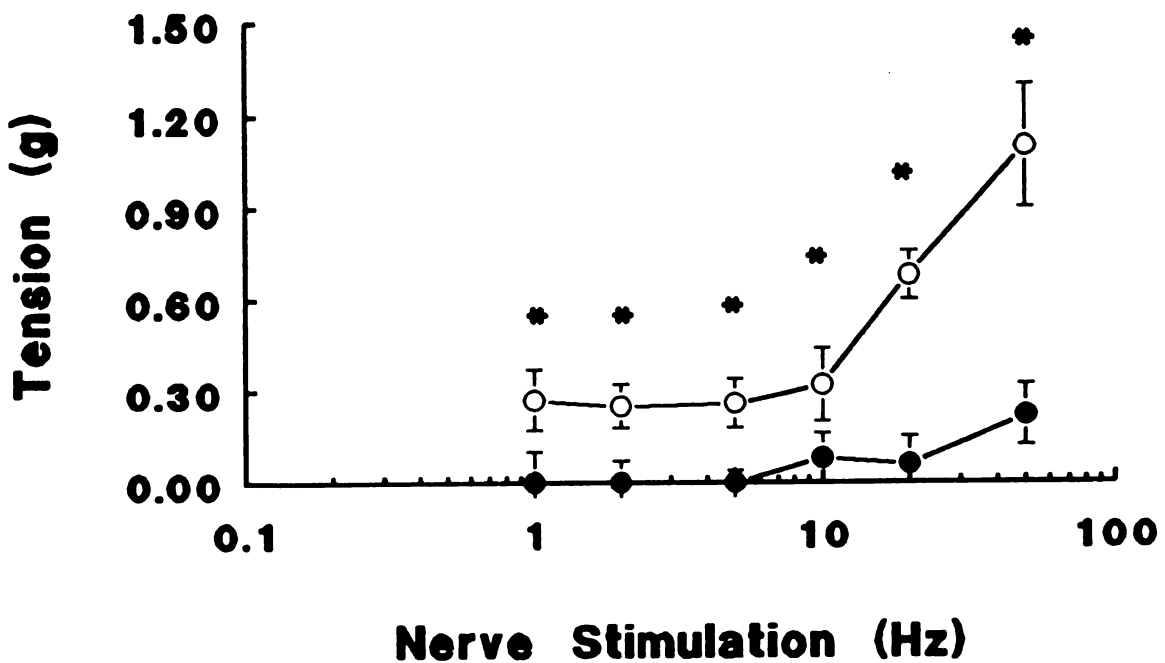
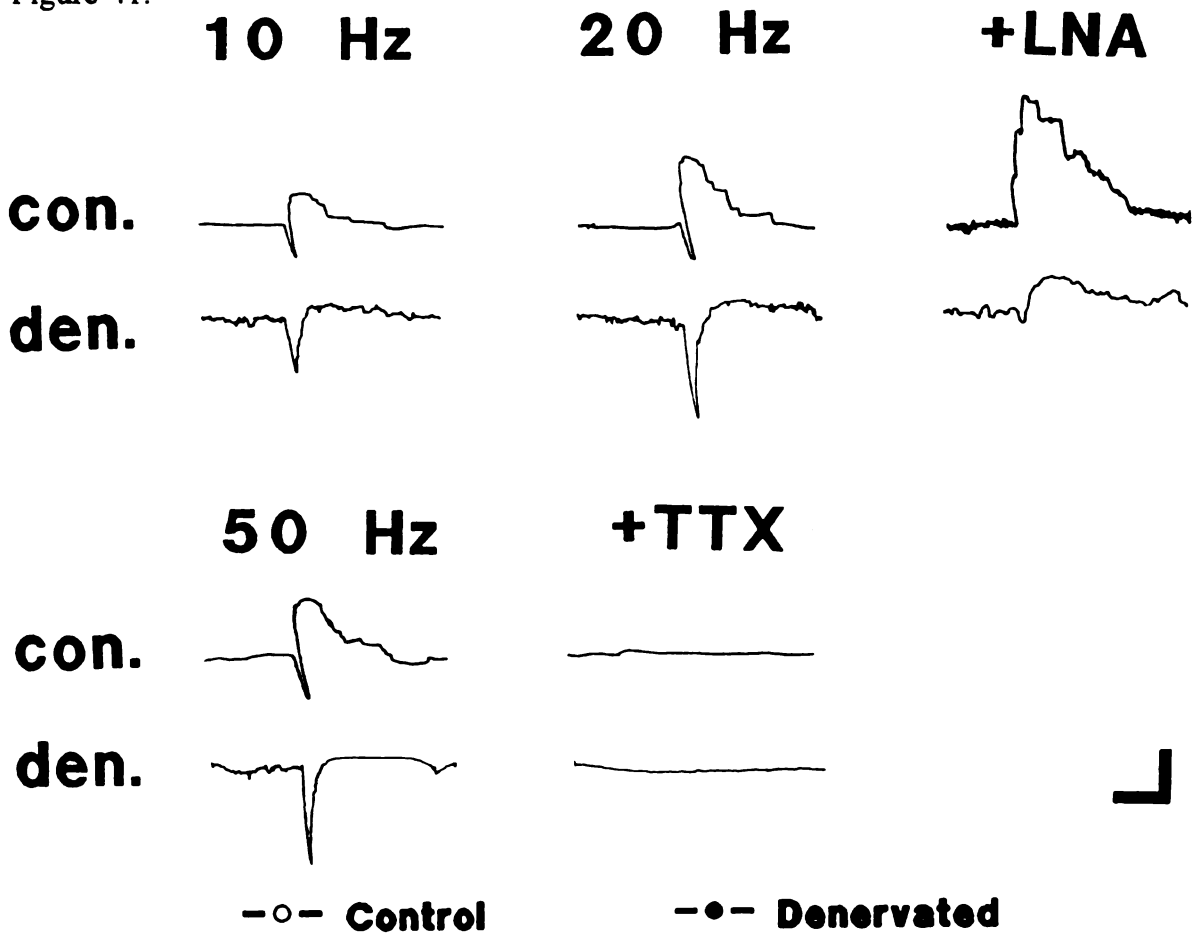
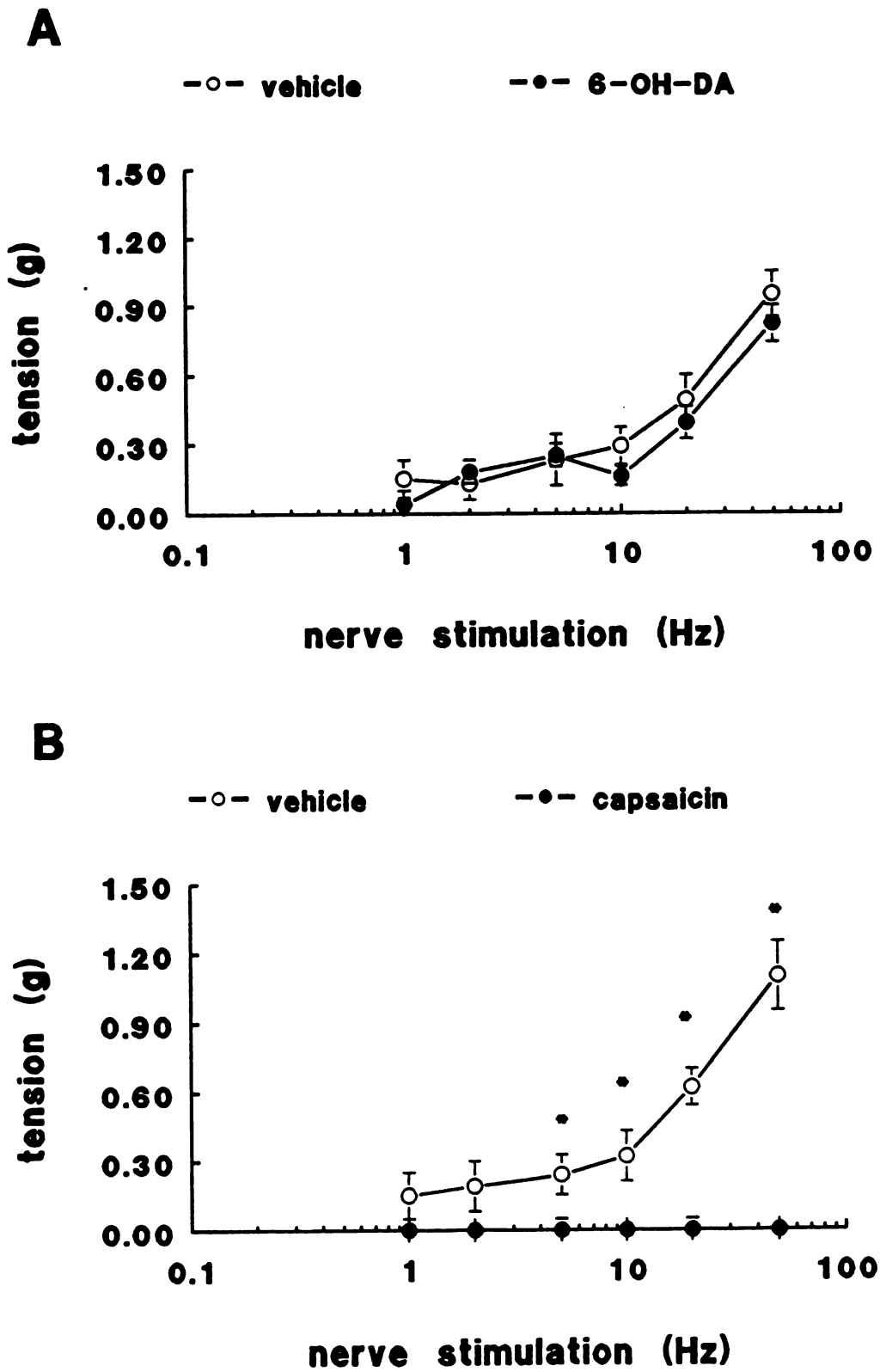


Figure 42. Neurogenic NANC contractions of longitudinal muscle-myenteric plexus preparations from animals treated with 6-OH-DA or capsaicin. A. Electrical stimulation of LMMPPs produced frequency-dependent increases in amplitude of contraction in vehicle- and 6-OH-DA-treated preparations. B. Electrical stimulation of LMMPPs produced frequency-dependent increases in amplitude of contractions in vehicle- and capsaicin-treated preparations. * represents $p < 0.05$ as compared to vehicle-treated controls.

Figure 42.



Interestingly, loss of primary afferent nerves also mimicked denervation-induced changes in nitrinergic innervation, suggesting common neurotransmitters may be involved in control of excitatory and inhibitory neurotransmitter release. L-nitro-arginine (300 μ M) potentiated rebound contractions by 240% in control preparations, but increased the amplitude of rebound contractions in denervated preparations only by 50% (Figure 41A). As loss of extrinsic sensory nerves increases NOS and NO, decreased excitatory NANC neurotransmission may result from inhibition by NO. Therefore, as denervated preparations contain more NOS as compared to controls, LNA inhibits fewer NOS molecules, and NO can inhibit SP release. However, control tissues contain less NOS, and LNA inhibited a greater percentage of total NOS, thus eliminating nitrinergic inhibition of SP release. Alternatively, neurotransmitters from sensory nerves are very important in mediation of motility (see introduction), and denervation may eliminate excitatory input into non-cholinergic motor neurons (see introduction). In conclusion, the results in this section demonstrate that loss of a single population of extrinsic nerves has profound effects on anatomy and excitatory and inhibitory innervation of longitudinal muscle.

7. NITRIC OXIDE REGULATES SUBSTANCE P BUT NOT ACETYLCHOLINE RELEASE

Introduction and Experimental Rationale

As described in the previous section, transmural nerve stimulation of LMMPs results in relaxation of GI smooth muscle, followed by contractile events referred to as "off" or "rebound" contractions. Although NOS antagonists attenuate neurogenic NANC relaxations, numerous investigators have observed that the amplitude and/or duration of rebound contractions is potentiated by NOS antagonists. Gustafsson *et al.* (1990) were the first investigators to propose that NO acts at enteric perikarya or nerve varicosities to inhibit release of excitatory non-cholinergic neurotransmitters. Addition of NOS antagonists would attenuate this prejunctional inhibition, resulting in the observed potentiation of neurogenic rebound contractions. However, other investigators (Osthaus and Galligan, 1992) have suggested that potentiation of rebound contractions results from physiological antagonism by NO on the action of excitatory neurotransmitters on GI smooth muscle. Transmural nerve stimulation results in release of excitatory and inhibitory neurotransmitters that act directly on GI smooth muscle. However, addition of NOS antagonists would decrease inhibitory tone to the muscle, resulting in larger excitatory responses, although release of excitatory neurotransmitters has not been altered.

The circular muscle receives a dense nitrinergic innervation from myenteric neurons, as well as excitory innervation from ChAt-/SP-ir myenteric neurons (Brookes *et al.*, 1991). Therefore, circular muscle-myenteric plexus preparations (CMMPPs) were used to determine if endogenous NO modulates SP and ACh release from the same enteric nerves. Circular muscle preparations were made by attaching strips of circular muscle-

myenteric plexus or rings of ileum parallel to the circular muscle to force transducers. As these preparations gave similar responses, rings of ileum were used for the majority of the experiments. Preliminary experiments indicated that CMMPPs do not maintain active tension in response to histamine (1-300 μ M) or prostaglandin $F_{2\alpha}$ (1-300 μ M), allowing contractile activity to be studied in the absence of neurogenic relaxations of smooth muscle. Furthermore, as contractions of circular muscle propel intestinal contents, nitrinergic modulation of excitatory neurotransmitter release would have profound effects on motility.

Transmural Stimulation Produces Monophasic Contractions of Circular Muscle

In the presence of scopolamine (1 μ M) and guanethidine (3 μ M), transmural stimulation caused monophasic NANC contractions of circular muscle (40 preparations, 10 animals) (Figure 43A). The amplitude of contractions was frequency-dependent (Figure 43B), and contractions were abolished by tetrodotoxin (0.3 μ M) (Figures 43A and 43B). Spontaneous contractile activity was observed in 5/40 preparations, and this activity was abolished as the frequency of nerve stimulation increased. NANC contractions were attenuated in a concentration-dependent manner by CP-96,345 (1-300 nM) (16 preparations, 3 animals) (Figure 43A and Figure 43C), a non-peptide neurokinin-1 (NK_1) receptor antagonist (Synder *et al.*, 1991), and were mimicked by SPME (1-1000 nM) (22 preparations, 4 animals), a NK_1 agonist (Figure 44A and Figure 44B) (Hall and Morton, 1991). The concentration of CP-96,345 that produced half-maximal inhibition of 20 Hz contractions was 12 ± 5 nM and the SPME EC_{50} was 29 ± 8 nM. The concentration-response relationship for SPME was abolished by addition of CP-96,345 (300 nM) (Figure 44B), but was not altered by TTX (300 nM) (Figure 44B).

Figure 43. Characterization of NANC contractions of circular muscle in the presence of scopolamine (1 μ M) and guanethidine (3 μ M). A. *Top Panel*: Increasing frequency of nerve stimulation (1-50 Hz, 1 sec, 0.3 msec pulse duration) increases the amplitude of circular muscle contractions; contractions are inhibited by TTX (300 nM). *Bottom Panel*: The NK₁ receptor antagonist CP-96,345 inhibits 20 Hz NANC contractions of circular muscle in a concentration-dependent manner. Horizontal axis is 25 sec, and vertical axis is 1 g. B. Frequency-response curve for NANC contractions in the absence and presence of TTX (300 nM). Data are mean $\pm$ SEM of maximum contraction amplitude expressed in grams of tension. C. Concentration-response curve for the NK₁ receptor antagonist CP-96,345. Data are mean $\pm$ SEM of percent of control 20 Hz NANC contractions.

Figure 43.

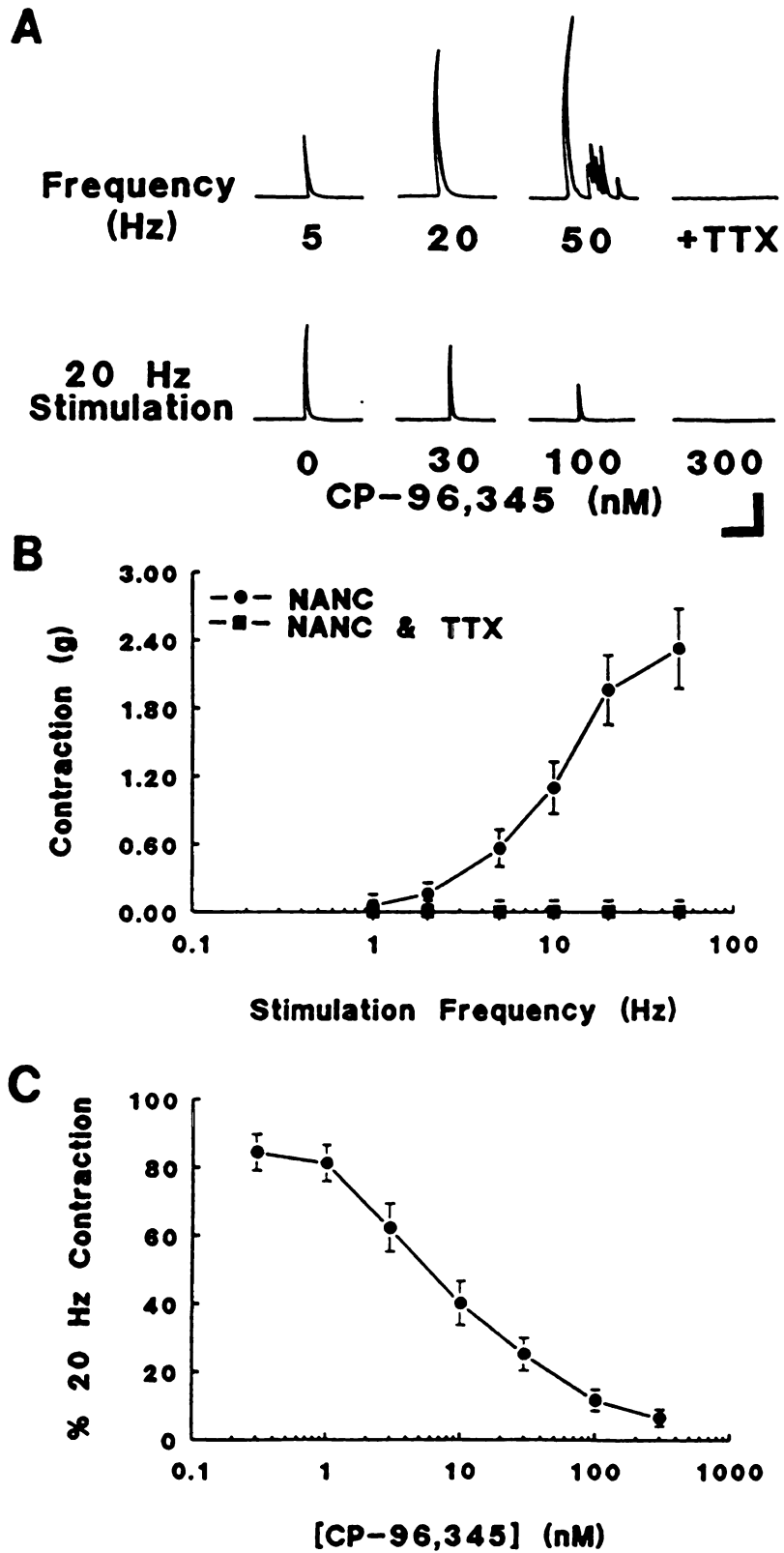


Figure 44. SPME mimics NANC contractions of circular muscle. **A. *Top Panel:*** Increasing concentrations of SPME increase the amplitude of contractions of circular muscle; contractions are not inhibited by TTX (300 nM). ***Bottom Panel:*** The NK₁ receptor antagonist CP-96,345 inhibits SPME-evoked contractions of circular muscle in a concentration-dependent manner. Horizontal axis is 25 sec, and vertical axis is 1 g. **B.** Concentration-response curve for the NK₁ receptor agonist SPME in the presence of TTX (300 nM) or CP-96,345 (300 nM). Data are mean $\pm$ SEM of maximum contraction amplitude expressed in grams. Guanethidine (3 μ M) and scopolamine (1 μ M) are present throughout this experiment.

Figure 44.

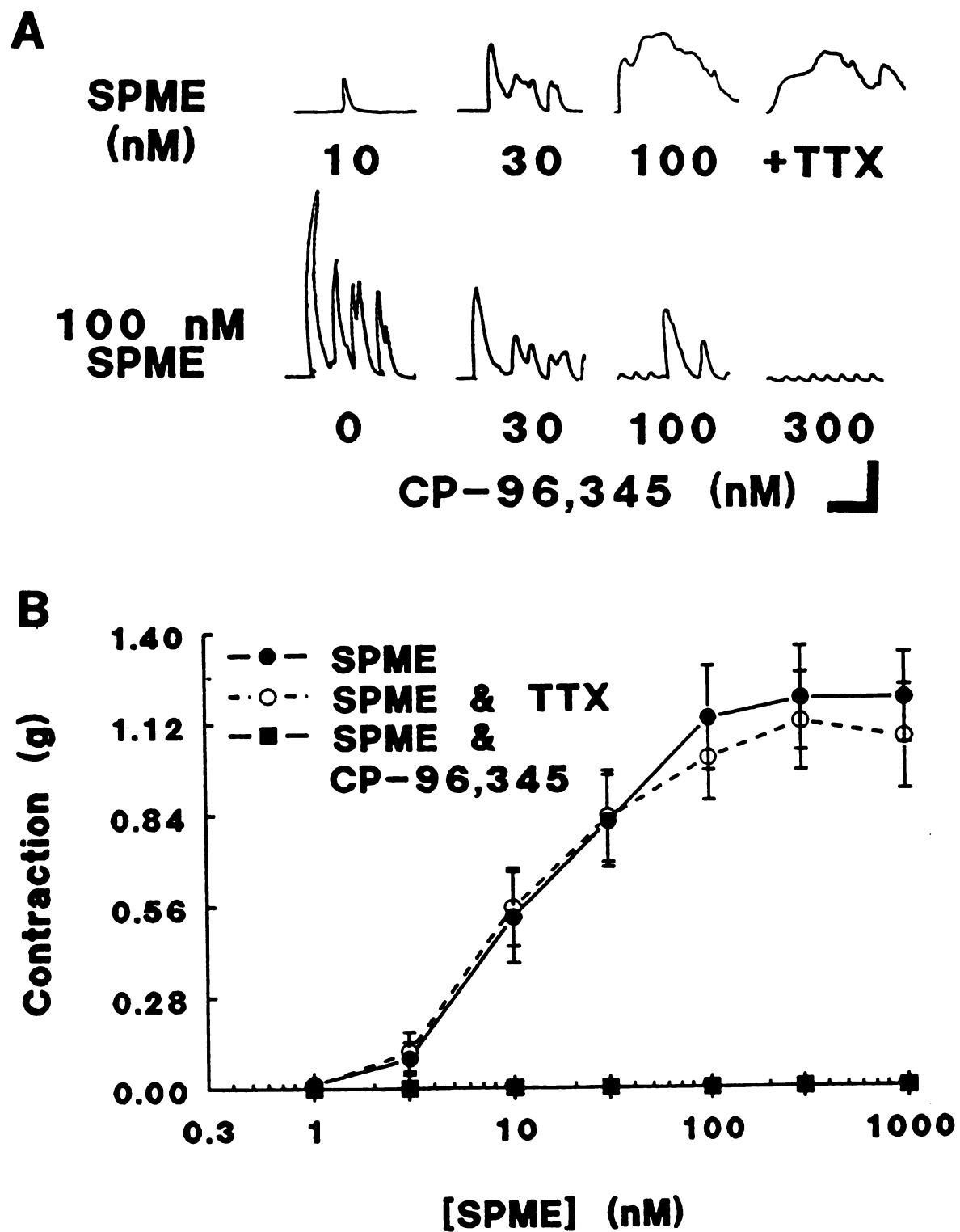


Figure 45. Characterization of cholinergic contractions of circular muscle in the presence of CP-96,345 (300 nM) and guanethidine (3 μ M). A. *Top Panel*: Increasing frequency of nerve stimulation (1-50 Hz, 1 sec, 0.3 msec pulse duration) increases the amplitude of circular muscle contractions; contractions are inhibited by TTX (300 nM). *Bottom Panel*: The mACh receptor antagonist scopolamine inhibits 20 Hz cholinergic contractions of circular muscle in a concentration-dependent manner. Horizontal axis is 25 sec, and vertical axis is 1 g. B. Frequency-response curve for cholinergic contractions in the absence and presence of TTX (300 nM). Data are mean $\pm$ SEM of maximum contraction amplitude expressed in grams. C. Concentration-response curve for the mACh receptor antagonist scopolamine. Data are mean $\pm$ SEM of percent of control 20 Hz cholinergic contractions.

Figure 45.

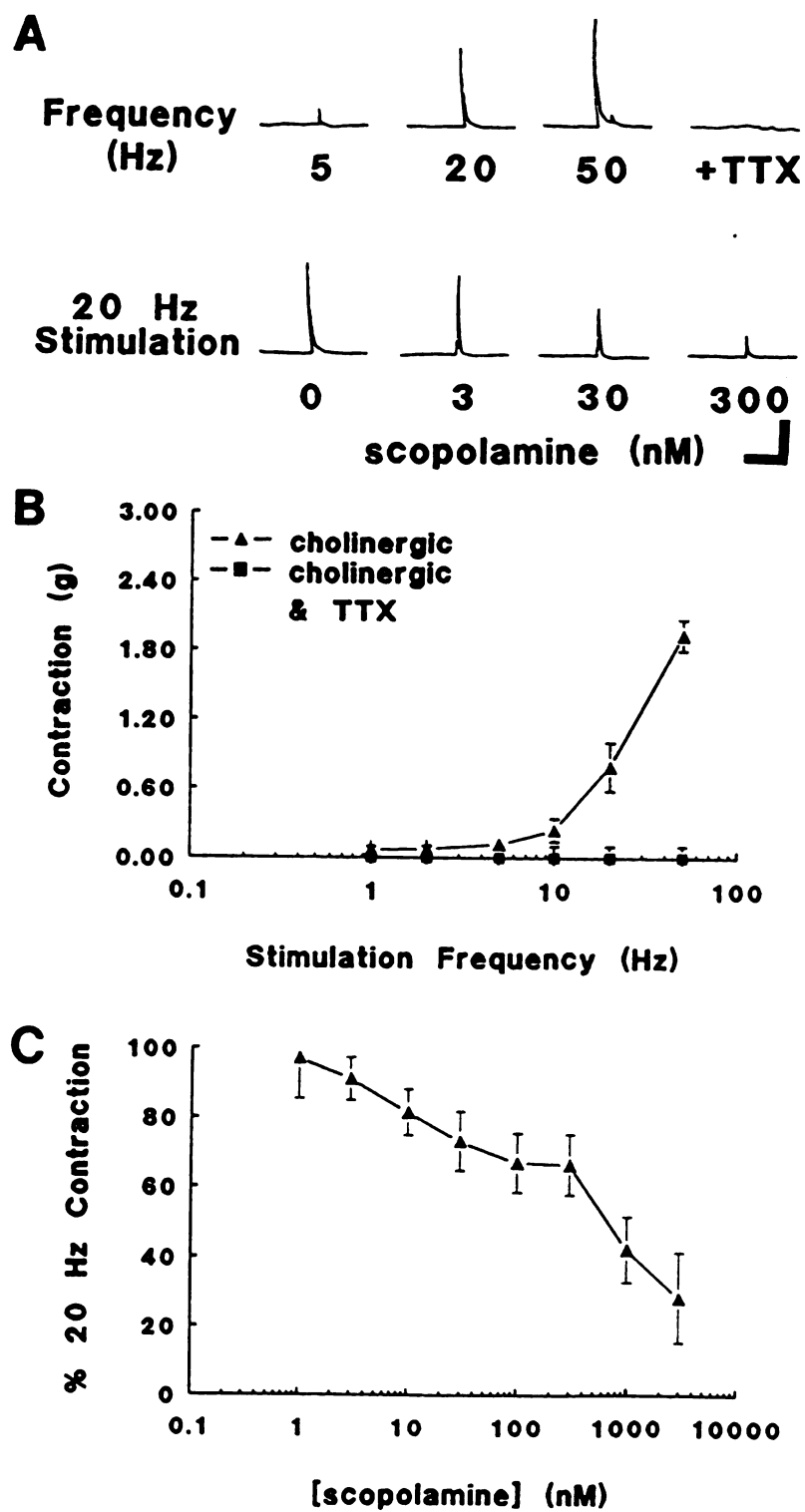
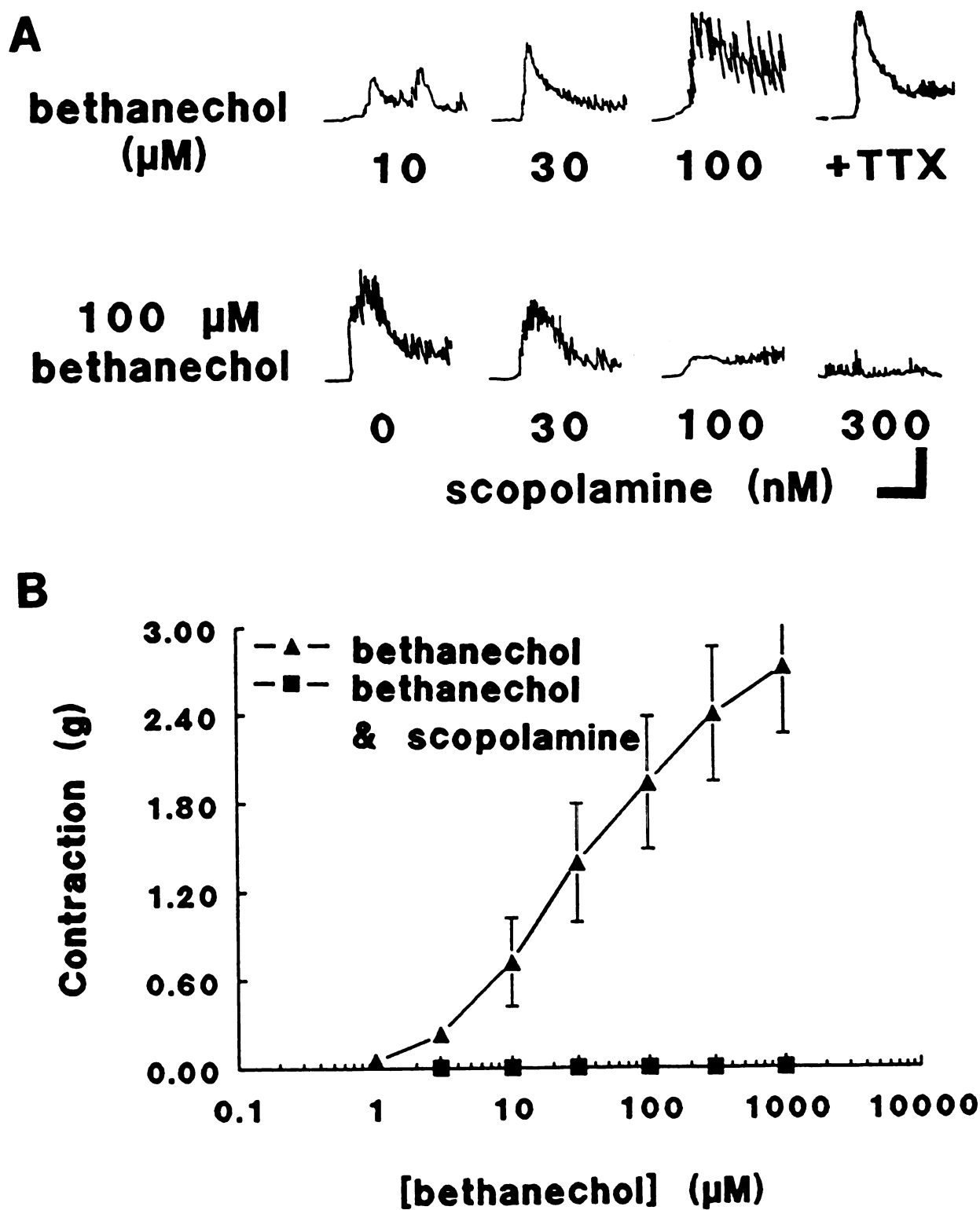


Figure 46. Bethanechol mimics cholinergic contractions of circular muscle. *A. Top Panel:* Increasing concentrations of bethanechol increase the amplitude of contractions of circular muscle; contractions are not inhibited by TTX (300 nM). *Bottom Panel:* The mACh receptor antagonist scopolamine inhibits bethanechol-evoked contractions of circular muscle in a concentration-dependent manner. Horizontal axis is 25 sec, and vertical axis is 1 g. *B.* Concentration-response curve for the mACh agonist bethanechol in the presence of TTX (300 nM) or scopolamine (1 μ M). Data are mean $\pm$ SEM of maximum contraction amplitude expressed in grams. Guanethidine (3 μ M) and CP-96,345 (300 nM) are present throughout this experiment.

Figure 46.



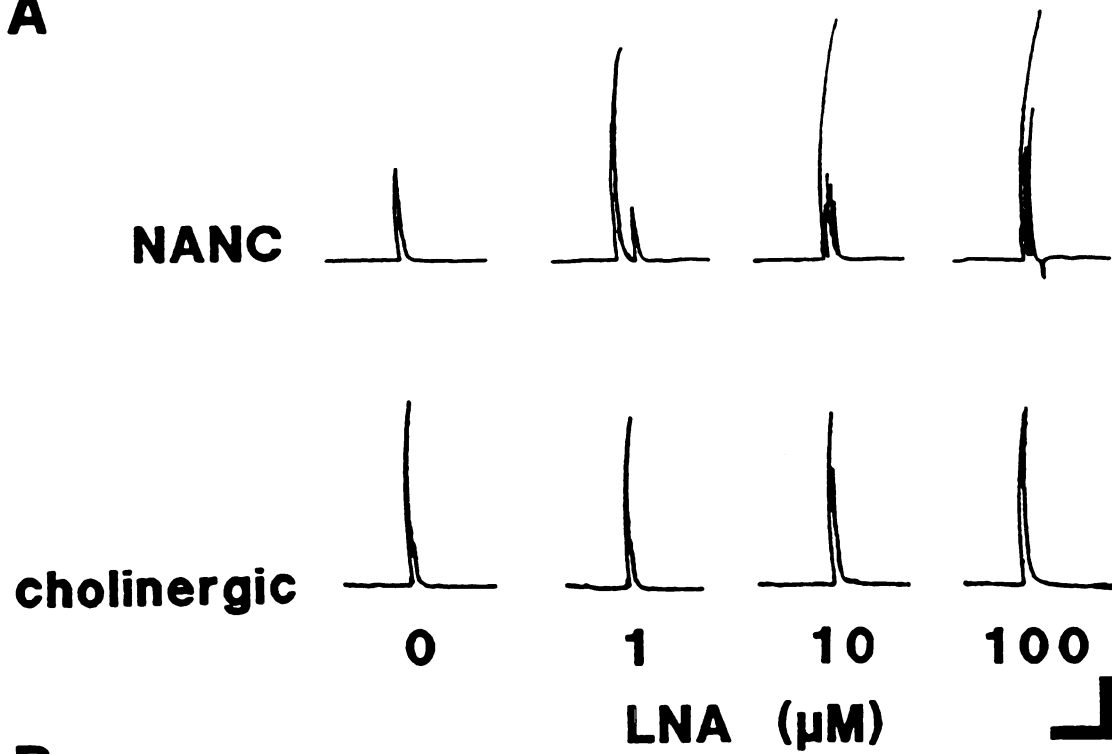
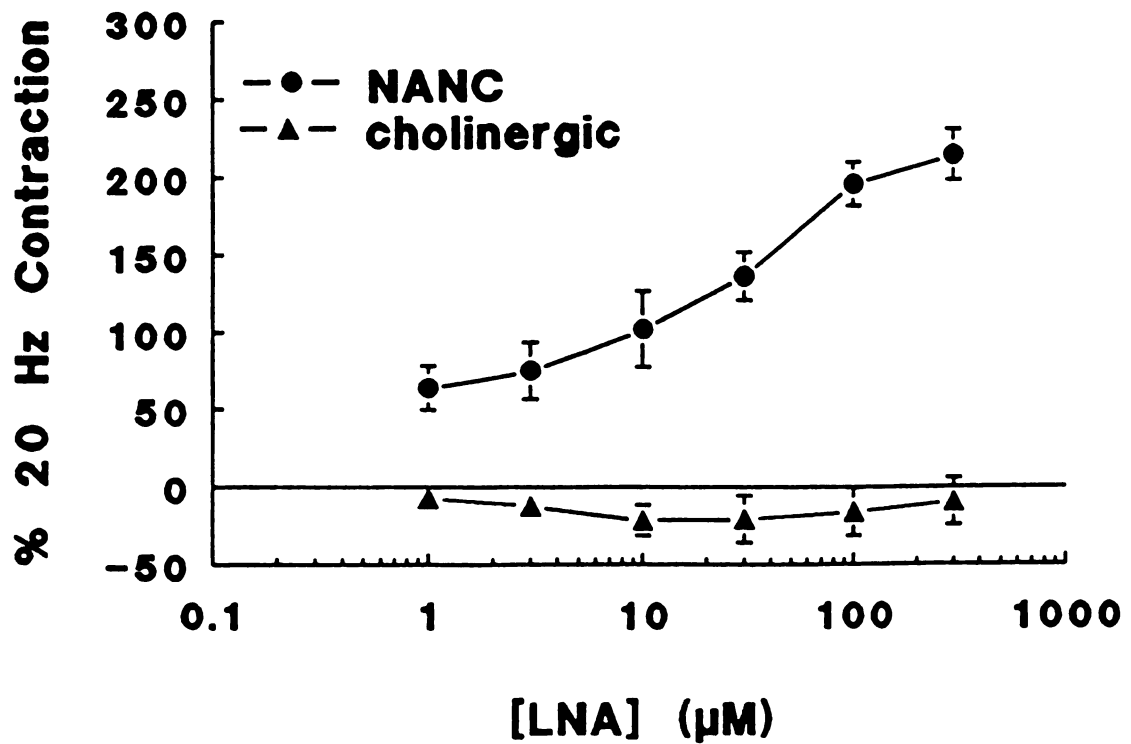
In the presence of guanethidine (3 μ M) and CP-96,345 (300 nM), transmural stimulation evoked monophasic contractions of circular muscle (18 preparations, 4 animals). Contraction amplitude was frequency-dependent (Figure 45A and Figure 45B), abolished by tetrodotoxin (300 nM) (Figure 45A and Figure 45B), and attenuated in a concentration-dependent manner by scopolamine (1-3000 nM) (22 preparations, 5 animals) (Figure 45C). Finally, bethanechol (0.3 μ M-300 μ M) mimicked these contractions (17 preparations, 3 animals) (Figure 46A and Figure 46B), with an EC_{50} of 20 ± 3 μ M. Although bethanechol produced contractions in the presence of TTX (300 nM) (Figure 46A), addition of 1 μ M scopolamine prior to bethanechol inhibited all agonist-induced contractile activity (Figure 46B). As neurogenic responses recorded in the presence of guanethidine and CP-96,345 were mimicked by bethanechol and attenuated by scopolamine, these contractions were mediated largely by ACh, and referred to as "cholinergic".

Nitric Oxide Synthase Inhibitors Potentiate NANC but not Cholinergic Contractions

N^G -nitro-L-arginine (LNA) potentiated 20 Hz NANC contractions in a concentration-dependent manner, with an EC_{50} of 18 ± 4 μ M (Figure 47). To determine if potentiation of NANC contractions was dependent on frequency of nerve stimulation, frequency-response relationships were generated in the absence and presence of 300 μ M LNA. As shown in Figure 48, LNA did not significantly alter NANC contractions obtained at 1 or 2 Hz, although these parameters were sufficient to elicit NANC contractions. Low frequency nerve stimulation (1 Hz) produced contractions (0.19 ± 0.06 g, maximum amplitude) in 12/40 tissues, whereas NANC contractions (0.28 ± 0.05 g, maximum amplitude) were observed in 24/40 preparations stimulated at 2 Hz. However,

Figure 47. Effects of LNA on NANC and cholinergic contractions of circular muscle. A. *Top Panel:* LNA potentiates NANC contractions (20 Hz, 1 sec, 0.3 msec pulse duration) of circular muscle in a concentration-dependent manner. *Bottom Panel:* LNA does not affect cholinergic contractions (20 Hz, 1 sec, 0.3 msec pulse duration). Horizontal axis is 20 sec, and vertical axis is 1 g. B. Concentration-response curves for the effects of LNA on NANC and cholinergic contractions. Data are mean $\pm$ SEM of percent of control 20 Hz NANC or cholinergic contractions recorded in the presence of guanethidine (3 μ M) and scopolamine (1 μ M) or CP-96,345 (300 nM), respectively.

Figure 47.

A**B**

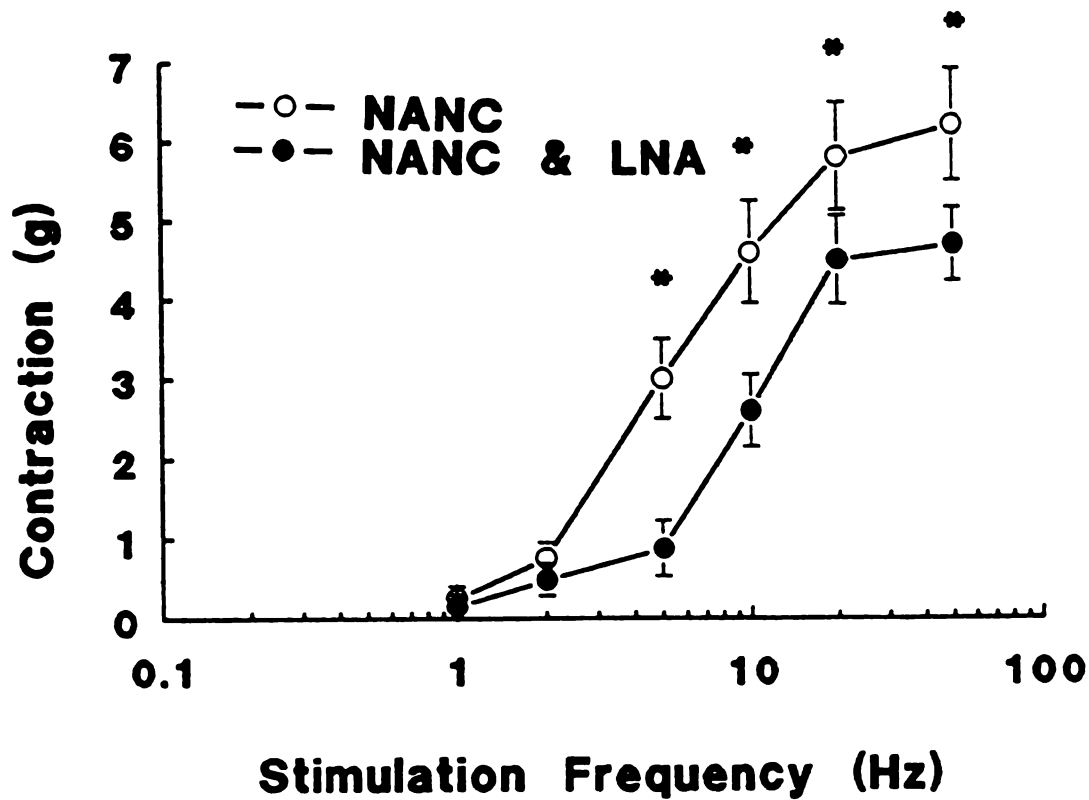


Figure 48. Potentiation of neurogenic NANC contractions of circular muscle by LNA is frequency dependent. Frequency-response curves were generated in the absence and presence of 300 μ M LNA. Data are mean $\pm$ SEM of maximum contraction amplitudes from 14 preparations in 3 animals. Guanethidine (3 μ M) and scopolamine (1 μ M) are present throughout this experiment. Asterisks indicate differences from control (Student's *t* test for paired data; $p < 0.05$).

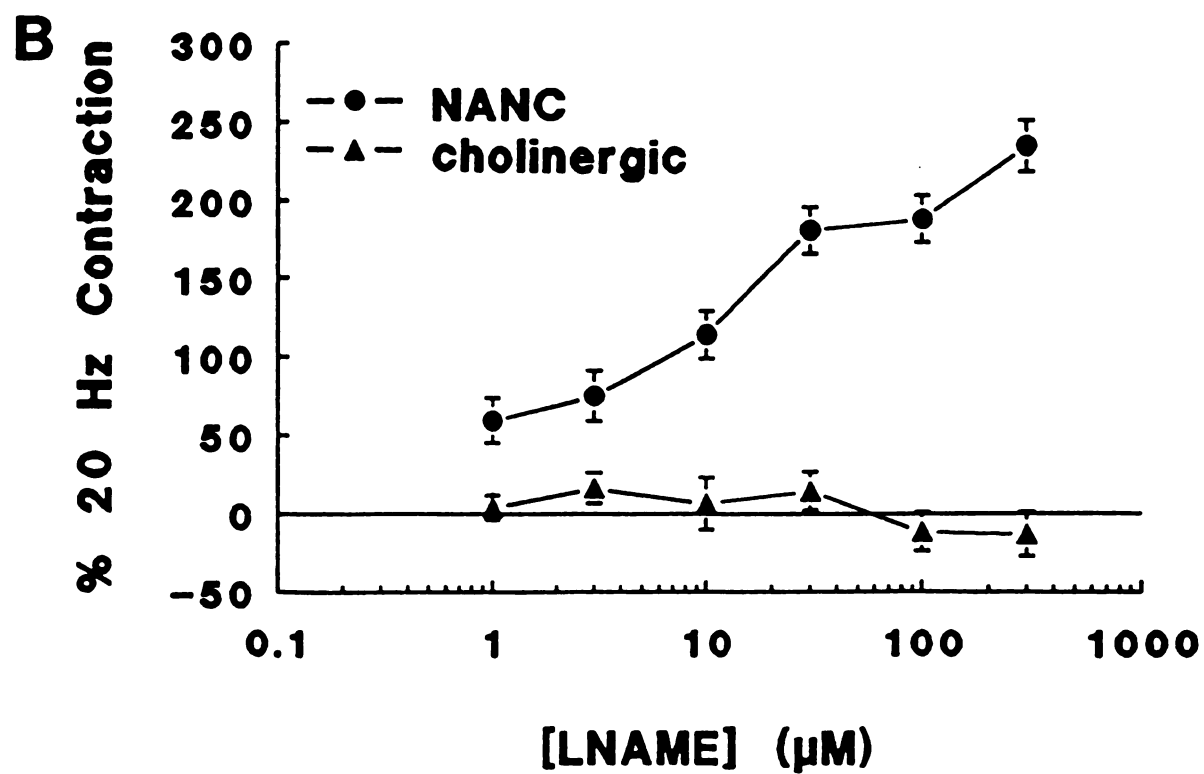
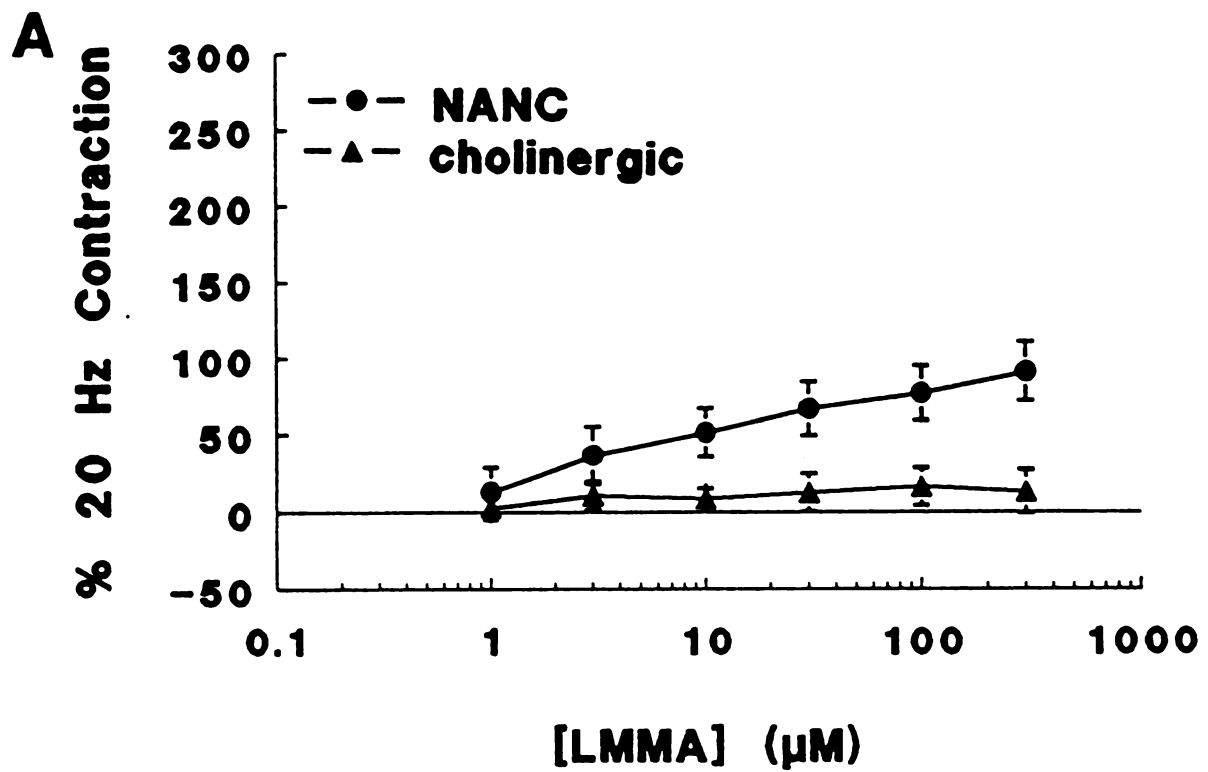
LNA potentiated the amplitude of NANC contractions at 5, 10, 20, and 50 Hz. The NOSynthase inhibitors N^G-methyl-L-arginine (LMMA) (1-300 μ M) (14 preparations, 3 animals) and N^G-nitro-L-arginine methyl ester (LNAME) (1-300 μ M) (16 preparations, 3 animals) also potentiated NANC contractions in a concentration-dependent manner (Figure 49). LNAME (21 ± 5 μ M, EC₅₀) potentiated 20 Hz responses in 12/16 tissues (Figure 49A), and revealed contractions in 2/4 tissues previously not exhibiting contractile activity, whereas LMMA (38 ± 10 μ M, EC₅₀) potentiated NANC contractions in 11/14 tissues (Figure 49B). Unlike LNAME, addition of LMMA did not reveal neurogenic contractile activity in unresponsive tissues. Finally, addition of NOS antagonists increased spontaneous activity of preparations. LNA increased spontaneous muscle activity in 6/16 preparations previously not exhibiting fluctuations in basal tone. Similar results were observed with LNAME and LMMA, as spontaneous activity was observed in 5/16 and 6/14 preparations respectively (Figure 50); most of this activity was inhibited by TTX (300 nM) (Figure 50).

To determine if NOS antagonists also potentiated cholinergic contractions, neurogenic cholinergic contractions were recorded in the presence of guanethidine and CP-96,345. LMMA (1-300 μ M) (12 preparations, 2 animals) (Figure 49A), LNAME (1-300 μ M) (14 preparations, 3 animals) (Figure 49B), and LNA (1-300 μ M) (16 preparations, 3 animals) (Figure 47A and Figure 47B) did not significantly alter the amplitude or time of duration of 20 Hz cholinergic contractions. Furthermore, LNA did not alter the amplitude of cholinergic contractions generated by any other frequency of stimulation (data not shown).

The site of action of NOS antagonists was elucidated by examining responses of

Figure 49. Concentration-response curves for the effects of LMMA and LNAME on NANC and cholinergic contractions of circular muscle. A. Concentration-response curves for the effects of LMMA on NANC and cholinergic contractions (20 Hz nerve stimulation). B. Concentration-response curves for the effects of LNAME on NANC and cholinergic contractions (20 Hz nerve stimulation). Data are mean $\pm$ SEM of percent of control 20 Hz NANC or cholinergic contractions recorded in the presence of guanethidine (3 μ M) and scopolamine (1 μ M) or CP-96,345 (300 nM), respectively.

Figure 49.



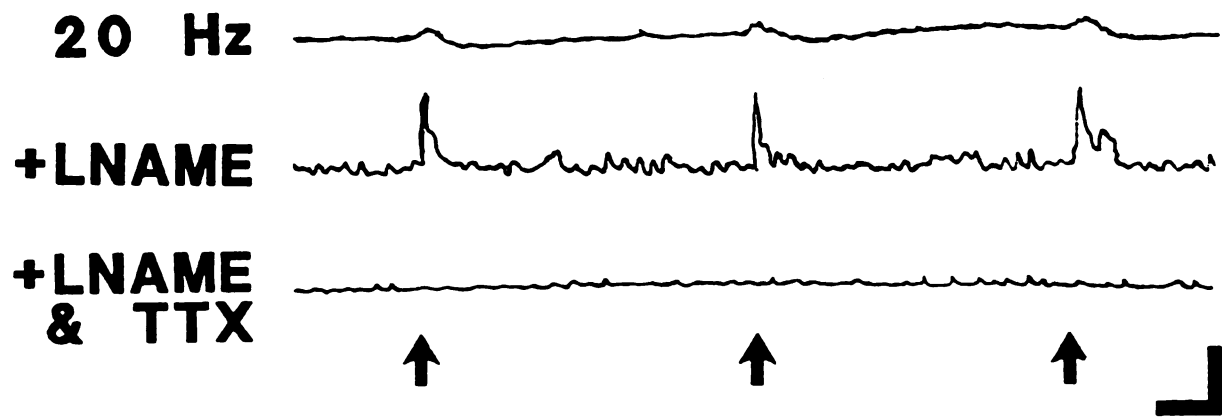


Figure 50. LNAME potentiates NANC contractions and spontaneous activity of circular muscle. A. Nerve stimulation (20 Hz) contracts circular muscle. B. LNAME (300 μ M) added 10 min prior to the experiment potentiates NANC contractions and increases spontaneous activity of circular muscle. C. TTX (300 nM) added 10 min prior to the experiment inhibits NANC contractions and spontaneous activity of circular muscle. Arrows indicate neurogenic contractions. Horizontal axis is 25 sec and vertical axis is 0.5 g. Guanethidine (3 μ M) and scopolamine (1 μ M) are present throughout this experiment.

circular muscle to SPME in the absence and presence of 10 (8 preparations, 2 animals) or 100 μ M LNA (8 preparations, 2 animals). SPME contractions were not altered by 10 or 100 μ M LNA (Table 8).

To verify the stereo-specificity of the NOS antagonists, 1-300 μ M N^G-methyl-D-arginine (DMMA) (11 preparations, 2 animals) and N^G-nitro-D-arginine methyl ester (DNAME) (17 preparations, 3 animals) were tested against 20 Hz NANC contractions. Neither DMMA nor DNAME altered NANC contractions, as compared to equal concentrations of LMMA (Figure 51A) or LNAME (Figure 51B).

L-Arginine (LArg) (21 preparations, 4 animals) and D-arginine (DArg) (20 preparations, 4 animals) were used to determine specificity and reversibility of NOS antagonists. Neither LArg nor DArg (1-3000 μ M) affected 20 Hz NANC contractions (Figure 52). Furthermore, neither numerous washes (10, 10 min each), nor addition of LArg or DArg (1 or 3 mM) reversed potentiation of NANC neurogenic contractions by LNA (300 μ M) (data not shown) or LNAME (300 μ M) if added after application of the NOS antagonist. However, if LArg (1 or 3 mM) was added to the tissues prior to application of NOS antagonists (14 preparations, 3 animals), neither LNA (data not shown) or LNAME (Figure 52) potentiated NANC contractions. Equal concentrations of DArg did not prevent potentiation of 20 Hz NANC contractions by LNA (data not shown) or LNAME (Figure 52).

CP-96,345 Attenuates NANC Contractions Following Potentiation by Nitric Oxide Synthase Antagonists

As described previously, 1-300 nM CP-96,345 attenuated neurogenic NANC contractions generated at 20 Hz with an IC₅₀ of 12 ± 5 nM. CP-96,345 (1-300 nM) also

Figure 51. Concentration-response curves for the effects of L- and D-enantiomers of XNNA and XNAME on NANC contractions of circular muscle. A. Concentration-response curves for the effects of LMMA and DMMA on NANC contractions (20 Hz nerve stimulation). Note the L-isomer data have been replotted from Fig. 49A. B. Concentration-response curves for the effects of LNAME and DNAME on NANC contractions (20 Hz). Note the L-isomer data have been replotted from Fig. 49B. Data are means $\pm$ SEM of percent of control 20 Hz NANC contractions. Guanethidine (3 μ M) and scopolamine (1 μ M) are present throughout this experiment.

Figure 51.

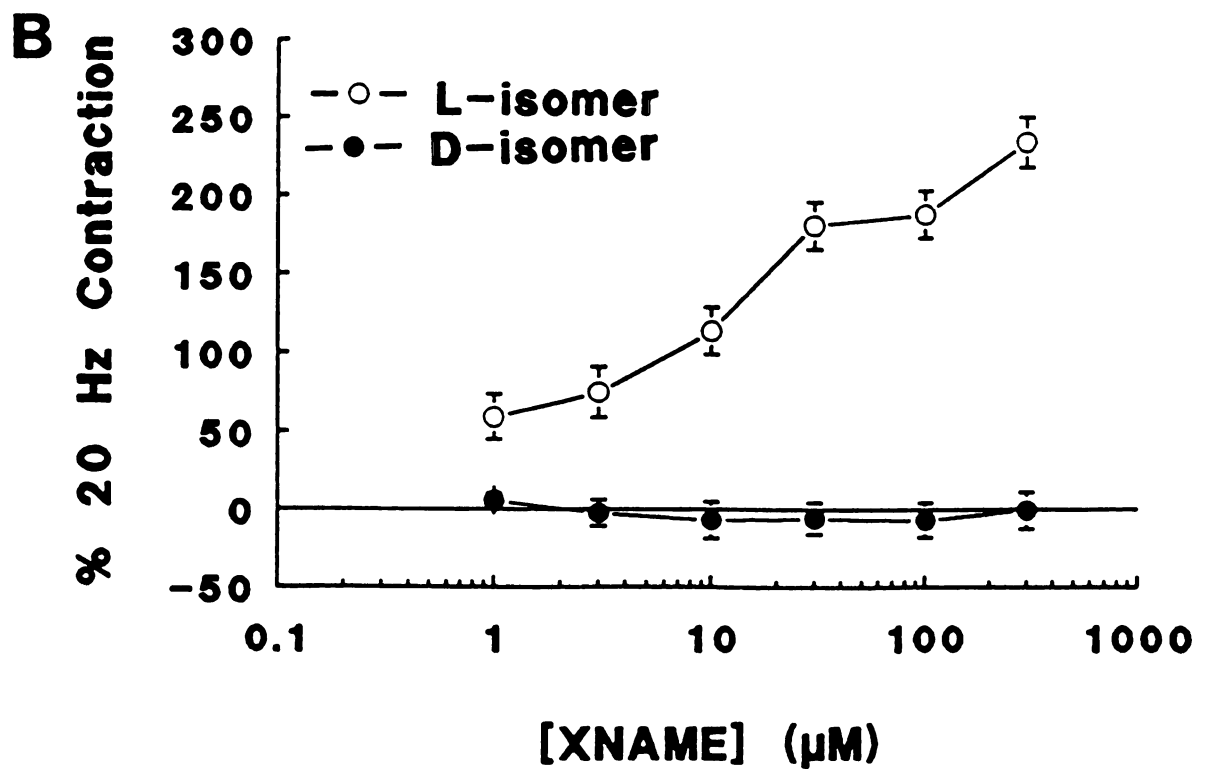
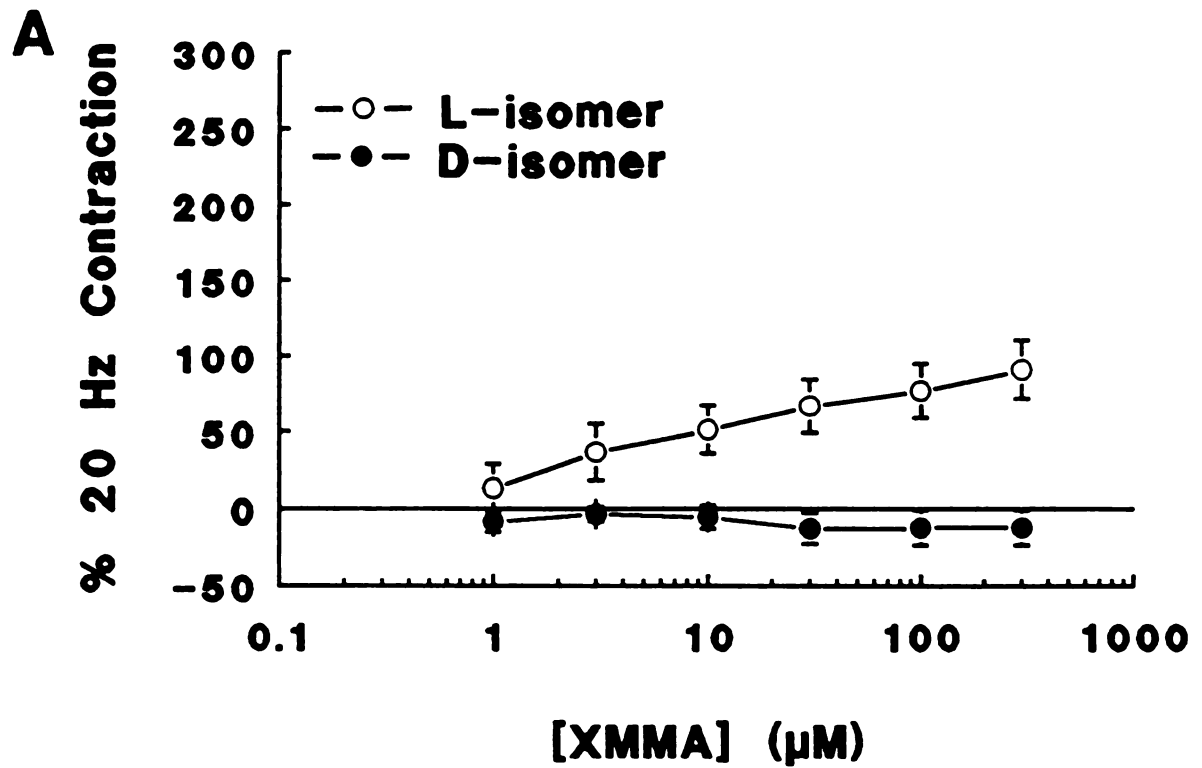


Figure 52. Concentration-response curves for the effects of L- and D-arginine and LNAME in the absence and presence of DArg or LArg (1 mM) on NANC contractions.

A. Concentration-response relationships for DArg and LArg on NANC contractions.

B. LArg but not DArg prevented potentiation of NANC contractions (20 Hz stimulation) by LNAME when added 10 min prior, but not after, LNAME. LNAME potentiated NANC contractions evoked by 20 Hz stimulation. Data are means $\pm$ SEM of percent potentiation of control 20 Hz NANC contractions. Guanethidine (3 μ M) and scopolamine (1 μ M) are present throughout this experiment.

Figure 52.

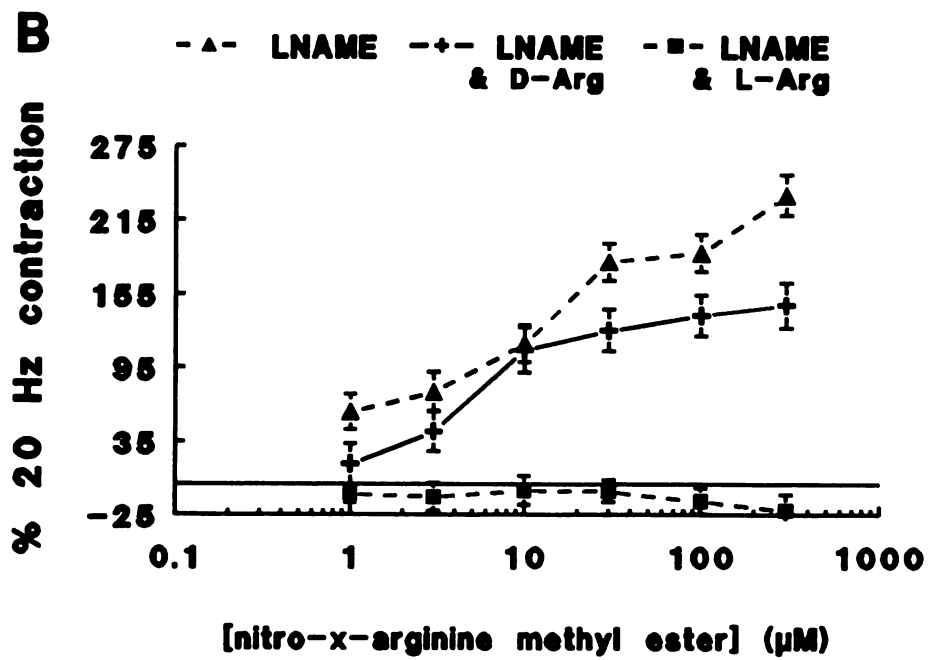
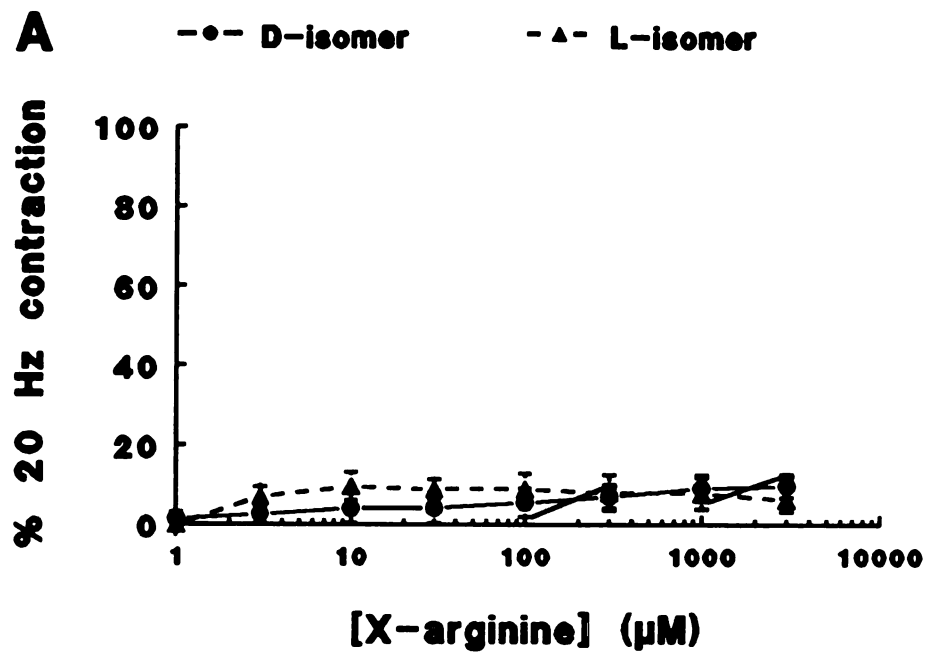


Table 8. Nitro-L-arginine does not alter the amplitude of circular muscle contractile responses to substance P methyl ester.

LNA (μ M)	Substance P Methyl Ester (nM)				
	10	30	100	300	1000
0	0.12 $\pm$ 0.04	0.21 $\pm$ 0.05	0.45 $\pm$ 0.23	0.92 $\pm$ 0.29	1.00 $\pm$ 0.24
10	0.13 $\pm$ 0.07	0.23 $\pm$ 0.07	0.51 $\pm$ 0.23	0.82 $\pm$ 0.28	0.92 $\pm$ 0.27
0	0.29 $\pm$ 0.11	0.70 $\pm$ 0.15	0.68 $\pm$ 0.23	0.69 $\pm$ 0.32	0.70 $\pm$ 0.28
100	0.26 $\pm$ 0.09	0.74 $\pm$ 0.12	0.68 $\pm$ 0.22	0.68 $\pm$ 0.22	0.67 $\pm$ 0.12

Non-cumulative concentration-response data were generated in the absence and presence of LNA added 10 min prior to SPME. Values represent mean $\pm$ SEM for amplitude of agonist-induced contractions (g) of 8 preparations from 2 animals for 10 and 100 μ M LNA.

attenuated LNA- and LNAME-induced potentiation of NANC contractions. CP-96,345 (300 nM) reduced LNA- and LNAME-induced potentiation of NANC contractions by 93 $\pm$ 3% and 95 $\pm$ 2%, respectively, with a combined IC₅₀ of 14 $\pm$ 3 nM (19 preparations, 5 animals).

Oxyhemoglobin Attenuates NANC but not Cholinergic Contractions

Hemoglobin (1-10 μ M) potentiated NANC contractions in a concentration-dependent manner in 11/15 preparations (Figure 53). Transmural stimulation failed to produce contractions in 3/15 preparations. However, monophasic contractions (0.27 $\pm$ 0.10 g, maximum amplitude) were observed in these tissues after addition of 1 μ M hemoglobin, and the amplitude of the contractions increased in a concentration-dependent manner upon addition of 3 μ M (0.39 $\pm$ 0.14 g) or 10 μ M (0.44 $\pm$ 0.15 g) hemoglobin. Interestingly, hemoglobin did not significantly affect cholinergic contractions (Figure 53). Hemoglobin (10 μ M) did not alter contractions generated by addition of 30 (0.47 $\pm$ 0.13

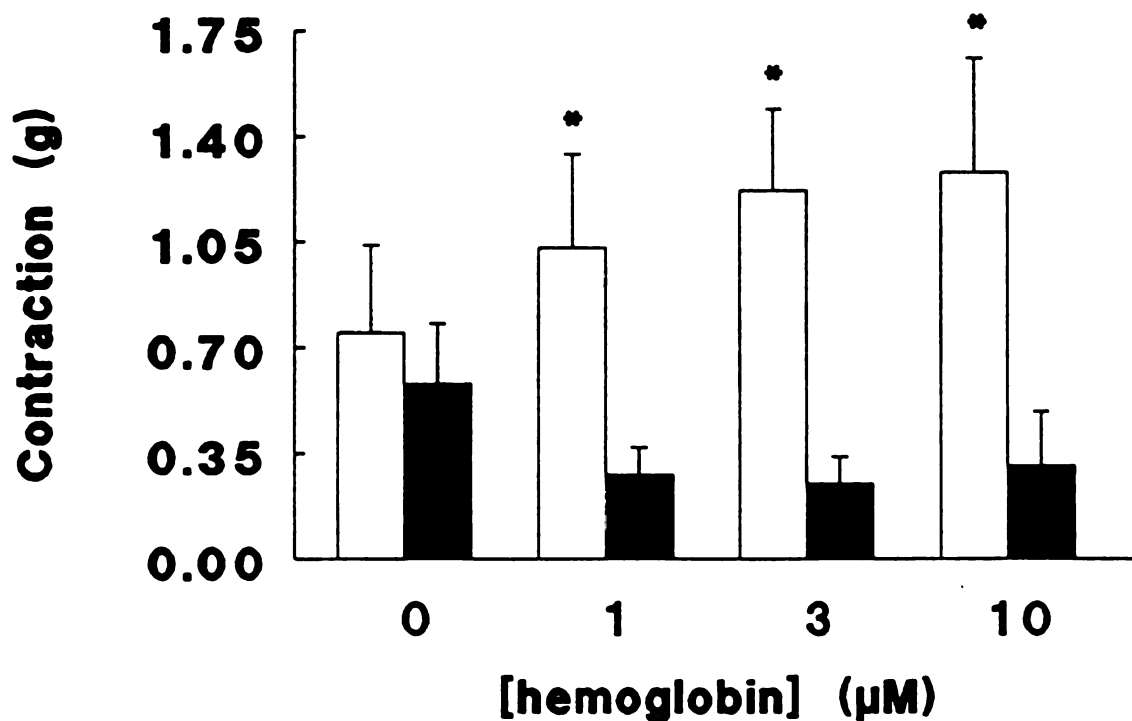


Figure 53. Concentration-response relationships for the effects of hemoglobin on NANC and cholinergic contractions of circular muscle. NANC (open bars) and cholinergic (filled bars) contractions (20 Hz) were affected differentially by hemoglobin. Data are means $\pm$ SEM of maximum contraction amplitude expressed in grams of tension. NANC and cholinergic contractions were recorded in the presence of guanethidine (3 μM) and scopolamine (1 μM) or CP-96,345 (300 nM), respectively. Asterisks indicate differences from control (Analysis of Variance proceeded by Test for Least Significant Differences; $p < 0.05$).

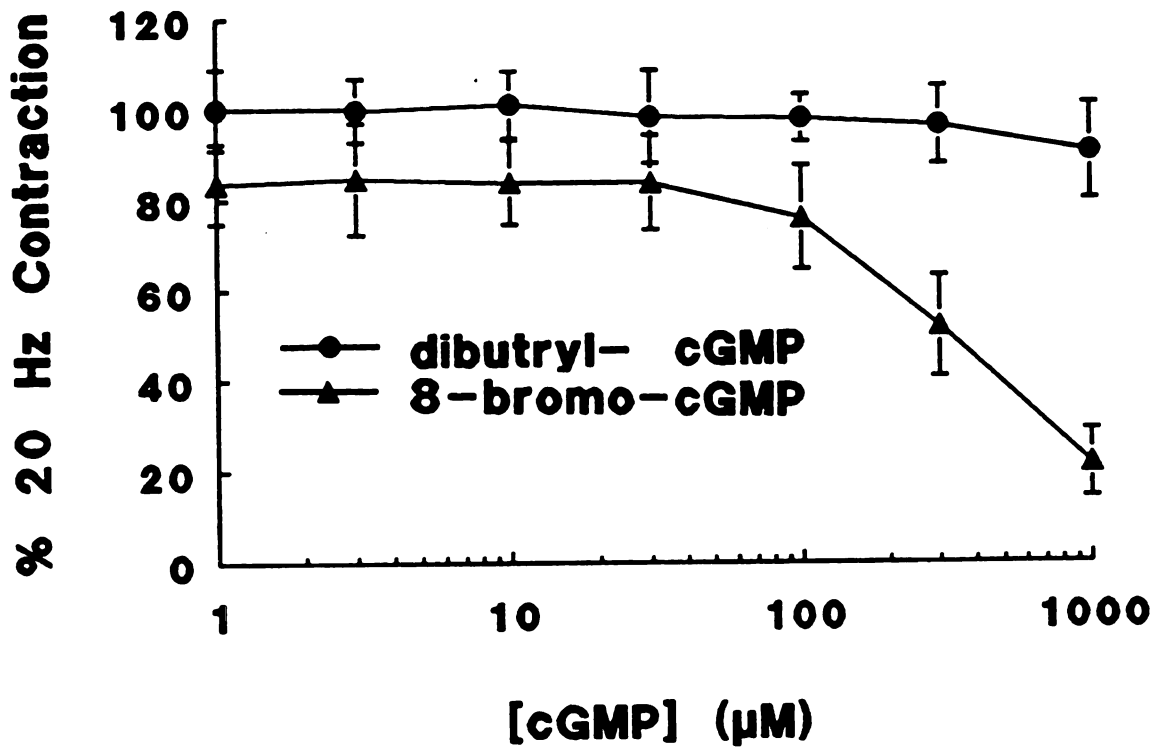


Figure 54. Concentration-response curves for the effects of 8-bromo-cGMP and dibutyl-cGMP on NANC contractions of circular muscle. Cumulative concentrations of 8-bromo-cGMP or dibutyl-cGMP are added at 20 min increments. Data are mean $\pm$ SEM of percent of control 20 Hz contractions. Guanethidine (3 μM) and scopolamine (1 μM) are present throughout this experiment.

g) or 300 nM (0.88 ± 0.16 g) SPME as compared to control responses (0.57 ± 0.11 ; 1.18 ± 0.27 g) (10 preparations, 2 animals). Finally, hemoglobin (10 μ M) increased spontaneous activity of 4/11 CMMPPs (data not shown).

cGMP Inhibits NANC Contractions

8-Bromo-cGMP (100-1000 μ M) (12 preparations, 2 animals), but not dibutyl-cGMP (1-1000 μ M) (11 preparations, 2 animals), produced a concentration-dependent decrease in NANC contractions produced by 20 Hz transmural nerve stimulation (Figure 54), with an EC_{50} of 189 ± 24 μ m. The inhibition caused by 8-bromo-cGMP did not exhibit tachyphylaxis, and contractions did not recover after repeated washing (10 washes, 10 min each) (data not shown).

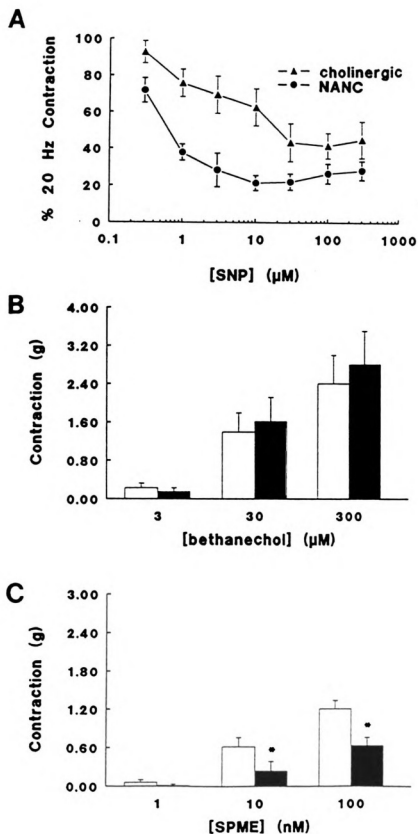
Papaverine (1-10 μ M) inhibits phosphodiesterases, and produced a concentration-dependent decrease in the amplitude of neurogenic NANC contractions (data not shown), with an IC_{50} value of 1.59 ± 0.23 μ M (13 preparations, 2 animals). The site of action of papaverine was determined by examining CMMPPs responses to SPME. Papaverine (10 μ M) inhibited 100 ± 0 % of contractile activity in response to 100 nM SPME (10 preparations, 2 animals). These data indicate that papaverine inhibits NANC contractions by a direct mechanism on circular smooth muscle.

Sodium Nitroprusside Inhibits NANC Contractions

Sodium nitroprusside (SNP) inhibited NANC contractions in a concentration-dependent manner (0.1-300 μ M) (Figure 55), with an EC_{50} of 390 ± 33 nM (16 preparations, 3 animals). SNP also inhibited nerve-evoked cholinergic contractions in a concentration-dependent manner (12 preparations, 2 animals), although the EC_{50} ($1098 \pm$

Figure 55. Effects of SNP on neurogenic and agonist-induced contractions of circular muscle. A. Concentration-response curves for the effects of SNP on NANC and cholinergic contractions of circular muscle. NANC and cholinergic contractions (20 Hz nerve stimulation) are attenuated by SNP in a concentration-dependent manner. Data are mean $\pm$ SEM of percent of control 20 Hz NANC or cholinergic contractions recorded in the presence of guanethidine (3 μ M) and scopolamine (1 μ M) or CP-96,345 (300 nM), respectively. B. Concentration-response relationship for bethanechol in the absence (open bars) and presence (closed bars) of 100 μ M SNP. Data are mean $\pm$ SEM of maximum contraction amplitude expressed in grams of tension. Guanethidine (3 μ M) and scopolamine (1 μ M) were present throughout the experiment. C. Concentration-response relationship for SPME in the absence (open bars) and presence (closed bars) of 100 μ M SNP. Data are mean $\pm$ SEM of maximum contraction amplitude expressed in grams of tension. Guanethidine (3 μ M) and CP-96,345 (300 nM) were present throughout the experiment. Asterisks indicate differences from control (Student's *t* test for paired data; $p < 0.05$).

Figure 55.



46 nM) for inhibition of cholinergic contractions was significantly greater than that for inhibition of NANC contractions.

The site of action of SNP was determined by examining the effects of SNP on SPME and bethanechol responses in tissues pretreated with guanethidine (3 μ M) and scopolamine (1 μ M), and guanethidine (3 μ M) and CP-96,345 (300 nM), respectively. In 4/10 tissues (2 animals), SNP (10 and 100 μ M) did not alter the amplitude of responses of circular muscle to 10 or 100 nM SPME. However, SNP (100 nM) significantly reduced the amplitude of SPME responses in 6/10 tissues (Figure 55). Sodium nitroprusside (100 μ M) did not alter the duration of contractions of circular muscle produced in response to 3, 30, or 300 μ M bethanechol (11 preparations, 2 animals) (Figure 55). The EC_{50} for inhibition of NANC contractions by SNP increased to 2600 ± 70 nM when preparations were treated with apamin (100 nM) (14 preparations, 3 animals) (Figure 56). As shown in Figure 56, addition of apamin resulted in an upwards shift of the SNP concentration-response curve. In the absence of SNP, apamin (1-300 nM) produced a concentration-dependent increase in the amplitude of neurogenic NANC contractions (16 preparations, 3 animals), with an EC_{50} of 69 ± 9 nM (Figure 56).

Superoxide Dismutase Does Not Alter the Amplitude of NANC Contractions

The physiological activity of NO is very short, as NO is broken down by free radicals. Superoxide dismutase (SOD) can scavenge free radicals, thus prolonging the actions of NO (reviewed in Moncada *et al.*, 1991). Superoxide dismutase (10-300 units/ml) did not have any effect on amplitude or duration of NANC contractions produced by 20 Hz transmural nerve stimulation (10 preparations, 2 animals) (Figure 57).

Vasoactive Intestinal Polypeptide Does Not Alter the Amplitude of NANC Contractions

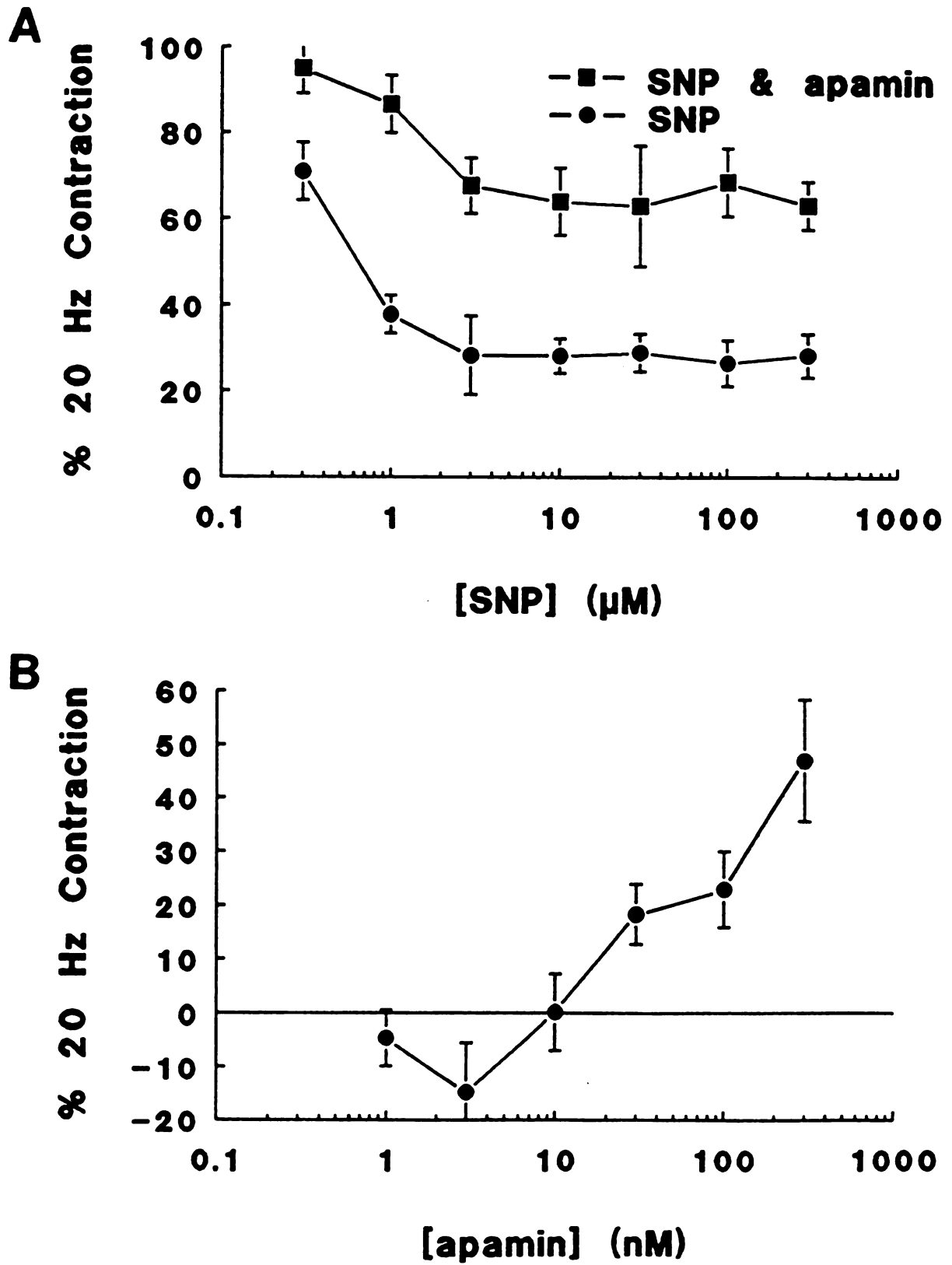
Vasoactive intestinal polypeptide (VIP) (1-300 nM) did not alter the amplitude of NANC contractions produced by 20 Hz transmural stimulation (16 preparations, 3 animals) (Figure 58). However, VIP caused a transient (< 2 min) relaxation of circular muscle in 8/15 preparations. At 1, 10, and 100 nM, VIP relaxed circular muscle by 0.18 ± 0.07 , 0.19 ± 0.05 , and 0.24 ± 0.06 g, respectively. These data indicate that unlike NO, VIP does not regulate release of SP.

Nitric Oxide Synthase- and Substance P-Immunoreactivity Are Localized in Different Nerves of the Circular Muscle Plexus

Preparations of submucosal plexus with attached circular muscle plexus were incubated in antiserum raised against NOS and SP to determine the anatomical relationship between NOS- and SP-ir nerves. In five preparations examined, bright SP-ir was localized in nerve fibers with large, strongly-immunoreactive varicosities. These nerves ran parallel to the circular muscle, and ramified extensively (Figure 60). Intense NOS-ir was also visualized in these preparations, as many nerves contained strong NOS-ir (Figure 59). The NOS-ir nerves also were orientated parallel to the muscle, and few NOS-ir varicosities were observed. Although it was impossible to quantify the number of nerves due to extensive branching, SP- and NOS-ir were never observed in the same nerve fibers (Figure 59).

Figure 56. Concentration-response curves for the effects of SNP and apamin on NANC contractions of circular muscle. A. Concentration-response curves for the effects of SNP on NANC contractions in the absence and presence of apamin (300 nM). Data are mean and SEM of percent of control 20 Hz NANC contractions. B. Concentration-response curve for the effect of apamin on NANC contractions of circular muscle. Data are mean and SEM and are expressed as percent of control 20 Hz NANC contractions. Guanethidine (3 μ M) and scopolamine (1 μ M) are present throughout this experiment.

Figure 56.



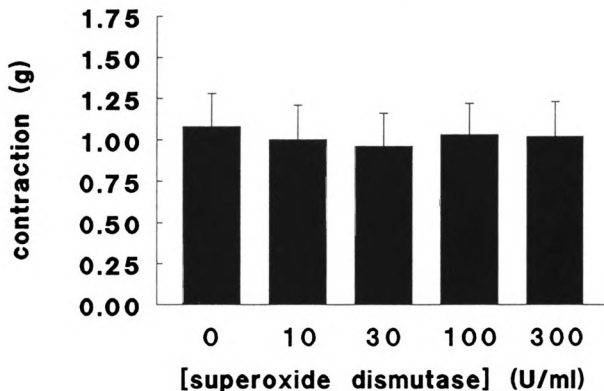


Figure 57. Concentration-response curve for the effects of superoxide dismutase on NANC contractions of circular muscle. Transmural stimulation (20 Hz) of circular muscle-longitudinal muscle preparations resulted in NANC contractions, and addition of superoxide dismutase did not alter amplitude or duration of contractions. Data are amplitude of contractions measured in grams, and are plotted as mean $\pm$ SEM.

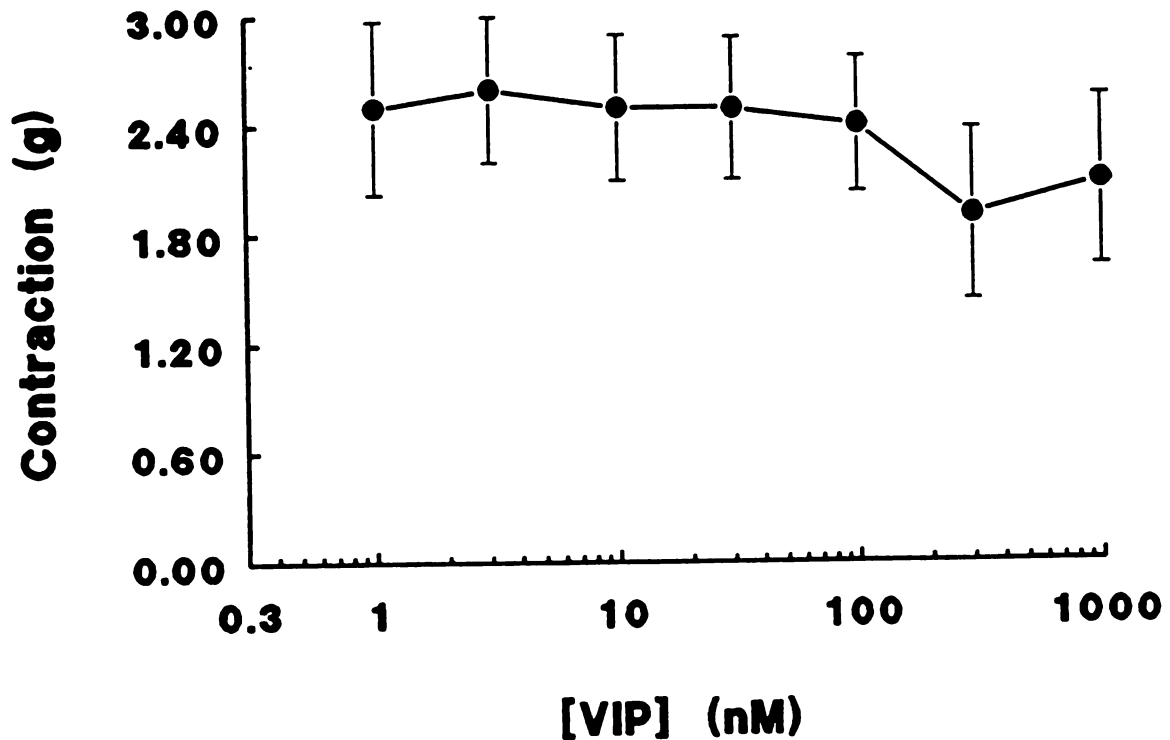
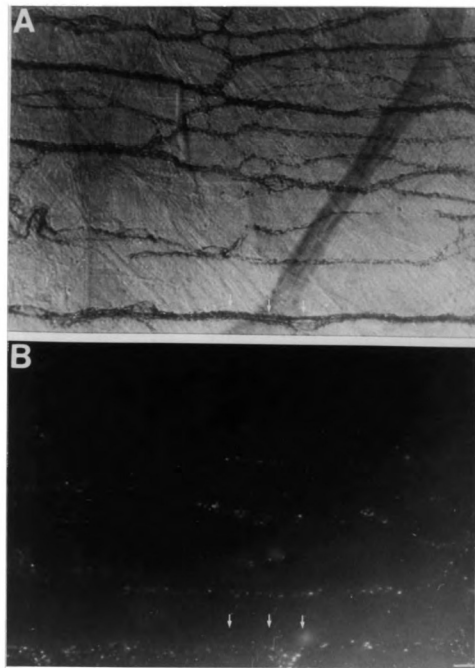


Figure 58. Concentration-response curve for the effects of vasoactive intestinal polypeptide on NANC contractions of circular muscle. Data are mean $\pm$ SEM of maximum contraction amplitude. Guanethidine (3 μ M) and scopolamine (1 μ M) are present throughout this experiment.

Figure 59. Colocalization of nitric oxide synthase- and substance P-immunoreactivity in circular muscle plexus of normal ileum. A. Whole mount preparation of circular muscle associated with submucosal layer. NOS-ir is observed in bright, varicose nerves which ramify extensively. B. Colocalization of SP-ir. SP-ir is also observed in multiple nerves, but these nerves do not contain NOS-ir.

Figure 59.



8. EXTRINSIC DENERVATION ALTERS NITRIC OXIDE PRODUCTION AS ASSAYED BY CITRULLINE PRODUCTION

Introduction and Experimental Rationale

As described in the introduction, NOS is the rate-limiting enzyme that catalyzes a 5-electron oxidation of one of two chemically equivalent guanidino nitrogens of L-arginine to form equimolar concentrations of L-citrulline and NO in the presence of molecular oxygen and NADPH (Palmer, 1988; Palmer, 1989; reviewed in Moncada *et al.*, 1991; Sanders and Ward, 1992). Concentrations of NO have been measured by electron paramagnetic resonance using trapping compounds such as nitroso compounds or reduced hemoglobin, spectrophotometric detection of formation of methemoglobin from hemoglobin, chemiluminescence detection of ozone and NO intermediates, indirect measurement of nitrites and nitrates using Griess' reagent, and more recently, using new amperometric microelectrode assays. However, the most common indirect measurement of NO is the widely-accepted assay of formation of citrulline from radioactive arginine (Bredt and Synder, 1989). This assay uses ion-exchange chromatography to separate [^{14}C]citrulline from [^{14}C]arginine, which remains bound on the cation exchange resin. The [^{14}C]citrulline concentration of tissues of interest can then be measured by counting the number of β -particles that decay per given time. As citrulline and NO are formed in equimolar ratios, alterations in citrulline production are indicative of changes in NO concentrations. Therefore, it was hypothesized that the increased number of NOS-ir neurons in myenteric ganglia would result in greater NO production in preparations made from denervated segments of ileum as compared to control segments. [^{14}C]Citrulline production was measured in normal and surgically-denervated ileum to determine if loss

of extrinsic nerves altered production of [^{14}C]citrulline and NO.

Characterization of [^{14}C]Citrulline Formation in Normal LMMP Homogenates

In the absence of tissue homogenate, cation-ion exchange columns bound $97 \pm 1\%$ ($n=3$) of [^{14}C]arginine applied to the column when de-ionized water was used to wash the columns, indicating that the majority of measured radioactivity was not free arginine. However, if the total volume of eluant or homogenate contained 40% or more HEPES buffer, $60 \pm 7\%$ of [^{14}C]arginine was unable to bind. As HEPES is positively charged, it may compete with arginine molecules for binding, resulting in displacement of [^{14}C]arginine from cation-exchange columns and extremely high backgrounds. Therefore, columns were eluted with water, and tissue homogenization buffer was used to bring homogenates to volume.

Preliminary experiments were performed to determine the optimum assay conditions for incubation of LMMP homogenates with [^{14}C]arginine. In the presence of $3 \mu\text{M}$ [^{14}C]arginine, 86.2 ± 16.4 (9 preparations, 3 animals), 66.4 ± 12.7 (9 preparations, 3 animals), and 49.6 ± 14.3 (3 preparations, 3 animals) pmoles [^{14}C]citrulline/mg protein was measured 25, 40, and 60 min after incubation of tissue homogenates with [^{14}C]arginine. All subsequent experiments were incubated for 25 min at 37°C .

The effects of varying the concentration of [^{14}C]arginine on the quantity of [^{14}C]citrulline formed/mg protein/min was determined in normal LMMP homogenates (9 preparations, 3 animals). A linear relationship was observed for small concentrations (1-10 μM) of [^{14}C]arginine, as increasing the concentration of [^{14}C]arginine resulted in greater production of [^{14}C]citrulline/mg protein/min (Figure 60). However, [^{14}C]arginine was no longer limiting by 20 μM , and [^{14}C]production was not dependent on the

Figure 60. Analysis of kinetics of [^{14}C]citrulline production by homogenates of longitudinal muscle-myenteric plexus preparations. A. Effects of increasing concentrations of [^{14}C]arginine on citrulline production in the presence of 1 mM NADPH. B. Double reciprocal (Lineweaver-Burke) plot of data from A. K_M is 7.2 μM arginine and $V_{\max}$ is 18.6 pmoles / mg protein/min. Homogenates were incubated with varying concentrations of [^{14}C]arginine for 25 min at 37°C.

Figure 60.

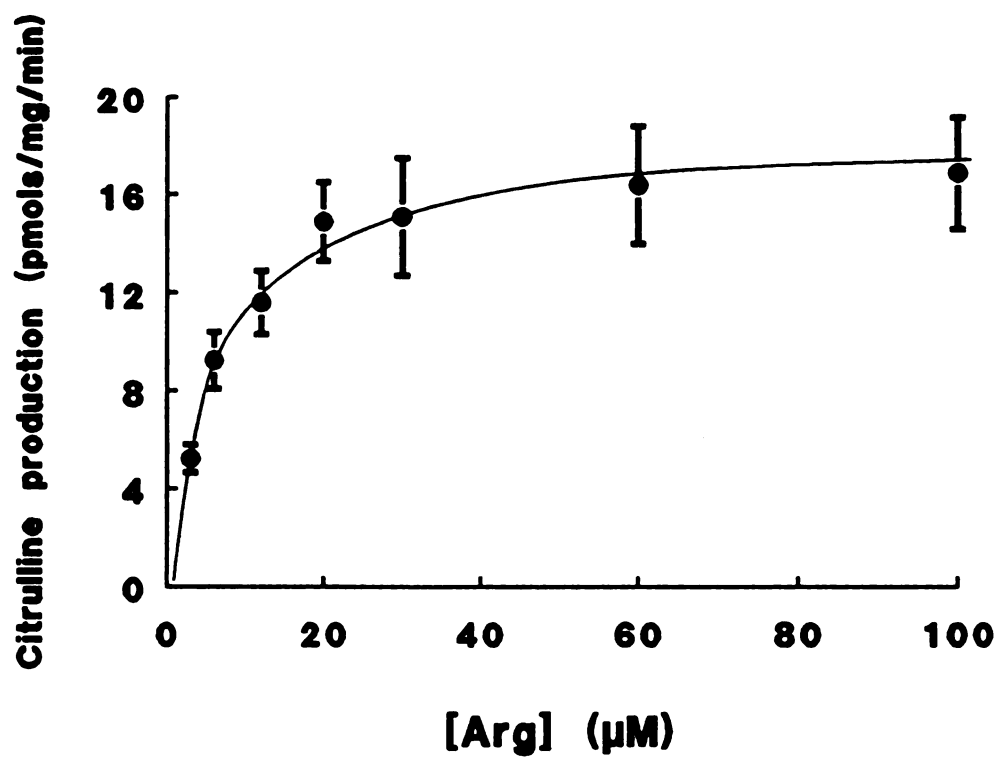
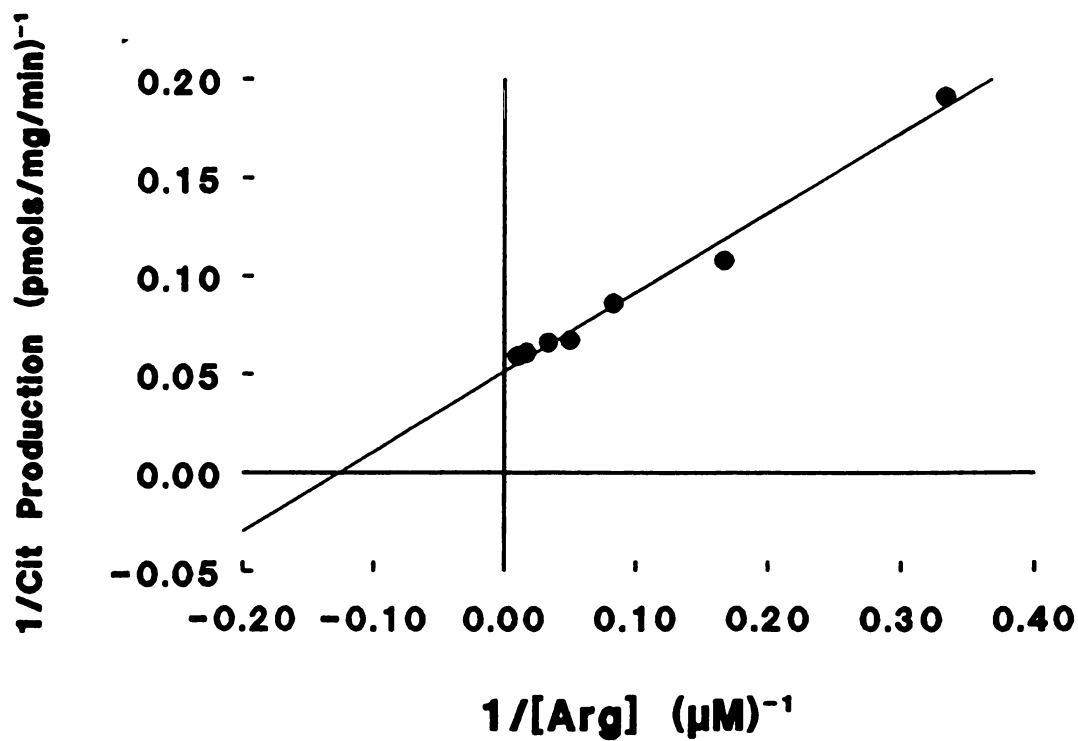
A**B**

Table 9. Characterization of [^{14}C]citrulline production in longitudinal muscle-myenteric plexus homogenates prepared from normal ileum.

Homogenate	[^{14}C]Arginine	
	3 μM	5 μM
Basal	69.5 $\pm$ 10.6 (9)	152 $\pm$ 18.7 (8)
+ NADPH (1 mM)	267 $\pm$ 44.3 (9) *	309 $\pm$ 37.2 (8) *
+ Calcium (2 mM)	40.3 $\pm$ 11.3 (9)	82.1 $\pm$ 21.7 (8)

Homogenates were incubated with [^{14}C]Arginine for 25 min. Values are mean $\pm$ SEM for (n) replicates from 3-4 animals, and are expressed as pmole [^{14}C]citrulline / mg protein. *, values for homogenates containing NADPH are significantly different ($p < 0.05$) from basal production, as well as from production in the presence of an additional 2 mM calcium.

concentration of [^{14}C]arginine. A double-reciprocal (Lineweaver-Burke) plot of these data revealed a Michaelis constant (K_M) of 7.2 μM , and a maximum initial velocity (V_{max}) of 18.6 pmole [^{14}C]citrulline/mg protein/min (Figure 1B). As 3 μM [^{14}C]arginine was on the linear portion of the substrate-velocity curve, this concentration was used for all

Thin layer chromatography was used to identify the column flow-through. The eluant components were visualized with ninhydrin spray, and the majority of the eluant had a similar migration distance as L-citrulline standards, whereas the components of tissue-free homogenates had a similar migration distance as L-arginine standards. These data are consistent with the formation of [^{14}C]citrulline from [^{14}C]arginine (Bredt and Synder, 1989).

Ca $^{2+}$ and Calmodulin Effects [^{14}C]Citrulline Formation in Normal LMMP Homogenates

Addition of 0.1 mM Ca $^{2+}$ (as CaCl $_2$) to normal LMMP homogenates did not increase basal [^{14}C]citrulline production, as 70.2 $\pm$ 11.6 pmoles [^{14}C]citrulline/mg protein

Figure 61. Effects of Ca^{2+} , NADPH, and calmodulin on [^{14}C]citrulline production by homogenates of longitudinal muscle-myenteric plexus preparations. A. Effects of 1 mM Ca^{2+} (as CaCl_2) on [^{14}C]citrulline production in the absence and presence of 1 mM NADPH. Asterisk (*) indicates $p < 0.05$ as compared to basal production, and cross (+) indicates $p < 0.05$ as compared to [^{14}C]citrulline production in the presence of 1 mM NADPH. B. Effects of 0.1 mM Ca^{2+} and 30 $\mu\text{g}/\mu\text{l}$ calmodulin (CaM) on [^{14}C]citrulline production in the presence of 1 mM NADPH. Asterisk (*) indicates $p < 0.05$ as compared to production in the presence of NADPH and CaM. Homogenates were incubated with 3 μM [^{14}C]arginine for 25 min at 37°C.

Figure 61.

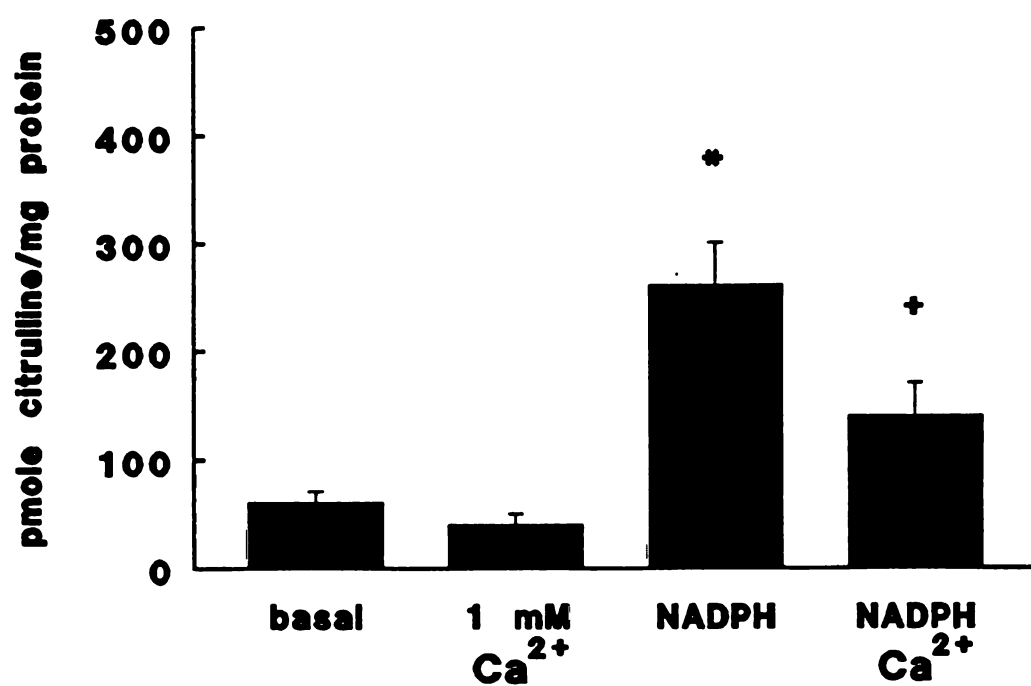
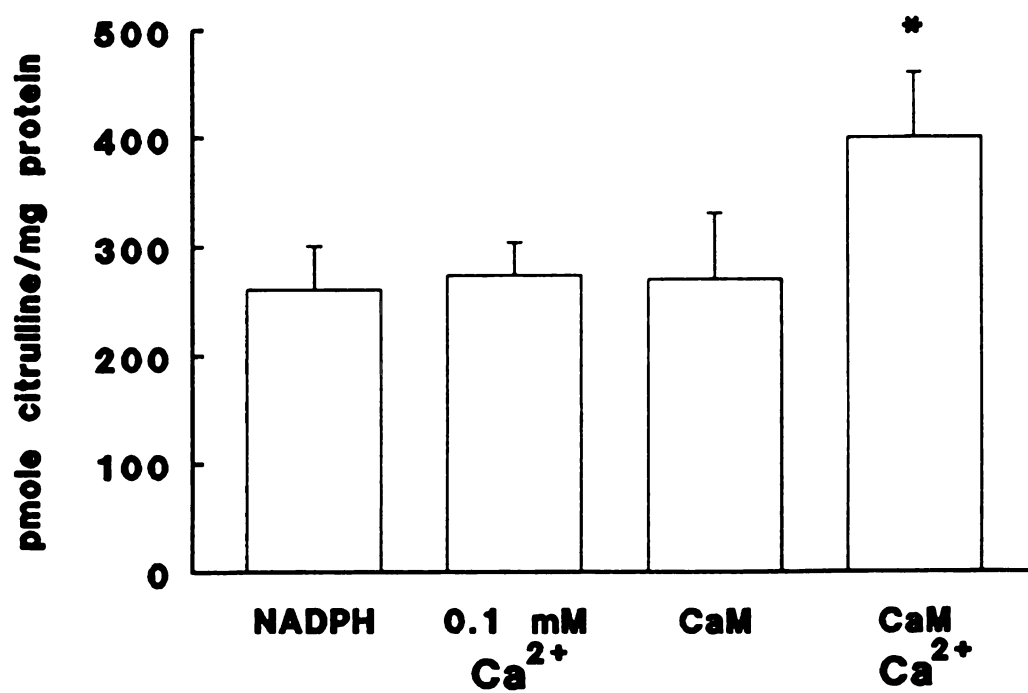
A**B**

Table 10. Effects of NADPH and calcium on [^{14}C]citrulline production by nitric oxide synthase in longitudinal muscle-myenteric plexus homogenates from normal ileum.

NADPH	Calcium (mM)	
	0 mM	2 mM
0 mM	69.5 $\pm$ 10.6 (9)	40.3 $\pm$ 13.0 (9)
1 mM	267 $\pm$ 44.3 (9) ^{*+}	143 $\pm$ 35.7 (9) ^{*+}

Homogenates were incubated with 3 μM [^{14}C]arginine for 25 min. Values are mean $\pm$ SEM for (n) replications from 4 animals, and are expressed as pmole [^{14}C]citrulline / 100 μg protein. *, values of homogenates containing NADPH are significantly different ($p < 0.05$) from values of homogenates without NADPH. +, values of homogenates containing Ca^{2+} and NADPH are significantly different ($p < 0.05$) from values of homogenates containing only NADPH.

was produced by homogenates in the presence of Ca^{2+} (9 preparations, 3 animals), and 69.5 $\pm$ 10.6 pmoles [^{14}C]citrulline/mg protein was produced in the absence of putative cofactors (9 preparations, 3 animals). Similarly, elevation of Ca^{2+} to 1 mM did not alter basal [^{14}C]citrulline production (Figure 61A). Although Ca^{2+} did not affect [^{14}C]citrulline production (Table 9), addition of 1 mM NADPH significantly increased [^{14}C]citrulline production by 285% (Figure 61A and Table 9), and was included in all subsequent experiments. The low concentration of Ca^{2+} (0.1 mM) did not alter NADPH-enhanced [^{14}C]citrulline production (274 $\pm$ 31.3 pmoles/mg protein, 9 preparations, 3 animals) as compared to NADPH-enhanced production in the absence of exogenous Ca^{2+} (267 $\pm$ 44.3 pmoles/mg protein, 9 preparations, 3 animals). However, elevation of exogenous Ca^{2+} to 1 mM significantly decreased NADPH-enhanced [^{14}C]citrulline production (8 preparations, 3 animals) (Figure 61A and Table 10).

The effects of exogenous calmodulin on [^{14}C]citrulline formation was examined, as the type I isoform of NOS is localized in enteric neurons (Bredt *et al.*, 1990; Belai *et*

Table 11. Addition of exogenous calmodulin does not alter nitric oxide synthase activity of longitudinal muscle-myenteric plexus preparations from normal ileum.

Nitric Oxide Synthase Activity (pmole [14 C] citrulline / mg protein)					
Concentration of Calmodulin (μ g/ μ l)					
0	0.01	0.1	1	10	30
254 $\pm$ 49.6 (8)	265 $\pm$ 48.7 (6)	241 $\pm$ 38.0 (6)	180 $\pm$ 67.9 (8)	266 $\pm$ 71.9 (6)	271 $\pm$ 66.7 (6)

Homogenates were incubated with 3 μ M [14 C]arginine, 1 mM NADPH, and varying concentrations of calmodulin for 25 min. Data are mean $\pm$ SEM for (n) replicates from 2 or 3 animals.

al., 1992; Costa *et al.*, 1992; Young *et al.*, 1992), and activity of this isoform is dependent on Ca^{2+} and calmodulin (Bredt and Synder, 1990). In the presence of 1 mM NADPH, 0.01 to 30 $\mu\text{g}/\mu\text{l}$ calmodulin did not affect [^{14}C]citrulline production (6-8 preparations/calmodulin concentration) (Figure 61B and Table 11). If 0.1 mM Ca^{2+} was included with 30 $\mu\text{g}/\mu\text{l}$ calmodulin, then [^{14}C]citrulline production increased as compared to NADPH-enhanced citrulline formation (7 preparations, 3 animals) (Figure 61B). Finally, EGTA was used to chelate Ca^{2+} in homogenates and to determine if endogenous Ca^{2+} was important in NADPH-enhanced [^{14}C]citrulline production. As shown in Figure 62, 0.1 and 1 mM EGTA attenuated [^{14}C]citrulline formation by 71 and 99%, respectively (9 preparations, 3 animals), further demonstrating the importance of Ca^{2+} in [^{14}C]citrulline formation.

[^{14}C]Citrulline Formation in the Presence of NOS Antagonists

Substituted L-arginine analogs such as N^{G} -nitro-L-arginine (LNA) and N^{G} -nitro-L-arginine methyl ester (LNAME) inhibit formation of NO (reviewed in Moncada *et al.*, 1991) and prevent NO-mediated responses of a number of tissues, including neurogenic relaxation of GI smooth muscle (reviewed in Sanders and Ward, 1992). In the presence of 1 mM NADPH, LNAME (0.01 mM) reduced [^{14}C]citrulline production by 38%, and 0.1 mM LNAME attenuated [^{14}C]citrulline formation by 95% as compared to NADPH-enhanced production in the absence of NOS antagonists (9 preparations, 3 animals) (Figure 62). Similar results were observed when LNA (0.01 or 0.1 mM) was included in homogenates, as [^{14}C]citrulline production decreased by 42 and 96%, respectively (Figure 62). However, the D-enantiomer of LNAME did not affect [^{14}C]citrulline formation as 232 ± 47.3 pmoles/mg protein was formed in the presence of 1 mM NADPH

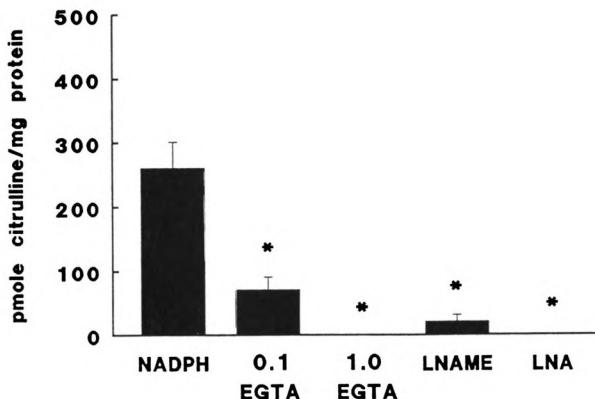


Figure 62. [¹⁴C]Citrulline production by homogenates of longitudinal muscle-myenteric plexus preparations in the presence of EGTA and NOS antagonists. Effects of EGTA (0.1 and 1 mM), 0.1 mM N^G-nitro-L-arginine methyl ester (LNAME), or 0.1 mM N^G-nitro-L-arginine (LNA) on [¹⁴C]citrulline production in the presence of 1 mM NADPH. Asterisks (*) indicate $p < 0.05$ as compared to production in the presence of NADPH. Homogenates were incubated with 3 μ M [¹⁴C]arginine for 25 min at 37°C.

and 0.1 mM DNAME (6 preparations, 2 animals). This was not different from production in the presence of 1 mM NADPH only.

[¹⁴C]Citrulline Formation in LMMP Homogenates From Extrinsically-Denervated Ileum

Basal [¹⁴C]citrulline formation was similar in extrinsically-denervated, control, and normal homogenates, as 40.0 ± 10.4 , 51.5 ± 11.5 , and 49.5 ± 10.6 pmoles/mg protein were produced by extrinsically-denervated (3 preparations), control (3 preparations), and normal (3 preparations) LMMP homogenates, respectively. NADPH (1 mM) significantly increased [¹⁴C]citrulline formation by 100% and 160% in homogenates prepared from extrinsically-denervated (3 preparations) and control (3 preparations) ileum. However, NADPH-enhanced [¹⁴C]citrulline production in homogenates from extrinsically-denervated ileum (9 preparations, 4 animals) was decreased as compared to production in homogenates from control ileum (9 preparations, 4 animals) (Figure 63). [¹⁴C]Citrulline production in homogenates of control ileum was not significantly different from that of normal homogenates, as 138.8 ± 26.2 and 100.8 ± 20.8 pmoles/mg protein were measured from control and normal homogenates, respectively (Table 12).

NADPH-enhanced [¹⁴C]citrulline formation in extrinsically-denervated LMMP homogenates was significantly inhibited by LNA (0.1 mM) (7.8 ± 4.4 pmoles citrulline/mg protein, 8 preparations, 3 animals) and LNAME (0.1 mM) (9 preparations, 3 animals) (Figure 63A). EGTA at concentrations of 0.1 and 1 mM attenuated [¹⁴C]citrulline production in control homogenates by 75% (9 preparations, 3 animals) and 99% (9 preparations, 3 animals), respectively. However, 0.1 and 1 mM EGTA only

Table 12. [^{14}C]Citrulline production in normal and control longitudinal muscle-myenteric plexus preparation homogenates.

Source	1 mM NADPH		
		100 μM L-NAME	1 mM EGTA
normal	100.8 $\pm$ 20.8 (6)	4.2 $\pm$ 0.2* (6)	0.2 $\pm$ 0.4* (6)
control	138.8 $\pm$ 26.2 (6)	4 $\pm$ 2.0* (6)	3.7 $\pm$ 2.3* (6)

Homogenates were incubated with 3 μM [^{14}C]arginine for 25 min. Values are means $\pm$ SEM for (n) replications from 3 animals, and are expressed as pmole [^{14}C]citrulline/mg protein. *, values of homogenates are significantly different ($p < 0.05$) from values of homogenates with NADPH. Significant differences between normal and control groups were not detected.

attenuated [^{14}C]citrulline formation by 48 and 70% in homogenates made from denervated LMMP preparations (9 preparations, 3 animals) (Figure 63A). Furthermore, although [^{14}C]citrulline production in the presence of 1 mM EGTA was a small proportion (30%) of total NADPH-enhanced [^{14}C]citrulline production, EGTA-insensitive [^{14}C]citrulline production was observed in all homogenates made from denervated segments of ileum.

As [^{14}C]citrulline production was normalized for the amount of protein in LMMP homogenates, [^{14}C]citrulline production may have appeared to decrease because surgical denervation induced muscle thickening, resulting in a greater concentration of protein in denervated homogenates as compared to controls. This hypothesis was tested by weighing LMMP preparations in a standard volume of homogenization buffer as described previously. LMMP preparations from control ileum (9 preparations) weighed 0.021 ± 0.001 g/cm, and this value was not different from LMMP preparations made from denervated ileum (0.024 ± 0.002 g/cm, 9 preparations) (Figure 9).

[¹⁴C]Citrulline Formation in LMMP Homogenates From Capsaicin-Treated Animals

As described previously, systemic administration of capsaicin was used to selectively destroy thin, unmyelinated sensory afferent nerves (Papka *et al.*, 1984; Galligan *et al.*, 1988). Ca^{2+} (0.1 mM) increased [¹⁴C]citrulline production by 30%, and NADPH (1 mM) significantly potentiated [¹⁴C]citrulline formation by 245% as compared to basal production in chemically-denervated LMMP homogenates. Similar results were observed in homogenates made from ileum in vehicle-treated animals (data not shown). However, in the presence of 1 mM NADPH, [¹⁴C]citrulline production was 50% less in homogenates from capsaicin-treated animals (7 preparations, 3 animals) than in homogenates from vehicle-treated animals (6 preparations, 2 animals) (Figure 63). [¹⁴C]Citrulline production was attenuated by LNA (0.1 mM) in homogenates made from capsaicin-treated and vehicle-treated animals, as citrulline production was decreased by 96% and 98% as compared to NADPH-enhanced production by homogenates from capsaicin- or vehicle-treated animals, respectively. LNAME (0.1 mM) also inhibited 97% and 98% of [¹⁴C]citrulline formation in LMMPs from capsaicin- and vehicle-treated animals (Figure 63B). Finally, removal of Ca^{2+} by EGTA (1 mM) attenuated citrulline production in homogenates from capsaicin- and vehicle-treated animals by 99% and 98%, respectively (Figure 63B).

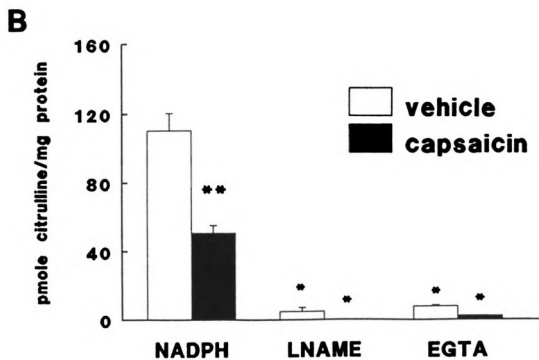
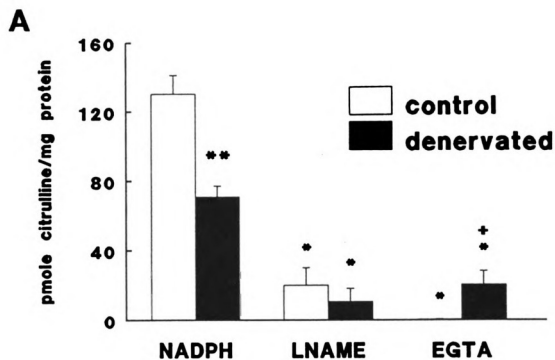
Myenteric Neurons Do Not Contain Inducible Nitric Oxide Synthase-Immunoreactivity After Extrinsic Denervation

After extrinsic denervation, approximately 33% of citrulline production was insensitive to removal of extracellular Ca^{2+} . Therefore, polyclonal antiserum against inducible NOS (iNOS) isolated from macrophages was used to determine if iNOS-ir was

localized in myenteric ganglia seven weeks after loss of extrinsic nerves. Inducible NOS-ir was not observed in myenteric or submucosal ganglia, nerve strands, arterioles, GI smooth muscle, or extraganglionic cells. Positive immunoreactivity was observed in extraganglionic macrophage-like structures (data not shown) after 3 hr incubation of normal ileum with 100 ng/ml lipopolysaccharide (serotype 0128:B12), suggesting correct fixatives and dilutions of reagents had been utilized. However, it was impossible to determine if lack of immunoreactivity resulted from absence of the antigen, or from the absence of binding of antiserum raised against macrophage iNOS although type II isoform of NOS was present.

Figure 63. Comparison of [^{14}C]citrulline production by homogenates in longitudinal muscle-myenteric plexus preparations from control and denervated ileum. A. Effects of 0.1 mM LNAME, and 1 mM EGTA on control and surgically-denervated ileum. Loops of ileum were denervated 7 weeks prior to the experiment and control segments were taken oral or aboral to the denervated segment. NADPH (1 mM) was present in all homogenates. B. Effects of 0.1 mM LNAME, and 1 mM EGTA on ileum from vehicle- and capsaicin-treated animals. Loops of ileum were taken 7 weeks after administration of vehicle or capsaicin. NADPH (1 mM) was present in all homogenates. Double asterisks (**) indicate $p < 0.05$ as compared to production by control homogenates. Single asterisks (*) indicate $p < 0.05$ as compared to production by control or denervated LMMP homogenates in the presence of 1 mM NADPH. Cross (+) indicates $p < 0.01$ as compared to control homogenates in the presence of 1 mM EGTA. Homogenates were incubated with 3 μM [^{14}C]arginine for 25 min at 37°C.

Figure 63.



9. FOS-LIKE PROTEIN INCREASES AFTER EXTRINSIC DENERVATION

Introduction and Experimental Rationale

Recently, expression of the intranuclear phosphoprotein Fos has been found to increase following manipulations such as exposure to growth factors (Milbrant, 1986) and induction of seizures (Sager *et al.*, 1988) (see introduction). Fos has been used as an indicator of increased (or increasing) nuclear activity, although the role of Fos has not been elucidated. Fos has been proposed to be a third messenger between extracellular signals and nuclear events (Sager *et al.*, 1988), as Fos is involved in initiation of transcription of cell-specific target genes ultimately resulting in a cellular response (Lau and Nathans, 1987; see introduction). Therefore, expression of Fos may indicate alterations in nuclear events of neurons.

Previous studies on enteric neurons have revealed that Fos-like-ir can not be detected in control submucosal and myenteric ganglia. However, stimulation of mucosa induces Fos-ir in a population of submucosal neurons (Kirchgessner *et al.*, 1992), suggesting some enteric neurons can express Fos protein in response to stimuli. As loss of extrinsic innervation induces a number of changes in submucosal and myenteric ganglia, some signal must communicate between the exterior *milieu* and the nucleus; *c-fos*, the early immediate gene (EIG) that encodes Fos protein would be a plausible candidate (Wessel *et al.*, 1991). Furthermore, increased protein synthesis would be expected to occur after increased nuclear events, which would in turn be correlated with increased Fos-ir. Therefore, immunocytochemistry was used to localize Fos-ir in submucosal and myenteric ganglionic nuclei to test the hypothesis that loss of extrinsic innervation induced EIG expression. Fos-ir could then be used to determine if extrinsic denervation effects

all enteric neurons, or if specific subpopulations of enteric neurons respond to loss of extrinsic nerves.

Fos-ir Is Not Found In Normal Submucosal or Myenteric Plexus Preparations

Three antiserum were used to localize Fos-ir in enteric nuclei (Table 13). Polyclonal antiserum SC-52 (Santa-Cruz), monoclonal antibody AB-1 (Oncogene Sciences), and polyclonal antiserum OA-11-823 (Cambridge Research Biochemicals) failed to recognize epitopes in normal submucosal (5 preparations/antibody) and myenteric plexus preparations (2-5 preparations/antibody) fixed in Zamboni's solution or 4% paraformaldehyde. As previous work has demonstrated that high concentrations of K^+ depolarize neurons and induce expression of Fos-ir *in vitro* (Morgan and Curran, 1986), segments of ileum were incubated in Krebs' solution containing 50 mM K^+ (as KCl) to determine if enteric neurons can express Fos. OA-11-823 and AB-1 did not recognize any epitopes on submucosal or myenteric ganglia after 1, 2, 3, or 4 h in depolarizing medium (3 submucosal and 3 myenteric plexus preparations/antibody/time point) (Table 14). However, one hour after incubation in high K^+ Krebs' solution, SC-52-ir was observed in submucosal (3 preparations) and myenteric (3 preparations) ganglionic nuclei in tissues fixed in Zamboni's solution (Figure 64). SC-52 binding was localized in ovoid-shaped organelles in submucosal and myenteric plexus preparations fixed in Zamboni's solution but not 4% paraformaldehyde. These organelles were also stained by the nuclear dye bisBenzimide (Hoechst dye 33528) (data not shown), indicating the antibody recognized a nuclear protein. Furthermore, Fos-ir was only localized in ganglionic nuclei, as nuclei from muscle cells, fibroblasts, and other extraganglionic cells [reviewed in 16] were not fluorescent. Similarly, structures such as submucosal arterioles, nerve fibers,

Figure 64. Fos-ir in submucosal and myenteric ganglia in whole mount preparations from control and surgically-denervated ileum. A. Fos-ir is not observed in submucosal ganglia from control ileum taken oral or aboral to denervated segments. B. Fos-ir in submucosal ganglia from loops of ileum denervated 1 week prior to experiment. C. Fos-ir is not observed in myenteric ganglia from control ileum. D. Fos-ir in myenteric ganglia from loops of ileum denervated 1 week prior to experiment. g, ganglion, arrows indicated Fos-ir nuclei. Calibration bar in D is approximately 40 μ m.

Figure 64.

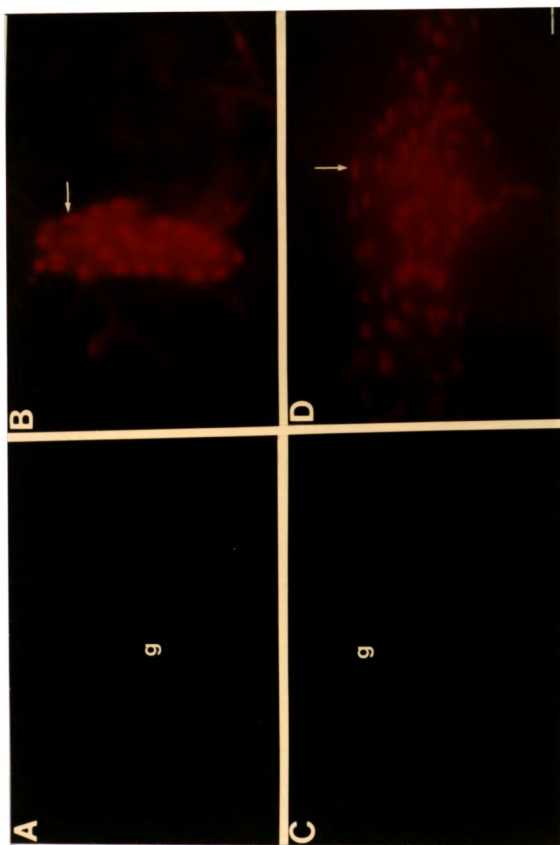


Table 13. Antiserum used to localize c-Fos protein in enteric nuclei.

Antibody Code Host	Source Target Antigen	Sequence of Synthetic Peptide Antigen
AB-1 mouse monoclonal	Oncogene Sciences human c-Fos, 128-152	KVEQLSPEEEEKRRIRR ERNKMAAA
OA-11-823 sheep polyclonal	Cambridge Research Biochemicals human c-Fos, 1-16	MFSGFNADYEASSSR [Cys]
SC-52 rabbit polyclonal	Santa Cruz Biotechnology human c-Fos, 3-16	SGFNADYEASSSRC

interganglionic nerve bundles, and muscle cells did not contain Fos-ir. In submucosal plexus preparations, submucosal ganglion contained an average of 8.2 ± 0.6 Fos-ir nuclei (3 preparations, 898 nuclei, 115 ganglia) (Table 14), with an average size of 11.3 ± 0.3 μm (long axis) and 8.5 ± 0.3 μm (short axis). Myenteric plexus preparations contained 110 ± 8 Fos-ir nuclei (3 preparations, 1,337 nuclei, 13 ganglia) and nuclei had an average size of 13.5 ± 0.3 μm (long axis) and 9.2 ± 0.3 μm (short axis). Binding of SC-52 was prevented by pre-incubating the antiserum with 10-fold excess (by weight) of synthetic c-Fos peptide prior to application of antiserum to tissues samples, and immunoreactive nuclei were not observed if the primary antiserum was not applied before secondary antiserum.

Although Fos-ir was observed in preparations made from segments of ileum incubated in Krebs' solution containing 50 mM K^+ , Fos-ir nuclei were also present in preparations made from segments of ileum incubated in normal Krebs' solution (Table 14). The number of Fos-ir nuclei/submucosal ganglion (4 preparations) and Fos-ir nuclei/myenteric ganglion (4 preparations) from segments of ileum incubated in Krebs'

Table 14. The number of c-Fos-ir nuclei in submucosal and myenteric ganglia in normal guinea pig ileum.

Treatment	Submucosal Ganglion (Fos-ir nuclei, ganglia)	Myenteric Ganglion (Fos-ir nuclei, ganglia)
SC-52		
normal ileum	0 ± 0	0 ± 0
zamboni fixation	(0,216)	(0,27)
normal ileum	0 ± 0	0 ± 0
4% paraformaldehyde	(0,234)	(0,20)
control medium	8.15 ± 0.59	110 ± 8
1 hour	(896,115)	(1337,13)
K ⁺ -medium	8.14 ± 0.43	126 ± 6
1 hour	(750,93)	(2050,18)
control medium	9.04 ± 0.47	123 ± 9
2 hours	(1924,216)	(3290,27)
K ⁺ -medium	7.54 ± 0.56	121 ± 8
2 hours	(1101,148)	(5862,62)
control medium	9.44 ± 0.40	123 ± 12
4 hours	(4194,450)	(9153,79)
K ⁺ -medium	9.84 ± 0.65	124 ± 8
4 hours	(2270,234)	(5990,49)
AB-1		
control medium	0 ± 0	0 ± 0
4 hours	(0,123)	(0,30)
K ⁺ -medium	0 ± 0	0 ± 0
4 hours	(0,92)	(0,35)
OA-11-823		
control medium	0 ± 0	0 ± 0
4 hours	(0,142)	(0,45)
K ⁺ -medium	0 ± 0	0 ± 0
4 hours	(0,78)	(0,37)

Number of Fos-ir nuclei/ganglion in normal guinea pig ileum. Data are means of Fos-ir nuclei/ganglion $\pm$ SEM for 3 preparations from each treatment.

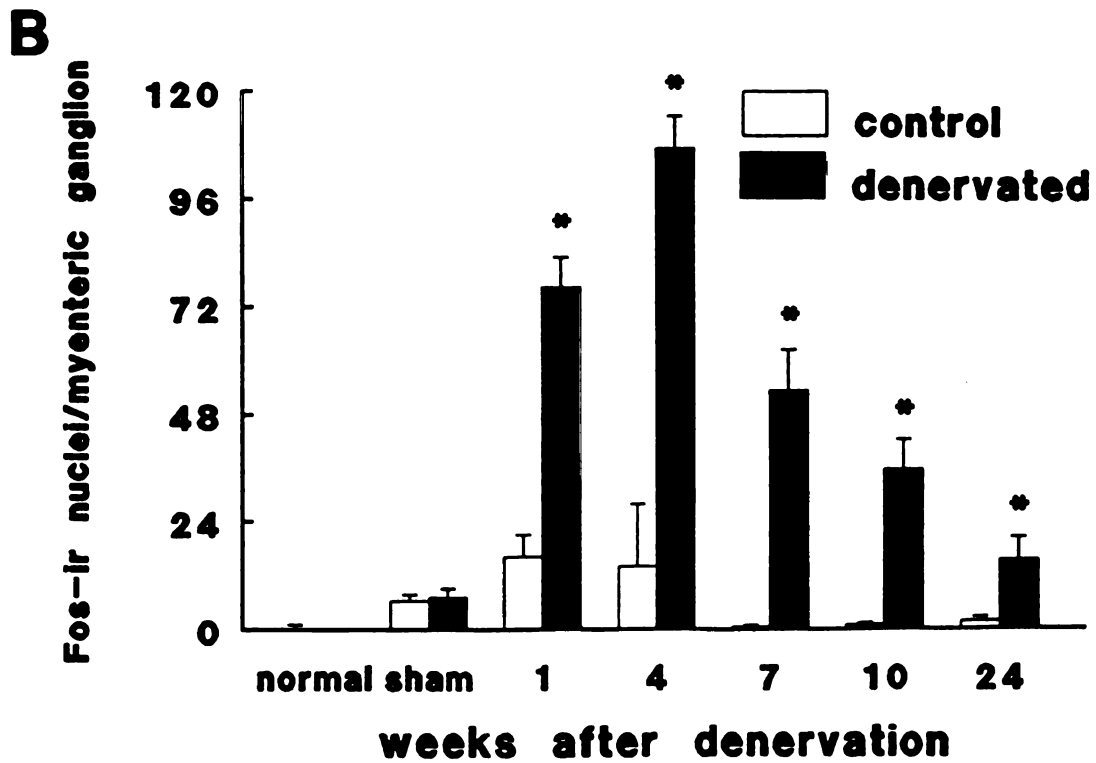
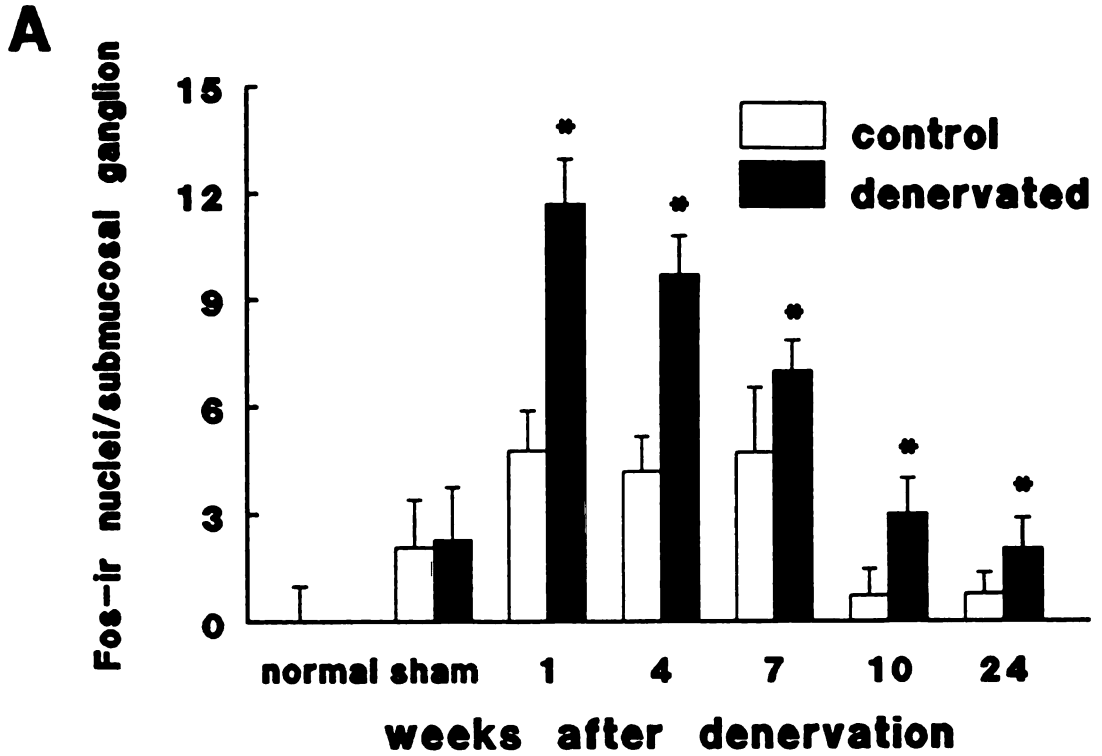
solution (6 preparations) was not different from the number of Fos-ir nuclei/ganglion in preparations incubated in high K^+ Krebs' solution (6 preparations) (Table 14). Furthermore, the number of Fos-ir nuclei/submucosal ganglion and Fos-ir nuclei/myenteric ganglion was not altered by the length of incubation (1-4 hr) in normal or high K^+ Krebs' solution (4-6 preparations/time point) (Table 14).

Surgical Extrinsic Denervation Increases Fos-ir in Submucosal and Myenteric Ganglia

A greater number of Fos-ir nuclei were observed in submucosal and myenteric ganglia one week after loss of extrinsic innervation as compared to control preparations taken from segments oral or aboral to the denervated region in animal-matched controls (Figure 65). Fos-ir was observed in ganglionic nuclei in submucosal and myenteric ganglia, but not in other structures (Figure 64). Significantly more nuclei contained Fos-ir in submucosal plexus preparations from extrinsically-denervated ileum, as 11.7 ± 1.2 submucosal nuclei/ganglion (9,727 Fos-ir nuclei, 851 ganglia, 8 preparations, 3 animals) were observed in denervated preparations, and control preparations contained 3.7 ± 1.1 Fos-ir submucosal nuclei/ganglion (2,100 Fos-ir nuclei, 559 ganglia, 8 preparations, 3 animals) (Figure 65A). An increase was observed also in myenteric plexus preparations, as 114 ± 6 myenteric nuclei/ganglion were found in extrinsically-denervated preparations (1,424 Fos-ir nuclei, 150 myenteric ganglia, 13 preparations, 4 animals), and control preparations contained 13 ± 4 nuclei/ganglion (2,469 Fos-ir nuclei, 231 ganglia, 10 preparations, 4 animals) (Figure 65B). However, the number of Fos-ir nuclei/ganglion did not increase in submucosal or myenteric preparations taken from sham-denervated segments of ileum as compared to controls taken from the same animals (Figure 65).

Figure 65. Number of Fos-ir nuclei in submucosal and myenteric ganglia from segments of ileum denervated 1-24 weeks prior to experiment. A. Number of Fos-ir nuclei in submucosal ganglia from sham and denervated ileum. B. Number of Fos-ir myenteric ganglia from sham and denervated ileum. Data are 8-10 tissues from 3-4 animals for each time point. Asterisks (*) indicate $p < 0.05$ as compared to segments taken oral or aboral to denervated segments.

Figure 65.



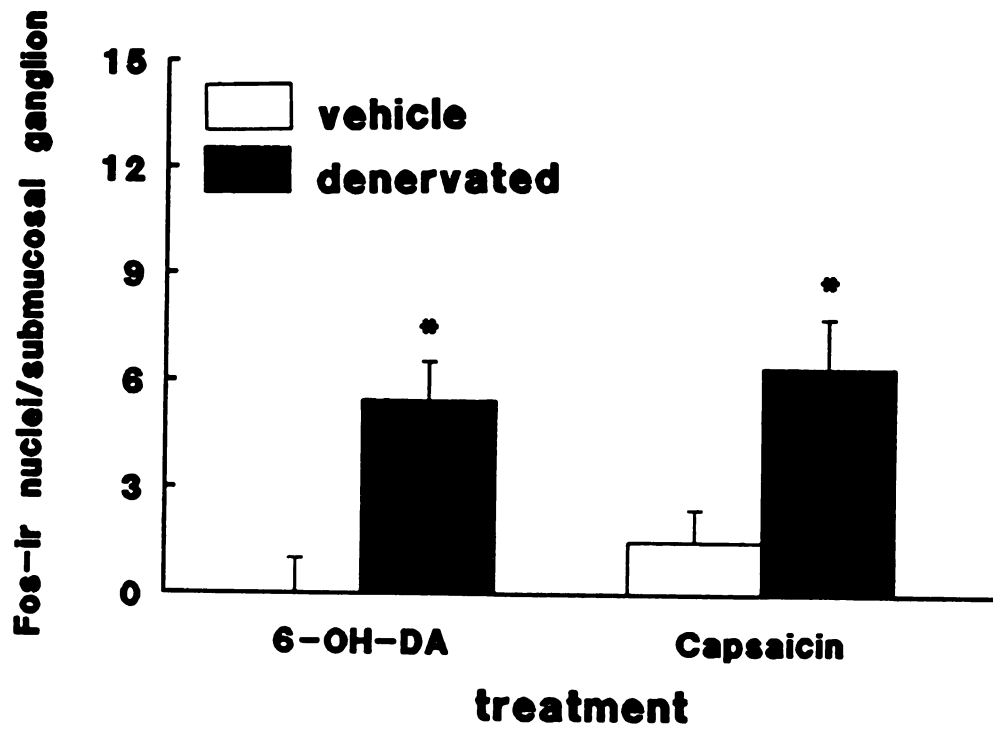
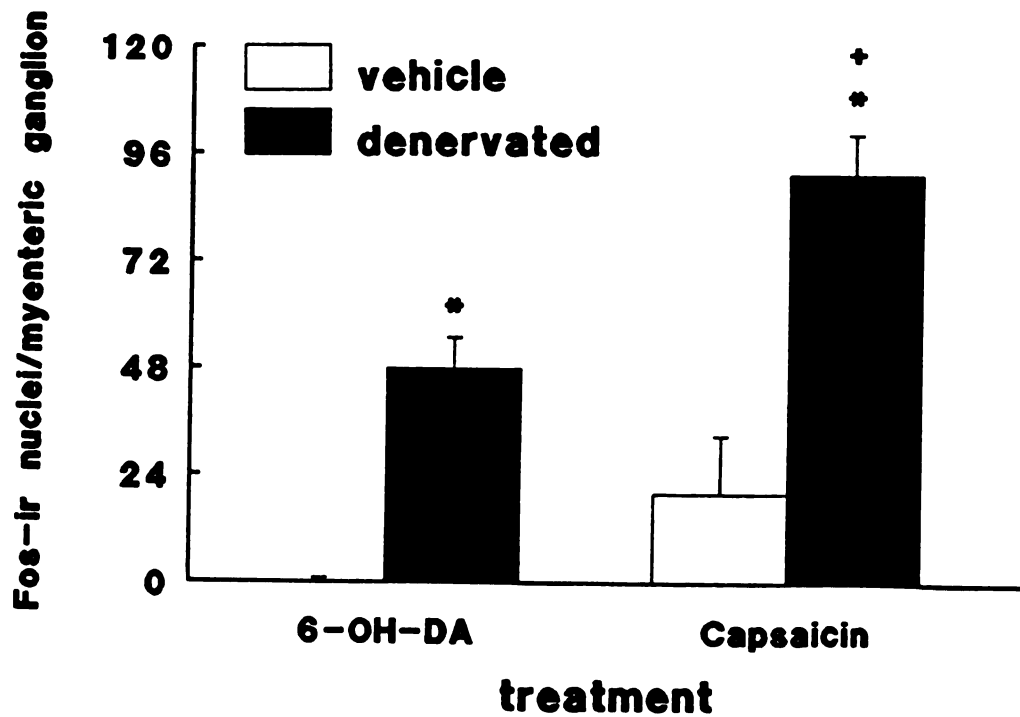
Similar results were observed in submucosal and myenteric ganglia 4, 7, 10, and 24 weeks after loss of extrinsic nerves (Figure 65), as the number of Fos-ir nuclei/submucosal ganglion and Fos-ir nuclei/myenteric ganglion was significantly greater in segments from denervated tissues, as compared to preparations from control segments of ileum. However, the number of Fos-ir nuclei/ganglion decreased over time. For example, 1 week after denervation, submucosal ganglion contained 11.7 ± 1.25 Fos-ir nuclei, whereas 2.05 ± 0.85 Fos-ir nuclei/ganglion were observed 24 weeks after extrinsic denervation.

Loss of Extrinsic Sympathetic or Sensory Nerves Increases Fos-ir in Submucosal and Myenteric Ganglia

Guinea pigs were treated with capsaicin or 6-OH-DA to destroy extrinsic sensory or sympathetic nerves, respectively. One week after treatment with 6-OH-DA, the number of Fos-ir nuclei increased significantly in submucosal and myenteric ganglia as compared to preparations from vehicle-treated animals (Figure 66). Similar results were obtained after systemic administration of capsaicin, as the number of Fos-ir nuclei increased by 350% and 700% in submucosal and myenteric ganglia, respectively, as compared to tissues taken from vehicle-treated animals (Figure 66).

Figure 66. Number of Fos-ir nuclei in submucosal and myenteric ganglia from segments of ileum taken from animals systemically-treated with 6-OH-DA or capsaicin. A. Number of Fos-ir nuclei in submucosal ganglia from animals treated with 6-OH-DA or capsaicin 1 wk prior to experiment. B. Number of Fos-ir nuclei in myenteric ganglia from animals treated with 6-OH-DA or capsaicin 1 week prior to experiment. Data are 3-6 tissues from 3 animals for each time point. Asterisks (*) indicate $p < 0.05$ as compared to segments taken from vehicle-treated animals, and cross (+) indicates $p < 0.05$ as compared to segments taken from 6-OH-DA-treated animals.

Figure 66.

A**B**

DISCUSSION

1. EXTRINSIC DENERVATION ALTERS ARTERIOLAR INNERVATION

Although VIP- or SP-without CGRP-ir nerve fibers are rarely associated with submucosal arterioles in normal ileum, Galligan *et al.* (1988) found new SP- and VIP-ir nerve fibers associated with arterioles as soon as 4 weeks after elimination of extrinsic nerves to loops of guinea pig ileum. Substance P- and VIP-ir were not found to be colocalized in the same nerve fibers, indicating that at least two populations of enteric neurons were altered by loss of extrinsic nerves. These SP- and VIP-ir nerve fibers were unlikely to result from axonal sprouting or regrowth of extrinsic nerves, as new SP- or VIP-ir nerves were not sensitive to capsaicin or 6-hydroxydopamine. Furthermore, sympathetic and primary afferent nerve fibers were not localized in the denervated segment of ileum during the appearance of new VIP- and SP-ir nerve fibers, suggesting the altered innervation originated from enteric neurons.

In 1990, Galligan *et al.* demonstrated functional importance of the new SP-ir nerves, as the neurotransmitter responsible for vasodilation of submucosal arterioles was altered by surgical extrinsic denervation. As described in the introduction, neurogenic vasodilation of arterioles was mediated by ACh; however, following loss of extrinsic nerves, vasodilations were inhibited by NK receptor antagonists, indicating vasodilation were mediated by SP. However, these anatomical and functional studies did not determine the mechanism for appearance of new innervation, nor did they identify the

origin of these enteric neurons. Therefore, the first specific aim of this dissertation was to determine the mechanism and origin of new SP- and VIP-ir vascular nerves.

New Vascular Nerves Originate From Myenteric Ganglia

These questions were answered by using the retrograde dye Fluoro-gold (Fg) to identify vasomotor neurons in submucosal ganglia. As described previously, Fg was injected into arterioles, retrograde transport was allowed to occur, and Fg-labelled neurons were visualized and counted. Preliminary experiments demonstrated that submucosal neurons only contained Fg if the dye was injected into the lumen of arterioles, and not over the surface of the arterioles, indicating axons of passage did not transport Fg. As a result of these experiments, the presence of Fg labelled submucosal neurons was interpreted as identification of vasomotor neurons.

New SP- or VIP-ir nerves could result from axonal sprouting of enteric neurons and formation of new synapses and neuroeffector junctions. Alternatively, new nerve fibers could result from changes in phenotypic expression of existing nerve fibers. If submucosal neurons provided the source of new innervation, then axonal sprouting would increase the number of sites for uptake of Fg, resulting in a greater number of Fg-labelled neurons/submucosal ganglia. However, if submucosal neurons provided the source of new innervation, and axonal sprouting did not occur, then the ratio of Fg-labelled neurons/submucosal ganglia would not differ. The new SP- or VIP-ir nerve fibers would result from either upregulation of SP or VIP, or *de novo* synthesis of SP or VIP in neurons normally not expressing these peptides. In this model, increased SP or VIP-ir would be manifested as increased colocalization of SP- or VIP-ir in Fg-labelled neurons. Although Fg-labelling was observed in submucosal neurons in whole mount submucosal

preparations made from normal, control, and denervated loops of ileum, increased Fg-labelled neurons/submucosal ganglia was never observed at any time point examined. These results indicate that loss of extrinsic nerves did not induce axonal sprouting from submucosal neurons. Furthermore, extrinsic denervation did not increase SP-ir/Fg-labelled neurons, nor VIP-ir/Fg labelled neurons within submucosal ganglia, implying that loss of extrinsic nerves did not change phenotypic expression of submucosal neurons. Therefore, although these data did not identify the mechanism by which new SP- or VIP-ir nerves were associated with submucosal arterioles, these Fg studies strongly suggest the source of SP- and VIP-ir nerves originates from myenteric neurons. Unfortunately, due to great technical difficulties, it was impossible to examine fluoro-gold labelling in preparations which contained the myenteric plexus, in addition to the submucosal layer.

In designing experiments to identify the mechanism for the appearance of new SP- or VIP-ir nerve fibers, it was assumed that axonal sprouts from submucosal ganglia would uptake and retrogradely transport Fg in a similar manner to adult axons. However, if this postulate was incorrect, then interpretation of Fg labelling in submucosal neurons would lead to false rejection of the hypothesis that SP- and VIP-ir nerve fibers result from axonal sprouts of submucosal neurons. Recently, Jiang and Surprenant (1992) have shown that surgical removal of the myenteric plexus prior to denervation prevents the appearance of SP-ir nerve fibers, as well as neurogenic, non-cholinergic vasodilation of submucosal arterioles. As the myenteric plexus is crucial in the regulation of the rate of cell proliferation and cell growth in the rat jejunum (See *et al.*, 1990), removal of the myenteric plexus may somehow prevent denervation-induced alterations in the submucosal plexus of the ileum. However, the more plausible interpretation of these data indicates

that extrinsic denervation induces axonal sprouting of SP- and VIP-ir nerve fibers. Alternatively, myenteric nerve fibers may associate with submucosal arterioles in normal tissue, and loss of extrinsic nerve fibers alters the phenotypic expression of these nerves, resulting in upregulation or new transcription and translation of SP. Therefore, the conclusions from retrograde tracing studies agree with the conclusions of Jiang and Surprenant (1992), providing strong evidence that extrinsic denervation induces alterations in myenteric neurons, resulting in new vascular innervation.

Extrinsic Denervation Induces Axonal Sprouting of Myenteric Nerves

Although retrograde tracing studies suggested that myenteric neurons as the source of new vascular innervation, neither retrograde tracing nor Jiang and Surprenant (1992) identified the mechanisms responsible for new SP- or VIP-ir nerve fibers to become associated with arterioles. As discussed previously, during development and regeneration, immature axons contain nonphosphorylated (np) neurofilament heavy chain (NF-H)-ir, and mature axons contain phosphorylated (p)-NF-H-ir. Therefore, antiserum raised against npNF-H (SMI 31), pNF-H-ir (SMI 32), and pnpNF-H-ir (SMI 33) were used to discriminate between axonal sprouting and alterations in phenotypic expression of myenteric neurons as putative mechanisms. Extrinsic denervation may increase SP- or VIP-ir through increased transcription or translation of pre-existing peptides, or *de novo* synthesis and expression of SP or VIP. Increased or new expression of neuropeptides in axons previously associated with arterioles would then result in denervation-induced appearance of SP- or VIP-ir nerve fibers. If intrinsic vascular nerve fibers contain pNF-H-ir (see results), then SP- or VIP-ir should colocalize with pNF-H-ir. However, if SP- or VIP-ir nerves do not contain pNF-H-ir, then appearance of SP- and VIP-ir nerves

would be consistent with axonal sprouting.

Four weeks after extrinsic denervation, almost all SP- and VIP-ir nerve fibers did not contain pNF-H-ir, indicating the appearance of SP-ir and VIP-ir nerve fibers resulted from axonal sprouting, or growth, of myenteric nerves. Unfortunately, these studies were unable to determine if axonal sprouting occurred by collateral branching of axons normally innervating mucosa and submucosal ganglia, or by new nerves entering the submucosal plexus. Although numerous extrinsic nerve fibers had returned by 26 weeks after surgery, some SP- or VIP-ir nerves did not contain pNF-H-ir, suggesting some axonal growth occurred at this late time. Interestingly, other VIP- or SP-ir nerve fibers did contain detectable pNF-H-ir, possibly indicating stabilization and maturation of new innervation (reviewed in Shaw, 1991). Finally, VIP- and SP-ir nerve fibers without pNF-H-ir frequently were closely associated with arterioles, and appeared to use arterioles as a tract, whereas pNF-H-ir nerves were frequently further away from arteriolar walls. These results are consistent with the pattern of reinnervation described by Hill (1982), suggesting the arteriolar wall may secrete trophic molecules to promote axonal growth (see Sternini, 1992).

Nonphosphorylated NF-H-ir was only observed in submucosal neurons and in occasional nerve-like structures consistent with retracting or dying distal processes of extrinsic nerve fibers. These structures did not contain pNF-H-ir, suggesting that extrinsic denervation resulted in dephosphorylation, or interrupted intracellular machinery, resulting in failure to maintain post-translational modifications such as phosphorylation of KSP sequences. Furthermore, npNF-H-ir was never observed in vascular innervation, suggesting axonal sprouts from myenteric ganglia, or regenerating extrinsic nerves, do not

contain heavy molecular weight neurofilament protein (NF-H). However, once NF-H becomes incorporated into axons, it is rapidly phosphorylated, thus accounting for the sudden appearance of pNF-H-ir. This conclusion is consistent with reports of appearance of pNF-H-ir in development and regeneration (Nasal *et al.*, 1986; Shaw, 1991), although NF-H- may remain nonphosphorylated for a greater length of time in sciatic nerve models of regeneration (Schilling and Pilgrim, 1990; Pestronk *et al.*, 1990).

Sympathetic and Sensory Nerves Mediate Axonal Sprouting of VIP-ir and SP-ir Nerves, Respectively

Anatomical data suggests that every submucosal and myenteric ganglionic neuron may receive sympathetic innervation (Furness and Costa, 1974a). Therefore, loss of sympathetic nerves following surgery or administration of 6-OH-DA may affect many populations of enteric neurons. Although little information exists on regulation of phenotypic expression or axonal sprouting, one of two general mechanisms must occur. Sympathetic nerves may release inhibitory substances such as cytokines (Rao and Landis, 1992) that down-regulate or inhibit expression of cellular oncogenes, regulatory proteins, or *trans*-acting factors that control transcription. Removal of this inhibition then allows these programs to become activated, resulting in axonal sprouting of VIP-ir nerves. At this time, few inhibitory molecules have been identified. Alternatively, during degeneration and withdrawal of sympathetic nerves, stimulatory growth factors and cytokines may be released to promote axonal growth and increased nuclear activity. For example, decentralization of superior cervical ganglionic (SCG) neurons releases NGF from sympathetic nerves, resulting in increased tachykinin mRNA and protein (SP) (Kessler *et al.*, 1983; Hendry, 1992).

Although these studies did not attempt to identify the cellular messengers responsible for increased SP- and VIP-ir nerves, neurotoxins were used to selectively eliminate sympathetic or sensory nerves to determine if loss of a single population of nerves could alter vascular innervation. As described in the introduction, decentralization of SCG increased SP-ir. However, SP-/without CGRP-ir were not observed after sympathectomy. This observation suggests that responsiveness of a tissue to loss of innervation must depend on the source of innervation, as well as the type of receptors expressed on target neurons. Removal of preganglionic nerve fibers to SCG also increased VIP-ir (see introduction). Interestingly, VIP-ir paravascular plexus formed after elimination of sympathetic nerves, similar to the results obtained after systemic sympathectomy of adult rats (Aberdeen *et al.*, 1992). Taken together, these results suggest that sympathetic nerves normally inhibit expression of VIP. Therefore, destruction of sympathetic nerves allows upregulation of VIP, as well as axonal sprouting of myenteric nerve fibers.

It was surprising that loss of extrinsic sensory nerves increased SP-ir vascular nerves, as few SP-/CGRP-ir nerves are observed in myenteric ganglia (Gibbins *et al.*, 1985; this dissertation). Similar thin, faint, SP-ir nerves have been observed by other investigators (K. Sharkey, personal communications), supporting the conclusion that new, SP-ir appear after loss of extrinsic sensory nerve fibers. Taken together, these data indicate that loss of specific populations of extrinsic nerve fibers can account for appearance of SP- or VIP-ir vascular innervation, implying regulation of enteric neurons is more complex than simple inhibition of α_2 or NK₁ receptors to control excitability of neurotransmitter release.

Speculation on the Significance of Increased SP- and VIP-Ir Vascular Innervation

Although Galligan *et al.* (1990) demonstrated new SP-ir nerve fibers regulate non-cholinergic vasodilation of submucosal arterioles, it has been impossible to answer the ultimate question: Why do myenteric nerves send new SP- and VIP-ir nerve fibers to associate with submucosal arterioles? As discussed in the introduction of this dissertation, neurogenic vasodilation of submucosal arterioles is mediated by ACh release from submucosal neurons. Anatomical tracing studies localize few parasympathetic nerves within the GI wall (Kirchgessner and Gershon, 1989), and stimulation of vagal nerves does not alter submucosal blood flow (Kewenter, 1965). Stimulation of submucosal ganglia or interganglionic nerve tracts produces marked vasodilations (Surprenant *et al.*, 1989), indicating that ChAt-ir submucosal neurons control blood flow. However, following surgical destruction of extrinsic nerves, ChAt-ir nerves have not been destroyed, yet vasodilation is mediated not by ACh, but by SP. This finding raises three questions: (1) Why does functional innervation change from cholinergic to non-cholinergic, even though cholinergic innervation has not been destroyed? (2) What is the mechanism of these alterations? and (3) What is the result of loss of cholinergic regulation of blood flow?

At this time, these questions can not be definitively answered. In the most simplistic analysis, one of two possibilities exist. (1) The alterations occur for a specific reason, *via* defined mechanisms, resulting in altered phenotypic and pharmacological innervation of submucosal arterioles. (2) The alterations occur by a non-specific pathway *via* defined mechanisms. Although it is impossible to answer either question, it is possible that changes in innervation of arterioles corresponds to changes in blood flow as

a result of loss of extrinsic innervation. Regeneration in the peripheral nervous system can be associated with induction of schwann cell proliferation, influx of macrophages, digestion of myelin, and axonal growth or sprouting (Hall, 1989; Zochodne and Ho, 1992). Many of these processes may occur in the GI tract following loss of extrinsic innervation, and each response must have a metabolic requirement. Therefore, alterations in innervation of submucosal arterioles may not occur because ChAt-ir nerve fibers or mACh receptors are down-regulated or destroyed, but because demands in blood flow have been altered.

Extrinsic sympathetic nerves constrict submucosal arterioles *via* release of ATP (Evans and Surprenant, 1992) whereas all known extrinsic and intrinsic nerves release vasodilatory agents (Lembeck and Holzer, 1979; reviewed by Surprenant, 1994). At this time, it is not known how loss of the only known source of vasoconstriction affects submucosal blood flow. As the microvasculature accounts for 20-30% of resistance blood flow, loss of sympathetic tone would be predicted to result in increased blood flow. It will be of interest to determine if enteric neurons can mediate vasoconstriction after loss of extrinsic nerves.

Throughout most of this dissertation, it has been assumed that extrinsic nerves mediate the changes in innervation of submucosal arterioles. Histological studies have verified that surgery does not result in gross morphological changes of the GI tract. However, loss of extrinsic nerve fibers may induce alterations in endothelial cells or smooth muscle, resulting in stimulatory production of trophic molecules that induce axonal sprouting from enteric neurons. It is interesting to note that intrinsic SP- and VIP-ir nerves, as well as extrinsic nerves, associate with submucosal arterioles before

innervating submucosal ganglia, or forming loose paravascular plexuses that are not in direct contact with arterioles. These results are consistent with the presence of a positive trophic molecule. Interestingly, low levels of NGF is associated with normal submucosal arterioles (Sternini, 1993), indicating arterioles may help to recruit their own innervation.

Although SP-ir nerve fibers are important in regulation of blood flow after denervation, a physiological function for VIP-ir nerve fibers has not been discovered. These nerves do not appear to regulate blood flow (Galligan *et al.*, 1990), but may play a role in control of secretion (reviewed in Furness and Costa, 1987). Alternatively, VIP may act as a trophic molecule on some unidentified target, ultimately allowing enteric neurons to control blood flow, secretion, and absorption in the absence of extrinsic innervation. Only further experiments will determine the significance of new SP- and VIP-ir, and describe other adaptations for regulation of blood flow in the absence of sympathetic and primary afferent nerves. Finally, it will be of interest to determine if the density of extrinsic nerve fibers returns after denervation, as well as the source of extrinsic innervation that re-innervates the ileum.

2. THERE IS MICROHETEROGENITY IN THE DISTRIBUTION OF NF-H IN ENTERIC NERVES

Although numerous investigators have described localization of neurofilament triplet proteins in the CNS, there is a general paucity of information regarding NF-ir in enteric nerves. Therefore, this study used antiserum raised against phosphorylated, nonphosphorylated, or phosphorylation-independent epitopes found primarily on NF-H to stain extrinsic and intrinsic innervation of the GI tract. The results from this study illustrate that the distribution of NF-H-ir is heterologous in the ENS, as binding of SMI

31, SMI 32, and SMI 33 results in unique patterns of immunoreactivity which are dependent on phosphorylation status of NF-H protein. This heterogeneity results from post-translational phosphorylation of NF-H, providing a differential distribution of pNF-H-ir within individual enteric neurons, as well as in populations of nerves. Finally, as in other tissues, only a subpopulation of myenteric and submucosal neurons contain npNF-H-ir.

Putative Functions of Phosphorylated NF-H

Phosphorylated NF-H-ir was observed in extrinsic nerves originating from sensory ganglia (*i.e.*, dorsal root ganglia), and prevertebral ganglia (*i.e.*, celiac ganglion), and in distal processes of nerves originating from enteric ganglia. These data suggest that phosphorylation of neurofilament proteins is not dependent simply on length or caliber of the axon, origin or location of the cell body, or specific functions of the neuron. Although the physiological functions of phosphorylation of neurofilaments are not clear, phosphorylation of NF-H may contribute to axonal maturation and stability (Willard and Simon, 1981; see introduction). Alternatively, phosphorylation may increase the space between neurofilament proteins, resulting in increased axon caliber necessary for high velocity conduction of action potentials, without requiring large quantities of cytoskeletal proteins (see Shaw, 1991).

All Enteric Neurons Do Not Contain NF-H

Although pNF-ir was found primarily in axons associated with muscle and in interganglionic bundles of axons, npNF-H-ir was localized in enteric perikarya of neurons in submucosal and myenteric plexuses. These results are consistent with previous studies

on central (Sternberger and Sternberger, 1983; Goldstein *et al.*, 1983) peripheral (Lee, Carden, and Trojanowski, 1986) and enteric (Eaker *et al.*, 1990; Trojanowski *et al.*, 1986) neurons. Such studies have observed similar microheterogeneity in localization of pNF-H-ir in single cells, as pNF-H-ir was observed in axons, but not in perikarya, and npNF-H-ir was observed in perikarya, but not axons.

In the brain and peripheral ganglia, nonphosphorylated NF-ir can be localized to subpopulations of neurons in central (Campbell and Morrison, 1989) and peripheral ganglia. Similar results are observed in enteric ganglia, as only some submucosal and myenteric neurons contained npNF-H-ir. Previous immunohistochemical studies have found that subpopulations of enteric neurons share common functions, morphology, and neuropeptide content (Furness *et al.*, 1988; Costa *et al.*, 1987). As described previously, the submucosal plexus of guinea pig ileum is unique, for only four classes of neurons can be described based on neurochemical content (also known as chemical coding) and putative functions. Data in this dissertation strongly suggest that NF-H-ir is found in only a single subpopulation of pNF-H-ir submucosal neurons, as almost all npNF-H-ir cells co-localized with SP, but not with NPY, CGRP, or VIP. Substance P-ir submucosal cells are thought to function as intrinsic sensory cells (reviewed in Furness and Costa, 1987), and the presence of npNF-H-ir may be related to neurotransmitter content or function of the neurons, such as reception and transduction of mechanical signals from mucosa. If this hypothesis is correct, then neurofilaments have a greater role in cell function than simply regulation of cell morphology. Alternatively, the presence of npNF-H-ir may allow neurons greater adaptability and plasticity as compared to neurons that lack neurofilament proteins. Nonphosphorylated NF-H-ir was also observed in a

population of neurons in myenteric ganglia. Co-localization studies of SMI 32 and neuropeptides indicate that npNF-H-ir was observed in some neurons containing VIP-ir or NOS-ir, but never NPY-ir or CGRP-ir. Myenteric ganglia contain approximately 105 neurons/ganglion (Young *et al.*, 1993), thus approximately 30% (32 npNF-H-ir neurons/ganglion / 105 total neurons/ganglion) of myenteric neurons contain npNF-H-ir. Although VIP-ir or NOS-ir was observed in some npNF-H-ir neurons, the identity of many npNF-H-ir neurons was not elucidated. However, approximately 44% of all myenteric neurons contain SP-ir and some NF-triplet-ir neurons contain SP-ir in myenteric ganglia in guinea pig ileum (Costa *et al.*, 1992). Although SP-ir cell bodies were difficult to resolve from bright SP-ir nerve fibers located over myenteric ganglia, rare SP-ir neurons could be observed to contain npNF-H-ir. Furthermore, the morphology of many npNF-H-ir neurons which did not contain VIP- or NOS-ir was similar to the morphology of Dogiel type II or small SP-ir neurons that lack observable dendrites (reviewed in Furness and Costa, 1987). Therefore, it is probable that some npNF-H-ir neurons contain SP-ir, and Dogiel I (VIP- and NOS-ir neurons), Dogiel II (SP-ir myenteric neurons), and Dogiel III (SP-ir submucosal neurons) contain npNF-H-ir.

As described above, npNF-H-ir is found in a population of neurons containing NOS or VIP-ir. These cells have morphologies that are consistent with Dogiel type I neurons, and are likely to represent interneurons and motor neurons (reviewed in Furness *et al.*, 1993). Anatomical tracing combined with immunohistochemical studies using antiserum against npNF-M and -H proteins (Vitadello, 1987; Brookes *et al.*, 1991) also suggest that the majority of npNF-ir cells are motor neurons and project to the circular muscle. Furthermore, as the circular muscle receives a dense SP innervation from

myenteric neurons (Brookes *et al.*, 1991), some npNF-H-ir neurons may contain SP-ir. If npNF-H-ir is found in neurons with differing morphology and neurotransmitter content, then the presence of npNF-H-ir may be indicative of common effector tissue and function. In agreement with previous reports (Costa *et al.*, 1992; Eaker *et al.*, 1990; Eaker *et al.*, 1993; Björklund *et al.*, 1984), some myenteric neurons do not contain detectable NF-H-ir, further suggesting that neurofilament proteins have additional functions other than regulation of morphology.

The pattern of immunoreactivity within myenteric cells exhibited heterogeneity, as some cells contained very filamentous staining throughout the cell soma and dendrites. However, other myenteric cells contained a pattern of npNF-H-ir that was more similar to that observed in the submucosal plexus. This second population of myenteric and submucosal cells contained more uniform npNF-H-ir, and did not have immunoreactive dendrites. However, some cells in submucosal and myenteric plexuses had short axons with npNF-H-ir, with brightest staining in axon hillock, and decreased immunoreactivity over the proximal process. Taken together, these observations agree with other published reports of microheterogeneity in the cellular distribution of npNF-H-ir, as NF-H is relatively nonphosphorylated in perikarya, and becomes phosphorylated as it is transported into the axon (Sternberger and Sternberger, 1983; Nixon *et al.*, 1987; Pestronk *et al.*, 1990).

There are several possible explanations for the lack of npNF-H-ir in some enteric neurons. In central and peripheral tissues, small cells frequently do not contain NF triplet-ir (Shaw *et al.*, 1981; Sharp *et al.*, 1982; Trojanowski *et al.*, 1986; reviewed in Shaw, 1991), suggesting small cells do not require the functions provided by

neurofilament triplet proteins. Alternatively, cells without NF-triplet-ir may contain other proteins that assume functions normally mediated by neurofilament proteins. For example, some autonomic neurons without NF-triplet-ir contain peripherin, an intermediate filament protein homologous to type-III intermediate filament proteins (Vickers *et al.*, 1990; Parysek *et al.*, 1988), or the intermediate filament α -internexin (Vickers *et al.*, 1992). This suggests neurofilament triplet proteins are not required by all cells. However, Trojanowski *et al.* (1986) estimated that 99% of all peripheral neurons contain NF-triplet-ir. Their observation suggests that localization of NF-triplet proteins may depend on the protocol used to prepare the antibody and on identity of the antigen. As NF-triplet proteins may be masked or altered by post-translational modifications such as phosphorylation or proteolytic cleavage (reviewed in Nixon *et al.*, 1982), an antibody raised against rat NF-H may not recognize a modified epitope in guinea pig NF-H, although NF-H is present in the cell. Finally, NF-H may be synthesized and rapidly transported out of the perikaryon, resulting in a lack of npNF-H-ir in some enteric neurons.

Localization of Phosphorylation-Independent NF-H-ir is Similar to p- and npNF-H-ir

Although SMI 31 and SMI 32 recognize specific phosphorylated or nonphosphorylated epitopes, SMI 33 identifies a nonphosphorylated epitope that is accessible in phosphorylated and nonphosphorylated NF-H. In the submucosal and myenteric plexuses, pnpNF-H-ir was similar to pNF-H- and npNF-H-ir, as some neurons and interganglionic nerve fibers contained pnpNF-H-ir. This staining was similar in appearance to previous reports using antibodies against pnpNF-H (Eaker *et al.*, 1990) or NF antiserum (Björklund *et al.*, 1984). However, unlike pNF-H-ir, pnpNF-H-ir was not

observed in submucosal or myenteric ganglionic nuclei, further suggesting that nuclear SMI 31-ir was the result of unique cross-reactivity with a phosphorylated protein found in enteric nuclei.

Summary of Distribution of NF-H-ir in Enteric Neurons

In conclusion, SMI 31, SMI 32, and SMI 33 staining demonstrate a unique distribution of phosphorylated, nonphosphorylated, and phosphorylation-independent neurofilament heavy chain protein in extrinsic and intrinsic innervation of the GI tract. Subclasses of enteric neurons contain npNF-H-ir, suggesting NF-H may have roles in cell processes that are not related to cell structure, but may be related to neurotransmitter content or cell functions. The presence of npNF-H-ir may be used to distinguish subpopulations of cells based on the cytoskeletal content of the neurons. However, only additional studies will determine the significance of the presence, or absence, of neurofilaments in normal tissue.

3. EXTRINSIC DENERVATION ALTERS ANATOMY OF MYENTERIC GANGLIA

NADPH diaphorase histochemistry was used to determine if extrinsic denervation induced alterations in nitrinergic nerves associated with submucosal arterioles. NADPH diaphorase histochemistry produces insoluble formazan precipitate found in paravascular nerves in submucosal plexus of rat small and large intestine (Nichols *et al.*, 1993), guinea pig small and large intestine (Nichols *et al.*, 1993), and guinea pig (Nichols *et al.*, 1993), rat (McConalogue and Furness, 1992), and dog (Ward *et al.*, 1992) colon. Submucosal plexus preparations of colon contain a population of NADPH diaphorase positive neurons

which co-localize NOS-ir (Ward *et al.*, 1992), and these neurons have been hypothesized to be involved in pacemaker activity and regulation of blood flow (Nichols *et al.*, 1992). However, studies in this dissertation did not demonstrate NADPH diaphorase staining in vascular nerves or submucosal ganglia in normal, control, or denervated guinea pig submucosal plexus. Costa *et al.* (1992) and Young *et al.* (1992) also failed to demonstrate NADPH diaphorase staining in submucosal plexus preparations of guinea pig ileum. This discrepancy may result from differences in animal species, section of GI tract examined, and fixative used to prepare submucosal plexus preparations. (Nichols and co-workers used a modified Zamboni's solution of 4% paraformaldehyde and 0.4% picric acid in PBS, whereas all others used paraformaldehyde only.) Regardless of the cause of differences in staining of submucosal plexus, nitrinergic nerves were not associated with submucosal arterioles at any time after denervation. However, myenteric plexus longitudinal muscle preparations were simultaneously processed to verify the reaction mixture was made correctly. The resulting data indicated that loss of extrinsic nerves increases NADPH diaphorase, and the second portion of this dissertation was dedicated to characterizing alterations in inhibitory neurotransmitters using longitudinal and circular muscle-myenteric plexus preparations.

NADPH Diaphorase Is A Reliable Marker for NOS

Although Bredt and Synder raised antiserum against NOS, neither a private nor a commercial source of NOS antiserum was available until the last year of these studies, requiring NADPH diaphorase histochemistry to be used as an index of NOS. Therefore, a number of control studies were performed to determine the validity of this approach. First, NADPH diaphorase staining had an absolute requirement for NADPH, as the

staining pattern changed drastically when an equimolar concentration of NADH was used in place of NADPH. Secondly, dicumarol, an inhibitor of non-specific dehydrogenase activity, inhibited NADH diaphorase staining, and NADPH diaphorase staining of mucosal crypts, but not neurons. Third, although extrinsic denervation significantly increased the number of NADPH diaphorase positive myenteric neurons/ganglion, submucosal neurons were not altered, implying a selective phenomenon occurred. Finally, although the reaction was enzymatically-catalyzed, optimum NADPH diaphorase staining occurred in fixed, not fresh, tissues, similar to cholinesterase histochemistry. Similar results have been obtained by Young *et al.* (1992) and Hope and Vincent (1987).

Final verification of the validity of NADPH diaphorase staining as a marker for NOS came after obtaining NOS antiserum. Approximately 80-100% of NADPH diaphorase-positive myenteric neurons/ganglia contained NOS-ir. It is likely that some neurons do not contain NOS and NADPH, as occasionally bright NOS-ir neurons were observed without detectable NADPH diaphorase staining. Dark NADPH diaphorase-stained neurons were observed also without detectable corresponding NOS-ir. These results suggest that some neurons contain NOS, but may not contain NADPH diaphorase staining because NOS is unable to catalyze the diaphorase reaction in that neuron. Alternatively, occasional neurons may contain non-specific NADPH diaphorase staining, but not NOS protein. However, it is also probable that dark NADPH diaphorase masked faint NOS-ir, and that bright NOS-ir quenched NADPH diaphorase staining, accounting for less than 100% colocalization of NOS-ir and NADPH diaphorase staining. It should be noted that the data in this dissertation agrees with previously published reports that demonstrated a close association of NADPH diaphorase staining and NOS-ir (Young *et*

al., 1992; Belai *et al.*, 1992; Ward *et al.*, 1992).

The Number of NOS-ir and NADPH Diaphorase Myenteric Neurons/Ganglia Increases After Loss of Extrinsic Innervation

As soon as 1 week after loss of extrinsic nerve fibers, the number of neurons containing NADPH diaphorase staining and NOS-ir was significantly elevated as compared to control tissues taken from the same animals. This increase in neuronal number resulted from loss of extrinsic nerve fibers, as the number of NOS-ir neurons was not altered in segments of ileum that were exposed, but whose nerve fibers were not crushed (sham surgeries). Furthermore, it is unlikely that increased NADPH diaphorase or NOS-ir results from axotomy of viscerofugal neurons, as only 1.025% of ileal myenteric neurons project to superior mesenteric ganglia and celiac ganglia (Messenger and Furness, 1992; Messenger and Furness, 1993; but see Fiallos-Estrada, 1993; Hökfelt, 1994). Finally, changes in NADPH diaphorase staining and NOS-ir are unlikely to occur as at result of ischemia, as similar results were obtained after systemic administration of capsaicin, but not 6-OH-DA, indicating loss of extrinsic sensory nerve fibers mediates the increase in myenteric neurons containing NOS-ir and NADPH diaphorase staining.

NADH histochemistry and immunohistochemistry for SMI 31-ir ganglionic nuclei were used to determine if increased NADPH diaphorase staining and NOS-ir resulted from stimulation of progenitor cells. Furthermore, following avulsion of ventral roots, NADPH diaphorase and NOS-ir increases in motor nuclei, after which time neuronal death ensues (Wu *et al.*, 1992; Wu *et al.*, 1993), suggesting increased NOS-ir/NADPH diaphorase staining may precede programmed cell death. Using histochemical staining for NADH histochemistry and immunohistochemical localization of SMI 31-ir, the number of

myenteric neurons/ganglia could be monitored after extrinsic denervation. As described previously, the number of myenteric neurons and neuronal nuclei were not altered at any time after extrinsic denervation, indicating that neuronal death did not occur; furthermore, these data imply that increased NOS-ir did not result from increased neurons/ganglia.

Although the mechanisms for increased NOS-ir were not addressed, two mechanisms could account for the elevated number of NADPH diaphorase and NOS-ir neurons/myenteric ganglia. Loss of extrinsic nerves may induce NADPH diaphorase activity and NOS-ir in neurons which normally do not express the type I isoform of NOS. For example, increased transcription of pre-existing NOS mRNA would result in the appearance of more NOS-ir neurons, as immunohistochemical methods could detect a greater amount of protein in a greater quantity of neurons. Similarly, increased translation of NOS would increase the intracellular levels of NOS, resulting in stronger immunoreactivity and NADPH diaphorase staining. Alternatively, extrinsic denervation may induce previously non-detectable NADPH diaphorase activity and NOS-ir by increasing mRNA stability and basal transcription and/or translation. Unfortunately, the polyclonal NOS antiserum was raised in rabbit, thus preventing many colocalization studies with antiserum raised against ChAt, NPY, CGRP, and VIP. In normal ileum, some interneurons and most inhibitory motor neurons contain NOS-ir (see introduction). However, only further experiments will determine the phenotypic classes of NOS-ir neurons after extrinsic denervation and if the increased quantity of NOS-ir neurons/myenteric ganglia results from *de novo* synthesis or upregulation of pre-existing NOS.

The Number of VIP-ir Myenteric Neurons/Ganglia Does Not Increase After Extrinsic Denervation

As VIP-ir colocalizes with NOS-ir (Costa *et al.*, 1992) and NADPH diaphorase staining (Aimi *et al.*, 1993; Talmage and Mawe, 1993), VIP-ir was examined in control and denervated preparations to determine if this inhibitory neurotransmitter was also affected. Interestingly, extrinsic denervation did not alter the number of VIP-ir myenteric neurons/ganglia at any time examined, indicating extrinsic denervation did not increase all inhibitory neurotransmitters but may have selectively affected NOS.

Although NADPH diaphorase staining and VIP-ir have been reported in the same population of myenteric neurons in guinea pig ileum (Costa *et al.*, 1992), colocalization studies with NADPH diaphorase and antiserum raised against VIP failed to identify VIP-ir and NADPH diaphorase positive neurons in normal or denervated ileum. However, anatomical studies reporting colocalization of NOS-ir or NADPH diaphorase staining and VIP-ir (Costa *et al.*, 1992; Aimi *et al.*, 1993; Talmage and Mawe, 1993), used colchicine-pretreated tissues. Colchicine inhibits microtubule elongation, and prevents anterograde axonal transport, resulting in accumulation of neuropeptides in cell somata. However, colchicine treatment can increase neuropeptide levels in the brain by inducing *de novo* synthesis of mRNA for some neuropeptides, although the mRNA was not found in these cells prior to colchicine treatment. This observation indicates that colchicine can alter transcription of mRNA encoding neuropeptides (Cortés *et al.*, 1990; Ceccatelli *et al.*, 1991). A similar phenomena may occur in the GI tract, as VIP-ir is found in 3 ± 0.5 myenteric cells/ganglion in freshly-fixed tissue, in agreement with the first published reports of VIP-ir by Furness and Costa (1984). Interestingly, these VIP-ir neurons do not contain NOS-ir, and are classified as Dogiel III cells, unlike the Dogiel I morphology of

NOS-ir cells. However, following colchicine treatment (80 μ M) in organotypic culture medium, 10-20 VIP-ir cells/ganglion can be detected, and these Dogiel type I neurons contain NOS-ir (Costa *et al.*, 1992). *In situ* hybridization studies of VIP mRNA in the ENS suggests that the relationship between VIP- and NOS-ir may not be as common as originally thought, as colchicine can induce *de novo* synthesis of VIP mRNA (Eckblad, personal communications). Furthermore, following decentralization of autonomic ganglia, or after placement into culture for 24 hrs, VIP-ir can increase by 240x (Rao and Landis, 1992) providing further evidence of phenotypic plasticity in the ANS, as well as further questioning the conclusion that VIP and NOS are found normally in the same population of myenteric neurons in guinea pig ileum.

4. EXTRINSIC DENERVATION ALTERS LONGITUDINAL MUSCLE INNERVATION

Functional Assay of Nitrinergic Innervation Indicates Increased NO Production Proceeding Extrinsic Denervation

Although strong NOS-ir and NADPH diaphorase staining have been found in myenteric neuron perikarya, NOS-ir and NADPH diaphorase staining have been found in varicose nerve fibers by some studies (Belai *et al.*, 1992; Aimi *et al.*, 1993; Nichols *et al.*, 1993), although other studies did not observe NOS-ir or NADPH diaphorase staining in nerve fibers (Aimi *et al.*, 1993) such as those that form the tertiary plexus on the longitudinal muscle (Costa *et al.*, 1992). However, as described in the introduction, neurogenic nitrinergic relaxations have been identified in all regions of the GI tract in all species examined to date, indicating that nerves release NO (see Shuttleworth, 1994; Wiklund, 1993).

The number of NADPH diaphorase and NOS-ir neurons increased approximately 90% as compared to control preparations after extrinsic denervation. As neurogenic relaxations of longitudinal muscle are mediated by release of NO in normal ileum, it was hypothesized that the increased quantity of NOS-ir myenteric neurons would release a greater amount of NO and produce larger relaxations as compared to control preparations. Therefore, longitudinal muscle-myenteric plexus preparations were used as a bioassay to determine if NO production was potentiated after extrinsic denervation.

Transmural stimulation (20 Hz) relaxed precontracted LMMPPs from normal, control, and denervated ileum. However, the relaxations were significantly larger in denervated preparations, as the amplitude and duration of relaxations was potentiated after loss of extrinsic nerves. The alterations in neurogenic relaxations did not result from denervation-induced changes in the properties of smooth muscle, as similar responses to histamine and SNP were observed in preparations from denervated and control segments of ileum. Furthermore, the muscle thickness of circular and longitudinal muscle, the wet weight of longitudinal muscle, and the passive and active muscle properties were not affected by loss of extrinsic nerves. These data provide anatomical and functional evidence that surgical destruction of extrinsic nerves and manipulations of the GI tract do not produce detectable changes in muscle cells, such as mitosis or hyperplasia, or alter contractibility of longitudinal muscle.

Substituted L-arginine analogs such as nitro-L-arginine bind and inhibit catalytic activity of NOS (see introduction). Therefore, NLA was used to determine if neurogenic relaxations of preparations from denervated ileum were mediated by NO. LNA inhibited relaxations of normal preparations in a concentration-dependent manner and attenuated

relaxations of control and denervated LMMPPs, verifying relaxations were mediated by NO. As discussed in the results, LNA did not attenuate relaxations generated at low frequency nerve stimulation, suggesting that NO is preferentially released during high frequency nerve stimulation. Similar results were observed in circular muscle preparations, and these data suggest different neurotransmitters are released by low- or high-frequency nerve stimulation. Although low-frequency nerve stimulation of sympathetic and parasympathetic nerves releases the classical neurotransmitters NE and ACh, high frequency nerve stimulation releases NPY and VIP, respectively, from large dense-cored vesicles (reviewed in Lundberg and Hökfelt, 1986). Therefore, an unidentified neurotransmitter may be released at low frequency nerve stimulation, whereas NO is released at greater frequencies of nerve stimulation.

Although electrophysiological studies have demonstrated that high-frequency nerve stimulation can release different neurotransmitters as compared to low-frequency nerve stimulation, concern has been raised that the high-frequency nerve stimulation necessary to evoke release of NPY from sympathetic nerve fibers, or VIP from parasympathetic nerve fibers, is not in the physiological range of which most nerves can fire for long periods of time. For example, vagal efferent nerves fire between 6 and 10 Hz (Miolan and Roman, 1978), and these nerves must be stimulated at frequencies greater than 20 Hz for VIP release. However, extracellular recordings of autonomic nerves have demonstrated that many nerves may spontaneously fire at high frequencies. For example, some myenteric neurons of the cat small intestine discharge at 36 Hz (Wood, 1989), although other myenteric neurons discharged at lower frequencies. Similar observations have been made from myenteric neurons in guinea pig ileum, as bursting-type neurons fire

over 20 Hz (Wood, 1989). Therefore, these data indicate that nerves may fire action potentials over a wide-range of nerve frequencies, which may preferentially release some neurotransmitters.

Finally, frequency-response curves were obtained in control and denervation preparations to determine if relaxations were potentiated at all frequencies. As described in the results, potentiation of relaxations were only observed at 10, 20 and 50 Hz. These frequencies produce NO-mediated relaxations in normal tissues, as the relaxations generated at these frequencies were attenuated by LNA (see above). These data are consistent with increased NO production and release by myenteric nerves after extrinsic denervation. Furthermore, as described in the preceding paragraph, relaxations generated at low frequency nerve stimulation were not sensitive to LNA, suggesting a neurotransmitter other than NO was responsible for observed relaxations. As the low-frequency relaxations were not altered by extrinsic denervation, this may be further evidence of preferential increase of NOS, and not simply an elevation in all inhibitory neurotransmitters.

Loss of Extrinsic Sensory Nerves Mediates Increased NOS-ir and Neurogenic Relaxations

Increased NOS-ir and larger relaxations were observed in surgically-denervated and capsaicin-denervated ileum, indicating alterations in NOS resulted from loss of extrinsic sensory nerves. Capsaicin selectively destroys unmyelinated or lightly-myelinated SP-/CGRP-ir nerves originating from small, dark-bodied neurons in DRG (see introduction). Although sympathetic nerves are found over the entire surface of myenteric ganglia, loss of sympathetic nerves did not alter NOS-ir, relaxations of longitudinal muscle, or active

or passive properties of longitudinal muscle. Interestingly, few SP-/CGRP-ir nerves are found in myenteric ganglia (Gibbins *et al.*, 1985; this study), although stimulation of primary sensory nerves evokes sEPSPs in some myenteric neurons (Takaki and Nokayama, 1988). Therefore, changes in NOS-ir may result from direct loss of trans-synaptic activity of extrinsic sensory nerves. As described in the introduction, denervation of SCG neurons increases SP- and VIP-ir. Release of neurotransmitters from extrinsic sensory nerves may repress expression of NOS; alternatively, damage of sensory nerves may release cytokines, trophic molecules, or some other unidentified molecule, that initiates transcription or translation of NOS. Unfortunately, little information exists in the literature to discriminate between these possibilities. However, as discussed in the introduction, decentralization of SCG increases transcription of TH and SP, resulting in elevated immunoreactivity and hydroxylation of tyrosine. Only molecular biological studies will be able to determine the proximal cause of elevated NOS in myenteric ganglia, or the ultimate result of increased neurogenic nitrinergic relaxations of ileum following extrinsic denervation.

Extrinsic Denervation Alters Rebound Contractions of Longitudinal Muscle

Surgical denervations of loops of intestine also altered rebound contractions of LMMPPs. In normal, control, and denervated ileum, these contractions are inhibited by TTX and CP96345-1, a nonpeptide NK₁ antagonist, indicating they are mediated by release of SP. After denervation or capsaicin treatment, but not 6-OH-DA treatment, rebound contractions were significantly smaller at all frequencies, and rebound contractions were not seen in tissues taken from capsaicin-treated animals. As described in the introduction, primary afferent nerves release SP, and local application of capsaicin

contracts LMMPPs by releasing SP (Szolcsányi and Barthó, 1978; Barthó and Szolcsányi, 1982). Therefore, contractions may be smaller, or non-existent, because extrinsic sensory nerves release SP and contract longitudinal muscle. However, few extrinsic sensory nerve fibers are found in longitudinal muscle, and mACh antagonists attenuate approximately 75% of capsaicin-induced contractions, indicating that primary afferent nerves innervate excitatory motor neurons (see introduction). A second, or alternative, explanation is based on work by Boeckxstaens (Bult *et al.*, 1990; Boeckxstaens *et al.*, 1990). They proposed that NO regulated SP release, as NLA potentiated rebound contractions but not exogenously applied SP. Osthau and Galligan (1991) also observed NOS antagonists attenuated rebound contractions of guinea pig ileum, although they hypothesized that attenuation of SP-mediated contractions resulted from physiological antagonism of excitatory neurotransmitters at longitudinal muscle. Therefore, motility of LMMPPs depended on temporal summation of excitatory and inhibitory innervation. Increased nitrinergic innervation would then antagonize SP release, resulting in smaller rebound contractions.

Normal LMMPPs were used to determine if NO actually inhibits SP release. However these data were difficult to interpret, as NOS antagonists affected relaxations and rebound contractions. Therefore, CMMPPs were used to test the hypothesis that NO inhibits SP from myenteric neurons. The results generated from CMMPPs support this hypothesis, indicating rebound contractions are attenuated after denervation as a result of increased inhibition of release of SP by elevated NOS.

5. ENDOGENOUS NO REGULATES SP BUT NOT ACh RELEASE

Motility of GI smooth muscle is partially controlled by myenteric neurons that release excitatory and inhibitory neurotransmitters (reviewed by Furness and Costa, 1987). Postjunctional responses are dependent on the identity, quantity, and temporal release of enteric neurotransmitters. For example, stimulation of LMMP preparations releases (among other neurotransmitters) NO and SP, resulting in a relaxation followed by a rebound contraction (Osthaus and Galligan, 1992). NOS antagonists attenuate neurogenic relaxations and potentiate rebound contractions, suggesting that release of NO normally masks action of excitatory neurotransmitters. However, others (Gustaffson *et al.*, 1990; Hryorenko *et al.*, 1994) have provided evidence that NO may inhibit release of excitatory neurotransmitters from myenteric nerves. Therefore, NO may affect enteric motor neurons, in addition to its well-characterized action on smooth muscle (see introduction).

Circular muscle of guinea pig ileum is innervated by myenteric neurons that contain both choline acetyltransferase (ChAt)- and SP-immunoreactivity (ir) (Brookes *et al.*, 1991). As ChAt catalyzes formation of ACh, these results suggest that cholinergic and noncholinergic input to circular muscle of guinea pig ileum is provided by a single population of neurons. Electrical stimulation of myenteric nerves evokes release of ACh and SP, resulting in a fast, cholinergic contraction, followed by a slower, noncholinergic contraction of circular muscle that is inhibited by SP antagonists (see introduction for review). Circular muscle also receives a dense innervation of NOS-ir myenteric nerves (Costa *et al.*, 1992), and electrical stimulation of myenteric nerves evokes release of NO (Shuttleworth *et al.*, 1994), resulting in relaxation of pre-contracted circular muscle. Therefore, as circular muscle is innervated by inhibitory nitrinergic and excitatory

cholinergic and noncholinergic nerves, circular muscle preparations were used to determine if NO inhibits release of SP and ACh from myenteric neurons.

Endogenous NO Inhibits NANC Contractions

Pharmacological antagonists were used to block NK₁ or muscarinic (mACh) receptors, allowing cholinergic and noncholinergic responses to be studied independently. In the presence of scopolamine, a mACh receptor antagonist, and guanethidine, an indirect-acting sympatholytic drug, transmural stimulation produced monophasic contractions that were nerve-mediated, as contractions were inhibited by TTX. NANC contractions were also inhibited by the competitive antagonist CP-96,345 and the estimated CP-96,345 EC₅₀ (12 nM) is consistent with a specific interaction at NK₁ receptors (Snider *et al.*, 1991). These data indicate that NANC contractions are mediated predominantly by nerve-released tachykinins such as SP. This conclusion was supported by studies using SPME, as SPME mimicked NANC contractions with an estimated EC₅₀ of 20 nM, suggesting SPME interacted specifically with NK₁ receptors on circular muscle (Hall and Morton, 1991). Finally, as CP-96,345 inhibited both neurogenic and SPME responses, these results indicate that nerve-released tachykinins activate NK₁ receptors, culminating in contractions of circular muscle.

The substituted LArg analogs LNA, LNAME, and LMMA are specific inhibitors of NO synthesis and NO-mediated responses in a number of tissues (see introduction), including GI smooth muscle. These drugs produced concentration-dependent potentiation of neurogenic NANC contractions in a manner consistent with inhibition of NOS. First, D-enantiomers DNAME and DMMA failed to potentiate NANC contractions, indicating potentiation by LArg analogs exhibited stereo-specificity. Secondly, LArg but not DArg

prevented potentiation by LNAME and LNA. These data agree with previous studies that suggested LArg analogs competitively inhibit NO synthesis. Furthermore, the observation that DArg was ineffective at preventing potentiation by LArg analogs further demonstrates stereo-specificity of LArg analogs. Therefore, NOS antagonists potentiate NANC contractions by specific and reversible inhibitors of NO synthesis.

The conclusion that NO inhibits NANC contractions was supported by data from studies using hemoglobin to bind and inactivate extracellular NO, and SNP to liberate NO in solution. Hemoglobin potentiated NANC contractions in a concentration-dependent manner, suggesting that decreased availability of NO results in larger NANC contractions. If endogenous NO inhibits NANC contractions, then SNP should attenuate NANC contractions. Although SNP did not appear to alter basal tone of circular muscle, SNP attenuated NANC contractions in a concentration-dependent manner. These results may indicate that NO donors, such as SNP, inhibit NANC contractions by attenuating tachykinin release from enteric neurons, whereas decreased NO production by NOS antagonists, or decreased NO availability by hemoglobin, potentiates tachykinin release.

SPME mimicked NANC contractions, and responses to SPME were used to determine if NOS antagonists, hemoglobin, and SNP affected contractibility and basal tone of circular muscle. Contractions of circular muscle induced by SPME were not altered by concentrations of LNA, as contractions of equal amplitude were observed in the absence and presence of LNA. Therefore, NOS antagonists do not alter contractibility of circular muscle, but potentiate NANC contractions by inhibiting NO synthesis. Similar results were observed with hemoglobin, as contractions induced by SPME were not affected by 10 μ M hemoglobin. These data indicate that hemoglobin does not alter

contractibility or basal tone of circular muscle, but potentiates NANC contractions by binding and inactivating NO. Therefore, NOS antagonists and hemoglobin potentiate NANC contractions by specific inhibition of NO synthesis or availability, respectively. Furthermore, these data suggest that NO inhibits tachykinin release from enteric excitatory motor neurons. Removal of the inhibitory nitrinergic tone by decreasing NO production or extracellular concentration should enhance tachykinin release, resulting in potentiation of NANC contractions.

If NO inhibits tachykinin release from myenteric neurons, then exogenous NO applied as SNP should attenuate NANC contractions. As described previously, SNP attenuated NANC contractions in a concentration-dependent manner. However, SNP significantly attenuated contractions elicited by SPME, indicating that SNP may alter signal transduction pathways coupled to NK₁ receptors, resulting in inhibition of contractions of circular muscle. Alternatively, SNP may relax circular muscle, resulting in decreased amplitude of SPME-evoked contractions.

Apamin blocks small conductance Ca²⁺-activated K⁺ channels found on smooth muscle (Daniel, 1985) and inhibits NO-mediated relaxation of GI smooth muscle (reviewed in Osthau and Galligan, 1992). Apamin potentiated neurogenic NANC contractions in a concentration-dependent manner. However, apamin shifted the SNP concentration-response curve upwards, indicating that apamin blocked some of the effects of SNP. This apamin-sensitive component represented the actions of SNP on smooth muscle, as apamin-sensitive ionic channels are not found on enteric neurons (Bornstein *et al.*, 1994). As a consequence of this direct effect of SNP, SNP attenuated SPME-induced contractions of circular muscle. In contrast, the apamin-insensitive component

of SNP-induced attenuation of NANC contractions represented the action of SNP on enteric neurons. This conclusion is supported by electrophysiological studies, as SNP suppresses noncholinergic, slow excitatory post-synaptic potentials (EPSPs) recorded from myenteric neurons (Tamura *et al.*, 1993). Finally, although SNP attenuated both neurogenic NANC and SPME-evoked contractions, SNP inhibited a greater proportion of neurogenic NANC contractions as compared to SPME responses. As discussed previously, SNP alters the properties of circular muscle, resulting in inhibition of SPME-induced contractions. However, if SNP attenuated neurogenic contractions by a postjunctional mechanism only, then SNP should attenuate neurogenic and SPME-evoked contractions equally, as NANC contractions are mediated by release of tachykinins. A greater proportion (approximately 80%) of neurogenic NANC contractions were inhibited by SNP as compared to SPME responses (approximately 40%). This observation further suggests that in addition to postjunctional inhibition of contractions, SNP affects excitatory motor neurons, resulting in inhibition of tachykinin release.

The final piece of evidence to support the conclusion of nitrinergic inhibition of tachykinin release resulted from use of CP-96,345. The NK₁ receptor antagonist also attenuated neurogenic NANC contractions after potentiation by NOS antagonists, with an estimated EC₅₀ (14 nM) consistent with a specific interaction at NK₁ receptors. Furthermore, CP-96,345 inhibited over 90% of NANC contractions after treatment with NOS antagonists. These results suggest that the effects of neurotransmitter released after potentiation are mediated by NK₁ receptors, consistent with enhanced release of tachykinins.

VIP Does Not Inhibit NANC Contractions

VIP-ir is closely associated with NOS-ir in myenteric neurons, and VIP is an inhibitory neurotransmitter in some segments of the GI tract (see introduction). However, VIP produced small, rapidly-desensitizing relaxations of circular muscle, and did not affect neurogenic NANC contractions. These data indicate that unlike NO, VIP does not regulate tachykinin release or significantly affect contractibility of circular muscle.

Endogenous NO Does Not Inhibit Cholinergic Contractions

In the presence of guanethidine and CP-96,345, transmural stimulation of circular muscle preparations produced monophasic contractions which were blocked by scopolamine and TTX, indicating contractions resulted from stimulation of cholinergic motor neurons. This conclusion was supported by use of bethanechol, a mACh receptor agonist, as bethanechol mimicked neurogenic contractions. Finally, scopolamine inhibited bethanechol-induced contractions, indicating nerve-released ACh activates mACh receptors, culminating in contractions of circular muscle.

Although NOS antagonists potentiated NANC contractions, inhibition of NO formation by LNA, LNAME, and LMMA did not affect cholinergic contractions, as contractions of equal amplitude were observed in the absence and presence of NOS antagonists. Similarly, hemoglobin did not significantly alter cholinergic contractions, indicating NANC but not cholinergic contractions are potentiated by NOS antagonists. These data are consistent with the conclusion that endogenous NO inhibits release of tachykinins but not ACh from excitatory motor neurons.

The studies using NOS antagonists and hemoglobin suggest that unlike NANC contractions, neurogenic cholinergic contractions are not inhibited by endogenous NO.

Surprisingly, SNP inhibited neurogenic cholinergic contractions, although the estimated EC_{50} and maximum effect of SNP were different for neurogenic NANC and cholinergic contractions. SNP inhibited cholinergic contractions with an estimated EC_{50} of 1098 nM, whereas attenuation of NANC contractions had an estimated SNP EC_{50} of 390 nM. These data indicate that a larger concentration of SNP is necessary to inhibit the same proportion of cholinergic contractions as compared to NANC contractions. Furthermore, equal concentrations of SNP inhibited a smaller proportion of cholinergic contractions as compared to NANC contractions. Therefore, although SNP attenuates neurogenic NANC and cholinergic contractions, cholinergic contractions are less sensitive to inhibition by the NO donor SNP.

The mACh receptor agonist bethanechol mimicked neurogenic cholinergic contractions, and responses to bethanechol were used to determine if SNP affected contractibility and basal tone of circular muscle. Although SNP attenuated SPME-induced contractions, bethanechol-induced contractions were not altered by SNP. These data indicate that SNP attenuates cholinergic contractions by inhibiting release of ACh from excitatory motor neurons, as basal tone and signal transduction pathways coupled to mACh receptors are not affected by SNP. However, NOS antagonists and hemoglobin failed to potentiate neurogenic cholinergic contractions, suggesting that endogenous NO present under these experimental conditions does not regulate ACh release. Greater concentrations of NO obtained by long duration of trains of nerve stimulation (Hryhorenko *et al.*, 1994) or application of NO donors such as SNP may provide sufficient NO for regulation of SP *and* ACh release from the same nerve varicosities.

As discussed previously, SNP attenuated SPME responses, indicating that SNP

altered signal transduction pathways coupled to NK_1 receptors. SNP did not affect bethanechol responses, suggesting that signal transduction pathways coupled to mACh receptors are not sensitive to inhibition by NO. Furthermore, these results strengthen the conclusion that a specific receptor or biochemical pathway is attenuated by SNP. If SNP inhibited SPME responses by decreasing basal tone of circular muscle, then mACh responses should be affected by SNP. As bethanechol responses were not altered by SNP, these data indicate SPME contractions of circular muscle are specifically inhibited by SNP. Finally, LNA and hemoglobin did not significantly alter SPME-induced contractions, suggesting that endogenous NO does not modulate NK_1 receptor mediated responses. The nerve-evoked (1 min train length, 32 Hz, 1 msec pulse duration) NO concentration (3000 pmol/g tissue/min) from circular and longitudinal muscles (Wiklund *et al.*, 1993) is much less than the amount of SNP added to circular muscle preparations in the present study, indicating that large concentrations of NO are necessary to attenuate cholinergic contractions.

Regulation of Release of Tachykinins and ACh

Circular muscle is innervated by myenteric neurons that contain both ACh and SP. The results presented in this paper indicate that NO differentially affects release of ACh and tachykinins from the same nerve, as NOS antagonists and hemoglobin potentiated NANC but not cholinergic neurogenic contractions. Furthermore, these data indicate that release of tachykinins is more sensitive to inhibition by NO. Although these studies did not determine the mechanism for inhibition of tachykinin release by NO, NOS-ir nerves are closely associated with other neurons. Over 40% of myenteric neurons contain SP-ir and these neurons do not contain NOS-ir (Llewellyn-Smith *et al.*, 1992), indicating that

many NOS-ir nerves are located near SP-ir nerves. This close proximity may allow NO released from interneurons or inhibitory motor neurons to diffuse across a short distance to effect excitatory motor neurons. Both NO and SNP bind and activate guanylate cyclase, resulting in elevated cGMP concentrations. Although cell permeable 8-bromo-cGMP inhibited NANC contractions in a concentration-dependent manner, exogenous cGMP was unlikely to affect excitatory motor neurons directly. SP-ir myenteric neurons in guinea pig ileum do not contain cGMP-ir after treatment with SNP (Young *et al.*, 1993), suggesting an alternative mechanism is responsible for inhibition of neurotransmitter release.

LMMP homogenates contain distinct vesicles with ACh or SP, comparable to small synaptic cholinergic vesicles (SSV) and large, dense-core peptidergic vesicles (LDCV) (Agoston *et al.*, 1985) found in other autonomic nerves. Differences in nitrinergic regulation of SP and ACh release may result from spatial localization of noncholinergic and cholinergic vesicles, as more numerous SSVs cluster near specialized regions of nerve varicosities, and LDCVs are found further away from the plasmalemma (reviewed in de Camilli and Jahn, 1990). If NOS-ir nerves are located preferentially near vesicles containing tachykinins, then endogenous NO may inhibit release of LDCVs; concentrations of endogenous NO may be too low to affect cholinergic SSVs found near release sites of varicosities. Alternatively, differential regulation of release of excitatory neurotransmitters by endogenous NO and differential sensitivity to exogenous NO may occur as a result of differences in docking proteins, spatial association with Ca^{2+} channels, or other unidentified requirements for exocytosis that differ between SSVs and LDCVs (Matteoli *et al.*, 1988).

Tonic and Stimulated Release of NO

Although nerve-evoked relaxations of circular muscle were not observed in this study, stimulation of myenteric nerves releases NO as measured by bioassay, high-performance liquid chromatography, and functional assays (see introduction). In this study, it was likely that nerve stimulation evoked release of NO, as potentiation of NANC contractions was dependent on the frequency of nerve stimulation. Contractions generated at 1 and 2 Hz were not potentiated by LNA, suggesting low frequency nerve stimulation is not sufficient to activate NOS. Most NO production by LMMP homogenates is Ca^{2+} -dependent (Kosta *et al.*, 1993), with an estimated EC_{50} of 350 nM. Furthermore, NO release is dependent on entry of extracellular Ca^{2+} , as ω -conotoxin GVIA preferentially attenuates NO released at low frequency nerve stimulation (Boeckxstaens *et al.*, 1993). Therefore, low frequency stimulation may not allow entry of sufficient Ca^{2+} to stimulate NO production or release from enteric nerves.

As discussed previously, NOS antagonists and hemoglobin potentiated neurogenic NANC contractions, implying that NO must be synthesized and released either prior to, or during, nerve stimulation. NO may continually be released to modulate neurotransmitter release and circular muscle tone, as basal levels of NO metabolites are detected prior to nerve stimulation. Furthermore, in this study, addition of NOS antagonists or hemoglobin increased spontaneous activity of circular muscle, indicating that NO is released under basal conditions to inhibit muscle tone. Alternatively, tonic release of NO inhibited release of excitatory neurotransmitters from myenteric nerves. NOS antagonists or hemoglobin remove this inhibition, resulting in increased spontaneous release of excitatory neurotransmitters, and increased activity of circular muscle. TTX

inhibited spontaneous activity of circular muscle after addition of NOS antagonists, indicating that endogenous NO inhibits release of excitatory neurotransmitters from enteric motor neurons. Removal of inhibitory nitrinergic tone increases neurotransmitter release, resulting in increased motor activity. Therefore, these data further support the general conclusion that endogenous NO inhibits excitatory noncholinergic neurotransmitter release from enteric neurons.

6. EXTRINSIC DENERVATION ALTERS CITRULLINE PRODUCTION

[¹⁴C]Citrulline is Produced by NOS in Normal Homogenates

When homogenates of LMMP from normal animals were incubated with [¹⁴C]arginine, the majority of column flow-through migrated on silica thin layer chromatography plates with the citrulline standard. As 98% of [¹⁴C]arginine remained bound to the cation-exchange column, these data suggest that [¹⁴C]arginine is converted to [¹⁴C]citrulline, consistent with the conversion of arginine to citrulline and NO by NOS (Bredt and Synder, 1989).

A number of experiments supported the conclusion that [¹⁴C]citrulline formation was catalyzed by NOS in LMMP homogenates. [¹⁴C]Citrulline formation by LMMP homogenates had a number of similarities to [¹⁴C]citrulline formation in cerebellum (Bredt and Synder, 1989; Bredt and Synder, 1990) and circular muscle-deep myenteric plexus of canine ileum (Kostka *et al.*, 1993). Although basal levels of [¹⁴C]citrulline could be measured, [¹⁴C]citrulline production increased over 2-fold the addition of 1 mM NADPH. As formation of NO requires oxidation of NADPH (reviewed in Moncada *et al.*, 1991), the NADPH-dependence of [¹⁴C]citrulline formation is consistent with NOS activity (Bredt

and Synder, 1990; reviewed in Schmidt *et al.*, 1992; reviewed in Dawson and Synder, 1994). Furthermore, [^{14}C]citrulline production was inhibited by the well-characterized NOS antagonists LNAME and LNA. However, the D-enantiomer of LNAME did not affect [^{14}C]citrulline production, suggesting LNAME acted through a stereospecific mechanism, such as binding to NOS.

The kinetics for [^{14}C]citrulline formation in unpurified LMMP homogenates are similar to the kinetics of unpurified cerebellar NOS (Bredt and Synder, 1989). [^{14}C]Citrulline formation in LMMP homogenates was linear in the range of substrate concentrations of 1-10 μM , with a K_M of 7.2 μM and $V_{\max}$ of 18.6 pmole/mg protein/min. These values are very similar to those of cerebellar homogenates (Bredt and Synder, 1989) (K_M of 6 μM and $V_{\max}$ of 150 pmole [^{14}C]citrulline/mg protein/min), although the lower $V_{\max}$ value in this study may indicate a greater concentration of proteins in LMMP homogenates that are not involved in the formation of [^{14}C]citrulline. Therefore, the ratio of NOS/total protein may be decreased in LMMP preparations as compared to cerebellar homogenates, culminating in a lower $V_{\max}$.

[^{14}C]Citrulline production was dependent on the presence of endogenous Ca^{2+} . EGTA preferentially chelates Ca^{2+} over other divalent cations (reviewed in Bartfai *et al.*, 1979), and addition of EGTA decreased [^{14}C]citrulline production by 71% (0.1 mM EGTA) and 99% (1 mM EGTA). These data indicate that sufficient endogenous Ca^{2+} is present in LMMP preparations to support [^{14}C]citrulline production, similar to activation of type I isoform of NOS described previously (reviewed in Schmidt *et al.*, 1992; Dawson and Synder, 1994). Addition of 0.1 mM exogenous Ca^{2+} did not significantly affect basal or NADPH-enhanced [^{14}C]citrulline production. These results are identical to studies of

circular muscle-deep muscular plexus (Kostka *et al.*, 1993), and may indicate that endogenous Ca^{2+} released during homogenization of neurons and smooth muscle cells is sufficient to activate NOS (Ca^{2+} EC_{50} 200 nM, (Bredt and Snyder, 1990)). Although low concentrations of Ca^{2+} did not alter basal [^{14}C]citrulline production, 1 mM Ca^{2+} attenuated [^{14}C]citrulline production in the presence of 1 mM NADPH. These data suggest that high concentrations of Ca^{2+} inhibit NOS. Nitric oxide synthase has multiple sites for phosphorylation by protein kinase C, and Ca^{2+} /calmodulin protein kinase (Bredt *et al.*, 1992) and phosphorylation of NOS decreases enzyme activity (Bredt *et al.*, 1992; reviewed in Schmidt *et al.*, 1992; Dawson and Snyder, 1994). As the presence of Ca^{2+} (1 mM) is necessary for activity of protein kinase C and Ca^{2+} /calmodulin kinase (Bredt *et al.*, 1992), 1 mM Ca^{2+} may activate kinases, resulting in decreased activity of NOS. Alternatively, Ca^{2+} may directly affect NOS activity, as 1 mM Ca^{2+} decreases purified enzyme activity (Bredt *et al.*, 1992). Therefore, large concentrations of Ca^{2+} may decrease [^{14}C]citrulline (and NO production) by activating kinases in the homogenate, as well as directly affecting NOS. Such a mechanism may be important to limit the duration of activity of NOS after nerve stimulation.

[^{14}C]Citrulline production was not altered by addition of 0.1 - 30 $\mu\text{g}/\mu\text{l}$ calmodulin. These data may suggest that the endogenous concentration of calmodulin released by homogenization is sufficient for NOS activity. Alternatively, [^{14}C]citrulline production may not require exogenous calmodulin. The type II isoform of NOS induced in macrophages, neutrophils, and kupffer cells (reviewed in Moncada *et al.*, 1991) is not dependent on Ca^{2+} or calmodulin, as calmodulin is normally bound to this form of NOS. However, when 0.1 mM Ca^{2+} was included with 30 $\mu\text{g}/\mu\text{l}$ calmodulin, greater citrulline

formation was observed as compared to homogenates containing only NADPH, implying that [^{14}C]citrulline production in LMMP homogenates required calmodulin.

[^{14}C]Citrulline production in normal LMMP homogenates occurred by a pathway that was dependent on NADPH, Ca^{2+} , and calmodulin, and was inhibited by L-but not D-substituted arginine analogs. Finally, the Michaelis constant was similar to that of cerebellar NOS, indicating [^{14}C]citrulline production in normal LMMP homogenates was mediated by type I isoform of NOS. Although this study did not determine the source of NOS, type I NOS-ir has been described in enteric neurons with morphology and chemical coding consistent with motor neurons (Costa *et al.*, 1992; Young *et al.*, 1992), and NOS-ir is not found in smooth muscle or enteric glia. In some regions of the GI tract, NO may be released from smooth muscle in response to VIP (Grider *et al.*, 1992); this does not appear to be true for small intestine (Daniel *et al.*, 1994). Finally, saxitoxin binds to neuronal Na^+ channels, and NOS activity and saxitoxin binding are found in enteric synaptosomes but not smooth muscle cells from canine ileum (Kostka *et al.*, 1993). Taken together, these data suggest that citrulline and NO are produced by NOS in enteric nerves.

Extrinsic Denervation Alters NOS Activity as Assayed by [^{14}C]Citrulline Formation

[^{14}C]Citrulline production in homogenates prepared from denervated loops of ileum was similar to production in normal homogenates, as citrulline formation was enhanced by exogenous NADPH. Furthermore, the NOS antagonists LNA and LNAME inhibited [^{14}C]citrulline production. However, extrinsic denervation significantly affected citrulline production as the quantity of [^{14}C]citrulline formed in denervated LMMP homogenates decreased by 50%. As citrulline production was expressed as a fraction of the total

amount of protein in LMMP homogenates, [^{14}C]citrulline production may have appeared to decrease if surgical denervation induced thickening of the longitudinal muscle. Muscle thickening can result from hyperplasia (increased cell number) or hypertrophy (increased cell volume) (Gabella, 1984), and could result in a greater protein content/homogenate. However, attenuation of [^{14}C]citrulline production was unlikely the result of increased protein content of homogenates, as the wet weight of muscle/cm was not affected by denervation, suggesting similar protein concentrations in control and denervated LMMP preparations.

Decreased [^{14}C]citrulline production was observed also in LMMP homogenates taken from animals treated systemically with capsaicin. Capsaicin is a well-characterized neurotoxin that selectively destroys thin, unmyelinated extrinsic sensory nerves without affecting enteric neurons (reviewed in Holzer, 1991) or sympathetic nerves (Galligan *et al.*, 1988; Yunker and Galligan, 1994a). This observation suggests that changes in [^{14}C]citrulline production observed after surgical denervation did not occur as a result of surgical trauma or handling of the ileum, but from loss of a specific subpopulation of extrinsic nerves. Interestingly, as shown in this dissertation, extrinsic denervation increases the number of myenteric neurons containing NADPH diaphorase and NOS-ir, and these anatomical changes are mimicked by systemic administration of capsaicin. Therefore, although extrinsic denervation increases NOS-ir and NADPH diaphorase staining, loss of extrinsic sensory nerves decreases [^{14}C]citrulline production by NOS.

Citrulline and NO are produced in equimolar concentrations (Bredt and Synder, 1989; Bredt and Synder, 1990), and [^{14}C]citrulline formation in this study was consistent with NOS activity. The data presented in the present study indicate that loss of extrinsic

sensory nerves decreases NADPH-enhanced production of NO in LMMP homogenates without affecting basal NO formation. However, in functional studies of innervation of longitudinal muscle, the amplitude and duration of NO-mediated neurogenic relaxations of LMMP preparations are potentiated after loss of extrinsic sensory nerves (Yunker and Galligan, 1994b). The biochemical data presented here are not consistent with denervation-induced increases in NOS-ir and NO-mediated neurogenic relaxations discussed previously. These data may indicate that loss of extrinsic innervation simultaneously increases NOS and alters regulation of NO production in homogenates. For example, [^{14}C]citrulline production may decrease as a result of differences in sensitivity to NADPH. If NADPH is less effective at stimulating NO and citrulline production in homogenates from denervated ileum as compared to controls, NADPH-enhanced [^{14}C]citrulline formation may be greater in control homogenates, although extrinsic denervation has increased NOS. Furthermore, NOS activity is regulated by a number of factors including Ca^{2+} , calmodulin, NADPH, cAMP dependent kinase, protein kinase C, and Ca^{2+} /calmodulin kinase (Bredt *et al.*, 1992; reviewed by Dawson and Snyder, 1994). Extrinsic denervation may affect one or more of these factors, resulting in altered regulation of NOS activity.

Paradoxical data from the biochemical assay and functional assay may also occur by different regulation of NOS under *in vitro* and *in situ* conditions. Activity of type I NOS is dependent on Ca^{2+} released from ryanodine-sensitive intracellular stores (Publicover *et al.*, 1993). If extrinsic denervation elevates sequestered Ca^{2+} , then nerve stimulation may cause greater increases in intracellular Ca^{2+} , culminating in elevated NO production and release, consistent with greater nitrinergic relaxations. However,

homogenization of these preparations would release intracellular Ca^{2+} stores, resulting in greater Ca^{2+} concentrations in homogenates from denervated tissues as compared to controls. Although low concentrations of Ca^{2+} stimulate NOS activity, elevated Ca^{2+} in homogenates from denervated tissues would inhibit NOS directly and stimulate Ca^{2+} -dependent phosphorylation of NOS (Bredt *et al.*, 1992), resulting in decreased NOS activity in homogenates from denervated tissues.

Although 1 mM EGTA inhibited [^{14}C]citrulline formation in normal and control preparations, 30% of NOS activity was unaffected by EGTA after extrinsic denervation. Kostka *et al.* (1993) observed that 40% of NOS activity was unaffected by 0.1 mM EGTA in synaptosomes prepared from circular muscle and deep muscular plexus of canine ileum. However, it is unlikely that Ca^{2+} -independent NOS activity in LMMP homogenates and enteric synaptosomes is mediated by the type II isoform of NOS localized in macrophages and liver cells (reviewed in Moncada *et al.*, 1991). Salter *et al.* (1991) also reported a Ca^{2+} -independent NOS in ileal homogenates from rat that was only partially inhibited by glucocorticoids, a known inhibitor of type II NOS (reviewed in Moncada *et al.*, 1991). Furthermore, the Ca^{2+} -independent form of NOS is not inhibited by L-canavanine (Kostka *et al.*, 1993), a selective type II NOS antagonist (Moncada *et al.*, 1991). These data, as well as that presented in the present study may be evidence of an undefined isoform of NOS, in addition to type I NOS, in LMMP homogenates.

7. EXTRINSIC DENERVATION INCREASES FOS PROTEIN

SC-52 Recognizes a Nuclear Protein in Enteric Neurons from Segments of Ileum Incubated in Krebs' Solution

Three antiserum were tested to determine if enteric neurons express Fos. Fos-ir was not constitutively expressed in enteric neurons of guinea pig ileum, as immunoreactivity was not observed in preparations that were placed in Zamboni's fixative or 4% paraformaldehyde immediately after dissection. As high K^+ -evoked depolarization can induce expression of Fos (see introduction), segments of ileum were incubated in Krebs' solution containing 50 mM KCl to determine if the absence of Fos-ir resulted from the true absence of Fos protein, or if the antibodies raised against Fos were unable to bind to Fos in enteric nuclei. SC-52-ir was observed in submucosal and myenteric ganglia, but not in any other structures, whereas AB-1 or OA-11-823 did not recognize any antigens. Furthermore, SC-52-ir was co-localized with staining by the nuclear dye bisBenzimide [40], indicating that SC-52 recognized a protein found exclusively in nuclei of ganglionic cells that was consistent with Fos protein.

Although SC-52-ir recognized a nuclear protein, immunoreactivity was not observed with antibodies AB-1 or OA-11-823. Comparisons between the target antigens indicated that SC-52 recognized a 14 amino-acid sequence found at the N-terminus of human c-Fos, whereas AB-1 was raised against a human C-terminus sequence. These results suggest that the human C-terminus sequence may not be shared by Fos in guinea pig enteric neurons, thus potentially accounting for the lack of immunoreactivity. However, tracing studies in the CNS have also established that antiserum raised against the N-terminus of Fos are more selective against Fos, as compared to proteins raised

against the C-terminus of Fos (see introduction), suggesting Fos protein is similar in neurons from the central and autonomic nervous systems. Furthermore, although OA-11-823 was raised against residues 1-16 of the N-terminus of synthetic c-Fos and SC-52 was raised against residues 3-16, OA-11-823 did not recognize enteric antigens. This discrepancy in binding suggests that residues 1 and 2 are crucial in determining binding of OA-11-823 and these residues are not available in guinea pig enteric Fos.

As described previously, ganglionic nuclei in submucosal and myenteric ganglia contained Fos-ir after segments of ileum were incubated for 1-4 hr in oxygenated Krebs' solution or Krebs' solution containing 50 mM KCl. Preparation of segments of ileum results in axotomies of enteric neurons projecting in aboral and oral pathways (reviewed in Furness and Costa, 1987), suggesting transection of enteric neurons may be sufficient stimulus to induce Fos. Similar results are seen in other peripheral nerves, as transection of sciatic nerves increases Fos-ir in ipsilateral neurons of lumbar spinal column (Chi *et al*, 1991) but not neurons in contralateral laminae. However, axotomies of enteric nerves are unlikely to account for all of the observed Fos-ir, as many enteric neurons project to other neurons, mucosa, or smooth muscle found within the segment and would not be directly affected by sectioning of the ileum into segments. As extrinsic nerves innervating segments of ileum are transected during dissection, loss of extrinsic nerves may cause expression of Fos protein in enteric neurons.

Extrinsic Denervation Induces c-Fos-ir in Enteric Neurons

The hypothesis that loss of extrinsic nerves increases expression of Fos protein was tested by eliminating extrinsic nerves to loops of ileum *in vivo*. By one week after surgical denervation, submucosal and myenteric ganglionic nuclei contained Fos-ir,

although Fos-ir was not observed in nuclei of extraganglionic cells. The majority of enteric neuronal nuclei expressed Fos, as approximately 12 submucosal neurons/ganglion and 114 myenteric neurons/ganglion contained Fos-ir, consistent with previous reports of the total number of cells/ganglion in guinea pig ileum (Young *et al.*, 1993a). Few Fos-ir nuclei were observed in ganglia from sham-denervated ileum, indicating that handling of loops of ileum and surgical procedures did not account for the large increase in Fos-ir observed after surgical extrinsic denervation. These data suggest that expression of Fos-ir in enteric nuclei may result partly from direct loss of extrinsic innervation of individual neurons. Alternatively, appearance of Fos-ir may occur as loss of extrinsic nerves induces alterations in enteric neurons, which in turn, synapse on enteric neurons which do not receive direct extrinsic innervation. Loss of extrinsic innervation could then alter neurotransmission by enteric nerves, resulting in Fos-ir in neurons without direct extrinsic innervation. Kirchgeßner *et al.* (1992) observed Fos-ir was expressed in some enteric neurons as a result of cholinergic synaptic transmission, as the nicotinic receptor antagonist hexamethonium prevented induction of Fos-ir in response to sensory stimuli. This result suggests that enteric neurons can induce Fos-ir in other enteric neurons by normal neurotransmission. Therefore, it is feasible that changes in enteric neurons previously innervated by extrinsic nerves can influence enteric neurons that do not receive extrinsic innervation, resulting in expression of Fos.

As surgical destruction of extrinsic nerves eliminated sympathetic and extrinsic sensory nerves, well-characterized neurotoxins were used to selectively destroy a single population of extrinsic nerves. Similar to the results of surgical extrinsic denervation, systemic capsaicin or 6-OH-DA treatment increased Fos-ir in submucosal and myenteric

nuclei, as a greater number of Fos-ir ganglionic nuclei was observed in submucosal and myenteric ganglia as compared to control preparations from vehicle-treated ileum. These results indicate that loss of extrinsic sensory or sympathetic nerves is sufficient for induction of Fos protein. Taken together, these results also support the conclusion that increased Fos protein results from loss of extrinsic nerves, and does not occur in response to surgical procedures used to destroy extrinsic nerves.

Most enteric neurons expressed Fos-ir after surgical extrinsic denervation or administration of capsaicin. Although an equal number of Fos-ir submucosal nuclei/ganglion was observed after 6-OH-DA or capsaicin administration, 6-OH-DA was less effective at inducing Fos-ir in myenteric ganglia as compared to ileum taken from capsaicin-treated animals. An average of 50 myenteric nuclei/ganglion contained Fos-ir after systemic 6-OH-DA treatment, whereas an average of 92 myenteric nuclei/ganglion contained Fos-ir after systemic capsaicin administration. These results indicate that loss of extrinsic sympathetic nerves induces Fos-ir in most submucosal ganglionic nuclei, and many myenteric ganglionic nuclei; however, loss of extrinsic sensory nerves may induce Fos-ir in all nuclei of enteric neurons. These results are surprising, as relatively few extrinsic sensory nerves are observed in submucosal or myenteric ganglia (see introduction). However, stimulation of extrinsic sensory nerves by capsaicin has profound effects on digestion and peristalsis (reviewed in introduction), indicating stimulation of few extrinsic sensory nerves has many effects on function. Therefore, although extrinsic nerves do not directly innervate all enteric neurons, loss of a single population of extrinsic nerves may alter nuclear events of many enteric neurons.

Time Course of c-Fos Expression

In submucosal and myenteric plexus preparations, Fos-ir remained elevated up to 24 weeks after surgical extrinsic innervation. However, the number of Fos-ir nuclei in submucosal and myenteric plexus preparations decreased over time in a time course similar to that required for return of extrinsic innervation. These results suggest that extrinsic nerves may repress expression of Fos-ir, and elimination of extrinsic nerves permits translation of *fos* mRNA. Alternatively, degenerating extrinsic nerves may release factors that increase expression of Fos (see introduction). Irrespective of the mechanism for induction of Fos-ir, the length of expression of Fos-ir in ganglionic nuclei was surprising, as Fos expression is rapidly expressed in most systems examined (reviewed in introduction) including the *in vitro* model used in some of the studies presented in this paper. For example, Fos-ir was detected in segments of ileum within 1 hr of incubating in oxygenated Krebs' solution. However, expression of Fos is also transient in many tissues, although some immediate early genes (IEGs) can be detected for over twenty weeks after transection of sciatic nerve (Herdegen *et al.*, 1992). As some IEGs are expressed during sciatic nerve regeneration, and decrease after successful regeneration, these data may suggest that IEGs are important in initiating and maintaining regenerative processes (Herdegen *et al.*, 1992). Similarly, as extrinsic innervation has not returned 24 weeks after surgical extrinsic denervation, some enteric neurons may continue to form new connections and express Fos. At this time, however, it is unknown if the same population of enteric neurons express Fos-ir at all times after loss of extrinsic innervation, or if subpopulations of enteric neurons only transiently express Fos-ir at certain time after denervation.

8. PLASTICITY OF THE ENS

Although many neurons of the CNS are unable to form new synapses following injury, autonomic neurons can survive axonal damage. Studies by previous investigators, as well as this dissertation, have shown that enteric neurons have a high degree of plasticity following trauma such as stenosis, obstruction, and loss of extrinsic innervation. Simplistically, the digestive tract is the oldest organ in mammals, with evolution adding discrete viscera, exoskeleton, and limbs. Therefore, some of the first neurons to be developed during evolution were the ancestors of enteric neurons, and may be related to transiently catecholaminergic adrenochromaffin cells. In the presence of trophic molecules, sympathetic, parasympathetic, or sensory neurons develop from these precursor cells. However, in the absence of environmental signals, transiently catecholaminergic cells develop into enteric neurons. As a result, enteric neurons may not undergo the linear maturation process of other neurons, and represent the oldest neurons in vertebrates. Although maturation and development of neurons is not well-understood, specializations of central and autonomic nerves may then limit the ability of the neuron to respond to traumatic injury, or to regrow after damage. However, enteric neurons have never differentiated; therefore, they can exhibit more plasticity than other neurons in the central or autonomic nervous systems. Such rationale may someday allow enteric neurons from a donor to be transplanted into damaged spinal cord or brain. If correct hormones and growth factors are applied, enteric neurons may be induced to form new connections and function as central, parasympathetic, or sympathetic neurons. However, such studies would require a better understanding of development of the nervous system, including a categorization of alterations of enteric neurons following loss of extrinsic innervation.

Growth Associated Protein 43 is Found in Enteric Nerves

Growth associated protein 43 (GAP 43) (Protein B50) is a membrane bound phosphoprotein identical to a phosphoprotein found in growth cone membranes during embryogenesis (phosphoprotein 46), in peripheral neurons undergoing regeneration (Benowitz and Routtenberg, 1987), and in brain regions associated with synaptic plasticity and synaptogenesis (Verhaagen *et al.*, 1989). Although most adult nerves do not contain GAP 43, the ENS of rats, ferrets, and humans contain GAP 43-ir in nerve fibers of the submucosal and myenteric plexuses, as well as in neurons of the myenteric plexus (Sharkey *et al.*, 1990). This observation suggests that the ENS may have a greater degree of plasticity than other nervous systems.

Intermediate Filament Proteins Are Found in Enteric Nerves

As described previously, neurofilament proteins belong to a larger family of proteins known as intermediate filaments that act as structural proteins and form 10 nm filaments *in vivo* and *in vitro*. Antiserum raised against α -internexin, an intermediate filament protein, has revealed that this 66 kDa protein is expressed at its highest levels in developing neurons in the CNS. It is hypothesized that α -internexin provides a scaffold for neuronal intermediate filament network in early development. Eventually, α -internexin is replaced during development by neurofilament proteins in many neurons. However, submucosal and myenteric neurons from adult animals contain α -internexin, suggesting the protein has some function that is not required in other mature neurons.

Ubiquitin is Found in Enteric Nerves

Approximately 80% of myenteric neurons contain a cytosolic protein termed protein gene product (PGP) 9.5 (Eaker *et al.*, 1994). Although the function of PGP 9.5 was not known, recent sequence homology comparisons reveal that ubiquitin and PGP 9.5 share significant sequence homology (Eaker, personal communications). Ubiquitin is a 76 amino acid polypeptide thought to be involved in essential cytoplasmic, non-lysosomal degradation of naturally short-lived or damaged proteins (Jentsch *et al.*, 1990), and extremely high levels are found in enteric, but not central nerves. Interestingly, the most responsive nerves in the periphery to regeneration contain high amounts of ubiquitin.

In the late 1960s and 1970s multiple researchers reported the presence of RNA in axons of squid and vertebrates, although ribosomes were rarely observed (reviewed in Chakraborty and Ingogkua, 1993). Unlike somata, which contain 85% ribosomal RNA, 10-15% transfer RNA, and 0-5% messenger RNA, the proportion of RNA in axons was 80-90% transfer RNA (Leseck *et al.*, 1973; Black and Leseck, 1977). Biochemical studies using Edman degradation reactions found that proteins with N-terminal acidic amino acid sequences underwent tRNA-dependent post-translational modifications by addition of arginine (reviewed in Chakraborty and Ingogkua, 1993). Arginylation is necessary for degradation of protein by ubiquitin, as addition of arginine allows binding of ubiquitin to internal lysine residues, while simultaneously weakening the structure of the protein to be degraded (Chakraborty and Ingogkua, 1993). Interestingly, although peripheral nerves arginylate and ubiquitinate cellular proteins within 12 h after lesion, nerves in the central nervous system do not respond for 3-5 days (Schyne-Athwal *et al.*, 1988). Differences in concentrations of ubiquitin and tRNA, and time of activation of these proteins may then

account for significant differences in the rate and ability to degrade unnecessary proteins, ultimately resulting in regeneration in peripheral nerves, and axonal death in the CNS. Therefore, enteric neurons may send axonal sprouts, form new connections, and change neuronal phenotype after extrinsic denervation because they are more plastic than other central or autonomic nerves.

9. SIGNIFICANCE

These studies are significant in three areas. Gastrointestinal diseases such as Crohn's disease and ulcerative colitis frequently are treated by surgical removal of normal and inflamed segments of intestine, resulting in a condition referred to as short bowel syndrome (SBS). Currently, intestinal transplantation is considered for treatment of SBS, but little is known about enteric adaptations that allow the transplanted segment to function normally in the absence of extrinsic nerves. Interestingly, increased NADPH diaphorase staining is observed in transverse sections of jejunum transplanted into recipient or donor animals (Stadelmann *et al.*, 1994; Heeckt *et al.*, 1994). Although marked ischemic is associated with transplantations, this observed increase in diaphorase staining may result from similar mechanisms such as those studied in this dissertation. If the hypothesis is correct, then altered transit and motility (Nakada *et al.*, 1994) results from denervation-induced potentiation of nitrinergic relaxations, and attenuation of rebound contractions. Second, although the anatomical, neurochemical, and basic electrophysiological characteristics of enteric neurons in normal gut have been documented, the responses of individual neurons to extrinsic denervation have not been examined. The study provided some of the first data to described alterations in subpopulations of enteric neurons following loss of extrinsic innervation, as well as

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providing some of the first studies to address mechanisms responsible for alterations in enteric nerves. Finally, although thousands of patients suffer from disorders of GI motility, little is known about the etiology of such diseases as irritable bowel syndrome. These diseases may actually be neurological problems that can be attributed to defects in transmission between the ENS and extrinsic nerves, or in alterations in neurotransmitter content of enteric neurons. In Hirschsprung's disease, diabetes, and colitis, alterations in enteric neurons include intense NADPH diaphorase staining in myenteric neurons, possibly resulting from decreased extrinsic innervation (Eliakim *et al.*, 1994; Ratcliffe *et al.*, 1994). Therefore, this dissertation provides some of the first evidence of specific enteric adaptations following extrinsic denervation, hopefully leading to a better understanding of GI neurology, as well as providing a useful model in the study of regulation of neuronal phenotype.

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