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Myc, Fos and Jun Proteins in Enteric and Sympathetic Neurons that Supply the Intestines

by

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Abstract

Nervous control of gastrointestinal functions is mediated by intrinsic enteric nerves and by extrinsic autonomic and afferent nerves but their anatomy and physiology remain subjects of active investigation. A possible approach to analyzing their phenotypic features and synaptic interactions has been offered by observations that neurons can often express characteristic constitutive and inducible nuclear proteins such as c-Myc, c-Fos, c-Jun and JunB. In this study, all mammalian enteric neurons tested exhibited constitutive c-Myc-immunoreactivity and guinea pig enteric neurons also expressed a constitutive nuclear c-Fos-like immunoreactivity. Constitutive c-Myc- and Fos-like antigens were found in all guinea pig enteric neurons, and their potential for quantitative analyses of neurochemical subsets was demonstrated. Immunoreactivity for inducible c-Fos and JunB, but not c-Jun, was upregulated by depolarizing stimuli in all tested neurochemical subsets of enteric neurons from isolated ileal segments, but some neuronal and glial expression of these antigens may have resulted from the isolation of intestinal segments. In order to extend these studies to subsets of sympathetic neurons in the inferior mesenteric ganglia, a detailed analysis of the neurochemical subsets in that ganglion was first performed. Those data supported a previously suggested possibility that the neurochemistry and electrophysiological properties of these cells may be correlated. The responses of Fos and Jun proteins in this sympathetic ganglion and the colonic nerves were then tested in a model of acetic acid-induced colitis. In some animals that had been refed immediately following induction of acute colitis, expression of c-Fos and JunB (but not c-Jun) was sometimes activated in subsets of sympathetic neurons that are most closely associated with fibres from

enteric neurons. This effect was more reliably produced by acute intracolonic capsaicin (0.2%) combined with balloon stimulation to mimic fecal transit, suggesting that it was mediated by convergence of enteric and capsaicin-sensitive inputs. Hence, enteric neurons express a characteristic set of constitutive nuclear antigens that are also useful as standards in immunohistochemistry, while inducible oncoproteins have so far proven most useful to reveal pathophysiological interactions of intestinal nerves.

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plexus of a ballon stimulated and capsaicin treated animal.

List of Abbreviations

ACh	-Acetylcholine
CG	-Celiac ganglion
CM	-Circular muscle
CNS	-Central nervous system
DRG	-Dorsal root ganglion
EGTA	-Ethylene glycol tetraacetic acid
ENS	-Enteric nervous system
FITC	-Fluorescein isothiocyanate
GI	-Gastrointestinal
IEG	-Immediate early gene
IMG	-Inferior mesenteric ganglion
IR	-Immunoreactivity
LM	-Longitudinal muscle
M	-Mucosa
MP	-Myenteric plexus
NOS	-Nitric oxide synthase
NPY	-Neuropeptide Y
NSE	-Neuron specific enolase
PBS	-Phosphate buffered saline
PGP 9.5	-Protein gene product 9.5
PREVERT. G	-Prevertebral ganglion
SDS-PAGE	-Sodium dodecylsulfate polyacrylamide gel electrophoresis
SIF	-Small intensely fluorescent
SMG	-Superior mesenteric ganglion
SOM	-Somatostatin
SP	-Substance P

SPLANC. N	-Splanchnic nerve
TH	-Tyrosine hydroxylase
TRITC	-Tetramethylrhodamine isothiocyanate
VIP	-Vasoactive intestinal polypeptide

CHAPTER ONE

INTRODUCTION

Nervous regulation has long been recognized as an important factor in propulsive motility, mucosal secretion and regulation of vasculature in the gastrointestinal (GI) tract but the anatomical and physiological bases of nerve functions are still subjects of active investigation. Intestinal pathology is often associated with motor, mucosal and vascular disturbances, and intestinal nerves are thus potentially important targets for clinical intervention but even less is known about the responses and roles of nerves in GI pathology. In fact, clinical disturbances of these important effector functions may also implicate nervous abnormalities as factors in some pathologies.

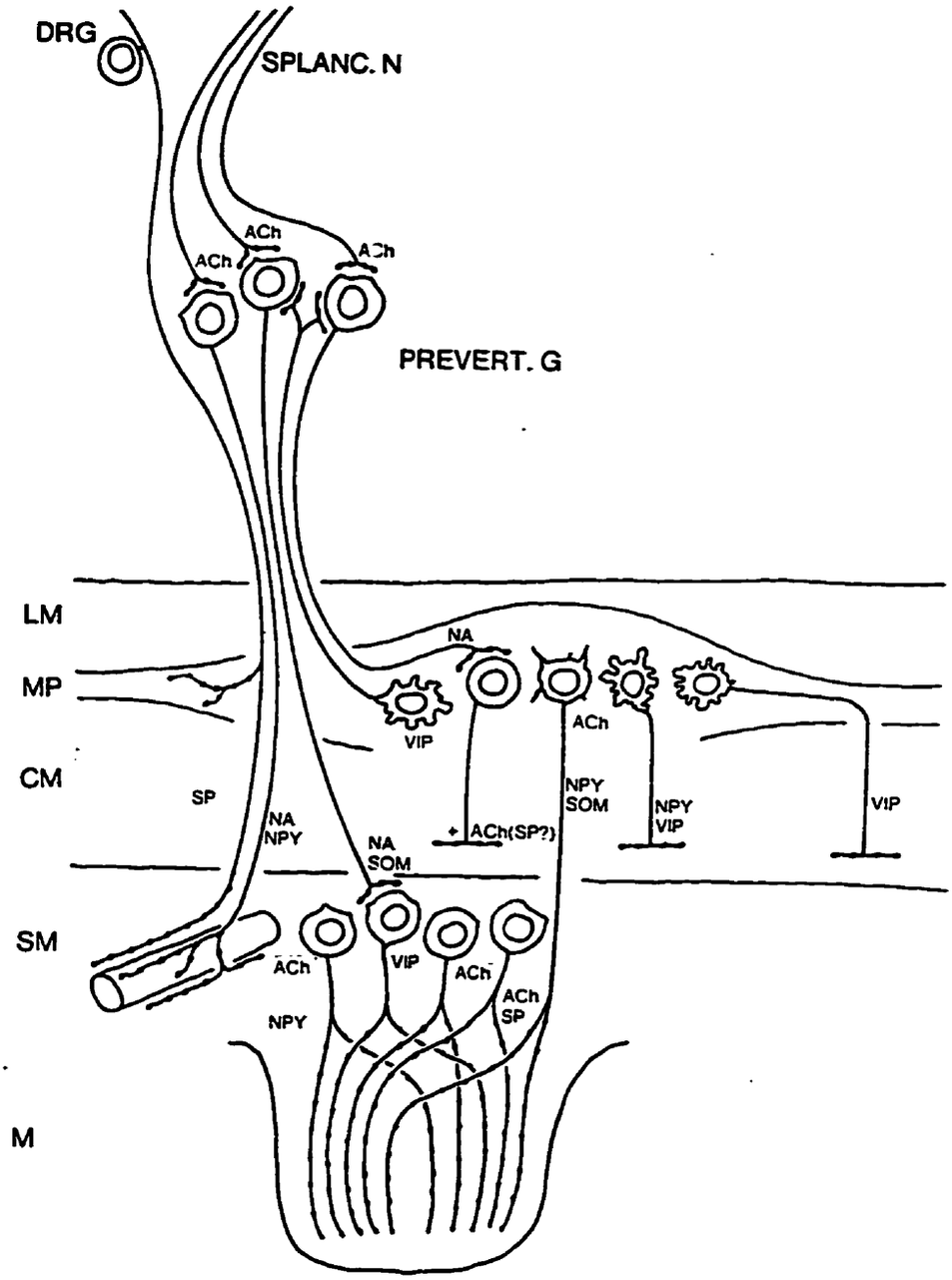
Neuroanatomical and electrophysiological studies have revealed organizational principles of the intestinal nerves that presumably reflect their functions and interactions. However, direct tests of neuronal activity in physiological preparations have been difficult to achieve. A recently developed strategy exploits the activity-related expression of nuclear proteins in neurons. Such an approach offers the possibility to investigate the activity of large numbers of neurons with cellular resolution, and may also reveal molecular mechanisms underlying the plasticity of these nerves. However, various subsets of neurons express considerable basal levels of some "inducible" nuclear proteins and may have characteristic thresholds for activating the expression of others, perhaps reflecting differences in the phenotype and/or environment of these cells. Hence, analysis of their expression may be useful not only to define nervous activity but also to identify populations of neurons and possibly to elucidate novel functions of these proteins.

1.1 Assessing the Structure and Function of the Intestinal Nerves

Afferent and efferent autonomic nerves from the central nervous system (CNS) play indispensable roles in some propagating intestinal activity, but nervous regulation of the intestines appears to be complex and multilayered. Parasympathetic fibres from the brain and sacral spinal cord directly innervate the intestines, and sympathetic fibres from the thoracolumbar spinal cord project to abdominal ganglia which in turn provide most of the postganglionic sympathetic fibres to the intestines (1). Cranial and sacral primary afferent fibres are also abundant in parasympathetic nerves to the intestines while thoracolumbar afferent fibres follow sympathetic pathways to the gut. In addition, an intrinsic ganglionated nervous network, the enteric nervous system (ENS), exists within the wall of the gut, pancreas and gall bladder (2). The ENS contains parasympathetic neurons directly innervated by the CNS and, as in parasympathetic ganglia, enteric neurons mostly contain acetylcholine (ACh). Nevertheless, the size and complexity of this ENS has led to its classification as a distinct subdivision of the autonomic nervous system. In fact, the ENS contains as many neurons as the spinal cord, and the intestinal ENS contains local circuits of sensory, internuncial and motor neurons capable of integrative reflex responses in the absence of extrinsic nervous input (2). In addition, sympathetic neurons that supply the intestines are not all simple relays for spinal sympathetic outflow as many also appear to integrate inputs from enteric and other sympathetic neurons, and perhaps from axon collaterals of thoracolumbar afferent fibres (3,4). Hence, considerable regulation of intestinal functions probably occurs through peripheral autonomic reflexes.

Mounting evidence has supported the idea that peripheral circuits within and between the ENS and sympathetic ganglia are formed by distinct functional subsets of neurons with characteristic electrophysiology, morphology and neurochemistry (2-5). This model is as yet incomplete but it has the potential to provide the anatomical basis for peripheral nervous regulation. The guinea pig contains the best characterized model of enteric and GI sympathetic neurons, largely because laminar "whole mount" preparations of enteric ganglia are relatively easy to dissect (see Chapter 2) and because sympathetic ganglia supplying the gut are discrete and accessible. In these animals, neurochemical subsets defined by immunohistochemical localization of neuropeptides, enzymes and other neural antigens may account for all enteric neurons in the stomach, small intestine and sympathetic ganglia (3,4; see Fig 1.1). Assessment of each proportion has been a painstaking process often involving indirect comparisons of neuronal density in similar preparations and so, perhaps not surprisingly, some discrepancies have been reported. Ideally, a standard method for labelling all neurons might be combined with immunohistochemical analysis of specific subsets to directly assess their proportions in each preparation but no such technique has been previously reported.

Neurochemical studies have also been combined with lesioning to demonstrate that some subsets of enteric and sympathetic neurons have characteristic projections and are associated with selected groups of peripheral fibres (2-5). These observations further support the idea that these subsets have functional relevance but anatomical and histological techniques alone give no information regarding functional responses of


DRG
SPLANC. N
ACh
ACh
ACh
PREVERT. G
LM
MP
CM
SM
M
NA
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ACh
NPY
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NPY
VIP
VIP
SP
NA
NPY
+ ACh(SP?)
NA
SOM
ACh
VIP
ACh
ACh
SP
NPY

Figure 1.1 Diagram of the enteric, sympathetic and primary afferent fibres supplying the guinea pig small intestine as well as some details of their neurochemical coding. See text for further details. Abbreviations: ACh, acetylcholine; CM, circular muscle; DRG, dorsal root ganglion; LM, longitudinal muscle; M, mucosa; MP, myenteric plexus; NA, noradrenaline; NPY, neuropeptide Y; PREVERT. G, prevertebral ganglion; SM, submucosa; SOM, somatostatin; SP, substance P; SPLANC.N, splanchnic nerve; VIP, vasoactive intestinal polypeptide. (Adapted from Costa M, Furness JB, Llewellyn-Smith IJ. Histochemistry of the enteric nervous system. In Johnson LR (ed) Physiology of the Gastrointestinal Tract, 2nd ed., Vol 1, Raven Press, New York, 1987, pp 1-40.)

neurons. Electrophysiological studies have provided valuable information regarding membrane properties and major synaptic inputs, and combinations of these studies with neurochemical and morphological analysis has revealed correlations between these features of enteric and sympathetic neurons (2-5). However, the dissection required for this approach usually removes any tonic inputs from the CNS and other peripheral nerves, and only few neurons can be analyzed by this technique in a given preparation. Furthermore, the sensitivity of this technique to disruption by motion of the dissected preparation often precludes analysis of neuronal activity directly associated with mechanosensory and visceromotor functions. These factors have undoubtedly contributed to the dearth of its application in models of intestinal pathophysiology.

An alternative approach that might circumvent many of these difficulties has been suggested by the observations that neurons often respond to stimulation by expressing a group of nuclear proteins (6). The proteins are encoded by a battery of cellular "immediate early genes" (IEGs), so called by analogy with viral IEGs because rapid stimulation of their transcription is achieved by modification of preexisting transcription factors. The largely discrete nuclear localization of IEG protein products, including c-Myc, c-Fos, c-Jun and JunB, also facilitates quantitative analysis of large numbers of neurochemically defined neurons by immunohistochemistry.

These IEG proteins all have transcriptional regulatory activity and thus they are ideally situated to markedly influence intracellular responses according to extracellular signals. In fact, considerable "basal" expression of some of these proteins is also detectable in select neuronal populations of normal animals where they presumably regulate genes

associated with specialized functions or capabilities of those neurons (7,8). A wide range of stimuli can elicit the appearance or increased expression of these proteins although the repertoire that are expressed may depend on the nature and magnitude of the stimulus as well as the phenotype of the cells. The precise functions of these proteins are as yet unclear, and in fact the c-myc, c-fos and c-jun genes had previously been identified as cellular "proto-oncogenes" because their deregulated expression or activity may contribute to neoplasia in proliferative tissues (9-11). Obviously, these "oncoproteins" are unlikely to affect proliferative mechanisms in post-mitotic neurons, but their inducible expression make them likely candidates for mediating transcriptional responses to various stimuli. Differences between the basal transcriptional activity of various neurons may also be reflected by distinct basally expressed proteins. Aside from the implications of basally expressed nuclear proteins for neuronal function, this phenomenon might also be technically exploited. Immunoreactive nuclei could serve as a useful standard in double-labelling immunohistochemistry to directly assess the proportions of neurons expressing other nuclear or cytoplasmic antigens.

Expression of these proteins may provide a useful tool to examine neuronal responses and interactions in pathology. For example, nerves appear to play important roles in inflammatory responses including regulation of vascular responses, release of immunomodulatory substances and conveying the sensation of pain (12). The role of nerves in intestinal inflammation is less clear, in part due to the anatomical complexity of the innervation and the number of potential immunomodulatory substances contained in various subsets of these nerves (13). The sensation of pain during active inflammatory

episodes indicates involvement of afferent fibres, and vascular, motor and secretory disturbances that often result in diarrhea strongly suggests that ENS function is altered as well. It is as yet unclear how any of the various nerves respond either acutely or chronically to inflammatory events but such knowledge might allow rational intervention to control symptoms and should facilitate assessment of any active neural contribution. Immunohistochemical analysis of inducible nuclear oncoprotein expression may provide a tool to address these issues in an animal model of intestinal inflammation.

The remainder of this chapter provides a more detailed description of the intestinal innervation. The intracellular roles of Myc, Fos and Jun proteins are considered and approaches to exploit their expression to analyze intestinal nerves are outlined. Finally, the objectives of the research performed to address these possibilities will be outlined.

1.2 Extrinsic Innervation of the Intestines

All divisions of the autonomic nervous system (parasympathetic, sympathetic and enteric) contribute to the regulation of GI functions. Parasympathetic preganglionic fibres from cranial and sacral levels of the CNS project to visceral targets via the vagus and pelvic nerves, respectively (1). Cranial parasympathetic neurons in the dorsal vagal nucleus project to subdiaphramatic GI structures including the stomach, pancreas and the small and large intestines. Sacral parasympathetic neurons in the intermediolateral cell column of sacral spinal levels (the exact levels of parasympathetic and sympathetic neurons within the spinal cord may vary slightly in different animals) innervate the pelvic ganglia where post ganglionic neurons continue to pelvic organs including the descending colon and rectum.

Both pre- and postganglionic neurons mediate most of their effects by release of ACh which acts at nicotinic receptors of post-ganglionic and enteric neurons and at muscarinic ACh receptors of visceral smooth muscle.

Sympathetic preganglionic neurons in the zona intermedia of the most caudal cervical levels to upper lumbar segments of the spinal cord affect subdiaphramatic GI targets via projections to sympathetic prevertebral ganglia (a small amount of the GI innervation by sympathetic nerves occurs via spinal projections to the paravertebral chain ganglia; 4,5,14). The superior mesenteric/ celiac ganglion complex (SMG/CG), located between the branchpoints of the superior mesenteric and celiac arteries from the aorta, contains postganglionic neurons that project to the upper gut including the stomach, small intestine and proximal colon. The inferior mesenteric ganglion (IMG) surrounds the inferior mesenteric artery at its branchpoint from the aorta and neurons in the IMG project to the lower large intestine. Sympathetic postganglionic neurons are also present in small ganglia along the intermesenteric nerve that connects the SMG/CG to the IMG, and a small number of cholinergic neurons also reside in the IMG (15). Guinea pigs appear to be unique in that their proximal colon also contains about 10,000 sympathetic neurons (16).

Although the spinal input to sympathetic ganglia is predominantly cholinergic, a variety of neuroactive substances have also been found in these nerves, including enkephalins, substance P (SP), luteinizing hormone-releasing hormone and neurotensin (4,5). Noradrenaline (NA) is the classically recognized neurotransmitter of sympathetic neurons in the prevertebral ganglia but neuropeptides are also colocalized in many of these neurons in a projection specific manner. In the guinea pig SMG/CG, neuropeptide Y is

contained in neurons projecting to intestinal blood vessels (NA/NPY neurons) and somatostatin is contained in projections to the enteric submucosal plexus (NA/SOM neurons) while no neuropeptides have been found in sympathetic projections to the myenteric plexus of the ENS (NA/- neurons; 17-23). However, the subsets and projections of neurons in the IMG have not been as clearly defined.

There is both considerable divergence and convergence of preganglionic inputs to the sympathetic prevertebral ganglia. The ratio of pre- to postganglionic neurons may range from 1:2 to 1:3 (5). Convergence of these inputs has been demonstrated by stimulation of central projections which elicits multiple post-synaptic events in individual ganglionic neurons. In addition to inputs from the CNS and neighbouring ganglia, prevertebral ganglia also receive convergent inputs from myenteric neurons within the wall of the gut (24-36). Populations of myenteric neurons within the distal colon project to the IMG and SMG/CG while myenteric neurons in more proximal intestines project mostly to the SMG/CG. These fibres are cholinergic but they also contain a variety of neuropeptides including vasoactive intestinal polypeptide (VIP). Indeed, enteric afferent fibres are likely to represent the major source of a dense VIP fibre network that is preferentially associated with NA/SOM and NA/- neurons in prevertebral ganglia (21,26-28). Another input to the sympathetic prevertebral ganglia may be provided by collaterals of primary afferent fibres that follow sympathetic pathways to the gut (33,35,37-39). In fact, the CNS is believed to supply a tonic drive to these ganglia but their activity is likely to reflect integration of input from these various sources (5).

Some distinction is apparent among primary afferent fibres in the viscera as they accompany either parasympathetic and sympathetic nerves and terminate at similar levels in the CNS. Afferent fibres that follow vagal and pelvic pathways may subserve any of a variety of different sensory modalities, including pain, and provide the afferent limb of many gut reflexes (40). However, most or all of the afferent fibres in sympathetic nerves are unmyelinated or thinly myelinated fibres that are believed to serve nociceptive functions. Indeed, these fibres are sensitive to capsaicin, the pungent extract of hot peppers that acutely activates and chronically inhibits peripheral nociceptive fibres in other tissues (40,41). They are believed to respond to noxious and/or high threshold mechanosensory stimuli (5,33) and might contribute to the sensation of visceral pain. Moreover, functional axon collaterals of those sympathetic afferent fibres may also project to the ENS and prevertebral ganglia (33,35,37-39) and so activation of these nerves may also reflexly alter peripheral nervous activity independently of its effects in the CNS. These fibres also project to submucosal vasculature and may mediate acute vascular responses by releasing proinflammatory substances such as substance P (SP; see below). The dense SP-immunoreactive fibre network in the abdominal sympathetic ganglia may be entirely comprised of axon collaterals from sympathetic afferent fibres to the viscera (37,38).

1.3 Intrinsic Innervation of the Intestines

The mammalian GI tract has its own intrinsic nervous system, the ENS, with neurons contained within two major ganglionated plexuses (2). The myenteric plexus includes an extensively interconnected network of ganglia and fibres between the outer

longitudinal and inner circular muscle layers. Various subsets of myenteric neurons project to the circular and longitudinal muscle, to the mucosa and to sympathetic ganglia. Myenteric ganglia are also extensively interconnected with the submucosal plexus in the submucosa. The submucosal plexus also contains interconnected ganglia and many of these neurons innervate intestinal blood vessels or extend processes to subepithelial sites. Neurons share the enteric plexus layers with numerous glia that envelope much of the nerves (42). The morphology and antigen expression of enteric glia appears to more closely resemble CNS astroglia than peripheral Schwann cells (43). These glia may provide trophic and metabolic support for enteric neurons and may also prevent ganglion infiltration by macrophage-like cells and lymphocytes that circulate through these layers (44). In fact, the close anatomical relationships between the nervous and immune systems in these layers suggest that opportunity also exists for neuroimmune interactions although the nature and implications of such interactions are not known.

As mentioned above, the ENS contains circuits of sensory, internuncial and effector neurons that can mediate independent reflex regulation of gut function. Neurogenic effects in isolated intestines of mechanical or electrical stimulation include ascending contractile and descending inhibitory activities, probably indicating activation of one or more intrinsic circuits that underly directional transit (2). Much of the excitatory enteric innervation is cholinergic, and ACh has a well recognized role in ENS functions, but a wide variety of neuroactive substances are also synthesized in the ENS including SP, NPY, SOM, VIP, serotonin, opioid peptides and nitric oxide (3). SP may be contained in intrinsic sensory neurons in both plexuses, while many neurons with VIP and/or neuronal nitric oxide

synthase (NOS) lack ACh and probably inhibit vascular and visceral smooth muscle activity (3,45,46).

Identification of mechanically gated sensory neurons has been controversial; these cells may exist in both the myenteric and submucosal plexuses but the tissue distortion required for activation of such neurons precludes simultaneous electrophysiological recording from these cells. It is possible that different sensory modalities are subserved by discrete neuronal populations as, for example, myenteric sensory neurons and/or collaterals of primary afferent fibres are believed to respond to intestinal distension while submucosal sensory neurons may respond preferentially to villus distortion (39,47). Nevertheless, the pathways used by each of these stimuli appear to converge on second order neural elements in the myenteric plexus (47). Putative myenteric sensory neurons were originally classified as such due to the lack of demonstrable presynaptic inputs (47) although subsequent studies have demonstrated some post-synaptic potentials in these cells (2). Putative submucosal sensory neurons have been reported on the basis of activity-related uptake of fluorescent dye (49) and activity-related c-Fos expression (see below).

A notable feature of the ENS is the remarkable plasticity of this network. Neurons establish and maintain functional connections despite extensive intestinal motility and turnover of some target tissues. Disruption of propagating motor activity by intestinal transection can be repaired, presumably by regeneration of functional enteric nerve circuits (50). This contrasts with CNS neurons that usually exhibit very limited regrowth of functional connections. Submucosal neurons can also respond to extrinsic denervation by increasing VIP- and SP-immunoreactive nerves along the submucosal vasculature (51).

Phenotypic changes in myenteric neurons of extrinsically denervated intestine include increased activity for NADPH diaphorase, a marker of NOS-containing neurons (52). Chronic obstruction of the rat ileum (53) and experimentally-induced inflammation (54) also increase VIP-immunoreactivity (-IR) in myenteric neurons although it is not clear this reflects increased or nascent expression by these nerve cells. Enteric nerves may also negatively regulate growth of surrounding tissues as many of those tissues become hypertrophic when enteric nerves are absent (44,55,56).

1.4 Immediate Early Gene Expression in Neurons

Expression of inducible immediate early genes in neurons has provided a potentially valuable experimental tool to analyze neuronal responses. The transcription factors encoded by those genes are also likely to fundamentally alter the transcriptional activity of cells. Nevertheless, the expression of some of these proteins may be regulated quite differently, perhaps reflecting unique transcriptional abilities or requirements of various neurons.

Despite the intense research initiated by the demonstration of neuronal c-fos expression, there currently exists few reports of neuronal expression of the c-myc gene in adult tissues. Products of the c-myc gene are also predominantly nuclear phosphoproteins that contain motifs found in other recognized transcriptional regulators and they are also believed to serve that function (57). C-Myc proteins form heterodimers with the smaller Max protein and associate with specific DNA sequences (58), and possible target genes for c-Myc regulation have been reported (59). On the other hand, other studies have also reported roles for c-Myc in post-transcriptional regulation (60) and DNA synthesis (61),

and so these proteins may serve a variety of roles.

Most previous investigations of neuronally expressed c-myc have been focused on developmental events, and c-myc mRNA has been found in proliferative neuronal progenitors (62) and transiently in some differentiating neurons (63). While c-myc mRNA is virtually undetectable in the CNS of adult animals (64), modest increases can be induced in neuronally differentiated PC12 cells by ACh receptor stimulation (65). However, no studies have previously examined c-myc expression in the ENS of any animal. In fact, substantial expression of c-myc has been reported in the intestines of adult mice (66), although almost certainly some of this occurs in the mucosa (67).

The c-fos gene is the prototypical IEG whose expression in neurons has been most widely reported. It encodes a nuclear phosphoprotein whose synthesis is transiently induced in a wide variety of cell types in response to a wide variety of stimuli (6,11). Although a role for the c-fos gene in cell division was originally suggested by its homology to the viral transforming oncogene v-fos, it is now clear that the c-Fos protein participates in a diversity of cellular processes. In fact, activity-related c-fos was first demonstrated in neuronally differentiated PC12 cells following depolarizing stimulation and activation of nicotinic ACh receptors (65,68). This was soon followed by demonstrations of c-Fos-IR in neurons of trans-synaptically activated CNS pathways (69,70). During the course of these investigations, it was discovered that the c-Fos protein was a constituent of the transcriptional activator protein-1 (AP-1)(10). Furthermore, another nuclear proto-oncogene product, c-Jun, was found to form heterodimers with c-Fos or homodimers with other c-Jun molecules that bind the consensus AP-1 DNA sequence. The discovery of other

members of the Fos family (Fra-1, Fra-2, FosB) and the Jun family (JunB, JunD) has led to the current view that AP-1 is not a static entity but actually represents an equilibrium of various Jun family homodimers and Fos/Jun heterodimers (11).

The levels of activity required for c-Fos induction appears to vary widely among different groups of neurons. For example, transynaptic induction of c-Fos-IR in the dorsal horn of the spinal cord requires noxious levels of stimulation, and even this fails to induce c-Fos in neurons of the dorsal root ganglia whose fibres convey the signals (70,71). On the other hand, resetting circadian rhythmns with flashes of light is sufficient to induce c-fos within suprachiasmatic neurons in the hypothalamus (72) and this is seemingly an innocuous stimulus. In the ENS, feeding was recently shown to be sufficient to stimulate the expression of c-fos mRNA in enteric neurons of the rat stomach (73), but it is not yet clear if physiological stimuli are sufficient to stimulate detectable levels of c-Fos protein in the intestinal ENS.

Characteristics of expression of these nuclear oncoproteins may also reveal distinct intracellular responses. In CNS neurons, levels of c-Jun, but not c-Fos, are more responsive to disruptions of nerve/target interactions that elicit regenerative and degenerative changes (74). Moreover, this response is sustained until regrowth is complete in some neurons that have the capacity to regenerate, but is only transient in those with more limited potential for regrowth (75). Interestingly, some CNS neurons degenerate following axotomy and c-Jun may also have a role in that process (76). However, neuronal activation of another Jun-family protein, JunB, often parallels that of c-Fos (77) but has not been examined in the ENS or sympathetic ganglia.

The large numbers of CNS and peripheral neurons that are capable of expressing c-Fos suggests that these nuclear proteins play important roles in the nervous system. Nevertheless, these roles are likely to be distinct in different populations of neurons. For example, c-Fos may help to regulate expression of tyrosine hydroxylase expression in catecholaminergic neurons (78) although c-Fos expression is not limited to those neurons. Moreover, suprathreshold stimulation of Fos and Jun expression in the spinal cord elicited by noxious stimulation (79) and in the hippocampus elicited by chemically-induced seizure (80), is followed by dramatically increased expression of proenkephalin. The kinetics of this response are consistent with a role for Fos and Jun proteins acting at a consensus AP-1 binding site in the promoter of the proenkephalin gene. However, no neural deficits have yet been described in transgenic mice in which the c-fos gene has been "knocked out" (81), and so the importance of c-Fos in neurons is less clear. This is likely due, at least in part, to the considerable redundancy that may be afforded by homologous Fos family proteins. Interestingly, the FosB protein has recently been shown to have even more specialized expression and functions in the CNS (8). This protein is expressed constitutively in some CNS neurons and is induced in others by nurturing behaviour. Moreover, in FosB knock out mice, nurturing behaviour is severely inhibited and pups born to these animals suffer considerable infant mortality. Therefore, expression of some of these proteins may be quite variable in different neuronal populations and may also be indispensible for normal function.

When this work was initially undertaken, only one previous study had examined c-Fos-IR to analyze the circuitry of the ENS of the guinea pig ileum (82). Kirchgessner and

her colleagues reported that villus distortion activated c-Fos-IR in enteric neurons of isolated guinea pig ileum that was restricted to submucosal sensory neurons when hexamethonium blocked secondary neuronal responses. This represents an apparent difference from spinal afferent neurons, where activity-dependent c-Fos expression has not been found (70) but the activation of submucosal sensory neurons was also inhibited by a serotonin receptor antagonist indicating an indirect response to mechanosensory stimulation. Moreover, it was not yet clear if all subsets of enteric and/or sympathetic neurons could express activity-related c-Fos. In fact, many c-Fos-IR cells in cholera toxin-treated positive control preparations from that study were not clearly identifiable as neurons. The possibility that glia express c-Fos-IR had not yet been addressed.

Fos-like proteins have since been reported to be induced in the ENS by extrinsic denervation (83), perhaps reflecting a molecular mechanism underlying the more chronic effects of that treatment (see above). Activation of c-Jun-IR has also been reported in isolated segments of guinea pig ileum incubated with colchicine to block axonal transport (54). Hence, enteric neurons are likely to have the capacity to express a variety of IEGs, but this phenomenon has yet to be fully characterized in the ENS.

Despite the fact that neuronal c-Fos expression was first demonstrated in cells with a sympathetic postganglionic phenotype (65,68), relatively few studies have yet examined its expression in sympathetic ganglia and no demonstration of its expression had yet been shown in the abdominal prevertebral ganglia. The superior cervical (sympathetic) ganglion of young rats express both c-Jun-IR, found within dying neurons, and Fos-like-IR in seemingly healthy neurons (63). The c-fos message is also increased in these ganglia with

increased age (85), and Fos-like antigens can also be induced by nicotine administration in that ganglion (86). However, it is not clear whether any of these observations involved transynaptic induction and, given the neurochemical differences between sympathetic neurons in paravertebral and prevertebral ganglia (4,5), it is also not clear if any of those observations are relevant to the prevertebral ganglia.

1.5 Intestinal Nerves in Inflammation

Inflammation represents a normally self-limiting response to such insults as pathogens or tissue damage. However, inflammatory events can occur inappropriately or be inefficiently controlled resulting in more damage to the tissue than was afforded by the initial stimulus. A role for nerves in inflammation of some tissues has been established (12) but the role of nerves in visceral inflammation is less clear. For example, it is not clear if anatomical abnormalities of intestinal nerves reported in inflammatory bowel diseases (IBDs) are the cause or effect of functional disturbances including acute episodes with pain, hemorrhaging and diarrhea (13).

The sensation of pain is mediated by thinly myelinated or unmyelinated, small diameter afferent fibres, similar to those that accompany sympathetic nerves to the gut (12,18). The sensation of pain in IBDs suggests that pain sensitive afferent pathways may be sensitized, as also occurs in hyperalgesia associated with other tissues. Activation of these nerves may also have efferent effects by local release of inflammatory mediators such as SP and CGRP from peripheral nerve terminals (12). This is likely to contribute to local inflammation, and release of these peptides from primary afferent axon collaterals might

also contribute to more distant inflammatory responses via an "axon reflex" mechanism. Since those afferent fibres that traverse the abdominal sympathetic ganglia also appear to have axon collaterals to sympathetic neurons, and may also have axon collaterals in enteric ganglia (see above), activation of these fibres may profoundly affect sympathetic and enteric nervous functions. However, the activity of intestinal nerves in GI inflammation has not yet been extensively examined.

1.6 Objectives

This study was undertaken to examine the expression of c-Myc, c-Fos, c-Jun and JunB in enteric neurons and in sympathetic neurons of the IMG. The hypothesis underlying this work was that analysis of the expression of these proteins could be exploited to examine anatomical and functional aspects of the intestinal nerves. The guinea pig was chosen for most of these studies because, as discussed above, it is the best characterized animal model of the ENS and prevertebral ganglia, and this choice also allowed a direct comparison with the previous report of c-Fos expression in the ENS. However, tissues from some other mammalian species were examined when appropriate. The characteristics of c-Myc antigens were examined first, including their presence in enteric neurons, their size, their stability, and their presence in a range of mammalian species (Chapter 3). The range of enteric neurons expressing constitutive c-Myc and c-Fos antigens in the guinea pig ileum was examined and their usefulness as neuroanatomical markers in double-labelling immunohistochemistry was assessed (Chapter 4). Activity-dependent expression of c-Fos, c-Jun and JunB was also analyzed in the ileum of these animals, including the propensity of

various subsets to express these antigens and some conditions that altered their expression (Chapter 5). The chemical neuroanatomy of the IMG was then examined (Chapter 6) as a basis for subsequent studies to interpret expression of these nuclear oncoproteins within sympathetic and enteric nerves in acute inflammation (Chapter 7).

CHAPTER TWO

METHODS AND MATERIALS

This chapter will outline the specific methods and materials that pertain to many or all research chapters. Details that are applicable to a particular chapter can be found in the "Methods and Materials" section of that chapter. Unless otherwise specified, general laboratory reagents were obtained from Sigma Chemical Company (St. Louis, MO) or BDH Inc. (Edmonton, Alberta).

2.1 Animal Studies

The guinea pig was used for most of this work because, as outlined in chapter 1, it is the best characterized animal model of enteric and sympathetic nerves, but tissues obtained from other animals were also used for analysis of c-Myc expression (see chapter 3). A total of 101 male albino guinea pigs (200-800g) were used throughout these studies. Animals were killed by overdose with sodium pentobarbitone (Somnotol, MTC Pharmaceuticals, Canada; >60mg/kg, i.p.) or anesthetized with halothane (4% in O_2) and exsanguinated. All procedures performed were approved by the University of Calgary Animal Care Committee and were conducted according to the guidelines established by the Canadian Council on Animal Care.

2.2 Tissue Preparations

Intestines were opened along the mesenteric border and fixed by overnight immersion in Zamboni's fixative (2% paraformaldehyde in picric acid, 4°C) or 4% paraformadehyde. After rinsing in phosphate-buffered saline (PBS), picric acid was cleared

from some Zamboni's fixed gut tissues by immersion in dimethylsulfoxide and subsequent rinses in PBS (3 X 10min each) and this substantially improved labelling by anti-Myc 033 (see Table 1 and chapters 3,4). All tissues for sectioning were rinsed, dehydrated in PBS containing 20% sucrose, mounted in tissue tek, frozen in 2-methylbutane at -70°C, and 12-14μm cryostat sections were mounted on slides coated with poly-D-lysine.

While sections are useful to examine some intestinal tissues, the small number of neurons in any given section limit its usefulness for that purpose. However, the laminar structure of the intestines also allows laminar preparations containing the myenteric plexus, which remains adherent to the longitudinal muscle, and the submucosal plexus, contained within the submucosal layer, to be physically dissected from the intestines of smaller animals (2,87). These so-called "whole mount preparations" (whole mounts) are prepared by stretching and pinning the opened intestines, with mucosa side up, on Petri dishes coated with Sylgard® (Dow Corning) in PBS containing 0.1μM nifedipine to block calcium-activated smooth muscle contraction. Whole mounts can also be dissected with forceps from live tissues, as has been done previously for electrophysiological analysis (see chapter 1) or in the present study for biochemical analysis (see chapter 3). In most of this work, tissues were fixed and rinsed prior to dissection of whole mounts for immunohistochemistry as described below.

2.3 Immunohistochemistry

Analysis of nuclear oncoprotein expression was performed by immunohistochemistry for three major reasons: first, this allowed for combination with

neurochemical analysis in double-and triple-labelling immunohistochemistry (see below); second, the nuclear localization of the proteins greatly facilitates assessment of the numbers of labelled cells; and third, the expertise and reagents were available.

Immunohistochemistry is a technique that exploits the specificity of antibody-antigen binding to reveal the histological and/or intracellular distribution of a given antigen (88). Antibodies are produced by differentiated clones of B cells, referred to as plasma cells, and provide a vital component of the humoral immune response (89). These molecules have conserved domains that are similar to that of all other antibodies and that interact with other components of the host immune system. In addition, antibodies have domains that interact with foreign bodies, or antigens, and the structure of this domain differs among different plasma cell clones. "Polyclonal" antibodies, so called because they are produced by a variety of plasma cell clones within the secondary host and bind multiple epitopes of a given antigen, are produced by immunization of a host animal and subsequent purification from its serum. Alternatively, monoclonal primary antibodies can be produced by antigen selection of fused spleen and tumor cells which yields hybridoma cells that secrete antibodies of a single epitope specificity. The specificity of labelling can often be tested by preabsorption of the antibody with excess of the cognate antigen to competitively inhibit specific antibody-antigen interactions. Technically, nonspecific interactions of the antibody with the tissue can often be competitively inhibited by inclusion of nonspecific proteins or sera.

For immunohistochemistry, tissues are incubated in primary antibodies, specific for the antigen of interest, under empirically determined conditions that allow antibody-antigen

interactions. While direct immunohistochemistry requires that the primary antibodies be conjugated to a detectable moiety, indirect immunohistochemistry employs a secondary antibody, produced by immunization of the "secondary" host with immunoglobulin of the "primary" host. Because multiple secondary antibody molecules can bind to a given primary antibody molecule (see below), any detectable signal is further amplified by this indirect method. The secondary antibody is usually conjugated either to enzymes, whose presence can be assessed with histological reactions, or to fluorochromes that can be detected by ultraviolet light-induced emission of visible fluorescence. This latter technique has the advantage that labelling in a given tissue by two or three primary antibodies, raised in different species, can be detected by species-specific secondary antibodies conjugated to distinct fluorochromes with characteristic absorption and emission spectra. Thus, in "double-" or "triple-labelled" tissues, the distribution of as many as three antigens can be analyzed simultaneously with appropriate fluorescence microscope filters.

In the present study, fixed tissues were rinsed in PBS containing 0.1% Triton X-100 (PBS-Triton, detergent added to permeablize tissue membranes) and incubated overnight in primary antibodies in solution containing PBS-Triton, bovine serum albumin (1mg/ml), sodium ethyldimethyltetra-acetate (a Ca^{++} chelator, 0.5mg/ml)) and .01% NaN_3 (a preservative, 0.4mg/ml). Commercially available primary antibodies (Table 2.1) were obtained from NCI/BCB Repository (Camden, NJ), American Type Culture Collection (Rockville, MD), Cambridge Biochemicals (Cambridge, MA), Santa Cruz Biotechnology Inc. (Santa Cruz, CA), Eugene Tech (Ridgefield Park, NJ), Polysciences (Warrington, PA), Chemicon (Temecula, CA), UltraClone (Isle of Wight, UK), Peninsula Laboratories,

Inc. (Belmont, CA) and Sigma Immunochemicals (St. Louis, MO). Other primary antibodies (Table 2.1) were kindly provided by Drs. J.H. Walsh, J.H. Rogers, K.T. Riabowol, R. Bravo, B. Mayer and the Medical Research Council of Canada Regulatory Peptide Group, Department of Physiology, University of British Columbia. The isotype of all polyclonal and monoclonal antibodies was IgG. Tissues were then rinsed in PBS-Triton (3 X 5-10min) before incubation in secondary antibodies (1h, room temperature). Secondary antibodies used were: goat anti-mouse IgG conjugated to fluorescein isothiocyanate- (FITC) or tetramethylrhodamine isothiocyanate- (TRITC) (Incstar, Stillwater, MN); goat anti-rabbit conjugated to FITC (Incstar); sheep anti-rabbit or anti-mouse conjugated to CY3 (Sigma Immunochemicals, St. Louis, MO); donkey anti-rat conjugated to FITC (Jackson ImmunoResearch Lab. Inc., West Grove, PA, USA; 712-165-150); donkey anti-mouse conjugated to aminomethylcoumarin (Jackson). For double- and triple-labelling protocols, both the primary and secondary antibody combinations were mixed prior to use. Tissues were then rinsed again, mounted on slides in phosphate-buffered glycerol (pH 8.6) and analyzed with a Zeiss Axioplan fluorescence microscope with appropriate filters and photographed using Kodak TMax 400 ASA film.

Table 2.1. Primary antibodies

Antigen	Host Species	Dilution	Source/Code
c-Myc	Mouse	1:500	NCI BCB/033
c-Myc	Mouse	1:5000	NCI BCB/070
c-Myc	Mouse	Medium*	ATCC/9E10
c-Myc	Mouse	1:500	Cambridge/904
c-Fos	Mouse	1:500	K. Riabowol/TF 161
c-Fos (FOS 4)	Rabbit	1:5000	Santa Cruz/SC 52
c-Fos	Rabbit	1:5000	K. Riabowol/TF 3
JunB	Rabbit	1:5000	R. Bravo/725
c-Jun	Rabbit	1:1000	K. Riabowol/c-Jun
TH	Rabbit	1:500	Eugene Tech/TE101
NSE	Rabbit	1:1000	Polysciences/17437
NSE	Mouse	1:100	Chemicon/MAB314
PGP 9.5	Rabbit	1:1000	UltraClone/RA95101
S-100	Rabbit	1:500	Sigma/S2644
VIP	Rabbit	1:1000	J.H. Walsh/7913
VIP	Mouse	1:500	UBC/VIP31
NPY	Rabbit	1:1000	Amersham/RPN.1702
NPY	Rat	1:500	Eugene Tech/NT115
SOM	Rabbit	1:1000	Peninsula/IHC 8001
SOM	Mouse	1:500	UBC/Soma 10
Calbindin	Mouse	1:500	Sigma/C8666
Calretinin	Rabbit	1:1000	J.H. Rogers/Calretinin
NOS	Rabbit	1:1000	B. Mayer/NOS 1

(medium* - Indicates antibody used as undiluted hybridoma cell culture medium)
Abbreviations: TH, tyrosine hydroxylase; NSE, neuron specific enolase; PGP 9.5, protein gene product 9.5; VIP, vasoactive intestinal polypeptide; NPY, neuropeptide Y; SOM, somatostatin; NOS, nitric oxide synthase; UBC, University of British Columbia.

CHAPTER THREE

C-MYC ANTIGENS IN THE MAMMALIAN ENTERIC NERVOUS SYSTEM.

3.1 Introduction

The c-myc proto-oncogene encodes proteins that appear to play important roles in normal cell growth, differentiation and death, and whose deregulated expression is associated with cell transformation and tumorigenesis (reviewed in 9,57). Despite a strong correlation between the expression of c-myc and cell cycle events, several examples of the dissociation of c-myc expression and mitotic events have been described (63,65,90-92). For example, receptor activation elicits modest increases of c-myc transcripts in some neurons (65). While c-myc mRNA is negligible in the adult central nervous system (CNS) (93), expression of myc-related genes in the CNS includes that of B-myc (Brain myc) (94). C-Myc proteins may be more stable in quiescent cells (95), possibly including post-mitotic neurons, and so considerable levels of c-Myc proteins in those cells need not even be accompanied by abundant c-myc transcripts.

The enteric nervous system (ENS), including the ganglionated myenteric and submucosal plexuses of the intestines, contains neurons whose development and environment are distinct from CNS neurons (2). However, the expression of c-Myc had not previously been reported in any peripheral neurons of mature animals. In fact, expression of the c-myc gene in full thickness segments of intestines persists into adulthood (64,66), and while some almost certainly originates in the mucosa (67,96-101), the expression of c-Myc proteins in the enteric nervous system (ENS) had not been examined.

Antibodies are an invaluable tool for determination of protein expression (see Chapter 2) and several monoclonal anti-c-Myc antibodies are commercially available. This study was undertaken to examine the presence and some properties of c-Myc antigens

contained in the ENS.

3.2 Hypothesis

C-Myc proteins will be detectable in the ENS. Their identity would be supported by demonstration with a range of antibodies and in a range of species. They might also be expected to share some characteristics, including size and stability, with c-Myc proteins in at least some other cells.

3.3 Methods and Materials

3.3a Animals and Tissues

C-Myc immunoreactivity (IR) was examined in the ileum and colon of 12 male albino guinea pigs (250-750g) and 3 adult male Sprague-Dawley rats (250-500g, Charles River). Animals were either overdosed with sodium pentabarbitone (10 animals), anesthetized with halothane (1 animal) or stunned (1 animal) and exsanguinated, the ileum and colon were removed, stretched and pinned for dissection of whole mounts (see Chapter 2) and fixed overnight with Zamboni's fixative, or fixed without stretching for cryostat sections. Pieces of ileum and colon were obtained post mortem (<20 min) from one male dog and one female sheep, as well as a piece of ileum from one male Cynomolgus monkey (*Macaca fascicularis*). A small piece (resection margin) of full thickness, grossly normal, human colon removed at surgery (kindly provided by Dr. R.M. Preshaw) was also examined. One guinea pig was perfused through the heart with Kreb's solution (200ml) followed by Zamboni's fixative (500ml) before the brain and spinal cord were removed and

fixed by overnight immersion in the same fixative. Whole mounts of the enteric plexus layers dissected from guinea pigs, rats, and monkey, and cryostat sections (12μm) of all tissues were processed for immunohistochemistry as described in Chapter 2.

3.3b Antibodies and Peptides

Murine monoclonal antibodies raised against peptides representing distinct regions of the human c-Myc protein were used to examine the presence of c-Myc-IR in the ENS (see also Table 2.1). Anti-MYC 9E10 (102) was raised against a peptide sequence corresponding to residues 408-439 of the human c-Myc protein and was used as undiluted cell culture medium from hybridoma cells. Anti-MYC 070 was raised against residues 171-188 and was used as ascites fluid (1:5000). Anti-MYC 904 and anti-MYC 033 were raised against residues 42/43-55 and were used as hybridoma supernatants or ascites fluid (1:500). On one whole mount of guinea pig ileum, double-labelling was performed using anti-MYC 033 together with a rabbit antiserum against vasoactive intestinal peptide (VIP). One whole mount preparation of monkey ileum was concurrently labelled with both anti-MYC 033 and anti-MYC 904.

3.3c Assessment of Antigen Stability

Segments of ileum (from approximately 5-10 cm rostral to the ileocecal junction) were removed from two guinea pigs and incubated in either oxygenated Kreb's solution (1 segment- 1 h, 1 segment-3 h, 1 segment-4 h; all at 37°C) or in Kreb's containing 10μg/ml cycloheximide (as for Kreb's alone). Tissues were then fixed by immersion in Zamboni's

fixative and processed for immunohistochemistry.

3.3d Specificity of Antibodies

In the control series for anti-MYC 033, anti-MYC 070 and anti-MYC 904, tissues were incubated in the primary antibody preabsorbed with the cognate peptide (033 and 904, 3μg/ml diluted antibody; 070, 0.5μg/ml diluted antibody). In controls for labelling by anti-MYC 9E10, incubation in the primary antibody was omitted. In addition, immunohistochemical analysis of the guinea pig cerebellum and spinal cord was performed in order to assess possible cross-reactivity with antigens contained in CNS neurons.

3.3e Immunoprecipitations of Radio-Labelled c-Myc Antigens

Longitudinal muscle/myenteric plexus and circular muscle layers from approximately 10 cm segments of fresh guinea pig colon (from between 10-20 cm from the internal anal sphincter) and ileum (from between 10-20 cm from the ileocecal junction) were dissected and visual inspection confirmed that most of the myenteric plexus remained attached to the longitudinal muscle. Tissues were then provided to Dr. Alan Gibson for radiolabelling, immunoprecipitation and separation of c-Myc antigens by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Tissue pieces were incubated at 37°C for 1h in medium containing 150-200 μCi/ml ^{35}S-methionine (Amersham, Oakville, Ont., Canada) or Tran-^{35} label (ICN Biochemicals, Mississauga, Ont., Canada) and subsequent procedures are described in detail elsewhere (103). Briefly, they were washed in ice cold PBS, homogenized, centrifuged at 12,000X g for 20 minutes and supernatants

were precleared with unbound protein A-agarose (Gibco BRL, Burlington, Ont., Canada) before incubation with anti-MYC 070 (colonic tissue), anti-MYC 033 (ileal tissue) or antibodies preabsorbed with their cognate peptides. Antibody-antigen complexes were precipitated with protein A-agarose-bound rabbit anti-mouse IgG, washed, boiled in sample buffer and fractionated on 10% Bio-Rad Mini-polyacrylamide gels. Gels were then processed and exposed to Kodak X-Omat film at -70°C for 4-14 days.

3.4 Results

3.4a Immunohistochemistry

Anti-MYC 033 labelled nuclei in whole mounts of the myenteric plexus and the submucous plexus in the ileum and colon of the guinea pig (Figs 3.1A,E, 3.2A,B). Although faint labelling of smooth muscle nuclei was also evident in sections, this labelling was obscured in whole mounts by the tissue thickness. Neuronal nuclei were also labelled in the ENS of the rat (Figs 3.3E-F), dog (Fig 3.3D), sheep (not shown) and the monkey ileum (Fig 3.3A). The nucleoli were often evident as unlabelled regions of the nuclei (see Figs 3.1E, 3.2A,E, 3.3A,D,F). Enteric neurons of the human colon were not labelled by this antibody (data not shown). In all cases, preincubation with the cognate peptide completely abolished labelling with anti-Myc 033 (see Fig 3.1B). This

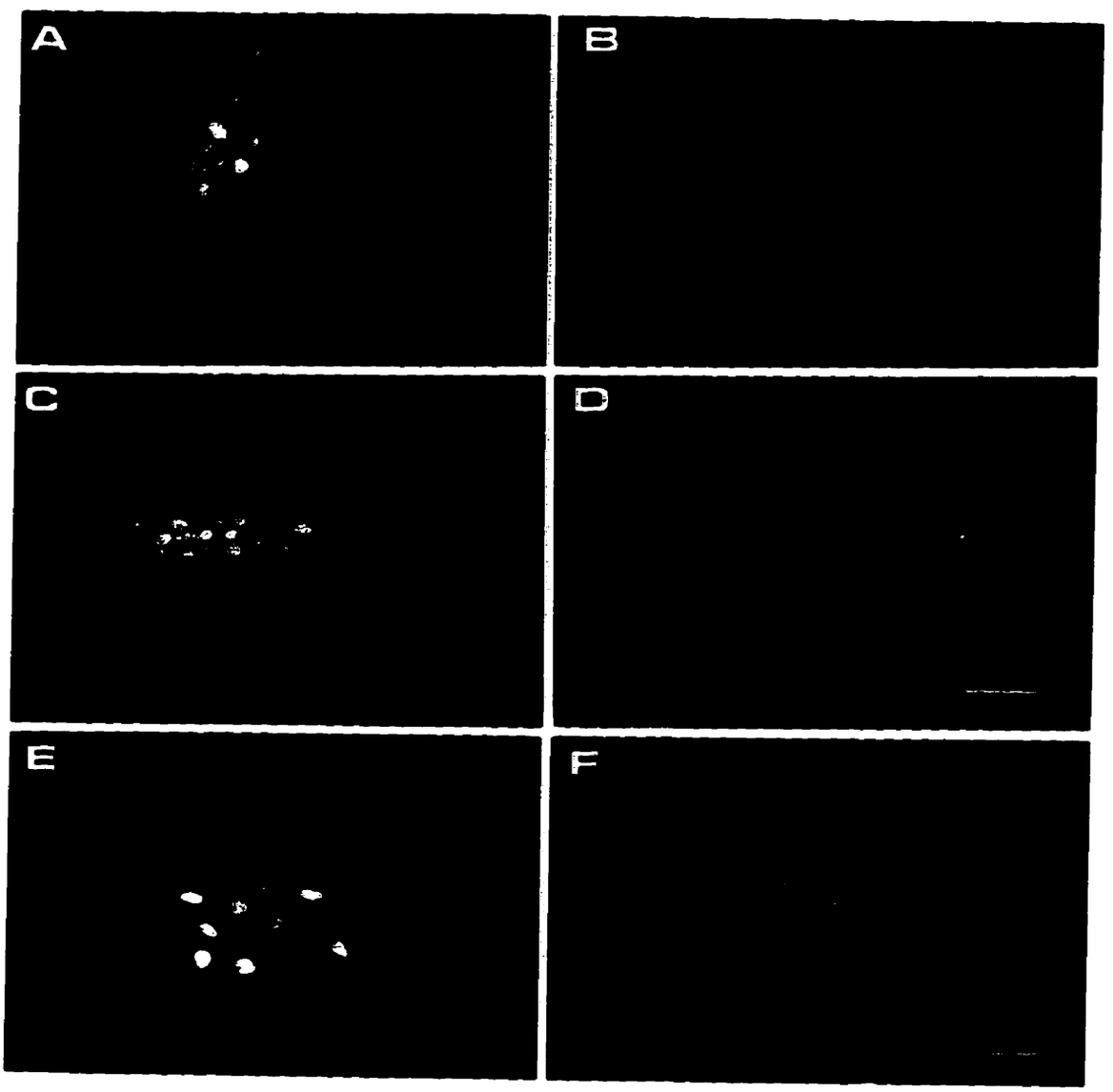
A
B
C
D
E
F

Figure 3.1 Fluorescence micrographs of c-Myc-immunoreactivity in whole mount preparations of the myenteric plexus (A-D) and submucous plexus (E,F) of the guinea pig ileum. Labelling of ganglionic nuclei by both anti-MYC 033 (A) and anti-MYC 070 (C) was abolished by preabsorption with their cognate peptides (B and D, respectively). The sizes of nuclei labelled by anti-MYC 033 (E) were less variable than those labelled by anti-MYC 070 (F). Neuronal nuclei labelled by anti-MYC 070 appeared more intensely stained than glia and other submucous layer cells (F) as well as longitudinal muscle cells (C). Scale bars: 50μm.

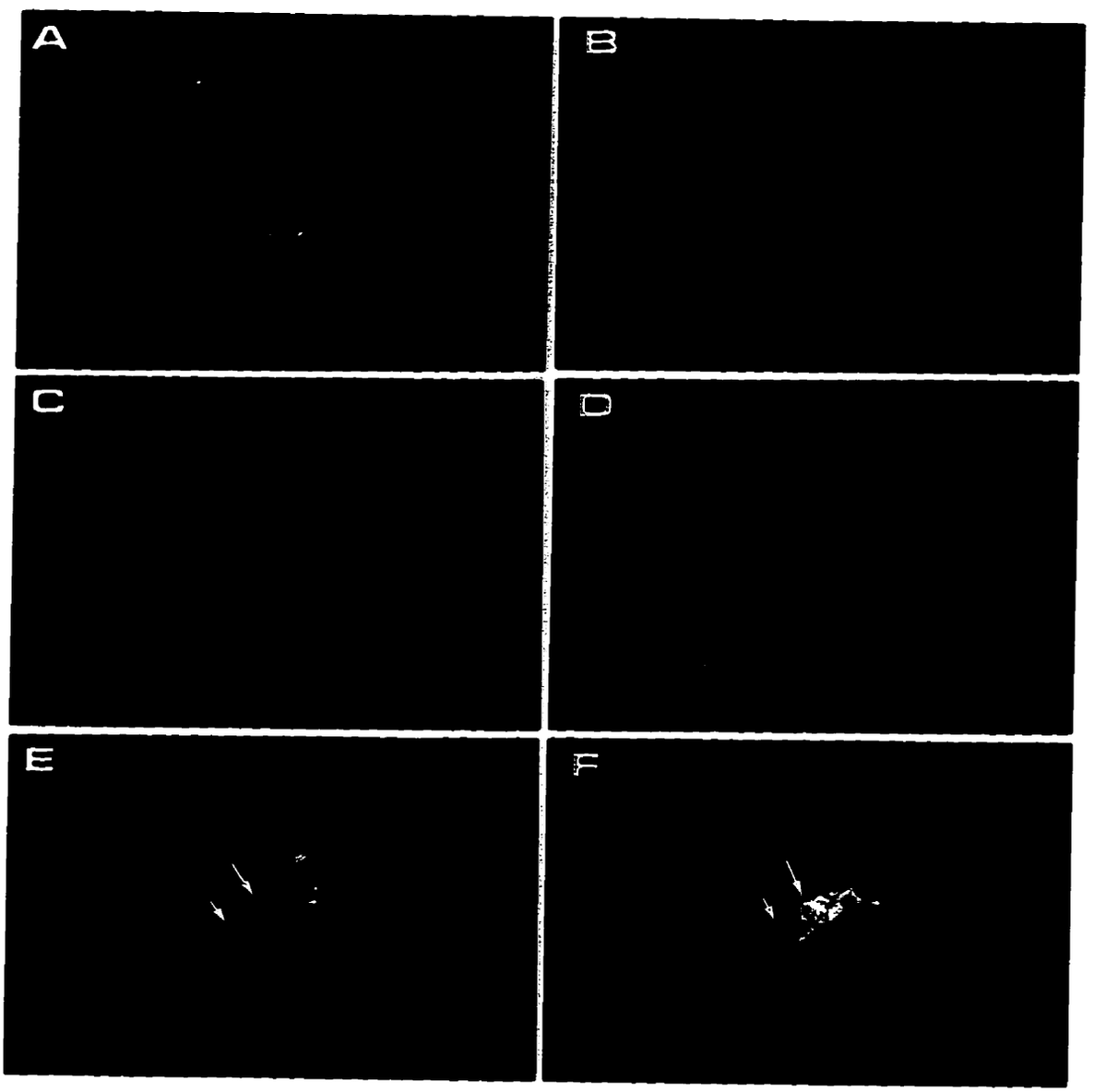
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Figure 3.2 Fluorescence micrographs of c-Myc-immunoreactivity in whole mount preparations of the guinea pig colon (A-D) and double labelling in the submucous plexus of the guinea pig ileum (E,F). Anti-MYC 070 also labelled myenteric neurons and glia (A), submucosal neurons and glia (C) as well as the nuclei of associated muscle cells (A) and other submucous layer cells (C). Labelling by anti-MYC 033 was restricted to myenteric neurons (B) and submucosal neurons (D). Double labelling with anti-MYC 033 (E) and anti-VIP (F) revealed that c-Myc-like immunoreactive neurons included those that contain VIP (arrows). Scale bar: 50μm.

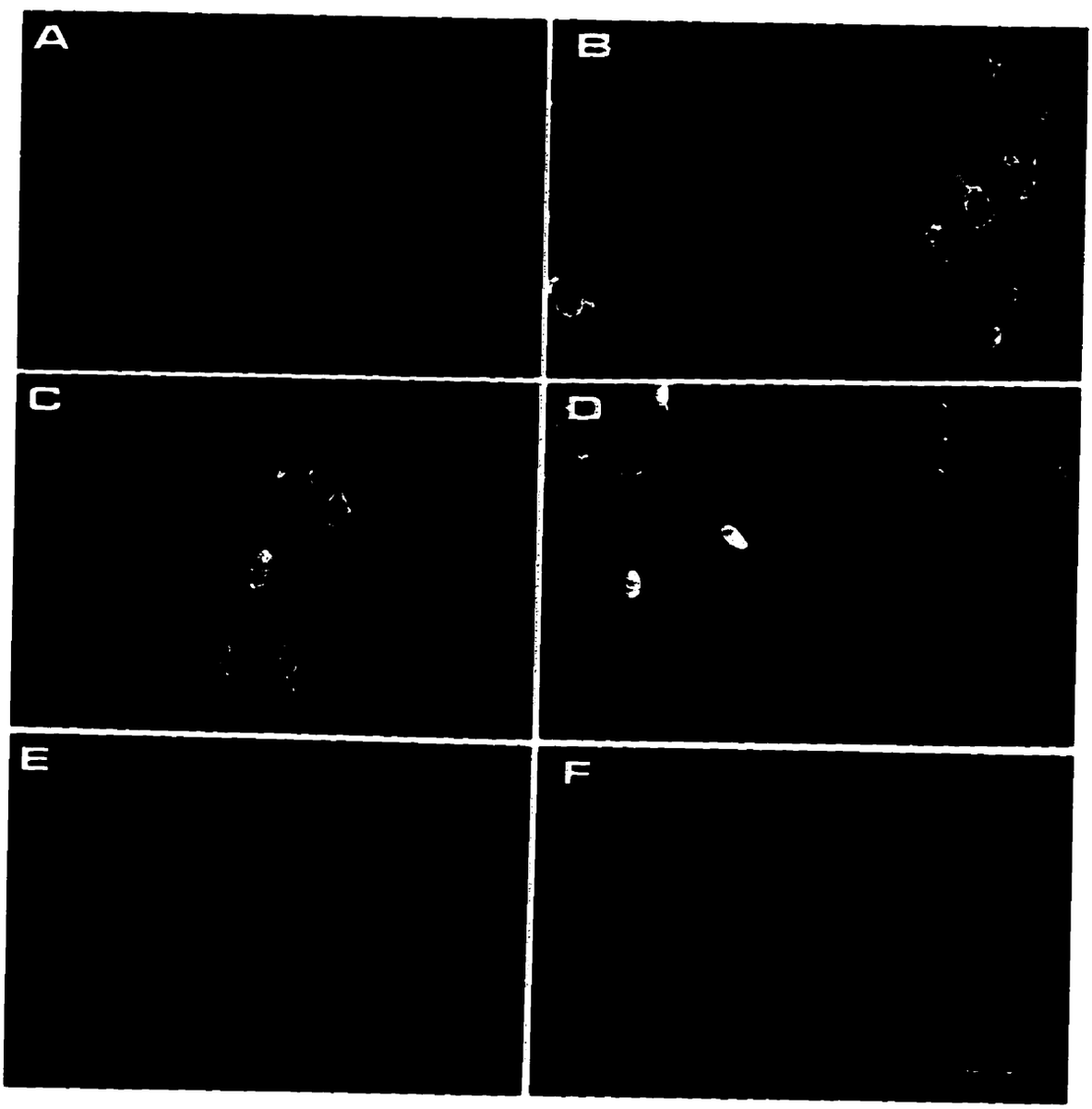
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Figure 3.3 Fluorescence micrographs of c-Myc-immunoreactivity in whole mount preparations of the myenteric plexus of the monkey ileum (A-C), rat ileum (E) and rat colon (F) and in a section of dog ileum (D). Anti-MYC 033 labelled nuclei in tissues from all these species (A,D-F). Anti-MYC 904 labelled cytoplasmic structures in enteric neurons of the monkey ileum (B) that were co-localized in cells labelled by anti-MYC 033 (C). Scale bar: 50μm.

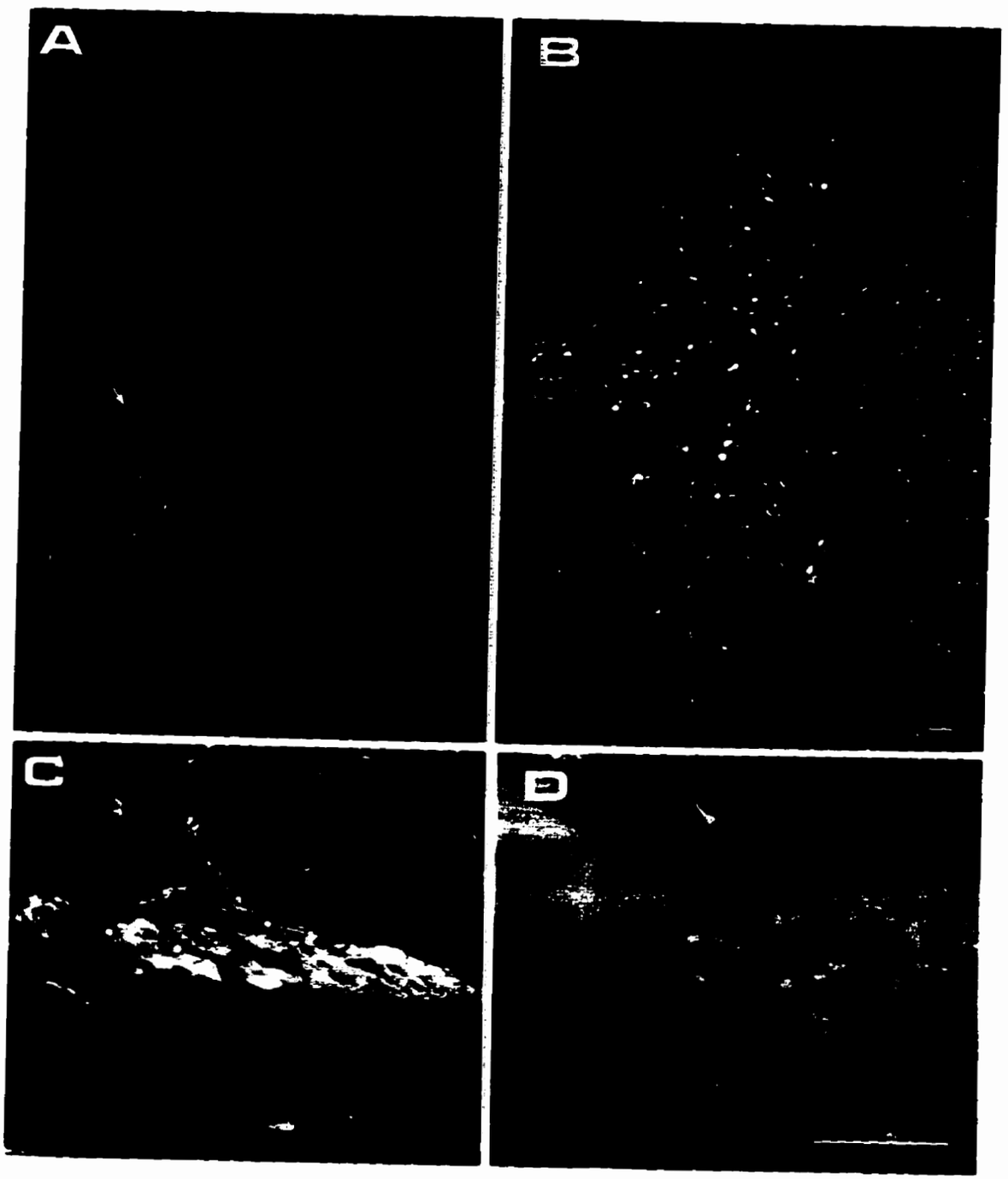

A
B
C
D

Figure 3.4 Flourescence micrographs of c-Myc-immunoreactivity in sections of the lumbar spinal cord (A,B) and the myenteric plexus of the human colon (C,D). Anti-MYC 033 only infrequently labelled the cytoplasm of spinal cord neurons (A, arrow) while anti-MYC 070 labelled most or all nuclei in the spinal cord (B). Cytoplasmic or cytoplasmic and nuclear labelling of the human myenteric plexus by anti-MYC 070 (C) contrasted with the exclusively nuclear labelling of these cells by anti-MYC 9E10 (D). Scale bars: 50μm.

antibody did not label nuclear antigens in the guinea pig cerebellum (data not shown) or spinal cord (Fig 3.4A) although occasionally cytoplasmic staining of neurons was observed in both tissues.

Anti-MYC 070 also labelled nuclear antigens in the ENS of the guinea pig (Figs 3.1C,F, 3.2C,D) and sheep (data not shown). Nuclei of guinea pig longitudinal muscle cells as well as many cells in the submucosal layer were also labelled, although this labelling appeared less intense than that within the enteric ganglia (see Figs 3.1C,F, 3.2C,D). In sections of the human colon, this antibody labelled cytoplasmic or cytoplasmic and nuclear antigens in the myenteric plexus (Fig. 3.4C) but failed to label antigens in the enteric ganglia of the rat, dog and monkey (data not shown). Anti-Myc 070 labelled most or all of the nuclei of neurons and glia throughout the guinea pig cerebellum (data not shown) and spinal cord (Fig 3.4B). All labelling was abolished by preincubation with the cognate peptide (see Fig 3.1D)

Anti-MYC 904 labelled only cytoplasmic structures in the enteric ganglia of the monkey ileum (Fig 3.3B). Double labelling with anti-MYC 033 and anti-MYC 904 confirmed that they were labelling overlapping, if not identical, cell populations (Fig 3.3C). Preincubation with the cognate peptide abolished labelling by both antibodies (data not shown).

Anti-MYC 9E10 labelled nuclei in the myenteric plexus of the adult human colon (Fig 3.4D). This labelling was not apparent in sections that were processed identically except that the primary antibody was not added (data not shown). No labelling of ENS cells was apparent in the other species (data not shown).

3.4b The Identity of Labelled Cells

In order to confirm the identity of cells in the guinea pig ENS that were labelled by anti-MYC 033 and anti-MYC 070, we measured the size and density of labelled nuclei in the submucosal plexus. The average density of nuclei labelled by anti-MYC 033 in preparations of ileal submucosal plexus was 6.9 $\pm$ 0.5 (mean $\pm$ SE; range 1 - 24) nuclei per ganglion (n=99 ganglia). The size of these nuclei ranged from 10.6 to 16.7μm with a mean size of 13.7 $\pm$ 0.3μm (n=28 nuclei, from 4 ganglia). The density of nuclei in preparations labelled by anti-MYC 070 was 17.2 $\pm$ 1.2 (range 4 - 41) nuclei per ganglion (n=67 ganglia). The sizes of nuclei were also more heterogenous than those labelled with anti-Myc 033, ranging from 7.1 to 16.5μm with a mean size of 11.2 $\pm$ 0.3μm (n=72 nuclei).

In one whole mount preparation of guinea pig ileum, double labelling with anti-MYC 033 and an antiserum against the neuropeptide vasoactive intestinal polypeptide (VIP, contained exclusively in neurons in these preparations, see Chapter 1) revealed that a subset of enteric neurons with c-Myc-IR were also immunoreactive for VIP (Figs 3.2E,F).

3.4c Stability of c-Myc Antigens

There were no apparent differences in labelling by either anti-MYC 033 or anti-MYC 070 following incubation of guinea pig ileum in the presence or absence of cycloheximide (data not shown). In ganglia of the submucous plexus, the density of anti-MYC 033-labelled nuclei ranged from 1 to 24 with an average of 6.4 $\pm$ 0.5 (n=80

ganglia). There were also no detected differences between c-Myc-IR in most of the guinea pigs that were anesthetized and 1 animal that was stunned and killed by exsanguination and whose tissues were removed and placed into cold buffer within 5 min (not shown).

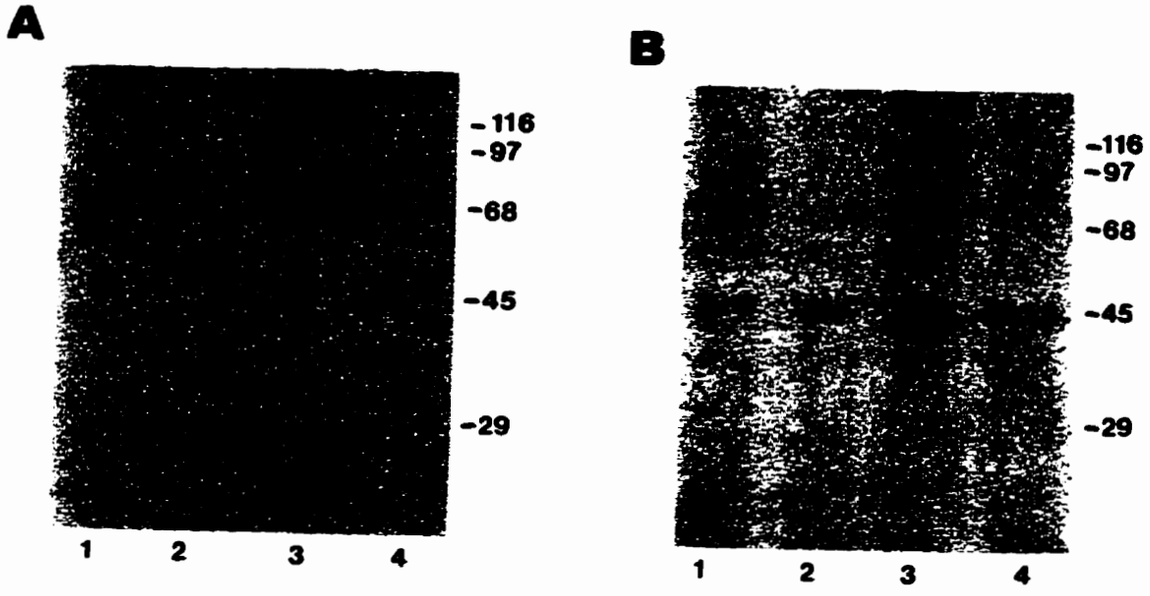
3.4d Immunoprecipitations

Anti-Myc 033 immunoprecipitated an apparently abundant antigen doublet that migrated in SDS-PAGE at approximately 120kDa, as well as a small cluster of antigens that migrated between 65 and 80kDa, from ileal preparations (Fig 3.5A). All these antigens appeared to be more abundant in the extract of longitudinal muscle/myenteric plexus than in the extract of circular muscle. Anti-MYC 070 specifically immunoprecipitated radiolabelled antigens of approximately 75kDa in addition to antigens of approximately 60kDa and 30kDa from both muscle layers of the guinea pig colon (Fig 3.5B). As with the anti-Myc 033 immunoprecipitated antigens, the signals from anti-Myc 070 antigens precipitated from the longitudinal muscle/myenteric plexus extract appeared stronger than from the circular muscle extract.

3.5 Discussion

3.5a Immunohistochemistry

The recognition of antigens in the ENS of six different mammalian species by one or more of four monoclonal antibodies raised against widely spaced peptide sequences of the human c-Myc protein supports the expression of c-Myc proteins in those cells.


A
-116
-97
-68
-45
-29
1
2
3
4
B
-116
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-45
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1
2
3
4

Figure 3.5 Autoradiograph of radiolabelled antigens immunoprecipitated by anti-MYC 033 from the ileum (A) and by anti-MYC 070 from the colon (B) of the guinea pig. The distribution of signal density between specifically precipitated antigens from longitudinal muscle/myenteric plexus preparations (lane 3 in A,B) and from circular muscle preparations (lane 1 in A,B) appeared to reflect the distribution of the myenteric plexus. Precipitation of antigens by anti-MYC 033 (A) of approximately 130, 120, 85, 75 and 68kd as well as a low molecular weight antigen of undetermined size (lanes 1 and 3) was prevented by preincubation with the cognate peptide (lanes 2 and 4). Precipitation of antigens by anti-MYC 070 (B) of approximately 75, 58 and 40kd (lanes 1 and 3) was also prevented by the cognate peptide (lanes 2 and 4) while antigens of approximately 40-50kd seemed unaffected (lanes 1-4).

Anti-MYC 033, which recognized nuclear antigens in the widest range of species, was raised against a peptide whose sequence is highly conserved among members of the Myc family (104,105) but nuclei of guinea pig CNS neurons were not labelled and so it probably does not recognize B-myc proteins (94) or non-specific neuronal antigens. The sequences recognized by anti-MYC 9E10, which only labelled nuclei in the human ENS, and anti-MYC 070, which labelled the ENS of the human as well as nuclei in the ENS of the guinea pig and the sheep, are not shared by identified Myc family members. On the other hand, enteric glia in the guinea pig and sheep intestine were also strongly labelled by anti-MYC 070 (see below) as were nuclei in the guinea pig CNS and so this antibody also seems likely to recognize some non-c-Myc antigens in those species. Interestingly, anti-MYC 904 and anti-MYC 033 were raised against almost identical peptide sequences but they labelled distinct intracellular compartments of monkey neurons and so they are likely to recognize distinct epitopes within that sequence.

Although labelling of the ENS by most of the anti-c-Myc antibodies was nuclear, cytoplasmic antigens were apparent in the myenteric plexus of the human colon labelled with anti-MYC 070 and in enteric neurons of the monkey ileum labelled with anti-MYC 904. Cytoplasmic c-Myc antigens have been reported previously (63,67,95-97,100,103,106) and it has also been suggested that the fixation and processing can affect the apparent localization of c-Myc antigens (97). On the other hand, cytoplasmic c-Myc proteins are abundant in non-proliferative *Xenopus laevis* oocytes (95) and have been detected by anti-MYC 904 in cytoplasmic extracts of human fibroblasts (103).

3.5b The Identity of Labelled Cells

Several of our observations support the suggestion that the anti-Myc antibodies label neurons in the ENS. First, although the average size of anti-Myc 033-labelled nuclei in the guinea pig submucous plexus (13.7μm) slightly exceeded a previous measure for neurons in this plexus (9.0μm) (107), possibly due to different methods used to visualize them, the extent of the range of sizes for labelled nuclei (10.6 - 16.7μm) was comparable with previous measurements (6.2 - 13.3μm) (107). Second, the density of anti-Myc 033-labelled nuclei (6.9/ganglion) was only slightly less than a previous measure of neuronal density (8.2/ganglion) (107) in the submucous plexus and is much less than total cell density. The difference in density between anti-Myc 033 labelled nuclei and the estimate for neurons may reflect some regional variation or observational bias (108), although it remains possible that a small subset of neurons were not labelled by anti-Myc 033. Third, the conspicuous nucleoli visible in cells labelled with anti-Myc 033 also suggests that the labelled cells are neurons as nucleoli in enteric glia are reported to be small or absent (109). Finally, double labelling with anti-MYC 033 and anti-VIP confirmed that at least a subset of the c-Myc immunoreactive neurons included those containing the widely distributed neuropeptide VIP (2,3). Thus the size, density and morphology of cells in the enteric ganglia labelled with anti-Myc 033 are consistent with their identity as neurons.

In contrast to the results with anti-Myc 033, the wide range of sizes, together with the high density of nuclei, labelled by anti-MYC 070 in the guinea pig submucous plexus led us to conclude that this antibody labelled nuclei of enteric glia as well as enteric

neurons. Nevertheless, the more intense labelling of nuclei in neurons compared to presumptive glia, muscle or submucous layer cells suggested that antigens recognized by anti-Myc 070 in these other cell types may differ quantitatively and/or qualitatively from the neuronal antigens.

3.5c The Stability of c-Myc Antigens

The apparent stability of c-Myc antigens in the guinea pig enteric ganglia in the presence of cycloheximide suggests that they are not as rapidly degraded as are Myc antigens in some cultured cell lines (91,110-113). Nevertheless, most previous studies have focused on proliferative cells and rapid turnover of c-Myc proteins may be an important component of cell division. Indeed, non-dividing *Xenopus* oocytes accumulate high levels of c-Myc proteins that are considerably more stable than similar proteins in proliferative *Xenopus* cells, possibly owing to post-translational differences (101). Furthermore, differentiation in Friend murine erythroleukemia cells is manifested by a withdrawal from the cell cycle and the stabilization of c-Myc proteins (114). In any event, the apparent stability of c-Myc antigens in enteric neurons makes it unlikely that levels of c-Myc proteins would substantially increase in an activity-related manner as is the case for expression of certain other proto-oncogene products in the ENS (82).

3.5d Immunoprecipitated c-Myc Antigens

Whereas both anti-MYC 070 and anti-MYC 033 precipitated antigens that migrated in the 60 - 80kDa range, which correspond with previous reports of the apparent molecular

mass of the most abundant c-Myc antigens (110,115-118), both antibodies precipitated antigens outside this range. A wide range of apparent sizes of c-Myc proteins has also been found previously in human cells (103,106,110,115-118), possibly due to potential transcriptional, post-transcriptional and post-translational events that might occur during c-myc expression in a cell type-specific manner and these events might also result in differences in the epitopes available for recognition by these antibodies. The different antigens immunoprecipitated by the two anti-Myc antibodies may partly reflect the different tissue sources (ileum and colon). However, as no differences in immunohistochemical labelling were apparent between the colon and ileum with either antibody this seems unlikely. The distribution between the guinea pig circular and longitudinal muscle preparations of antigens specifically precipitated by both antibodies appeared to reflect the distribution of the myenteric plexus. Interestingly, the pattern of c-Myc antigens specifically precipitated by anti-MYC 070 from the guinea pig colonic longitudinal muscle/myenteric plexus preparation appeared similar to c-Myc antigens precipitated by this antibody from c-myc over-expressing human cells (103).

The 120KDa doublet immunoprecipitated by anti-Myc 033 resembles similar sized doublets precipitated from human cells and *Xenopus* oocytes (103). However, the human and *Xenopus* antigens have been shown to be cytoplasmic and so the 120kDa antigens may not contribute to the nuclear c-Myc-IR of enteric neurons. The 60-80kDa antigens are then more likely candidates for the nuclear proteins. Indeed, the much weaker incorporation of radiolabel within those antigens may also be consistent with a low turnover rate of these proteins.

3.5e Conclusions

These data have demonstrated that proteins with antigenic properties, molecular sizes and the stability characteristic of some c-myc gene products are present in the ENS of a diverse range of mammals. This is the first reported evidence that c-Myc proteins are expressed in any differentiated neuronal population, although expression of c-myc mRNA has been found in neuronal cell types (63,93). Further work will be required to assess the functions of c-Myc proteins in the ENS. It is tempting to speculate that some previously suggested consequences of c-myc expression may have some relevance in the gut. For example, the regulation of genes affecting cell adhesion that has been attributed to c-myc expression (119-121) would seem vital in these cells in order that they might balance maintenance of synaptic contact with the flexibility to withstand the motility of the gut. In any event, the ENS might provide a setting for investigating the functions and regulation of c-Myc proteins in post-mitotic neurons.

CHAPTER FOUR

THE USE OF CONSTITUTIVE NUCLEAR ONCOPROTEINS TO LABEL NEURONS IN THE ENTERIC NERVOUS SYSTEM OF THE GUINEA PIG ILEUM

4.1 Introduction

Extensive studies have classified neurons of the enteric nervous system (ENS) according to electrophysiological, morphological and histochemical criteria (2,3). The use of neuron-specific markers visualized by immunohistochemistry has provided a particularly powerful tool to classify neurons. This has led to the concept of chemical coding of neurons on the basis of their content of one or more putative transmitters or other neuron-specific substances (3). However, the fraction of total neurons represented by each subtype is not completely clear. This is due in part to the lack of a simple but definitive method to allow accurate neuronal counts that can be combined with immunohistochemical subtyping. Methods which have been employed to count the total number of enteric neurons, such as toluidine blue (122,123) or NADH diaphorase staining (108), are not readily combined with immunohistochemistry. Immunohistochemical labelling of neuron-specific antigens such as neuron-specific enolase (NSE) also provides a method to observe elements of the ENS (124) and could be readily combined with immunohistochemical subtyping of neurons, but the dense labelling of the nerve fibres by these antibodies might interfere with the discrimination of individual cells. Immunohistochemical labelling with a "nerve cell body" antibody has recently been proposed as an appropriate technique for counting the total neuronal density in the guinea-pig small intestine because of its apparent specificity for neurons and the lack of nerve fibre staining (125-128). Nevertheless, like the other aforementioned techniques, the cytoplasmic labelling afforded by this antibody does not allow easy discrimination of adjacent and overlapping neurons. Nuclear or nucleolar counts are the preferred method of counting neurons in the central nervous system in order to

overcome this difficulty (129) and so an immunohistochemical method of labelling neuronal nuclei would be a potentially useful tool for quantifying neuronal subtypes in the ENS.

The products of the c-myc and c-fos proto-oncogenes (c-Myc and c-Fos, respectively) are nuclear phosphoproteins that participate in gene regulation (6,9). Stable (or constitutive) c-Myc antigens have been detected in neurons in the guinea-pig ENS (see Chapter 3), but only inducible c-Fos antigens had so far been reported in this tissue (81). In this study, the possibility of using anti-c-Myc antibodies to distinguish enteric neurons and glia and to count neurons was explored. The possibility that stable c-Fos-like-immunoreactivity (-IR) existed in the guinea-pig ENS and could be exploited in the same way was also investigated. Finally, double-labelling of enteric neurons with antibodies against these nuclear antigens and cytoplasmic antigens contained within specific subsets was performed to determine whether this approach might be useful as a counterstain to directly assess the proportions of those subsets.

4.2 Hypothesis

Labelling of constitutive nuclear Myc and Fos-like antigens will be useful to count neurons in the guinea pig ENS. This would require that they are contained in all enteric neurons but not in glia and that they can be demonstrated in any given subtype of neuron in double-labelled preparations.

4.3 Methods and Materials

4.3a Tissues and Antibodies

Twelve male albino guinea-pigs (250-450g) were used. Animals were anesthetized with halothane before segments of ileum were removed and then killed by exsanguination. Tissues were immediately fixed and whole mount preparations of myenteric plexus and submucosal plexus were dissected for immunohistochemical analysis as described in Chapter 2. Primary antibodies used were mouse anti-MYC 033, mouse anti-MYC 070, rabbit anti-FOS 4 and the peptide against which it was raised (representing amino acid residues 3-16 of the human c-Fos protein; cat# sc-52p), rabbit anti-human neuron specific enolase, rabbit anti-human protein gene product 9.5 (PGP 9.5), rabbit anti-S-100, rabbit anti-neuropeptide Y (NPY), rabbit anti-calretinin and mouse anti-calbindin. One whole mount preparation each of submucosal plexus was incubated in anti-MYC 070, anti-FOS 4 and either anti-NSE or anti-PGP 9.5. Whole mount preparations from 2 animals were double-labelled with anti-Fos 4 and anti-calbindin or with anti-MYC 033 and either anti-NPY or anti-calretinin.

The c-Myc-IR in the guinea pig ENS has previously been demonstrated to be abolished by preincubation of the primary antibody with its cognate peptide (see Chapter 3). In the control series for anti-FOS 4, tissues were also incubated in the primary antibody preabsorbed with the cognate peptide (2μg/ml).

4.3b Quantitative Data

The average neuronal density of ganglia labelled by anti-FOS 4 was assessed by counting these nuclei in each of 60 ganglia/animal on whole mount preparations of

longitudinal muscle/myenteric plexus from 4 animals and in each of 100 ganglia/animal on whole mount preparations of submucous plexus from 5 animals.

4.4 Results

4.4a Cytoplasmic and Nuclear Antigens in the ENS

As previously reported by others (124,130), both anti-NSE and anti-PGP 9.5 appeared to label neuronal elements of the submucosal plexus (Fig 4.1A,C) and the myenteric plexus (not shown). However, as also previously reported for NSE-IR in the pig small intestine (131), different levels of immunoreactivity were apparent in cell bodies labelled by either antisera. This ranged from almost no immunoreactivity to labelling that was so intense as to virtually obscure unlabelled neuronal nuclei (Fig 4.1A,C). In both cases visible ovoid or circular gaps in the labelling within ganglia and the ganglion interconnectives appeared to correspond to neuronal nuclei and glia (see below). The varying levels of immunoreactivity, the intense staining of nerve fibres and overlap of labelled cells in the ganglia all prevented accurate counts of neurons labelled by either of these antisera.

S-100-IR, which has been reported in enteric glia (132-134), was also apparent in the submucosal plexus (not shown) and the myenteric plexus (Fig 4.1E). As has been previously described (134), immunoreactive glial processes were abundant and formed basket-like networks around large gaps in the ganglionic labelling that appeared to correspond to neuronal cell bodies (Fig 4.1E). Glial cell bodies in ganglia of the submucosal plexus (not shown) and the ganglion interconnectives of both plexuses (see

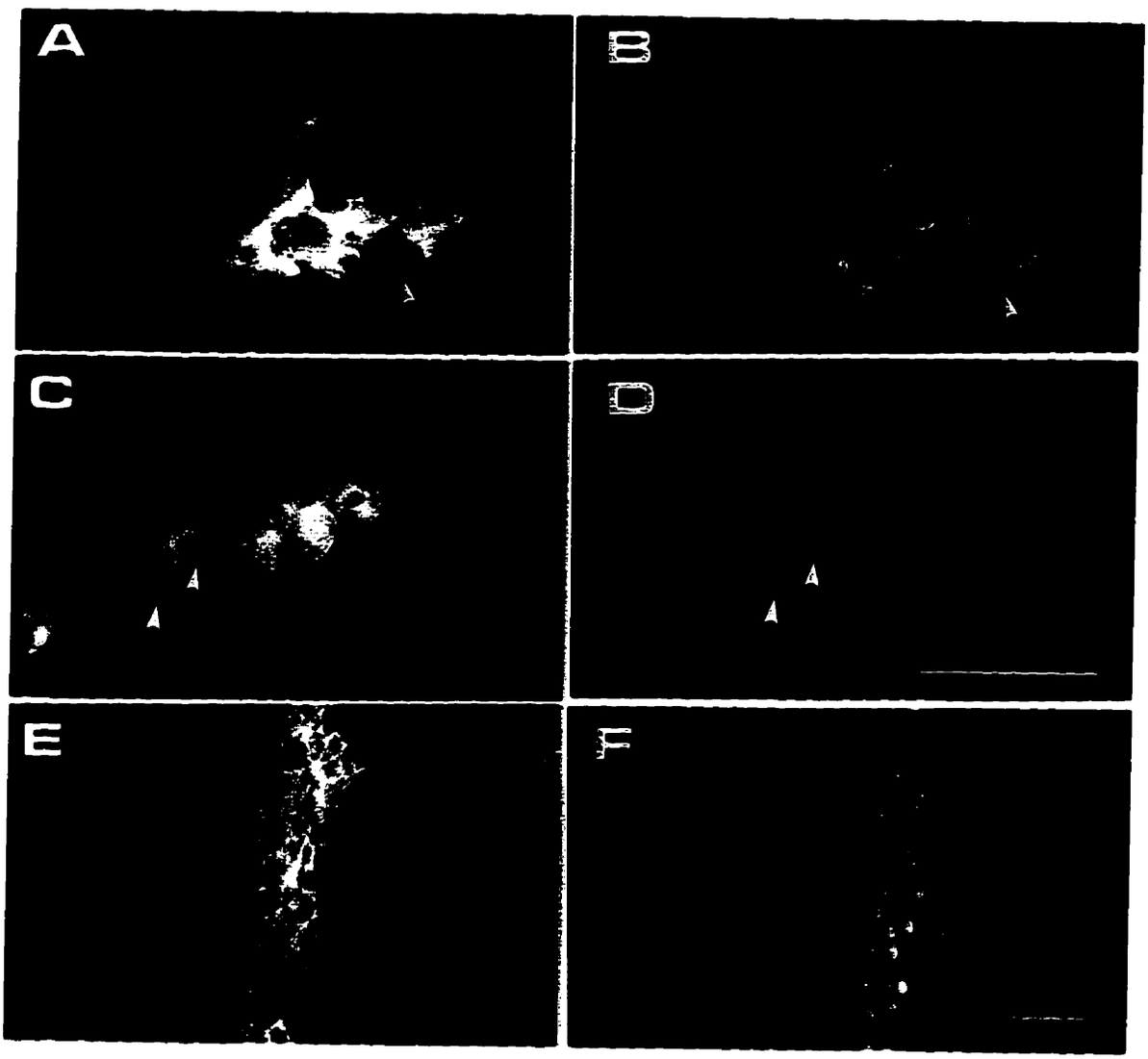
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Figure 4.1 Fluorescence micrographs of submucosal ganglia (A-D) and myenteric ganglia (E,F) labelled with anti-NSE (A), anti-PGP 9.5 (C), anti-S-100 (E) or anti-MYC 033 (B,D,F). Submucosal ganglia labelled with anti-NSE (A) and anti-MYC 033 (B) demonstrated that all NSE-immunopositive neurons were also labelled by anti-MYC 033 but not all neurons were strongly NSE-immunoreactive (arrows). Submucosal ganglia labelled with anti-PGP 9.5 (C) and anti-MYC 033 (D) showed that all PGP 9.5-immunopositive neurons were also labelled by anti-MYC 033 but PGP 9.5-immunoreactivity was also weak in some neurons (arrows). Labelling of the myenteric plexus by anti-S-100 (E) and anti-MYC 033 (F) confirmed that S-100-immunopositive glia, clearly evident in the ganglion interconnectives, were not labelled by anti-MYC 033 and that gaps in the ganglionic S-100-immunoreactivity that corresponded to the positions of neurons contained anti-MYC 033-labelled nuclei. Scale bars: 100μm (Bar in D is for panels A-D, bar in F is for panels E,F).

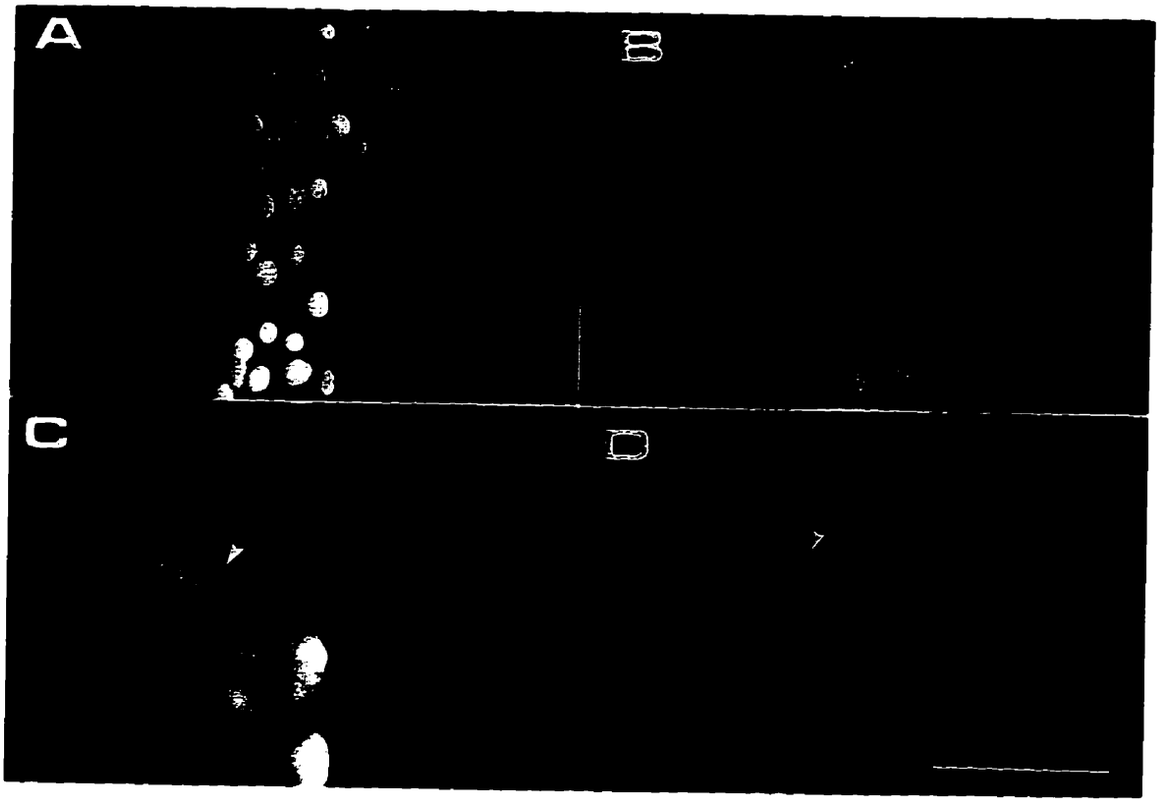
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Figure 4.2 Fluorescence micrographs of a myenteric ganglion (A,B) and a submucosal ganglion (C,D) labelled with anti-FOS 4 (A), anti-MYC 033 (B), a combination of anti-FOS 4 and anti-NSE (C) and anti-MYC 070 (D). Nuclei in myenteric ganglion that were labelled by anti-FOS 4 (A) were identical to those labelled by anti-MYC 033 (B) but anti-FOS 4 also weakly labelled the nuclei of smooth muscle cells (A). Gaps left in the submucosal ganglion labelled by anti-NSE and anti-FOS 4 (C, arrow) contained small nuclei that appeared to be glial when labelled by anti-MYC 070 (D, arrow). Scale bar: 100μm.

Fig 4.1E) were readily identifiable by a thin layer of labelled cytoplasm surrounding the nuclei. Within the myenteric ganglia, however, the intense fibre labelling interfered with discrimination of all but a few glial cell bodies (Fig 4.1E).

As previously reported (Chapter 3), anti-MYC 033 labelled nuclei throughout the ganglia of the myenteric (Figs 4.1F, 4.2B) and submucosal plexuses (Fig 4.1B,D). The labelled nuclear profiles were predominantly large and circular or ovoid with visible nucleoli. Small variations in the intensity of the labelling between nuclei were occasionally observed. In contrast, anti-MYC 070 labelled a population of nuclei in the myenteric plexus (not shown) and submucosal plexus (Fig 4.2D) that appeared to include smaller nuclei with more variable profiles and lacking visible nucleoli. Labelling of extraganglionic nuclei of smooth muscle (not shown) and other unidentified cell types in both plexuses labelled by anti-MYC 070 was also evident (see Fig 4.2D). In sections, but not whole mounts, labelled with anti-MYC 033 using the more sensitive fluorochrome, CY3, very faint smooth muscle nuclei labelling could also be seen. These observations are consistent with predominantly neuronal labelling by anti-MYC 033 and labelling of perhaps all cell nuclei by anti-MYC 070 (see also Chapter 3). Double-labelling with anti-MYC 033 and anti-S-100 established that nuclei labelled by anti-MYC 033 did not include any S-100-immunoreactive cells. All visible gaps in the S-100-immunoreactivity in ganglia, corresponding to neuronal cell bodies, contained anti-MYC 033 labelled nuclei (Fig 4.1E,F). In addition, double-labelling with anti-MYC 070 and anti-S-100 confirmed that the smaller nuclei labelled in the ganglia and interconnectives by anti-MYC 070 included all visible S-100-IR glia (not shown).

Anti-FOS 4 also labelled a population of nuclei in the enteric plexuses whose appearance, sizes and distribution appeared identical to those labelled by anti-MYC 033 (Fig 4.2A). Anti-FOS 4 also faintly labelled the nuclei of smooth muscle cells. While in most cases the labelling was uniformly distributed in the nucleus, a small number of nuclei displayed a punctate pattern of labelling and a small variation in the intensity of labelling was occasionally observed (not shown). Double-labelling with anti-FOS 4 and anti-MYC 033 confirmed that the cell populations in the enteric ganglia labelled by these antibodies were identical (Fig 4.2A,B). Preincubation with the cognate peptide abolished the labelling by anti-FOS 4 (not shown).

4.4b Constitutive Myc and Fos Antigens as Pan-Neuronal Markers

In order to establish the proportion of neurons labelled by anti-MYC 033 (and hence also anti-FOS 4), double labelling with anti-MYC 033 and anti-NSE or anti-PGP 9.5 were performed. In both cases, all strongly immunoreactive cell bodies were found to contain nuclei labelled by anti-MYC 033 (Fig 4.1A-D). However, because neither anti-NSE nor anti-PGP 9.5 labelled all neurons, it often could not be determined from these preparations whether the small ovoid gaps in both plexuses that lacked anti-MYC 033-IR belonged to glia or weakly NSE- or PGP 9.5-immunoreactive neurons (see Fig 4.2C). In order to address this problem, a combination of anti-FOS 4 and anti-NSE or anti-PGP 9.5 together with anti-MYC 070 was used so that nuclei within the gaps left by the anti-FOS 4 and the cytoplasmic marker could be observed by their immunoreactivity with the anti-MYC 070. Following this procedure, these gaps were found to contain small nuclei that were typical of

glia (Fig 4.2C,D).

In order to establish a quantitative estimate of the ganglionic neuronal density in the guinea pig ileum, neuronal nuclei labelled by anti-FOS 4 were counted in whole mount preparations of longitudinal muscle/myenteric plexus and submucosal plexus. Ganglionic neuronal density was chosen in order to avoid differences arising from variations in the degree of tissue stretch that might confound measures of density according to area or length of the intestine. Ganglia were defined as components of the plexus containing one or more nucleated nerve cell profiles (108). The ganglionic density of nuclei labelled by anti-FOS 4 in the myenteric plexus was found to be 52.5 $\pm$ 2.9 nuclei/ganglion (mean $\pm$ SEM; n=4 animals; range, 47.6 - 59.8 nuclei/ganglion). The range of nuclei in each ganglion from all animals was from 1 to 461 nuclei/ganglion. In the submucous plexus the mean ganglionic density was 7.02 $\pm$ 0.12 nuclei/ganglion (n=5 animals; range, 6.56 - 7.23 nuclei/ganglion). The range of nuclei in each ganglion from all animals was from 1 to 34 nuclei/ganglion.

4.4c Constitutive Myc and Fos Antigens as Counterstains in Double-Labelling Immunohistochemistry

Neurons immunoreactive for NPY, calretinin and calbindin were also found in both plexuses as previously described (2,3,18,125,135,136). Briefly, NPY neurons could rarely be discerned in the myenteric plexus but were more abundant in the submucosal plexus where they were usually situated along the periphery of ganglia. Both calbindin and calretinin neurons were abundant in myenteric ganglia and calretinin neurons were also

plentiful in submucosal ganglia but calbindin neurons were only rarely seen in the submucosal plexus.

In Chapter 3, c-Myc-IR had been found to exist within neurons that contained VIP-IR. In order to establish whether c-Myc was also contained within other neurochemical subsets of these cells, whole mounts of both plexuses were double-labelled with anti-MYC 033 and one of anti-VIP, anti-NPY or anti-calretinin, or with anti-Fos 4 and anti-calbindin. Anti-MYC 033 also effectively labelled nuclei of neurons with NPY- or calretinin-IR, while anti-FOS 4 was similary effective in counterstaining nuclei of preparations labelled with anti-calbindin (Fig 4.3).

4.5 Discussion

4.5a Myc and Fos Antigens as Pan-Neuronal Markers

Previous attempts to measure the density or numbers of enteric neurons have employed cytoplasmic labels that in general do not afford either optimal resolution or convenience for combination with immunohistochemical subtyping. We have provided evidence that constitutively expressed oncoproteins can be used as non-selective immunohistochemical markers of guinea pig enteric neurons. The discrete nuclear immunoreactivity of these markers reduces interference with immunohistochemical analysis of cytoplasmic antigens. Furthermore, because the antibodies anti-MYC 033 and anti-FOS 4 give identical results and have been raised in different species (mouse and rabbit, respectively), there exists options for combining them with a range of primary antisera.

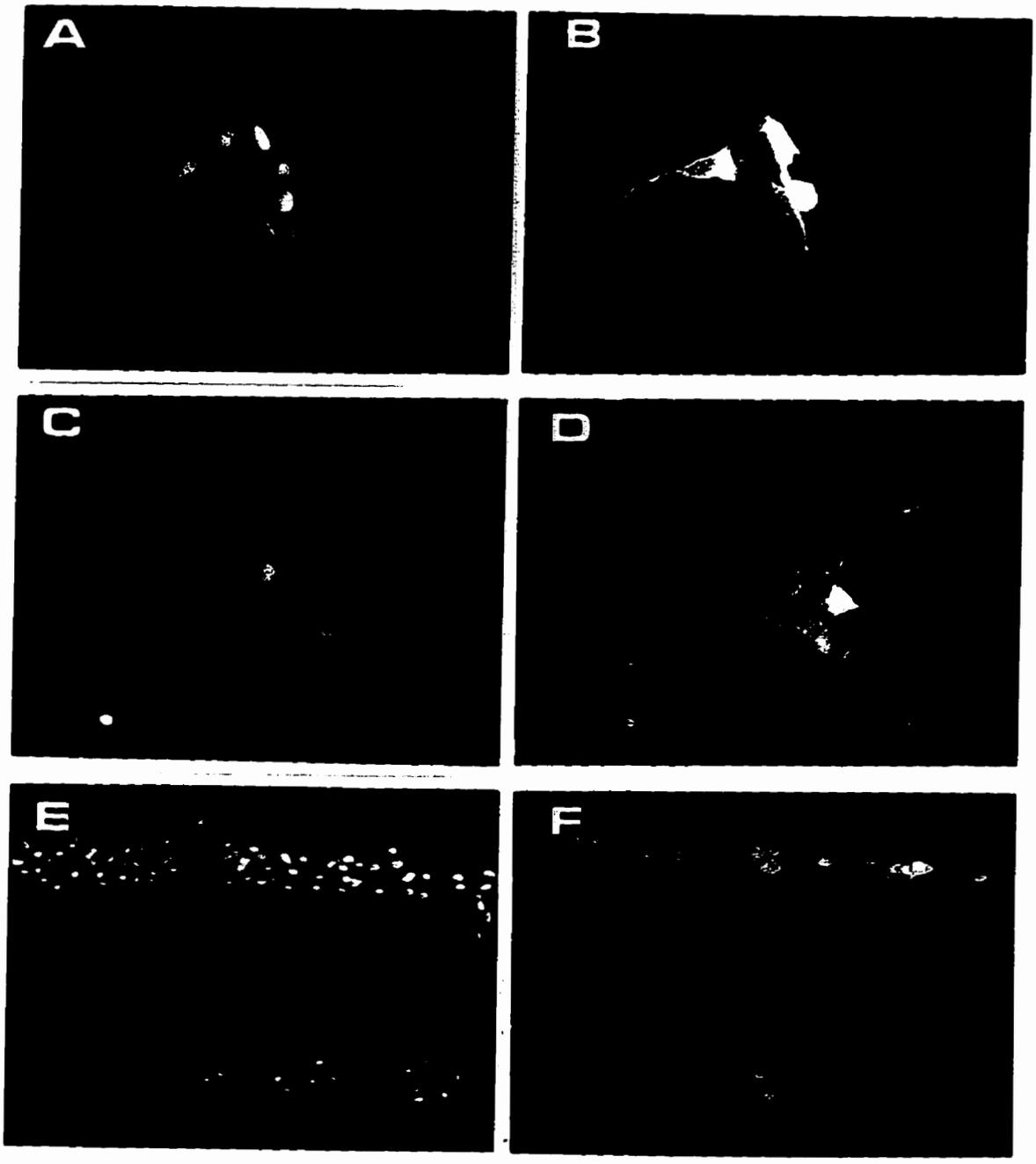
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Figure 4.3 Fluorescence micrographs of immunohistochemical double-labelling of submucosal (A-D) and myenteric ganglia (E,F) double-labelled with anti-MYC 033 (A,C) and anti-NPY (B) or anti-calretinin (D), or with anti-FOS 4 (E) and anti-calbindin (F). Immunoreactivity for these nuclear antigens was contained in all tested subsets and the nuclear labelling appeared to provide a suitable counterstain to visualize all neurons. Scale bar: A-D = 100μm, E,F = 200μm.

Anti-MYC 070 appeared to label every nucleus in each preparation. While this apparent lack of specificity precludes its use as a specific cell-type marker, the characteristics of the labelled nuclei still appear to allow neurons and glia to be distinguished with some reliability. Furthermore, use of this antibody may also allow observation of cells that are associated with the enteric plexuses but that are not labelled by neuronal- or glial-specific markers.

Both NSE and PGP 9.5 appear to be excellent markers of gastrointestinal nerve fibres, but because of this they are of limited use for visualizing individual elements in enteric ganglia (124,127,130,137). In addition, the levels of both NSE- and PGP 9.5-LI were clearly variable among ENS neurons. Possible reasons for variable levels of NSE labelling in the pig ENS have been discussed (131) and it might be interesting to determine whether the levels of PGP 9.5 antigens parallel those of NSE antigens in individual enteric neurons.

Our measured density of neurons in the myenteric ganglia of the guinea pig is markedly different from that of Young et al. (128) who reported about 105 neurons/ganglion. This difference was unlikely to have resulted from different labelling by the nerve cell body antibody used by those authors and the nuclear antibodies used in the present study because we have found that the nerve cell body antibody and anti-MYC 033 label the identical cells in double-labelled preparations (data not shown). In fact, the approach those authors employed was based on a small sample biased toward intermediate or large ganglia, and single neurons were considered as extraganglionic. Indeed, when myenteric ganglia containing less than 4 neurons were omitted from our data, we obtained a

value of 111.5 ± 9.1 neurons/ganglia which more closely approximates values from those previous studies. However, since small ganglia in the submucosal plexus are not similarly excluded, we felt this was unjustified. Our reported values more closely resemble those of Furness and Costa (122) and Wilson et al. (123) who reported about 45 neurons/ganglion for the myenteric plexus and 8 neurons/ganglion for the submucous plexus. Since subjectivity is unavoidable when determining the boundaries of ganglia (108), it may also account for some of the differences between previous data and our own, particularly because the boundaries of the nerve cell profiles were not as conspicuous using a nuclear label as they might have been with a cytoplasmic label.

4.5b The Identity of the c-Fos Antigen

The pan-neuronal distribution of the c-Fos antigen recognized by anti-FOS 4 in guinea pig enteric ganglia suggests that it is not identical to the inducible c-Fos antigen reported by Kirchgessner et al. (82). Attempts to identify the antigen(s) labelled by this antibody in Western blot analysis have been unsuccessful (A.W. Gibson, unpublished data), and this antibody did not react similarly with other species (data not shown). It therefore seems likely that this nuclear labelling represents a spurious, albeit fortuitous, cross-reactivity of the anti-FOS 4 antibody, although for the purpose of brevity it will henceforth be referred to as Fos-like.

4.5c Myc and Fos Antigens as Counterstains

The pan-neuronal distribution of c-Myc and Fos-like antigens is further supported by their presence in all neurochemical subsets tested to date. Indeed, neurons with VIP, NPY or calretinin are likely to account for more than 80% of submucosal neurons in the guinea pig ileum (2,3,18,125,135) while calbindin is present in about 20% of myenteric neurons (136). These double-labelled preparations also offer a more precise technique to directly assess the proportions of these neurochemical subsets than is afforded by indirect comparisons of neuronal density. In fact, Krista Amundson in our laboratory has since extended this work by using this technique to reassess proportions of various neurochemical subsets in the ileum, to compare those with similar subsets in other gastrointestinal organs, and to detect changes in those proportions in a model of inflammation (54). On the other hand, the reassessment of tested subsets in the ileum has mostly agreed with previous estimates from single-labelled preparations, thus attesting to the great care with which those previous studies had been performed.

4.5d Conclusions

These data have demonstrated that c-Myc and c-Fos-like antigens are present in all enteric neurons of the guinea pig ileum, although the role of c-Myc antigens and the identity of constitutive c-Fos-like antigens are both unclear. Notwithstanding this uncertainty, both anti-FOS 4 and anti-MYC 033 are potentially quite useful anatomical tools as pan-neuronal markers and counterstains for immunohistochemical subtyping of enteric neurons in the guinea pig intestines.

CHAPTER FIVE

NUCLEAR FOS AND JUN ANTIGENS IN THE ENTERIC NERVOUS SYSTEM OF THE GUINEA PIG ILEUM

5.1 Introduction

Immunohistochemistry is being used in a growing number of studies to demonstrate activation of inducible Fos and Jun proteins in neurons. Identification and quantification of positively stained cells is facilitated by the nuclear localization of Fos and Jun antigens, and "double-labelling" techiques can also be employed to distinguish between their expression in various subsets of neurons and other cells (see Chapters 1, 2). Fos and Jun proteins form interactive components of a transcriptional regulatory complex whose activity is likely affected differently by various Fos and Jun family proteins (6,11). Basal levels of c-Fos- and JunB-immunoreactivity (-IR) are usually undetectable in the central nervous system (CNS) but are often coordinately upregulated in many neurons following depolarizing stimulation and might then be useful to detect transynaptic activity in a variety of paradigms (70-72,74). On the other hand, basal levels of c-Jun-IR can often be detected in at least some CNS neurons and these levels may be preferentially affected by signals associated with regeneration, degeneration and apoptosis (7,74-76).

Immunohistochemistry has also previously been used to identify "chemically coded" subsets of neurons in the enteric nervous system (ENS). Various combinations of neural antigens are contained in neuronal subsets with characteristic morphology, electrophysiology and presumably function (2,3). Studies of chemical coding in the ENS of the guinea pig ileum may have accounted for all neurons in both the submucosal and myenteric plexuses (3). For example, the submucosal plexus appears to be comprised of four major exclusive subsets of neurons that contain either vasoactive intestinal polypeptide (VIP), neuropeptide Y (NPY), calretinin or substance P, while large subsets of myenteric

neurons include about 20% that contain calbindin. Putative functions have been assigned to various of these subsets based on these anatomical and functional characteristics although direct demonstrations of neurons with mechanosensory and visceromotor functions have been difficult to achieve.

Kirchgessner and her colleagues reported that submucosal sensory neurons with substance P and calbindin activated c-Fos-IR in response to villus distortion in isolated ileal preparations (82). Hence, it seemed likely that detection of c-Fos could be readily applied to analysis of ENS activity, although it remained to be determined whether all neuronal subsets were likely to share a similar capacity to express c-Fos. Moreover, it was also unclear whether c-Fos activation in those isolated preparations was limited to neurons, and the distribution and responses of Jun family proteins in the ENS had not yet been described.

Since the only report of acute c-Fos activation in the ENS (82) suggested that this approach was compatible with analysis of isolated ileum, this study was initiated to examine the properties of inducible Fos and Jun expression in those preparations. Some limitations to this approach were noted as dissection was associated with considerable basal expression of some nuclear antigens. Notwithstanding these difficulties, depolarizing stimuli were used to test activity-related expression of c-Fos-, JunB- and c-Jun-IR in various subsets of enteric neurons and in enteric glia.

5.2 Hypothesis

Expression of nuclear c-Fos- and JunB-IR will be activated within all subsets of enteric neurons by depolarizing stimulation of ileal segments and they may also be

expressed in enteric glia. Some basal expression of c-Jun-IR may be evident but it might not be detectably altered by those stimuli.

5.3 Methods and Materials

5.3a Animals and Tissues

Experiments were performed on 10 adult male albino guinea pigs (180-550g). Prior to use all animals were fasted for 12h, but were allowed water ad libitum. Animals were anesthetized with halothane (4% in oxygen) and 3 segments of approximately 5cm, from about 5 to 20cm from the ileocecal junction, were removed. These segments were either fixed and processed as whole mounts of myenteric and submucous plexuses for immunohistochemistry (see Chapter 2) or incubated as described below.

5.3b Incubation of Isolated Ileal Segments

In preliminary experiments, c-Fos was found to be induced in perhaps all enteric neurons of incubated preparations when the enteric plexus layers had been dissected, when the intestines were opened to expose the lumen or when the ends of the segment were ligated with a suture. Therefore, all subsequent experiments were performed on ileal segments that were open at both ends, incubated for 5min in an oxygenated Krebs' solution at 37°C and then treated for one hour in one of the following ways: (a) Krebs' solution was maintained; (b) Krebs' solution was changed every 5min; (b) 20μM veratridine in Krebs' solution for 5min intervals separated by 5min incubations in normal Krebs' solution; (c) 2μM veratridine in Krebs' solution for 5min intervals separated by 5min incubations in

normal Krebs' solution; (d) Krebs' solution containing 50mM K^+ replacing equimolar Na^+ was added; (e) 50mM K^+ Krebs' solution for 5min intervals separated by 5min incubations in normal Krebs' solution; (d) 50mM K^+ Krebs' solution containing 0 Ca^{++} and 1mM ethylene glycol tetraacetic acid (EGTA) was added; (e) 50mM K^+ Krebs' solution containing 100μM hexamethonium was added. All incubating solutions also contained 1μM nifedipine to block motility. Tissues were then incubated in normal Krebs' solution (or Ca^{++}-free Krebs' with EGTA for (d)) for an additional 2h to allow for IEG expression before they were cut open, pinned, fixed and processed for immunohistochemistry in the same way as untreated tissues.

5.3c Primary Antibodies

Primary antibodies used were: mouse anti-MYC 033 and rabbit anti-FOS 4 (used as counterstains in double-labelling experiments to count enteric neurons); mouse anti-FOS TF161; rabbit anti-FOS TF3; rabbit anti-c-JUN; rabbit anti-JUNB; rabbit anti-VIP; rabbit anti-calretinin; rabbit anti-NPY; mouse anti-calbindin (see Chapter 2, Table 1).

5.4 Results

5.4a C-Fos, JunB and c-Jun in Untreated Intestine

We examined c-Fos- and JunB-IR in the enteric nervous system of normal, unincubated ileal segments in order to compare this with labelling in segments incubated as outlined below. In the myenteric and submucosal plexuses of normal guinea pig ileum, nuclei labelled by polyclonal anti-FOS TF3 and anti-JUNB were rare. In 6 of 10 animals

no labelling was observed in either plexus by anti-FOS TF161. Very light nuclear labelling by anti-FOS TF161 was sometimes found in some neuronal nuclei in either myenteric or submucosal ganglia (2 animals each). Anti-c-JUN faintly labelled a very small proportion of nuclei in the myenteric plexus but labelled many or all nuclei in the submucosal plexus (n = 3 animals; Fig 5.1). However, this labelling was not affected by any of the depolarizing stimulation (n = 2 each of veratradine and K^+ stimulated segments; see below) and so it was not tested further. Anti-FOS TF3 and anti-JUNB lightly labelled the cytoplasm and ganglionic interconnectives and more heavily labelled the cytoplasm of a small number of neurons in both plexuses of all normal and incubated segments (see Figs 5.2, 5.3F). No labelling by any of the antisera was apparent in muscle cells or extraganglionic submucosal cells.

Neurons immunoreactive for VIP, NPY, calretinin and calbindin were apparent in both plexuses (see Chapters 3, 4). In double-labelled tissues, calbindin-IR was confirmed to exist in a population of neurons mostly distinct from those containing VIP- or NPY-IR, although a small proportion of myenteric neurons contained both calbindin- and calretinin-IR (not shown). In double-labelled tissues from those untreated animals in which submucosal neurons expressed c-Fos-IR, that was found to occur exclusively in VIP-immunoreactive neurons.

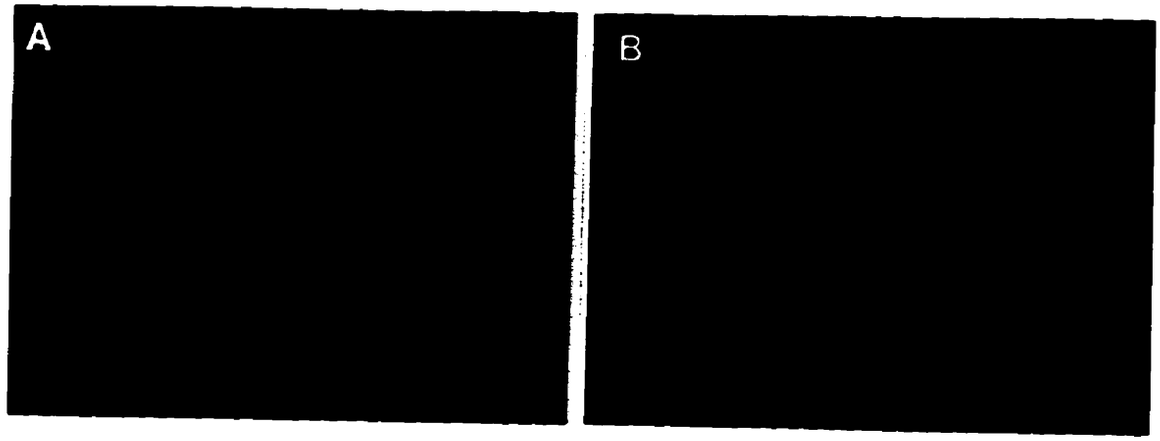
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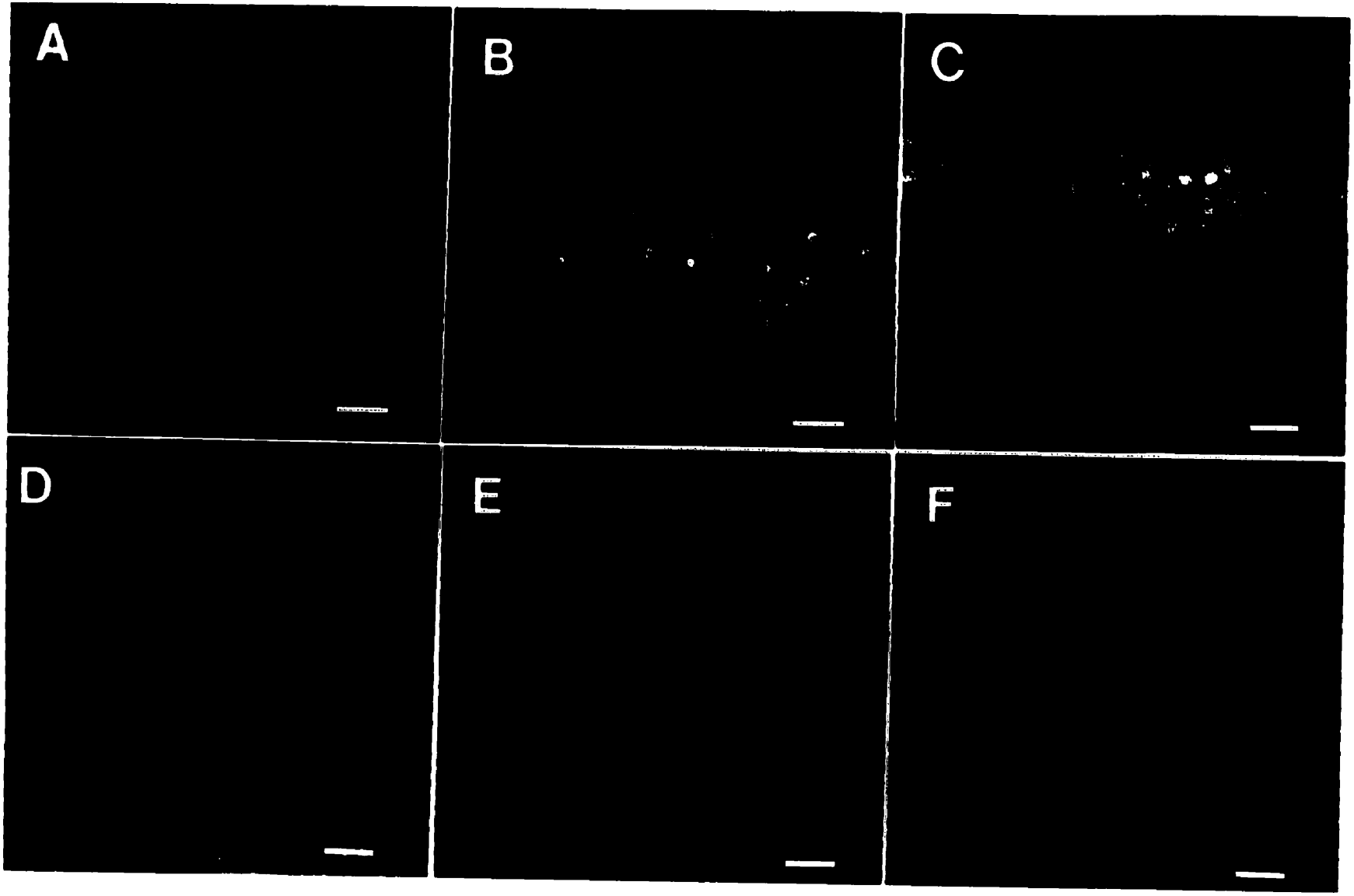
Figure 5.1 Fluorescent micrographs of immunoreactivity for c-Jun in the myenteric and submucosal plexuses of untreated guinea pig ileum. Very little labelling was found in the myenteric plexus (A) while most or all nuclei of submucosal neurons were labelled (B). Scale bars: A = 200μm, B = 100μm.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E
F

Figure 5.2 Fluorescent micrographs of c-Fos- and JunB-immunoreactivity in the myenteric plexus. Labelling of smooth muscle nuclei was apparent in all incubated segments. Labelling by anti-FOS TF161 was largely absent in ganglia of segments incubated in normal Krebs' solution (A). Neuronal and glial nuclei of segments incubated with 20μM veratridine were labelled by anti-FOS TF161 (B). Anti-FOS TF3 labelled neurons in segments stimulated by 50mM K^+ (C). Labelling by anti-JunB was largely absent in segments incubated in normal Krebs' solution (D). 100μM hexamethonium did not prevent the appearance of JunB-immunoreactivity in K^+-stimulated segments (E). Neuronal, but not glial, JunB-immunoreactivity was mostly abolished when extracellular Ca^{++} was removed from the incubation solution (F). Scale bars: 50μm.

A
B
C
D
E
F

Figure 5.3 Fluorescent micrographs of c-Fos- and JunB-immunoreactivity in the submucosal plexus. In segments incubated in normal Kreb's solution, neurons immunoreactive for VIP (A) were predominantly those that expressed Fos (B; anti-FOS TF161). In K^+-stimulated segments, the number of JunB-immunoreactive neuronal nuclei was increased (C). The labelled nuclei of muscle cells in a blood vessel can also be seen. In segments stimulated with veratridine, neurons immunoreactive for NPY (D) were among those labelled by anti-FOS TF161 (E, arrows). K^+-stimulation also increased the number of neuronal nuclei labelled by anti-FOS TF3 (F) but did not appear to alter the number of neurons with cytoplasmic labelling (arrow). Scale bars: 50μm.

5.4b C-Fos and JunB in Incubated, Unstimulated Tissues

In the myenteric plexus of segments incubated for 3h in normal Krebs' solution (8 animals), c-Fos- and JunB-IR in most ganglia was not different from untreated controls (Figs 5.2A, D). However, a few ganglia contained robust nuclear c-Fos- and JunB-IR in neurons and glia. Ganglia at the mesenteric border and both cut ends of all incubated segments, including those with visible damage, more frequently displayed this enhanced labelling. In areas of the myenteric plexus lacking visible damage, only 0.46 $\pm$ 0.31% (mean $\pm$ SEM; 90 ganglia from 3 animals) of myenteric neurons were labelled by anti-Fos TF161 while 3.8 $\pm$ 3.0% (120 ganglia from 4 animals) were labelled by anti-JUNB. No clear preference of c-Fos or JunB expression for VIP, NPY, calretinin or calbindin neurons was observed in any ganglia containing labelled nuclei. Glia were recognizable by their nuclear morphology and their identity was confirmed in tissues double-labelled with antibodies that labelled constitutive neuronal nuclear antigens and with anti-S100 (see Chapter 4). Glia and weakly immunoreactive neurons were always more clearly visible with anti-FOS TF161 than with either of the polyclonal antisera. Nevertheless, in myenteric plexus and submucosal plexus preparations from all incubated segments that were double-labelled with anti-FOS TF161 together with anti-FOS TF3 or anti-JUNB, it was apparent that these antigens were contained in overlapping and perhaps identical cell populations. In addition, nuclei of muscle cells both in the blood vessels and in the muscle coats of all incubated segments were immunoreactive for c-Fos and JunB (see Figs 5.2, 5.3C).

In the submucosal plexus of these segments a larger proportion of neurons in areas lacking visible damage expressed c-Fos- (25.4 ± 4.3%; 90 ganglia from 3 animals; anti-FOS TF161) and JunB-IR (13.4 ± 7.7%; 120 ganglia from 4 animals; Fig 2B). Anti-FOS TF161 labelling was preferentially localized in VIP neurons (Figs 5.3A,B) but small numbers of NPY and calretinin neurons also expressed this antigen. Calbindin neurons only rarely expressed antigens detectable by anti-FOS TF3 and anti-JUNB. Glial expression of c-Fos- and JunB-IR was usually rare but, as in the myenteric plexus, ganglia nearer the mesenteric border and those in visibly damaged areas of all incubated segments more frequently displayed c-Fos and JunB antigens in neurons and glia. Small numbers of unidentified extraganglionic cells in the submucosa of all incubated segments also expressed c-Fos- and JunB-IR.

5.4c C-Fos and JunB in Segments Incubated with Depolarizing Stimulation

Stimulation by 20μM veratridine (5min on, 5min recovery; segments from 3 animals) for the first hour of incubation resulted in a marked increase in the numbers of neuronal nuclei in myenteric ganglia that expressed c-Fos and JunB-IR (Fig 1B). Approximately 83% and 71% (60 ganglia each from 2 animals) of myenteric neurons were labelled by anti-FOS TF161 and anti-JUNB, respectively. Many neurons of all tested subsets expressed these antigens with no obvious subset preference. Glial labelling by anti-FOS TF161 was also apparent in myenteric ganglia and ganglionic interconnectives.

Increased numbers of c-Fos- and JunB-immunoreactive neurons were also observed in the submucosal plexus of these segments (Fig 5.3E). Approximately 82% and 59% (60

ganglia each from 2 animals) of submucosal neurons were labelled by anti-FOS TF161 and anti-JUNB, respectively. All neuronal subsets exhibited enhanced c-Fos or JunB labelling with no obvious subset preference (Figs 5.3D, E). Furthermore, there appeared to be a greater number of unidentified extraganglionic cells that expressed c-Fos and JunB antigens. The number of labelled glia did not appear to be markedly elevated.

In order to test for dose dependency of veratridine stimulated IEG expression, the effect of 2μM veratridine was tested in one experiment. The apparent proportions of JunB- and c-Fos-immunoreactive neurons were again elevated in both plexuses compared to paired unstimulated controls but not to the levels seen in segments treated with 20μM veratridine.

Stimulation by 50mM K^+ for the first hour of the incubation period also elicited a marked increase in the numbers of neuronal nuclei that expressed c-Fos and JunB antigens in the myenteric plexus (Fig 5.2C). For example, 28.5 $\pm$ 0.5% (90 ganglia from 3 animals) of myenteric neurons were labelled by anti-JUNB following continuous exposure to this stimulus for 1h. No qualitative differences were observed when the stimulus was delivered periodically (5min on, 5min recovery; 3 animals). Similar to the veratridine stimulated segments, neurons of all tested subsets expressed these antigens with no obvious subset preference. Anti-FOS TF161 also labelled many glial nuclei in myenteric ganglia and ganglionic interconnectives.

In the submucosal plexus, periodic administration of 50mM K^+ Krebs' solution did not markedly alter the density of neurons that expressed nuclear c-Fos- or JunB-IR compared to paired unstimulated controls. When the stimulus was maintained for 1h, however, marked increases in the numbers of both c-Fos- and JunB-immunoreactive

submucosal neurons were apparent (Figs 5.3C, F). For example, 37.9 ± 9.0% (90 ganglia from 3 animals) of submucosal neurons were labelled by anti-JUNB. Increases were also observed in the proportions of all neuronal subsets that expressed c-Fos- or JunB-IR and, again, no subset preference was apparent.

Hexamethonium (100μM) was used to block nicotinic acetylcholine receptors in K^+-stimulated segments but this did not appear to affect the labelling in either the myenteric plexus or the submucosal plexus preparations compared to paired stimulated controls (Fig 5.2E). Nevertheless, when the K^+ stimulus was delivered in the absence of extracellular Ca^{++} together with a compensating increase in Mg^{++} and the addition of 1mM EGTA (3 animals), the nuclear c-Fos- and JunB-IR in neurons of both plexuses was almost totally abolished. Interestingly, c-Fos-immunoreactive glial nuclei in the myenteric plexus were still apparent in these segments and in one segment they appeared to increase (Fig 5.2F).

5.5 Discussion

5.5a Activity-Related c-Fos and JunB in enteric neurons

This study has demonstrated that Fos- and JunB-IR are inducible in at least four immunohistochemically defined subsets of enteric neurons in the guinea pig ileum. A generalized neuronal activation was likely to have resulted from the depolarizing stimuli used in this study thus supporting the idea that expression of both c-Fos and JunB in enteric neurons was associated with increased neuronal activity. This finding tends to support the idea that these responses can be exploited to examine synaptic interactions between these cells. In fact, neuronal activation of these antigens by K^+-stimulation was largely abolished

in the absence of extracellular Ca^{++}, probably due in part to inhibition of synaptic transmission. On the other hand, both antigens appeared unaffected by hexamethonium and so nicotinic acetylcholine receptors do not constitute an indispensable component of this response.

Depletion of extracellular Ca^{++} may also have more directly antagonized neuronal c-Fos expression. In central neurons, entry of Ca^{++} can induce c-fos transcription by at least two distinct biochemical pathways. Activation of Ca^{++}-calmodulin-dependent protein kinases induces phosphorylation of the cyclic AMP response element binding protein which acts through the cyclic AMP response element and/or the Ca^{++} response element in the c-fos promoter to upregulate c-fos expression (138,139). Ca^{++} entry can also induce c-fos transcription by a less well characterized mechanism that appears to function through a different promoter sequence, the serum response element, perhaps by activating the mitogen activated protein kinase cascade (138).

Previous work has suggested that submucosal neurons in the guinea pig ileum can be classified into four major subgroups (3) whereby VIP neurons account for 45%, NPY neurons for 30%, calretinin neurons for 15% and those containing substance P and perhaps calbindin (82) make up the remaining 10% of these cells. If this scheme is correct, then the potential for IEG expression appears to exist in all submucosal neurons. Furthermore, in some myenteric ganglia of stimulated segments, virtually all neurons expressed c-Fos and so perhaps all subtypes of these cells exhibit activity-related expression of these proteins as well. However, the proportions of calbindin neurons measured in this laboratory (54) more closely agrees with the original observations of Furness et al. (136) who reported them to be

scarce in the submucosal plexus. Hence, it is likely that this study has not accounted for all of the submucosal neurons. Furthermore, these antisera probably label less than 50% of myenteric neurons and so the capacity of the remaining neuronal subsets to express these antigens has yet to be precisely determined.

Interestingly, periodic stimulation of ileal segments with 50mM K^+-containing Krebs' solution did not markedly affect the numbers of c-Fos- and JunB-immunoreactive neurons in the submucosal plexus. This may indicate a greater refractoriness of submucosal neurons or, alternatively, it may reflect a greater buffering capacity of the submucosal plexus due to the presence of extensive ion transport systems. The less conspicuous differences between the numbers of c-Fos-and JunB-immunoreactive neurons labelled in each plexus following veratridine stimulation may support the latter view. Nevertheless, more unidentified extraganglionic cells in the submucosa layer were found to express c-Fos and JunB-IR following this stimulus and so a less direct mechanism of veratridine-stimulated antigen expression in submucosal neurons cannot be excluded.

5.5b The Identity of Fos and Jun Antigens

These data do not firmly establish the identity of the various Fos and Jun antigens. Nevertheless, both the polyclonal and the monoclonal anti-c-Fos antibodies labelled nuclear antigens that reacted similarly to the stimuli and it seems likely that at least one of these may be bona fide c-Fos. Moreover, activation of JunB-IR largely paralleled that of c-Fos and was distinct from the expression of c-Jun-IR, similar to the reported patterns of expression of Jun antigens in many CNS neurons (74,75). Interestingly, basal expression of

c-Jun-IR was detected in perhaps all submucosal neurons but was less evident in myenteric neurons. This may be due in part to technical considerations as the immunofluorescence might be less evident in the thicker myenteric plexus whole mounts. Nevertheless, it is tempting to speculate that this may also be due in part to more frequent interruptions of nerve-target interactions resulting from the more rapid tissue turnover in the mucosa.

5.5c Other Contributions to c-Fos and JunB in Neurons and Other Cells

It is also important to note that a substantial amount of c-Fos and JunB expression in neurons and other cell types appeared to be related to the isolation and/or incubation procedures. Physical damage and/or discharges of fibres during dissection may have been responsible for the seemingly generalized expression of these antigens in some ganglia, and so isolated intestinal segments may not provide an ideal model system to continue this type of study. In fact, the "basal" expression in preparations such as described by Kirschgessner et al. (82) precluded their use in the present study (see Methods and Materials), perhaps indicative of differences in the sensitivity of the techniques or the specificity of the antibodies between these reports. C-Fos-IR was preferentially localized in VIP submucosal neurons both in unstimulated segments and a small number of untreated animals but it is not clear that this reflected increased activity of these cells. Indeed, a physiologically relevant stimulus that activates detectable levels of c-Fos-IR in enteric neurons remains to be found.

Previous work has shown that Fos and Jun antigens are induced by damage in vascular smooth muscle cells (140) and this may account for the present observations in the vasculature. The consistent c-Fos and JunB expression we observed in the intestinal smooth

muscle coats of all incubated segments suggests that their expression may be similarly regulated in visceral smooth muscle. The stimulus for the observed glial expression of c-Fos has not been determined but may involve activation of extrinsic fibres by the dissection (see also Chapter 7). Furthermore, this underscores the importance of using a counterstain to determine the identity of labelled cells. Indeed, the maintained or increased numbers of c-Fos immunoreactive glia we observed in segments stimulated in the absence of extracellular Ca^{++} may explain the increased ganglionic c-Fos-IR reported by Kirchgessner et al. (82) in tissues incubated without extracellular Ca^{++}. In fact, the distribution and morphology of many of the c-Fos-immunoreactive neurons that they showed in cholera toxin-treated tissues were remarkably reminiscent of glia.

5.5d Conclusions

These studies have demonstrated that depolarizing stimuli induce Fos-, JunB-, but not c-Jun-IR in at least four immunohistochemically identified subtypes of neurons in the guinea pig ileum. Their activity-related expression supports the idea that they may be useful to examine neuronal activity and neuronal interactions. However, the in vitro isolation and/or incubation of ileal segments also induces expression of these antigens in some neurons and in other cells. Hence, stimuli other than activation may contribute to their expression, and their expression is not limited to enteric neurons.

CHAPTER SIX

IMMUNOHISTOCHEMICALLY-DEFINED SUBTYPES OF NEURONS IN THE INFERIOR MESENTERIC GANGLION OF THE GUINEA-PIG.

6.1 Introduction

The previous chapters have characterized expression of Myc, Fos and Jun proteins in a well described neuronal network, the enteric nervous system of the guinea pig ileum. It would also be useful to examine their expression in sympathetic nerves but, when this work began, the description of neuronal subsets in the inferior mesenteric ganglion (IMG) was not well documented.

Much of the sympathetic outflow to the abdominal viscera is supplied by the abdominal prevertebral ganglia (reviewed in 4,5): the IMG contains postganglionic neurons that supply the distal colon and pelvic organs whereas neurons in the coeliac/superior mesenteric ganglion (CG/SMG) complex innervate much of the upper gastrointestinal tract. Prevertebral neurons may receive inputs from other ganglionic neurons, from preganglionic neurons in the thoracolumbar spinal cord, from neighbouring prevertebral ganglia, from neurons in the myenteric plexus of the intestines and from axon collaterals of primary afferent fibres that supply the viscera (2,4,5,14,21-23,26-30,37,38,141).

Although noradrenaline (NA) was the first recognized sympathetic transmitter, biologically active peptides have been found in many sympathetic neurons and these neuropeptides may also play an important role in the regulation of sympathetic targets. In prevertebral ganglia of the guinea pig, for example, neurons containing somatostatin- (SOM-) and neuropeptide Y- (NPY-) immunoreactivity (IR) have been found in largely exclusive subgroups together with NA synthetic enzymes (NA/SOM and NA/NPY neurons, respectively) such as tyrosine hydroxylase (TH) (17,20-22). A third major group contains no known neuropeptide (NA/- neurons). These major subgroups and small groups of

NPY/SOM and vasoactive intestinal polypeptide (VIP) neurons have been carefully documented in the CG/SMG (21,22). While similar groups of neurons also exist in the IMG (19,21,29,142), estimates of the SOM neuron population have ranged from 45% (141) to 63% (19). In addition, the proportions of NPY/SOM and VIP neurons had not assessed.

Discrete subgroups of extrinsic fibres within the prevertebral ganglia also appear to contain distinct neuropeptides. For example, a network of varicose fibres containing various neuropeptides, including VIP, arises predominantly from myenteric neurons in the gut wall with "intestinofugal" projections (26-30); they surround NA/SOM and NA/- neurons but largely exclude NA/NPY neurons (21,30) but it is not known whether their distribution is biased with respect to NPY/SOM or VIP neurons. Collateral projections of primary afferent fibres that supply the viscera constitute the largest source of substance P- (SP-) immunoreactive fibres (19,21,37). SP fibres form particularly dense baskets around VIP neurons in other sympathetic ganglia (143,144) and while they also form some dense pericellular baskets in the IMG (37,38,145), no specific immunohistochemical subtype had been identified within these structures.

Growing evidence also indicates a role for nitric oxide (NO) in the regulation of neuronal targets (146). The NO synthetic enzyme, nitric oxide synthase (NOS), has recently been identified by antibodies and NADPH-diaphorase activity in the IMG of the guinea pig prevertebral ganglia (147,148). NOS fibres largely arise from myenteric and spinal preganglionic neurons with perhaps a minor contribution by infrequent NOS reactive perikarya, but it was not known whether NOS in fibres or perikarya colocalizes with any

neuropeptides.

In the CG/SMG, different neuronal subgroups project to distinct target tissues in the upper GI tract: NA/SOM fibres innervate submucous ganglia, mucosa and circular muscle, NA/NPY fibres supply the intestinal blood vessels, NA/- fibres project to myenteric ganglia and the mucosa (17,18) and no NPY/SOM neurons have been found to project to the upper GI tract (20). However, sympathetic NA/SOM fibres may have more sparse projections to the colonic submucous plexus (142). The distribution of sympathetic fibre subtypes in the myenteric plexus of the distal colon had not been reported in these animals.

In order to determine the proportions of neuronal subtypes and the fibres with which they are associated in the guinea pig IMG, double- and triple-labelling immunohistochemistry were employed to examine the distribution and colocalization of TH-, SOM-, NPY-, VIP- and SP-IR. The possible colocalization of NOS-IR with VIP-IR in fibres and perikarya was also addressed. In addition, colocalization of TH, SOM and NPY in fibres of the colonic myenteric plexus was investigated.

6.2 Hypothesis

The proportions, projections and associated fibres of IMG neuronal subtypes will differ somewhat from those in the CG/SMG, but the distribution of these properties among IMG neurons may still be consistent with a model of correlated neurochemistry and electrophysiology of these cells.

6.3 Methods and Materials

6.3a Animals and Tissues

The distal colon and IMG of 13 male guinea pigs (200-810g) were examined. Each animal was anaesthetized and the IMG and the distal colon (about 5-15cm from the anus) were removed. The colon was fixed and dissected as whole mount preparations of myenteric and submucosal plexuses as described in Chapter 2. The ganglia were sagittally sectioned (in the plane of the inferior mesenteric artery and the aorta; 12μm) and serially mounted on coated glass slides. Slides from 3 animals were processed for NADPH-diaphorase histochemistry (149).

6.3b Primary Antibodies

Primary antibodies used were: mouse anti-neuron specific enolase (NSE), rabbit anti-TH, rabbit anti-SOM, mouse anti-SOM, Rabbit anti-NPY, rat anti-NPY, rabbit anti-VIP, mouse anti-VIP, rabbit anti-SP, rat anti-SP and rabbit anti-NOS (see also Table 2.1, Chapter 2).

6.3c Specificity Controls

We noted that among all pairs of antibodies specific for a given antigen (see Table 2.1, Chapter 2), the labelling by monoclonal antibodies was generally less robust. Nevertheless, the overall pattern of sections single-labelled by either of that antibody pair was otherwise similar and labelling of identical structures was observed in sections double-labelled with any given antibody pair with two exceptions: the monoclonal rat anti-NPY

also faintly labelled nuclei of perhaps all IMG neurons, and the rabbit anti-SP polyclonal antiserum labelled a small number of neurons. In specificity controls for each of the primary antisera (except anti-TH and anti-NOS) performed by preincubation with the cognate antigen (10nmol/ml), neither of those exceptions were blocked by addition of the cognate antigen and these presumably non-specific phenomenon were characterized further. In order to control for nonspecific labelling by the secondary antibody combinations, they were also tested on sections preincubated with a single primary antibody.

6.3d Quantitative Data

Quantitative data is shown as mean ± SEM. Proportions of IMG neuronal subtypes (Table 6.1) were counted on 1 slide from each animal of serial sections (8-15 sections/slide), separated by at least 96μm, that were double-labelled for TH and SOM, TH and NPY, SOM and NPY as well as SOM and VIP (see Results).

6.4 Results

6.4a Distribution of TH-, SOM-, NPY-, VIP- and SP-IR

In single labelled sections of the IMG, the labelling by antisera for TH, SOM, NPY, VIP and SP was largely as has been previously described (4,18,19,21,23,26,27, 29,37,38,145,150). Briefly, TH-IR was found in most neurons and in some fibres of all nerve trunks. TH-IR was homogenously distributed throughout perikarya of neurons and the apparent levels of TH-IR in neurons and fibres was sometimes quite variable in neurons and fibres. SOM-IR was found in a large proportion of neurons (Fig 6.1A) while a smaller

proportion contained NPY-IR. Within neuronal perikarya, both SOM- and NPY-IR were found in perinuclear structures, probably Golgi bodies (151), and the neurons often contained 2, or infrequently, 3 nuclei (Fig 6.1B). NPY neurons were observed either singly or groups that were usually around the periphery of the ganglion, especially near major nerve trunks, blood vessels and islands of extra-adrenal chromaffin cells, or small intensely fluorescent (SIF) cells. They were also observed more frequently in the distal lobe, but this was in part a function of the size difference between the distal and proximal lobes, as the former was almost invariably larger than the latter. We acknowledge that some subjectivity was introduced when determining the extent of each lobe because a clear gap between them extending through all sections was rarely found. In addition, small groups of neurons near nerve trunks were considered to be IMG neurons if they were closely associated with the rest of the ganglion. Perivascular fibres in the IMG contained robust NPY- and TH-IR. Occasional dense, pericellular baskets of fibres were found with all SOM and NPY antibodies but both SOM- and NPY-IR appeared weak in nerve trunks.

A dense varicose VIP fibre network was observed throughout most of the ganglion but usually appeared weaker in nerve trunks and was mostly excluded from splanchnic nerves, SIF cell islands and regions of NPY neuron clusters. A small population of neurons was found either singly or, less frequently, in small clusters (≤5 cells) in the distal lobe near the colonic, hypogastric and splanchnic nerve trunks. A network of varicose and nonvaricose SP fibres extended throughout almost the entire

p.l.
d.l.
A
B

Figure 6.1 Fluorescence micrographs of SOM-IR in the IMG. (A) A low power micrograph reveals the predominance of SOM neurons in the proximal lobe (p.l.) and distal lobe (d.l.) of the IMG. (B) Although many neurons appeared to be binucleate, a rare, apparently trinucleate neuron can be seen. Scale bar: A=200μm, B=50μm.

IMG except SIF cell islands. Nonvaricose SP fibres were abundant in all major nerve trunks. TH-, NPY- and SP-IR were also found in some SIF cells, either singly or in SIF cell islands, while SOM- and VIP-IR were found only in rare singly occuring SIF cells.

6.4b Colocalization of TH-, SOM- and NPY-IR

In sections labelled for TH and either SOM or NPY, both SOM-IR and NPY-IR were found in subgroups of TH neurons (Figs 6.2A-D). Of all TH neurons, 79.8±3.5% (mean±SEM; n=5 animals) contained SOM-IR and 22.4±1.9% (n=5) contained NPY-IR. In all cases, neurons were only counted when perinuclear labelling was evident although binucleate (and trinucleate) neurons were counted as one cell. These populations were not exclusive and so these data do not account for those neurons with TH-, NPY- and SOM-IR (see below). In addition, 1.7±0.7% of SOM neurons and 6.5±2.9% of NPY neurons (each about 1.5% relative to TH neurons) lacked detectable TH-IR. SOM- and NPY-IR within ganglionic fibres or nerve trunks were often colocalized with TH-IR (not shown). In sections from 2 animals that were double-labelled with anti-TH and anti-neuron specific enolase (NSE), we confirmed that a small proportion of neurons lacked TH-IR but the intense labelling of fibres by anti-NSE did not provide optimum conditions for counting individual neurons (not shown).

In sections from 8 animals double-labelled for SOM and NPY, a small but not negligible proportion of neurons and fibres contained both antigens (Figs 6.2E,F, 6.5C,D). NPY/SOM neurons, alone or in clusters (≤8 cells/section), were most frequently found in the distal lobe, particularly near the hypogastric and colonic nerve

A
B
C
D

Figure 6.2 Fluorescence micrographs of TH-, SOM- and NPY-IR in double-labelled sections of IMG. Although most neurons contained TH-IR, some neurons with SOM- or NPY-IR (A, C) lacked detectable TH-IR (B, D, arrows). The light labelling of nuclei by the NPY antibody was apparently nonspecific (see Methods and Materials). Some neurons with SOM-IR (E) also contained NPY-IR (F, arrows). Scale bar: 50μm.

A
SOM
B
NPY
C
III
D
SOM
E
NPY
F
III

Figure 6.3 Fluorescence micrographs of SOM-, NPY- and TH-IR in triple-labelled sections of IMG. Neurons with SOM- and NPY-IR (A, B, arrows) often lacked TH-IR (C, arrows), and rare neurons contained NPY- but neither SOM- nor TH-IR (A, B, C, double arrows). Other neurons with SOM- and NPY-IR (D, E, arrows) clearly contained TH-IR (F, arrows). Scale bar: 50μm.

trunks. In sections from 5 animals, 3844 SOM neurons, 1063 NPY neurons and 285 NPY/SOM neurons were counted, yielding a ratio of about 15:4:1. Both SOM- and NPY-IR were also found colocalized in a small proportion of intraganglionic fibres (not shown).

In sections from 4 animals triple-labelled for TH, SOM and NPY, NPY/SOM neurons sometimes lacked TH-IR and infrequent NPY neurons were found to lack both TH- and SOM-IR (Fig 6.3A-C). However, other NPY/SOM neurons clearly contained TH-IR (Fig 6.3D-F) and TH/- neurons were only rarely found (Fig 6.4A-C). For example, in sections from one animal in which adjacent sections contained 1071 SOM neurons, only 4 such neurons were found. Most or all of the NPY/SOM and NPY neurons that lacked TH-IR probably contained VIP-IR (see below). Therefore, at least six subgroups of IMG neurons could be recognized; NA/SOM, NA/NPY, NA/-, NA/NPY/SOM, VIP/NPY/SOM and VIP/NPY (in accordance with convention, we use NA rather than TH to designate the noradrenergic subgroups).

6.4c Distribution of VIP, SP and NOS in Fibres and Perikarya

In adjacent labelled sections and in sections triple-labelled for VIP, SOM and NPY, the VIP neurons invariably contained NPY-IR and most of these neurons (48 of 59 neurons from 3 animals) also contained SOM-IR (Figs 6.4D-F). VIP neurons were often clearly associated with dense pericellular baskets of SP-immunoreactive fibres (Figs 6.5A,B). VIP- and SP-IR were also found colocalized within rare fibres and VIP fibres were mostly excluded from regions of NPY neurons, including those with SOM-IR (Figs 6.5C-F).

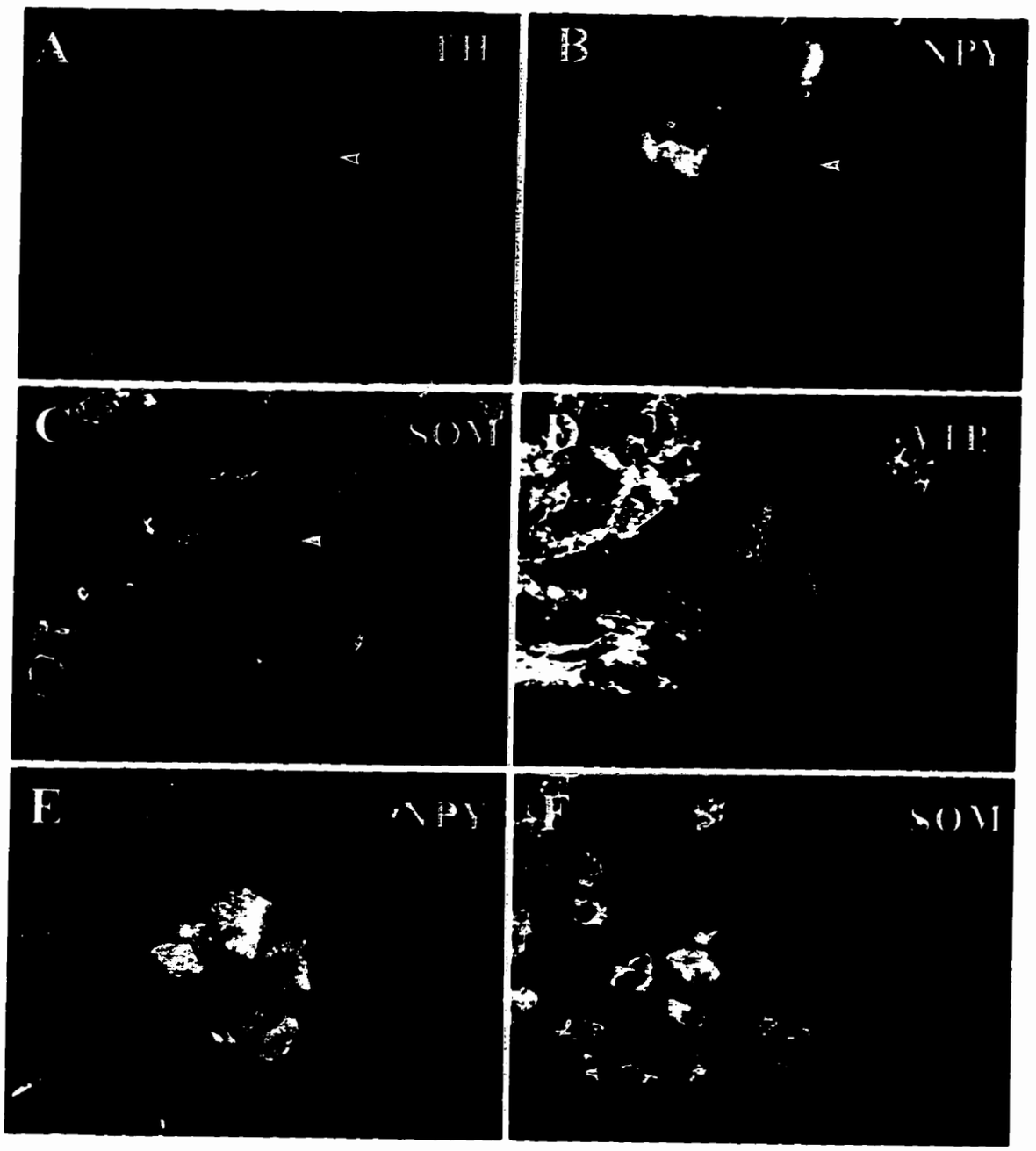
A
TH
B
NPY
C
SOM
D
VIP
E
NPY
F
SOM

Figure 6.4 Fluorescence micrographs of TH-, SOM-, NPY- and VIP-IR in triple-labelled sections of IMG. In sections triple-labelled for TH, SOM and NPY (A-C), small numbers of neurons with TH-IR (A, arrow) lacked both NPY- and SOM-IR (B, C, arrows). In sections triple-labelled for VIP, SOM and NPY (D-F), the dense VIP fibre network was largely excluded from regions of VIP neurons and most neurons with VIP-IR also contained both SOM- and NPY-IR. Scale bar: 50μm.

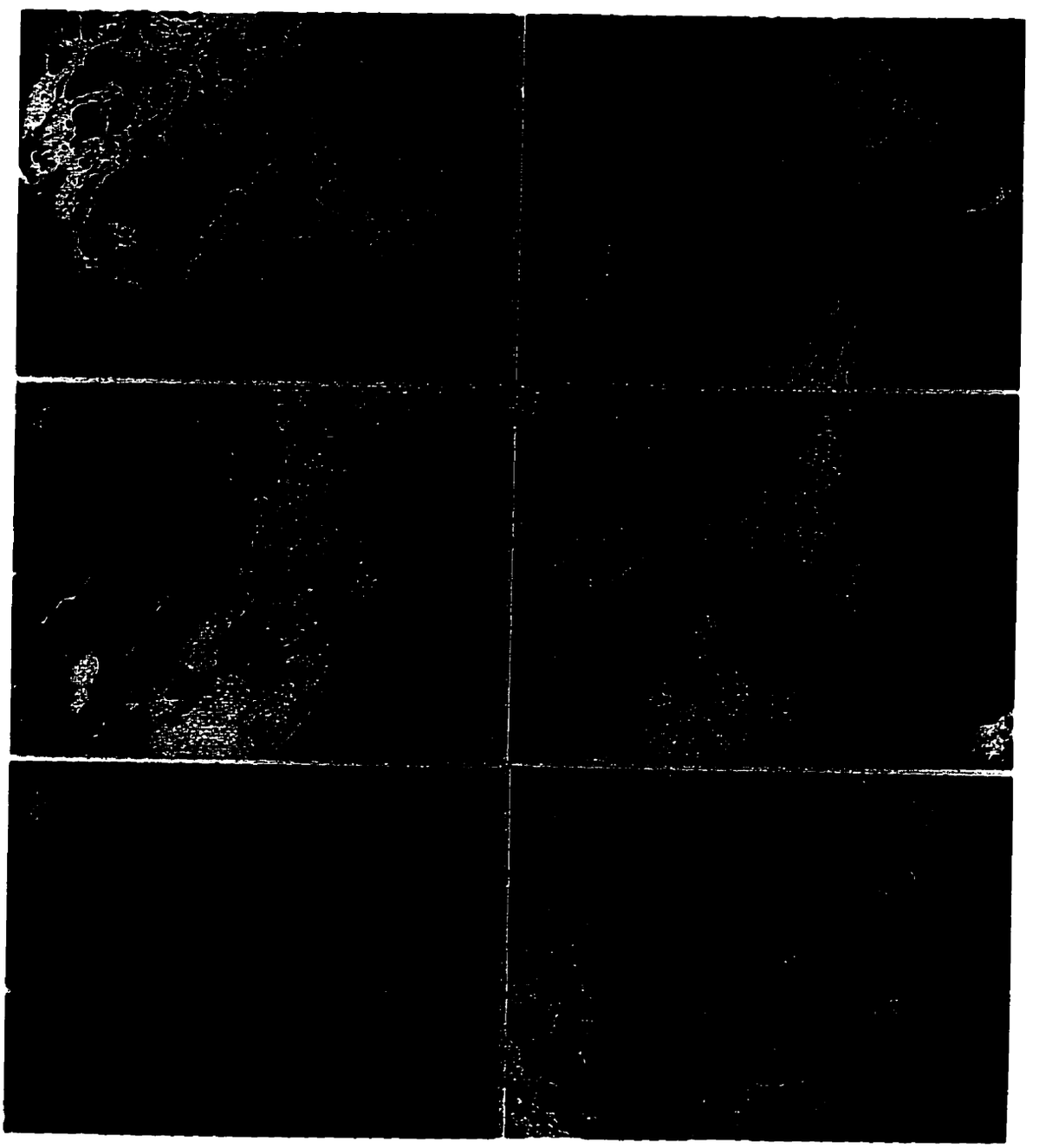

Figure 6.5 Fluorescence micrographs of VIP-, SP-, SOM- and NPY-IR in double-labelled sections of IMG. In a section near the splanchnic nerve double-labelled for VIP and SP (A, B), neurons with VIP-IR can be seen to receive a particularly rich supply of SP fibres (arrows). In adjacent sections (C,D and E,F, respectively) along the periphery of the IMG between the hypogastric and colonic nerves that were double-labelled for either SOM and NPY (C, D) or VIP and SP (E, F), VIP fibres (E), but not SP fibres (F), were mostly excluded from regions of NPY/SOM neurons (C, D). Fibres with both SOM- and NPY-IR (C, D, arrows) or VIP- and SP-IR (E, F, arrows) can also be seen. Labelling of some neurons by the SP antibody was apparently nonspecific (see Methods and Materials). Scale bar: 50μm.

As recently reported by others (147), varicose NOS fibres and infrequent perikarya were detected by NOS antiserum and NADPH-diaphorase staining. Intense NOS-IR was apparent in varicose fibres whose distribution resembled that of VIP fibres while weaker NOS-IR was apparent throughout the ganglia. In sections double-labelled for NOS and VIP, some colocalization of NOS and VIP was found in varicosities but much of the NOS- and VIP- appeared to be contained in distinct varicosities (Figs 6.6A,B). Many of the NOS neurons also contained detectable VIP-IR (Figs 6.6C,D) but some NOS- and VIP-IR were found in different neurons. No colocalization of NOS-IR with SP-IR was found (not shown).

6.4d Distribution of IMG Neuronal Subtypes

The quantitative data were obtained from sections double-labelled for TH and SOM, TH and NPY as well as SOM and NPY (Table 6.1). In view of the distinction between NA/NPY/SOM and VIP/NPY/SOM neurons revealed in triple labelled sections, the proportions from each animal were adjusted to account for those neurons. First, all of the SOM and NPY neurons that lacked TH-IR were assumed to represent VIP/NPY/SOM neurons. Although this assumption was largely true, a few VIP/NPY neurons lacked SOM-IR (see above) but the small proportion they represented were not considered. Since the TH neurons accounted for almost all neurons, the proportions of each of the non-TH-SOM and non-TH-NPY groups were expressed as proportions of TH neurons and averaged to obtain the proportion of VIP/NPY/SOM neurons (Table 6.1).

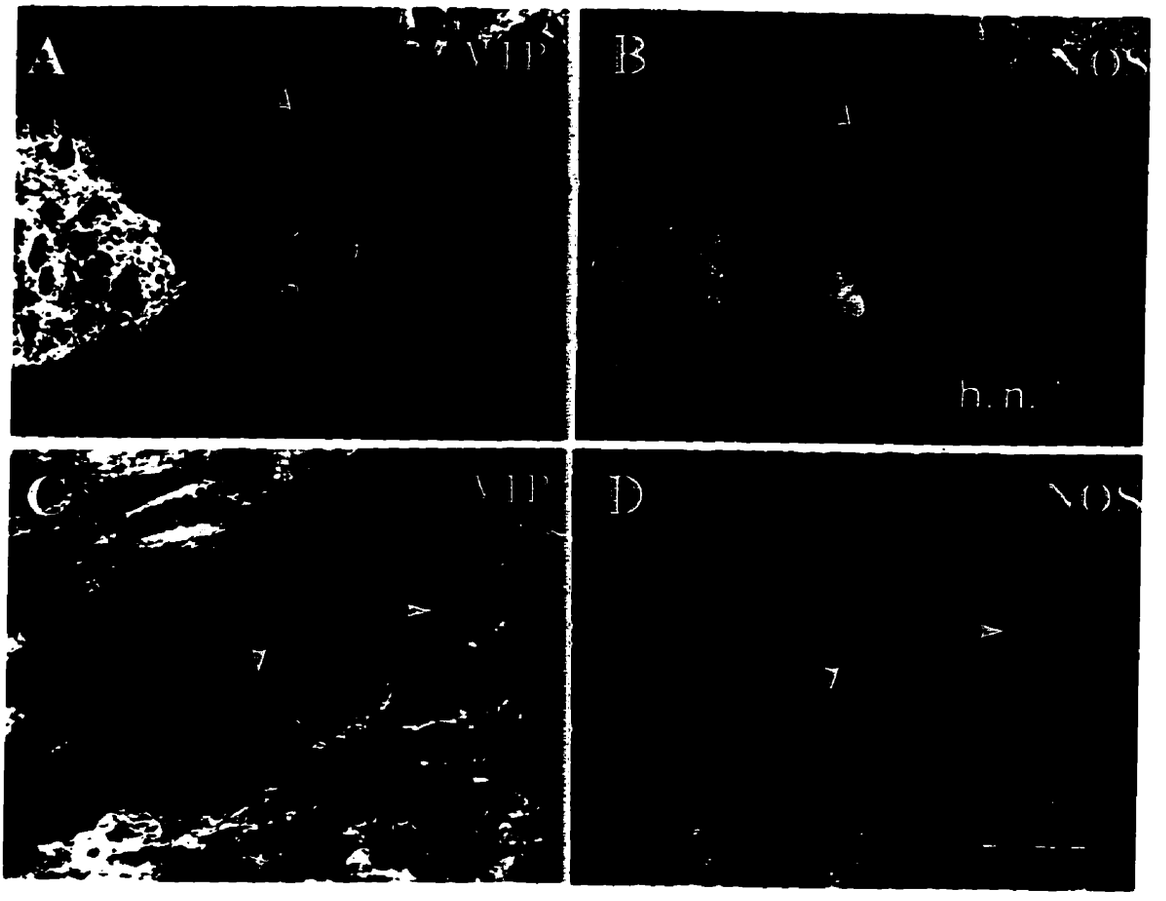
A
B
h.n.
C
D

Figure 6.6 Fluorescence micrographs of VIP- and NOS-IR in double-labelled sections of IMG. Many neurons with VIP-IR (A) also contained NOS-IR (B) and some NOS neurons did not contain detectable VIP-IR (A,B, arrows). Strongly immunoreactive NOS varicosities (B,D) resembled those with VIP-IR (A,C), but weaker NOS-IR was apparent throughout most of the ganglia and can also be seen in a hypogastric nerve (B, h.n.). Some of the VIP varicosities (C, arrows) also contained strong NOS-IR (D, arrows) but much of each appears to be in distinct varicosities. Scale bar: A,B=100μm, C,D=50μm.

Although these calculations introduced small errors, the relative proportions obtained were consistent with our qualitative observations in triple-labelled sections. Furthermore, similar proportions were obtained when the proportions of VIP neurons in sections double-labelled for VIP and SOM were expressed relative to the TH neurons in sections double-labelled for TH and SOM (Table 6.1).

The proportion of SOM neurons that contained NPY were then calculated and, from this, the proportion that lacked TH was subtracted to obtain the proportion of SOM neurons that contained both NPY- and TH-IR in each animal. The proportion of NPY neurons that contained both SOM- and TH-IR was calculated similarly. These numbers were then expressed as proportions of TH neurons and averaged to obtain the proportion of NA/NPY/SOM neurons (Table 6.1).

The proportions of NA/NPY/SOM neurons were then subtracted from each of the proportions of TH/SOM and TH/NPY neurons to arrive at corrected figures for NA/SOM and NA/NPY neurons, respectively. Finally, the proportions of NA/- neurons were those TH neurons that remained. No clear differences associated with animal size were noted but one large animal (632g) had both the least NA/SOM (65%) and the most NA/- (13%) neurons. Nevertheless, the proportions of other subtypes did not vary markedly from the average in other animals (not shown) and so these data were pooled.

6.4e Distribution of Sympathetic Fibres in the Myenteric Plexus of the Distal Colon

Since these observations revealed that NA/- neurons were rare in the IMG, the compared the colocalization of TH-IR with SOM-IR and with NPY-IR in the myenteric

plexus of the distal colon. As previously described in single-labelled whole mount segments (2,124), NPY- and SOM-IR were found in some myenteric neurons and many varicose fibres, while TH-IR was found in varicose and non-varicose fibres. In double-labelled segments, most NPY neurons and fibres were also found to contain SOM-IR while some SOM neurons and fibres did not have detectable NPY-IR (not shown). NPY-IR was found in only rare fibres colocalized with TH-IR but a considerable proportion of TH fibres also contained SOM both in myenteric ganglia and in the tertiary plexus (Fig 6.7). In segments triple-labelled for SOM, NPY and TH, none of the TH or TH/SOM fibres were found to contain NPY-IR (not shown).

Table 6.1. Proportions of IMG neuronal subtypes as percentage of NA neurons (mean ± SEM, n=5 animals).

	NA/SOM	NA/NPY	NA/-	NA/NPY/SOM	VIP/NPY/SOM
pIMG	81.4±6.1	14.4±3.1	5.5±4.1	1.2±0.2	0.5±0.3*
dIMG	74.3±2.6	20.1±1.3	2.0±1.5	4.9±1.8	1.9±0.6*
tIMG	76.2±3.2	18.8±1.0	3.1±2.4	3.6±1.2	1.6±0.5* /2.0±0.5**
Range	67-88	18.4-29.3	0-12.6	0.7-7.6	0.4-3.3*/ 1.3-2.8**
# neurons counted	4000	1049	250***	285	78*/59**

pIMG = proximal lobe, dIMG = distal lobe, tIMG = total of IMG.

* Neurons counted as non-TH-SOM/NPY.

** VIP neurons counted by comparison with SOM in double-labelled sections (n=3 animals)

*** Approximate number obtained by subtraction.

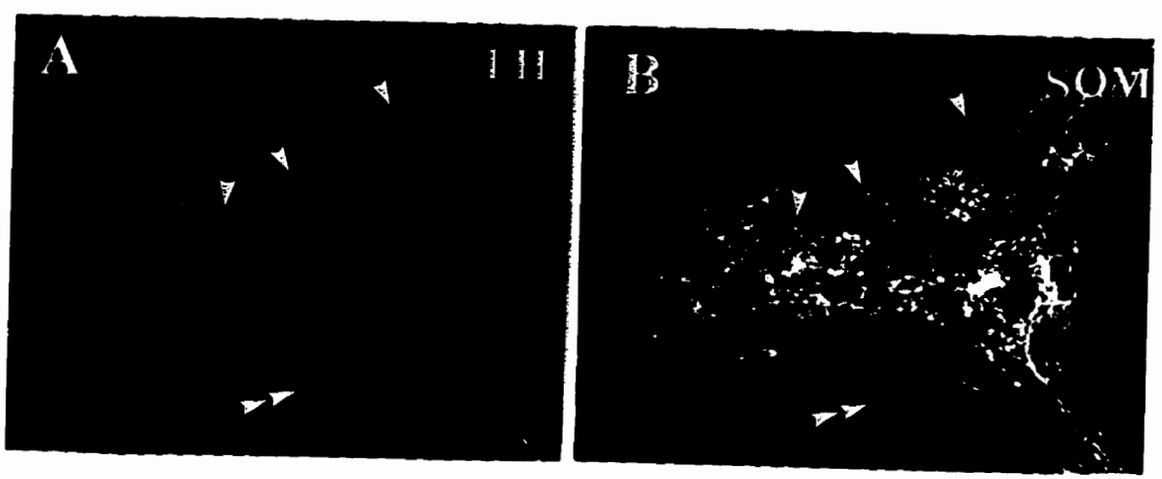
A
III
B
SQM

Figure 6.7 Fluorescence micrographs of TH- and SOM-IR in double-labelled myenteric plexus of the distal colon. Some fibres in the tertiary plexus (double arrows) and in ganglia (arrows) contained both TH- (A) and SOM-IR (B). Scale bar:50μm.

6.5 Discussion

6.5a Major Neuronal Subtypes in the IMG

Our data confirm that NA neurons account for the vast majority of guinea pig IMG neurons (4,19,145) and indicate that NA/SOM and NA/NPY neurons comprise about 75% and 20%, respectively, of these neurons. The remaining 5% is composed of NA/- and NA/NPY/SOM neurons while about 2-3% of all IMG neurons lacked TH-IR and most or all of these neurons also contained VIP. Small subdivisions within this VIP subgroup of neurons were also apparent as a few lacked SOM-IR, some contained NOS-IR and a small proportion of NOS neurons lacked detectable VIP-IR. Given the small proportion represented by all of these small subgroups (about 2-3%), it was not possible to establish reliable estimates regarding their respective sizes.

Our data indicating that about 75% of neurons are NA/SOM neurons differ markedly from the 45% reported by Keast et al. (142). Since the proportion of NA/- neurons were obtained by subtraction, it is perhaps not surprising that the present estimate of about 1-5% for these neurons is also substantially different than the 30-40% estimated by those investigators. While some difference might be attributable to variability in labelling intensity, variability between animals and the presence of multinucleate neurons, these factors seem unlikely to account for these discrepancies because our measured proportion of total NPY neurons is remarkably consistent with their own and another previous estimate (23). In addition, SIF cells in the IMG may contain various combinations of neuropeptides and NA (150) but these cells are usually morphologically distinct from neurons and the possibility that a small number were mistakenly identified as neurons in the present study

seems unlikely to account for this large difference. Even more enigmatic is that Keast and her coworkers examined ganglia treated with colchicine which increases the apparent levels of NPY-IR (23), probably by inhibiting peptide transit out of neuronal perikarya (151), but the effects of colchicine on SOM-IR in these neurons have not been examined. It is possible that SOM-IR is depleted by extirpation and/or incubation of the ganglion as Keast et al. (131) examined only ganglia incubated in vitro. In this respect, it is perhaps noteworthy that our measure more closely approaches the initial rough estimate of 63% reported by Hökfelt et al. (19) in ganglia treated similar to those in the present study. Finally, differences in antibody sensitivity or specificity may account for these differences. Nevertheless, both SOM antibodies used in the present study labelled similarly large proportions of IMG neurons. Furthermore, a report published after the completion of this work described similarly large proportions of SOM neurons in this ganglion (152).

Multinucleate neurons in prevertebral ganglia are likely to result from abrogation of cytokinesis during the final mitotic events in those cells (153), but their significance of their occurence remains to be determined. The proportions of heterochromatin and euchromatin in nuclei of binucleate neurons of the CG/SMG are not different from those in mononuclear neurons (153), suggesting that this phenomenon might provide a means fro increased overall gene expression. On the other hand, protein synthesis and axonal transport rates in binucleate neurons of the rabbit superior cervical ganglion are not different from their mononuclear counterparts (154). Interestingly, binucleate cells are common in cleavage arrested human embryos (155), and the numbers of binucleate rat hepatocytes are reduced during regeneration (156), suggesting that multiple nuclei may somehow contribute to

maintenance of a non-mitotic state.

6.5b VIP, NOS and SP in the IMG

Interestingly, VIP neuron subsets exhibit some similarities to, and some differences from, those in other abdominal ganglia. For example, NPY and SOM are distributed similarly in NPY/SOM and VIP neurons in both the CG/SMG (20,22) and the IMG. However, subgroups of VIP neurons with and without NA have been reported in the CG/SMG (20) but TH-IR appeared to be lacking from all VIP neurons in the IMG. Unlike the case for NPY and SOM neurons where the discrete subcellular distribution allowed unambiguous identification of immunoreactive cells, the homogenous cytoplasmic distribution and variable levels of TH-IR in the present study may have allowed a small number of NA/VIP neurons to be overlooked. On the other hand, although some NOS- and VIP-IR were found separately in some neurons, as they are in the CG/SMG (148), they were also frequently found colocalized suggesting that this population more closely resembles VIP/NOS neurons in pelvic ganglia (149). This may support the idea that most or all VIP neurons in the IMG are identical to the cholinergic neurons in this ganglion (4,152). Finally, the selective association of SP fibres with VIP neurons in the IMG resembles a similar association in the stellate (144) and paravertebral ganglia (143) of these animals. However, the significance of the biased distributions of SP and VIP fibres is unclear because many IMG neurons appear to receive peripheral inputs from both primary afferent and intestinofugal fibres (5).

The exclusion of the VIP fibre network from regions of NPY neurons (30) appears to include all NPY neurons including those with SOM-IR (Fig 6.5). While this may be coincidence, it is tempting to speculate that NPY may directly affect the growth of these fibres. Some of these VIP fibres also contained NOS-IR (Fig 6.6) but much of the VIP- and NOS-IR were found in distinct varicosities. Presumably this reflects the heterogeneous nature of the neuronal populations contributing these fibres which may include VIP/NOS neurons in the pelvic plexus (149) or colonic myenteric neurons in which colocalization has not been reported. At least some of these fibres may have originated from any of the VIP and/or NOS neurons within the IMG. Indeed, ganglion neurons are a potential source for any of the fibres reported herein as intraganglionic projections may not be inconsiderable (157).

6.5c Projections of IMG Neurons in the Distal Colon

The abundance of NA/SOM neurons in the IMG also appears to be reflected by the distribution of SOM in many TH fibres of the distal colon. It is possible that some of these colonic NA/SOM fibres originated in other sympathetic ganglia (14), but most are likely to have arisen from the IMG. Hence, the projections of NA/SOM neurons to much of the enteric nervous system appears to differ between the large and small bowel (17,142). It may be more than coincidence that the projections of both intrinsic (123) and extrinsic SOM neurons appear to differ between the myenteric plexuses of the ileum and the colon.

6.5d Relationship of Immunohistochemical Subtypes to Electrophysiological Properties in IMG Neurons

Interestingly, the discovery of three major electrophysiological subtypes of prevertebral ganglion neurons distinguished by membrane properties and major extrinsic inputs (31,142,158-160) raised the possibility that neuropeptide content and electrical behaviour are correlated and may identify functional subsets (142,159), analogous to the "chemical coding" described for enteric neurons (3). Based on their occurrence and distribution, it had been proposed that NA/SOM neurons are those that behave as "tonic" firing neurons, NA/NPY neurons are "phasic" firing neurons and NA/- neurons are "long after-hyperpolarizing" (LAH) neurons (142,159). Consistent with this idea, Keast *et al.* (142) found that SOM-IR was preferentially found in tonic firing neurons in both the CG and IMG, but some phasic firing neurons in the IMG also contained SOM-IR. Given our observation that about one fifth of NPY neurons also contain SOM, it is possible that these phasic SOM neurons also contained NPY. This idea may also explain why phasic SOM neurons were not found in the CG by Keast et al. (142) because NPY/SOM neurons are also more rare (20). The scarcity of NA/- neurons in the IMG also supports the idea that these neurons are identical to the rare LAH neurons (142,159). It may be interesting to determine if subclasses of phasic and tonic neurons (161) reflect different combinations of peptide-IR.

It is also noteworthy that the exclusion of VIP-immunoreactive intestinofugal fibres from NPY neurons appears to be reflected by weak or absent peripheral inputs to phasic neurons of the CG (31,160). However, a similar bias of VIP fibres in the IMG, including

their exclusion from NPY/SOM neurons, is more difficult to reconcile with the large proportion of IMG neurons (about 90%; 141), including phasic neurons (142), that receive inputs via the colonic nerves. Since NA/SOM and NA/- neurons are surrounded by VIP fibres, any neighbouring NA/NPY or NPY/SOM neurons might also then be accessible and the greater density of NA/NPY neurons in the CG may then simply allow for more efficient exclusion of these fibres.

6.5e Conclusions

These data indicate that the proportions, projections and associated fibres of immunohistochemically-defined IMG neuronal subsets are not identical to those in more rostral prevertebral ganglia. Nevertheless, these subtle differences may also reflect functional distinctions that have also been found, and so these data also tend to support the idea that prevertebral ganglion neurons are "chemically coded" with respect to their immunohistochemical content, membrane properties and their major inputs.

CHAPTER SEVEN

ACTIVATION OF FOS- AND JUN-IMMUNOREACTIVITY IN THE GUINEA PIG INFERIOR MESENTERIC GANGLION BY CONVERGENT STIMULATION OF ENTERIC AND PRIMARY AFFERENT NERVES

7.1 Introduction

Neurons in the guinea pig inferior mesenteric ganglion (IMG) supply most of the sympathetic fibres in the distal colon (4,14). As in other sympathetic ganglia, most IMG neurons are noradrenergic, receive inputs from the thoracolumbar spinal cord, and some contain neuropeptide Y-immunoreactivity (NPY neurons, about 20% of IMG neurons) but other subsets of IMG neurons include those containing somatostatin (SOM neurons, about 75%), both NPY and SOM (NPY/SOM neurons, 4-7%) or neither NPY nor SOM (-/- neurons; 1-5%; see Chapter 6). Most or all NPY neurons project to vasculature (18), NPY/SOM neurons include some cholinergic neurons and may innervate pelvic viscera (149,152), and SOM and -/- neurons project to the myenteric and submucosal plexuses of the enteric nervous system (ENS) in the wall of the colon (142,Chapter 6).

The ENS contains its own peripheral reflex circuits of sensory, interneuncial and effector neurons that regulate intestinal functions, and some myenteric neurons also have "intestinofugal" projections to the prevertebral ganglia where they may reflexly regulate the sympathetic outflow to the gut (2,5,21,24-36,141,142,148,160,161). These peripheral inputs to the IMG form dense varicose networks of neuropeptide-containing fibres that are most closely associated with SOM and -/- neurons (Chapter 6), although it is not clear whether any IMG neuronal subsets are preferentially affected by colonic stimulation.

Primary afferent fibres that accompany sympathetic nerves to the viscera also give rise to axon collaterals throughout the ENS and IMG (27,37,38,145). The ultrastructure and neurochemistry of these fibres are similar to nociceptive afferent fibres in other tissues and they are thought to contribute to the sensation of gastrointestinal pain (12,13,40). Like

other peripheral nociceptive fibres, these afferent fibres can also be acutely activated and chronically inhibited by capsaicin, the neurotoxic extract of hot peppers (41), and they are also believed to contribute to local and surrounding responses to noxious stimuli by release of inflammatory mediators from peripheral nerve terminals and axon collaterals (12).

Axon collaterals of these sympathetic afferent fibres may also activate peripheral reflexes in the ENS and IMG. For example, stimulation of colonic nerves or mechanosensory stimulation of isolated colonic segments has been shown to increase synaptic activity in neurons of the attached, decentralized IMG, probably reflecting release of acetylcholine (ACh) and neuroactive peptides from intestinofugal fibres and release of neuroactive peptides from primary afferent fibres (24,25,31,33,35,36,141,142, 160,162). In fact, the activity of most IMG neurons is thought to reflect integration of inputs from the central nervous system, from neighbouring prevertebral ganglia and from both groups of peripheral fibres (5). Nevertheless, it has not been possible to demonstrate electrophysiological responses of the IMG and ENS neurons in intact animals. Furthermore, the extensive dissection and manipulation required for electrophysiological analyses may well have contributed to the considerable "basal" activity in those preparations and this may also confound considerations of activation thresholds.

An alternative approach exploits the rapidly induced expression of nuclear Fos and Jun proteins in activated neurons. For example, enteric neurons in the ileum may all share the capacity to express activity-related c-Fos- and JunB-immunoreactivity (-IR) (Chapter 5) although basal levels of c-Jun-IR are seemingly unaffected by neuronal activity and it is not yet clear if those responses occur in the colonic ENS under any physiological conditions. In

fact, visceral (intraperitoneal) inflammation has also been shown to activate transynaptic c-Fos expression in spinal cord neurons that appear to reflect activation of primary afferent fibres (71). Presumably, more focal intestinal inflammation might also activate c-Fos in neurons within peripheral enteric and sympathetic reflex pathways that involve these fibres, but this has not previously been examined.

This study was initially undertaken to examine acute responses of c-Fos, JunB and c-Jun antigens in characterized neuronal subsets of the IMG, and in the colonic ENS, elicited by acute acetic acid-induced colitis. However, refeeding of colitic animals was found to be required for any response, and the sporadic nature of responses in that model required the development of a more reliable model system to costimulate low threshold neural activity and capsaicin-sensitive primary afferent fibres in the colon. Based on these studies, a model for activation of c-Fos- and JunB-IR in IMG neurons by convergent stimulation of primary afferent and intestinofugal fibres has been proposed.

7.2 Hypothesis

Immunoreactivity for c-Fos and JunB will be activated within SOM and -/- neurons in the IMG, and perhaps within neurons and/or glia of the colonic ENS, by appropriate colonic stimulation. Activation of these proteins may be differently affected by low threshold, mechanosensory stimulation and/or by activation of capsaicin-sensitive afferent fibres. Some basal expression of c-Jun may also be evident in the IMG but may not be acutely altered by those stimuli.

7.3 Methods and Materials

7.3a Animals and Tissues

A total of 54 male albino guinea pigs (200-550g) were used. All animals were fasted 18-24h before use but allowed water ad libitum. Colon tissues were fixed and dissected as described in Chapter 2. The IMGs were serially mounted on slides that contained representative sections, spaced by at least 12μm, throughout the entire ganglia (see also Chapter 6).

Sections of IMG were labelled with rabbit anti-Fos TF3, anti-c-JUN or anti-JUNB. In double- and triple-labelled sections, these antisera were combined with mouse anti-SOM and/or rat anti-NPY. Colon tissues were double-labelled with mouse anti-Fos TF161 together with either the anti-FOS 4 (to label all neuronal nuclei, see Chapter 4) or rabbit anti-S-100 to label enteric glia.

7.3b Studies of Acute Colitis

For induction of acute colitis, animals were lightly anesthetized with halothane (4% in O_2) and an enema of 0.5ml of 2% acetic acid in 0.9% saline was delivered approximately 7-8cm from the anus. The same volume of saline alone was used as a control. Groups of acetic acid-treated and saline treated animals were allowed to recover and either refed (chow and/or lettuce and carrots) or continued to fast. After 3-4h, they were reanesthetized, the IMG and distal colon were removed, and the animals were killed by exsanguination.

7.3c Capsaicin and Balloon Stimulation

In one set of experiments performed by Cathy MacNaughton, animals were anesthetized and capsaicin (0.2% dissolved in a vehicle of 80% saline, 10% Tween-80, 10% ethanol) was delivered intracolonically (approximately 8cm from the anus) or the distal colon was removed and capsaicin or vehicle was applied to the mesenteric attachment (between approximately 4-8cm from the anus). In all following experiments, the effects of intracolonic capsaicin treatment alone was compared to capsaicin or vehicle combined with balloon stimulation of the distal colon. For these experiments, a small balloon, made from the fingertip of a latex glove, was inserted in the colon about 8cm from the anus following capsaicin or vehicle treatment. The balloon was inflated with 50μl of saline such that the diameter was similar to that of a fecal pellet (approximately 0.5cm). The balloon was slowly withdrawn from the colon over a period of about a minute, and this procedure was repeated every 5min (6 times) in 0.5h. Animals treated with capsaicin alone also remained anesthetized for 0.5h, all animals were allowed to recover and they continued to fast for another 2h. Animals were then reanesthetized and the IMG and distal colon were removed, grossly examined and fixed.

7.3d Quantitative Analysis

Quantitative data are shown as mean±SEM and were obtained from one slide from each animal triple-labelled for c-Fos, SOM and NPY (see Table 7.1). Neurons were defined as c-Fos-positive when nuclear labelling was clearly evident and the nonspecific

nuclear labelling of IMG neurons by the NPY antibody was used to identify the -/- neurons. The average proportion of each neuronal subset with c-Fos-IR within an experimental group was compared to all other subsets within that group and to the same subset in all other experimental groups. Statistical significance was assessed using the Tukey Kramer Multiple Comparisons Test.

7.4 Results

7.4a C-Fos, c-Jun and JunB in Untreated Animals

In sections of the IMG from untreated animals, the c-Fos and JunB antisera were found to label some nerve fibres and to faintly label the cytoplasm of all neurons, but nuclear labelling was not found (n=3 animals). The c-Jun antiserum also labelled the cytoplasm of IMG neurons, and nuclear c-Jun-IR was also evident in IMG neurons of smaller animals (<250g, n=8 animals, see Fig 7.1) but less evident or undetectable in larger animals (>300g, n=6 animals). The smaller chromaffin cells sometimes contained nuclear c-Jun-, c-Fos- and JunB-IR as well (not shown). In sections triple-labelled for SOM, NPY and c-Jun, large subsets of SOM neurons and NPY neurons, and smaller groups of NPY/SOM neurons and -/- neurons, were visible. When present, nuclear c-Jun-IR in double- and triple-labelled sections was found in some neurons of all of these subsets but was usually most evident in SOM and -/- neurons (Fig 7.1E,F). However, no clear association was found between any of the experimental procedures (see below) and c-Jun expression and so this was not characterized further. In the ENS of the distal colon, c-Fos-IR was absent in both the myenteric and submucosal plexus of untreated animals (not

shown).

7.4b C-Fos and JunB in Acute Colitis

In preliminary experiments, 2% acetic acid (but not saline) was found to consistently induce a mild colitis within 3-4h with obvious mucosal hemorrhaging but animals showed no obvious signs of distress and would usually eat when allowed. Nuclear c-Fos and JunB were induced in IMG neurons within 3-4h only when the animals were fed following treatment. Therefore, experimental groups in subsequent studies included fasted animals that were treated with saline and continued to fast (n=4 animals), treated with saline and refed (n=4), treated with acetic acid and continued to fast (n=8), or treated with acetic acid and refed (n=8). Animals were allowed to recover 3-4h before dissection in order to provide time for refeeding and induction of these nuclear antigens. Nuclear c-Fos- and JunB-IR were mostly absent from IMG neurons of saline treated animals although a small proportion of neuronal nuclei contained both antigens in one animal. In triple-labelled sections from this animal, these immunoreactive neurons included some of all neurochemical subsets (not shown). Similarly, in acetic acid-treated animals that continued to fast, these nuclear antigens were mostly absent (Fig 7.1A) although c-Fos was found in a small number of all neuronal subsets from one animal. In acetic acid-treated animals that were refed, nuclear c-Fos- and JunB-IR were found in many IMG neurons of some animals (Fig 7.1B,D). In double- and triple-labelled sections from these animals, both c-Fos and JunB were found to be most evident in SOM and -/- neurons (Fig 7.1B,C). However, this response was highly variable: in 1 animal, nuclear c-Fos was found in 79 of 234 SOM

neurons and 3 of 6 -/- neurons but only 3 of 51 NPY neurons and 0 of 4 NPY/SOM neurons. Both antigens were substantially less apparent in 3 other animals of this group while no c-Fos or JunB-IR was seen in the IMG of another 3 animals. In the treated segments of distal colon, c-Fos was never found in the myenteric plexus of any animals although some very faint labelling was sometimes seen in rare neurons and more abundant extraneuronal nuclei, probably belonging to glia, within the submucosal plexus from acetic acid treated animals (Fig 7.2).

7.4c C-Fos in Capsaicin and Balloon Stimulated Animals

In order to distinguish whether activation of capsaicin-sensitive nerves might have a role in the responses of IMG neurons, the distal colon of 3 animals was surgically exposed and 0.2% capsaicin (n=2 animals) or vehicle (n=1) was applied to the mesenteric border of the distal colon to activate the fibres supplying the colon. Both c-Fos- and JunB-IR were apparent in a small proportion of IMG neurons, mostly within SOM and -/- neurons, of the animal treated with mesenteric vehicle but this response was markedly elevated in the animals treated with mesenteric capsaicin (not shown). In the intestines, c-Fos-IR was also found in some glial nuclei and fewer neurons within both plexuses of the vehicle-treated animal, and this labelling was increased in the capsaicin- treated animal. Since this procedure involved considerable manipulation of the colon that itself appeared to activate some IMG neurons, a similar dose of capsaicin was delivered intraluminally in another animal. Despite obvious physiological effects of this treatment, including transiently elevated respiration, heartrate and faint traces of blood on the mucosal suface, intraluminal

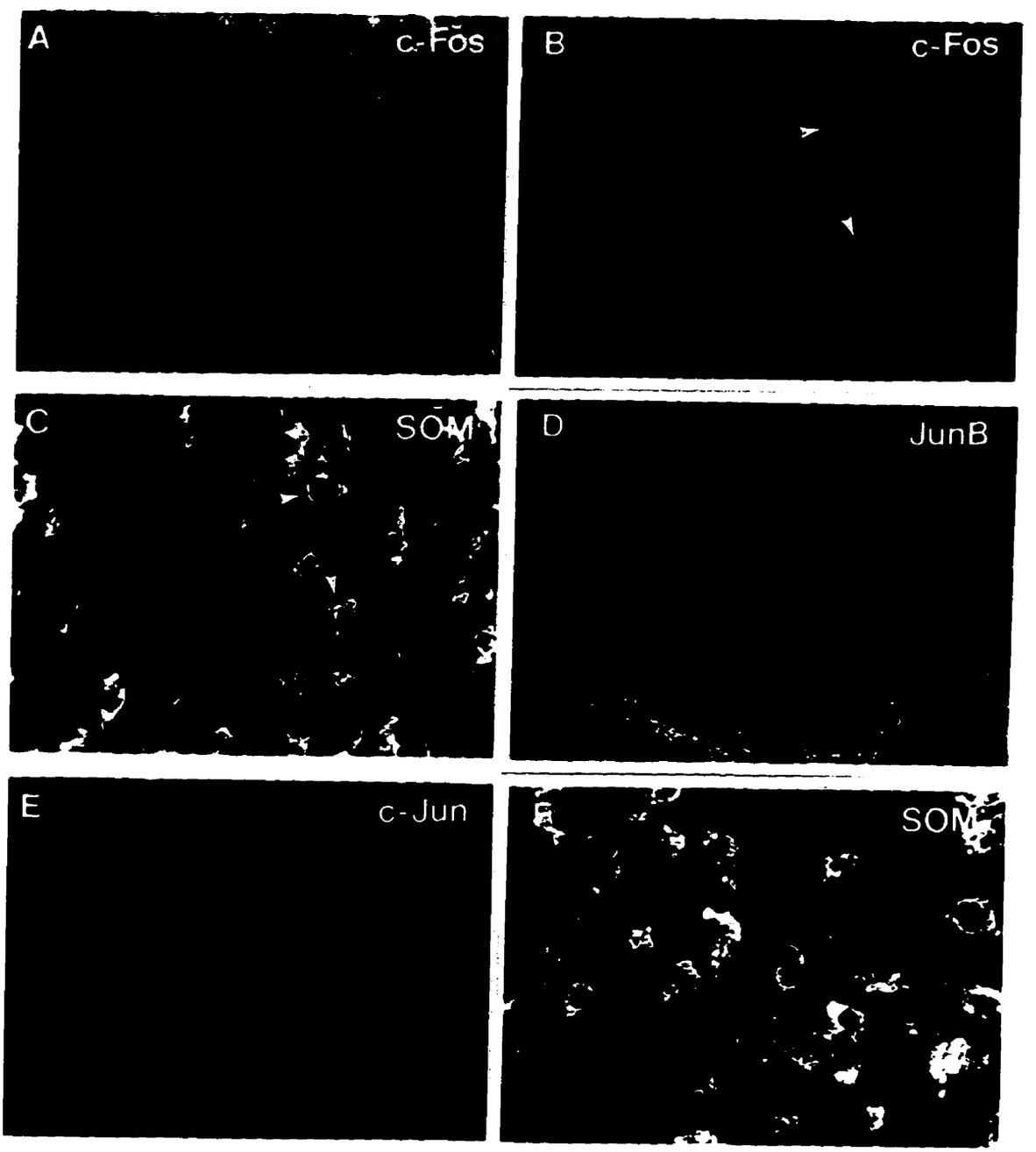
A
c-Fos
B
c-Fos
C
SOM
D
JunB
E
c-Jun
F
SOM

Figure 7.1 Fluorescent micrographs of c-Fos-, JunB- and c-Jun-immunoreactivity in the inferior mesenteric ganglion. Nuclear c-Fos was absent from the ganglia of acetic acid-treated animals that continued to fast (A) but was found in some neurons from animals that had also been refed (B). C-Fos-immunoreactive nuclei from those animals usually belonged to SOM neurons (B,C). JunB-immunoreactivity was also found in the nuclei of ganglionic neurons from those animals (D). C-Jun-immunoreactivity was found in ganglia from smaller animals wherein it was also usually most evident in SOM neurons (E,F). Scale bar: 100μm.

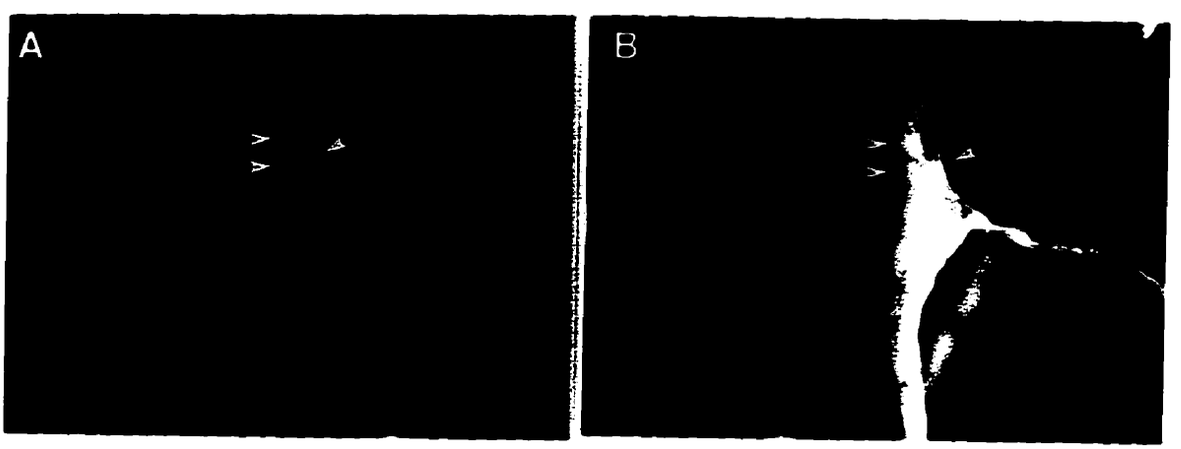
A
B

Figure 7.2 Fluorescent micrographs of c-Fos-immunoreactivity (A, arrows) in S-100-immunoreactive glia (B, arrows) in the submucosal plexus of an acetic acid treated animal. Scale bar: 100μm.

capsaicin alone failed to induce c-Fos or JunB in the IMG, but c-Fos labelling was found in mostly glia within both plexuses of the colon (not shown).

These data indicated that paired stimulation provided by colitis and refeeding was necessary, but not always sufficient, for activation of c-Fos in IMG neurons. However, activation of capsaicin-sensitive fibres itself seemed inadequate to activate this response, which occured preferentially in those neurons most closely associated with the enteric afferent fibres. Since refeeding proved to be so variable a stimulus, an intraluminal balloon was used in subsequent experiments to activate low threshold colonic nerves by imitating the passage of fecal pellets (see Methods and Materials). This was combined with intraluminal capsaicin or vehicle treatment to investigate the effects of these paired stimuli. None of the animals showed obvious discomfort following recovery from anesthesia and only faint traces of blood were ever found on the colonic mucosal surface of both capsaicin-treated groups. Since activation of JunB-IR in the IMG had previously been found to parallel that of c-Fos (see above), JunB-IR was not considered further.

Nuclear c-Fos-IR was absent from most IMG neurons of animals that had been treated with capsaicin alone, although 3 SOM neurons of approximately 300 were labelled in the IMG of 1 animal (Table 7.1). In animals treated with vehicle and balloon stimulation, c-Fos was again absent from most neurons but was consistently found in almost half of the rare -/- neurons (Fig 7.3A,B,C). In animals treated with both capsaicin and the balloon stimulation, nuclear c-Fos-IR was also significantly increased in SOM neurons but was still mostly absent from NPY and NPY/SOM neurons (Fig 7.3D,E,F). In the treated segments of colon from these animals, c-Fos was not present in either plexus from vehicle-

treated/balloon stimulated animals but was again evident in mostly glial nuclei of both plexuses from animals in both capsaicin-treated groups (Fig 7.4).

Table 7.1 The percentage of each of the IMG neurochemical subsets with c-Fos in animals treated with colonic balloon stimulation, intraluminal capsaicin or both (shown as mean ± SEM, n = 3 animals each).

Treatment Group	SOM Neurons	-/- Neurons	NPY Neurons	NPY/SOM Neurons
Balloon Stimulation	0 ± 0	38.2 ± 12.4*	0 ± 0	0 ± 0
Intracolonic Capsaicin	0.33 ± 0.33	0 ± 0	0 ± 0	0 ± 0
Capsaicin and Balloon	37.6 ± 16.1**	47.9 ± 11.3†	2.9 ± 2.9	1.7 ± 1.7

* $P < 0.05$ compared to SOM neurons, NPY neurons and NPY/SOM neurons within the balloon stimulated group, and compared to -\- neurons of the capsaicin treated group.

** $P < 0.05$ compared to NPY neurons and NPY/SOM neurons within the capsaicin treated, balloon stimulated group, and compared to SOM neurons of both of the other groups.

† $P < 0.01$ compared to NPY neurons and NPY/SOM neurons within the capsaicin treated, balloon stimulated group, and compared to -/- neurons of the capsaicin treated group.

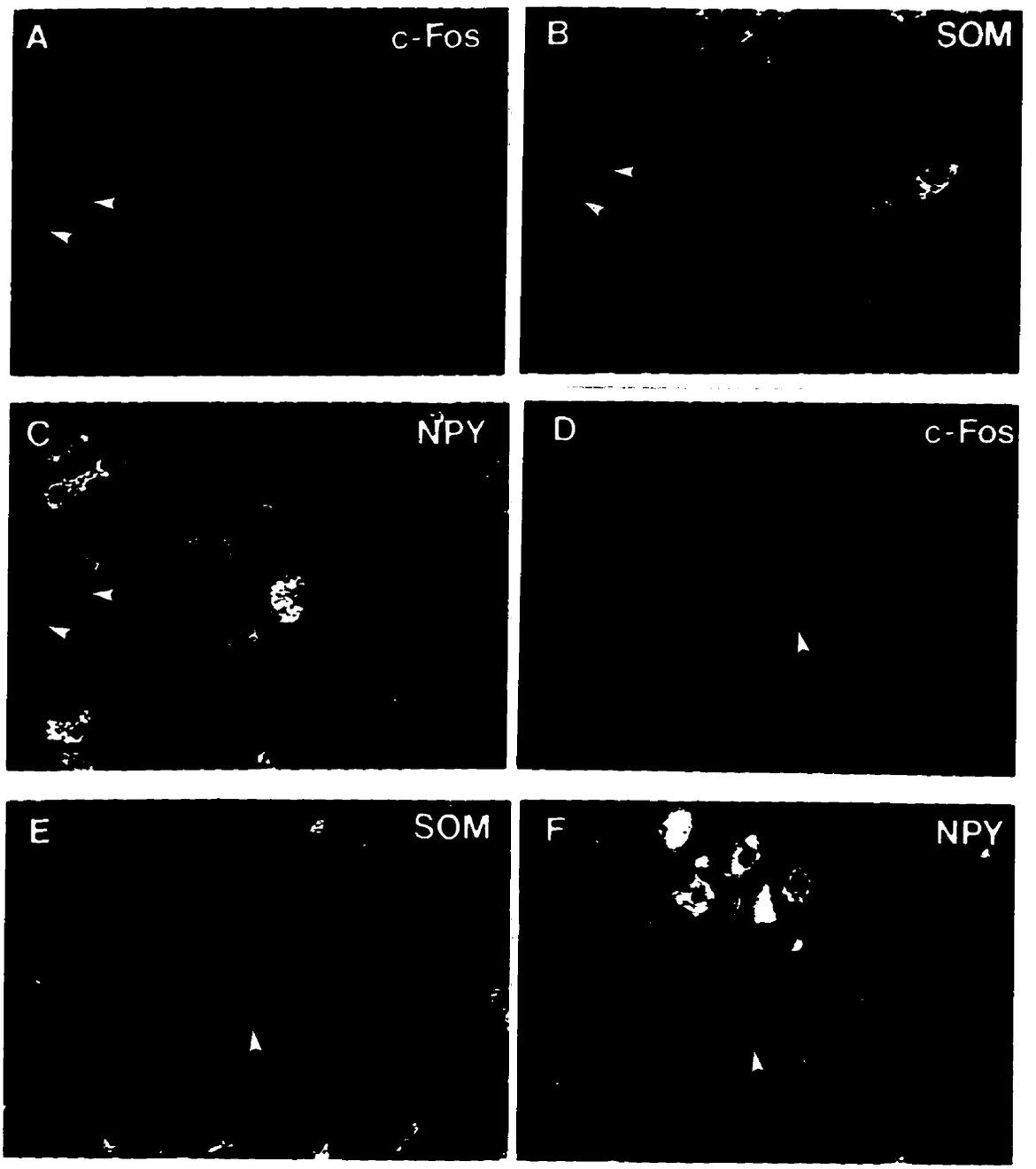
A
c-Fos
B
SOM
C
NPY
D
c-Fos
E
SOM
F
NPY

Figure 7.3 Fluorescent micrographs of c-Fos-immunoreactivity in immunohistochemical subsets of neurons in the inferior mesenteric ganglion of animals treated with balloon stimulation (A-C) or balloon stimulation and intraluminal capsaicin (D-F). In the IMG of animals treated with balloon stimulation, nuclear c-Fos (A, arrows) was found in neurons without SOM (B, arrows) or NPY (C, arrows). When this stimulus was paired with capsaicin, nuclear c-Fos (D, arrows) was also found in SOM neurons (E, arrows) but not NPY neurons (F, arrows). Scale bar: 100μm

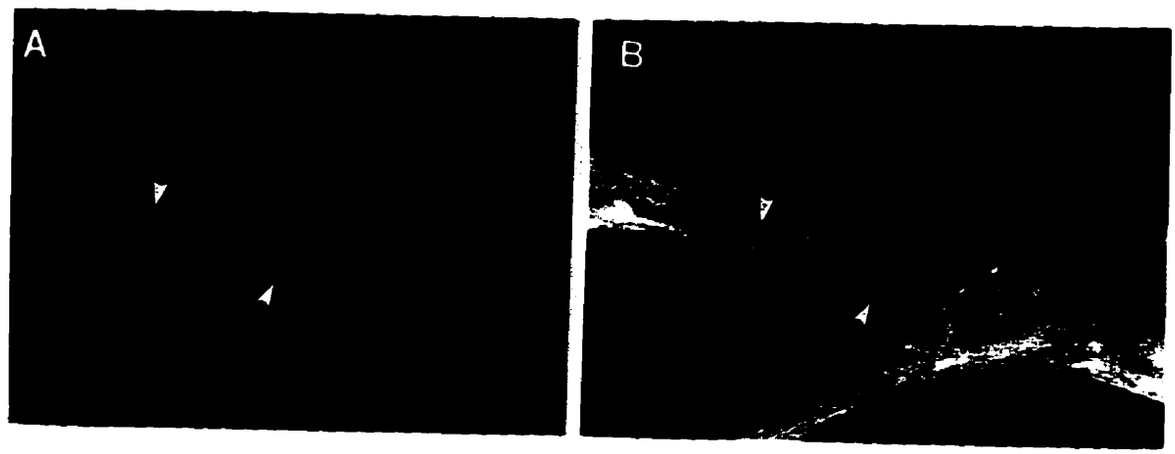
A
B

Figure 7.4 Fluorescent micrographs of c-Fos-immunoreactivity (A, arrows) in S-100-immunoreactive glia (B, arrows) in the myenteric plexus of a capsaicin treated animal. Scale bar: 100μm.

7.5 Discussion

7.5a Activation of IMG Neurons in Acute Colitis

These data indicate that acute acetic acid-induced colitis can contribute to rapid c-Fos and JunB activation in IMG neurons of the guinea pig. However, a highly variable effect of refeeding was also required for this response, and c-Fos activation was not observed in the IMG of almost half of those animals. Activation of primary afferent fibres may well have contributed to the mild mucosal hemorrhaging seen in all of those animals but the failure of intraluminal capsaicin to induce c-Fos in the IMG also indicates that activation of these colonic fibres is in itself insufficient to elicit this response (see below). On the other hand, capsaicin applied extrinsically to the intestinal nerves potentiated the responses of IMG neurons to stimulation that was probably provided by the surgical manipulation of the intestines. Furthermore, when c-Fos-IR was activated, it occured preferentially in SOM and -/- neurons which are the subsets most closely associated with intestinofugal fibres (Chapter 6) and so the variable stimulus provided by refeeding was likely to have originated in the ENS. One candidate for such a stimulus is the passage of fecal pellets as it involves enteric nerves, is likely to be stimulated by refeeding and is likely to vary between animals according to factors that are difficult to completely control.

The time course examined in these studies probably precedes some of the inflammatory reactions, such as recruitment and infiltration of immune cells, and therefore includes only early inflammatory responses to acetic acid-induced damage. Thus, neuronal expression of c-Fos and JunB proteins in these early stages may indicate neuronal activity that directly contributes to subsequent inflammatory events.

7.5b Activation of IMG Neurons by Capsaicin and Balloon Stimulation

Unfortunately, a comprehensive study of the effects of fecal transit would probably require surgical exposure of the colon, and this procedure would itself likely induce considerable c-Fos. Instead, a small intraluminal balloon was used to mimic the passage of fecal pellets in subsequent experiments. Interestingly, the balloon stimulation itself induced c-Fos in many of the rare -/- neurons. This response may be indicative of different thresholds and/or different inputs than any of the other subsets. This might also suggest that this stimulation was supraphysiological, but the grossly normal appearance of the colon and lack of c-Fos in the ENS suggests that primary afferent fibres were not involved (see below).

Intraluminal capsaicin may also have induced some inflammatory effects within the colon but the grossly normal appearance of the colon from these animals suggests that any such effect was markedly less than in the acetic acid treated group. The failure of intraluminal capsaicin alone to induce c-Fos in the IMG probably indicates that stimulation of the axon collaterals in the IMG via the colon is itself insufficient to substantially affect those neurons. While larger doses of capsaicin will also be tested in subsequent experiments, the systemic effects of such a treatment may also include more direct effects of capsaicin within the IMG. When balloon stimulation was paired with capsaicin treatment, c-Fos was also induced in SOM neurons thus duplicating the effects of acetic acid treatment in refed animals. Taken together, these observations support a model wherein convergent signals from the ENS and sympathetic afferent fibres in the colon are both necessary and

sufficient to induce c-Fos and JunB in SOM neurons of the IMG. In fact, intestinofugal fibres have been shown to provide cholinergic inputs that elicit fast excitatory potentials in IMG neurons, while a variety of neuroactive substances in intestinofugal fibres and in primary afferent fibres elicit slow excitatory potentials in those cells (5). While either of these events in isolation may fail to reach the threshold for action potential generation in IMG neurons, summation of these excitatory stimuli are likely to increase the chance for those neurons to reach their firing threshold (5). The present study suggests the possibility that this integrative response may be activated by intestinal inflammation when peristaltic activity is also stimulated in the affected segment of gut. While a possible role for spinal outflow cannot be excluded, those fibres are likely to provide substantial inputs to NPY neurons (23,142,160) but c-Fos was rarely ever found in those neurons.

These data support the largely separable nature of responses among these neuronal subsets, although this point would be further strengthened by a demonstration of selective c-Fos expression in NPY and/or NPY/SOM neurons. The few NPY and NPY/SOM neurons that sometimes exhibited c-Fos-IR support the idea that most or all of these neurons are capable of expressing these antigens although it was not possible to distinguish between cholinergic and noradrenergic NPY/SOM neurons. Furthermore, the possibility that the capsaicin-sensitive fibres mediate some lower threshold activity such as that provided by balloon stimulation has not yet been excluded. Future experiments to assess the contribution of primary afferent fibres to these acute responses will include animals treated to ablate these fibres and the use of receptor antagonists for neuroactive substances that are likely to be released from those fibres.

7.5c C-Fos in the Colonic ENS

Both refeeding and balloon stimulation were likely to have affected the activity of enteric nerves in the colon but the lack of c-Fos-IR in the colonic ENS of any of those animals suggests that supraphysiological levels of stimulation are required for activation of c-Fos in these tissues. Interestingly, when c-Fos was activated in the ENS of inflamed or capsaicin-treated colon, it occured primarily in glia. The meaning of this glial response remains to be determined although it is notable that synthesis of cytokines by glia may also be stimulated by inflammatory stimuli (165). It is also possible that activation of enteric neurons was suppressed by a balancing increase in reflex sympathetic activity that sometimes remained subthreshold for c-Fos expression in IMG neurons. Indeed, sympathetic nerves may directly inhibit submucosal neurons and presynaptically inhibit ACh release in the myenteric plexus (166,167). This latter effect may be a particularly important response to inflammation as ACh release is chronically inhibited in nematode-inflamed small intestine (168), but that effect appears to be due in part to inflammatory cytokines. In fact, impaired release of noradrenaline has also been demonstrated in the myenteric plexus of those animals (169), and this effect may also be due in part to actions of cytokines on sympathetic nerve fibres. Nevertheless, the present study also suggests that this may be preceded by effects that are manifest at the somata of sympathetic neurons. It is also notable that c-Fos has been implicated as a factor in the regulation of tyrosine hydroxylase (78), an enzyme required for noradrenaline synthesis, and so regulation of noradrenaline may be affected at various levels during intestinal inflammation.

7.5d Differential Expression of c-Fos, JunB and c-Jun

Unlike c-Fos and JunB, the expression of nuclear c-Jun was not detectably altered by any of the experimental manipulations but it was also preferentially localized in SOM and -/- neurons of smaller animals. It is possible that this reflects some developmental or phenotypic differences among these neuronal subsets in the IMG. Alternatively, this might reflect longer latency responses to the considerable ongoing inputs from the ENS that has been observed in isolated preparations (24,35,162). On the other hand, it is unclear how much of that ongoing activity has been induced by those experimental manipulations, and such a proposal would also imply age-dependent changes in that activity or the neuronal responses. In fact, age-associated decreases of inducible c-Fos and c-Jun expression have been found in some CNS neurons (170) and in some other excitable cells (171). Interestingly, however, c-fos mRNA is actually increased with age in sympathetic neurons of the superior cervical ganglion (85) although those neurons are likely to more closely resemble NPY neurons in the IMG (23,142,Chapter 6).

7.5e Conclusions

The rare -/- neurons in the IMG may preferentially express c-Fos in response to low threshold mechanosensory stimulation of the colon while expression of c-Fos (and JunB) in SOM neurons may require convergent stimulation of intestinofugal and primary afferent fibres. However, these stimuli do not markedly affect c-Fos in NPY or NPY/SOM neurons. In addition, no acute effects were demonstrated by changes in c-Jun expression but the expression of that antigen appears to be related to the age of the animals.

CHAPTER EIGHT

SUMMARY AND CONCLUSIONS

8.1 C-Myc in the Enteric Nervous System

The size and complex organization of the enteric nervous system (ENS) has led it to be considered as a unique subdivision of the autonomic nervous system (1). Enteric neurons are further unique from those in the central nervous system (CNS) in that they constitutively express c-Myc antigens. Presumably that reflects unique regulation of at least some genes in these neurons, although the identity of any such c-Myc regulated genes in enteric neurons are not known. It is also notable that enteric nerves also constitutively express other "growth-related" antigens, such as growth associated protein-43 (GAP-43)(172) that are usually down regulated in other nerves following maturation, and it is tempting to speculate that constitutive expression of various growth-related proteins by enteric nerves underlies their remarkable plasticity. Future experiments to assess the role of c-Myc in these tissues could potentially exploit cultured myenteric ganglia wherein its expression might be more accessible to manipulation. On the other hand, if c-Myc regulates genes that facilitate neuronal adaptation to intestinal motility, for example, demonstration of such a function might await the development of techniques to alter gene expression in vivo with temporal and cellular resolution.

8.2 Constitutive c-Myc and Fos-Like Antigens

The demonstration of constitutive c-Myc- and c-Fos-like-immunoreactivity (IR) in all enteric neurons of the guinea pig has also provided a potentially useful tool to counterstain preparations in double-labelling immunohistochemistry. Hence, the proportions of particular subsets of neurons that can also be identified by

immunohistochemistry can now be directly quantified in the same preparation. This work has already been extended by K.R. Amundson in this laboratory to reexamine the proportions of previously described subsets of enteric neurons throughout the guinea pig intestines, and to quantitate changes in the apparent proportions of these cells in ileitis (54). Immune cells in these layers have also since been demonstrated to lack these antigens (44), further supporting the idea that these antigens are unique to neurons in these tissues. Neuronal nuclear labelling by the anti-FOS 4 antibody has also since been used to quantitate the destructive effect of detergent on myenteric neurons in these animals (44), and to distinguish between neurons and mitotic glia in ganglia of inflamed ileum (173).

Interestingly, the pan-neuronal distribution of constitutive Fos-like-IR may have since been disputed. Karaosmanoglu et al. (174) reported seemingly identical labelling, albeit by a different c-Fos antiserum, that was in only about 60% of the neurons labelled with cuprolinic blue. That stain is thought to label rough endoplasmic reticulum which is abundant in enteric neurons although it is not yet clear that all other cells lack this feature. Unfortunately, the nucleolar labelling they also reported in an undetermined proportion of those cells might also suggest some technical imperfections. Indeed, it was also unclear if the small stained cells they described at the periphery of ganglia that lacked constitutive c-Fos also lacked other "neuronal" markers. These small "neurons" seem likely to account for the almost threefold higher estimate of small intestinal neuron population than had previously been estimated (108).

8.3 Inducible Fos and Jun Antigens

Peripheral intestinal reflexes have been extensively studied in acutely isolated intestinal tissues but analyses of circuits underlying those reflexes have been limited by the technology to concurrently analyze structure and functions of the nervous system. One possible approach exploits the activity-related neuronal expression of nuclear Fos and Jun antigens. Indeed, mechanosensory villus stimulation and cholera toxin were previously reported to activate c-Fos-IR in immunohistochemically characterized enteric neurons of isolated ileum (82). However, isolation and dissection of these tissues also removes tonic extrinsic nervous activity and necessarily damages many intestinal nerves, and those effects that may also explain the considerable basal levels of c-Fos- and JunB-IR observed in otherwise unstimulated segments in the present study. Depolarizing stimulation was capable of inducing c-Fos-IR in greater numbers of all tested neuronal subsets, but it is as yet unclear whether the threshold for c-Fos expression is similar within or between subsets. For example, the presence of c-Fos-IR in some submucosal neurons with vasoactive intestinal polypeptide-IR from untreated ileum and from incubated but unstimulated ileal segments suggests that those cells may respond to routine stimulation by expressing that antigen. However, the depolarizing stimulation used in the present study and the stimuli used by Kirchgessner and her colleagues (82) may well have exceeded the physiological "norm", despite which c-Fos expression was never found in all neurons of any given subset. Given the considerable isolation-induced expression of these antigens, future experiments may be better directed to establishing the nature and thresholds of stimuli that might be

associated with c-Fos and JunB expression in intact animals.

Despite the apparent plasticity of the ENS, it is notable that nuclear c-Jun was mostly restricted to submucosal ganglia. Nevertheless, these neurons have the capacity to rapidly induce nuclear c-Jun as it is strongly activated by colchicine in the ileal ENS (54). A preliminary experiment in this laboratory has also suggested that c-Jun is induced in the intestinofugally projecting myenteric neurons in the colon by extrinsic nerve section. However, it should be noted that the observations of small intestinal c-Jun expression in the present study were rather limited and were restricted to smaller animals. Given the age-dependance of c-Jun expression noted in the inferior mesenteric ganglion (IMG; see Chapter 7), it may also be useful to reexamine the basal and inducible levels of this antigen in older animals as well.

8.4 Chemical Coding of Neurons in the IMG

Since isolated ileal segments proved less than ideal as a model system, subsequent studies focused on whole animals. Unfortunately, the ileum is only accessible surgically, while the distal colon can be stimulated without wholly invasive measures and has a discrete sympathetic innervation supplied mostly by the inferior mesenteric ganglion (IMG). When these studies were undertaken, the previously extensive immunohistochemical studies of the guinea pig IMG still had not provided reliable estimates of the abundance of various neuronal subsets. Therefore, the abundance of those subtypes and some of the fibres with which they were associated were first examined. Interestingly, those data demonstrated target-specific differences between somatostatin-containing neurons (SOM neurons) in the

IMG and more rostral prevertebral ganglia, but nonetheless appeared to support a previously suggested model wherein "chemically coded" subsets of prevertebral ganglion neurons share correlated neurochemical, anatomical, electrophysiological, and presumably functional properties.

8.5 Fos and Jun Proteins in the IMG and Colonic ENS

In the last series of studies, various noxious and mechanosensory stimuli were used to activate expression of c-Fos in the IMG and colonic ENS. Acute colitis elicited only sporadic c-Fos in the ENS of the colon, mostly found in glia, and c-Fos and JunB were sometimes induced in SOM and -\- neurons within the IMG only when the animals were immediately refed. Hence, physiological levels of stimulation are likely to be insufficient to activate c-Fos in any of those tissues, and a pathophysiological stimulus had more variable effects that also required refeeding-associated signals in the IMG. This approach was abandoned in favor of a more reliable technique to stimulate colonic primary afferent fibres with capsaicin and low threshold colonic mechanosensory circuits with a balloon. Interestingly, only -/- neurons expressed c-Fos in response to balloon stimulation, thus indicating some fundamental differences between their responses and that of all the other subsets of IMG neurons. Costimulation of the colon with both capsaicin and the balloon was required to elicit c-Fos expression in SOM neurons, thus supporting the possibility that these neurons form part of reflex circuit that responds to noxious stimulation and/or higher threshold events by modifying intestinal functions stimulated by low threshold stimuli. Future experiments will examine the role of capsaicin-sensitive primar afferent nerves and

the substances they contain to these responses. It may also be interesting to determine if any of these effects also extend to more rostral prevertebral ganglia of these animals.

In contrast to c-Fos (and JunB), expression of c-Jun in IMG neurons was not found to be affected by those stimuli within the elapsed time. Longer latency effects have not yet been excluded, but the expression of c-Jun in the present study appeared to be more strongly associated with the age of the animals. It is possible that the presence of c-Jun in smaller (younger) animals reflects developmental modification of synaptic contacts and that this is largely complete in larger (older) animals. The lack of c-Jun apparent in the IMG of older animals might also suggest age-related losses in the plasticity of these neurons, but this has yet to be reported.

8.6 Conclusions

The expression of the transcriptionally active protein products of the immediate early genes has offered a novel approach to assessing the phenotype and interactions of a variety of nervous tissues. Constitutively expressed proteins in select groups of neurons seem likely to reflect developmental and/or environmental differences of various neurons and may ultimately enhance understanding of their functions. Nevertheless, inducible expression of c-Fos and JunB was most reliably elicited by pathophysiological levels of stimulation in the peripheral intestinal nerves. Therefore, while this approach is likely to prove useful to investigate neuronal actions and interactions in intestinal pathology, it may be of more limited use to detect synaptic activity associated with "normal" intestinal function. These studies might also support the idea that any indispensible functions of

inducible Fos and Jun proteins in these cells would only become apparent under pathological conditions, a suggestion that may be readily testable in c-fos knock out mice.

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